

Design, synthesis, and evaluation of the effect of potential peptides against CD38: An *in silico* and *in vitro* study

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ABSTRACT

The transmembrane glycoprotein known as CD38 (cluster of differentiation 38) is a desirable target for targeted therapeutics as it is essential for immune response and cell signaling, especially in hematological malignancies. The current study aimed to design antibody/nanobody-derived peptides using bioinformatic tools and experimentally investigate their specific binding and impact on the extracellular CD38 expressing cells. Following the synthesis of one of the peptides that indicated somewhat better *in silico* results, known as CDR3a23 (L-pep), and its circular version as C-pep, flow cytometry was used to assess each one's specific binding to CD38. MTT and trypan blue exclusion assays were then used to investigate the peptide's impact. The peptide's anti-proliferative mechanism was also confirmed by cell cycle analysis and apoptosis after annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining. Finally, the L-pep with K_d of 630 nM, indicated high affinity to CD38. The IC_{50} values of the L-pep and C-pep against the HL-60 cells were achieved as 540 nM and 780 nM ($P < 0.0001$), respectively. Also, when the peptides combined with 1 μ M ATRA, these values significantly decreased to 390 nM and 630 nM ($P < 0.0001$), respectively. During arresting the cells at the G1 phase, the total apoptosis of the L-pep and C-pep achieved as 47.5 % and 34.3 %, respectively, which significantly increased to 72.5 % and 55.3 %, respectively, in combination with 1 μ M ATRA. Through their specific binding and suppression of cell growth, our research identified potential peptides against cells exhibiting high levels of CD38.

1. Introduction

The type II transmembrane protein cyclic ADP ribose hydrolase, also referred to as cluster of differentiation 38 or CD38, appears on immune cells such as macrophages, T cells, B cells, and natural killer (NK) cells. It is significantly upregulated in activated immune cells and functions as a biomarker for inflammatory diseases, autoimmune disorders, aging-related inflammation, and hematologic malignancies [1,2].

Nicotinamide adenine dinucleotide (NAD^+) is hydrolyzed by the ectoenzyme CD38 to produce nicotinamide (NAM) and adenine diphosphate ribose (ADPR). The second stage creates cyclic adenine dinucleotide (cADPR) by using cyclase activity to break down NAD^+ . Nicotinamide mononucleotide (NMN), a crucial precursor of NAD^+ in the salvage pathway, and nicotinamide adenine dinucleotide phosphate

($NADP^+$) are additional CD38 substrates that block the absorption of NAD^+ into cells and its conversion to intracellular NAD^+ [3]. In this line, E146, E226, W125, and W189 are all part of the active site that performs all of these reactions. Several metabolic functions that preserve energy balance, DNA repair, and other processes depend on NAD^+ [4,5]. In animals, NAD^+ depletion is linked to normal aging, while NAD^+ augmentation is linked to longer lifespans and better health. On the other hand, via the calcium channel ryanodine receptor found on the endoplasmic reticulum of nerve cells, cADPR is essential for maintaining intracellular calcium homeostasis. Thus, the CD38 is essential for maintaining intracellular calcium homeostasis and extracellular NAD^+ [6]. This crucial cellular balance has been disrupted in several illnesses, including hematologic malignancies, by the overexpression and hyperactivation of CD38 [7]. In this regard, two high affinity

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anti-CD38 antibodies, isatuximab (SARCLISA, Sanofi-Aventis) and daratumumab (Darzalex, Janssen Pharmaceuticals), are intended to reduce the number of cancerous plasma cells [1,8]. Although daratumumab contributes by causing tumor cell lysis via antibody-dependent cytotoxicity (ADCC), complement-mediated cytotoxicity (CDC), body-dependent cell-dependent phagocytosis (ADCP), and apoptosis, isatuximab suppresses the CD38 enzymatic activity and induces direct apoptosis [9–11]. The diverse recognition epitopes on the CD38 may be the cause of the various processes exhibited by the anti-CD38 antibodies. Three distinct epitopes, namely epitopes 1, 2, and 3, are recognized by the CD38-specific antibodies. The crystal structures of isatuximab (PDB ID: 4CMH) and daratumumab (PDB ID: 7DHA) coupled to CD38 demonstrate that both antibodies have different functional characteristics and bind to two different epitopes on the CD38 (epitopes 2 and 1, respectively) [1,10,11].

On the other hand, nanobodies that bind to the three distinct functional epitopes on the CD38 were found in a prior study [12]. The study's findings demonstrated that, of the nanobodies found, MU523 and MU1067 significantly reduce the CD38 catalytic activity by attaching to the epitope 2 [11,12]. Therefore, a new avenue for the treatment or alleviation of the negative symptoms of linked disorders may be opened by the nanobodies with the lowest concentration and side effects and the highest specificity and action against the CD38. Despite being highly specific, antibodies' comparatively long biological half-life makes them unsuitable for longitudinal imaging studies. Additionally, issues with cost, availability, manufacturing, and stability make it uncertain if nanobodies will be widely used for clinical imaging [13–15]. These problems can be resolved by using peptides derived from nanobodies or antibodies, which have a similar action, affinity, and advantageous pharmacokinetics with significant tumor tissue absorption and quick clearance from non-target organs in vivo [16,17].

So, we are interested in using bioinformatic tools to design peptides derived from the antibodies and/or nanobodies against CD38 activity as a tool for rational drug discovery for this seemingly elusive target. Thankfully, structural information in the form of CD38 crystal structures in association with a variety of biological antibodies/nanobodies made it possible for us to verify the precision of the docking position predictions. Therefore, we created peptides derived from the CDR3 (complementarity-determining region 3) of the isatuximab antibody as well as the MU523 and MU1067 nanobodies for this investigation. We assessed the peptide's structure and affinity in association with the CD38 using bioinformatics tools, and one of the more appropriate peptides was chosen to test its binding and function in an in vitro experiment.

1.1. Chemicals and reagents

RPMI-1640 (Cat.: R-6504, Sigma-Aldrich, Germany), DMEM/F12 (Cat.: BI-1011, Bio Idea, UK), TrypsinEDTA 10x (Cat.: BI-1602, Bio Idea, UK), penicillin/streptomycin (Cat.: BI-1203, Bio Idea, UK), fetal bovine serum (FBS; Cat.: F-1001/100, Biosera, France); annexin V Apoptosis Detection Kit (BioLegend, Cat NO:640914), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Cat.: M5655, Sigma Aldrich, USA), DMSO (density: 1.1 g/cm³; Cat.: 102,952, Merk, USA), FITC (Cat.: F7250, Sigma-Aldrich, Germany), All-Trans Retinoic Acid (ATRA, Cat.: A2625, Sigma-Aldrich, Germany). Anti-CD38 antibody (Cat.: 303504, BioLegend, San Diego, CA, USA). All cell lines used in this study were provided from Pasteur Institute of Iran.

2. Methods

2.1. Sequence alignment

Previous studies have demonstrated that isatuximab antibody and MU1067 and MU523 nanobodies bind exclusively to CD38's epitope 2 and completely block its catalytic activity [1,11,12,18]. The antibody/nanobody sequences were aligned with one another to analyze the

sequence variances and similarities. The CDRs sequences from the isatuximab, MU1067, and MU523—important regions for epitope recognition and binding—were found and extracted thanks to this alignment. The molecules' sequences were first produced in FASTA format and uploaded to ClustalW for the alignment process [19]. After completing the alignment, the output files were analyzed using the online ESPrnt 3 server [20]. This final step allowed us a clearer visualization of the alignment results.

2.2. Modeling and validation of peptide structures

The CDR3 sequences from the isatuximab, MU1067 and MU523, refer to the designed peptides in this study, were subjected to molecular and structural modeling using AlphaFold2 [21] and I-TASSER [22–24]. The predicted peptide models were refined by the ModRefiner server [22–24] to improve structural accuracy. The quality assessment of the predicted structures was done using PROCHECK (<https://saves.mbi.ucla.edu>), Qmean(<https://swissmodel.expasy.org/qmean>), and PROSA (<https://prosa.services.came.sbg.ac.at>) and the most stable structures for each peptide were selected for subsequent docking studies with the CD38 structure. PROSA specifically addresses the needs encountered in the validation of protein structures obtained from X-ray analysis, NMR spectroscopy, and theoretical calculations, while QMEAN is a composite scoring function that can derive both global (i.e., for the entire structure) and local (i.e., per residue) error estimates based on one single model [25]. Additionally, the Z-Dope scores [26] for the peptide structures were calculated to assess the reliability of each model. the Z-Dope is derived from the normalized DOPE (Discrete Optimized Protein Energy) score, which evaluates the energy of a protein model based on atomic interactions.

2.3. Molecular docking

The structural files related to the CD38 and the peptide sequences (as ligands) were prepared for molecular docking. The CD38 structure (PDB ID: 1ZVM) [5] was downloaded in PDB format from the RCSB-PDB database (<https://www.rcsb.org>) and designated as the receptor. The refined peptide models, known as Isa, CDR3a23, and CDR3a67, were saved in PDB format. Before docking, structural optimization was performed: the CD38 file was optimized using UCSF Chimera v1.16 [27], and each peptide model was further optimized for conformational stability by UCSF Chimera v1.16. Molecular docking was performed using HADDOCK v2.4 [28], and the docking poses were evaluated based on binding affinity and interaction energy. The residues involved in the interaction between the CD38 and the peptides were visualized using LigPlos [29,30]. Additionally, the 3D structures of the CD38/Isa, CD38/CDR3a23, and CD38/CDR3a67 complexes were analyzed with UCSF Chimera v1.16 to accurately identify and characterize the peptide binding sites on CD38.

2.4. Protein binding energy prediction (PRODIGY)

Following molecular docking, the CD38/Isa, CD38/CDR3a23, and CD38/CDR3a67 complexes were evaluated using the PRODIGY web server [31,32] to determine which complex exhibited the most favorable binding, stability, and optimal structural features (ΔG and K_d).

2.5. Molecular dynamics simulation

Although both modeling tools (I-TASSER and AlphaFold2) produced acceptable results, the optimized peptide models from AlphaFold2, was selected as more appropriate models for molecular dynamics (MD) simulation based on the PROCHECK and the Z-Dope scores. The CD38/Isa, CD38/CDR3a23, and CD38/CDR3a67 complexes were prepared as inputs for the MD simulation. MD simulations were conducted using the GROMACS software package version 2022 [33] with the CHARMM36

force field [34]. The complexes were placed in a cubic box solvated with TIP3P water molecules, and the system pH was maintained at 7. To neutralize the simulation system, Na^+ and Cl^- ions were added at appropriate concentrations. The NVT and NPT equilibration phases were set at 1000 ps each, followed by a 100 ns production run. The simulation temperature was controlled at 300 K and pressure at 1 bar. Electrostatic interactions were calculated using the particle mesh ewald (PME) method, while the Lennard-Jones potential was applied for van der Waals interactions. System trajectories were recorded at 2 ps intervals throughout the simulation. Post-simulation analyses included calculating root-mean-square deviation (RMSD), radius of gyration (R_g), root mean square fluctuation (RMSF), an abundance of secondary structure elements, hydrogen bonds, and MM-PBSA (molecular mechanics poisson-boltzmann surface area) for binding energy analysis.

