Modulation of Catalytic RNA Biological Activity by Small Molecule Effectors

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Abstract: Catalytic RNAs, known as ribozymes, act as true enzymes and are implicated in important biological processes, such as protein synthesis, mRNA splicing, transcriptional regulation and retroviral replication. Ribozymes are capable of serving as a new molecular target for a variety of drugs and as a reliable screening system for their biological activity.

Key Words: Ribozymes, RNase P, ribosome, drug target, aminoglycosides, macrolides, retinoids, calcipotriol.

INTRODUCTION

The discovery of catalytic RNA almost 25 years ago was one of the most unexpected findings since the postulation of the central dogma of biology [1]. Although RNA molecules were recognized from the beginning as essential and absolutely necessary components of the genetic information flow, they have been long considered as passive intermediates between the vocabulary of nucleotide sequences and the ribosome-based interpretation into proteins. Although some RNA species, like tRNA, seem to carry conserved features that are completely idiosyncratic (e.g. unusual modifications) indicating that they hold indeed a very special position during molecular evolution, RNA was in general believed to provide the appropriate structural and physico-chemical interface for interaction of proteins that were responsible for catalyzing the major metabolic pathways.

The discovery of catalytic RNAs (or ribozymes) shifted completely the current perspectives of catalysis and expanded our point of view on major cellular events. Among those events, protein synthesis is the most recently identified process that is catalyzed by a ribozyme *per se*. Today the "RNA world" hypothesis is the backbone of virtually any evolutionary-based discussion on the origin of the contemporary biochemical functions. The almost daily discovery of new roles that RNA is involved in, not only as passive carrier of information but also as essential regulator of cell's complexity, has established that ribonucleic acids are indeed ancestral molecules of outmost importance [2, 3].

The capability of RNA and especially catalytic RNA to act as a specific sensor of large or small molecules is central to its diverse biological functions. In addition its ability to interact through sequence or structure specific hybridization with other nucleic acids, gave the opportunity to the researchers worldwide to use catalytic RNA molecules as tools with enhanced catalytic properties as well as molecular targets for specific inactivation [4]. Much of the recent work on comparative structural genomics of ribozyme-catalyzed reactions revealed the dynamic features of RNA-ligand recognition events and the direct impact of these studies on our ability to control cell function at the RNA level. It also attracted serious attention and efforts to combat pathogens by specifically targeting their RNA or RNA-protein complexes [5].

Finally the discovery of RNA interference, a cellular response that leads to gene silencing and that was elusive until recently, gave new insights on RNA-based gene regulation and has been developed as a tool for monitoring gene expression [6].

SMALL RNA LIGANDS AND RIBOZYMES

The search for small molecule effectors as selective RNA ligands was driven by early observations that certain antibiotics could be inhibitors of protein synthesis. The ribosome, a synthesis machine of a 2.5 MDa ribonucleoprotein assembly, is the largest ribozyme in nature characterized so far. The recent crystallographic studies of the large ribosomal subunit have revealed that peptide bond synthesis is performed by an RNA catalyst (23S rRNA), without the direct involvement of protein [7]. However, since RNA molecules that participate in the transnational machinery were not appropriate in terms of size to easily probe RNA-ligand interactions, the discovery of small ribozymes (like the hammerhead ribozyme) provide suitable RNA molecules for these studies. Moreover, these small RNA molecules can catalyze specific reactions, thus providing a direct measurement of the ability of small molecule effectors to inhibit or enhance their catalytic potency. A wealth of information regarding hammerhead ribozymes, such as conserved nucleotide requirements, kinetics, mechanism and structure was available and that is the main reason why hammerhead ribozyme is one of the best characterized RNA enzymes so far [8-11]. One of the few ribozymes of ancestral origin still represented in contemporary cells is Ribonuclease P (RNase P). RNase P catalyzes a specific endonucleolytic cleavage of a phosphodiester bond, which removes the 5' leading sequence from all precursor tRNA molecules, thus participating in their complex maturation process. It is a ribonucleoprotein in mostly all forms studied, which consists of a single RNA

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component and one (Bacteria) or more (Archaea, Eukaryotes) protein subunits [12, 13]. The RNA component from bacteria, one of the first catalytic RNA molecules identified, is responsible for the main catalytic function of the RNase P holoenzyme [3, 15, 16] and is a well-studied target, along with the holoenzyme, for various effector molecules.

