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Design, Synthesis and Structure-Activity Relationship Analysis of Charged Biotinylated Arg-Gly-Asp Derivatives against MDA-MB-231 Cells

Bonaventure M[1], Shoufia J[2], Hemamalini V[2], Sivasamy R[3] and Suja S[1]*

[1]Department of Biochemistry, Bharathiar University, Coimbatore, India

[2]Department of Bioinformatics, Bharathiar University, Coimbatore, India

[3]Department of Human Genetics & Molecular Biology, Bharathiar University, Coimbatore, India

Abstract

Various studies have been done successfully to show the electrostatic interactions of charged biomolecules against the cancer cell surface but few have analyzed the effect of these electrostatic interactions on the ligand-receptor interactions. In this study, negatively charged N-Biotin-RGD and positively charged C-Biotin-RGD were designed, synthesized, and characterized with docking analysis. The fixation of MDA-MB-231 cells with formalin made their cell surface neutrally charged thus removing the electrostatic interactions between charged biotinylated RGD derivatives and MDA-MB-231 cells. The results of the binding affinity of biotinylated RGD derivatives against MDA-MB-231 cells showed that N-Biotin-RGD had higher binding affinity than C-Biotin-RGD. The cytotoxic effect was analyzed by incubating charged biotinylated RGD derivatives with live MDA-MB-231 cells. MDA-MB-231 cell surface is negatively charged due to high Hypersialylation of polyglycan and Warburg effect. The results of their cytotoxic activities against live MDA-MB-231 cells were found to be electrostatic in nature. C-Biotin-RGD had an attractive interaction with the MDA-MB-231 cell surface resulting in a higher cytotoxic effect. In comparison, N-Biotin-RGD had a repulsive interaction with the MDA-MB-231 cell surface resulting in a lower cytotoxic effect. Hence, positively charged C-Biotin-RGD is a better cytotoxic agent than a negatively charged N-Biotin-RGD against MDA-MB-231 cells.

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Correspondence:

Suja Samiappan, Department of Biochemistry, Bharathiar University, Coimbatore - 641 046, Tamil Nadu, India, Tel: 0422-242-8560; Mob: +91-8754425857

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Introduction

Cancer cells are caused by epigenetic and genetic changes that make the resulting abnormal cells resistant to the normal regulatory checkpoints [1,2]. These changes are continuously triggered by the cancer cells in order to take advantage to their ever-changing intracellular activities and their surrounding environment [3]. This includes the need for increased amounts of biomolecules that are involved in metabolism and proliferation [4]. Cancer cells maximize their energy production by adopting or altering the pathways that give high output of bioenergetic molecules. Aerobic glycolysis is used as main source of ATP molecules for highly dividing cancer with lactic acid as its byproduct [5]. Hypersialylation is recognized as a hallmark for various cancers including breast cancer. The addition of sialic acid on glycoconjugate chains results in promotion of tumor development, inhibition of cellular apoptosis, induction of cell detachment, improvement of cell invasion, enhancement of immune evasion, and induction of metastases [6]. The overexpression of lactic and sialic acids is directly proportional to the negative charges on the cancer cell surface [7].

The increased knowledge of the unique biochemical features of cancer cells have resulted in a predictive approach of designing small biomolecules that are complementary in shape to the binding sites of the intended targets. Among various approach, computer modeling is one of the leading techniques for designing drug candidates [8,9]. The application of drug design for diagnosis and treatment of various cancers usually focuses on features that are the source of the hallmarks of cancer [10]. This includes the biomolecular expressions that are either overexpressed or uniquely expressed in tumor cells. The extracellular receptors are the direct link of communication between the cells and its environment, they are the best options to target as they are easily accessible and can be analyze straightforwardly. Among the most involved receptors, integrins are unique as they are involved in most stages of cancer development including the formation of solid tumor, migration to neighboring local regions, anoikis resistance when transported in blood vessels and attachment

to distant organs during metastasis [11]. Integrins are classified into various subtypes depending on the sequence they recognize and a subset that binding with Arg-Gly-Asp (RGD) motif represent almost half of all the integrins [12].