2.6. Peptide synthesis

The L-pep and C-pep were chemically synthesized and purified by high-performance liquid chromatography (HPLC) at a purity of 90 %, analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF), and approved by electrospray ionization mass spectrometry (ESI-MS) analysis (Shine Gene Biotechnologies, Inc., Shanghai, China). The designed 22-mer peptide related to the L-pep possesses the following amino acid sequence: "AAQYQDRYYDEFTWKEKDMDYW" labeled with fluorescein 5-isothiocyanate (FITC) at its C-terminal. Also, the sequence of the designed 24-amino acid peptide related to the C-pep is "CAAQYQDRYYDEFTWKEKDMDYWC" (Figure S1).

2.7. Cell culture

HL-60 human myeloblastic leukemia cells and SH-SY5Y neuroblastoma cells were cultured in RPMI-1640 and DMEM/F12 mediums, respectively, supplemented with 10 % (v/v) heat-inactivated fetal bovine serum and 1 % (w/v) penicillin-streptomycin solution. The cells were maintained in a 95 % humidified incubator supplied with 5 % CO_2 , at 37 °C.

2.8. CD38 expression analysis and peptide binding assay

As shown in the human protein atlas (www.proteinatlas.org), we selected the HL-60 cell line as an extracellular CD38-expressing model and the SH-SY5Y cell line as a negative one. All-trans-retinoic acid (ATRA) was administered to the both cell lines at a final concentration of 1 μM for 12, 24, and 48 h to assess the overexpression of extracellular CD38 receptor. Concisely, a six-well plate was filled with 2×10^5 cells per well. FITC-labeled anti-CD38 antibody (5 μl of 200 $\mu\text{g}/\text{ml}$ stock incubated for 30 min on ice in the dark) was used to examine the extracellular CD38 level before and after treatment of the cells with ATRA using flow cytometry (BD Biosciences, San Jose, CA, USA).

The binding of the designed peptides (L-pep and C-pep) was assessed after it was confirmed that the HL60 cells are a positive model for the extracellular CD38 and the SH-SY5Y cells are the negative one, even after treatment with 1 μM ATRA for 48 h. Thus, in six-well plates, the HL60 and SH-SY5Y cells were cultured at a density of 2×10^5 cells per well. After 24 h cultivation, the mediums were supplemented with 2 ml of new medium containing 1 μM ATRA for 12 h. Next, 2 ml of new mediums containing various doses of the FITC-labeled L-pep (40, 400, and 800 nM for HL60 cells and 800 nM for SH-SY5Y cells) and 1 μM ATRA were replaced. The cells were then incubated for 4 h at 37 °C in the dark. It should be mentioned that the same condition was carried out on the HL60 cells without ATRA to ascertain the peptide binding specificity.

Also, to determine the binding of the C-pep (not labeled with FITC), the HL60 cells (2×10^5) were cultured in the medium containing 1 μM ATRA for 12 h. The medium was replaced by 2 ml of fresh medium

containing a high concentration of C-pep (3 μM) and 1 μM ATRA for 4 h at 37 °C. The cells were washed thrice with PBS (phosphate buffer saline) to remove the unbounded peptides. Then, the medium was replaced by 2 ml fresh mediums containing 400 or 800 nM FITC-labeled L-pep and 1 μM ATRA and incubated for 4 h at 37 °C in the dark. Moreover, to compare the affinity of the peptides (L-pep and C-pep) to CD38, the pre-treated cells with 1 μM ATRA were treated with 800 nM C-pep for 4 h at 37 °C. The cells were washed thrice with PBS to remove the unbounded peptides. Then, the medium was replaced by 2 ml of fresh medium containing 800 nM FITC-labeled L-pep and 1 μM ATRA and incubated in the same conditions. The binding of the peptides was investigated by flow cytometry (BD Biosciences, San Jose, CA, USA), and the raw data were analyzed by FlowJo software v10.10 and GraphPad Prism 8.2.1 software.

To calculate the K_d value (dissociation constant), HL60 cells (4×10^4 /well in a 96-well plate) were incubated with 1 μM ATRA for 12 h at 37 °C. Then, the culture media was replaced with fresh media containing 1 μM ATRA and different concentrations of FITC-labeled L-pep (100, 500, 600, 700, 800, 1000, 1200, 1500, and 2000 nM) and incubated for 4 h at 37 °C. After twice washing the cells with PBS, the peptide binding assay was performed at $\lambda_{\text{ex}} = 498 \text{ nm}$ and $\lambda_{\text{em}} = 517 \text{ nm}$ using Bio Tek Cytation 3 (USA).

2.9. Trypan blue exclusion assay

To determine the anti-proliferative effect of the designed peptide on both the cell lines, we performed the trypan blue exclusion assay time-dependently. In this line, 1) un-treated cells (control), 2) cell + ATRA, 3) cell + L-pep, 4) cell + C-pep, 5) cell + L-pep + ATRA, and 6) cell + C-pep + ATRA are the six conditions that the test was performed in a six-well plate during 3 days. In this assay, 4×10^5 cells per well (both of the cell lines) were cultured in their relative medium containing 5 % FBS (v/v), 1 μM ATRA, and 800 nM L-pep or C-pep (the highest concentration used for the binding assay). 0.2 % trypan blue (Sigma-Aldrich, Germany) was used to stain the cells counted on a Luna Dual Fluorescence Cell Counter. The cell viability was calculated using the below formula:

$$\text{Cell Viability} = ((\text{Total Cells} - \text{Dead Cells}) / \text{Total Cells}) \times 100.$$

2.10. Cell viability assay (MTT test)

Similarly, the MTT test was used for 48 h under the same six previously conditions listed to assess the peptides' dose-dependent anti-proliferative effect. Briefly, 96-well plates were seeded with 4×10^4 cells per well (both of the cell lines) at different dosages of 100, 300, 700, and 1000 nM of L-pep, C-pep, and ATRA supplemented with 5 % FBS (v/v). The ATRA concentration in the combination of ATRA with L-pep or C-pep conditions was 1 μM . Untreated wells, or the cells cultured in culture media only, were used as a control for cell viability. Following 48 h of incubation at 37 °C, 20 μl of MTT (5 mg/ml) was added, and the cells were then incubated for an additional 4 h at 37 °C. After discarding the supernatant, 100 μl of DMSO was added. The blue formazan crystals were completely dissolved by gently shaking the plate. A Bio Tek microplate reader was used to measure the absorbance (A) at 570 nm, and the formula " $(\text{Abs. of Test} / \text{Abs. of Neg. control}) \times 100$ " was used to determine the viability percentage. For every concentration, the test was conducted three times.

2.11. Apoptosis and cell cycle analysis

Given the results achieved from the MTT assay and trypan blue exclusion assay, double staining with FITC-Annexin V and PI was performed using a phosphatidyl serine detection kit (BioLegend, Cat NO:640914) on the HL60 cells. The six conditions mentioned above

were also reconsidered in the apoptosis test and cell cycle analysis. The HL60 cells (2×10^5) were incubated with IC_{50} values of the peptides (with or without 1 μ M ATRA) in a six-well plate. After 48 h, the cells were harvested, washed three times with PBS, centrifuged at 1200 rpm, and then re-suspended in the binding buffer (calcium buffer, 500 μ l). FITC Annexin V (3 μ l) was added to the cells, followed by the addition of 3 μ l PI. The samples were then incubated for 15 min in the dark at 4 °C and then subjected to a flow cytometer (BD Biosciences, San Jose, CA, USA) evaluation. The wavelength of Annexin V-FITC is equal to 520 nm, which is in the FL1-H channel, and the wavelength of PI dye is equal to 617 nm, which is in the FL2-H and FL3-H channels that were read in the device. In this procedure, annexin V attaches to phosphatidylserine on the outer surface of apoptotic cell membranes in the presence of calcium. PI dyes only enter cells with disrupted plasma membranes and cannot cross healthy cell membranes. Therefore, cells that are annexin-positive but PI-negative suggest early apoptosis, while those that are positive for both annexin V and PI indicate late-stage apoptosis. The cells that are PI-positive but annexin V-negative are recognized as necrosis.

The same conditions were also conducted to investigate the effect of the peptides on the cell cycle of HL60 cells. Briefly, the cells (2×10^5) were cultured with the IC_{50} values of the peptides (with or without 1 μ M ATRA) in a six-well plate for 48 h. After harvesting, washing, and re-suspending in 1 ml PBS, 1 ml of cold ethanol was added to fix the cells overnight. Then, the cells were centrifuged at 1500 rpm, ethanol was discarded, and the cells were washed with PBS three times. After centrifugation (1500 rpm) and discarding the supernatant, the PI MASTER MIX (PI: 1 mg/ml, RNase DNase free: 10 mg/ml, and PbS, Ca^{+2} , Mg^{+2} free: 950 μ l) was added to the cells and incubated at room temperature for 30 min and analyzed by a flow cytometry instrument (BD Biosciences, San Jose, CA, USA). It should be noted that at the same

conditions, the cells were treated with 1 μ M ATRA to investigate the effect of ATRA on apoptosis and cell cycle of the cells.

2.12. Statistical analysis

Prism 8.2.1 software from GraphPad Software Inc. was used for data analysis. Data are expressed as means and standard deviations (SDs) obtained from at least three experiments. The statistical analysis included two-tailed Student t-tests and two-way ANOVA to compare two or more groups. The P-value of 0.05 indicated a statistically significant difference in the findings of the analysis.

3. Results

3.1. Sequence alignment of anti-CD38 antibodies/nanobodies

For comparison, alignment similarity scores were calculated, revealing that the CDR3 region of isatuximab shares 31.8 % and 22.7 % sequence identity with the CDR3 region of MU1067 and MU523, respectively while the CDR3 region of MU1067 shares 40.9 % identity with the CDR3 region of MU523. We also included MU551 nanobody as a control in this analysis because its structure has been experimentally resolved through X-ray crystallography. MU551 nanobody (PDB ID: 5F1O), as a reference providing a reliable structural baseline, confirmed that our target CDR3 region chosen to design the CD38 inhibitory peptides is modeled on a structurally validated framework. Moreover, we compared the approximate CDRs regions of the nanobodies and the isatuximab with daratumumab to highlight their difference, which led to the recognition of different epitopes on the CD38 (Fig. 1).

