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REVIEW

The NeverEnding E-Story

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Abstract—The review discusses the functional role of the ribosomal E-site in the context of recent structural data. Traditionally, the E-site has been considered to serve only as a binding site for deacylated tRNA (E-tRNA) prior to its dissociation from the protein synthesis complex. Here, we examine specific contacts formed between E-tRNA and rRNA of the large ribosomal subunit in different organisms, as well as the sequence of their formation and disruption. The mechanism of translation suppression by inhibitors that bind to the ribosomal E-site is discussed. Based on current evidence regarding the location of aminoacyl-tRNA synthetases (ARSs) in the immediate vicinity of the ribosome, we propose a hypothesis that one of the primary functions of the ribosomal E-site is to prepare tRNA (through its modulation) for the formation of a specific complex with ARS, in the content of which it is released from the ribosome.

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INTRODUCTION

Ribosomes are macromolecular machines that synthesize proteins by a fundamentally conserved cellular mechanism across all three domains of life. They contain three binding sites for one of the key players in translation – transfer RNAs (tRNAs) – which are designated the A, P, and E sites. These sites serve to bind the substrates of the peptidyl transferase reaction (PTR), aminoacyl-tRNA (A-tRNA) and peptidyl-tRNA (P-tRNA), as well as deacylated tRNA released from the ribosome (E-tRNA). Each site is formed by the two parts located on the small (SSU) and large (LSU) ribosomal subunits, respectively [1].

From the earliest days of molecular biology, it has become clear that the A- and P-tRNAs, in their aminoacyl- and peptidyl-forms, respectively, are involved in decoding genetic information recorded in the nucleotide sequence of mRNA during translation and serve as the PTR substrates. In contrast, the function of the ribosomal E-site has been a subject of long debates, since it binds E-tRNA produced in the PTR (see reviews by Nierhaus [2] and Semenkov et al. [3]). Despite decades of studies, the functional significance of this site, which provides only a temporary refuge for tRNA dissociating from the ribosome, remains unclear. About twenty years ago, D. Wilson and K. Nierhaus published a comprehensive review entitled “The E-site story: the importance of maintaining two tRNAs on the ribosome during protein synthesis.” In it, the authors not only summarized the efforts aimed to elucidate the role of the ribosomal E-site, but also insisted that these studies are far from being over. The only conclusion they regarded as firmly established was that E-tRNA is involved in the maintenance of correct reading frame of mRNA [4]. To the best of our knowledge, no reviews have been since then that were focused specifically on this most crucial functional center of the ribosome. It should be noted that the review by Wilson and Nierhaus [4] appeared only a few years after a new era in the ribosome research had begun – the era of near-atomic resolution studies of the structure of a functional ribosome [5-8]. Initially, this era was dominated by X-ray crystallography of archaeal and bacterial ribosomes and their functional complexes. In the following years,

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the leading method has become cryogenic electron microscopy (cryo-EM), due to dramatic advances in the resolution and ability to study the structures from various organisms under conditions maximally close to the cellular environment [9-11]. More recently, cryo-electron tomography has made it possible to visualize the structure of functional ribosomes in their native state, in the cytoplasm or organelles. These developments supplemented by novel physical and computational approaches, have allowed to integrate structural ribosomal data with the results of decades of biochemical and genetic studies (see Flis et al. [12] and Nishima et al. [13]).

In this review, we discuss the problem of the ribosomal E-site based on the conclusion of recent studies and analysis of structural data deposited in the RCSB Protein Data Bank (PDB).

HOW DOES tRNA MOVE INTO THE E-SITE?

Even when studying the mechanism of protein synthesis by classical biochemical, biophysical, and genetic methods, researchers have established the view that ribosome, as a molecular machine, undergoes a series of diverse dynamic transformations at each step of polypeptide synthesis [14, 15]. These transformations are reversible structural rearrangements, such as partial rotation of the ribosomal SSU relative to the LSU, displacement of the SSU "head" relative to its "body," reversible large-scale movements of the LSU L1 and L7 protuberances, changes in the relative positions of the translation factor domains, and, finally, translocation of mRNA and tRNA from the A-site to the P-site and from the P-site to the E-site (see reviews by Noller et al. [16], Korostelev [17], and Lindahl [18]).

Recently developed methods have allowed not only to visualize these large-scale conformational changes, but also to reveal their previously unknown details [19].

In the classical ribosome configuration, when its subunits are not rotated relative to each other, the anticodon and acceptor segments of tRNA molecules interact with the corresponding A-, P-, and E-sites of the SSU and LSU. These arrangements are referred to as classical and designated as A/A, P/P, and E/E, where the first letter refers to the SSU and the second – to the LSU [20]. It is important to keep in mind that at any given time, only two tRNAs are present in the functional ribosome: either A- and P-tRNAs, or P- and E-tRNAs. (However, it should be noted, that in many structural studies, ribosome–tRNA complexes for subsequent analysis were obtained *in vitro* using a 3 to 4-fold excess of deacylated tRNA relative to the peptidyl- or aminoacyl-tRNA. In such "artificial" complexes, tRNAs may occupy all three sites; see Seely et al. [21]).

