Bioorganic Chemistry 52 (2014) 56–61

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Amino and carboxy functionalized modified nucleosides: A potential class of inhibitors for angiogenin

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ARTICLE INFO

Article history:
Received 13 July 2013
Available online 26 November 2013

Keywords:
Thymidine
Angiogenesis
Chorioallantoic membrane (CAM) assay
Docking

ABSTRACT

The 3′-amino and carboxy functionalize thymidines execute their ribonucleolytic inhibition activity for angiogenin. These modified nucleosidic molecules inhibit the ribonucleolytic activity of angiogenin in a competitive manner like the other conventional nucleotidic inhibitors, which have been confirmed from kinetic experiments. The improved inhibition constant (K_i) values 427 ± 7, 775 ± 6 μM clearly indicate modified nucleosides are an obvious option for the designing of inhibitors of angiogenesis process. The chorioallantoic membrane (CAM) assay qualitatively suggests that amino functionalized nucleosides have an effective potency to inhibited angiogenin-induced angiogenesis. Docking studies further demonstrate the interaction of their polar amino group with the P_1 site residues of angiogenin, i.e., His-13 and His-114 residues.

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1. Introduction

Angiogenin is a member of ribonuclease superfamily protein [1] and is a potent inducer of angiogenesis [2] (growth of new blood vessel). The angiogenesis process is found to operate at an alarming rate during the proliferation of tumor cells for the supply of nutrients necessary for their growth to solid tumor [3]. Angiogenin and ribonuclease A (RNase A) being the members of same superfamily they share a structural homology regarding their ribonucleolytic site but the ribonucleolytic activity of angiogenin is about 10^5-10^6 times weaker than that of RNase A [4]. However, ribonucleolytic activity of angiogenin is crucial for its angiogenic activity [5]. Therefore the enzymatic site (ribonucleolytic site) of angiogenin is an important target from therapeutic point of view. Abolition of the ribonucleolytic activity by low molecular weight inhibitors would therefore tantamount to inhibit the angiogenic activity of angiogenin leading to the inhibition of the undesired neovascularization. The ribonucleolytic site of RNase A and angiogenin is comprised of multiple subsites which bind the phosphate, base, and sugar components of RNA [6–8]. The P_1 subsite where cleavage of the phosphodiester bond occurs is comprised by His-12, Lys-41 and His-119 for RNase A and His-13, Lys-40 and His-114 for angiogenin and it serves as the main catalytic site (Fig. 1). There are two other important B_1 and B_2 subsites which recognize the nucleobases of normal RNA. Among these subsites, B_1 site shows pyrimidine specificity whereas the B_2 site has an affinity for purine nucleobase [9].

Most of the nucleotide-based inhibitors of angiogenin are functionalized with phosphate or pyrophosphate group. These inhibitors inhibit the ribonucleolytic activity of angiogenin by virtue of their structural similarities with its normal substrate [7,10]. However, these conventional nucleotidic inhibitors have a limited success towards angiogenin compared to RNase A [11], as well as, the high negative charge on the phosphate groups is a great disadvantage regarding their cell permeability [12]. Because of the limited success of the nucleotidic inhibitors different non-nucleosidic molecules are tried on angiogenin [13] and also dinucleosides with amide linkage [14].

In literature it has been reported that for RNase A the pK_a values of His-12 and His-119 changes from ~5.22/6.78 to ~6.30/8.10 during its binding with the substrate [15]. Keeping this point in mind we employed a series of 3′-modified nucleosides functionalized with amino and carboxy groups for angiogenin inhibition study. At the physiological pH these polar groups are likely to remain in their conjugate form. Now if these molecules are recognized at the ribonucleolytic site of angiogenin because of the presence of nucleodase and ribose moieties then amino and carboxylic groups have a possibility to interact with the P_1 site residues. However, replacement of the phosphate or pyrophosphate group by one amino or carboxylic group lowers the net negative charge of these molecules compared to their nucleotide analogs.