The functional roles of these CDR3 regions supported their usage in

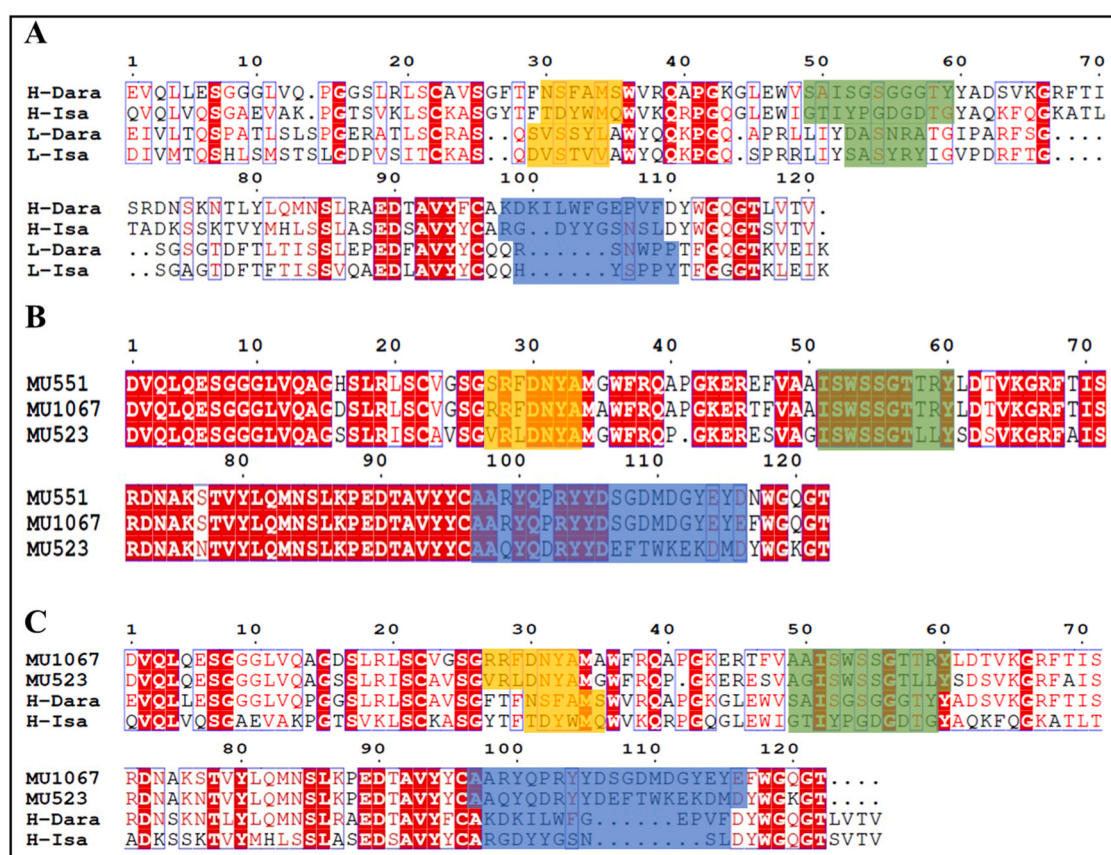


Fig. 1. Sequence alignment of A) antibodies (isatuximab and daratumumab), B) nanobodies (MU1067, MU551, and MU523), and C) antibodies and nanobodies. In all sequences, the approximate CDR1, CDR2, and CDR3 regions are highlighted in yellow, green, and blue, respectively. Note: “H-Dara/Isa” and “L-Dara/Isa” refer to the heavy chain and light chain of daratumumab/isatuximab antibody, respectively.

peptide design that targets CD38, despite their comparatively low sequence similarity. Because of their hypervariable character, which enables great adaptability and specificity to many epitopes, prior research has shown that the CDR3 loop is the most crucial CDR for binding specificity and affinity toward its target. Therefore, despite their considerable sequence variation, the CDR3 of MU523 and MU1067 nanobodies and isatuximab antibody enable us to capture unique binding features, which may result in new binding interactions and inhibitory profiles. Furthermore, a wider variety of structural conformations is offered by the heterogeneity among these sequences, which may be useful in locating peptides with the best binding affinities and stability for CD38 inhibition. Therefore, despite the sequence variability of the CDR3 regions of isatuximab, MU1067, and MU523, their structural flexibility and functional significance in CD38 binding justify their selection for constructing peptide-based inhibitors. This strategy may increase the possibility of discovering potent inhibitory peptides with high specificity that can target CD38. The CDR3 sequences of MU1067, MU523, and isatuximab were also achieved by Expasy (https://web.expasy.org/compute_pi/) shown in [table S1](#) along with their characteristics as potential peptides against CD38.

Moreover, to confirm the CDRs sequences, the MU1067 and MU523 nanobodies were also modeled by Chai-1 (lab.chaidiscovery.com), D-ITASSER (<https://zhanggroup.org/D-I-TASSER>) as strong modeling tools comparable or better than the Alphafold3 [35,36]. So, structural alignments for the models built for MU523 and MU1067 nanobodies using AlphaFold2, Chai-1, D-ITASSER, and I-TASSER were also performed with UCSF-Chimera v1.16 ([Figure S2](#)).

3.2. Modeling and validation of the designed peptide structures

The structure of the peptides Isa, CDR3a53, and CDR3a67 were generated/predicted using both I-TASSER and AlphaFold2 ([Figure S3](#)) subjected to optimization and refinement on the ModRefiner server and the 3D models estimated qualitatively by the three various independent servers (PROCHECK, Qmean, and PROSA) ([Table S2](#)).

The refined models were also further validated by calculating their Z-Dope scores, indicating the structural stability of each model. For the AlphaFold2-derived structures, the Z-Dope scores were as follows: Isa (-7.32×10^{-2}), CDR3a53 (-6.68×10^{-2}), and CDR3a67 (-5.16×10^{-2}). For the I-TASSER-derived structures, these values were -5.19×10^{-2} , -7.03×10^{-2} , and -3.72×10^{-2} , respectively ([Figure S4](#)). According to the PROCHECK and the Z-Dope scores, the AlphaFold2-obtained models seemed somewhat better than the I-TASSER obtained models in terms of stability and accuracy.

3.3. Molecular docking

The AlphaFold2 and I-TASSER-obtained models for the Isa, CDR3a23, and CDR3a67 peptides were also docked with the human CD38 structure using the HADDOCK service for molecular docking. The findings are shown in [Tables 1](#), and [2D](#) and [3D](#) structures of the spatial orientation and interaction residues between each peptide and CD38 have been shown in [Figures 2](#) and [S5](#).

Van der Waals, electrostatic, desolvation, and restraint violation

Table 2
MM-PBSA binding energy analysis between CD38 in complex with CDR3a23, CDR3a67, and Isa peptides.

Complexes	Δ WV	Δ EE	Δ G GAS	Δ G SOLV	Δ G Total
CD38/Isa	-29.81 $\pm$ 4.65	-65.14 $\pm$ 60.12	-94.95 $\pm$ 64.77	72.70 $\pm$ 51.65	-22.26 $\pm$ 11.19
CD38/ CDR3a67	-27.45 $\pm$ 7.73	21.76 $\pm$ 41.68	-5.71 $\pm$ 48.89	-25.68 $\pm$ 33.23	-31.37 $\pm$ 9.31
CD38/ CDR3a23 (L-pep)	-45.68 $\pm$ 5.57	-75.81 $\pm$ 68.26	-121.49 $\pm$ 73.83	86.38 $\pm$ 62.77	-35.11 $\pm$ 7.65
MU523	-305.51	-874.38	-1179.89	858.65	-324.81
Nanobody	$\pm$ 12.05	$\pm$ 84.02	$\pm$ 96.07	$\pm$ 73.16	$\pm$ 18.90

Δ WV: Van der waals energy, Δ EE: Electrostatic energy, Δ G SOLV: Solvation free energy, Δ G GAS: Δ WV + Δ EE, Δ G Total: Total binding free energy (Δ G GAS + Δ G SOLV). The unit of measurement for all energies is kcal/mol.

energies are among the several energetic components that are included in the HADDOCK score, which is a composite metric. It ranks the binding affinities of docked complexes using an overall score. Stronger and more stable binding is indicated by a lower (more negative) HADDOCK score. This part assesses the repulsive and attractive forces between non-directly linked atoms. It shows the degree of spatial complementarity between the receptor and peptide surfaces. Tighter and more stable binding interfaces are a result of favorable van der waals interactions. The interactions between the charged groups of the peptide and the receptor produce electrostatic energy. Binding stability is frequently dominated by favorable electrostatic interactions, especially when the peptide and receptor residues form hydrogen bonds or salt bridges. The energetic cost of eliminating water molecules from the interface to enable direct interactions between peptides and receptors is known as desolvation energy. When water displacement occurs, the binding interface generates effective and energetically advantageous contacts, as shown by a favorable desolvation energy. The degree to which the docked model complies with experimental or predetermined spatial constraints—such as those generated from NMR or cryo-EM data—is measured by this word. A low restraint violation energy indicates that the docking option is practical and conforms to biophysical or structural limitations. A docking solution's relative quality in relation to the whole ensemble of models produced during the docking process is gauged by the Z-score. The solution is among the most energetically beneficial and stands out from other less ideal models when the Z-score is more negative. Together with van der waals, electrostatic, desolvation, and restraint violation energies, the HADDOCK scores offer information about the stability and binding interactions of every peptide-CD38 complex. Out of all the docked models, the CDR3a23 (L-pep) peptide made with AlphaFold2 had the greatest HADDOCK score (-116.6 ± 8.3), and the most advantageous electrostatic energy (-350.9 ± 72.9). The I-TASSER-generated models exhibited somewhat less favorable electrostatic interactions and a typically higher restraint violation energy, even if they also produced good binding interactions. This finding demonstrates how AlphaFold2 has improved its ability to produce the structurally optimized peptides that are more closely aligned with the CD38 binding interface. The docking findings acquired from the I-TASSER-

Table 1
Molecular docking results using HADDOCK server for the AlphaFold2/I-TASSER.-modeled peptides.

Peptide	HADDOCK score	Van der Waals energy	Electrostatic energy	Desolvation energy	Restraints violation energy	Z-Score	Modelling tool
Isa	-66.7 $\pm$ 7.4	-27.8 $\pm$ 4.2	-182.8 $\pm$ 25.7	-6.4 $\pm$ 3.2	41.3 $\pm$ 30.9	-1.6	I-TASSER
CDR3a67	-99.9 $\pm$ 3.8	-57.3 $\pm$ 5.5	-157.7 $\pm$ 17.3	19.2 $\pm$ 3.1	81.7 $\pm$ 12.2	-1.9	
CDR3a23 (L-pep)	-109.7 $\pm$ 3.6	-43.3 $\pm$ 4.6	-333.2 $\pm$ 30.0	-11.2 $\pm$ 2.2	114.5 $\pm$ 19.2	-1.7	
Isa	-75.5 $\pm$ 2.9	-35.3 $\pm$ 4.2	-212.8 $\pm$ 40.3	-0.5 $\pm$ 1.8	28.7 $\pm$ 14.5	-1.7	Alpha-Fold2
CDR3a67	-90.7 $\pm$ 7.0	-44.6 $\pm$ 5.9	-231.2 $\pm$ 37.1	-5.5 $\pm$ 2.4	56.9 $\pm$ 34.6	-1.6	
CDR3a23 (L-pep)	-116.6 $\pm$ 8.3	-44.5 $\pm$ 6.9	-350.9 $\pm$ 72.9	-7.5 $\pm$ 4.3	55.5 $\pm$ 34.9	-2.0	
CDR3a23 (C-pep)	-101.8 $\pm$ 7.9	-36.9 $\pm$ 8.8	-318.9 $\pm$ 71.6	-9.4 $\pm$ 4.6	83.0 $\pm$ 44.7	-2.2	
MU523 Nanobody	-77.2 $\pm$ 11.5	-44.3 $\pm$ 4.6	-192.1 $\pm$ 50.5	-6.3 $\pm$ 4.8	118.8 $\pm$ 24.8	-1.8	

generated models also revealed the greatest HADDOCK score (-109.7 ± 3.6) for the CDR3a23, much like the data received from the AlphaFold2 models.