During the PTR, the growing polypeptide is transferred from the P-site tRNA to the amino acid residue of the A-site tRNA. Moreover, the acceptor end of the A-tRNA molecule, which is located on the LSU and now carries the growing polypeptide chain, moves toward the P-site of this subunit and adopts the so-called hybrid, or intermediate, A/P state (since the anticodon portion of this tRNA remains bound to the A-site of the SSU). At the same time, the 3′ acceptor end of the P-site tRNA moves into a specific functional site on the LSU – the E-site (its structure and characteristics will be discussed below), while the anticodon part remains associated with the P-site of the SSU. Therefore, the tRNA also adopts an intermediate state, in this case referred to as the P/E state. The formation of the intermediate states of the two tRNAs is accompanied by a limited rotation of the SSU relative to the LSU. The ribosome acquires a state poised for the translocation, i.e., becomes ready to move both tRNAs together with the bound mRNA by exactly one codon in a strictly defined direction. The elementary act of translocation ultimately ends with the transition of the peptidyl-tRNA and deacylated tRNA from the intermediate states into the classical P/P and E/E states, respectively (see review by Korostelev [17]).

CONTACTS OF E-tRNA WITH THE RIBOSOME

In the classical state, the E-site tRNA forms minimal contacts with the mRNA anticodon on the SSU and two specific segments of the rRNA on the LSU. Let us consider these contacts in detail. Figure 1 shows the structure of *Escherichia coli* tRNA and its principal contacts with mRNA and 23S rRNA in the E/E state. This structure was obtained by high-resolution cryo-EM [22] and selected from a series of functional ribosome complexes representing the early steps of the polypeptide chain elongation.

As can be seen in Fig. 1, the trinucleotide anticodon segment of the tRNA forms a full codon–anticodon complex with the complementary codon in the mRNA. This fully supports the main conclusion of Wilson and Nierhaus [4], who stated that the principal function of the deacylated tRNA–E-site complex is to maintain correct reading frame.

At the same time, our analysis of numerous structures of E-tRNAs in the E/E state in prokaryotic, eukaryotic, and mitochondrial ribosomes obtained by both X-ray crystallography and cryo-EM, has shown that a complete codon–anticodon complex is observed only in rare cases [22]. More often, there is only a single hydrogen bond between the tRNA anticodon and mRNA codon, or the contact between the E-tRNA and mRNA is absent. This can be explained by the fact that, unlike the codon–anticodon complexes formed

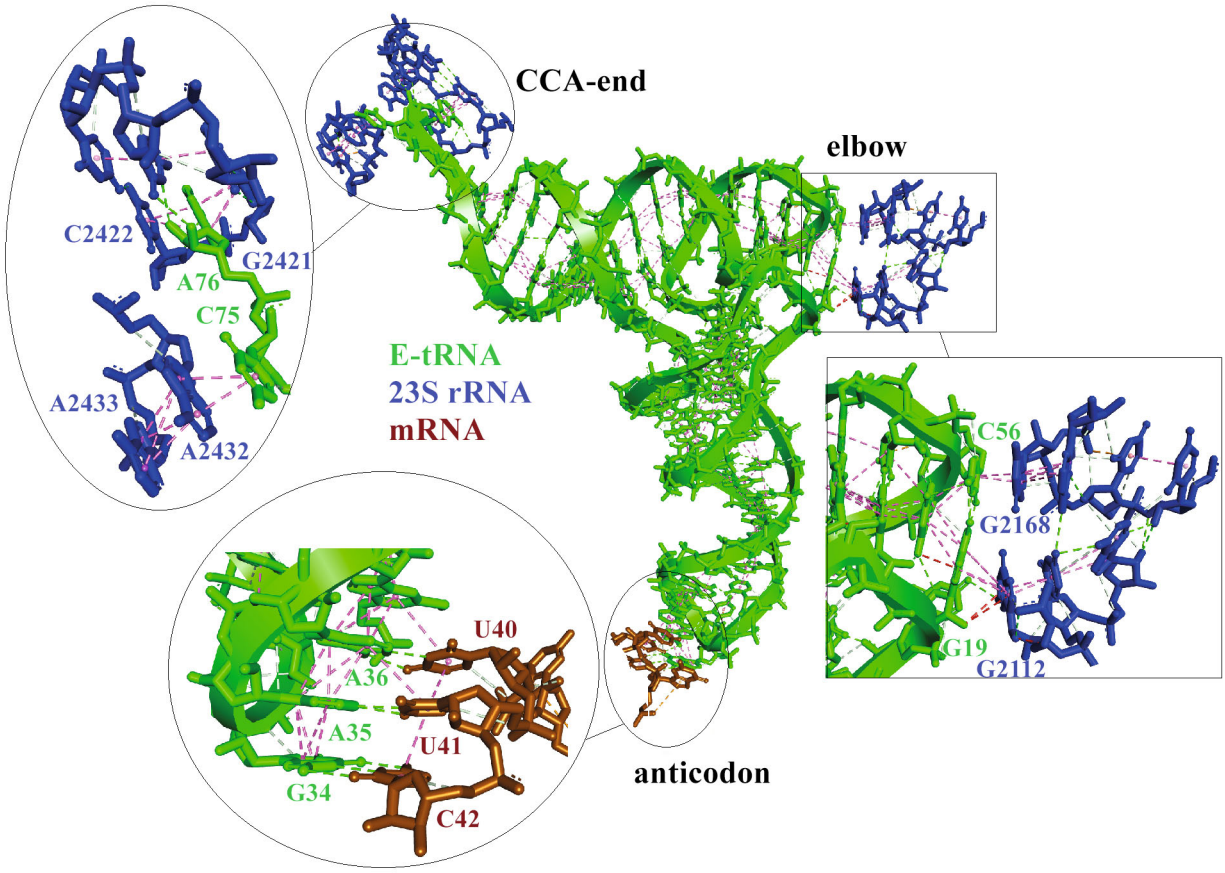

Fig. 1. Three main contacts of E-tRNA with mRNA and rRNA in the classical E/E state (*E. coli*; PDB ID: 7N31). In addition to the codon–anticodon complex (enlarged view, lower left inset), the figure shows the Watson–Crick GC base pair formed through the tertiary interactions in the tRNA "elbow," its stacking with G2112 and G2168 of the L1 protuberance of the 23S rRNA (enlarged view, right inset), and contacts of the 3′-CpA terminal group of E-tRNA with nucleotide residues of the 3′A pocket and A2432 of the 23S rRNA (enlarged view, upper left inset). In this and all subsequent figures, hydrogen bonds and intermolecular aromatic contacts are shown as green and red dashed lines, respectively. Construction, analysis, and visualization of RNA spatial structures were carried out with Discovery Studio Visualizer v.21.1.0.20298.