Much of the early work on small molecules effectors on ribozymes was focused specifically on aminoglycosides. Today it is known that, as well as being among compounds that bind to the 16S rRNA, aminoclycosides also act as inhibitors of other ribozymes than the hammerhead, such as the group I introns the hairpin ribozyme, the ribozyme from the hepatitis delta virus (HDV) and both bacterial and eukaryotic RNase P enzymes that carry an essential for activity RNA subunit.

The present report summarizes briefly the effect of small molecular weight ligands on catalytic RNAs.

EFFECT OF SMALL RNA LIGANDS ON RIBOSOME

Since it has been elucidated that peptidyl transferase center consists solely of RNA, the entire ribosome is considered as a ribozyme. Ribosomal RNA is the target of many antibiotics [16, 17]. The antibiotics developed so far are based on natural products including tetracycline, aminoclycosides, macrolides and several peptidyl transferase inhibitors among others (Fig. (**1**)), predominantly targeting 30S and 50S ribosomal subunits and blocking protein synthesis in specific pathogens but not in mammalian cells. Confirming earlier biochemical work, the detailed structural data of ribosome–antibiotic complexes have revealed that the natural products interact predominantly with the RNA components of the ribosome, and provide a useful tool for the design of specific inhibitors, even for each pathogen individually [7].

Tetracycline, the first broad-spectrum antibiotic discovered, is a classical inhibitor of A-site occupation [18]. Binding to the A-site in the 30S subunit sterically hinders the movement of the aminoacylated tRNA so that it cannot simultaneously interact with decoding site in the 30S subunit and the peptidyl transferase center in the 50S subunit. Recent crystal structure analysis of complexes of the small ribosomal subunit with tetracycline revealed that several sites of 16S rRNA and the ribosomal proteins S4, S7, S9, and S17 come into contact with tetracycline. The interaction of tetracycline with the site formed by the residues A964-G966, G1053, C1054 and A1196-G1198 of 16S rRNA, is functionally responsible for the blockage of the A-site [19, 20].

Antibiotics, which disturb the decoding process and induce misreading of the genetic code, interact with distinct sites in the 16S rRNA [21, 22]. Aminoglycoside antibiotics such as streptomycin and paromomycin bind with high affinity to the bacterial decoding site and increase the error rate of translation. Aminoglycosides are water soluble oligosaccharides, that possess a linked ring system (2-deoxystreptamine ring, DOS) consisting of aminosugars and aminocyclitol and contain a number of ammonium groups, mostly positively charged at neutral pH. Moreover, they contain a significant number of hydrogen bond donor groups that contribute to rRNA binding [5, 23]. Their binding site was found to be located at the aminoacyl-tRNA decoding site (A site) on the 16S ribosomal RNA [21]. Kinetic analysis showed that, during decoding, a correct tRNA-mRNA interaction induces a conformational change of the A site to permit translation [24]. Aminoglycosides disturb the fidelity of this selection step by stabilizing a similar conformation for near- or non-cognate complexes [25, 26]. Recent crystal structures show that the specific recognition of base-pairing geometry leads to a closure of the domains of the small subunit around cognate tRNA. This domain closure is likely to trigger subsequent steps in tRNA selection [26]. The decoding process is based on the formation of a minihelix between the codon of the mRNA and the anticodon of the cognate aminoacyl-tRNA [27]. This interaction is performed at the A site by adenines 1492 and 1493 [28-31]. When the cognate tRNA–mRNA complex is formed, the two adenines flip out from the A-site helix and form type I-type II interactions [32] with the first two base pairs of the minihelix [33]. This conformational change constitutes the molecular switch that irreversibly decides the continuation of translation [25, 34, 35]. Aminoglycosides like paromomycin stabilize a conformation of the A site that normally occurs only when a cognate tRNA-mRNA complex is bound. As a consequence, the stabilities of near cognate aminoacyl-tRNA binding to this site are increased and the kinetics controlling the translation fidelity are disturbed in such a way that the ribosome is not able to discriminate between cognate and near- or non-cognate tRNA-mRNA complexes any more [36]. The peptide antibiotic viomycin (Fig. (**2**)), a member of the turberactinomycin family widely used against turbeculosis, behaves in many respects like the aminoglycosides: it binds to the decoding site of 16S rRNA, thereby inducing miscoding. This compound is a cyclic peptide containing non-coded amino acids in addition to the amino acids arginine, lysine and serine, which often occur in RNA-binding domains of proteins [4].

