FEBS Letters 585 (2011) 3270–3276

journal homepage: www.FEBSLetters.org

Complex formation of rutin and quercetin with copper alters the mode of inhibition of Ribonuclease A

Debi Ranjan Tripathy, Atanu Singha Roy, Swagata Dasgupta *

Department of Chemistry, Indian Institute of Technology, Kharagpur 721 302, India

ARTICLE INFO

Article history:
Received 19 July 2011
Revised 19 August 2011
Accepted 4 September 2011
Available online 9 September 2011

Edited by Miguel De la Rosa

Keywords:
Ribonuclease A
Quercetin
Rutin
Copper complexes
Inhibition

ABSTRACT

Rutin and quercetin, both minor components of green tea and their Cu(II) complexes interact with Ribonuclease A (RNase A) in a novel way. The effects of rutin, quercetin and their copper complexes on the catalytic activity of the protein were investigated. Rutin shows an *enhancement* in the ribonucleolytic activity whereas the copper complexes and quercetin behave as non-competitive type inhibitors with K_i values in the μM range. The secondary structural changes of RNase A in presence of the ligands were measured by circular dichroism and Fourier transform infrared spectroscopy. The binding parameters were obtained using a fluorescence quenching analysis.

1. Introduction

There exists a crucial balance between RNA polymerases that synthesize RNA and RNA depolymerases, also known as ribonucleases (RNase) that degrade RNA [1]. The central thesis of DNA to RNA to protein is largely dependent on the action of these proteins. RNases can be cytotoxic at higher levels by entering the cytosol and retaining their ribonucleolytic activity and degrading cellular RNA, and in turn inhibiting protein synthesis that subsequently causes cell death [2]. Studies have shown that several pancreatic RNase A homologues, including angiogenin, eosinophil-derived neurotoxin, and bovine seminal RNase A, utilize their enzymatic activities to bring forth potent physiological effects [3–5]. This has led to widespread interest and increase in the development of synthetic or natural ribonuclease inhibitors [4]. Out of the four His residues in the protein His 12, 48, 105 and 119, His 12 and His 119 are present in the P1 subsite of the active site of the enzyme and are mainly engaged in the phosphodiester hydrolysis mechanism [6]. Of the homologues known, angiogenin, a potent blood vessel inducer, has a structural similarity of ~60% with RNase A [7]. The inhibition of angiogenin has shown suppression of growth and metastasis of solid tumors [4,8,9]. It has been demonstrated that ribonuclease activity increased in cancer cells, and in the serum and urine of the affected to a level much higher than the control. This was observed in humans as well as animals. Interestingly the activity of the ribonuclease inhibitor was found to decrease in cancer tissues [9]. Thus, the development of inhibitors has become essential.

A recent investigation has shown that natural polyphenols from red wine and green tea [10] prevent angiogenesis (new blood vessel formation) [11]. Rutin is the glycoside between the flavonol quercetin and the disaccharide rutinose. The two components rutin and the aglycone rutin (quercetin) (Fig. 1) are present in fruits, red wine, tea (black, green), grape, orange, wheat, onion and soybean. Both quercetin and rutin are known to exhibit antioxidant [11], anti-inflammatory [12] and anticancer [13] activities.

In the human diet a trace amount of copper is present which acts as a cofactor for angiogenesis [14]. Specific drugs are oftentimes used to decrease the concentration level of copper in blood via chelation. Penicillamine [15], captopril [16] and ammonium tetrathiomolybdate [17] are known chelators of copper that have been used for antiangiogenic therapy, though side effects have also been observed [18]. Thus, copper complexation by naturally occurring compounds from dietary constituents that have antiangiogenic activity can be exploited as potentially better antiangiogenic compounds [19].

We have investigated the effect of quercetin and rutin and their Cu(II) complexes [20] on the ribonucleolytic activity of RNase A. As

Abbreviations: RNase A, Ribonuclease A; Qu, Quercetin; Rut, Rutin; QuCu, quercetin-Cu(II) complex; RutCu, rutin-Cu(II) complex; 2′,3′-cCMP, cytidine 2′,3′ cyclic monophosphate; t-RNA, transfer ribonucleic acid; CD, circular dichroism; FTIR, Fourier transform infrared; ^{1}H NMR, proton nuclear magnetic resonance

* Corresponding author. Fax: +91 3222 255303.
E-mail addresses: swagata@iitkgp.ac.in, swagata_dasgupta@hotmail.com (S. Dasgupta).

0014-5793/$36.00
doi:10.1016/j.febslet.2011.09.005

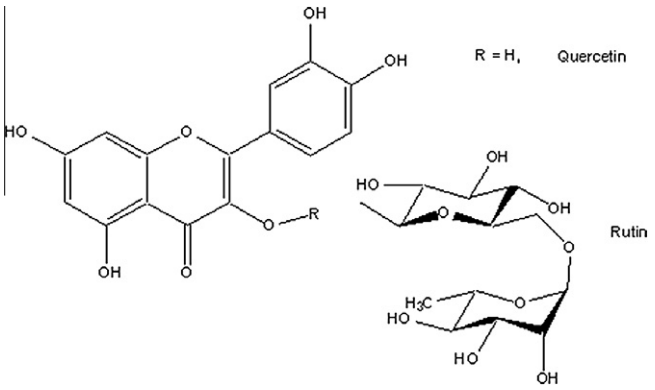


Fig. 1. The chemical structures of rutin and quercetin.

polyphenols exhibit inhibitory effects against a multitude of enzymes [21,22], agarose gel followed by a precipitation assay and kinetic experiments were performed. ^{1}H NMR experiments were performed to understand the mode of binding with the compounds. Secondary structural changes due to complexation were also studied by CD and FTIR spectroscopy. To determine the binding constant of the compounds with RNase A and number of binding sites per protein, fluorescence quenching studies were performed.

