ORIGINAL ARTICLE

A C G publications

Org. Commun. 16:3 (2023) 166-173

organic communications

Molecular modeling, synthesis and characterization of FOLR1 specific peptides for tumor targeting activity

Özgür Yılmaz *

Materials Technologies, Marmara Research Center, TUBITAK, 41470, Gebze, Kocaeli, Türkiye

(Received August 27, 2023; Revised September 22, 2023; Accepted September 24, 2023)

Abstract: Peptides have low oral bioavailability, low plasma stability, and short circulation time; therefore, they are used in targeted strategies in cancer. In this study, according to *in silico* analysis, novel small peptide sequences, which are consist of three amino acid residues, with high binding capacity against the human FOLR1 surface molecule were obtained. Modeling studies were carried out to determine peptide sequences. RhB-K*FFF, RhB-K*WFE, and RhB-K*YDY peptides have been synthesized by using the Solid Phase Peptide Synthesis (SPPS) method, purified by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) and characterized by Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS/MS) and proton nuclear magnetic resonance (^{1}H-NMR). The purity of RhB-K*FFF, RhB-K*WFE, and RhB-K*YDY peptide are 96%, 95%, and 92%, respectively. Also, cell viability test was performed for the peptides. In our further study, the peptide with highest binding affinity will be conjugated with chemotherapeutic agent in order to improve its anti-cancer activity.

Keywords: Cancer; FOLR1 protein; molecular modeling; peptide synthesis.

1. Introduction

Cancer, which is the second most common disease worldwide, has treatment strategies as radiotherapy, chemotherapy, surgery, and phototherapy.[1,2] Nowadays, peptide-based biomaterials and peptide drug investigations have increased.[3,4,5] To date, there are more than 100 peptide drugs used in the treatment of various diseases in the US, European and Japanese markets.[6]

Targeted cancer therapy has become increasingly important among cancer treatments in recent years that uses small molecules or antibodies to target specific genes and proteins that help cancer cells survive and grow. This therapy specifically targets cell surface molecules of cancerous cells with maximizing the potential to selectively destroy malignant cells, while minimizing the treatment-related toxicities on healthy tissues. As a result, reduced side effects resulted in better tolerated patients to cancer therapies can be exploited.[7]

In targeted cancer therapies, drug conjugates which consist of targeting unit and drug selectively carries the therapeutic agent to the specific area of the tumor. These targeting units can be small molecules, peptides, or antibodies.[8] Compared to antibodies; peptides have ease of synthesis, chemical stability, reduced immunogenicity, increased tissue penetration, and rapid blood clearance. These advantages cause peptides to be preferred more as carriers to allow targeted therapy to become widespread in clinics. Several peptide carriers have already been developed for cancer treatments and molecular imagings.[7-9]

* E-mail:yilmaz.ozgur@tubitak.gov.tr; Phone: +0(262) 677 2000/3165; Fax: +90(262) 641 2309.

The article was published by ACG Publications
http://www.acgpubs.org/journal/organic-communications © Month-Month 2023EISSN:1307-6175
DOI:http://doi.org/10.25135/acg.oc.158.2308.2887
Available online: September 27, 2023

Folate receptor (FR) is a promising biological target on cell surface which has been proved to be overexpressed on the vast majority type of cancers such as ovary, breast, lung, kidney, and column. Thus, folate receptor over-expression can be exploited by targeted therapy and molecular imaging in oncology.[10] Cellular folate uptake occurs via the Folate Receptor-1 (FOLR1) and the Reduced Folate Transporter (RFC). Folic acid plays an important role in DNA replication, repair, and synthesis, methylation reactions, and also protein synthesis.[11] FOLR1, which is a 38-kDa glycosylphosphatidylinositol-associated glycoprotein, is expressed on the apical surfaces of the epithelium in healthy individuals, usually in the kidney, choroid plexus, and lung.[12,13] It is also known that there is a relationship between FOLR1 expression and the malignant potential of cancer.[14]

In this study, we determined three novel peptides, which are RhB-K*FFF, RhB-K*WFE, and RhB-K*YDY (Figure 1), against cancer with using molecular modeling techniques. After that, all peptides were synthesized by using fluorenyl-methoxy-carbonyl based solid-phase peptide synthesis, purified with using High Performance Liquid Chromatography (HPLC) and characterized with mass spectrometry with Electrospray Ionization (ESI) source.