23S rRNA, the major constituent of peptidyl transferase center (PTC), is the target of many different classes of important antibiotics such as puromycin, blasticidin S, chloramphenicol, sparsomycin, streptogramins, and macrolides (Fig. (**1**)). The majority of them interact with the active site hydrophobic crevice in the peptidyl transferase region or with a second important hydrophobic crevice in the entrance of the exit channel that nascent polypeptide travels through. Crystallographic studies have yielded three-dimensional structures of several PTC-binding ligands complexed with the 50S ribosomal subunit [37]. In most cases, the structural data are not only in excellent agreement with findings from earlier biochemical and genetic studies, but also provide a molecular basis for the mechanism of action of the translation inhibitors.

Puromycin and blasticidin S are nucleoside antibiotics, which specifically inhibit ribosomal peptidyl transferase in both prokaryotic and eukaryotic systems [18]. Puromycin, a structural analog of the 3-end of aminoacyl-tRNA (although the aminoacyl residue is linked to the ribose *via* an amine bridge rather than an ester bond), has played a central role in our understanding of the mechanism of peptide bond formation [15]. It binds to an RNA structural motif that contains several conserved binding sites for the 3 termini of the acceptor and donor tRNAs [38], and elongation is terminated

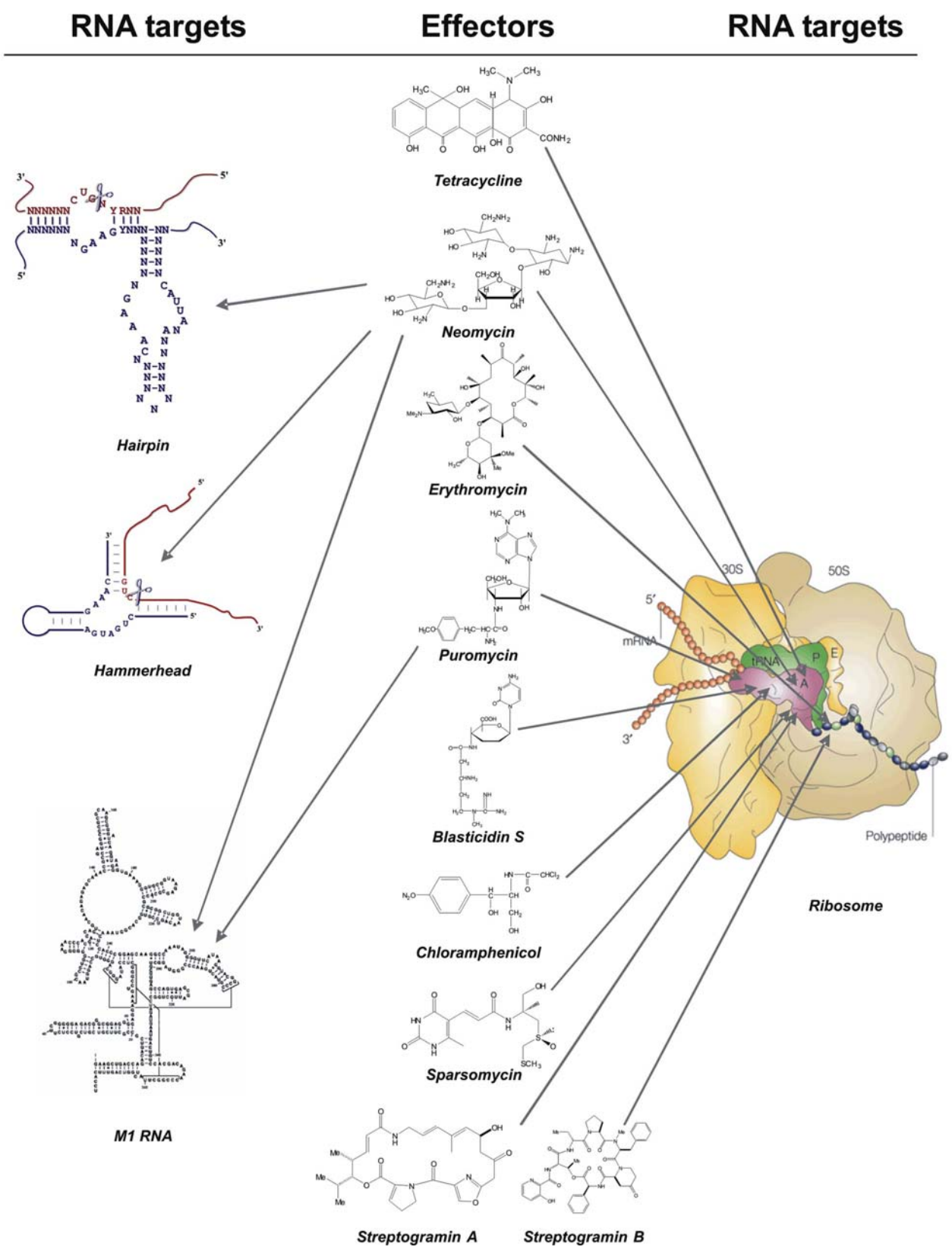

Fig. (1). Characteristic small molecular weight ligands and their respective catalytic RNA targets. Ribosome cartoon was adopted from http://histo.ipfw.edu/ images/ribosome.gif.

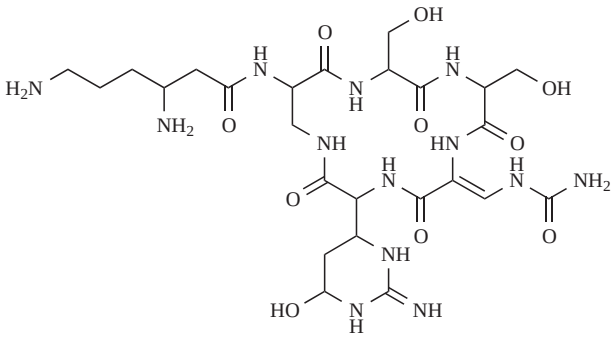

Fig. (2). Structure of viomycin.