2. Materials and methods

2.1. Materials

RNase A, yeast (t-RNA), cytidine 2′,3′-cyclic monophosphate (2′,3′-cCMP), quercetin and rutin were from Sigma–Aldrich (USA) and other reagents from SRL India. A Perkin–Elmer UV–vis spectrophotometer (Lambda 25) and a Horiba Jobin Yvon Spectrofluorometer (Fluoromax-4) were used for the spectral measurements. Concentrations were determined using the following data: RNase A $\varepsilon_{278.5} = 9800\ M^{-1}\ cm^{-1}$ [23]; 2′,3′-cCMP $\varepsilon_{268} = 8500\ M^{-1}\ cm^{-1}$ [24] in water.

2.2. Determination of metal polyphenol stoichometry

The preparation and characterization procedure including the geometry optimized structure of the complexes are included in the Supplementary data.

2.3. Agarose gel-based assay

RNase A (2 μM, 20 μl) was mixed with 10 μl of Tris-acetic acid buffer (pH 8) and 20 μl (200 μM) each rutin, quercetin and their copper complexes incubated for 5 h. Aliquots (20 μl) were incubated with 20 μl of t-RNA (16.6 mg/ml, freshly dissolved in RNase A free buffer) and incubated for 15 min. Sample buffer (10 μl) with 10% glycerol and 0.025% bromophenol blue was added to quench the reaction. Aliquots (15 μl) were loaded onto a 1.1% agarose gel and the undegraded t-RNA visualized by ethidium bromide staining.

2.4. Precipitation assay

The ribonucleolytic activity of RNase A was assayed by the precipitation method as described by Bond et al. [25]. RNase A (4 μM, 10 μl) was mixed with 90 μl of each ligand (0–280 μM) and incubated for 4 h. The solution was treated with 50 μl of t-RNA (20 mg/ml) and 50 μl of HSA (0.5 mg/ml) in 5 mM Tris–HCl buffer pH 7.5 for 30 min. Ice cold 1.14 N perchloric acid (200 μl) containing 6 mM uranyl acetate was added to quench the reaction. The solution was chilled at 0 °C for 30 min and centrifuged at 4 °C at 12 000 rpm for 5 min. The supernatant (50 μl) was diluted to 1000 μl and the change in absorbance measured at 260 nm with respect to a control set.

2.5. Inhibition kinetics

The effect of the polyphenols and their copper complexes on the ribonucleolytic activity of RNase A was checked following the method described by Anderson et al. [24].The assay was carried out in oligo vinylsulfonic acid free 0.1 M Mes-NaOH buffer, pH 6.0 containing 0.1 M NaCl and 2′,3′-cCMP as the substrate [26]. The substrate concentration was varied from 0.2 to 0.9 mM and the inhibitor concentration from 0 to 30 μM. The inhibition constant and the type of binding enzyme were established from Lineweaver–Burk plots.

2.6. Fluorescence spectroscopy

The emission spectra were recorded from 290 to 400 nm with excitation at 275 nm [27] with a 5 nm slit width. A quantitative analysis of the interaction of ligands [28] with RNase A was performed by titration in 20 mM phosphate buffer of pH 7. A 3 ml solution of RNase A was titrated with successive addition of the respective ligands (0–15 μM). Interaction studies of the ligands were performed with a maximum alcohol content of 4% for all studies, which does not cause any structural change to the protein [29].

2.7. Circular dichroism measurements

CD measurements were carried out on a Jasco-810 automatic recording spectrophotometer, using a 1 mm path length quartz cell. The spectra were recorded in the range of 190–240 nm with a scan rate of 50 nm/min and a response time of 4 s. Three scans were accumulated for each spectrum. Results were expressed as ellipticity and secondary structure determined using DICHROWEB, an online server [30].

2.8. Fourier Transform infrared (FT-IR) studies

RNase A (200 μM) was dissolved in 20 mM phosphate buffer of pH 7.0. RNase A:ligand ratios of 1:0.5 and 1:1 were prepared for these ligands. FT-IR measurements were carried out at room temperature on a Nexus-870 FT-IR spectrometer (Thermo Nicolet Corporation) equipped with a Zn/Se-attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. Secondary structure information was determined following the method of Byler and Susi [31].

2.9. Melting point determination

A fluorescence based approach [32] was used to measure the T_m of RNase A in the presence and absence of the ligand. The fluorescence intensity of Tyr was measured at various temperatures. RNase A (4 μM) was mixed with 4 μM of each ligand and heated from 40 to 75 °C in 5 °C increments and the fluorescence intensity measured following excitation at 275 nm and emission monitored at 308 nm. The change in fluorescence intensity (ΔF value) was plotted with respect to temperature and the T_m value determined from the derivative curve.

