Journal of Cell and Molecular Research (2021) 12 (2), 87-96 DOI: 10.22067/jcmr.v12i2.89670

Research Article

Comparative *in silico* Survey of Retinoic Acids as Putative Biomolecules Against VEGF Receptors: A Glycomics-based Approach

Ali Fallah, Esmaeil Samadian, Azadeh Mohammad-Hasani, Abasalt Hosseinzadeh Colagar*

Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran

Received 17 November 2020 Accepted 3 January 2021

Abstract

In this study, putative interactions between all of the retinoic acid (RA) ligands (all-trans (At), 9-cis (9c), and 13-cis (13c)), and VEGF receptors (VEGFR-1, -2 and -3) were investigated. It was performed considering the glycosylation status of the receptors to achieve a more reliable mode of interactions based on glycomics. We found that RAs may have a higher affinity for ligand-binding domains in VEGFRs. Furthermore, all RA isomers can strongly attach to VEGFR-3 receptor in comparison to other ones. It was also demonstrated that receptor dimerization of RAs may be less targeted. Moreover, regarding post-translational modifications, glycosylated structures showed conflicting binding energies. RAs may target the human vasculature, specifically lymph vessels, through VEGFR-3. In addition, the ligand binding-mediated activation of VEGFRs may be affected by these agents. Also, the glycosylation status of the receptors can interfere with these manners. Furthermore, our results confirmed that the consideration of carbohydrates in crystal structures is essential for a better interpretation of ligand/receptor interactions during drug discovery studies. Even though these observations improved our understanding of the binding patterns of RAs to VEGFRs, validation of these results needs further analysis to introduce these biomolecules as anti-VEGF remedies.

***Keywords*:** Retinoic acids, Angiogenesis, VEGF receptors, Glycomics, *in silico*

Introduction

Organization of blood and lymphatic vessels, vasculogenesis and angiogenesis, is critical to provide whole-body with fresh oxygen and nutrient supply and remove catabolites. At the cellular level, activation of vascular endothelial growth factor receptors (VEGFR-1, -2, and -3) by their cognate ligands (different isotypes of VEGF) is the most important receptor tyrosine kinase/RTK-related signaling pathway. This pathway adjusts multiple processes which are essential for developmental, physiological, and pathological neovascularization (Christensen et al. 2017; Lee et al. 2017; Qiao et al. 2006; Shibuya 2013). The VEGFs are signal proteins which are produced by cells to trigger the formation of vessel networks via binding to their corresponding RTKs. All isoforms of VEGF-A can bind to VEGFR-1 and VEGFR-2, while VEGF-B is special for VEGFR-1 (Álvarez-Aznar et al. 2017; Goel and Mercurio 2013; Jeltsch et al. 2006; Leppänen et al. 2010). VEGF-C and VEGF-D isoforms with cleaved C-terminal domain are high-affinity ligands for VEGFR-3. Upon elimination of both pro-peptides, they obtain binding affinity for VEGFR-2. VEGF-E which exists in poxviruses can specifically bind to VEGFR-2 (Mercer et al. 2002).

As the ligand binding to the extracellular domain (ECD) of VEGFRs is required for their stimulation, the disruption of these ligand/receptor complexes deregulates their RTK and normal physiological activities. For this reason, ligand trapping from VEGFR-1 is resulted in the placental loss of preeclampsia and abnormalities in retinal and corneal vascularization (Markovic-Mueller et al. 2017). In contrast, VEGFR-1 traps the main angiogenic ligand VEGF-A from VEGFR-2 and displays a negative regulatory role against VEGFR-2-induced blood vessel sprouting. Alternatively, VEGFR-2/VEGFR-3 heterodimerization is an additional signal to facilitate this sprouting. Moreover, VEGFR-3 homodimers mediate lymphogenesis. So, a defective VEGFR-3 cascade results embryonic death (Leppänen et al. 2013; Qiao et al. 2006).

Considering the fact that classical anti-VEGF compounds are clinically administrated to prevent

*Corresponding author's e-mail address:
ahcolagar@umz.ac.ir, acolagar@yahoo.com

particular disease (cancer, retinopathy, endometriosis, and so forth)-associated neovascularization, the discovery of detailed molecular mechanisms of blood vessel formation is essential to design advanced anti-angiogenic remedies (Hegde et al. 2018; Ma & Ning 2019).

