Molecular Biology Reports **30:** 9–14, 2003.

Kinetics of inhibition of ribonuclease P activity by peptidyltransferase inhibitors

Effect of antibiotics on RNase P

Dimitra Kalavrizioti, Anastassios Vourekas, Apostolos Tekos, Antigoni Tsagla, Constantinos Stathopoulos* & Denis Drainas**

*Department of Biochemistry, School of Medicine, University of Patras, 26500 Patras, Greece; *Present address: Department of Biochemistry and Biotechnology, University of Thessaly, 26 Ploutonos st. 412 21 Larissa, Greece; **Author for correspondence (Phone: +30-610-997746; Fax: +30-610-997690; E-mail: Drainas@med.upatras.gr)*

Accepted 3 October 2002

Key words: protein synthesis inhibitors, ribozymes, RNase P

Abstract

A cell-free system derived from *Dictyostelium discoideum* has been used to study the kinetics of inhibition of RNase P by puromycin, amicetin and blasticidin S. Detailed kinetic analysis showed that the type of inhibition of RNase P activity by puromycin is simple competitive, whereas the type of inhibition by amicetin and blasticidin S is simple non-competitive. On the basis of K_i values amicetin is stronger inhibitor than puromycin and blasticidin S.

Introduction

A dramatic appearance of multi-drug resistant pathogens during the recent years severely threatens human health care systems world-wide. In order to prevent the devastating spreading of infectious diseases, novel antibiotic drugs have to be developed. To be successful towards this goal, the selection of effective molecular targets is of outmost importance. The enzyme RNase P seems to be a promising target. This ubiquitous and essential endonuclease is a Mg^{2+}-dependent key enzyme in tRNA biogenesis that generates the mature 5′ termini of tRNA precursors. Although all known RNase P enzymes (with the exception of RNase P from spinach chloroplasts) [1] have the common feature of being composed of RNA and protein, the details of their architecture are highly diverse. In bacteria, the catalytic function of this enzyme resides entirely in its RNA subunit [2]. Catalytic activity of RNA subunits, has never been demonstrated so far for eukaryotic RNase P enzymes. However, comparative sequence analysis has established that these RNAs share high homology to bacterial RNA, suggesting a common ancestry [3]. Recently, we reported that after extensive deproteinization of *Dictyostelium discoideum* RNase P a new catalytic activity was revealed. The proteinase K/phenol/SDS treated enzyme cleaves tRNA precursors several nucleotides upstream of the normal cleavage site of RNase P, liberating products with 5′ hydroxyl ends [4]. Eukaryotic RNase P enzymes are composed of multiple protein components, which contribute about 70% to the enzyme's molar mass [5]. This high protein/RNA ratio is a common feature of all eukaryotic nuclear RNase P enzymes characterized to date [1], including nuclear *D. discoideum* RNase P [6]. In addition, human orthologs of the yeast RNase P protein subunits have been found [7]. These findings indicate that the structures of RNase P enzymes from different eukaryotes are similar. Thus, *D. discoideum* RNase P is a suitable model system for other eukaryotic RNase P enzymes, such as the human nuclear RNase P, and could become a promising system for the identification and development of novel inhibitors. Recently, we reported that natural and synthetic retinoids [8, 9], aminoclycosides [10], calcipotriol and anthralin, separate [11, 12] or in combination [13] inhibit RNase P activity from the slime mold *D. discoideum*.