2.10. NMR of proteins

Exchangeable hydrogen atoms of RNase A were replaced with deuterium by dissolving the protein in D_2O with incubation for

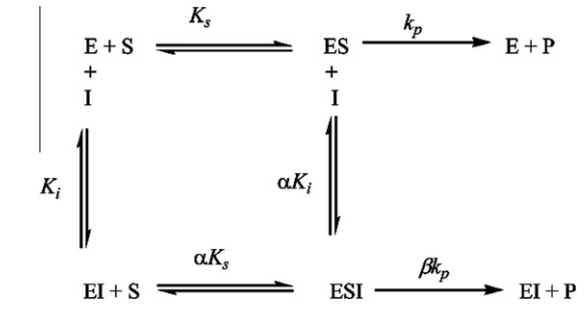
20 min at 55 °C and then lyophilized following the procedure of Quirk and Raines [33,34]. This procedure was performed thrice. The lyophilized protein was dissolved in D_2O. The [ligand] to [enzyme] molar ratio was maintained at 5:1 for all the compounds. ^1H NMR spectra were recorded on a Bruker 400 MHz spectrometer at 22.5 °C. The ligand was dissolved in CD_3CN (0.1 ml) and deutorated RNase A was dissolved in (0.6 ml D_2O). The acquisition time was 2 s with 100 scans. The δ value was recorded with respect to 3-trimethylsilylpropane sulfonic acid (DSS).

3. Results and discussion

In vivo, green tea and grape juice have the ability to inhibit the angiogenesis process. The effect of these compounds combined with promising results for copper reduction therapy provide a platform for the development of complexes with therapeutic potential. Previous studies prompted us to investigate the effect of quercetin and rutin (Fig. 1) and their copper complexes on the enzymatic activity of RNase A. This can be extended to study the enzymatic activity against specific proteins of the Ribonuclease superfamily that demonstrate unusual biological activity, such as angiogenin.

The effect of the polyphenols and their copper complexes on RNase A was assayed qualitatively by the degradation of t-RNA in an agarose gel based assay. The extent of degradation of t-RNA was estimated by observing the intensity of stained ethidium bromide as shown in Fig. 2. The ability of the ligand to affect enzyme activity is measured in terms of the extent of t-RNA degradation from the differential intensity observed. The most intense band observed in lane 6 corresponds to that of undegraded t-RNA. The intensity for the degradation of t-RNA by RNase A is shown in lane 5 and considered as the control since it is indicative of the maximum degradation possible by the protein alone. Results from the polyphenols and their complexes distinctly show that in lane 1 the intensity of the band is less intense with respect to lane 5. This indicates that in case of rutin the activity of RNase A is *enhanced*. This has been confirmed by the precipitation assay and enzyme kinetic studies. In case of Qu, RutCu and QuCu, the bands indicate varying degrees of inhibition of the enzyme by the ligands. A quantitative estimation of the relative inhibitory power of the polyphenols and their copper complexes on RNase A was estimated from the precipitation assay. The results obtained from this study also indicate *activation* of RNase A by rutin with quercetin and the copper complexes exhibiting inhibitory activity. The order of inhibition of RNase A by the compounds is RutCu < Qu < QuCu with IC_{50} values of 245 μM, 236 μM and 204 μM respectively (Fig. 3).

Specific constants obtained from kinetic experiments confirmed the nature of inhibition as shown in the panels of Fig. 4. Rutin showed activation with a K_i value of (−)28 ± 5 μM with quercetin exhibiting mixed type inhibition characteristics with a K_i value 74 ± 1 μM. The corresponding copper complexes of rutin and quercetin showed non-competitive inhibition with K_i values of 122 ± 2 and 14 ± 5 μM respectively. The scheme for the modes of inhibition is given in the following diagram:



where *E* is the enzyme, *S* the substrate, *I* the inhibitor and *P* the product; here $K_s = [E][S]/[ES]$, $\alpha K_s = [EI][S]/[ESI]$; $K_i = [E][I]/[EI]$, $\alpha K_i = [ES][I]/[ESI]$; k_p = rate constant for the breakdown of *ES* to *P*; $\beta\ k_p$ = rate constant for the breakdown of *ESI* to *P*. The simplest mixed system is one in which *EI* has a lower affinity than *E* for *S*, and the *ESI* complex is non-productive. This means $\alpha > 1$ and

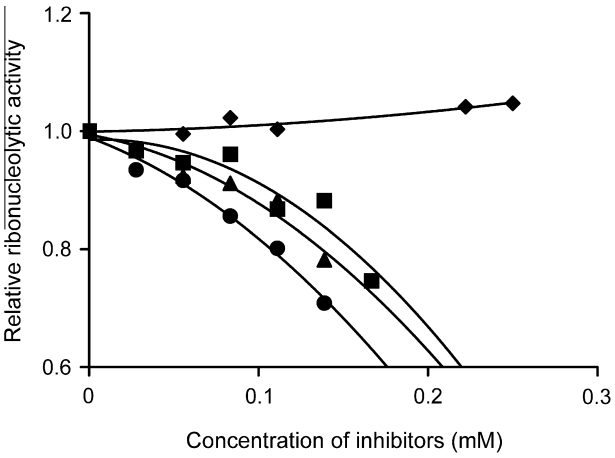


Fig. 3. Relative ribonucleolytic activity plots of RNase A by Rut (♦), Qu (▲), RutCu (■), QuCu (●).