The glycome is the entire complement of sugars, whether free or present in more complex molecules of an organism. Glycomics is a comprehensive and multi-aspect study of glycome (Rudd et al. 2017). 32 types of sugar linkages were reported in various Saccharides. These building blocks can increase the degree of complexity. Sugar structures, are highly branched. Glycans are highly dynamic as they can bound to proteins or conjugate with lipids to form modified structures (Aizpurua-Olaizola et al. 2018). Glycans play significant roles during viral/bacterial recognition, cell signaling events, intrinsic immunity modulation, prohibition of cell proliferation, cancer expansion, cell fate determination, invasion, circulation arrangement, protein folding and other important biological procedures (Aizpurua-Olaizola et al. 2018; Rudd et al. 2017). Hence, glycomics is widely applicable in the clinic as it was reported previously (Reid et al. 2012).

Currently, we know that glycosylation facilitates signal transfer from the surrounding microenvironment to inside the cell. The structure of carbohydrates has direct impact on ligand/receptor binding, dimerization, and internalization of various signaling complexes, and, thus activation of different cell receptors. RTKs are the most prevalent type of Asparagine (N)-linked glycans that transduce signals which are important for cell communications. Accordingly, it could be concluded that VEGFRs mediate main functional properties of the vascular endothelial cells during angiogenesis. There are 13, 18, and 12 N-glycosylation sites with different carbohydrate side-chains in VEGFR-1, -2, and -3, respectively (Contessa et al. 2010; Itkonen and Mills 2013; Lopez-Sambrooks et al. 2016). Although it has not been fully understood so far, these sugar residues, which are not completely detected in crystal structures, may also affect the conformational features of VEGFRs. Therefore, comprehending the effects of the sugar structures on the binding of a candidate drug VEGFRs improves our knowledge about the binding mode of action of drug/VEGFRs such complexes. also, this direct impact is important for a glycomics-based point of view in drug design against VEGFRs.

Retinoids are physiological derivatives of vitamin A which have several positive impacts on normal cell growth and differentiation, tissue homeostasis, developmental organogenesis, and visual performance (Khalil et al. 2017; Mallipattu and He 2015; Zhu et al. 2015). However, they may have destructive effects on the morphogenesis during human embryogenesis (Gudas, 1994; Rönn et al., 2015). All retinoids including all-trans (At), 9-cis (9c), and 13-cis (13c) retinoic acids (RAs) emerge more recently as antineoplastic agents. Accordingly, on the contrary to the harmful influence of At-RA, a main active isomer of RA, (Rühl et al. 2018; Tsuji et al. 2015; Hu et al., 2020) that frequently causes embryonic death due to developmental deformity, it has been used in cancer therapy towards acute promyelocytic leukemia (Huang et al., 1988) colorectal cancer, pancreatic cancer, oral leukoplakia and skin cancer (Applegate and Lane 2015; Di Masi et al. 2015; Lodi et al. 2016; Moon et al. 1997; Tarapcsák et al. 2017; Uray et al. 2016; Szymański et al., 2020).

Interestingly, although we know that RAs are diffusible biomolecules (Minkina et al. 2017) and exert their impressions on gene regulation via nuclear receptor RAR/RXR. However, several studies demonstrated that RAs can tune-up the angiogenesis through binding to VEGFRs with an unclear pattern (Njar et al. 2019; Simandi et al. 2018; Urushitani et al. 2018; Costantini et al., 2020).
In the present study, via a computational-based approach we investigated the binding manner of At-RA, 9c-RA, and 13c-RA with the extracellular regions of three human VEGFRs (R-1, R-2, and R-3). We found an alternative mechanism which is responsible for the VEGFR-mediated inhibitory implications of RAs during angiogenesis. Furthermore, regarding the importance of VEGFRs' saccharide residues inactivation, we also compared the possible binding sites of three isoforms of RAs to both glycosylated and unglycosylated crystal structures of VEGFRs introduced so far.