when it becomes incorporated into a nascent peptide chain. Blasticidin S, which structurally relates to puromycin and to aminoacyladenylyl terminus of aminoacyl-tRNA, consists of a cytosine bonded to a pyranose ring that has an amino acid-like appendage attached to it, is a partial mimetic of P-site bound tRNA. The cytosine derivative has been identified as binding to 23S rRNA at two non-overlapping sites [39].

Chloramphenicol inhibits several kinds of peptidyl transferase assays. It does not interfere with tRNA fragment binding to the P site of the peptidyl transferase center but competes with the binding of tRNA fragments to the A site and with puromycin. It is suggested that the drug inhibits the peptidyl transferase reaction by disturbing the binding of the 3-CCA end within the catalytic center without weakening tRNA binding. Crystallographic studies showed that chloramphenicol binds either to the hydrophobic crevice to the entrance of the peptide exit tunnel of *Haloarcula marismortui* [39], or to the active site hydrophobic crevice of *Deinococcus radiodurans* ribosomal subunits [40], suggesting that both of these sites may be physiologically relevant. It was suggested that these two different chloramphenicol binding sites observed in the structural studies of the archaeal (*H. marismortui*) and eubacterial (*D. radiodurans*) 50S subunits most likely correspond to the two binding sites on eubacterial 50S subunits that have been inferred previously from binding [41] and kinetic studies [42, 43].

Sparsomycin is a potent ribosome-targeted drug that acts in all cell types. It binds in the core of the peptidyl transferase center by staking to the flexible nucleotide (A2602) of 23S rRNA and induces substantial conformational alterations. Sparsomycin contacts primarily a P-site bound substrate, and extends also into the active site hydrophobic crevice affecting the correct positioning of both A-site and P-site tRNAs [39].