LigPlot analysis validates important residues involved in binding for each peptide, and binding orientations and interacting residues further depict the peptide-CD38 interactions in Figures 2 and S5. K69, Y70, E72, I73, H74, P75, R78, T136, Q137, V138, Q139, R140, D141, M142, F143, D147, Y152, V288, K289, N290, P291, and E292 are the essential residues of epitope 2 on CD38. All peptides modeled using AlphaFold2 and I-TASSER exhibited interactions with the residues within epitope 2 or

close to them (Figures 2, 3, and S5). Additionally, the whole MU523 nanobody (as the control for the CDR3a23 peptide) attaches to the CD38's epitope 2 completely. This binding specificity demonstrates how these peptides and MU523 nanobody may be used to specifically block CD38 by taking advantage of important residues in epitope 2. Therefore, based on the docking results, the CDR3a23 peptide is more appropriate as a CD38 inhibitory candidate.

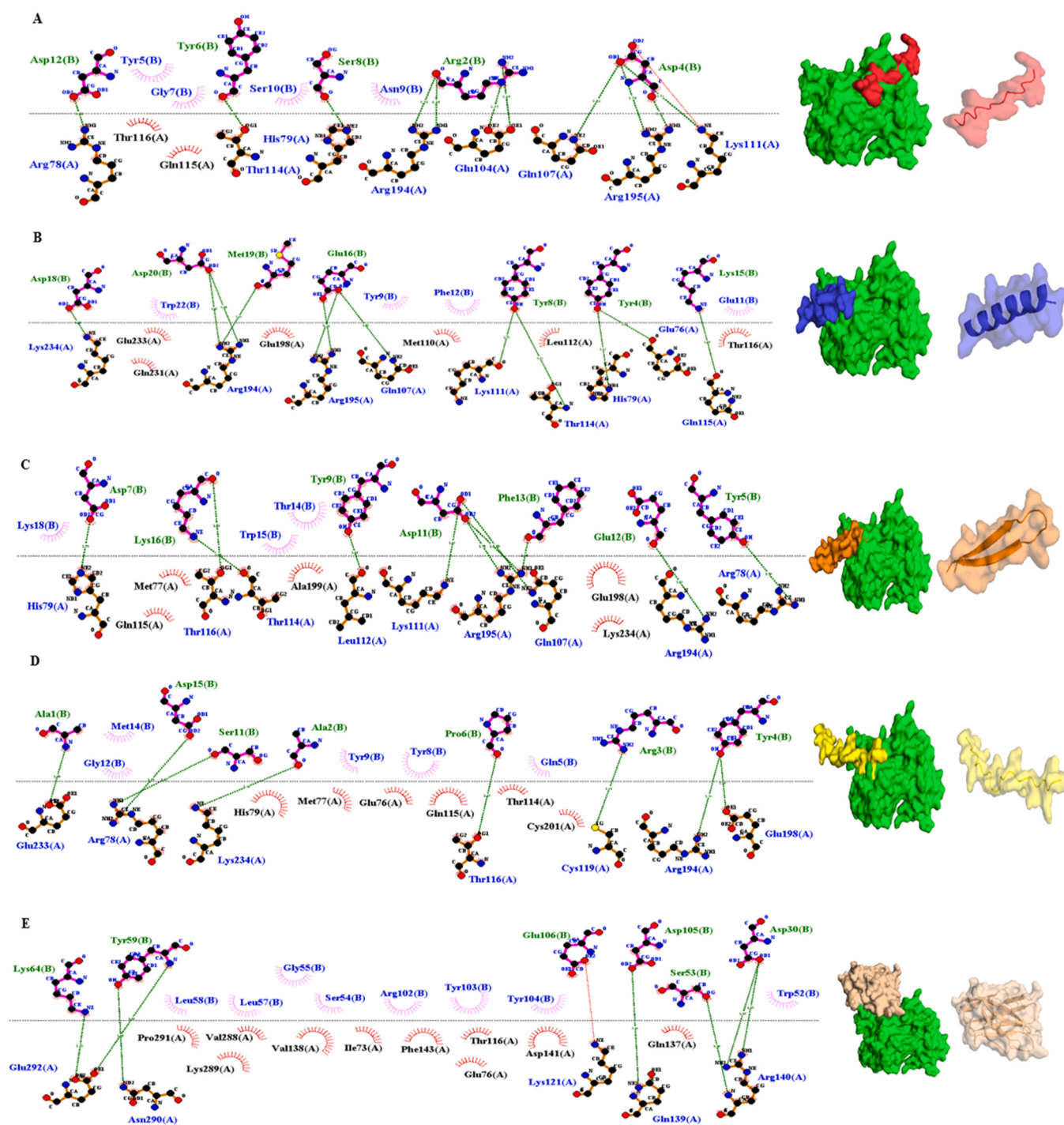


Fig. 2. Visualization of CD38 binding interfaces with the A) Isa, B) CDR3a23 (L-pep), C) CDR3a23 (C-pep), D) CDR3a67 peptides, and E) MU523 nanobody modeled by AlphaFold2, highlighting key interacting residues as identified by LigPlot analysis; "A" chains represent the residues related to CD38 protein and "B" chains represent the residues related to peptides.

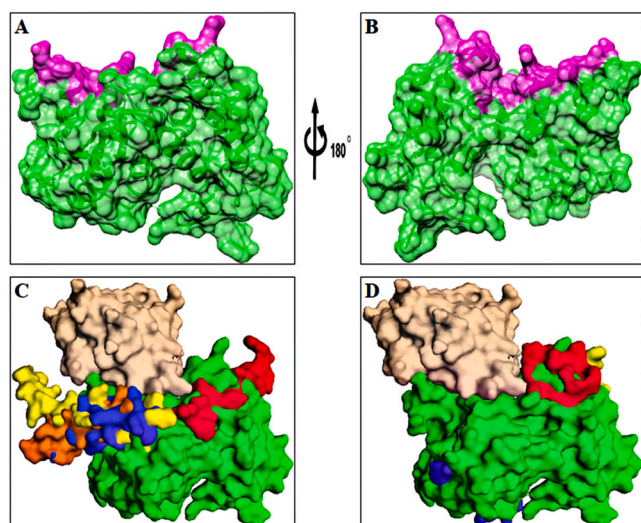


Fig. 3. The general structure of the CD38 and its epitope 2, as well as the binding sites of peptides to the CD38. A and B) the CD38 is shown in green and epitope 2 site in purple. C) The binding site of the designed peptides and the MU523 nanobody modeled with AlphaFold2 (as a control model) to the CD38. D) The binding site of the designed peptides and the MU523 nanobody modeled with I-TASSER (as a control model) to the CD38. The CD38 is shown in green, MU523 nanobody in beige, Isa in red, CDR3a23 (L-pep) in blue, CDR3a67 in yellow, and circular CDR3a23 (C-pep) in orange.

3.4. Protein binding energy prediction (PRODIGY)

Following molecular docking, the PRODIGY server was also utilized to assess the binding affinities of each CD38-peptide complex and determine the most stable interactions. Table S3 summarizes the binding free energy (ΔG) and dissociation constants (K_d) for each peptide, modeled both by I-TASSER and AlphaFold2. The PRODIGY results indicate that, among the designed peptides, the Isa peptide showed the highest binding affinity to CD38, as evidenced by a ΔG of $-12.0 \text{ kcal mol}^{-1}$ and a dissociation constant (K_d) of $1.6 \times 10^{-9} \text{ M}$, both of which reflect strong binding potential. In the case of AlphaFold2-modeled structures, the CDR3a67 and CDR3a23 peptides exhibited the ΔG values of -10.9 and $-10.3 \text{ kcal mol}^{-1}$, respectively. These values were also -10.2 and -10.0 for the CDR3a67 and CDR3a23 peptides modeled by I-TASSER, respectively.

So, to be sure of the best selection, we performed molecular dynamics as the PRODIGY results indicated close ΔG values for the designed peptides.

3.5. Molecular dynamics simulation

Molecular dynamics (MD) simulations were conducted on the peptides-CD38 complexes to assess dynamic behavior and stability in a biological environment. Analyses of root mean square deviation (RMSD), radius of gyration (Rg), root mean square fluctuation (RMSF), secondary structure content, hydrogen bonding, and MM-PBSA energies provided insight into the conformational stability and binding efficacy of these peptide-CD38 complexes.

The RMSD values for each CD38-peptide complex reveal the structural stability of the system throughout the simulation. The average RMSD values for CD38 alone were 0.21 nm , while these values for CD38 in complex with the CDR3a23, CDR3a67, and Isa peptides were 0.22 , 0.3 , and 0.22 nm , respectively (Fig. 4A). For individual peptides, CDR3a23 peptide exhibited a lower RMSD (0.19 nm), suggesting higher stability, while CDR3a67 and Isa peptides had higher RMSD values at 0.25 and 0.24 nm (Fig. 4D).

The Rg values were used to monitor the compactness of each

complex. The average Rg for the unbound CD38 was 1.97 nm , while its complex with CDR3a23, CDR3a67, and Isa peptides showed average Rg values of 1.77 , 1.90 , and 1.89 nm , respectively. The lower Rg value for the CD38/CDR3a23 complex suggests increased compactness conformation (Fig. 4B). For the peptides themselves, the CDR3a23 peptide demonstrated an Rg of 1.88 nm , further indicating structural compactness in its bound state, in contrast to the CDR3a67 and Isa peptides with values of 1.76 and 1.74 nm (Fig. 4E).

The RMSF analysis demonstrates the relative flexibility of CD38 residues when bound to the designed peptides compared to CD38 alone. Overall, the average RMSF for CD38 in complex with the CDR3a23 peptide is approximately 0.12 nm , whereas CD38 bound to CDR3a67 and Isa shows slightly lower average RMSF values of around 0.10 nm . These findings suggest that CD38, when bound to the CDR3a23 peptide, experiences somewhat greater flexibility in specific regions than that of the Isa and CDR3a67 peptides. From the RMSF plot, certain residues exhibit higher fluctuations than others across all complexes, particularly at the N-terminal region and a few other peaks around residues 180 and 250. These regions correspond to higher RMSF values of up to 0.6 – 0.7 nm , indicating more flexibility in these parts of the CD38 structure. Notably, these high-fluctuation regions are outside the main binding interface, as critical residues for binding tend to exhibit lower RMSF values due to the stabilization provided by interactions with the peptides or nanobody. For the core epitope 2 region of the CD38, which includes residues like K69, Y70, E72, H74, R78, and others around the binding interface, the RMSF values are generally lower, indicating reduced flexibility. This decreased fluctuation is expected, as binding interactions typically stabilize these residues, reducing their mobility.

The average number of hydrogen bonds between CD38 and the peptides supports binding stability, in which the CDR3a23 peptide exhibits the highest number of hydrogen bonds (6.9), followed by the CDR3a67 and Isa peptides (4.4 and 4.2 , respectively) (Fig. 4G).