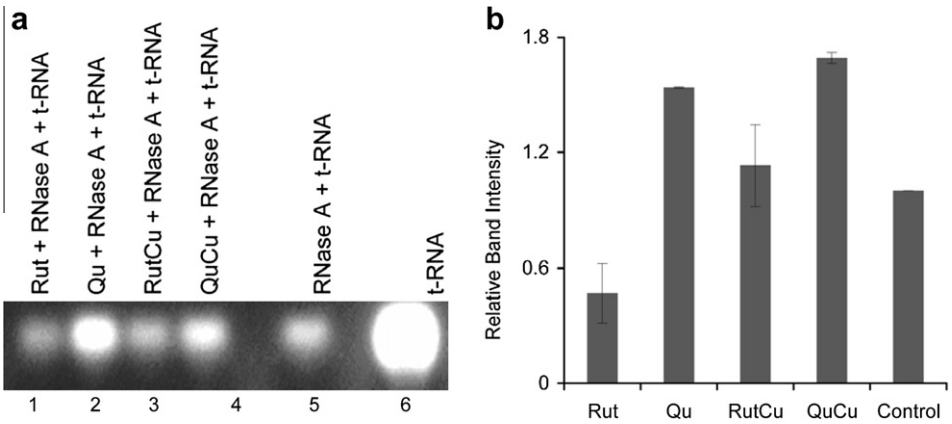


Fig. 2. (a) Agarose gel-based assay for the inhibition of RNase A by rutin, quercetin and their copper complexes and (b) the corresponding histogram band intensity.

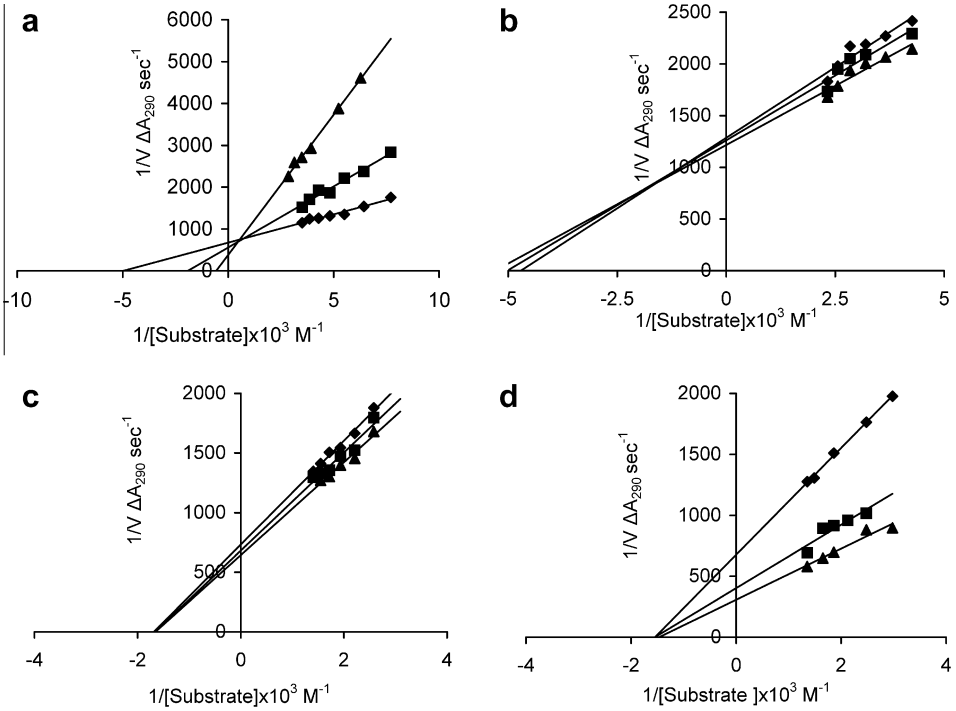


Fig. 4. Lineweaver–Burk plots for inhibition of RNase A by (a) Rutin, (b) Qu, (c) RutCu, and (d) QuCu. Concentrations of ligands are 30 μM (♦), 15 μM (■), 0 μM (▲) for Rutin and 14 μM (♦), 7 μM (■), 0 μM (▲) for other compounds.

Table 1
The inhibition constant values and mode of inhibition for the rutin, quercetin and their copper complexes against ribonucleolytic activity of RNase A.

Inhibitor	α	β	K_i (μM)	Mode of inhibition
Rut	0.92	0.99	(−)28 ± 5	Hyperbolic mixed-type
Qu	2.98	0	74 ± 1	Linear mixed-type
RutCu	1	0	122 ± 2	Non-competitive
QuCu	1	0	14 ± 5	Non-competitive

$\beta = 0$. The system may be considered a mixture of partial competitive inhibition and pure non-competitive inhibition. This system is referred to as a linear mixed-type inhibition. Another possibility arises when the inhibitor decreases the rate constant for product formation, but at the same time the affinity of the enzyme for the substrate. This can be described as $\alpha < 1$, $\beta < 1$, and also $\beta > \alpha$. In other words k_p has decreased, but K_s has decreased much more. There may be some doubt whether I in this case should be referred to as an inhibitor or an activator. This type of character is exhibited in a type of hyperbolic mixed type of inhibition [35]. The corresponding α, β, K_i values are tabulated in Table 1.

The CD spectra of RNase A in the presence and absence of increasing amounts of polyphenols and their copper complexes show significant changes at 208 and 215–222 nm indicating conformational changes of secondary structure of RNase A (Fig. 5). For CD studies the concentration of RNase A was 15 μM. Two sets of RNase A-ligand complexes were prepared maintaining an RNase A to ligand ratio of 1:0.5 and 1:1 respectively. Interestingly, the data for rutin RNase A displays a decreasing order and other compounds shown increasing in the α-helix (Supplementary data, Table S2). This trend is similar to that obtained from FTIR studies (Supplementary data).