by the A- and P-site tRNAs, the transient complex between the E-tRNA and mRNA is not stabilized by direct interactions with 16S rRNA or proteins of the SSU.

As a result, the anticodon loop of the E-tRNA is the first to lose the contact with the ribosome, which precedes its exit (Fig. 2) [23].

The second contact of the E-tRNA with the ribosome involves the so-called tRNA "elbow," formed by its T- and D-loops (Fig. 1). The tRNA elbow interacts with the tip of the L1 protuberance of the LSU, which is composed of protein L1, and with nucleotide residues of the 23S rRNA that are not immediate neighbors in the polynucleotide chain. These nucleotides belong to the loops flanking helix H77 in the secondary structure. In the 3D ribosome structure, these residues are brought into a close proximity and, according to several published ribosomal structures, form both Watson–Crick and non-canonical base pairs [24] (see Lehmann et al. [25]). The intermolecular tRNA–rRNA contacts that form in this structure are stacking interactions between heterocyclic bases of nucleotides that are not directly adjacent in the primary RNA structure. This motif is common among RNAs of various classes and plays an important role in shaping the 3D structure of tRNAs and rRNAs, as well as in their functioning [26-28].

As mentioned earlier, translation is accompanied by reversible large-scale movements of the L1 protuberance relative to the "body" of the LSU. The state in which the protuberance is maximally displaced from the LSU is referred to as the "out" state, while the maximally closed conformation is termed the "in" state. The elbow of the E-tRNA interacts with the L1 protuberance only in its "in" state. Structural analysis of ribosomes at various functional stages reveals either complete (double) or partial (single) contacts between the E-tRNA and the L1 protuberance. Single contacts most likely correspond to the early stages of deacylated tRNA entry into the E-site (i.e., the P/E state) and, definitively, to the initial steps of its

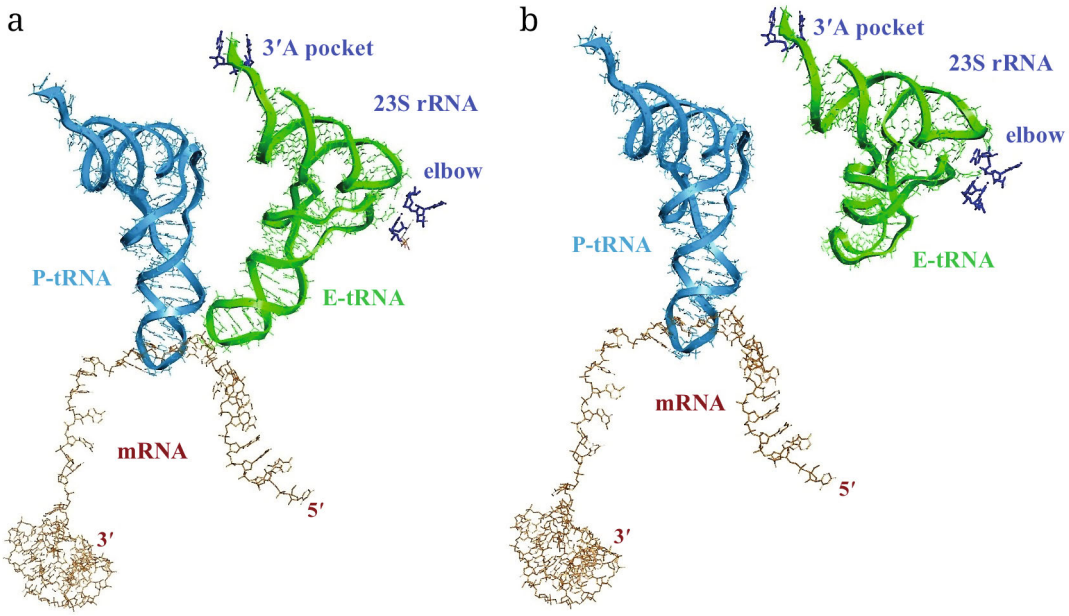

Fig. 2. The entry (a) of E-tRNA into the ribosomal E-site (PDB ID: 5QU7) and the beginning of its exit (b) (PDB ID: 5UQ8). (These events were captured because mRNA had a well-developed secondary structure near the 3′-end that slowed its entry into and movement through the ribosome [25]).