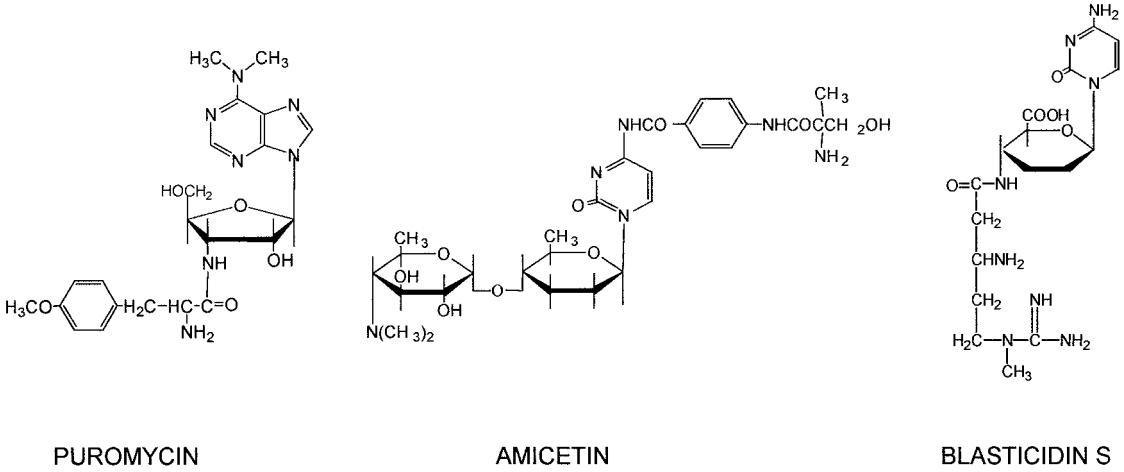

Figure 1. Structures of amicetin, puromycin and blasticidin S.

Natural antibiotics are products of the secondary metabolism of microorganisms and they possess the ability to kill bacteria and other microorganisms or to inhibit their growth. Many antibiotics in use interact with ribosomal RNA [14]. Recently, it was found that several of them interfere with other functional RNAs, such as the RNA component of *Escherichia coli* RNase P [15, 16], the self splicing group I introns, the hammerhead ribozyme, the human hepatitis delta virus ribozyme and HIV RNA [14, 17], although it was believed that they act specifically on bacterial ribosomes. Thus it seems likely that some of the antibiotics in use, in addition to their inhibitory effect on ribosome function, exert their biological effect by acting on RNase P.

Very little is known world-wide on the effect of protein synthesis inhibitors on RNase P activity. Puromycin, a peptidyltransferase inhibitor of prokaryotic and eukaryotic systems, has been shown to inhibit RNase P holoenzyme activity from mouse [18], *Tetrahymena thermophila* [19], human mitochondria [20], as well as catalytic activity of the RNA subunit of RNase P from *E. coli* [15]. Preliminary studies in our laboratory have shown that puromycin, amicetin and blasticidin S, exhibit a dose-dependent inhibition effect on RNase P activity from *D. discoideum* [21]. Recently, it has been reported that aminoglycoside antibiotics, known to disturb the decoding process, inhibit *E. coli* RNase P holoenzyme and M1 RNA [16] and as well as the *D. discoideum* RNase P [10]. Furthermore, aminoglycoside-arginine conjugates proved to be 500-fold more effective inhibitors of *E. coli* RNase P holoenzyme than aminoglycosides alone [22]. Also it is important to note that recently a new class of inhibitors of RNase P ribozyme has been reported, which effect their inhibition by binding to the substrate of the enzyme reaction [23].

In the present study we examined in depth the kinetics of inhibition of *D. discoideum* RNase P by puromycin, blasticidin S and amicetin. Detailed kinetic analysis showed that puromycin behaves as classical competitive inhibitor, whereas blasticidin S and amicetin behave as classical non-competitive inhibitors.

Materials and methods

Growth of *D. discoideum* cells (strain AX2 wild type), cell breakage, S-100 fraction preparation, and purification of *D. discoideum* nuclear RNase P were carried out as previously described [6, 24].

Enzyme assays were carried out at 37 °C in 20 μl buffer D (50 mM Tris/HCl pH 7.6, 10 mM (NH_4Cl), 5 mM $MgCl_2$ and 5 mM dithiothreitol) containing 2–5 fmol tRNA substrate [an *in vitro* labeled transcript of the *S. pombe* $tRNA^{ser}$ gene *SupSI*] and 1.3 μg protein from the RNase P fraction. The reactions were stopped by addition of 5 μl stop dye (80% formamide, 50 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). Reaction products were resolved on a denaturing 10% polyacrylamide/8M urea gel and visualized by autoradiography without drying. Activity was quantified by Cerenkov counting of excised gel slices.