Quenching and binding parameters K_{SV} (Stern-Volmer quenching constant), K_b (binding constant), K_a (association constant), n (the number of ligand molecule per protein) and f_a (the fraction of fluorophore accessible to the quencher)} were calculated from fluorescence quenching data at 298 K with the help of Stern-Volmer, modified Stern-Volmer and double-logarithm plots [36,37]. The fluorescence spectra of RNase A in the absence and presence of rutin and quercetin and their copper complexes at 25 °C are shown in Fig. 6(a)–(d), respectively. Interestingly, addition of quercetin and rutin and their copper complexes to RNase A leads to a significant quenching of the fluorescence intensity of tyrosine for all ligands despite differences observed in the mode of inhibition in case of rutin. Quenching and binding parameters for these ligands with RNase A are given in Table 2.

^{1}H NMR Proton nuclear magnetic resonance (NMR) spectra of RNase A are well characterized and the C(2)–H resonances of three His residues out of four observed separately [38]. When a competitive inhibitor binds to the enzyme, the chemical shifts (δ) of the C(2)–H of the active site His residues are expected to change. However, for a ligand not going to the active site, little or no change in the δ value of C(2)–H is expected [39]. As would be expected the chemical shift will not be affected for non-competitive ligands. In Fig. 7, the signals for the C(2) protons of His 12, His 105, His119 are indicated by a, b and c, respectively. No significant chemical shift of C(2)-H of His-12 or His-119 RNase A is observed after binding with rutin or quercetin (Fig. 7). For comparison, the δ values of His 12, 119 of 2′CMP, a known competitive inhibitor have been shown where a significant shift is observed when bound to RNase A. For RNase A bound to RutCu and QuCu (Supplementary data) the ^{1}H NMR spectra of RNase A does not show any change indicating that neither the polyphenols or their copper complexes are in the vicinity of the active site of the protein.

The effect of the polyphenols and their copper complexes on the melting point of RNase A was determined and is shown as a first derivative in Fig. 8 for clarity. It is observed that rutin has a thermal stabilization property showing a 2 °C increase in the melting point whereas the other ligands Qu, RutCu, QuCu show a decrease in the melting point.

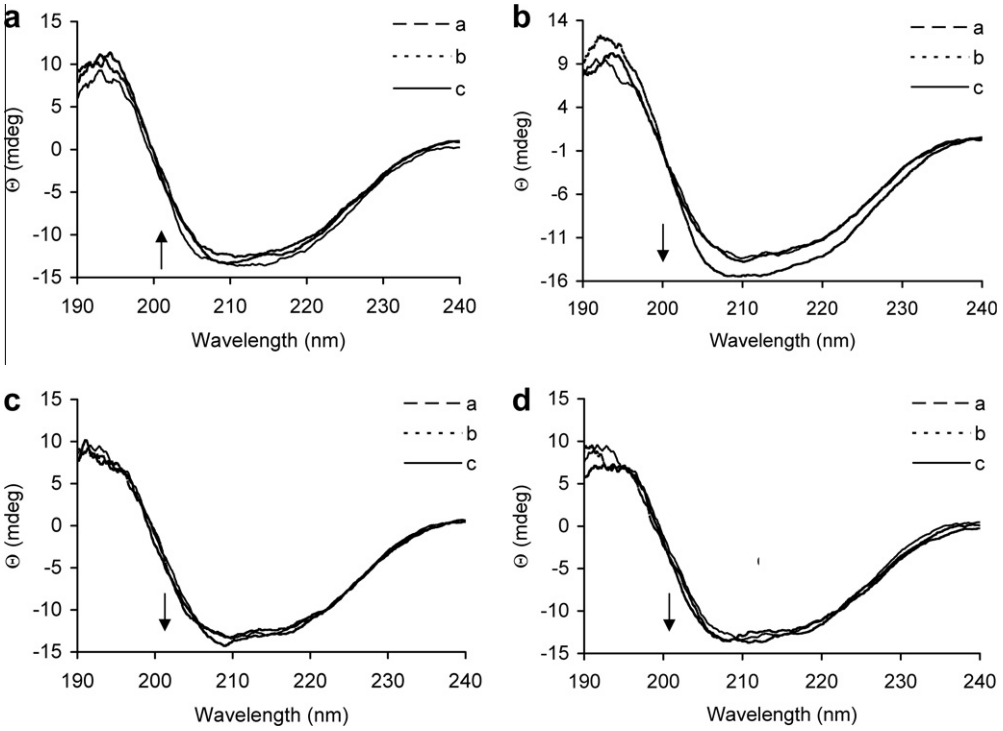


Fig. 5. CD spectra of (a) RNase A-Rut, (b) RNase A-Qu, (c) RNase A-RutCu, (d) RNase A-QuCu, (a) RNase A:ligand 1:1 (---), (b) RNase A:ligand 1:0.5 (. . .) and (c) Free RNase A (—).