In this particular report we have selected 2′-deoxynucleoside to protect the amide bond towards the hydrolysis so that they can deliver the free amino and carboxylic groups at the catalytic site.

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http://dx.doi.org/10.1016/j.bioorg.2013.11.005

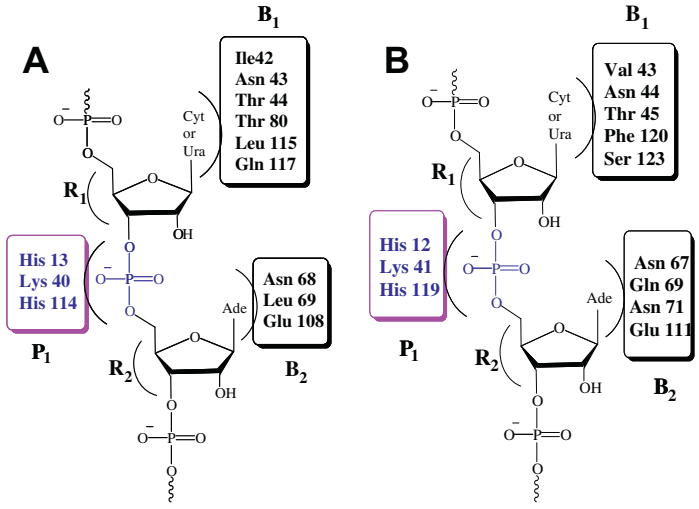

Fig. 1. Key residues of ribonucleolytic site of (A) Human angiogenin and (B) RNase A.

Otherwise in presence of the 2′-OH, the inhibitor molecules can undergo easy hydrolysis of its amide bond as found in case of angiogenin's normal substrate (RNA).

2. Results and discussion

Amino and carboxy functionalize modified nucleosides* showed their inhibitory activity on RNase A [16,17] and it encourages us to extend the inhibition study for angiogenin, a homologous protein of RNase A (structures of the inhibitor molecules of these series is given in Fig. 2). Therefore, the most potential compounds of those series were selected for this study. However, comparison of the inhibitory potential of these two series can also produce valuable information for the further modification of low molecular weight inhibitor of angiogenin.

The ribonucleolytic inhibitory activity of these compounds on angiogenin was quantitatively estimated by precipitation assay [18]. We observed the similar trend of inhibition in case of angiogenin as for RNase A for both class of compounds. Compound **2** and **5** from amino and carboxy functionalized nucleosides respectively showed the best inhibitory activity on angiogenin (22.00% and 23.42% respectively) (Fig. 3). It suggested both amino and carboxy functionalizations have an equal importance in angiogenin inhibition. The bar diagram obtained from this assay (Fig. 3) indicated that for amino functionalize nucleosides inhibitory activity depends upon the nature of the side chain part for respective amino acids. However, for the other set of compounds the chain length of

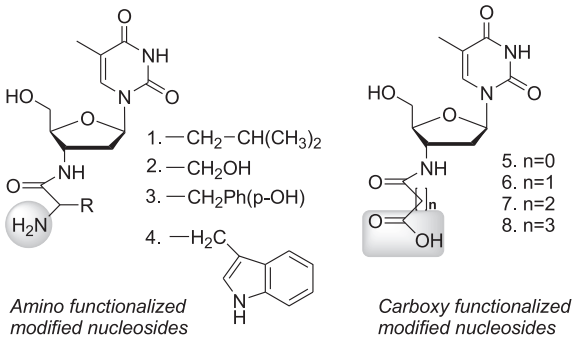

Fig. 2. Structures of amino and carboxy functionalize modified nucleosides. *Note:* Synthesis of these inhibitor molecules has been reported in Refs. [16,17].