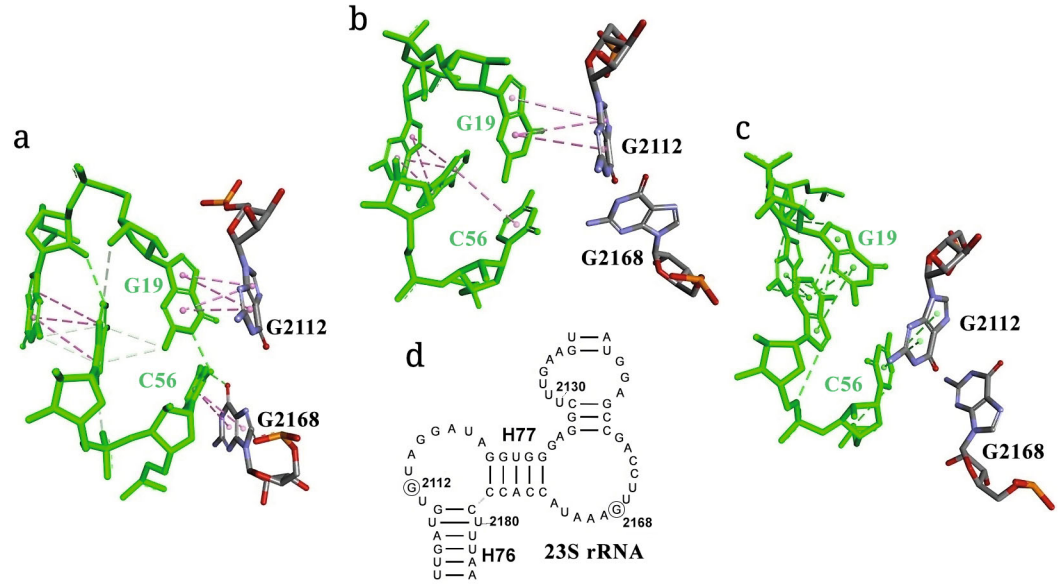

Fig. 3. Contacts between the E-tRNA elbow and nucleotide residues of the L1 protuberance. Full contact: a) PDB ID: 7SSN; partial contacts: b) PDB ID: 7ST6, c) PDB ID: 7ST2. d) Secondary structure of the 23S rRNA segment containing G2112 and G2168 [29].

departure from this site. Figure 3 shows examples of such states in *E. coli* ribosomes observed by cryo-EM [29]. Similar states have been previously detected by X-ray crystallography in archaeal ribosomes [30].

The third contact of deacylated tRNA with the E-site shown in Fig. 1 deserves special attention. It occurs within the functional center of the ribosome (hereafter referred to as the 3′A pocket) formed by a specific segment of the LSU rRNA that possesses a unique spatial configuration and serves to firmly anchor the 3′-terminal adenosine residue of the tRNA. This pocket is composed of two adjacent rRNA nucleotides (G2421 and C2422) and is stabilized by the hydrogen bonding with the trinucleotide CCA segment of the same rRNA, as well as by stacking interactions with neighboring rRNA bases [26, 28]. Remarkably, the structure of this pocket remains unchanged and can be observed even in ribosomes lacking tRNA.

The requirement for the 3′-terminal adenosine of the E-tRNA for its binding to the E-site was first demonstrated using tRNA molecule lacking this nucleotide [31] and later confirmed directly by chemical probing and mutational analysis [32-34]. However, the detailed structure of the 3′A pocket became known only after X-ray crystallography studies of ribosomes and their complexes with model poly- and oligonucleotides mimicking the 3′-terminus of tRNA [35]. These structural studies also showed that irreversible binding of synthetic tRNA mimetics to the 3′A pocket completely blocked translocation [35]. In other words, the CCA-end of the E-site tRNA must be completely released from the ribosome for a single translocation event to proceed without hindrance.

Conversely, any interference with the tRNA binding to the vacant 3′A pocket caused by disruption of its native structure (the effect of antibiotics and other E-site inhibitors on this pocket will be discussed below) also markedly suppresses or even completely halts translocation. This occurs, for example, when the structure of the 3′A pocket is altered by the deletion of a nitrogenous base from one of its forming nucleotides [36] or through mutagenesis of the 3′A pocket nucleotide residues critical for the interaction with the 3′-terminal adenosine of tRNA [37].

As already noted, the E-tRNA–3′A pocket complex is formed during the movement of deacylated tRNA from the P- to the E-site, i.e., it stabilizes tRNA in the P/E state. When the E-tRNA dissociates from the ribosome, its 3′-end is the last to be released from the 3′A pocket. This curious fact has been established when the resolution of cryo-EM ribosome structures reached 2 Å [38].

In the 3D structures of ribosomes from *E. coli* and several other bacteria, partial dissociation of the E-tRNA sometimes leaves only its 3′-terminal CA segment bound to the 3′A pocket in the same conformation as in the full E/E state (see Watson et al. [38]; PDB ID: 7K00). Such retention of the 3′-terminal dinucleotide of the E-tRNA is extremely rare in archaeal and eukaryotic ribosomes.

It should be noted that in archaeal and eukaryotic ribosomes (both cytoplasmic and mitochondrial), one of the LSU proteins is positioned in a close proximity to the 3′A pocket. In contrast, in eubacterial ribosomes, proteins are located at a considerable distance from this site. As a result, the conformation of the 3′-CA-end of the E-tRNA in eubacterial ribosomes differs markedly from that in archaeal, eukaryotic, and mitochondrial ribosomes (Fig. 4).

Indeed, comparison of structures shown in Fig. 4 reveals that in eubacterial ribosomes, the heterocyclic bases of the 3′-terminal CA segment of tRNA lie approximately in the same plane, with the nitrogenous base of C75 stacked against A2432 (*E. coli* numbering). In turn, A2432 stacks with A2433, thus forming (together with C75) a five-nucleotide stack. In ribosomes from non-bacterial sources, C75 is positioned roughly as in the 3′-terminus of free tRNA – its base plane is approximately parallel to that of C74. In all known cases, A76 occupies a similar position relative to the 3′A pocket bases with which it stacks. The identity of these rRNA bases may vary (and some can be modified), but the cytidine residue C2394 of the 23S rRNA, which forms a network of specific hydrogen bonds with A76 in the 3′A pocket, is strictly conserved.