Macrolide antibiotics are an important therapeutic class of ribosome targeted antibiotics. They inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit in domain V of the 23S rRNA, to the pocket located between the hydrophobic crevice and the tunnel constriction, and causing premature dissociation of the peptide during translation [44]. Crystallographic studies of bacterial and archaeal 50S subunits showed that 23S rRNA nucleotides A2058 and A2059 are located accessibly on the surface of the tunnel wall where they act as key contacts sites for macrolide binding [45, 46]. It is characteristic that A to G mutation or post translational methylation of A2058 [47] and as well as 2058G/2059G and 2058C/2059C dual mutations lend resistance to typical macrolides [46]. Chemically modified macrolides such as ketolides (products of an insertion of a ketone bone in the C3 position and the removal of the cladinose sugar) and azalides (semi synthetic derivatives of macrolides), overcome the above modifications by interacting with other regions of the ribosomal tunnel. Crystal structures of the large ribosomal subunit of *D. radiodurans* in complex with the azalide azithromycin [48] (Fig. (**3a**)) (a semi synthetic derivative of erythromycin, with a 15-membered lactone ring progressing an additional nitrogen at position C9,10), a slow-binding ligand of ribosomes [49], and the ketolide ABT-773 [48] (Fig. (**3b**)), indicate that both compounds exert their antimicrobial activity by blocking the protein exit tunnel. In contrast to all macrolides studied so far, two molecules of azithromycin bind simultaneously to the tunnel. The additional molecule also interacts with proteins L4 and L22, which are implicated in macrolide resistance. However, it is not clear whether the second azithromycin-binding site observed in the crystal structure is of biological importance or an artifact of *Deinococcus* complex.

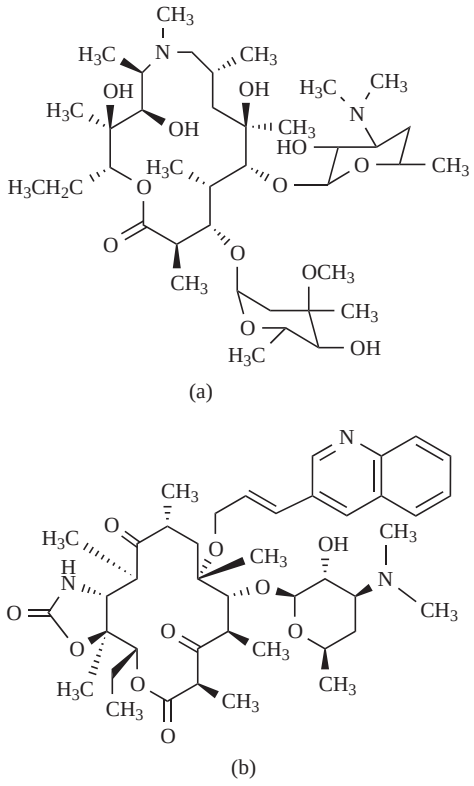

Fig. (3). Structures of **a)** azithromycin, and **b)** ABT-773 (cethromycin).

Streptogramins are produced as a mixture of two chemically unrelated compounds, type A and type B, that act synergistically *in vivo* and *in vitro* [16]. Type A streptogramins, which are unsaturated macrolactones, and type B streptogramins, which are macrocyclic peptides of unusual amino acids, act synergistically on the ribosome and convert weak bacteriostatic effects into lethal bactericidal activity [50].

Crystal structures of *D. radiodurans* 50S subunit complexed with Synecrid®, a 7:3 mixture of streptogramin A and B derivatives, the semi-synthetic dalfopristin and quinupristin respectively, have revealed the binding sites of streptogramins, and indicate that the synergistic effect derives from direct interaction between both compounds and shared contacts with a single nucleotide, A2062. The binding site of quinupristin is located in the common macrolide pocket and interacts with A2058 in the ribosomal exit tunnel. Dalfopristin binds directly within the peptidyl transferase center affecting both A- and P-site occupation by the tRNA molecules. The streptogramin complex with the *Deinococcus* 50S subunit reveals a localized conformational change of the peptidyl transferase center in the orientation of residue U2585, which has been hypothesized as a source of the bactericidal activity and the postantibiotic effect of the Synecrid® combination [51, 52].