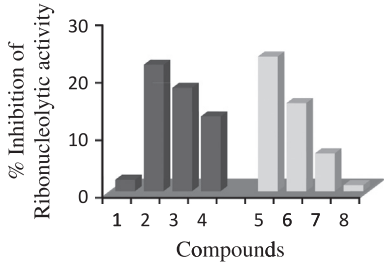

Fig. 3. Percentage inhibition of ribonucleolytic activity of angiogenin by compounds **1**, **2**, **3**, **4** (black) and **5**, **6**, **7**, **8** (gray).

the dibasic acid part played the determining role in inhibition process.

The mode of ribonucleolytic inhibition of angiogenin was further confirmed with the help of kinetic experiment where 6-FAM-dArUdAdA-6-TAMRA was used as the substrate of angiogenin protein [19]. This study was based on the fluorescence quenching of the fluorescein moiety attached to rhodamine by a phosphodiester bond. The enhanced fluorescence of fluorescein moiety was measured during the cleavage of facile phosphate bond by angiogenin. The inhibition constant value (K_i) for compounds **2**, **3**, **5** and **6** was found 427 ± 7, 1251 ± 4, 775 ± 6 and 1,114 ± 3 μM respectively and the nature of the Lineweaver-Burk plots confirmed their competitive mode of inhibition (Fig. 4). The K_i values of these four compounds are much better than 3′-CMP (12,703 ± 0.03 μM), a nucleotidic inhibitor of angiogenin (determined using the same substrate). However, the K_i values obtained from this experiment clearly show the preference of amino group over the carboxy one. The comparative K_i values of these inhibitors for RNase A and angiogenin are given in Table 1.

In view of the results obtained from inhibition of the ribonucleolytic activity of angiogenin based on the fluorimetric assay, it appeared pertinent to observe the effect of the amino functionalized modified nucleosides on the angiogenin induced angiogenesis. The chorioallantoic membrane (CAM) assay [1] was therefore conducted to verify the inhibition of angiogenin-induced angiogenesis by compounds **2** and **3**. As expected, vascularization was enhanced in presence of angiogenin (Fig. 5A). Vascular growth was comparable to the blank (Fig. 5B) for the cases when compounds mixed with angiogenin. It suggested that both **2** (Fig. 5C) and **3** (Fig. 5E) suppress the growth of blood vessels induced by angiogenin. This