RGD is a tripeptide of Arginine, Glycine and Aspartic acid residues. Arginyl residue is responsible for the positive charges due to the α-amino group and the guanidine side chain while aspartic acid residue provided the negative charge due to carboxyl groups of both the side chain and the C-terminal group. This makes RGD tripeptide a zwitterion as both charges neutralized each other to give a neutrally charged peptide. RGD tripeptide has a low cell attachment activity due to its highly flexible conformation when interacting with integrins. However, the blocking of either one of the N- or C-terminal ends or both terminals resulted in improved cellular activity [13]. Thus, biotin derivatives such as biotin-NHS and biotin hydrazide can be used respectively to block N- and C-terminal ends of biomolecules such as peptides [14,15]. This process could be used to improve the binding of RGD towards integrins but also to create positively charged as well as negatively charged derivatives. The presence of biotin is also useful for qualitative as well as quantitative analyses due to the fact that its interaction with streptavidin and its derivatives is among the most stable non-covalent interactions found in nature. Thus, as aerobic glycolysis is mostly suited to highly proliferating cancer cells, it can be targeted using positively charged biomolecules while slow growing cells could be targeted by focusing on their increased expression of integrins.

The hypotheses of this study mainly are; (i) *in-silico* drug design could improve the binding affinity of RGD tripeptide on RGD-recognizing integrins by conjugating it with biotin; (ii) The resulting charged biotinylated RGD derivatives could have electrostatic interactions with negatively charged cancer cells; (iii) molecular-based analysis with fixed cells resulting in neutrally charged cancer cells could be used to determine the binding affinities of these biotinylated RGD derivatives; (iv) cell-based analysis using live cells could be used to determine the involvement of electrostatic interaction in cytotoxic activities; (v) structure-activity relationship could be used to determine the best biotinylated RGD derivative for the treatment of breast cancer.

Materials and Methods

In-silico studies

***In-silico* design of charged biotinylated RGD derivatives:** The structure of biotinylated RGD tripeptides were drawn using Chemsketch freeware. N-Biotin-RGD was designed by taking N-atom of the amino terminal of RGD tripeptide and linking it with C atom of the carboxyl group of biotin. C-Biotin-RGD was designed by linking the last N atom of the hydrazyl group of biotin hydrazide with C atom of carboxyl end of RGD tripeptide.

Confirmation of charges for biotinylated RGD derivatives: At the physiological pH, the overall charges of RGD tripeptide and its biotinylated derivatives depends on the pKa values of ionizable groups (Table 1) and were calculated using modified Henderson-Hasselbalch equations:

- For the amino terminal: $(-NH_2) \times (10^{pKa-pH} / 10^{pKa-pH} + 1)$
- For the carboxyl terminal: $(-COOH) \times (10^{-(pKa-pH)} / 10^{-(pKa-pH)} + 1)$
- For positively charged R group: $(R) \times (10^{pKa-pH} / 10^{pKa-pH} + 1)$

Table 1: pKa values of N- and C-terminal residues of RGD tripeptide.

Amino acid	pKa		
	(-COOH)	$(-NH_2)$	R group
Arginine (R)	2	9	12.5
Aspartic acid (D)	2	9	3.9

- For negatively charged R group: $(R) \times (10^{-(pKa-pH)} / 10^{-(pKa-pH)} + 1)$

Calculation of isoelectric points: The isoelectric point of an aqueous peptide solution is the pH at which both the positively charged groups and the negatively charged groups of the molecules are at equilibrium. The calculation of pI was done using the following formula:

$$pI = (pK_{a1} + pK_{a2}) / 2$$

Where pK_{a1} and pK_{a2} correspond to the values within which the charge of biotinylated RGD derivatives was zero.

Molecular docking studies: ITGB1 was downloaded from the Protein Data Bank (PDB ID: 4WJK) and its 3D structure was energy minimized using the OPLS3e force field. The selected ligands were prepared using LigPrep in Schrodinger Maestro 11.8. The ligands tautomer's were computed using the specific OPLS3e force field energy. Then the lowest binding energy which conforms to the best structure of the docked complexes was selected.