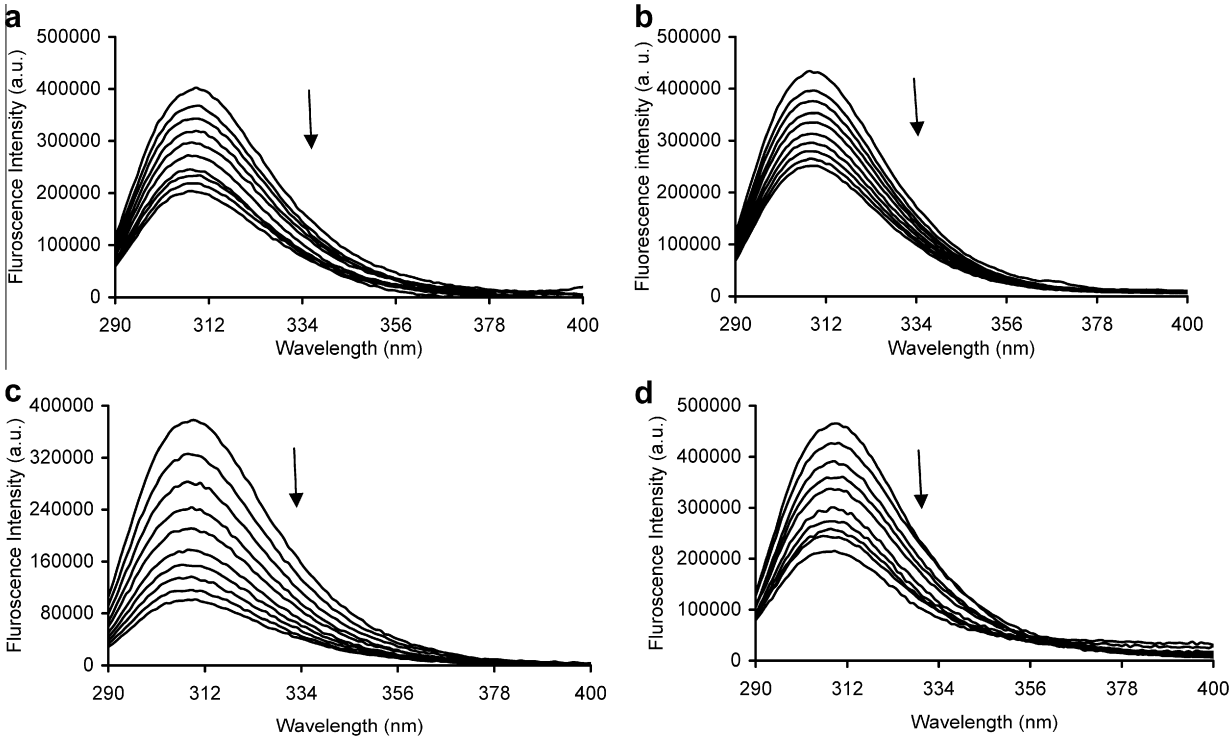


Fig. 6. Fluorescence quenching spectra of RNase A in absence (top) and presence of (a) Rut, (b) Qu, (c) RutCu and (d) QuCu in 20 mM phosphate buffer of pH 7.0; Ligand concentrations: 0 to 15 μM.

These studies show that the behavior of rutin in its interaction with RNase A is distinctly different than quercetin and the copper complexes. Between the two copper complexes, QuCu is the more potent inhibitor due to the presence of a greater stoichiometric ratio of Cu(II) in the QuCu complex in comparison to RutCu. This is reflected in the agarose gel studies and in the K_i values. All the

Table 2
The binding parameters for the interactions of quercetin, rutin and their copper complexes with RNase A.

Systems	K_{sv} (M^{-1})	K_a (M^{-1})	K_b (M^{-1})	n	f_a
Rut	6.41×10^4	4.90×10^4	4.85×10^4	1.10	1.12
Qu	5.10×10^4	3.27×10^4	3.73×10^4	1.13	1.76
RutCu	11.41×10^4	5.52×10^4	7.50×10^4	1.30	1.66
QuCu	7.20×10^4	3.42×10^4	4.86×10^4	1.15	1.58

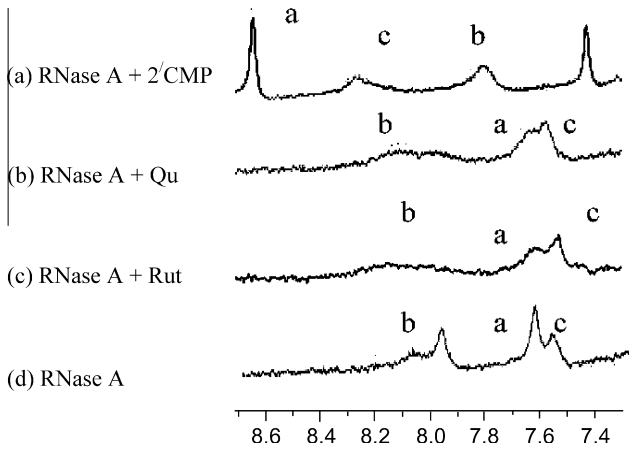


Fig. 7. ^{1}H NMR spectra of RNase A and its complexes with ligands the signal for C(2) protons of His 12, His 105 and His119 indicated by a, b and c respectively.

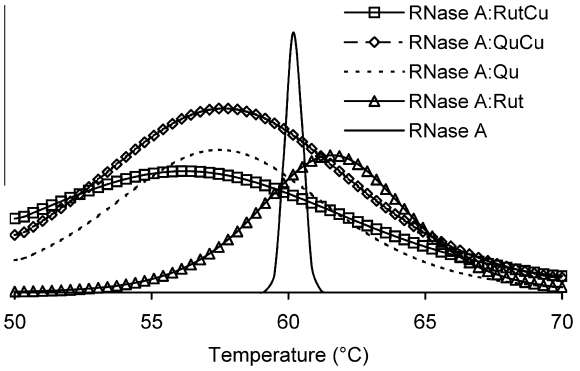


Fig. 8. First derivative plots for melting point determination of RNase A in presence of the ligands.