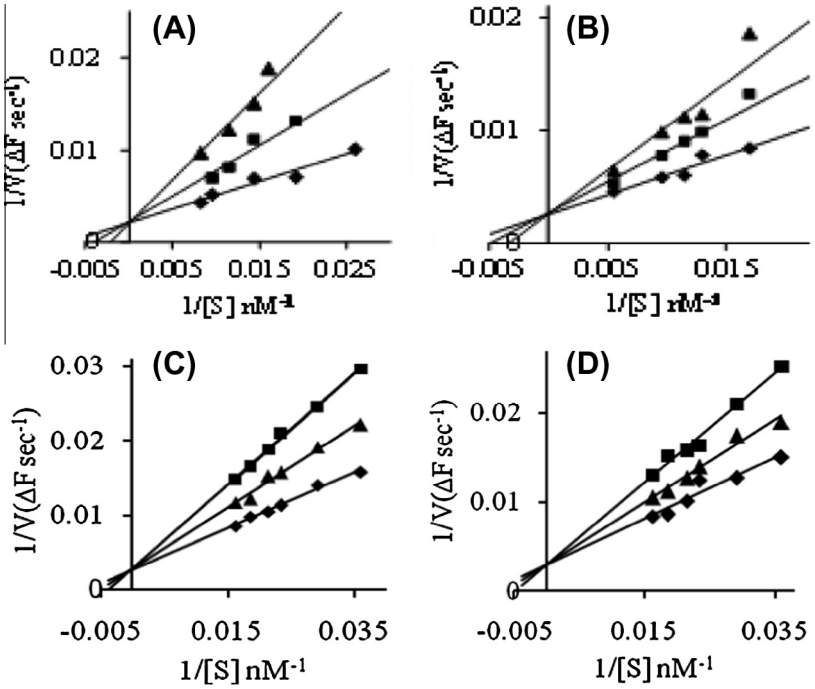

Fig. 4. Lineweaver-Burk plots for the inhibition of angiogenin by (A) **2**: 0 mM (♦), 0.2 mM (■) and 0.8 mM (▲); (B) **3**: 0 mM (♦), 0.4 mM (■) and 1.2 mM (▲); (C) **5**: 0 mM (♦), 0.3 mM (▲) and 1.2 mM (■); (D) **6**: 0 mM (♦), 0.7 mM (▲) and 2.5 mM (■). Angiogenin concentration: 0.36 μM.

Table 1
Comparative K_i values for RNase A and angiogenin with compounds **2**, **3**, **5** and **6**.

Compound	K_i value for RNase A (μM)	K_i value for angiogenin (μM)
2	80 ± 3	427 ± 7
3	451 ± 2	1251 ± 4
5	132 ± 2	775 ± 6
6	380 ± 2	1114 ± 3

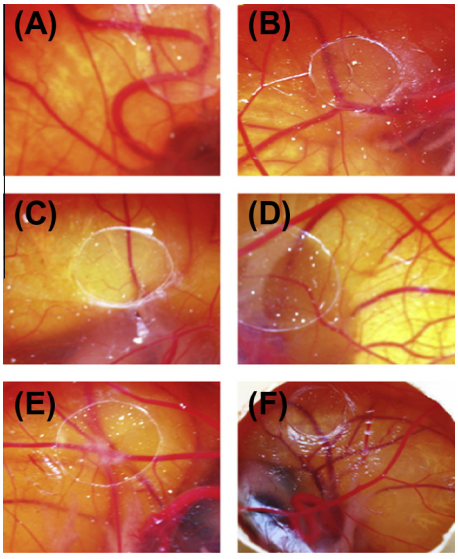

Fig. 5. CAM assay (A) 10 ng angiogenin; (B) control; (C) 0.1 μM compound **2** + 10 ng angiogenin; (D) 0.1 μM compound **2**; (E) 10 ng angiogenin + 0.1 μM compound **3**; (F) 0.1 μM compound **3**.

is more apparent for **2** than **3** indicating, albeit qualitatively, but it indicate compound **2** acts as a better inhibitor than compound **3**. On the other hand the compounds themselves inhibit vascular growth to a negligible extent (Fig. 5D and F).

Docking studies were then performed with the help of *FlexX* to get an insight about the possible interactions involved between the protein residues and ligand molecules. In the most preferred docking poses for **2** and **3** with human angiogenin (1B1I), we found that the amino groups were within hydrogen bonding distance of His-114 (Fig. 6A and B). Along with this possible interaction compound **2** also formed a hydrogen bonding network with His-13 with its 5′-OH and 3′-amide oxygen. However, the thymine nucleobase of **2** was found to interact with Lys-40 and Asp-41 residues of angiogenin. In case of compound **3** only the 3′-amide oxygen atom was within hydrogen bonding distance of His-13 and its nucleobase moiety was found to interact only with Asp-41 which is a residue of angiogenin near the B_1 subsite. The interactions with the ribonucleolytic site residues corroborated competitive nature of the synthesized inhibitors as observed in the Lineweaver-Burk plots for compounds **2** and **3**. The hydrogen bonding distance between the amino acid residues of angiogenin and compounds **2** and **3** are given in Table 2.