The MM-PBSA results (Table 2) also indicate the binding free energies for each complex. In line with the docking results, the CD38/CDR3a23 complex exhibited somewhat lower binding free energy ($-35.11 \text{ kcal mol}^{-1}$) than that of the CD38/CDR3a67 ($-31.37 \pm 9.31 \text{ kcal mol}^{-1}$) and the CD38/Isa (-22.26 ± 11.19) complexes, suggesting slightly more favorable binding interaction among the three complexes.

The MM-PBSA results provide detailed insights into the thermodynamics of binding interactions, breaking down the contributions of various energy components to the total binding free energy (ΔG Total). The CD38/CDR3a23 complex showed the most favorable van der Waals energy (ΔW) value of $-45.68 \pm 5.57 \text{ kcal mol}^{-1}$, significantly lower (more favorable) than the values for the CD38/Isa ($-29.81 \pm 4.65 \text{ kcal mol}^{-1}$) and CD38/CDR3a67 ($-27.45 \pm 7.73 \text{ kcal mol}^{-1}$) complexes. The ΔW energy reflects the contribution of non-covalent, attractive interactions between atoms at the binding interface. The highly favorable ΔW for the CD38/CDR3a23 complex indicates excellent spatial complementarity between the CDR3a23 peptide and CD38, resulting in stronger and more stable binding. The CD38/CDR3a23 complex also exhibited the most favorable electrostatic energy (ΔEE) value of $-75.81 \pm 68.26 \text{ kcal mol}^{-1}$, outperforming the CD38/Isa ($-65.14 \pm 60.12 \text{ kcal mol}^{-1}$) and CD38/CDR3a67 ($21.76 \pm 41.68 \text{ kcal mol}^{-1}$) complexes. Electrostatic energy reflects interactions between charged residues at the interface. A lower (more negative) ΔEE suggests stronger ionic interactions, such as salt bridges or hydrogen bonds. The favorable ΔEE for the CD38/CDR3a23 complex highlights the significant role of electrostatic interactions in stabilizing the CD38-peptide complex, further supported by the structural positioning of key charged residues. The solvation free energy (ΔG SOLV) for the CD38/CDR3a23 complex was also $86.38 \pm 62.77 \text{ kcal mol}^{-1}$, slightly higher than the CD38/Isa ($72.70 \pm 51.65 \text{ kcal mol}^{-1}$) and CD38/CDR3a67 ($-25.68 \pm 33.23 \text{ kcal mol}^{-1}$) complexes. ΔG SOLV represents the energetic cost of displacing water molecules at the binding interface. While a high ΔG SOLV value can indicate unfavorable solvation effects,

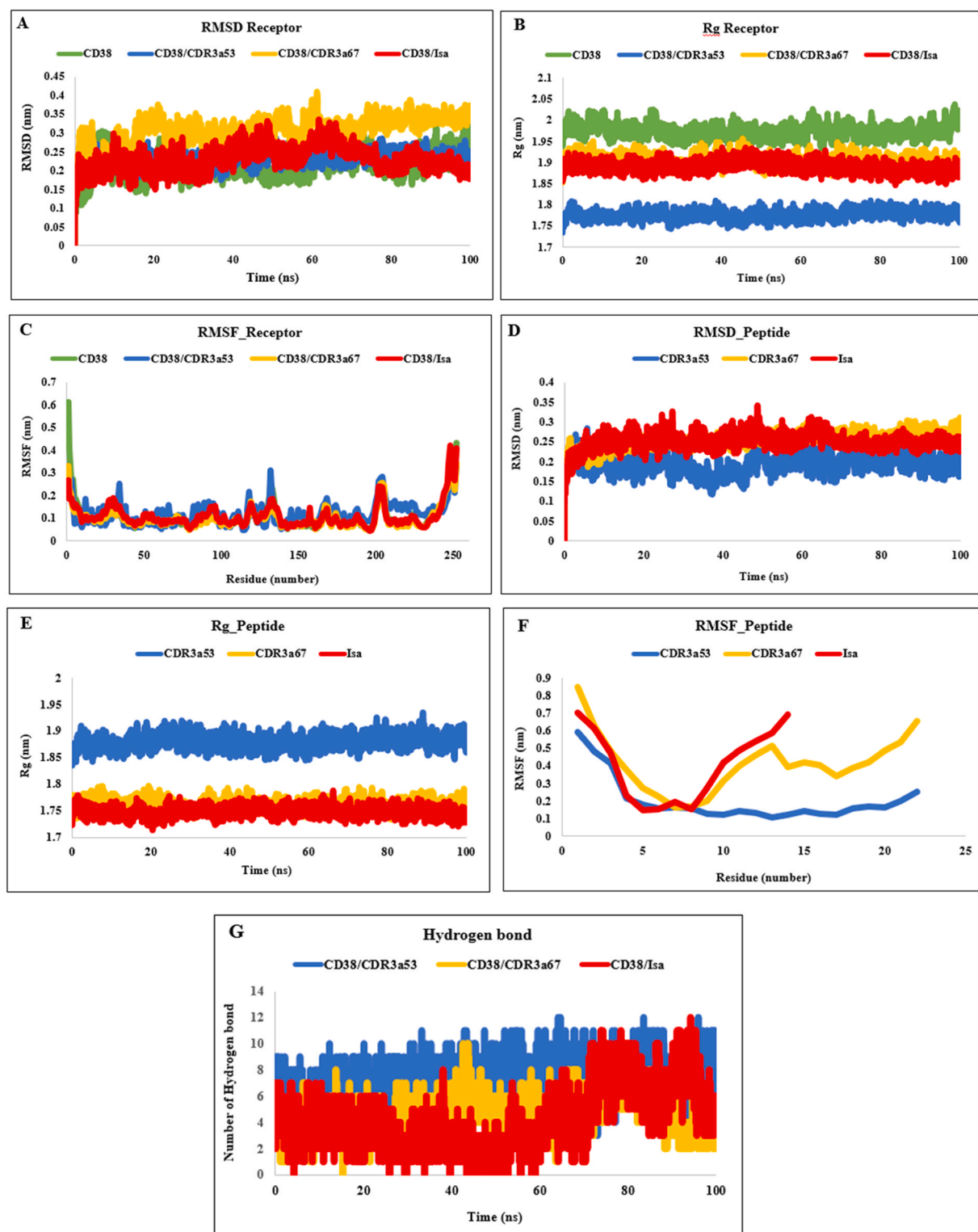


Fig. 4. The molecular dynamics simulation results of the CD38 alone and in complex with the designed peptides. A) The RMSD, B) Rg, and C) RMSF of the CD38 alone and in complex with the designed peptides (CDR3a23, CDR3a67, and Isa). D) The RMSD, E), Rg and F) RMSF of the designed peptides. G) The number of hydrogen bonds between the CD38 and the designed peptides.

the total binding free energy (ΔG Total) remains strongly negative for the CD38/CDR3a23 complex, indicating that other interactions (ΔW and ΔEE) compensate for the solvation cost. The higher solvation energy for the CD38/CDR3a23 complex might result from the displacement of a larger number of water molecules due to the compact and specific binding interface. Overall, the results confirmed that all of the designed

peptides are suitable candidates against CD38, but the CDR3a23 peptide is somewhat better inhibitor than that of the CDR3a67 and Isa peptides.

The secondary structure content was also analyzed to assess structural integrity during the simulation (Fig. 5). Alpha-helix content was slightly increased in the CD38/CDR3a23 complex (37.1 %) compared to the CD38 alone (34.3 %), suggesting that the binding interaction

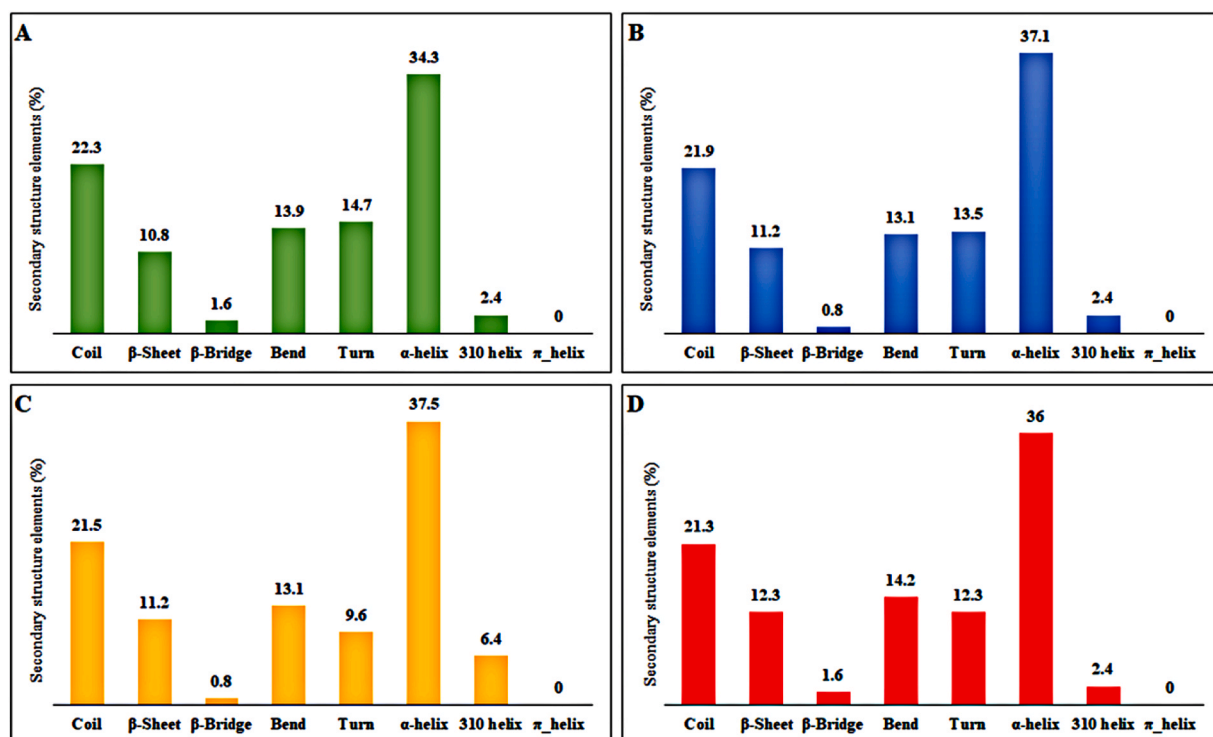


Fig. 5. The percentage of secondary structures of CD38 A) alone and in complex with B) CDR3a23, C) CDR3a67, and D) Isa peptides.

between the CDR3a23 peptide and CD38 supports structural stability. Similarly, the minor changes in β -sheet, coil, and turn percentages indicate the stability of secondary structure in all complexes.

We also used the same comprehensive analyses—structural modeling, molecular docking, PRODIGY, and molecular dynamics simulations—to confirm our preliminary results about the effectiveness of the CDR3a23 peptide for the whole MU523 nanobody. By using this method, we were able to confirm if the complete nanobody maintained the structural integrity, stability, and binding effectiveness observed with the peptide alone. We offer a thorough analysis of these findings and go over their ramifications in relation to CD38 inhibition below.