Therefore, after dissociating from its codon–anticodon complex with mRNA, the E-tRNA is not immediately released from the ribosome but retains specific contacts with the LSU and maintains a defined spatial position for some time. The possible functional significance of this phenomenon is discussed in the concluding section of this article.

E-SITE INHIBITORS

Ribosomes are targeted by a large number of natural and synthetic bioactive compounds, including numerous antibiotics. Most inhibitors of protein biosynthesis bind to the A- and P-sites of the ribosome within its decoding center, as well as in the peptidyl transferase center and the tunnel through which nascent polypeptide chains exit from the ribosome [39].

Antibiotics that specifically target the E-site are much less common [40]. However, some well-known antibiotics (e.g., aminoglycoside kasugamycin, pentapeptide edeine, and tetrapeptide GE81112) which had been originally assumed to act as translation initiation inhibitors [39], in fact overlap with a significant portion of the mRNA-binding channel on the SSU in the P-site and E-site regions [41].

The antibiotic amicoumacin A (AMI) exhibits a higher selectivity toward the E-site region of the mRNA-binding channel [42, 43]. AMI does not interact directly with E-tRNA. The mechanism of its inhibitory activity lies in the ability to form multiple contacts with rRNA residues constituting the E-site region of the SSU and simultaneously bind to mRNA within the decoding center of this region, thereby effectively “gluing” the mRNA to the rRNA and halting translocation [42, 43]. AMI inhibits translation not only in eubacteria and archaea but also in eukaryotes, and is therefore considered to be a potential anticancer agent [44].

In this regard, the well-known antibiotic cycloheximide (CHX) demonstrates a much greater selectivity toward ribosomes from the three major evolutionary domains of life: it suppresses protein synthesis in archaea and eukaryotes, but has little or no inhibitory effect on bacterial ribosomes [45-47].

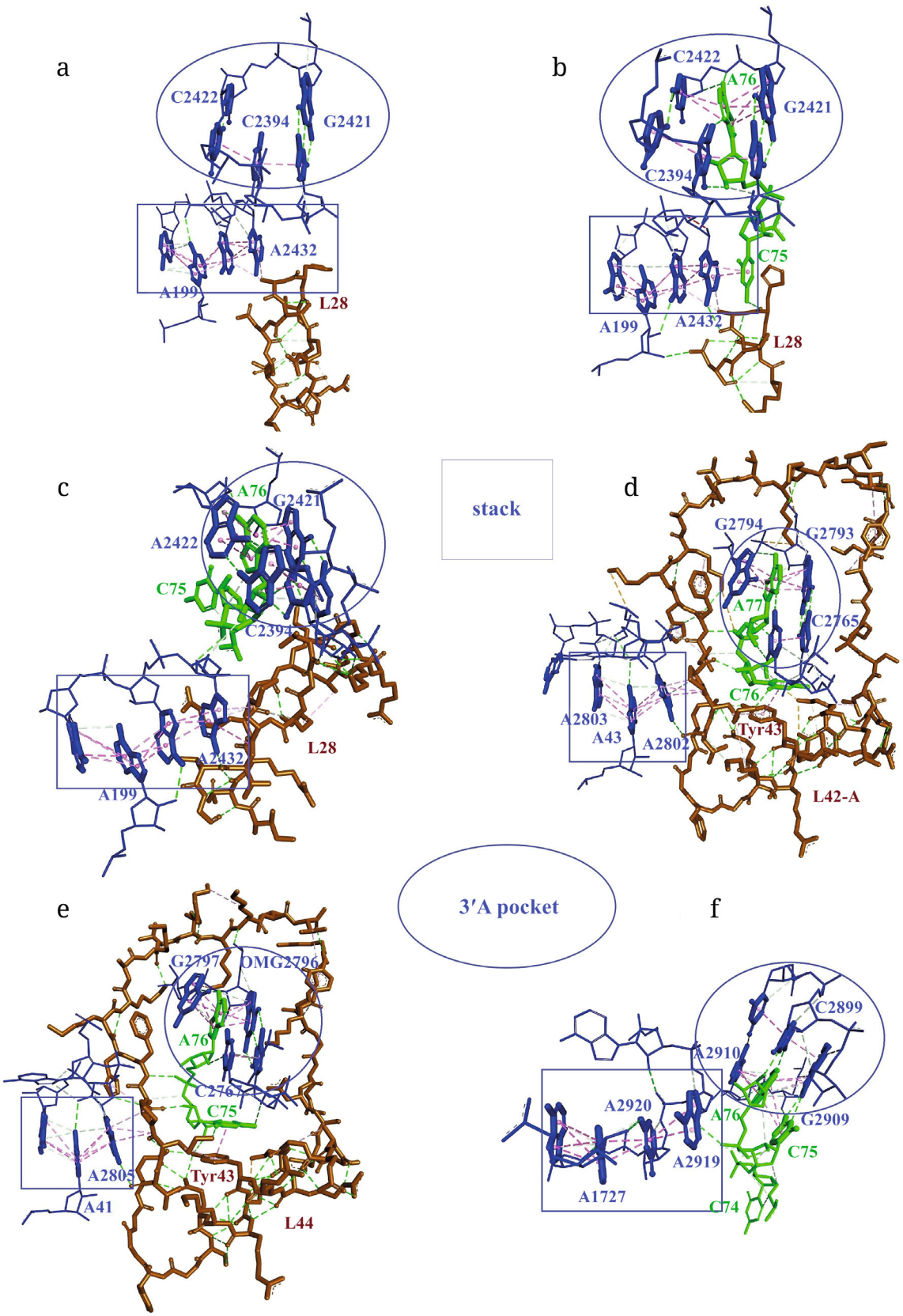

Fig. 4. The 3′A pocket and its environment in ribosomes from different sources. a) *E. coli* without E-tRNA (PDB ID: 9NL5); b) *E. coli* with E-tRNA (PDB ID: 7K00); c) *Thermus thermophilus* (PDB ID: 5UQ7); d) *Saccharomyces cerevisiae* (PDB ID: 8T3A); e) *Nicotiana tabacum* (PDB ID: 8B2L); f) human mitochondrial ribosome (PDB ID: 7QI5). Green, CpA; light brown, proteins; blue, rRNA of LSU.