On the other hand, for carboxy functionalized nucleoside **5** it was found that the carboxylic group was in a position to form a hydrogen bonding network with His-13 and His-114, like the phosphate groups of nucleotidic inhibitors. This also corroborated the competitive nature of **5** as obtained the nature of Lineweaver-Burk plot for **5**. The thymine moiety of **5** was found within the hydrogen bonding distance of Asp-41 and Arg-121. From the docked conformation of **6**, however, we found the free carboxylic group was projected in a different orientation than for **5** and formed a possible hydrogen bonding network with Ser-118 and Asp-116 (Fig. 7A and B). The ribonucleolytic site residues His-13 and His-114 were found within the hydrogen bonding distance of thymine nucleobase as well as with the carbonyl oxygen of the amide bond at the 3′-position. The most important thing found from docking experiment was that for amino functionalized modified nucleosides alternation of the amino acid side chain did not changed the binding patterns of the ligands with angiogenin. However, for carboxy functionalized modified nucleosides incorporation of additional CH_2 group drastically changed their binding pattern. The

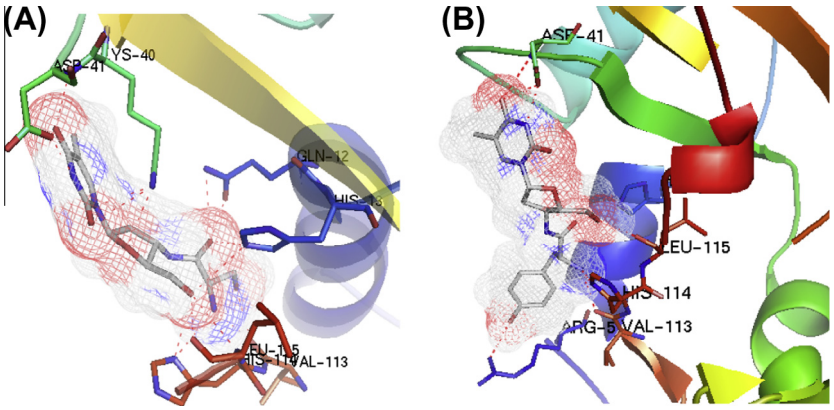

Fig. 6. Docking poses (A) compound **2** and (B) compound **3** with human angiogenin (1B1I).

Table 2
Hydrogen bonding distance between the amino acid residues of angiogenin and compounds **2** (A), **3** (A), **5** (B) and **6** (C).

Protein 1B1I	Compound **2**	Compound **3**
Amino acid residues of 1B1I		
(A)		
Arg-5 [$N^{\eta 1}$]		2.82 [O^{η}]
Gln-12 [$O^{\varepsilon 1}$]	2.54 [O amide of amino acid]	
Gln-12 [$N^{\varepsilon 2}$]	3.28 [O amide of amino acid]	
His-13 [$N^{\varepsilon 2}$]	3.21 [O amide of amino acid], 3.42 [O5′]	3.12 [O amide of amino acid]
Lys-40 [N^{ζ}]	3.28 [O2], 3.20 [N1], 3.02 [O(sugar)]	
Asp-41 [$O^{\delta 1}$]	2.51 [O4], 2.64 [N3]	2.57 [O4], 2.42 [N3]
His-114 [$N^{\delta 1}$]	3.37 [O5′], 3.10 [NH_2] amino acid	3.15 [NH_2] amino acid
Angiogenin (1B1I)		Compound **5**
Amino acid residue of 1B1I		
(B)		
His-13 [$N^{\varepsilon 2}$]		2.92 [O (COOH)]
Lys-40 [N^{ζ}]		2.87 [O (amide bond)]
Asp-41 [$O^{\delta 1}$]		3.33 [N (3)]
		3.13 [O (4)]
		3.08 [O (2)]
Asp-41 [$O^{\delta 2}$]		2.73 [N (3)]
His-114 [$N^{\delta 1}$]		2.66 [OH (COOH)]
Leu-115 [N]		3.23 [O (COOH)]
Leu-115 [O]		3.44 [O (COOH)]
Arg-121 [$N^{\eta 1}$]		2.96 [O(4)]
Arg-121 [$N^{\eta 2}$]		2.43 [O(4)]
Angiogenin (1B1I)		Compound **6**
Amino acid residue of 1B1I		
(C)		
His-13 [$N^{\varepsilon 2}$]		3.10 [O(4)]
His-114 [$N^{\varepsilon 2}$]		2.82 [O (amide bond)]
Leu-115 [O]		2.87 [O(2)]
		2.58 [N(3)]
Asp-116 [$O^{\delta 1}$]		2.99 [OH (COOH)]
Asp-116 [$O^{\delta 2}$]		3.44 [N (amide bond)]
		3.30 [O (amide bond)]
		2.37 [OH (COOH)]
Gln-117 [N (back bone)]		2.81 [O(2)]
Ser-118 [N]		3.09 [OH (5′)]
Ser-118 [O^{γ}]		2.56 [OH (COOH)]
Arg-121 [$N^{\eta 2}$]		2.81 [OH (5′)]
Arg-121 [N^{ε}]		2.87 [OH (5′)]