In-vitro studies

Materials: Arg–Gly–Asp (RGD) peptide, Biotin-NHS, Biotin-hydrazide, Cellulose acetate membrane (MWCO=500 Da), a single-sided magnetic biodialyzer, 1ethyl-3-Dimethylaminopropyl Carbodiimide hydrochloride (EDC) were purchased (Sigma Aldrich, India). DMEM, Fetal Bovine Serum (FBS), Bovine Serum Albumin (BSA), Penicillin-Streptomycin, Phosphate Buffer Saline (PBS) and Tween-20 were all purchased (HiMedia, India).

MBA-MD-231 cell culture: MBA-MD-231 cells were obtained from NCCS (Pune, India) and were cultured in T75 flasks with 10 ml of a medium consisting of high glucose DMEM with 10% FBS, and 1% Penicillin-Streptomycin at 37°C, under 5% CO_2 and 95% humidity. Subculturing was done approximately once every week at a ratio of 1:4, using 1 ml of 0.125% trypsin-EDTA.

Synthesis of N-Biotin-RGD: RGD peptide solution (2 mg in 1 ml of PBS) was mixed with biotin-NHS solution (20 mg in 1 ml of DMSO). The two solutions were mixed and incubated overnight at 4°C. The synthesized derivative was purified using a biodialyzer with a membrane of a molecular weight cutoff of 500 Da [14].

Synthesis of C-Biotin-RGD: RGD peptide solution (5 mg in 1 ml of 0.1M MES (2-N-Morpholino-Ethanesulfonic acid) at pH 5.5) was mixed with biotin hydrazide solution (13 mg in 1 ml of DMSO), then 250 µl of the EDC solution was added. The mixture was incubated overnight at room temperature under constant agitation. The synthesized derivative was purified using a biodialyzer with a membrane of a molecular weight cutoff of 500 Da [15].

Binding affinity assay: MDA-MB-231 cells were seeded in 96-well plate and incubated overnight at 37°C. The media was removed, the cells were washed with PBS-T, fixed with 4% formalin, blocked with 5% BSA, sample solutions were added and the plate was incubated overnight at 4°C. The cells were washed with PBS, stained

Table 2: Calculation of the charges of biotinylated RGD derivatives.

Ionizable groups	Guanidine side chain	Carboxyl terminal end	Amino terminal end	Carboxyl side chain	Net charge calculation
pKa and pH	12.5	2.0	9.0	3.9	**7.4**
Formulae for calculation of charges	$10^{pKa-pH}/10^{pKa-pH} + 1$	$10^{-(pKa-pH)}/10^{-(pKa-pH)} + 1$	$10^{pKa-pH}/10^{pKa-pH} + 1$	$10^{-(pKa-pH)}/10^{-(pKa-pH)} + 1$	**Sum total of all charges at pH 7.4**
RGD tripeptide	+1	-1	+1	-1	**0**
N-Biotin-RGD	+1	-1	NA	-1	**-1**
C-Biotin-RGD	+1	-1	+1	NA	**+1**

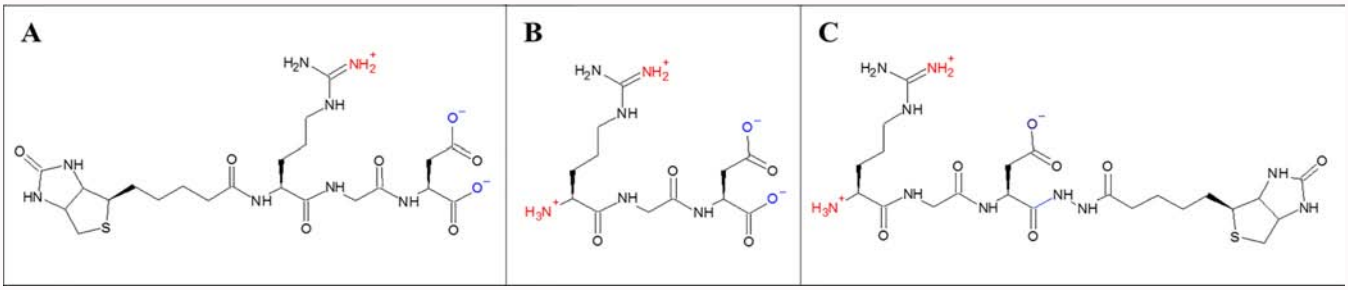

Figure 1: *In-silico* design of charged biotinylated RGD derivatives: A) N-Biotin-RGD; B) RGD tripeptide; C) C-Biotin-RGD.