With a low RMSD of 0.391 and a TM-score of 0.9919, the structural validation of the MU523 nanobody using AlphaFold2 produced remarkable modeling accuracy. A low RMSD indicates a little departure from the target structure, and a TM-score around 1 indicates a high degree of alignment with the native-like fold. These numbers are suggestive of a highly accurate model. It was shown that 88.3 % of the nanobody residues are found in core regions, 10.8 % are found in allowed regions, and none are found in disallowed zones. The model's dependability is supported by its excellent structural integrity, which is further supported by the Qmean (0.74) and PROSA (-6.58) scores (Table S2). These measurements show a nanobody model that is both energetically advantageous and structurally stable. The high proportion of residues in core regions aligns with structural robustness, indicating that the full MU523 nanobody structure closely resembles an ideal fold. Such stability is essential for therapeutic applications, as it predicts a minimized risk of misfolding under physiological conditions, which could enhance the nanobody's efficacy and pharmacological stability (Fig. 2E).

Molecular docking results showed a HADDOCK score of -77.2 ± 11.5 for the MU523 nanobody/CD38 complex, indicating a high binding affinity. Additionally, van der Waals and electrostatic contributions were notably favorable, with values of -44.3 ± 6.6 and -192.1 ± 50.5 , respectively (Table 1). Binding affinity (ΔG) calculated by PRODIGY was -9.9 kcal/mol, with a dissociation constant (K_d) of 5.7×10^{-8} M at 25°C (Table S3). The MD results indicated that the

average RMSD for the MU523 nanobody/CD38 complex is 0.34, which was slightly higher than the CDR3a23/CD38 complexes but still low enough to indicate overall stability. On the other hand, the RMSD values for the MU523 nanobody remained consistently low, averaging 0.18 nm throughout the 100 ns simulation. The RMSF analysis of the MU523 nanobody/CD38 complex revealed low fluctuation values (0.05–0.20 nm) across most residues, with slightly higher fluctuations observed in the CDR regions (up to 0.30 nm) as the disordered structures. The RMSF values for the CDR3a23 peptide were significantly higher, averaging 0.25–0.40 nm, with the N- and C-terminal residues showing the highest fluctuations (up to 0.60 nm). The lack of a stabilizing scaffold of the CDR3a23 peptide contribute to the higher RMSF values. The Rg values for the both CD38-bounded (2.36) and unbounded nanobody (1.3) forms also confirmed compactness, suggesting a stable, folded structure during the simulation. The Rg values remained stable around 2.1 nm, reflecting a compact and consistent tertiary structure throughout the simulation. The low and stable Rg values confirm the nanobody's structural integrity, essential for maintaining high binding specificity and stability. The Rg values fluctuated between 0.8 and 1.1 nm, reflecting the smaller and more flexible nature of the CDR3a23 peptide. These values indicate that the CDR3a23 peptide adopts a more dynamic and less compact conformation during the simulation, consistent with its linear structure. These results reinforce the full nanobody's stability and predict minimal degradation during therapeutic delivery (Fig. 6B–G). The high hydrogen bond number between the MU523 nanobody and CD38 (28.38) also highlights the formation of a strong and stable interface with CD38 relative to the CDR3a23 peptide (6.9). This significant number of hydrogen bonds suggests a high degree of specificity, with the MU523 nanobody creating extensive interactions that anchor it to the CD38 (Fig. 6H).

After MM-PBSA analysis, total binding energy for the MU523 nanobody/CD38 complex was -324.81 ± 18.90 kcal/mol, as a highly favorable energy profile (9-fold higher than that of the CDR3a23). The interaction energies, with strong van der Waals (-305.51 ± 12.05) and electrostatic contributions (-874.38 ± 84.02), underscore the robustness of the binding (Table 2). This stability is critical for applications

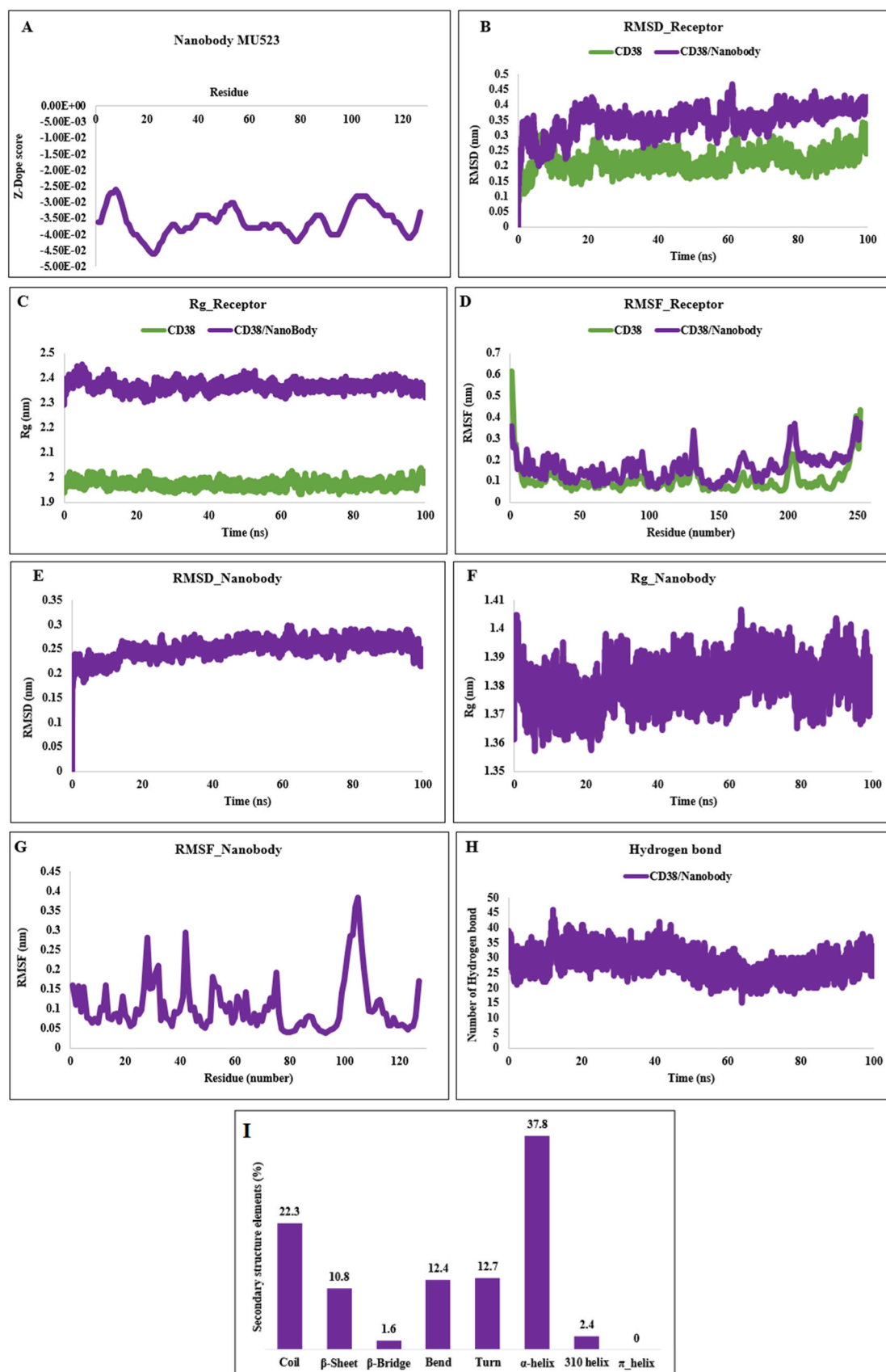


Fig. 6. The results of Z-Dope score and molecular dynamics simulation of the MU523 nanobody. A) Z-Dope score. B) RMSD, C) Rg, and D) RMSF of the CD38 complexed with the MU523 nanobody. E) The RMSD, F) Rg, and G) RMSF of the MU523 nanobody. H) Number of hydrogen bonds of the CD38/MU523 nanobody complex. I) The percentage of secondary structures of CD38 complexed with MU523 nanobody.

requiring prolonged CD38 inhibition, as a lower binding energy correlates with higher interaction persistence and reduced dissociation risk under physiological stressors.

Analysis of CD38's secondary structure in association with the MU523 nanobody also revealed that, like the CDR3a23 peptide, the α -helix content slightly increased to 37.8 %, while other secondary components stayed mostly steady. This conservation suggests that the CD38's normal folding is not much changed by the binding interaction. The MU523 nanobody attaches to CD38 without causing conformational changes that may compromise CD38's inherent activities, as evidenced by the stability of CD38's secondary structure in complex. This characteristic is critical to the effectiveness of inhibitors because it promotes selective inhibition while preserving CD38's structural integrity, reducing the possibility of adverse consequences associated with structural disruption (Fig. 6I). The nanobody retained its secondary structure, including stable beta sheets and alpha helices, throughout the simulation. The preservation of secondary structure elements underscores the nanobody's structural stability and robustness, crucial for its binding efficacy and resistance to conformational changes. The CDR3a23 peptide displayed minimal secondary structure, primarily adopting random coil conformations with occasional turns and bends. The lack of a stable secondary structure makes the CDR3a23 peptide more flexible but less stable, which could limit its performance in environments requiring rigidity. So, the nanobody MU523 demonstrates superior structural stability, with low RMSD and Rg values, while CDR3a23 peptide exhibits higher flexibility and less compactness. Both nanobody MU523 and CDR3a23 peptide bind effectively to CD38, but the nanobody achieves higher binding affinity and stability due to its well-structured framework. It should be noted that the discrepancy between the HADDOCK (more favorable HADDOCK score for the CDR3a23 peptide relative to the MU523 nanobody) and MM/PBSA results (much more negative binding energy for the MU523 nanobody than the CDR3a23 peptide) arises from the fundamental differences in what these methods measure. HADDOCK scores reflect the quality of the predicted docking pose and are influenced by the size, flexibility, and fit of the ligand in the binding pocket. This often leads to more favorable scores for small and flexible peptides. In contrast, MM/PBSA calculations, performed after molecular dynamics simulations, provide a more accurate estimate of binding free energy by considering the dynamic stability, solvent effects, and entropic contributions of the complex. Therefore, while the peptide achieves a better HADDOCK score, the nanobody forms a more stable and energetically favorable complex during MD simulations, as reflected in the MM/PBSA results [37–41].

In this regard, we also performed both structural modeling and docking using the Chai-1 Discovery to validate our results (Figure S6). Generally, the Chai-1 Discovery reveals two scores of pTM (predicted TM-score) and ipTM (interface predicted TM-score). The pTM is an integrated measure of how well the model has predicted the overall structure of a complex. Typically, a pTM score above 0.5 suggests structural similarity and that the predicted structure is reliable. On the other hand, the ipTM specifically evaluates the accuracy of the predicted relative positions of subunits within a complex. The ipTM scores above 0.6 are generally considered successful predictions of interface quality [36]. In our study, the structural modeling results from Chai-1 Discovery were satisfactory (pTM scores of 0.75, 0.69, 0.86, 0.86, and 0.88 for the MU523, MU1067, CDR3a23, CDR3a67, and Isa, respectively); however, the docking results did not indicate favorable findings (ipTM scores: 0.34, 0.21, 0.28, 0.23, and 0.26 for the MU523, MU1067, CDR3a23, CDR3a67, and Isa, respectively). This validation confirmed that our study, which employed AlphaFold2 were more accurate and in complete agreement with the HADDOCK results.