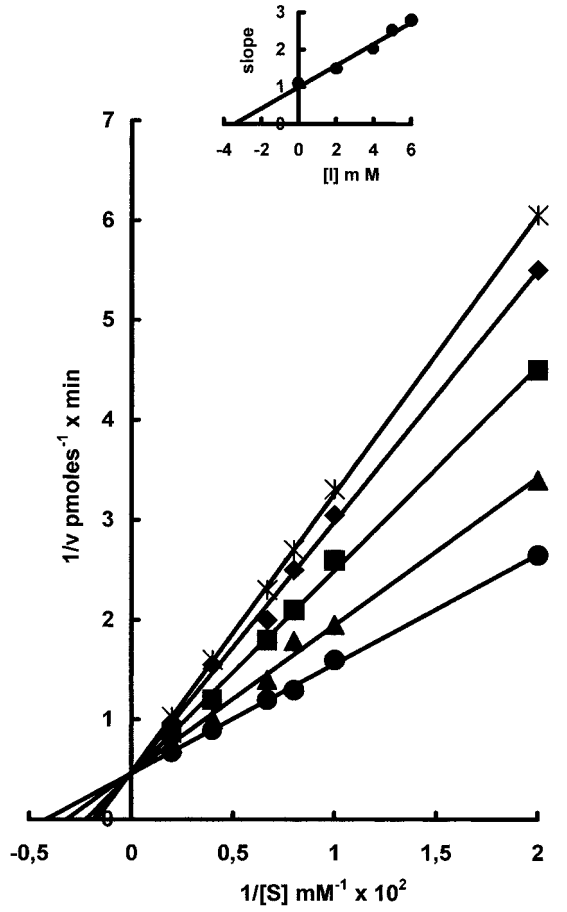

Figure 2. Double reciprocal plot (1/v *versus* 1/[pre-tRNA]) for RNase P reaction in the presence of puromycin. The reaction was carried out at each one of the indicated concentrations in the presence or in the absence of inhibitor. All reactions were carried out at 37 °C in 20 μl of buffer D. ●, without inhibitor; ▲, with puromycin at 2 mM; ■, with puromycin at 4 mM; ♦, with puromycin at 5 mM; ∗, with puromycin at 6 mM. Top panel: replot of the slopes of the double reciprocal lines *versus* inhibitor (I) concentrations.

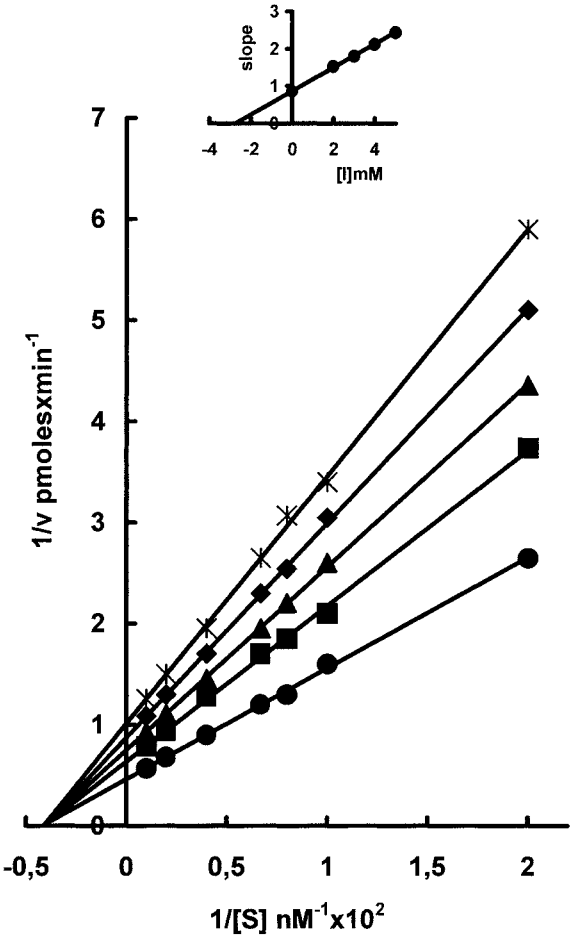

Figure 3. Double reciprocal plot (1/v *versus* 1/[pre-tRNA]) for RNase P reaction in the presence of amicetin. The reaction was carried out at each one of the indicated concentrations in the presence or in the absence of inhibitor. All reactions were carried out at 37 °C in 20 μl of buffer D. ●, without inhibitor; ■, with amicetin at 2 mM; ▲, with amicetin at 3 mM; ♦, with amicetin at 4 mM; ∗, with amicetin at 5 mM. Top panel: replot of the slopes of the double reciprocal lines *versus* inhibitor (I) concentrations.