experiments conducted with rutin indicate that on binding with the protein, the interaction pattern results in a situation that promotes the ribonucleolytic activity of RNase A. It may be speculated that the enzyme complex formed with rutin being more stable as observed from the melting studies facilitates the binding of the substrate resulting in a greater formation of the product. The kinetic parameters with β being non-zero *only for rutin* indicate that both pathways to form the product are probable thus, increasing the activity of the protein in the presence of rutin. With $\beta = 0$ for quercetin and the copper complexes the possibility of this particular route is ruled out. In these cases the formation of the ESI complex is non-productive thus, leading to inhibition due to depletion of available enzyme. Thus, regulation of enzymatic properties may be possible with the initiation of complexation with metal ion. Based on the structural homology between RNase A and angiogenin these complexes can be subsequently used for the antiangiogenic therapy. Further studies are underway to ascertain the effect of the complexes on the angiogenic activity of angiogenin. Similar glycone flavonoids may be further checked for their ability to activate/inhibit the catalytic activity of RNase A and angiogenin. Activation with enhancement of the ribonucleolytic activity of the protein that is possible with rutin may prove beneficial for the degradation of RNA and may also assist in the wound healing property of angiogenin since its angiogenic property is related to its catalytic potency [40–42].

Acknowledgements

SDG is grateful to Council of Scientific and Industrial Research (CSIR), India for financial support for the project [01(2115)/07/EMR-II] and CRF, IIT Kharagpur for instrumental facilities D.R.T. and A.S.R. thank CSIR, New Delhi for fellowships.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.09.005.

References

[1] Raines, R.T. (1998) Ribonuclease A. Chem. Rev. 98, 1045–1066.
[2] Ledoux, L. (1955) Action of ribonuclease on two solid tumors in vivo. Nature 176, 36–37.
[3] Shapiro, R., Riordan, J.F. and Vallee, B.L. (1986) Characteristic ribonucleolytic activity of human angiogenin. Biochemistry 25, 3527–3532.
[4] Ghosh, K.S., Maiti, T.K. and Dasgupta, S. (2004) Green tea polyphenols as inhibitors of ribonuclease A. Biochem. Biophys. Res. Commun. 325, 807–811.
[5] Sorrentino, S., Glitz, D.G., Hamann, K.J., Loegering, D.A., Checkel, J.L. and Gleich, G.J. (1992) Eosinophil-derived neurotoxin and human liver ribonuclease: identity of structure and linkage of neurotoxicity to nuclease activity. J. Biol. Chem. 267, 14859–14865.
[6] Iwahashi, K., Nakamura, K., Mitsui, Y., Ohgi, K. and Irie, M. (1981) Further evidence for the existence of the pO site in the active site of ribonuclease. The binding of thymidine 3′,5′-diphosphate to ribonuclease. J. Biochem. (Tokyo) 90 (6), 1685–1690.
[7] Vallee, B.L. and Riordan, J.F. (1997) Organogenesis and angiogenin. Cell. Mol. Life Sci. 53, 803–815.
[8] Fett, J.W., Olson, K.A. and Rybak, S.M. (1994) A monoclonal antibody to Human Angiogenin. Inhibition of ribonucleolytic and angiogenic activities and localization of the antigenic epitode. Biochemistry 33, 5421–5427.
[9] Olson, K.A., French, T.C., Vallee, T.L. and Fett, J.W. (1994) A monoclonal antibody to human angiogenin suppresses tumor growth in athymic mice. Cancer Res. 54, 4576–4579.
[10] Oaka, M., Bedouia, J.E. and Valere, B.S. (2005) Antiangiogenic properties of natural polyphenols from red wine and green tea. J. Nutr. Biochem. 16, 1–8.
[11] Stewart, Laura.K., Soileau, Jeff.L., Ribnicky, David., Wang, Zhong.Q., Raskin, Ilya., Poulev, Alexander., Majewski, Martin., Cefalu, William.T. and Gettys, Thomas W. (2008) Quercetin transiently increases energy expenditure but persistently decreases circulating markers of inflammation in C57BL/6J mice fed a high-fat diet. Metabolism 57, S39–S46.
[12] Mark Davis, J., Angela Murphy, E., Carmichael, Martin D. and Davis, Ben. (2009) Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. Am J. Physiol. Regul. Integr. Comp. Physiol. 296, R1071–R1077.
[13] Paliwal, S., Sundaram, J. and Mitragotri, S. (2005) Induction of cancer-specific cytotoxicity towards human prostate and skin cells using quercetin and ultrasound. Br. J. Cancer 92, 499–502.
[14] Hu, G.F. (1998) Copper stimulates proliferation of human endothelial cells under culture. J. Cell. Biochem. 69, 326–335.
[15] Brem, S., Zagzag, D. and Tsanaclis, A.M. (1990) Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. Am. J. Pathol. 137, 1121–1142.
[16] Kotsaki-Kovatsi, V.P., Koehler-Samouilidis, G. and Kovatsis, A. (1997) Fluctuation of zinc, copper, magnesium and calcium concentrations in guinea pig tissues after administration of captopril (SQ 14225). J. Trace Elem. Med. Biol. 11, 32–36.
[17] Brewer, G.J., Dick, R.D., Grover, D.K., LeClaire, V., Tseng, M., Wicha, M., Pienta, K., Redman, B.G., Jahan, T., Sondak, V.K., Strawderman, M., LeCarpentier, G. and Merajver, S.D. (2000) Treatment of metastatic cancer with tetrathiomolybdate, an anticopper, antiangiogenic agent: Phase I study. Clin. Cancer Res. 6, 1–10.
[18] Saari, J.T. and Schuschke, D.A. (1999) Cardiovascular effects of dietary copper deficiency. BioFactors (Oxford, England). 10, 359–375.
[19] Maiti, T.K., Chatterjee, J. and Dasgupta, S. (2003) Effect of green tea polyphenols on angiogenesis induced by an angiogenin-like protein. Biochem. Biophys. Res. Commun. 308, 64–67.