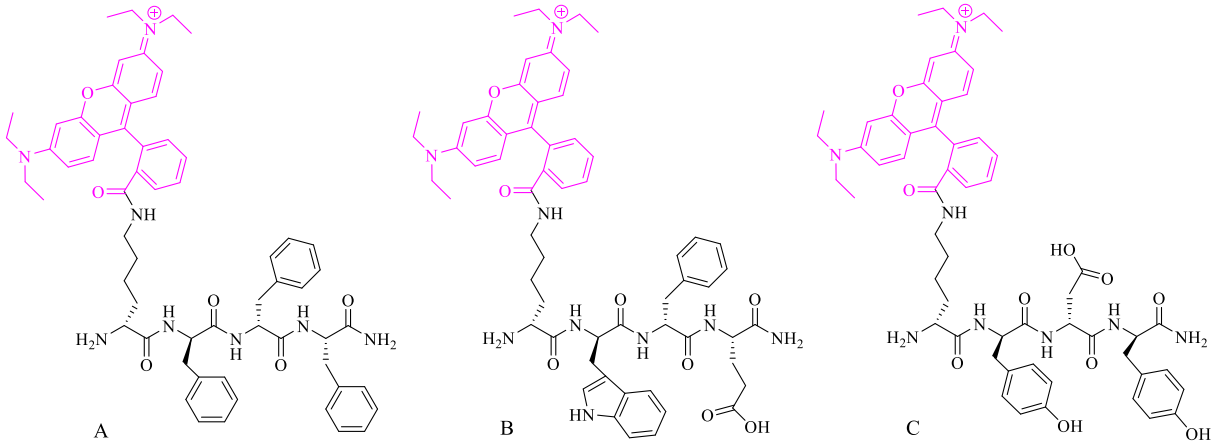

Figure 1. Chemical structure of **A)** RhB-K*FFF **B)** RhB-K*WFE **C)** RhB-K*YDY peptides

2. Experimental

2.1. Molecular Modeling Studies

The structure coded 4LRH for the FOLR1 was used from the protein database.[15] The binding site of folic acid was chosen as the binding site for docking calculations. All tripeptide combinations have been tried for docking calculations. Binding calculations of a total of 8000 triplet peptides to the FOLR1 were performed by virtual scanning method. Docking was done with HPEPDOCK software.[16] Receptor and ligands were visualized in the Visual Molecular Dynamics (VMD) application.[17]

2.2. Chemical Material and Apparatus

All Fmoc-protected-L-amino acids, coupling reagents, and rink amide MBHA resin (200-400 mesh, 0.52 meq/g loading capacity) were obtained from Chem-Impex (Illinois, USA). Dimethylformamide (DMF), dichloromethane (DCM), dimethyl sulfoxide-d6 (DMSO-d_6), tetramethylsilane (Me_4Si), diethyl ether and acetonitrile (ACN) were purchased from either Sigma-Aldrich (St. Louis, Missouri, USA) or Merck (Darmstadt, Germany). All other reagents were ACS grade or higher and purchased from commercial sources. MilliQ water was double deionized using a Milli-Q® Advantage A10® Water Purification Systems (18.2 MΩ.cm at 25°C, TOC: 7 ppb).

2.3. Synthesis, Purification and Characterization of Peptides

2.3.1 General Peptide Synthesis Procedure

All peptides were synthesized by using flurorenyl-methoxy-carbonyl based solid-phase peptide synthesis (Fmoc-SPPS) strategy on a Rink amide MBHA resin at 0.25 mmol. N-terminus of peptides was extended with a lysine residue. The resin (200-400 mesh, 0.52 meq/g loading capacity) swelled in 10 mL of dry DMF for 1 h and carefully drained. To the resin were added 1.5 eq of Fmoc-protected amino acid and 3.0 eq of *N,N*-diisopropylethylamine (DIEA). The coupling reaction was stirred overnight at room temperature, after which, the resin was washed with DMF, then DCM. Attachment of first amino acid was checked with treating three sets of approximately 1 mg of resin with 3 mL solution of 20% piperidine in DMF for 20 min. All amino acids were coupled with *N,N,N′,N′*-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU). Couplings were carried out with 3.0 equivalents of Fmoc-L-amino acids, 2.85 eq of coupling reagent, 6 eq of DIEA with the assistance of nitrogen gas. After each coupling unreacted N-terminal of peptides were capped with the solution of DMF/Ac2O/Pyridine (10 : 1 : 1) (10 min). When the polypeptide chain was completed, the resin was washed with DCM, and then peptide was cleaved from the resin and fully deprotected by treatment of the resin with a mixture of trifluoroacetic acid (TFA)/water (90 : 5 v/v) for 2 hours. In order to isolate linear peptides that contain Cys(Trt) residues, ethanedithiol was also added to the cleavage cocktail as a cation scavenger. Free peptides were separated from the resin by filtration and the resin was further washed with TFA. Collected filtrate was precipitated in diethyl ether and centrifuged. The supernatant was removed and diethyl ether was further added to repeat the washing step up to three times. The precipitate was dried and lyophilized in water.