Results

In a previous study, we have shown that the nucleoside-analog antibiotics puromycin, blasticidin S and amicetin, which specifically inhibit ribosomal peptidyltransferase in both prokaryotic and eukaryotic systems [25], exhibit a dose-dependent inhibition effect on RNase P activity from *D. discoideum*. The potency of these inhibitors calculated on the basis of IC_{50} values is approximately equal, with amicetin being slightly stronger and blasticidin S a slightly weaker effector than puromycin [21]. Our present study was designed to reveal the mechanism of RNase P inhibition by these antibiotics (Figure 1), in a cell free system derived from *D. discoideum*. The type of inhibition of *D. discoideum* RNase P activity by puromycin, amicetin and blasticidin S was elucidated by detailed kinetic analysis. The substrate for RNase P assays was an *in vitro* ^{32}P labeled transcript of the *S. pombe* tRNA gene *SupS1*. Enzyme assays were carried out in 37 °C in 20 μl buffer D. The initial velocity in the presence or absence of inhibitors was determined from the initial slopes of time plots (not shown). Figure 2 shows double reciprocal plots with increasing concentrations of puromycin. The lowest line in Figure 2 represents the data obtained in the absence of inhibitor (control). The slopes of the lines

in Figure 2 were replotted against the concentration of puromycin, and the results are shown in the top panel of Figure 2. The linearity of this plot is indicative of simple competitive inhibition and leads to the graphical determination of $K_i = 3.46$ mM from the negative intercept of the line with the [I] axis. The same kinetic analysis was carried out for amicetin (Figure 3) and blasticidin S (Figure 4). In contrast with puromycin, these compounds showed simple non-competitive inhibition. The slopes of the lines in Figures 3 and 4 were replotted against the concentration of amicetin or blasticidin S, and the results are shown in the top panel of Figures 3 and 4. The linearity of this plots is indicative of simple non-competitive inhibition and leads to the graphical determination of $K_i = 2.75$ mM for amicetin and $K_i = 7.4$ mM for blasticidin S from the negative intercept of the line with the I-axis. Further evidence for simple competitive kinetics for puromycin or simple non-competitive kinetics for amicetin or blasticidin S comes from the Dixon plots (Figure 5). It is important to note that preincubation of the antibiotics with the enzyme prior the addition of the substrate did not alter the inhibitory effect of the antibiotics on RNase P activity.