[20] Ghosh, K.S., Maiti, T.K., Mandal, A. and Dasgupta, S. (2006) Copper complexes of (−)-epicatechin gallate and (−)-epigallocatechin gallate act as inhibitors of Ribonuclease A. FEBS Lett. 580, 4703–4708.
[21] Chowa, J., Shen, S., Huan, S.K., Lin, H. and Chen, Y. (2005) Quercetin, but not rutin and quercitrin, prevention of H_2O_2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. Biochem. Pharmacol. 69, 1839–1851.
[22] Glossmann, H., Presek, P. and Eigenbrodt, K. (1981) Quercetin inhibits tyrosine phosphorylation by the cyclic nucleoside-independent, transforming protein kinase Naunyn–Schmiedeberg's. Arch. Pharmacol. 317, 100–102.
[23] Sela, M. and Anfinsen, C.B. (1957) Some spectrophotometric and polarimetric experiments with ribonuclease. Biochim. Biophys. Acta 24, 229–235.
[24] Anderson, D.G., Hammes, G.G. and Walz, F.G. (1968) Binding of phosphate ligands to Ribonuclease A. Biochemistry 7, 1637–1645.
[25] Bond, M.D. (1988) An in vitro binding assay for angiogenin using placental ribonuclease inhibitor. Anal. Biochem. 173, 166–173.
[26] Smith, B.D., Soellner, M.B. and Raines, R.T. (2003) Potent inhibition of Ribonuclease A by oligo (vinylsulfonic acid). J. Biol. Chem. 278, 20934–20938.
[27] Garcia-Borron, J.C., Escribano, J., Jimenez, M. and Iborra, J.L. (1982) Quantitative determination of tryptophanyl and tyrosyl residues of proteins by second-derivative fluorescence spectroscopy. Anal. Biochem. 125, 277–285.
[28] Maiti, T.K., Ghosh, K.S. and Dasgupta, S. (2006) Interaction of (−)-epigallocatechin-3-gallate with human serum albumin: fluorescence, Fourier transform infrared, circular dichroism, and docking studies. Proteins 64, 355–362.
[29] Lin, S., Li, M. and Wei, Y. (2004) Ethanol or /and captopril-induced precipitation and secondary conformational changes of human serum albumin. Spectrochim. Acta A 60, 3107–3111.
[30] Whitmore, L. and Wallace, B.A. (2004) DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res. 32, W668–W673.
[31] Byler, D.M. and Susi, H. (1986) Examination of the secondary structure of proteins by deconvolved FT-IR spectra. Biopolymers 25, 469–487.
[32] Vedadi, M., Niesen, F.H., Allali-Hassani, A., Fedorov, O.Y., Finerty Jr., P.J., Wasney, G.A., Yeung, R., Arrowsmith, C., Ball, L.J., Berglund, H., Hui, R., Marsden, B.D., Nordlund, P., Sundstrom, M., Weigelt, J. and Edwards, A.M. (2006) Chemical screening methods to identify ligands that promote protein stability, protein crystallization and structure determination. Proc. Natl. Acad. Sci. USA 103 (43), 15835–15840.
[33] Quirk, D.J. and Raines, R.T. (1999) His... Asp Catalytic Dyad of Ribonuclease A: Histidine Ka Values in the Wild-Type, D121N, and D121A. Enzym. Biophys. J. 76, 1571–1579.
[34] Luigi, S. and Vincenza, V. (1979) ^{1}H NMR study on the interaction of cupric ion with ribonuclease A. Biochim. Biophys. Acta 580, 100–107.
[35] Segel, I.H. (1993) Enzyme Kinetics, John Wiley & Sons Inc., New York.
[36] Lakowicz, J.R. (1991) Principles of Fluorescence Spectroscopy, 3rd ed, New York, Plenum Press.
[37] Jiang, M., Xie, M.X., Zheng, D., Liu, Y., Li, X.Y. and Cheng, X. (2004) Spectroscopic studies on the interaction of cinnamic acid and its hydroxyl derivatives with human serum albumin. J. Mol. Struct. 692, 71–80.
[38] Manuel, R., Marta, B., Jorge, S., Carlos, G., Jose, L.N., Josk, L.N. and Jose, H. (1989) Sequential ^{1}H-NMR assignment and solution structure of bovine pancreatic ribonuclease A. Eur. J. Biochem. 183, 623–638.
[39] Ghosh, K.S., Debnath, J., Pathak, T. and Dasgupta, S. (2008) Using Proton Nuclear Magnetic Resonance to study the mode of Ribonuclease A inhibition by competitive and noncompetitive inhibitors. Bioorg. Med. Chem. Lett. 18, 5503–5506.
[40] Rajnoch, J. and Viklicky, O. (2004) Angiogenesis and organ transplantation. Folia Microbiol. (Praha) 49 (5), 499–505.
[41] Marianna, K., Nobuyuki, F. and Dean, D.M. (2009) Human mast cells synthesize and release angiogenin, a member of the ribonuclease A (RNase A) superfamily. J. Leukoc. Biol. 86 (5), 1217–1226.
[42] King, T.V. and Vallee, B.T. (1991) Neovascularisation of the the meniscus with angiogenin an experimental study in rabbits. J. Bone Joint Surg. 73 (4), 587–590.