Materials and Methods

Preparation of structures

Retinoic acids are small biomolecules with an average mass of about 300.435 kDa. The structures of interest isoforms (At-RA, 9c-RA, and 13c-RA) have been retrieved from PubChem database (http://pubchem.ncbi.nlm.nih.gov/) in structure-Data file (SDF) format which is an acceptable input for our docking program. Identifier (ID)s of retrieved isoforms from public repository were as

follows: 6419707; 449171, and 5282379, respectively (Dunning 2018). Also, the PDB files of human VEGFR1-3 have been obtained from the Protein Database (http://www.pdb.org). All structures were prepared by WebLab (http://weblab.cbi.pku.edu.cn) (Liu et al., 2009) and the visual molecular dynamics (VMD 1.9.3) package (www.ks.uiuc.edu/Research/vmd/) (Zhang 2015). The Swiss-PdbViewer modeling program was applied to perform energy minimization of all investigated structures (http://www.expasy.org/swissmod) (Guex et al. 2009). As the cut-off for bond, torsion, angle, improper angles, non-bounded and electrostatic functions was set for 10 Å by default, energy (E) exchanges between two steps was stopped below 0.05 kJ/mol, and the acting forces between any atom stopped below 10 Å.

The structural details including PDB IDs, domains, length, and glycosylation status of the extracellular regions of our studied VEGF receptors are summarized in the Table 1.

In order to minimize the energy of all 3D structures prepared by the Web Lab, we performed the energy minimization procedure using Swiss-PdbViewer (DeepView). The first (E1) and the end (E2) energy amounts for each molecule were listed in the Table 2. Moreover, docking energies obtained for different RA-VEGFR complexes using HEX 8.0 were described in Table 1.

A comparison of assessed docking energies for various RA-VEGFR complexes in this work indicated that At-RA, 9c-RA, and 13c-RA (-256.10, -256.11, and -248.56 kJ/mol, respectively) are highly bound to VEGFR-3 4BSK (Table 1). This is also occurred at different amino acid residues in VEGFR-3 D2 (OA-133: ARG-CA to OA-190: GLY-CA) (Figure 2).

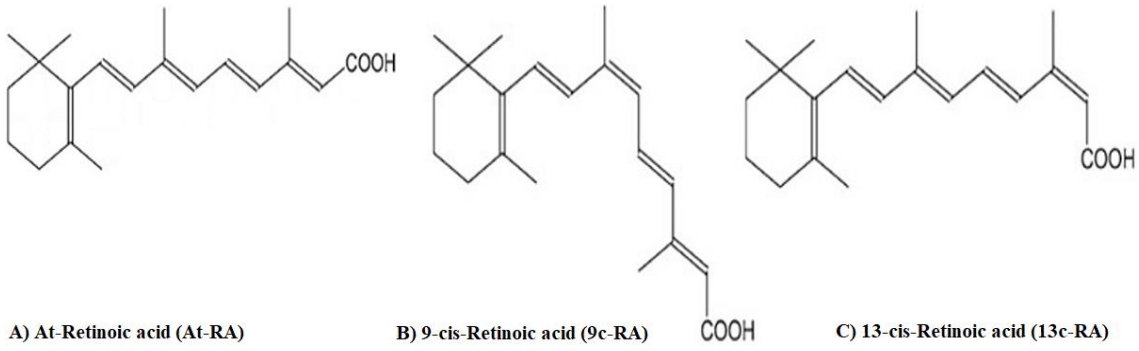

Figure 1. Schematic structural representation of retinoic acid isoforms including all-trans (At), 9-cis (9c), and 13-cis (13c) retinoic acids (RAs). At-RA is the main active isomer of RA (http://pubchem.ncbi.nlm.nih.gov/).

Docking parameters

Docking studies were carried out using HEX 8.0 (http://hexserver.loria.fr), which is an interactive molecular graphics program to calculate and display feasible docking modes of pairs of protein and ligand molecules (Roudini et al. 2020). Moreover, it can predict the position of ligand-receptor contacts approximately. All docking parameters were left as their default values (Pourhashem et al. 2017).

Results

The molecular structures of retinoic acid isoforms including At-RA, 9c-RA, and 13c-RA were represented in Figure 1 as they were obtained from PubChem. Chemical differences and reactive groups are shown for each structure.

Besides, our ligands can attach to other investigated structures with lower tendencies and they have the lowest binding mode in complex with VEGFR-1 D2 (1QTY), as demonstrated in Table 1. Also, we found At-RA, 9c-RA, and 13c-RA bind slightly to VEGFR-2 and VEGFR -1 D3 (residues from [O] R-143: ASN-CA to [O] R-278: LYS-CA and [O] X-241: LYS-CA to [O] X-331: LYS-CA, respectively) (Figure 2). In addition, as it was demonstrated in Figure 3 regarding the trends of binding energies for RA-VEGFR complexes, a decreased binding mode was detected for VEGFR-1 D3 (5T89) in comparison to VEGFR-2 D3 (2X1X) and also for VEGFR-2 D3 (2X1X) in comparison to

Table 1. Structural details (Chain, Domain, Glycosylation status) for VEGFRs and docking simulations for RAs-VEGFRs.