EFFECT OF SMALL RNA LIGANDS ON RIBOZYMES AND RNASE P

Aminoglycoside antibiotics have been reported to exhibit a diverse mode of effectiveness on different RNA molecules. When bound to the 16S rRNA they disturb the decoding process and induce misreading of the genetic code by conformational changes to the rRNA. On the other hand, neomycin B and tobramycin (Fig. (**4**)), aminoglycosides that predominantly interact with other ribozymes (M1 RNA, hammerhead, hairpin, HDV, group I intron) (Fig. (**1**)), choose a rather indirect way to inhibit their catalytic activity. They compete with Mg^{2+} ions that are an absolute requirement for ribozyme activity *in vitro*. It has been shown that this inhibitory effect is pH dependent indicating that there is a direct relationship between the available positively charged amino groups and their potentiality to inactivate a ribozyme catalyzed reaction [53-60]. Divalent metal ions have been proven essential components of ribozyme's structural integrity as well as their functional ability *in vitro*. Thus a divalent metal ion displacement mechanism could explain the inhibitory effect on ribozyme's activity. However it is still not clear if this displacement affects ions that are responsible for ribozyme's active structure or affects the appropriate number of divalent metal ions that are required by the catalytic mechanism [58]. In early reports it has been proposed for instance that RNase P RNA could not be inhibited by aminoglycosides since the number of divalent metal ions that are required for enzyme's activity *in vitro* contributes to the absence of inhibition by known cationic inhibitors. However, neomycin proved to be a very strong, although non-competitive, inhibitor among many aminoglycosides tested and this ability was attributed to its high number of amino groups [59]. In addition neomycin indeed displaces magnesium that is crucial for enzyme's activity although it was clear from these reports that this displacement had a more prominent effect when magnesium was deduced from the assay conditions. It has been suggested that neomycin B binds to the P-15 loop of the *E. coli* RNase P RNA subunit (M1 RNA) in such a way that it displaces a Mg^{2+} ion which is probably involved in the chemistry of the cleavage [60]. Recently, the crystal structure of the RNase P RNA subunits from *Thermotoga maritima* and *Bacillus stearothermophilus* [61, 62] has been solved. According to these crystallographic data, Mg^{2+} ion binding sites are predicted in the locality of the P-15 loop of the RNase P RNA. Moreover, it is worth mentioning that the conjugation of several arginine residues to the aminoglycosides, render these molecules more effective inhibitors compared to the parental ones [63], thus suggesting a way to improve the pharmacological potency of RNA binding effectors. Based on the crystallographic data, the structural features important for catalysis and metal ion binding are inferred. However, the modulation mechanisms of the aforementioned small molecules will be elucidated by the resolution of the crystal structure of RNase P in complex with these compounds.

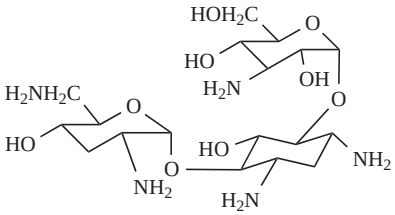

Fig. (4). Structure of tobramycin.

Detailed analysis of all the above mentioned reports reveals that the inhibitory mechanism that affects ribosome is different than the one that affects other catalytic RNAs. In brief, these differences are located in the different aminoglycoside binding sites that seem to exist. It has been recently proposed that there are two types of aminoglycoside binding sites: the first consists of asymmetric internal loops as in the decoding centre of the ribosome and in many artificial RNA molecules that were selected *in vitro*. The second type of binding site is located at the central metal-ion binding cores of ribozymes. In the first case the inhibitory effect comes from a slight distortion of RNA structure while in the second case the mainly responsible inhibitory event is the displacement of essential divalent metal ions [4].

Apart from aminoglycosides, there are several other small ligands like, puromycin, amicetin, blasticidin S, porphyrins, porphynes, retinoids, arotinoids, calcipotriol and anthralin that modulate RNase P activity. Puromycin, a mimic of the 3' terminal end of the aminoacyl-tRNA, was the first inhibitor of RNase P activity reported [64] (Fig. (**1**)). The sensitivity to this antibiotic, which is considered to be a common feature among various RNase P enzymes, has been used as a tool to determine [65-67] and further investigate [68, 69] the enzyme's reaction from diverse sources. Puromycin, as well as amicetin (Fig. (**5**)) and blasticidin S (which belong in the same group of inhibitors) inhibit tRNA maturation by eukaryal RNase P in a dose-dependent manner. These inhibitory effects were independent of a pre-incubation step, which implies that these compounds do not cause conformational changes to the enzyme structure and therefore do not act as slow binding inhibitors, as in the ribosome complex. Detailed kinetic analysis established their modes of inhibition as simple competitive in the case of puromycin, and as non-competitive for amicetin and blasticidin S, therefore giving evidence for two distinct inhibitory sites in the RNase P holoenzyme. Among the three molecules, amicetin is the