hydrogen bonding distance between the amino acid residues of angiogenin and compounds **5** and **6** are given in Table 2.

Theoretical calculations of binding energies along with docking studies had been performed for the better understanding of inhibition by compounds **2** and **3** compared to that of 3′-CMP as observed experimentally. These two compounds were selected for their more or less similar binding pattern with angiogenin and to find out the reason for better inhibitory activity of amino functionalize modified nucleosides over their phosphate analogs. The total ligand-receptor interaction energy values obtained from

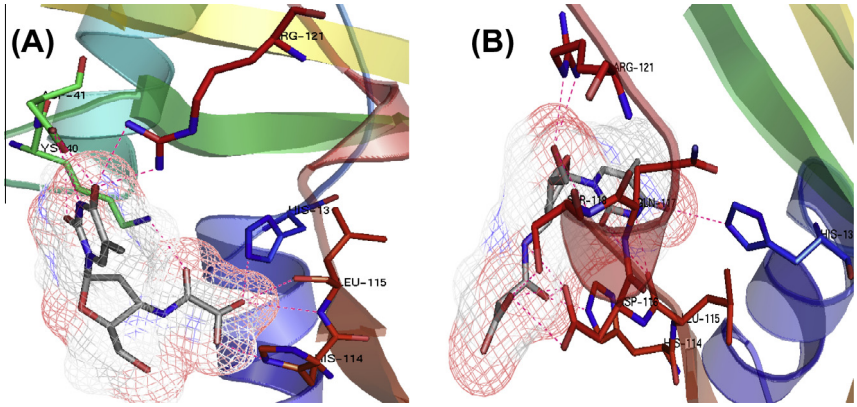

Fig. 7. Docking poses (A) compound **5** and (B) compound **6** with human angiogenin (1B1I).

PEARLS [20] were found to be -8.75, -6.25, -2.13 kcal/mole for compounds **2**, **3** and 3′-CMP respectively. This implies that **2** and **3** bind more strongly with angiogenin than 3′-CMP which was in agreement with the experimental observations. Gln-117 of angiogenin produced a hindrance during pyrimidine binding at the B_1 site, which resulted in the relatively lower ribonucleolytic activity of the protein [21]. In the docked conformation of 3′-CMP with angiogenin the phosphate group was within hydrogen bonding distance of His-13 (2.76 Å) and His-114 (3.00 Å). The presence of the sugar moiety along with phosphate anchoring renders the compound less effective as an inhibitor compared to **2** and **3** (Fig. 8). For the compounds **2** and **3**, the longer chain and inherent flexibility provides a positional advantage of the thymine nucleobase to avoid the hindrance offered by Gln-117 during binding at the B_1 site. This is most likely the cause for the better inhibition of **2** and **3**.