Receptors	PDB IDs	Chain	Domain (amino acid positions)	Glycosylation status		Docking Energy (kJ/mol)		
				Sugars and aa positions	Number of glycosylation sites	At-RA	9c-RA	13c-RA
VEGFR1	1FLT	95aa (Phe X135-Thr X226)	D2 (aa151 – aa 214)	-	-	-228.38	-223.9	-209.82
	1QTY	101aa (Phe T135-Gln T225)	D2 (aa151 – aa 214)	-	-	-15.76	-17.29	-18.19
	5T89	646aa (Asp 31-Thr Y654)	D1 (aa32-aa123), D2 (aa151-aa214), D3 (aa230-aa327), D4 (aa335-aa421), D5 (aa428-aa553), D6 (aa556-aa 654)	ASN X 100 (NAG X 701 & NAG X 702), ASN X 196 (NAG X 703), ASN X 251 (NAG X 704), ASN X 323 (NAG X 705), ASN X 402 (NAG X 706), ASN X 417 (NAG X 707 & NAG X 710), ASN X 574 (NAG X 708), ASN X 625 (NAG X 709) / ASN Y 100 (NAG Y 701), ASN Y 164 (NAG Y 702), ASN Y 196 (NAG Y 703 & NAG Y 704), ASN Y 251 (NAG Y 705), ASN Y 402 (NAG Y 707), ASN Y 417 (NAG Y 708), ASN Y 547 (NAG Y 709), ASN Y 625(NAG Y 710)	16	-217.79	-231.50	-218.01
VEGFR2	2X1W	213aa (Phe L 125-Glu L 326)	D2 (aa141-aa207), D3 (aa224-aa320)	ASN L 143 (NAG L 2401), ASN L 245 (NAG L 2601 & NAG L 2602), ASN L 318 (NAG L 2701)/ ASN M 245 (NAG M 1201 & NAG M 1201), ASN M 318 (NAG M 1301, NAG M 1302 & BMA M 1303)/ ASN N 143 (NAG N 1401), ASN N 245 (NAG N 1601), ASN N 318 (NAG N 1701 & NAG N 1702)/ ASN O 143 (NAG O 2001), ASN O 158 (NAG O 2101), ASN O 245 (NAG O 2201 & NAG O 2202), ASN O 318 (NAG O 2301).	12	-244.06	-244.45	-241.28
	2X1X	213aa (Pro R124-Glu R326)	D2 (aa141-aa207), D3 (aa224-aa320)	ASN R 143 (NAG R 404), ASN R 245 (NAG R 402 & NAG R 403), ASN R 318 (NAG R 405 & NAG R 406).	3	-251.99	-245.43	-242.62
	3KVQ	108aa (Ile A669-Gly A756)	D7 (aa667-aa753)	-	-	-241.31	-247.96	-240.43
	3V6B	424aa (His R131-Phe R329)	D2 (aa141-aa207), D3 (aa224-aa320)	-	-	-189.43	-206.67	-186.63
VEGFR3	4BSJ	232aa (Asp A328-His A554)	D4 (aa331-aa415), D5 (aa422-aa552)	ASN A 411 (NAG A 601), ASN A 515 (NAG A 602)	2	-51.50	-49.20	-43.38
	4BSK	214aa (Pro A30-Ile A224)	D1 (aa30-aa127), D2 (aa151-aa213)	ASN A 33 (NAG A 301), ASN A 104 (NAG A 302), ASN A 166 (NAG A 303)	3	-256.10	-256.11	-248.56

*ASN: Asparagine

* NAG: N-acetyl glucose

Table 2. Energy minimization (first and end energy) for VEGFRs.