Table 3: pI values of biotinylated RGD derivatives.

RGD tripeptide and its derivatives	Net charges between certain pH values					pI values
	0–2.0	2.0–3.9	3.9–9.0	9.0–12.5	12.5–14	
RGD tripeptide	+2	+1	**0**	-1	-2	**6.45**
N-Biotin-RGD	+1	**0**	-1		-2	**2.95**
C-Biotin-RGD	+2		+1	**0**	-1	**10.75**

with Streptavidin-HRP for 1 hour and incubated with TMB solution for 30 min. The enzyme activity was stopped and the optical densities were read at 590 nm [16].

Cytotoxicity assay: MDA-MB-231 cells were seeded in 96-well plate and incubated overnight at 37 °C. The media was removed and the sample solutions were added and the plate was incubated for 24 h at 37 °C. The media was removed and the cells were incubated with MTT solution for 4 h. The formazan crystals were solubilized with DMSO and the optical densities were read at 540 nm [17]. The cell death percentage was calculated using the following formula:

Cell death % = 100 - [(OD of treated cells/OD of control cells) × 100]

Where, OD refers to the Optical Density at 540 nm.

Statistical analysis: All experiments were carried out in triplicate and the results were expressed as Mean ± SD using GraphPad Prism 8.0.2. (263). Statistical comparison of mean values was performed with one-way Analysis of Variance (ANOVA), $p \leq 0.05$ was considered to be statistically significant and was represented by an asterisks (*).

Structure-activity relationship analysis

The relationship between charges of biotinylated RGD derivatives and their cytotoxic activities against MDA-MB-231 cells was analyzed by comparing the overall charges of each biotinylated RGD derivatives as well as their pI with their IC_{50} values.

Results and Discussion

In-silico studies

Design of charged biotinylated RGD derivatives: The designing of biotinylated RGD derivatives was done by adding biotin on N-terminal end of RGD tripeptide to form N-biotinylated RGD derivative while biotin hydrazide was added on C-terminal end of RGD tripeptide to form C-biotinylated RGD derivative (Figure 1).

Confirmation of the net charges of biotinylated RGD derivatives: The charges of biotinylated RGD derivatives was found structurally by finding the sum of all charges present on each derivative and empirically using formulae derived from Henderson-Hasselbalch equations. Structurally and empirically, the overall charge of C-Biotin-RGD was +1, while N-Biotin-RGD has an overall charge of -1. RGD tripeptide had 0 as the overall charge (Figure 1 and Table 2).

Isoelectric potential of charged RGD biotinylated derivatives: At the physiological pH, the results showed that N-Biotin-RGD was acidic in nature while C-Biotin-RGD was basic in nature and RGD tripeptide was slightly neutral (Table 3).

Docking of biotinylated RGD derivatives against ITGB1: The molecular docking results in 2D structures were generated for better interpretation of the bond formations and to determine which amino acids were involved in the interactions. 3D images were created to determine the extends to which conformational changes were produced within the ligands and the receptor (Figure 2). The results showed that each ligand had its own unique conformation and the same was observed with the receptor for each interaction. The bonds formed were also unique to each interaction so as the amino acids which were involved in the bond formation (Table 4).