2.3.2 Purification of the Synthesized Peptides

Reverse phase preparative HPLC was performed by on a Dionex UltiMate 3000 HPLC system, employing a Thermo Scientific Hypersil Gold C18 column (250 × 10 mm, 5 μm) at flow rate of 2 mL/min at 40°C. ACN/water gradient containing 0.1% TFA (5 - 100%, 1 - 70 min) was utilized as eluent. All peptides were dissolved in MilliQ water. The concentration of the peptides was adjusted as 20 mg/mL. 1.5 mL of this peptide solution was purified for one batch by using 2 mL injection loop and purification repeated consecutively. The purity of each fraction was assessed with analytical RP-HPLC, before combining pure fractions.

Analytical RP-HPLC was performed by a Dionex UltiMate 3000 HPLC system equipped with a Thermo Scientific AcclaimTM 120 C18 column (46 x 150 mm, 3 μm). Elution of the peptides was achieved using an ACN/water gradient containing 0.1% TFA (5 - 100%, 1 - 30 min, flow 0.4 mL/min) (see supporting information). After the purity of collected fractions confirmed by analytical HPLC, lyophilization was achieved using a Telstar Cryodos Freeze Dryer and peptides were stored at -20°C.

2.3.3 Characterization of the Synthesized Peptides

The synthesized peptide characterized by mass spectrometry by an Agilent 6530 Q-TOF mass spectrometer equipped with Electrospray Ionization (ESI) source. ^{1}H NMR spectra was recorded at 500 MHz. DMSO d_6 was used as a solvent, and Me_4Si was used as the internal standard. (See supporting information for the NMR and mass spectra).

*{9-[2-(5-Amino-5-{1-[1-(1-carbamoyl-2-phenyl-ethylcarbamoyl)-2-phenyl-ethylcarbamoyl]-2-phenyl-ethylcarbamoyl}-pentylcarbamoyl)-phenyl]-6-diethylamino-xanthen-3-ylidene}-diethyl-ammonium **[RhB-K*FFF] (A)***: Pink powder, ^{1}H NMR (600 MHz, d_6-DMSO; δ, ppm): 1.03-1.08 (4xC$\underline{H}_3$ and C$\underline{H}_2$, m, 14H), 1.23 (N$\underline{H}_2$, s, 2H), 1.40 (2xC$\underline{H}_2$, m, 4H), 2.08 (3xC$\underline{H}_2$, m, 6H), 2.65-2.77 (2xC$\underline{H}_2$, m, 4H), 2.60-2.62 (C$\underline{H}$, m, 1H), 2.89-3.02 (3xC$\underline{H}_2$, m, 6H) 4.43-4.55 (3xC$\underline{H}$ and N$\underline{H}_2$, m, 5H), 6.32-6.38 (m, 6H), 7.11-7.17 (m, 7H), 7.18-7.20 (m, 4H), 7.22-7.24 (m, 4H), 7.47-7.51 (m, 2H), 7.75-7.78 (m, 1H), 7.83-7.91 (4xN$\underline{H}$, m, 4H), 8.06 (d, J=8.1 Hz, 1H), 8.30 (d, J=8.2 Hz, 1H), 8.37 (d, J=8.3 Hz, 1H). LC-HRMS (*m/z*): 1011.52.