Therefore, while the peptide achieves a better HADDOCK score, the nanobody forms a more stable and energetically favorable complex during MD simulations, as reflected in the MM/PBSA results.

It should be noted that by modifying the CDR3a23 peptide (L-pep) with cysteine residues at both the N- and C-termini to create a circular

peptide structure (C-pep), we observed some improvements in its stability compared to the L-pep while its binding was lower than that of the L-pep. The C-pep has an RMSD of 0.378, indicating enhanced stability compared to the linear peptide. Cyclization typically boosts structural stability by reducing conformational flexibility, and in this case, it contributes to a more rigid, stable peptide structure. Additionally, the PROSA score of 0.88 is an improvement, indicating a better fit within the database of known stable protein structures (Table S2). The addition of cysteines and the resulting disulfide bond contribute to fewer disallowed regions in the peptide structure, helping to maintain a stable and well-folded conformation (Fig. 2C and Table S2). The HADDOCK score of -101.8 for the C-pep suggests some lower binding characteristics to CD38 relative to the L-pep. Also, less negative van der Waals and electrostatic energy values indicate weaker binding interactions (Table 1). However, ΔG and K_d values for the circular peptide were achieved as -10.5 kcal/mol and 1.98×10^8 , respectively, compared to the L-pep. The TM-score of 0.8531 and a high core percentage of 95.5 % reflect the structural integrity and accuracy of the circular peptide (Table S3). In summary, the C-pep demonstrates superior stability but lower binding affinity to the CD38 compared to the L-pep. These advantages/disadvantages make the C-pep more experimentally evaluated and compared with L-pep. So, we selected and synthesized either the L-pep or the C-pep and compared their bindings and functions experimentally. It should be noted that for further convenience we apply the "L-pep" and "C-pep" for the linear and circle CDR3a23 peptides, respectively in the following sections.

3.6. Expression evaluation of the extracellular CD38 receptor

It is well known that ATRA can cause human myeloid leukemia cells to differentiate, especially in the HL-60 cell line, where it dramatically increases the expression of the extracellular CD38. The start of myeloid differentiation processes, such as G1/G0 phase cell cycle arrest and increased oxidative metabolism, is linked to increase the extracellular CD38. As explained in the methods section, we treated the HL60 cells with $1 \mu\text{M}$ ATRA for 12, 24, and 48 h to increase the extracellular CD38. Remarkably, the expression of extracellular CD38 noticeably increased from 94.9 % (at the FITC intensity between 10^1 and 10^2), as the basic level, to 99.2 %, 99.5 %, and 99.8 % (at the FITC intensity between 10^2 and 10^3) following the cells treatment with $1 \mu\text{M}$ ATRA after 12, 24, and 48 h, respectively (Fig. 7A). So, because no difference was observed among the treatment times, we applied the treatment time of 12 h in the next tests. Up-regulation of the extracellular CD38 on the SH-SY5Y cell was also examined. As seen in Fig. 7B, the results showed that even after 48 h treatment with $1 \mu\text{M}$ ATRA, the SH-SY5Y cells had nearly no extracellular CD38 (0.28 % at the FITC intensity of 10^0 – 10^1). Therefore, to assess the peptides' binding and function (L-pep and C-pep), we used SH-SY5Y cells as the negative cells for the extracellular CD38 and the HL60 cells as the positive one.

3.7. Binding evaluation of the designed peptides on the extracellular CD38

To evaluate the specific binding of the designed peptides, L-pep and C-pep, we conducted four experiments in the following order: 1) binding evaluation of FITC-labeled L-pep (40, 400, and 800 nM) on the HL60 cells at basic expression of the extracellular CD38 and, 2) at a high expression level of the extracellular CD38 (pre-treated with $1 \mu\text{M}$ ATRA for 12 h), 3) binding evaluation of FITC-labeled L-pep (800 nM) on SH-SY5Y cells pre-treated with $1 \mu\text{M}$ ATRA for 12 h, and 4) binding evaluation of the C-pep peptide on HL60 cells pre-treated with $1 \mu\text{M}$ ATRA for 12 h. When HL60 cells expressed a high level of extracellular CD38, the FITC-labeled L-pep indicated noticeable binding to the receptor by the values of 2.7 %, 26.1 %, and 81.2 % at concentrations of 40, 400, and 800 nM, respectively (Fig. 8A). at the basic expression of the extracellular CD38, these values significantly decreased to 1.53 %, 3.92 %, and

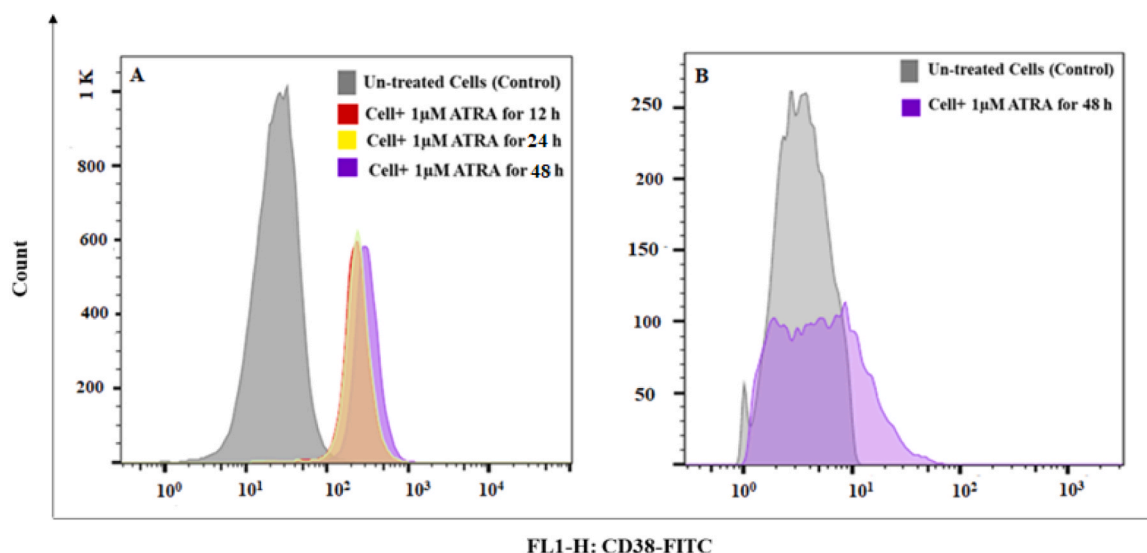


Fig. 7. Evaluation of the extracellular CD38 expression on the A) HL60 and B) SH-SY5Y cells before and after treatment with 1 μ M ATRA. The HL60 cells had a basic expression for the extracellular CD38, which was significantly increased after treatment with 1 μ M ATRA for 12, 24, and 48 h. In contrast, the SH-SY5Y cells had nearly no extracellular CD38 even after 48 h treatment with 1 μ M ATRA. All experiments were done in triplicate.

65.3 %, respectively (Fig. 8B). For further confirmation of the peptide specificity, the binding value of 800 nM FITC-labeled L-pep on the SH-SY5Y cells was 0.36 %, indicating the peptide-specific binding to the extracellular CD38 (Fig. 8C). Moreover, the FITC-labeled L-pep, approximately indicated no binding (0.93 % and 1.58 % at 400 and 800 nM, respectively) when HL60 cells were pretreated with high concentration of the C-pep peptide (3 μ M). This result shows the specificity of the C-pep peptide against the extracellular CD38, similar to the L-pep. In a competitive experiment conducted with the same concentrations of the both peptides (800 nM), the FITC-labeled L-pep indicated a binding value of 54.6 %, while its binding affinity was 81.2 % when the cells were not pretreated with the C-pep (Fig. 8D). This result indicates that the L-pep has a higher binding affinity to the extracellular CD38 than that of the C-pep, which is in line with our *in silico* results. On the other hand, the K_d for the L-pep was experimentally achieved as 630 nM, while the *in silico* data revealed it as 29 nM. This difference reveals the importance of experimentally validation of the *in silico* data.

3.8. The designed peptides demonstrate potential anti-proliferative effect

3.8.1. Trypan blue exclusion assay

The trypan blue exclusion experiment demonstrated that, the peptides, especially L-pep, had a substantial and time-dependent impact on the proliferation of the HL60 cells after 24 h treatment, as seen in the Fig. 9A and S7. Additionally, the peptides' combination with 1 μ M ATRA greatly suppressed the proliferation of the cells, which is comparable to 1 μ M ATRA alone. Remarkably, the population of SH-SY5Y cells were unaffected by either ATRA or the peptides even after 72 h (Fig. 9B and S7). So, we conducted a dose-dependent 48 h MTT experiment to validate these findings.

3.9. MTT assay

After setting up the best effective time of the peptides via the trypan blue exclusion assay (48 h), an MTT assay was used to detect the anti-proliferative effect of the designed peptides dose-dependently (100, 300, 500, 800, and 10000), alone or in combination with 1 μ M ATRA, on the HL-60 and SH-SY5Y cells. It should be noted that the same concentrations were used to evaluate the anti-proliferative effect of ATRA on the cells. As shown in the Fig. 10A, the proliferation of the HL-60 cells was found to be noticeably inhibited by the peptides in a dose-

dependent manner, while they had no effect on the SH-SY5Y cells (Fig. 10B). The inhibition rate of HL-60 cells significantly increased following incubation the cells with the peptides in combination with 1 μ M ATRA. However, the effect of C-pep was lower than that of the L-pep, confirming the *in-silico* and the binding results. The IC_{50} values of the L-pep and C-pep against the HL-60 cells were achieved as 540 nM and 780 nM ($P < 0.0001$), respectively. Also, when the peptides combined with 1 μ M ATRA, these values significantly decreased to 390 nM and 630 nM ($P < 0.0001$), respectively. It should be noted that ATRA inhibited 30 % of the HL60 cells at the final concentration (1 μ M) while, similar to the peptides, it had no anti-proliferative effect on the SH-SY5Y cells.

3.10. Apoptosis and cell cycle analysis

Annexin V-FITC/PI double staining was also utilized to assess the mechanism of the peptides as shown in Fig. 11. The early apoptotic rates of HL-60 cells treated with the IC_{50} values of L-pep and C-pep for 48 h were 13.9 % and 10.2 %, respectively, which were significantly higher than that of the control or un-treated cells (3.09 %). Also, the late apoptotic rates for the peptides were achieved as 33.6 % and 24.1 %, respectively, which are comparable relative to the control (0.53 %). At the same condition, when the peptides combined with 1 μ M ATRA, these values increased to 30.1 % and 12.5 % for early apoptosis and 42.4 % and 42.8 % for late apoptosis, respectively. Also, total apoptosis of the L-pep and C-pep achieved as 47.5 % and 34.3 %, respectively, which significantly increased to 72.5 % and 55.3 %, respectively, when were combined with 1 μ M ATRA. This is while, under the same conditions, the early, late, and total apoptosis for 1 μ M ATRA achieved as 14.5 %, 30.4 %, and 44.9 %, respectively. Similar to trypan blue and MTT assays, the L-pep indicated more apoptotic effect against the HL60 cells relative to the C-pep. However, the both peptides demonstrated more apoptotic effect when combined with ATRA which is in line with the previous results.