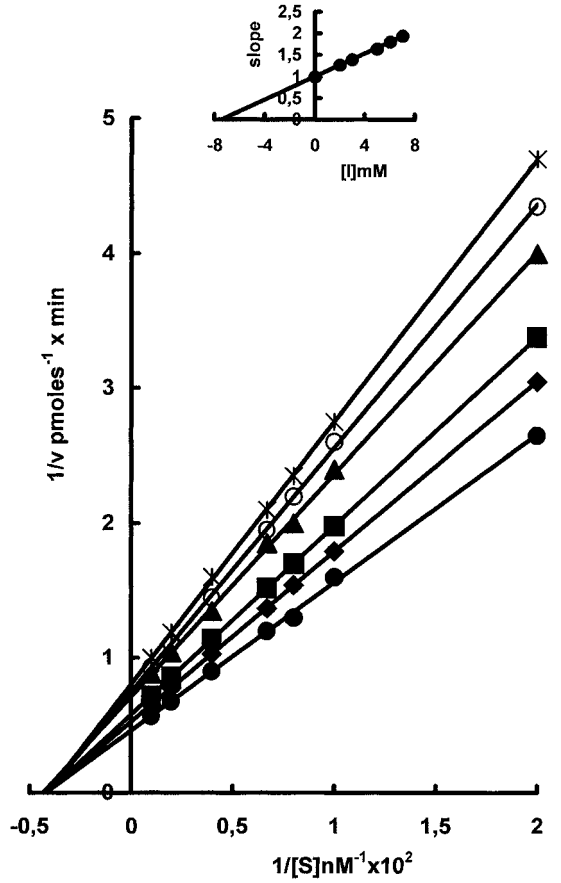

Figure 4. Double reciprocal plot (1/v *versus* 1/[pre-tRNA]) for RNase P reaction in the presence of blasticidin S. The reaction was carried out at each one of the indicated concentrations in the presence or in the absence of inhibitor. All reactions were carried out at 37 °C in 20 μl of buffer D. ●, without inhibitor; ♦, with blasticidin S at 2 mM; ■, with blasticidin S at 3 mM; ▲, with blasticidin S at 5 mM; ○, with blasticidin S at 6 mM; ∗, with blasticidin S at 7 mM. Top panel: replot of the slopes of the double reciprocal lines *versus* inhibitor (I) concentrations.

Discussion

Puromycin inhibits protein synthesis by causing nascent polypeptide chains to be released before their synthesis is completed. It resembles the aminoacyl terminus of an aminoacyl-tRNA. Its amino group joins the carbonyl group of the growing polypeptide to form a product that dissociates from the ribosome. Puromycin reaction has been used as a model reaction for investigating ribosomal peptidyltransferase [26]. This reaction is inhibited by amicetin and blasticidin S [27, 28], whose structural relationship to puromycin is apparent (Figure 1). The kinetic analysis of the inhibition of puromycin reaction by amicetin or blasticidin S revealed two different types of inhibition; a competitive inhibition when the ribosome complex (AcPhe-tRNA - poly(U) - ribosome) is not preincubated with the inhibitor, and a mixed non-competitive inhibition when the ribosome complex is preincubated with the inhibitor prior the addition of the substrate [27, 28], supporting that the ribosome can be subjected to modulation of its activity by certain ligands. In our system, preincubation of the antibiotics with RNase P prior the addition of precursor tRNA, did not alter the effect of the antibiotics on RNase P activity, which means that they do not cause conformational changes to RNase P, as they do to ribosome complex. On the other hand, amicetin and blasticidin S, in spite their resemblance to puromycin, behave as classical non-competitive inhibitors, whereas puromycin behave as classical competitive inhibitor. This is evidence for two distinct inhibitory sites on the RNase P holoenzyme.

Several data show that the amino acid acceptor-stem and the T-stem of precursor tRNA are important determinants in the selection of the cleavage site [29]. A model substrate, consisting only of a stem and loop structure derived from the acceptor stem, T-stem, and T-loop is a good substrate for RNase P from *E. coli in*