*[9-(2-{5-Amino-5-[1-[1-(1-carbamoyl-3-carboxy-propylcarbamoyl)-2-phenyl-ethylcarbamoyl]-2-(1H-indol-3-yl)-ethylcarbamoyl]-pentylcarbamoyl}-phenyl)-6-diethylamino-xanthen-3-ylidene]-diethyl-ammonium [**RhB-K*WFE**] (**B**):* Pink powder, ^{1}H NMR (600 MHz, d_6-DMSO; δ, ppm): 0.85 (C<u>H</u>$_3$, t, J=7.0 Hz, 3H), 1.04-1.09 (2xC<u>H</u>$_3$, m, 6H), 1.22-1.25 (C<u>H</u>$_3$ and C<u>H</u>$_2$, m, 5H), 1.98 (N<u>H</u>$_2$, brs, 2H), 1.38-1.45 (C<u>H</u>$_2$, m, 2H), 1.47-1.54 (C<u>H</u>$_2$, m, 2H)*, 2.09 (N<u>H</u>$_2$, brs, 2H)*, 1.70-1.79 (C<u>H</u>$_2$, m, 2H), 1.89-1.96 (C<u>H</u>$_2$, m, 2H)*, 2.20-2.25 (C<u>H</u>$_2$, m, 2H),2.26-2.31 (C<u>H</u>$_2$, t, J=7.4 Hz, 2H), 2.37-2.40 (C<u>H</u>$_2$, m, 2H), 2.60-2.63 (C<u>H</u>$_2$, m, 2H), 2.75-2.83 (C<u>H</u>$_2$, m, 2H), 2.85-2.91 (C<u>H</u>$_2$, m, 2H)*, 2.91-2.96 (C<u>H</u>$_2$, m, 2H), 2.99-3.04 (C<u>H</u>$_2$, m, 2H), 3.06-3.12 (C<u>H</u>$_2$, m, 2H)*, 3.51-3.56 (C<u>H</u>$_2$, m, 2H), 3.59-3.64 (C<u>H</u>$_2$, m, 2H), 3.86-3.91 (C<u>H</u>, m, 1H), 4.01-4.05 (C<u>H</u>, m, 1H), 4.20-4.24 (C<u>H</u>, m, 1H), 4.53-4.61 (C<u>H</u> and N<u>H</u>$_2$, m, 3H), 6.33-6.38 (m, 3H), 6.94-6.97 (m, 1H), 6.99-7.01 (m, 1H), 7.02-7.06 (m, 2H), 7.10-7.15 (m, 3H), 7.16-7.21 (m, 2H), 7.30 (d, J=8.1 Hz, 1H), 7.46-7.52 (m, 2H), 7.66 (d, J=7.9 Hz, 1H), 7.75-7.78 (m, 1H), 7.84-7.92 (4xN<u>H</u>, m, 4H), 7.97 (d, J=7.9 Hz, 1H), 8.39-8.44 (m, 2H), 10.76 (N<u>H</u>, s, 1H), 12.14 (O<u>H</u>, s, 1H). LC-HRMS (*m/z*): 1032.50.

*[9-(2-{5-Amino-5-[1-{1-[1-carbamoyl-2-(4-hydroxy-phenyl)-ethylcarbamoyl]-2-carboxy ethylcarbamoyl}-2-(4-hydroxy-phenyl)-ethylcarbamoyl]-pentylcarbamoyl}-phenyl)-6-diethylamino-xanthen-3-ylidene]-diethyl-ammonium [**RhB-K*YDY**](**C**)*: Pink powder, ^{1}H NMR (500 MHz, d_6-DMSO; δ, ppm): 0.85 (C<u>H</u>$_2$, t, J= 6.9 Hz, 2H), 1.07 (C<u>H</u>$_3$, t, J= 7.0 Hz, 3H), 1.19-1.31 (3xC<u>H</u>$_3$ and C<u>H</u>$_2$, m, 11H), 1.47-1.55 (C<u>H</u>$_2$, m, 2H), 2.08 (N<u>H</u>$_2$, brs, 2H), 2.26-2.30 (C<u>H</u>$_2$, m, 2H), 2.37-2.39 (C<u>H</u>$_2$, m, 2H), 2.60-2.63 (C<u>H</u>$_2$, m, 2H), 2.69-2.75 (C<u>H</u>$_2$, m, 2H), 2.83-2.90 (C<u>H</u>$_2$, m, 2H),2.94-3.00 (C<u>H</u>$_2$, m, 2H), 3.59-3.64 (C<u>H</u>$_2$, m, 2H), 3.87-3.91 (C<u>H</u>$_2$, m, 2H), 4.01-4.04 (C<u>H</u>$_2$, m, 1H), 4.27-4.31 (C<u>H</u>$_2$, m, 1H), 4.40-4.46 (C<u>H</u>$_2$, m, 2H), 4.48-4.53 (C<u>H</u> and N<u>H</u>$_2$, m, 3H), 5.32 (O<u>H</u>, s, 1H), 5.76 (O<u>H</u>, s, 1H), 6.36 (d, J= 11.9 Hz, 3H), 6.58-6.64 (m, 2H), 6.97 (d, J=8.4 Hz, 1H), 7.00 (d, J=7.8 Hz, 1H), 7.04 (d, J=8.3 Hz, 1H), 7.13 (s, 1H), 7.27 (s, 1H), 7.45-7.56 (m, 2H), 7.73-7.78 (m, 2H), 7.83-7.91 (4xN<u>H</u>, m, 4H), 8.44 (d, J=8.1 Hz, 1H), 8.48 (d, J=7.8 Hz, 1H), 9.13 (s, 1H), 9.18 (s,1H), 12.42 (O<u>H</u>, s, 1H). LC-HRMS (*m/z*): 1011.51