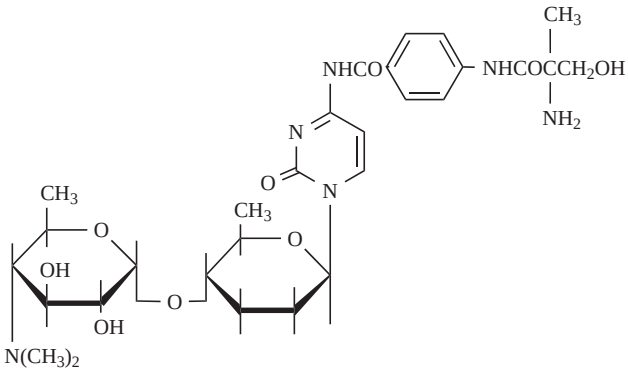

Fig. (5). Structure of amicetin.

stronger inhibitor as judged by its lower K_i value. Moreover, it was shown that simultaneous presence of amicetin and blasticidin S in the reaction mixture had no additive inhibitory effect, suggesting that these compounds compete for binding on a common site. The localization of these two inhibitory sites on the RNase P ribonucleoprotein complex is still unknown, but it seems logical to assume that these ribosomal RNA binding antibiotics also bind to the RNA component of the RNase P holoenzyme.

Inhibition of bacterial RNase P activity by porphyrins and porphines is a complex phenomenon, as these molecules bind strongly to the M1 RNA as well as the pre-tRNA substrate and tRNA product of RNase P reaction [70]. The mechanism of inhibition seems independent of the net charge of these molecules, and it is possible that no crucial Mg^{2+} displacement takes place either, as it is the case with aminoglycoside antibiotics. Porphyrins and porphines bind to tRNA structural elements that are required for substrate recognition by RNase P, such as the T-loop, and therefore, it is possible that stereochemical hindrance of enzyme-substrate interaction is responsible for their inhibitory effect.

Retinoids, belong to a group of natural and synthetic analogues of vitamin A, and are implicated in cell proliferation, differentiation, and pattern formation during development. They represent the drugs of choice for various skin disorders [71, 72], and have been used as chemopreventive and chemotherapeutic agents in cancers of skin and other organs [73, 74]. It is well known that retinoids mediate their regulatory functions through binding to respective nuclear receptors, which act as transcription factors [75]. Arotinoids comprise the promising, third generation of retinoids, which exhibits less toxicity combined with increased potency, and have found use in dermatology and other fields of clinical medicine [76, 77]. It has been shown that retinoids and arotinoids can also directly inhibit RNase P catalysis *in vitro* by a mechanism that does not involve retinoid nuclear receptors [78-80]. The type of inhibition in all cases studied is simple competitive, and among the compounds assayed, acitretin from retinoids (Fig. (**6a**)) and Ro 13-7410 from arotinoids (Fig. (**6b**)) exhibited the lowest K_i values. The interpretation of the experimental results suggest that these compounds bind to allosteric sites on the RNase P holoenzyme by hydrophobic interactions, and possibly form complexes with a molar ratio of 1:1.

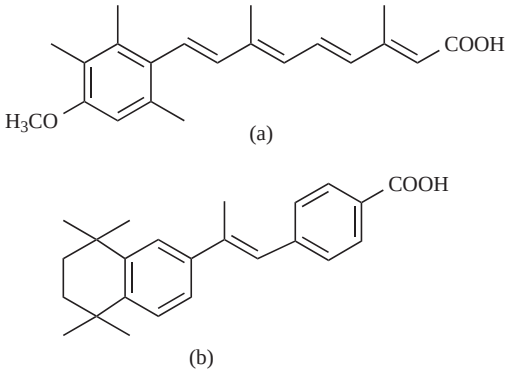

Fig. (6). Structures of **a)** acitretin, and **b)** Ro-7410.