Receptor	PDB IDs	Chain	E_1 (first energy minimization kJ/mol)	E_2 (end energy minimization kJ/mol)
VEGFR1	1FLT	X, Y	-6040.213	-10363.84
	1QTY	T, U, X, Y	-4588.174	-22151.435
	5T89	X, Y	-40754.605	-71025.164
VEGFR2	2X1W	L, M, N, O	-23284.633	-37331.891
	2X1X	R	+343794.188	-11717.921
	3KVQ	A	-1688.371	- 4945.834
	3V6B	R	-2886.585	-10666.744
VEGFR3	4BSK	A	16982.123	-9005.400
	4BSJ	A	-7782.256	-12937.562

Figure 2. Docking of three retinoid types (At-RA, 9c-RA, and 13c-RA) with VEGFR-1, 2, and 3 complexes: A1-3, B1-3, and C1-3 are docking results of retinoid types with VEGFR-1 (PDB-ID 5T89), VEGFR-2 (PDB-ID 2X1X), and VEGFR-3 (PDB-ID 4BSK), respectively. RA isoforms differentially bind to different domains of VEGFRs.

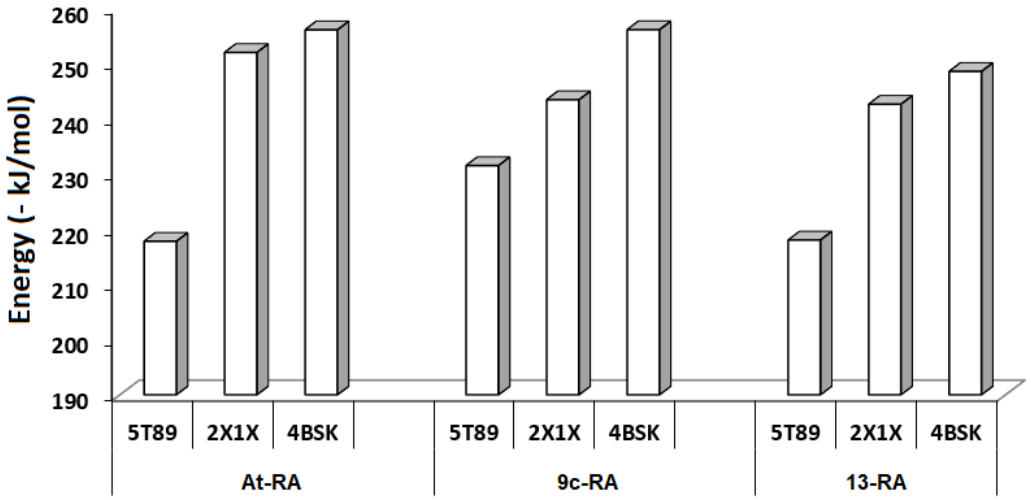

Figure 3. Docking energy amounts are shown for various complexes of At-RA, 9c-RA, and 13c-RA isoforms with different VEGF receptors, including VEGFR-1 D3 (5T89), VEGFR-2 D3 (2X1X), and VEGFR-2 D3 (2X1X).

VEGFR-3 D2 (4BSK) in a gradual mode in case of 9cRA isoform. A similar trend was observed for both At-RA and 13-RA isoforms, while differences were more remarkable for 5T89 in comparison to 2X1X and 4BSK. According to crystal structures of three VEGFRs, structures 5T89, 2X1W, and 4BSK, for VEGFR-1, -2, and -3, have resolved the higher number of carbohydrate residues among other structures studded up-to-now, respectively (Table 1). Considering the glycosylated VEGFR-2 structures, 2X1W (with a higher glycosylation status) has a lower docking tendency (At-RA: -244.06; 9c-RA: -244.45; 13c-RA: -241.28, kJ/mol) in comparison to a less glycosylated structure 2X1X (At-RA: -251.99; 9c-RA: -245.43; 13c-RA: -242.62, kJ/mol). These observations were not confirmed for other receptors such as glycosylated 2X1X with a lower energy versus non-glycosylated 3V6B with a higher energy (Table 1).

Discussion

The regulatory function of the VEGF signaling system has comprehensively been established in normal *de novo* and pathological revascularization. This cascade is modulated by the activation of VEGF receptor family members (VEGFR-1, -2, and -3). VEGFR-2 triggers the blood vessels organization whereas VEGFR-1 plays a negative feedback for VEGFR-2 function (Greenberg et al. 2008; Stuttfeld and Ballmer-Hofer 2009). In VEGFR-3-related signaling pathways, VEGFR-3 (only) induces lymphatic vascularization and also in co-operation with VEGFR-2 mediates blood angiogenesis (Leppänen et al. 2013). These trans-membrane RTKs have seven extra-cellular immunoglobulins (Ig)-like domains (D1-7) and their activation process involves the ligand binding through D1-3, dimerization through D4-7, and auto-phosphorylation of the cytoplasmic kinase domains. The ligand-mediated induction mode via a membrane-distal domain 2 (D2) is conserved for all VEGF receptors. Additionally, for both VEGFR-1 and 2, D3 (not mainly) is used for ligand attachment (Leppänen et al. 2010; Leppänen et al. 2013; Markovic-Mueller et al. 2017).