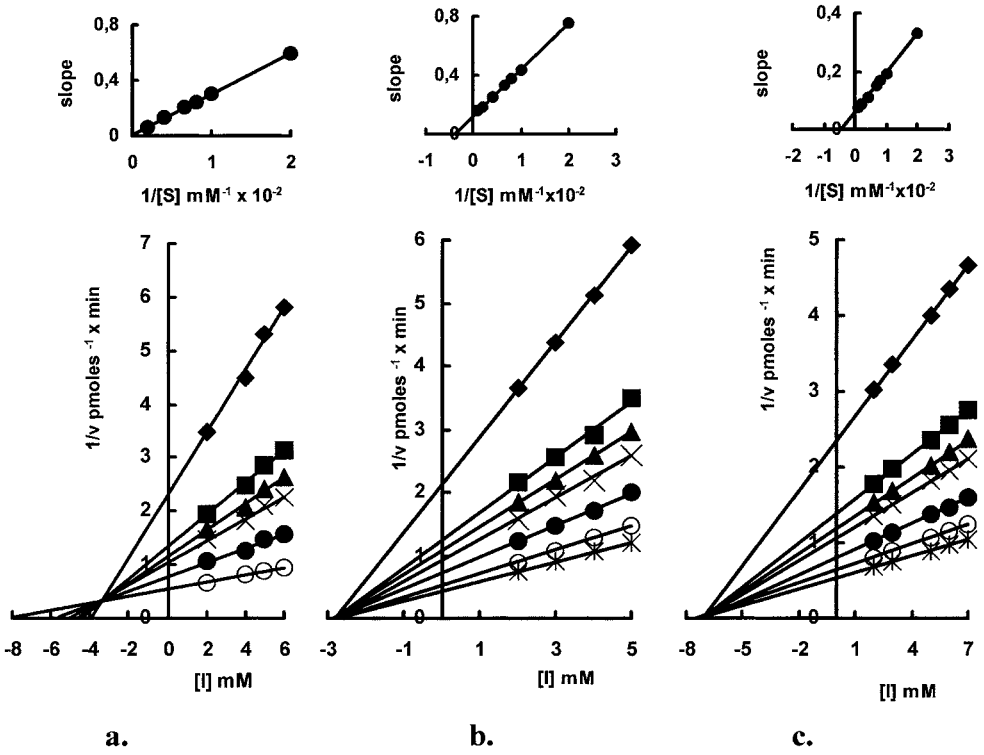

Figure 5. Dixon plot for the RNase P reaction in the presence of puromycin (a), amicetin (b) and blasticidin S (c). The concentrations of pre-tRNA were: 50 nM (♦), 100 nM (■), 125 nM (▲), 150 nM (×) and 250 nM (●), 500 nM (◯), 1000 nM (∗). Top panels: Replots of the slopes of the Dixon lines *versus* 1/[pre-tRNA].

vitro [30]. Since puromycin possesses structural similarity to aminoacyl-adenylyl 3′ terminus of aminoacyl-tRNA [25], the competitive character of puromycin inhibition support the notion that puromycin interferes with tRNA binding on *D. discoideum* RNase P. In a previous study we have shown that the simultaneous presence of puromycin and blasticidin S or amicetin causes stronger inhibition than in the presence of each antibiotic alone at the same concentration, indicating that the inhibition mode is synergetic due to the presence of two not mutually exclusive binding sites [21]. Furthermore, the inhibition caused by the simultaneous presence of amicetin and blasticidin S suggested that these two antibiotics have overlapping mutually exclusive binding sites on *D. discoideum* RNase P [21]. The detailed kinetic analysis carried out in the present study has confirmed this initial observation. The competitive character of puromycin inhibition and the non-competitive character of amicetin and blasticidin S inhibition of *D. discoideum* RNase P activity clearly show that there are two not mutually exclusive inhibitory sites on the enzyme. On the basis of K_i values, amicetin is a stronger inhibitor than blasticidin S on RNase P activity, in contrast to what has been previously reported for peptidyltransferase reaction [27, 28]. This could be explained by the fact that peptidyltransferase catalyzes a different reaction. Furthermore, amicetin has lower K_i value than that of puromycin, meaning that amicetin behaves as a stronger inhibitor. The non-competitive character of amicetin, compared to puromycin which behaves as a competitive inhibitor, enhances its inhibitory potency. It should be noticed that a non-competitive inhibitor behaves as a stronger effector than a competitive one even if the two inhibitors have the same K_i value. Taking into account that amicetin and blasticidin S are known to bind ribosomal RNA [31] their effect on RNase P activity may also be due to interaction with the RNA component of *D. discoideum* RNase P.

The effect of puromycin, blasticidin S and amicetin on RNase P activity indicates that these compounds, in addition to their inhibitory effect on peptidyltransferase activity, exert a direct effect on tRNA biogenesis. RNase P is an enzyme essential for cell viability and presumably co-evolved with the ribosome. The fact that they share common structural

characteristics (both RNA-protein complexes) and the fact that they share tRNA molecules as substrates, make RNase P a more simple and promising molecular target for *in vitro* studies on antibiotic inhibition, which may provide a much more differentiated picture of the molecular basis of antibiotics action. *D. discoideum* represents a lower eukaryote that engulfs an RNase P holoenzyme with unique characteristics. The RNA subunit of the enzyme seems to have catalytic properties [4] although the holoenzyme shares general eukaryotic properties [low buyonant density [6], multi-protein subunits, unpublished data]. Although few reports on antibiotic-RNase P interactions have been focused on the bacterial catalytic RNA subunit, our recent reports on this particular eukaryotic holoenzyme give a more differentiated picture [10, 21]. Also, it is a good model system for comparison with other eukaryotic RNase P enzymes, such as the human nuclear RNase P, and could become a promising system to evaluate toxic side effects of antimicrobial inhibitors. Finally, it provides a new target for the identification and development of novel inhibitors.