In-vitro studies

Synthesis of N-Biotin-RGD: The synthesis of N-Biotin-RGD was

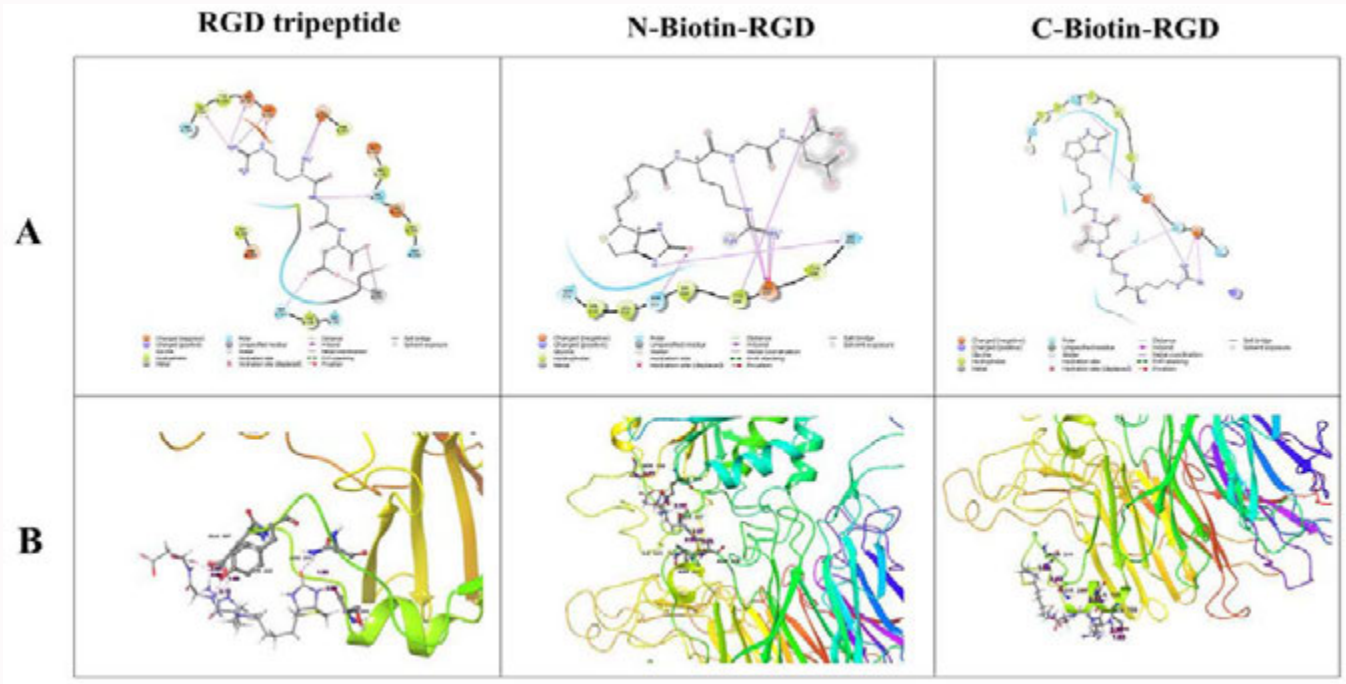

Figure 2: Docking analysis of biotinylated RGD derivatives against ITGB1. A) 2D structural conformations; B) 3D structural conformations.

Table 4: The docking analysis of biotinylated RGD derivatives against ITGB1.

Ligand	Bonds involved	Involvement of amino acid Residues	involvement of Biotin ring	Involvement of ionizable Side Chains	Docking Score	Glide Score
RGD tripeptide	10	SER B:227 GLU B:320 (2) MG B:501	-	SER B:134 ILE A:225 ASP A:227 ASP A:228 (2) MG B:501	-7.53	-7.66
N-Biotin-RGD	7	GLU207 TYR208	SER203 ASN211	GLU207 (3)	-5.86	-39.601
C-Biotin-RGD	6	GLN 199	SER203 ASN211	GLU198 (2) GLU202	-6.427	-45.498

achieved by adding RGD tripeptide with biotin-NHS which resulted in the formation of amide bond between amino group of RGD tripeptide and the carboxyl group from biotin and the formation of NHS as a byproduct (Figure 3).

Synthesis of C-Biotin-RGD: The synthesis of C-Biotin-RGD was done using RGD tripeptide and biotin hydrazide with the involvement of EDC. The carboxyl group of RGD tripeptide was activated by using EDC which resulted in the formation of an unstable O-acylisourea intermediate (Figure 4). The interaction of biotin hydrazide with the unstable O-acylisourea intermediate resulted in the formation of C-Biotin-RGD and the formation of an isourea byproduct.