Calcipotriol, a vitamin D_3 analogue (Fig. (**7a**)), is widely used for the treatment of psoriasis and other keratinization disorders. It binds to vitamin D_3 receptors in target cells increasing differentiation and inhibiting proliferation of keratinocytes by transcriptional regulation [81, 82]. Calcipotriol exhibits a bimodal, dose-dependent action on RNase P activity *in vitro*: up to a concentration of 50 μM it has an activating effect, but further increase of its concentration results in enzyme inhibition that could be due to multisite (activating/inhibitory) interaction [83].

Anthralin is a chrysarobine derivative (Fig. (**7b**)), and has been long used in the effective treatment of chronic plaque psoriasis [84]. This drug inhibits key enzymes in various metabolic pathways, which is possibly due to its redox activity [85, 86]. Anthralin can also inhibit tRNA maturation by RNase P in a dose-dependent manner [87]. When anthralin and calcipotriol are combined *in vitro*, their inhibitory effects on RNase P activity are additive, indicating that these inhibitors are not mutually exclusive [88]. It should be noted here, that it is unknown whether calcipotriol, anthralin, retinoids and arotinoids target the RNA, the protein subunits or even an unidentified component (a fatty acid or lipid) of eukaryal RNase P ribonucleoprotein complex.

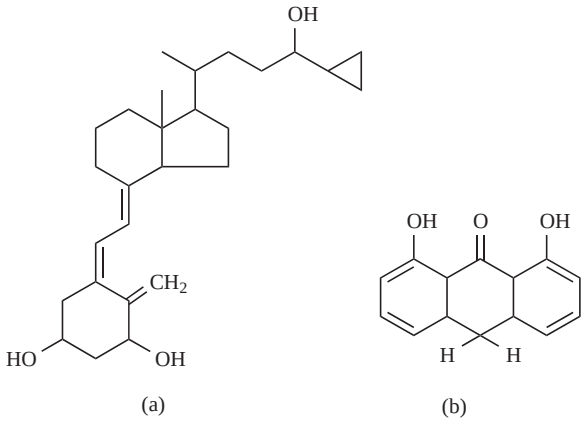

Fig. (7). Structures of **a)** calcipotriol, and **b)** anthralin.

Almost 15 years ago it was suggested that antibiotics, like other secondary metabolites, should be seen as effector molecules in the regulation of many biological activities and surely not only as inhibitors but rather as modulators. A very

good example supporting this notion was known for many years (since 1965). Streptomycin-dependent ribosomes that show altered translation accuracy leading to hyperaccurate ribosomes can confer bacterial resistance and sometimes dependence on streptomycin [89]. From this point of view the antibiotic is a positive effector molecule, because it is absolutely required for viability. In good agreement to this observation it has been reported that aminoglycoside antibiotics instead of inhibiting they can actually promote the cleavage reaction that is catalyzed by the hairpin ribozyme. This ribozyme does not depend on the presence of divalent metal ions for catalysis, but only for proper folding. It has been shown that especially neomycin B can stimulate the activity of this specific ribozyme, by playing the role of the structurally required positively charged environment that normally the magnesium provides. Neomycin B in the appropriate pH environment carries all the necessary positively charged groups that are important for ribozyme's proper folding and activity. This observation is in contrast to the action of the same antibiotic as it was described above for the cases of ribosomal decoding site and other small ribozymes. Furthermore the antibiotic viomycin enhances the self-cleavage of the *Neurospora crassa VS* ribozyme and at the same time decreases the amount of magnesium that is required for activity. It also stimulates the trans-cleavage reaction by facilitating the interaction between negatively charged RNA molecules [90].

Antibiotics can bind numerous RNA targets with similar, albeit modest affinity. Additionally, multiple binding sites on RNA molecules are likely to coexist. These selectivity issues are particularly important where future therapeutic applications of RNA ligands are concerned. High concentrations of competing RNA targets might scavenge potential RNA binders thus altering their therapeutic properties and causing side effects.

All the above-mentioned results lead to the assumption that rather small molecules such as an aminoglycoside antibiotic are able to modulate the catalytic efficiency of ribozymes and in many cases to increase their catalytic potential by stimulating their catalytic assembly forms and thus increasing the number of possible reaction partners. Small molecular weight effectors with such properties might have played an important role in the transition from an RNA world to an RNA-protein world [91].

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