2.4. Biological Assay

2.4.1. Cell Viability Assay

Peripheral blood was obtained from healthy donors who did not have inflammatory diseases or infections for at least ten days (n=3). PBMCs were isolated by density gradient centrifugation (Histopaque 1077, Sigma, St. Louis, MO, USA). To evaluate the effects of the molecules on the cell viability, tripeptides treated with different doses (0.01 uM, 0.1 uM, 10 uM).[18] After 24 hours the viability of PBMCs was assessed with propidium iodide (25 ng/mL) staining and analyses were performed with flow cytometry (FACSAria II, Becton Dickinson, San Jose, CA, USA).

3. Results and Discussion

3.1. Molecular Modeling Studies

The rapid development of peptide-based biomaterials and the success of peptide drugs have increased the interest in synthetic peptides prepared by Fmoc solid-phase peptide synthesis (SPPS).[3,4] The shape of the structure of the folic acid-linked human FOLR1 is as in Figure 2, and the binding site of folic acid was chosen as the binding site for docking calculations. All tripeptide combinations, which are FFF, WFE, and YDY, have been tried for docking calculations. Binding calculations of a total of 8000 triplet peptides to the FOLR1 were performed by virtual scanning method, docking was done with HPEPDOCK software,[16] and the relationship with the receptor and ligands were visualized in the VMD application.[17]

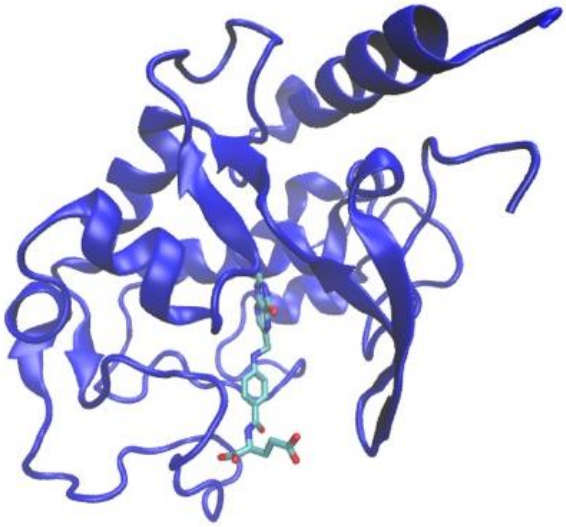

Figure 2. Structure of folic acid-linked human FOLR1 (4LRH.pdb)

The three sequences with the best scores (FFF, WFE, and YDY) are listed in Table 1. The image of the FOLR1 receptor-ligand complexes in the VMD program is shown as in Figure 3. Among the peptides targeting the tumor microenvironment, the focus was on the FOLR1 protein.[18] Unique and small peptide sequences that bind to FOLR1 with high capacity have been synthesized. An efficient and hierarchical protein-peptide docking has been realized with the ModPep algorithm in HPEPDOCK. Thanks to this algorithm, faster results are obtained in short linear peptide sequences.[19] As listed in Table 1, docking score for RhB-K*FFF is -274.561 kcal/mol, for RhB-K*WFE is -224.540 kcal/mol, and for RhB-K*YDY is -189.890 kcal/mol. Higher negative scores indicate more successful docking. However, comparison is more important to us than this value. Peptide sequences are shown in the Table 1.