Binding affinities of biotinylated RGD derivatives against fixed MBA-MD-231 cells: The binding affinities using biotinylated RGD derivatives against MBA-MD-231 was performed against fixed cells which removed the possibility of electrostatic interaction of charges due to the facts that fixed cells are not biologically active to produce lactate nor cause the Hypersialylation of glycans responsible for the negative charge on most cancer cells but also the washing process using detergent such as Tween-20 removed the remaining lactate that was present on the cell surface. This resulted in an interaction that involves only ligands and receptors. The results show that N-Biotin-RGD had the higher binding affinity than C-Biotin-RGD (Figure 5).

Cytotoxicity assay: The cytotoxic effect of biotinylated RGD derivatives resulted in the IC_{50} values of 13.1 ± 2.43 μM for C-Biotin-RGD and 47.58 ± 5.43 μM for N-Biotin-RGD. RGD tripeptide had no significant inhibitory effect on MDA-MB-231 cells in the micromolar range (Figure 6). C-Biotin-RGD showed also inhibitive activities against MDA-MB-231 cells in nanomolar concentration even though it was not very significant. This is not the case for N-Biotin-RGD in nanomolar concentration. These effects showed that C-Biotin-RGD had higher cytotoxic activities against MDA-MB-231 cells than N-Biotin-RGD.

Structure-activity relationship analysis

The relationship between the charges of biotinylated RGD derivatives and their biological activities against MDA-MB-231 cells was analyzed by comparing their isoelectric points with their cytotoxic effects. Ionic interactions act at a longer distance and are stronger to break than other intermolecular bonds including Hydrogen bonds that are involved in the interaction of ligands and their specific receptors. After IC_{50} calculations, it was observed that positively charged C-Biotin-RGD had higher killing effect and negatively charged N-Biotin-RGD had the lower killing effect. The comparison of pI values of both biotinylated RGD derivative showed C-Biotin-RGD to be more basic at the physiological pH while N-Biotin-RGD

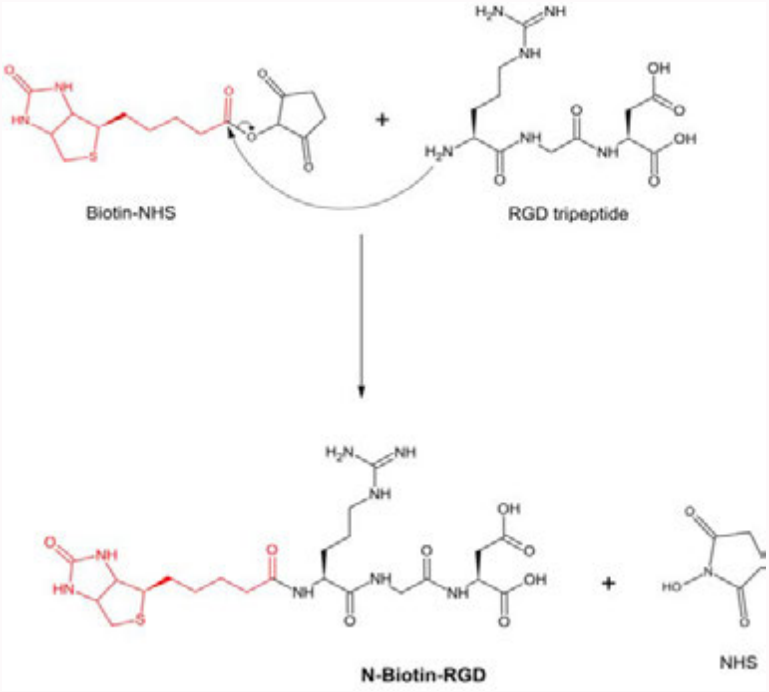

Figure 3: Synthetic scheme of N-Biotin-RGD.