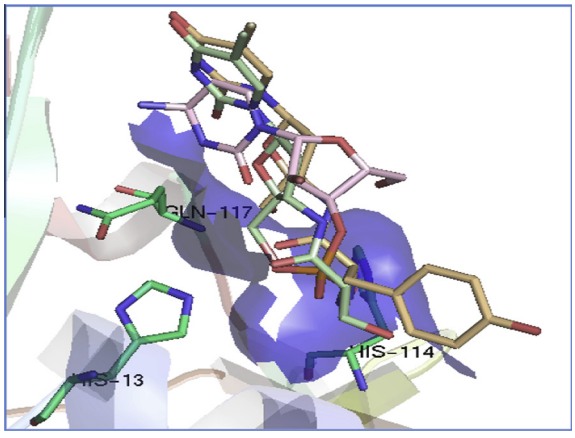

Fig. 8. Combined docking poses of **2**, **3** and 3′-CMP with human angiogenin (1B1I).

From the accessible surface area (ASA) calculation between angiogenin (uncomplexed) and the respective docked complexes with compounds **2**, **3**, **5**, **6** it is clearly evident that these ligands binds with the active site residues and make them less accessible towards the solvent molecules (Table 3). From the table it is well documented that compounds **2** and **5** preferentially interact with His-13 and Lys-41 compared with His-114.

Amino and carboxy functionalized nucleosides showed to inhibit the ribonucleolytic activity of angiogenin. The inhibitory activity of these two set of compounds was found to be dependent on the polarity of the amino acid side chain part and on the hydrophobicity of the dibasic acid part. Inhibition constant values of **2**, **3**, **5** and **6** are 427 ± 7, 1251 ± 4, 775 ± 6 and 1,114 ± 3 μM respectively, which are much lower than 2′-CMP, 3′-CMP, 2′-UMP and 5′-AMP (K_i values are 8700, 13000, 13100 and 7200 μM respectively) [8]. CAM assay explored that angiogenin-induced angiogenesis was also inhibited by amino functionalize nucleosides **2** and **3**. Along with the lower K_i values, these compounds are less polar compared with their nucleotidic analogs. Therefore, these synthesized compounds can be used *in vivo* more effectively as a potential antiangiogenic drugs.

3. Materials and methods

3.1. Materials

Recombinant human angiogenin, human serum albumin (HSA), were from Sigma-Aldrich. 6-FAM-dArUdAdA-6-TAMRA was purchased from Integrated DNA Technologies (Coralville, IA). All other reagents were from SRL India. UV-Vis measurements were made

Table 3
Change in accessible surface area (ΔASA) in Å^2 of interacting residues of angiogenin (Uncomplexed), and its complexes with compounds **2**, **3**, **5** and **6**.

Amino acid residues	Compound **2**	Compound **3**	Compound **5**	Compound **6**
Arg-5	00	22.25	00	00
Gln-12	34.36	00	00	00
His-13	39.62	17.31	32.63	6.23
Lys-40	17.25	00	18.32	00
Asp-41	21.33	9.36	21.59	00
His-114	16.49	15.28	7.61	16.51
Leu-115	00	00	8.48	28.74
Asp-116	00	00	00	13.65
Gln-117	00	00	00	6.06
Ser-118	00	00	00	10.92
Arg-121	00	00	11.24	6.85
ΣΔACS	129.05	64.2	99.87	88.96

using a Perkin Elmer UV–Vis spectrophotometer (Model Lambda 25). Fluorescence measurements were recorded on a Spex Fluorolog-3 machine. Concentrations of the solutions were determined spectrophotometrically using the following data: ε_{278} (angiogenin) [22], ε_{260} (6-FAM-dArUdAdA-6-TAMRA) [23] are 12500 and 102,400 M^{-1} cm^{-1} respectively.

3.2. Precipitation assay with angiogenin

Inhibition of the ribonucleolytic activity of angiogenin was quantified following the method as described by Bond [18]. In this method 5 μl of angiogenin (0.2 μM) was mixed with 10 μl of 20 μM of **1**, **2**, **3**, **4**, **5**, **6**, **7** and **8** individually along with 20 μl of 6-FAM-dArUdAdA-6-TAMRA (0.1 mg/ml) and 20 μl of HSA (0.02 mg/ml) to a final volume of 55 μl and incubated for 5 h at 37 °C. After incubation, the reaction mixture was quenched with 100 μl of ice-cold 1.14 N perchloric acid containing 6 mM uranyl acetate. The solution was then kept in ice for another 30 min and centrifuged at 4 °C at 12000 rpm for 5 min. 100 μl of the supernatant was taken and diluted to 550 μl. The change in absorbance at 260 nm was measured and compared with a control set.