Table 1. General information and molecular docking results for synthesized peptides

Peptide Sequence	Chemical Formula	Molecular Weight	Exact Mass (g/mol)	Purity (%)	Docking Score (HPEPDOCK) (kcal/mol)
RhB-K*FFF	$C_{61}H_{71}N_8O_6^+$	1222.2	1011.55	96	-274.561
RhB-K*WFE	$C_{59}H_{70}N_9O_8^+$	986.06	1032.53	95	-224.540
RhB-K*YDY	$C_{56}H_{67}N_8O_{10}^+$	1127.1	1011.50	92	-189.890

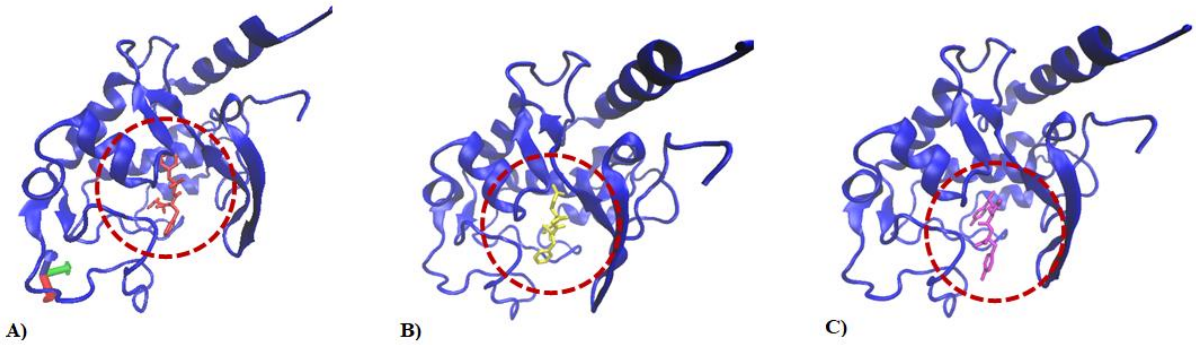

Figure 3. Images of the FOLR1 protein formed in the VMD program with ligands **A)** FFF (red), **B)** WFE (yellow), and **C)** YDY (magenta)

3.2. Purification and Characterization of Peptides

The purity and exact mass values of three synthesized sequences (FFF, WFE, and YDY) are listed in Table 1. The purity of the synthesized RhB-K*FFF peptide, which has poor water solubility, is 96%. The purity of the synthesized and characterized RhB-K*WFE peptide, which has poor water solubility, is 95%, and the purity of the synthesized RhB-K*YDY peptide, which has good water solubility, is 92%. According to the calculations made from the mass spectra, the monoisotopic mass peak [M^+] = 1011.50 for the RhB-K*FFF peptide was measured as an exact value to the theoretical molecular weight of 1011.55 g/mol. Furthermore, it has been confirmed that these experimentally calculated molecular weights for all other peptides (RhB-K*WFE and RhB-K*YDY) are exact value to the theoretical molecular weight values of the peptides (Table 1). All peptides have been successfully synthesized.

3.3. Biological Assay

Following the design and the synthesis of RhB-K*FFF, RhB-K*WFE, and RhB-K*YDY peptides to target FOLR1 cell viability test was examined on peripheral blood mononuclear cells (PBMCs) with respect to previous study of our group.[20] In the viability assays, the cells have abnormal cell membrane function, uptake viability reagent and stained. These stained cells were measured via flow cytometry and percentage of viable cells were calculated. According to the viability assay, when the concentration of these molecules was 2.5 uM, 5.0 uM, and 10 uM, they did not hinder the viability of PBMCs for 24 hours of incubation (Figure 4). Overall, our results suggest that the tripeptides can selectively target the intermediate level of FOLR1 displayed on cancer cell surfaces, suggesting the RhB-K*FFF peptide as the most promising tumor-driven peptide. With its use with RhB-K*FFF-conjugated therapeutic agents, it will enable cancer cell-specific treatment with *in vitro* and *in vivo* evaluation in further studies.