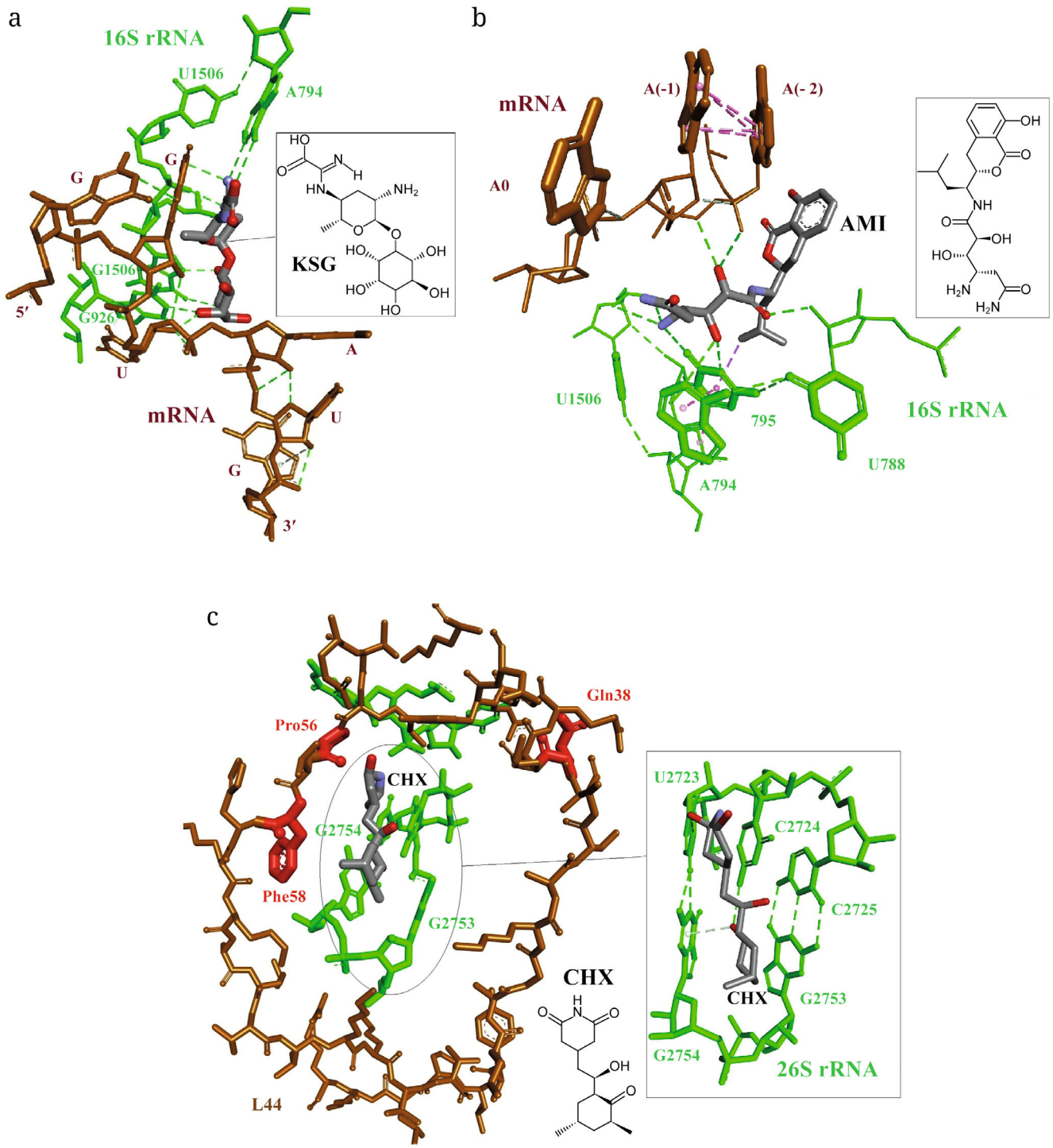

Fig. 5. Antibiotics interacting with the mRNA region of the ribosomal E-site: a) kasugamycin (KSG), PDB ID: 9FCO; b) amicoumacin A (AMI), PDB ID: 4W2F; c) cycloheximide (CHX), PDB ID: 7R81. Amino acid residues in protein L44 whose mutations result in the resistance to CHX are highlighted in red.

According to the data from PubMed Central (PMC), the number of publications mentioning CHX has exceeded 3000 annually over the past decade. Therefore, here we briefly summarize only the main details of its interaction with the ribosomes sensitive to this antibiotic.