Recent studies disclose the potential of retinoids to interrupt with angiogenic processes. Weninger et al. in 1993 illustrated that retinoids are potent inhibitors of VEGF/VPF production by normal human keratinocytes. These cells may contribute to the therapeutic effects of retinoids (Weninger et al. 1998). Also, in 2007 Noonan et al. showed that the synthetic retinoid 4-hydroxy fenretinide (4HPR) displays anti-angiogenic effects (Noonan et al. 2007). In addition, microarray data by Albini et al. demonstrated that some anti-angiogenic agents, such as N-acetyl-cysteine and 9c-RA, induce molecular indices in endothelial cells which mimic *in vitro* senescence (Albini et al. 2012). Tsuji et al. showed evident data for the binding of retinoic acid isomers (At-RA, 9c-RA, and 13c-RA) with RXRs and RARs (Tsuji et al. 2015). Moreover, Zhong et al. confirmed that CYP26C1 is a 4-oxo-atRA hydroxylase and probably be important for adjusting the condensation of active retinoids in human tissues (Zhong et al. 2018).

Taken together, there is no clear data indicating that how RAs affect VEGFRs from outside the cell.

Here for the first time, the binding patterns of RAs with VEGFRs were clarified using a docking-based approach. Results revealed that all three RA isomers (At-, 9c-, and 13c-RA) can remarkably bound to VEGFR-3. In comparison to interactions with other domains, RAs have a higher affinity to D2 in VEGFR-3 (Figure 2). This may suggest a novel mechanism for these agents which targets the vasculature, especially through the ligand (VEGF-C) detachment from its respective receptor which leads to the lymph/blood vessels deterioration. These biomolecules can also interfere with blood vessels formation through the inhibition of VEGF-A-VEGFR-2 and (with a lower affinity) VEGFR-1 (Table 1). Although RAs may have strong contacts with similar domains, as depicted in VEGFR-3 D2 (Figure 2) or VEGFR-1/-2 D3 (Figure 2), they can't interact with similar residues, may be owing to variations in RA isomers (Figure 1). Actually, structural remodeling for receptor dimerization in D4-7 could not be sharply affected by RAs, considering the competed energies which were demonstrated in Table 1. As the kinase activity of VEGFRs is fundamentally concerted by their glycosylation status (Gomes Ferreira et al. 2018), their glycosylated 3D structures were also considered in the current study. In this regard, glycosylation may lead to a lower affinity of RAs for VEGFR-2 (2X1W) when compared with 2X1X. Conversely, glycosylation may affect the 5T89 folding in a way that different modes achieve in RA-VEGFR-1 docking assessments. These inconsistencies in binding energies between glycosylated and unglycosylated structures suggest that sugar residues can alter the ligand-binding manners. Accordingly, post-translational modifications like glycosylation should be considered in such docking studies to reach a reliable behavior. It implies the importance of glycomics-based approaches in ligand/receptor binding studies.

Taking a computational analysis, instead of a real experiment, is easy and cost-benefit, though, validation of the outputs by real experiments are inevitable. It is clear that all factors applied during simulations are based on approximation and are under control by users. Currently, by advances in computational biology docking of several thousands of ligands were performed which can be applied in pharmaceutical industries to design improved structure-dependent drugs (Yadav et al. 2018). Nevertheless, we can have a better interpretation of the applications of RAs in tissue regeneration, wound repair, and cancer therapy or other VEGF-associated system uses in the future. thus, molecular dynamics and experimental analysis in further investigations may confirm our findings.

Acknowledgments
Thanks to Zeinab Pourhashem (School of Advanced Technologies in Medicine, Golestan University of Medical Sciences, Gorgan, Iran) for their scientific support. We are grateful to our colleagues for the critical reviews of the manuscript.

Conflicts of interest
The authors declare that they have no conflict of interest.

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