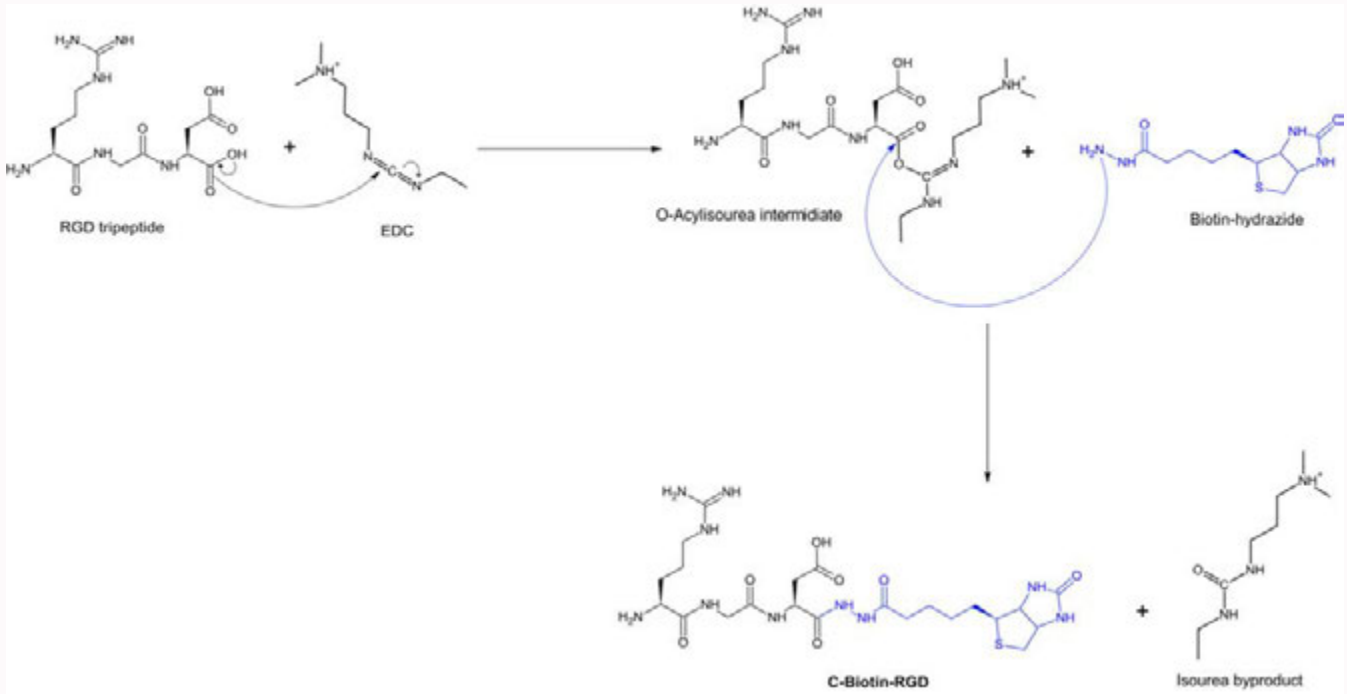

Figure 4: Synthetic scheme of C-Biotin-RGD.

was acidic at pH 7.4 and RGD tripeptide was slightly neutral at the same pH (Table 5). The relationship between the structure, shown by the presence of charges as well as through pI, and activity through their cytotoxic effects against MDA-MB-231 cells was confirmed in this study.

Conclusion

The present study showed the importance of *in-silico* studies in designing and testing molecular prospects before their analysis in laboratory settings. The charges created by biotinylation of end terminals of RGD tripeptide resulted in a positively charged C-Biotin-RGD and a negatively charged N-Biotin-RGD. Even though ligand-receptor interactions may involve electrostatic interactions between them, here the term electrostatic interaction was used for ionic interactions between the ligands and the cancer cell surface. Ionic interactions are stronger and act at a longer distance compare to other

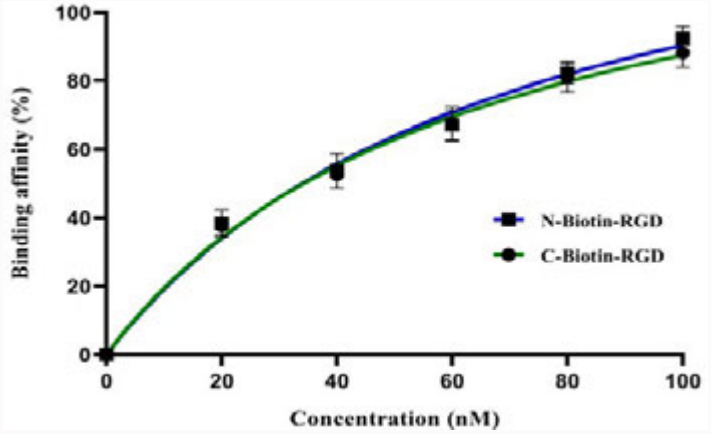

Figure 5: The binding affinity of biotinylated RGD derivatives to fixed MDA-MB231 cells.