Acknowledgements

We are very grateful to Prof. Dimitrios Kalpaxis for critical reading of the manuscript. This work was supported in part by the Research Committee of Patras University, program 'K. Karatheodoris'.

References

1. Frank DN & Pace NR (1998) Annu. Rev. Biochem. 67: 153–180.
2. Guerrier-Takada C, Gardiner K, Marsh T, Pace N & Altman S (1983) Cell 35: 849–857.
3. Chen J-L & Pace NR (1997) RNA 3: 557–560.
4. Stathopoulos C, Tekos A, Zarkadis IK & Drainas D (2001) Eur. J. Biochem. 268: 2134–2140.
5. Chamberlain JR, Lee Y, Lane WS & Engelke D (1998) Genes Dev. 12: 1678–1690.
6. Stathopoulos C, Kalpaxis DL & Drainas D (1995) Eur. J. Biochem. 228: 976–980.
7. Eder PS, Kekuda R, Stolc V & Altman S (1997) Pro. Natl. Acad. Sci USA 94: 1101–1106.
8. Papadimou E, Georgiou S, Tsambaos D & Drainas D (1998) J. Biol. Chem. 273: 24375–24378.
9. Papadimou E, Monastirli A, Tsambaos D, Merk HF & Drainas D (2000) Skin Pharmacol. Appl. Skin Physiol. 13: 345–51.
10. Tekos A, Tsagla A, Stathopoulos C & Drainas D (2000) FEBS Lett. 485: 71–75.
11. Papadimou E, Monastirli A, Stathopoulos C, Tsambaos D & Drainas D (2000) Eur. J. Biochem. 267: 1173–1177.
12. Drainas D, Papadimou E, Monastirli A, Tsambaos D & Merk HF (2000) Skin Pharmacol. Appl. Skin Physiol. 13: 128–132.
13. Papadimou E, Monastirli A, Tsambaos D & Drainas D (2000) Biochem. Pharmacol. 60: 91–94.
14. Wallis MG & Schroeder R (1997) Prog. Biophys. Molec. Biol. 67: 141–154.
15. Vioque A (1989) FEBS Lett. 246: 137–139.
16. Mikkelsen NE, Mathias B, Virtanan A & Kirsebom LA (1999) Proc. Natl. Acad. Sci. USA 96: 6155–6160.
17. Walter F, Vicens Q & Westhof E (1999) Curr. Opin. Chem. Biol. 3: 694–704.
18. Potuschak T, Rossmanith W & Karwan R (1993) Nucl. Acids Res. 21: 3239–3243.
19. True HL & Celander DW (1996) J. Biol. Chem. 28: 16559–16566.
20. Rossmanith W & Karwan R (1998) Biol. Bioph. Res. Com. 247: 234–241.
21. Stathopoulos C, Tsagla A, Tekos A & Drainas D (2000) Mol. Biol. Rep. 27: 107–111.
22. Eubank TD, Biswas R, Jovanovic M, Litovchick A, Lapidot A & Gopalan V (2002) FEBS Lett. 511: 107–112.
23. Hori Y, Bichenkova EV, Wilton AN, El–Attug MN, Sabat-Ebrahimi S, Tanaka T, Kikuchi Y, Araki M, Sugiura Y & Douglas KT (2001) Biochemistry 40: 603–608.
24. Tekos A, Stathopoulos C & Drainas D (1998) Biochemistry 37: 15474–15480.
25. Vázquez D (1979) Inhibitors of protein biosynthesis. Springer-Verlag, Heidelberg New York.
26. Monro RE & Marcker KA (1967) J. Biol. Chem. 25: 347–350.
27. Theocharis DA, Kalpaxis DL & Coutsogeorgopoulos C (1986) Eur. J. Biochem. 159: 479–483.
28. Kalpaxis DL, Theocharis DA & Coutsogeorgopoulos C (1986) Eur. J. Biochem. 154: 267–271.
29. Altman S, Kirsebom L & Talbot S. (1993) FASEB J. 7: 7–14.
30. McClain WH, Guerrier-Takada C & Altman S (1987) Science 238: 527–530.
31. Spahn CMT & Prescot CD (1996) J. Mol. Med. 74: 423–439.