The binding site of CHX within the ribosomal E-site is located in the 3′A pocket and its environment. As noted above, the architecture of the 3′A pocket is highly conserved. However, unlike in bacteria, the 3′A pocket in archaeal and eukaryotic ribosomes (including mitochondrial ones) is surrounded by proteins,

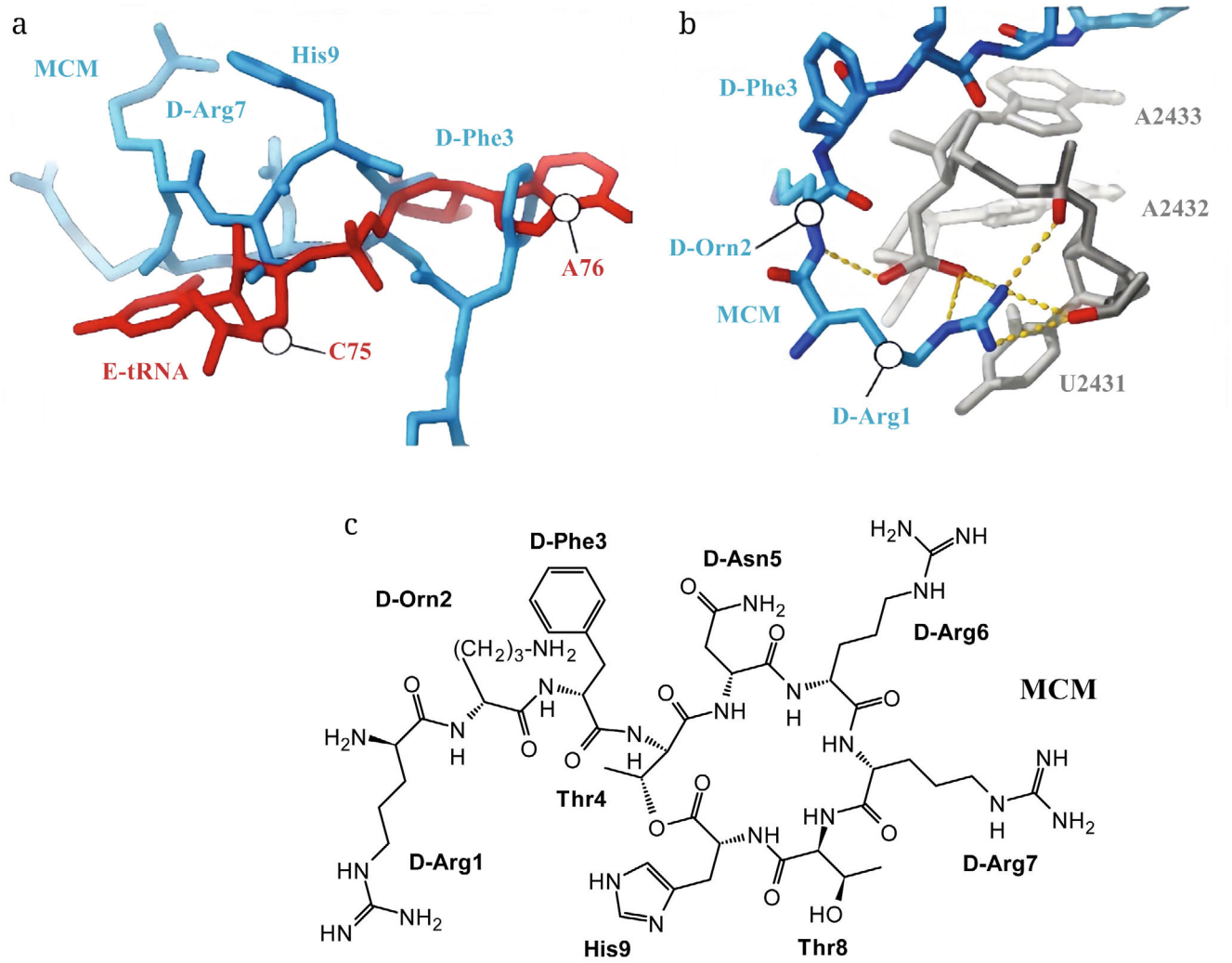

Fig. 6. Interaction of the antibiotic manikomycin A (MCM) with *E. coli* ribosome: a) MCM overlapping with the 3′CA-end of E-tRNA; b) shielding of A2432, which otherwise forms a stacking interaction with C75 of E-tRNA; c) chemical structure of MCM.

and at least one of these proteins is directly involved in specific CHX recognition. This is evidenced by the fact that mutations in several amino acid residues of protein L44, situated near the 3′A pocket, result in the resistance to CHX (Fig. 5c).

Until very recently, no protein biosynthesis inhibitors acting selectively on the E-site of the LSU of bacterial ribosome have been known (although theoretical analysis suggested that such selective inhibitors could be designed [47, 48]). Only recently, Wright et al. [49] identified an inhibitor produced by *Streptomyces rimosus*. This compound, a depsipeptide named manikomycin A (MCM), binds selectively to the 3′A pocket, thereby preventing interaction of the CCA-end of E-tRNA with its primary binding site within the E-site of bacterial ribosome (Fig. 6).

At the same time, proteins surrounding this pocket in archaeal and eukaryotic ribosomes either hinder MCM binding or make it entirely impossible. Analysis of regions of ribosome stalling induced by MCM revealed the enrichment of Pro codons in the P- and A-sites of ribosomes blocked by this compound. [49]. So far, there is no explanation for this phenomenon. The authors suggest that since translation of mRNA regions containing Pro codons proceeds more slowly, the period during which the ribosomal E-site remains unoccupied by E-tRNA (and, therefore, remains accessible to the antibiotic) is prolonged. It is also possible that the observed effects are related to the function of bacterial translation factor EF-P, which is activated when the PTR rate markedly decreases due to the presence of Pro or other amino acids forming the so-called “problematic” amino acid sequences. EF-P restores the PTR rate within a time period that is shorter than a single translocation step and is released from the ribosome [50]. The binding of EF-P to the ribosome leaves the 3′A pocket accessible for the MCM binding [51].