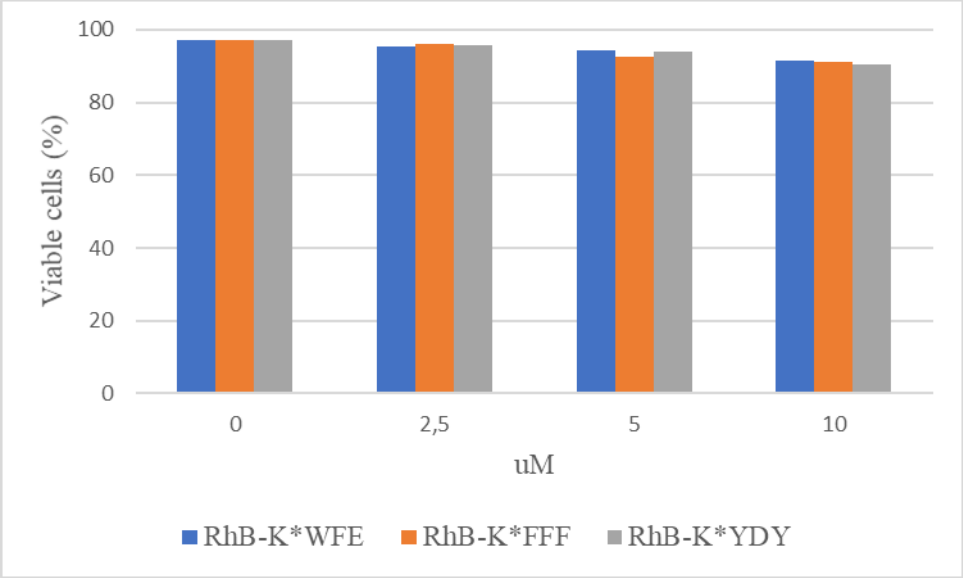

Figure 4. Peripheral blood mononuclear cells (PBMCs) were treated with the different concentrations of the molecules for 24 hours

4. Conclusion

Unique and novel small peptide sequences with high binding capacity against the human FOLR1 surface molecule, stable in biological fluids. According to docking score, RhB-K*YDY has more successful docking score (-189.890 kcal/mol) than other synthesized peptides (RhB-K*FFF and

RhB-K*WFE) for human FOLR1 protein. All peptides have been successfully synthesized, purified above 90%, and then characterized. The purity of RhB-K*FFF, RhB-K*WFE, and RhB-K*YDY peptide are 96%, 95%, and 92%, respectively. The peptides were tested by the viability of Peripheral Blood Mononuclear Cell. According to the cell viability test of synthesized peptides for 24 hours, cell viability was slightly affected up to certain concentrations. This study showed that this peptide sequences present promising results for cancer targeting studies. In further study, the synthesized peptides will be examined for their binding affinity to the FOLR1 protein on the Isothermal Titration Calorimetry (ITC) device. The binding affinity of the peptides to the cancer cells can be determined by fluorescence dye in the cell test. The selected peptide will be stained with fluorescence dye to proceed *in vitro* analysis. Then, we will perform conjugations between selected peptide and chemotherapeutic agents to increase its anticancer activity.

Acknowledgments

The author would like to thank the Turkish Scientific and Technological Council (Project Number: 116E661) for partial financial support.

ORCID

Özgür Yılmaz: 0000-0003-3892-2775

References

[1] Yanık, H.; Ayan, S.; Akdemir, A.; Erdoğan, Ö.; Ustundag, C.; Çevik, Ö.; Yılmaz, Ö. Synthesis, cytotoxic activities and molecular modeling studies of some 2-aminonaphtho[2,3-d][1,3]thiazole-4,9-dione derivatives. *Org. Commun*. **2020**, *13*, 184–193.

[2] Gürbüz, B.; Ayan, S.; Bozlar, M.; Üstündağ, C. B. Carbonaceous nanomaterials for phototherapy: a review. *Emergent Mater*. **2020**, *3*, 479–502.

[3] Behrendt, R.; White, P.; Offer, J. Advances in Fmoc solid-phase peptide synthesis. *J. Peptide Sci*. **2016**, *1*, 4-27.

[4] Fields, G. B.; Noble, R. L. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Protein Res*. **1990**, *35*, 161-214.

[5] Muttenthaler, M.; King, G. F.; Adams, D. J.; Alewood, P. F. Trends in peptide drug discovery. In Nature reviews drug discovery. *Nat. Rev. Drug Discov*. **2021**, *20* (*4*), 309-325.

[6] Cooper, B. M.; Iegre, J.; O'Donovan, D. H.; Ölwegård Halvarsson, M.; Spring, D. R. Peptides as a platform for targeted therapeutics for cancer: Peptide-drug conjugates (PDCs). *Chem. Soc. Rev*. **2021**, *50*, 1480-1494.