3.3. Inhibition kinetics with angiogenin

Purchased recombinant human angiogenin contains a substantial amount of bovine serum albumin (BSA). Pure angiogenin was obtained after removal of BSA using Amicon Ultra centrifugal filter devices (Millipore) with a 30kD molecular weight cutoff. Purified angiogenin was checked to be free of contaminating ribonucleases by zymogram electrophoresis [24,25]. 6-FAM-dArUdAdA-6-TAMRA was used as substrate where 6-FAM is 5′,6-carboxyfluorescein and 6-TAMRA is 3′,6-carboxytetramethylrhodamine. Kinetic experiments were carried out in oligovinylsulfonic acid free [21] 0.1 M Mes-NaOH buffer (pH 6.0) containing 0.1 M NaCl using substrate concentration from 20 to 122 nM. For **2**, **3**, **4** and **5** the concentration ranged from 0 to 0.8, 0 to 1.2, 0 to 1.2 and 0 to 2.5 mM respectively. The angiogenin concentration was 0.36 μM. An increase in fluorescence emission intensity at 515 nm, upon excitation at 490 nm, indicated the progress of the reaction [19]. The inhibition constants (K_i) were determined from initial velocity data using Lineweaver-Burk plots.

3.4. Angiogenic activity

The effect of **2** and **3** on angiogenin-induced angiogenesis was assessed by the chick embryo chorioallantoic membrane (CAM) assay as described by Fett et al. [1] The chorioallantoic membrane of the chicken egg was exposed by carefully drilling a small hole through the shell on day 2. 10 μl aliquots of angiogenin, angiogenin mixed with **2** and **3** separately were placed on transparent plastic disks and dried under laminar flow. The disks were inverted over the chorioallantoic membrane on day 10 and response was assayed microscopically after 48 h. The density of blood vessel formation was compared with a control set.

3.5. Docking studies of compounds 2, 3, 5 and 6 with angiogenin and calculations of interaction energetic

The crystal structure of human angiogenin (PDB entry 1B1I) was downloaded from the Protein Data Bank [26]. The 3D structures of the nucleoside-amino acid conjugates were generated by Sybyl6.92 (Tripos Inc., St. Louis, USA) and their energy-minimized conformations were obtained with the help of the TRIPOS force field using Gasteiger-Hückel charges with a gradient of 0.005 kcal/mole. The *FlexX* software as part of the Sybyl suite was used for docking of the nucleoside-amino acid conjugates with RNase A. PyMol [27] was used for visualization of the docked conformations. The total ligand-receptor interaction energies of the docked structures for protein-ligand complexes was computed using *PEARLS* [20].

3.6. Accessible surface area calculation

The accessible surface area (ASA) of angiogenin (uncomplexed) and their docked complexes with compounds **2**, **3**, **5**, **6** were calculated using the program NACCESS [28]. The structures corresponding to the minimum score as obtained from the FlexX analysis of the protein-ligand docked structures were chosen in each case. Composite coordinates of the angiogenin and inhibitors were generated to form the docked complex. The change in ASA for residue, ***i*** was calculated using: $\Delta ASA^i = ASA^i_{angiogenin} - ASA^i_{angiogenin\text{-}inhibitor}$. If a residue lost more than 10 $Å^2$ ASA when going from the uncomplexed to the complexed state it was considered as being involved in the interaction.

Acknowledgments

J.D. thanks CSIR, India for a fellowship. S.D. and T.P. are grateful to the Department of Science and Technology (DST), New Delhi, India, for financial support.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.11.005.

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