Finally, we would like to draw attention to the observation which, at the first glance, may seem

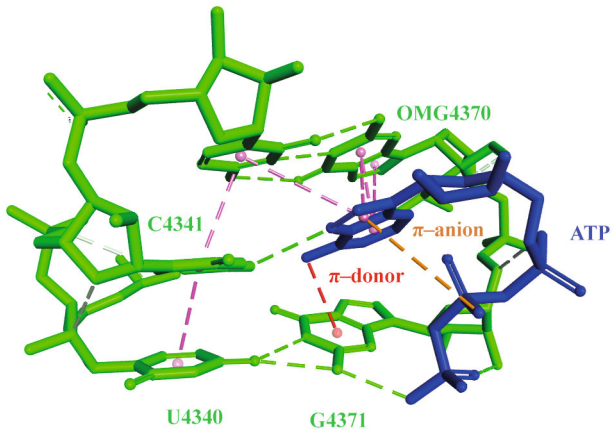

Fig. 7. ATP molecule in the 3′A pocket of the human ribosome (PDB ID: 8QYX). Note the interaction between the amino group of ATP and one of the G-pocket residues, as well as the π–anion interaction between the same adenine base and the phosphate group.

unrelated to the inhibitors of ribosomal E-site. While analyzing the 3D structure of the isolated human 60S ribosomal subunit (PDB ID: 8QYX), we unexpectedly found an ATP molecule within its 3′A pocket (Fig. 7) [52]. The ribosomal preparation used for the structural analysis was obtained in the studies of subunit biogenesis, and the authors of the original paper did not comment on this finding.

Interestingly, although the complexes formed by the 3′A pocket with the 3′-terminal adenosine of the E-site tRNA and with adenosine moiety of ATP share certain structural similarities, their conformations differ markedly. Although adenine bases of both nucleosides exist in a weakly pronounced *anti*-conformation, they are oriented in the opposite directions relative to the bases of the 23S rRNA that form the pocket. Consequently, the nature of hydrogen bonds formed by these heterocyclic bases with the cytosine residue of the pocket (C4341) also differs.

Cellular extracts used for the isolation of 60S subunits undoubtedly contained free ATP [52]. Nevertheless, even if the observed ATP–3′A pocket complex in the 60S subunit represented a crystallography artifact, the obtained structural information may prove to be valuable for the rational design of new synthetic antibiotics targeting protein biosynthesis.

INSTEAD OF A CONCLUSION

In this review, we would like to emphasize once again that the translocation of tRNA from the P-site to the E-site represents one of the key stages of protein biosynthesis. This step is accomplished through the large-scale movements of ribosomal subunits and domains relative to each other, as well as through the formation of numerous strong and specific contacts between the E-tRNA and specialized functional centers of the ribosome. First, E-tRNA participates in the movement of the codon–anticodon complex along the ribosome while maintaining the correct reading frame [4]. Second, we assume that the precisely defined topology and dynamics of tRNA within the E-site contribute to the ribosomal functioning after tRNA dissociation from the E-site. It should be noted that tRNA occupies this position even after the translation termination, when its participation in the mRNA translocation is no longer required [53].

It is also important to mention numerous translation initiation, elongation, and termination factors, various quality control factors that ensure proper ribosome function [54], and a group of protein factors finalizing maturation of newly synthesized polypeptides directly on the ribosomal surface. Together, these components constitute the so-called ribosome interactome, which, in the case of eukaryotic ribosomes, includes over a hundred proteins and RNAs [55].

The attempts to establish a direct functional link between the ribosome and ARSs have been made already in the early studies into the mechanism of protein synthesis [56]. With accumulation of data on the ribosomal interactomes, it has become clear that ARSs are not only components of these complexes (as was expected) but can also associate with various ribosomal components [57-60]. It was proposed that, as soon as free deacylated tRNA is released from the ribosome, it is immediately captured by the cognate ARS [58].

Analysis of tRNA positioned in the E-site in the classic E/E state shows that it lies within a shallow depression and remains accessible from the ribosomal surface. Its anticodon loop is also accessible. As noted above, it loses contact with mRNA shortly after transition from the P/E to the E/E state. It is well established that the anticodon within this loop is the first and most important structural element recognized by an ARS [61]. Therefore, it is reasonable to suggest that ARSs can interact with their cognate tRNAs while the latter are still located in the E-site. Furthermore, ARS might dissociate together with tRNA into the periribosomal space, where it can complete the aminoacylation reaction and, at an appropriate moment, deliver tRNA to the elongation factor I (EF-Tu in prokaryotes). Such process will undoubtedly be advantageous both kinetically and thermodynamically.

If our hypothesis is experimentally confirmed, this would imply that an important, perhaps, even the primary function of the ribosomal E-site is direct preparation of tRNA for participation in the new rounds of translation.

Abbreviations

ARS	aminoacyl-tRNA synthetases
CHX	cycloheximide
E-tRNA	deacylated tRNA
LSU	large ribosomal subunit
MCM	manikomycin
PTR	peptidyl transferase reaction
SSU	small ribosomal subunit

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Contributions
V.M. analyzed the data and prepared all figures; A.B. designed the project. Both authors contributed substantially to the analysis of literature and writing of the manuscript.

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Ethics approval and consent to participate
This work does not contain any studies involving human and animal subjects.

Conflict of interest
The authors of this work declare that they have no conflicts of interest.

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