[7] Chavda, V.P.; Solanki, H.K.; Davidson, M.; Apostolopoulos, V.; Bojarska, J. Peptide-drug conjugates: a new hope for cancer management. *Molecules* **2022**, *27*(*21*),7232.

[8] Worm, D.J.; Els-Heindl, S.; Beck-Sickinger, A.G. Targeting of peptide-binding receptors on cancer cells with peptide-drug conjugates. *Peptide Sci.* **2020**, *112*, e24171.

[9] Xing, L.; Xu, Y.; Sun, K.; Wang, H.; Zhang, F.; Zhou, Zhang, J.; Zhang, F.; Caliskan, B.; Qiu, Z.; Wang, M. Identification of a peptide for folate receptor alpha by phage display and its tumor targeting activity in ovary cancer xenograft. *Sci. Rep*. **2018**, *8*, 8426.

[10] Frigerio B.; Bizzoni, C.; Jansen, G.; Leamon, C.P.; Peters, G.J.; Low, P.S.; Matherly, L.H; Figini M. Folate receptors and transporters: biological role and diagnostic/therapeutic targets in cancer and other diseases. *J. Exp. Clin. Cancer Res*. **2019**, *38*(*1*), 125.

[11] Notaro, S.; Reimer, D.; Fiegl, H.; Schmid, G.; Wiedemair, A.; Rössler, J.; Marth, C.; Zeimet, A. G. Evaluation of folate receptor 1 (FOLR1) mRNA expression, its specific promoter methylation and global DNA hypomethylation in type I and type II ovarian cancers. *BMC Cancer* **2016**, *16*, 589.

[12] Leung, F.; Dimitromanolakis, A.; Kobayashi, H.; Diamandis, E. P.; Kulasingam, V. Folate-receptor 1 (FOLR1) protein is elevated in the serum of ovarian cancer patients. *Clinical Biochem*. **2013**, *46*, 1462-1468.

[13] Liu, Y.; Lian, T.; Yao, Y. A systematic review and meta-analysis of higher expression of folate receptor alpha (FOLR1) predicts poor cancer prognosis. *Biomarkers* **2020**, *25*(*5*), 367-374.

[14] Matsunaga, Y.; Yamaoka, T.; Ohba, M.; Miura, S.; Masuda, H.; Sangai, T.; Takimoto, M.; Nakamura, S.; Tsurutani, J. Novel anti-FOLR1 antibody–drug conjugate MORAb-202 in breast cancer and non-small cell lung cancer cells. *Antibodies* **2021**, *10* (*1*), 6.

[15] Zardecki, C.; Dutta, S.; Goodsell, D. S.; Lowe, R.; Voigt, M.; Burley, S. K. PDB-101: Educational resources supporting molecular explorations through biology and medicine. *Protein Sci*. **2022**, *31*(*1*), 129-140.

[16] Zhou, P.; Jin, B.; Li, H.; Huang, S. Y. HPEPDOCK: A web server for blind peptide-protein docking based on a hierarchical algorithm. *Nucleic Acids Res*. **2018**, *2*, 443-450.

[17] Humphrey, W.; Andrew, D.; Klaus, S. VMD: visual molecular dynamics. *J. Mol. Graph*. **1996**, 14, 33-38.

[18] Liang, Z.; Dong, J.; Yang, N.; Li, S.D.; Yang, Z. Y.; Huang, R.; Li, F. J.; Wang, W. T.; Ren, J.K.; Lei, J.; et. al. Tandem car-t cells targeting folr1 and msln enhance the antitumor effects in ovarian cancer. *Int. J. Biol. Sci*. **2021**, *17* (*15*), 4365-4376.

[19] Zhou, P.; Li, B.; Yan, Y.; Jin, B.; Wang, L.; Huang, S. Y. Hierarchical flexible peptide docking by conformer generation and ensemble docking of peptides. *J. Chem. Inf. Model*. **2018**, *58*(*6*), 1292-1302.

[20] Köse, C.; Uysal, E.; Yazıcı, B.; Tuğay, Z.; Dingiş Birgül, S.İ.; Yanık, H.; Tavukçuoğlu, E.; Gülyüz, S.; Akdemir, A.; Esendağlı, G.; Yılmaz, Ö.; Alptürk, O. Design and synthesis of novel peptidomimetics for cancer immunotherapy. *Org. Commun*. **2020**, *13(3*), 89-102.