Table 5: Structure-activity relationship analysis of charged biotinylated RGD derivatives.

Biological activities	RGD tripeptide	N-Biotin-RGD	C-Biotin-RGD
Binding affinity	-	+ + +	+ +
Cytotoxic activities	-	+ +	+ + +

The biological activities were symbolized with "-" for negative effects; "+" for the positive effects; "+ +" for more positive and "+ + +" for the most positive effects

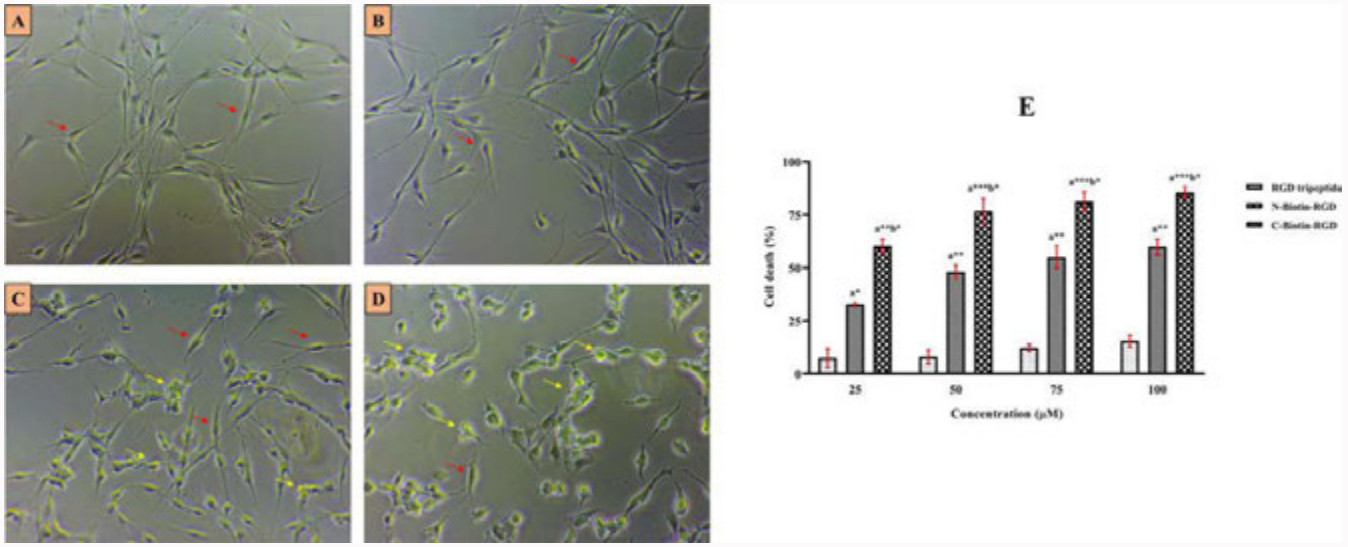

Figure 6: The cytotoxic effect of biotinylated RGD derivatives on MDA-MB-231 cells: (A) Control (B) RGD tripeptide (C) N-Biotin-RGD (D) C-Biotin-RGD and (E) Graphical representation of the cytotoxic effects of biotinylated RGD derivatives.

intermolecular bonds. Thus, they would be more effective than ligand-receptor interactions in biological activities where both are involved. Increased lactate production and Hypersialylation of glycans are important factors in the cancer cell proliferation and multiplication [18,19]. Even though, the inhibition of integrin activities results in cell death, the involvement of electrostatic interactions shows that integrin inhibition is not the main inhibitor of cell viability [20]. This can be confirmed by comparing the binding affinities of biotinylated RGD derivatives with their cytotoxic activities respectively. N-Biotin-RGD had higher binding affinity and lower cytotoxic activity while C-Biotin-RGD had lower binding affinity and higher cytotoxic activity. Although further studies are required, with the pave of these present findings, our work provided an evidential possibility for correlating the charges of a drug candidate and their effectiveness as cytotoxic agents.

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