It is commonly acknowledged that DNA breakdown and subsequent leaking from apoptotic cells result in decreased DNA stability. PI can be used to stain this DNA. As a result, the emergence of cells with limited stability in cultures—such as hypoploid or sub-G1 cells—has been regarded as an additional indicator of apoptosis and has been utilized to measure its severity. Nuclear DNA distribution was examined using flow cytometry to demonstrate in further detail how the peptides'

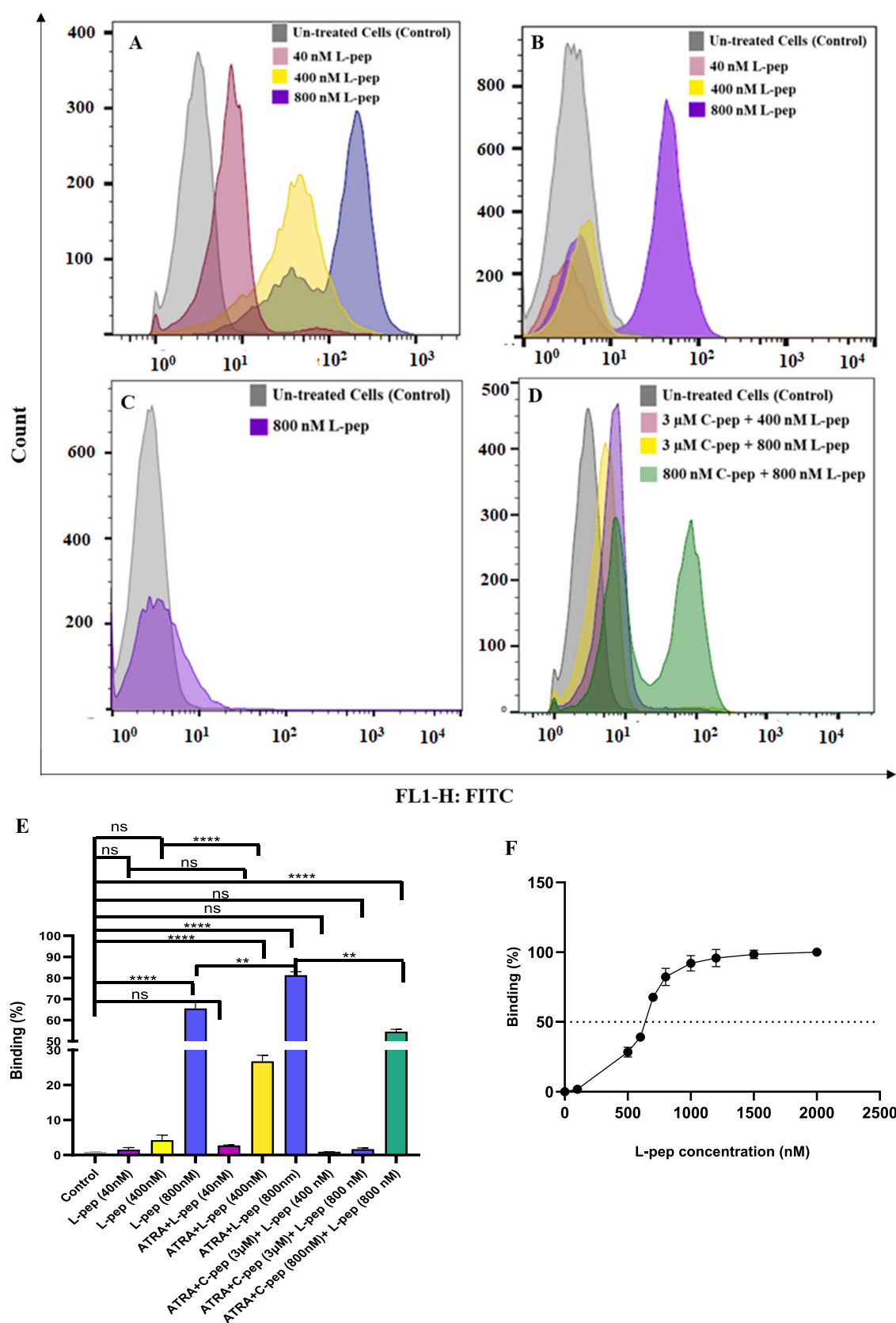


Fig. 8. The specific binding of FITC-labeled L-pep on A) HL60 cells with high expression of extracellular CD38 achieved by pre-treating the cells with 1 μ M ATRA for 12 h, B) HL60 cells with basic expression of extracellular CD38, C) SH-SY5Y cells pre-treated with 1 μ M ATRA for 12 h. D) The competitive binding of C-pep and L-pep on the extracellular CD38 of HL60 cells. E) Quantitative binding of the C-pep and L-pep at the mentioned conditions. F) K_d determination of the L-pep using Bio TeK Cytation 3. Fluorescence of cells incubated without peptides was minimal and subtracted from the presented data. All experiments were done in triplicate. ns: not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

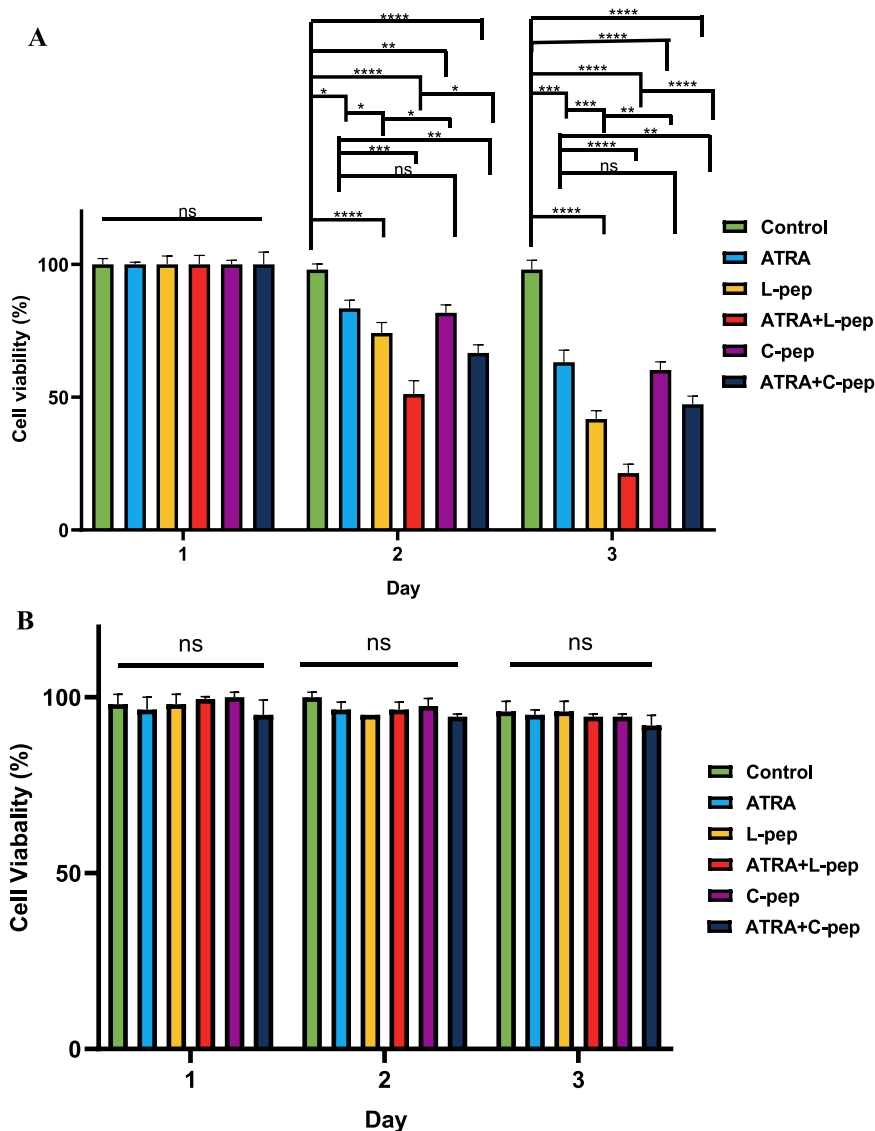


Fig. 9. Evaluation of anti-proliferative effects of the designed peptides, ATRA, and their combination together on the A) HL60 and B) SH-SY5Y cells using trypan blue exclusion assay. All experiments were done in triplicate. ns: not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

suppression of cell growth is intimately linked to apoptosis. Therefore, we also looked at how the peptides L-pep and C-pep, ATRA, and their combinations affected the G1, S, and G2 phases of the HL-60 cell cycle.

The cells were treated with each peptide alone and also in combination with 1 μ M ATRA for 48 h at their achieved IC_{50} . Cell cycle distribution was assessed using flow cytometry after staining with PI, allowing us to quantify the percentage of cells in each phase. The results indicated that the cell population in G1 phase increased from 32.14 % (un-treated cells as the control) to 52.21 %, 42.41 %, and 44.23 % after treatment of the HL60 cells with L-pep, C-pep, and ATRA, respectively. The results were also accompanied by a significant decrease in the S (36.31 %, 32.56 %, and 28.71 %) and G2/M (11.19 %, 23.45 %, and 23.79 %) phases via treatment of the cells with L-pep, C-pep, and ATRA, respectively, while these values for the control were 50.43 % and 19.68 %, respectively. These results indicated that the peptides and ATRA induced post-G1-arrest apoptosis in the HL60 cells. Similar to the results achieved from other methods, the combination of L-pep with 1 μ M ATRA increased the G1 phase to 65.9 %, accompanied by a significant reduction in the cell populations in the S (18.04 %) and G2 phases (6.39 %). This result was similar for the combination of C-pep with 1 μ M ATRA with the values of 63.78 %, 19.84 %, and 8.35 % for

G1, S, and G2 phases, respectively. It should be noted that, under the same conditions, G1, S, and G2 values were achieved as 44.23 %, 28.71 %, and 23.79 %, respectively, when the cells were treated with 1 μ M ATRA alone (Fig. 12).

4. Discussion

Several hematologic malignancies, including multiple myeloma (MM), acute myeloid leukemia (AML), and T-cell acute lymphoblastic leukemia (T-ALL), have shown the overexpressed CD38 as a validated tumor antigen [42]. Furthermore, recent studies have focused on the possible role of CD38 in neurodegenerative diseases like Parkinson's and Alzheimer's disorders. Its expression in brain cells, such as microglia, astrocytes, and neurons, points to a major role in the pathophysiology of neurodegeneration as well as neuronal health [43,44]. Further sustaining neuroinflammation and neuronal damage, elevated that CD38 levels can result in increased release of pro-inflammatory cytokines and a senescence-associated secretory phenotype (SASP) in astrocytes and microglia [43]. In this regard, for the first time, our lab also indicated that CD38 plays a significant role in epilepsy [6]. Sadeghi et al. also discovered a novel mechanism effect for phenytoin to potentially block

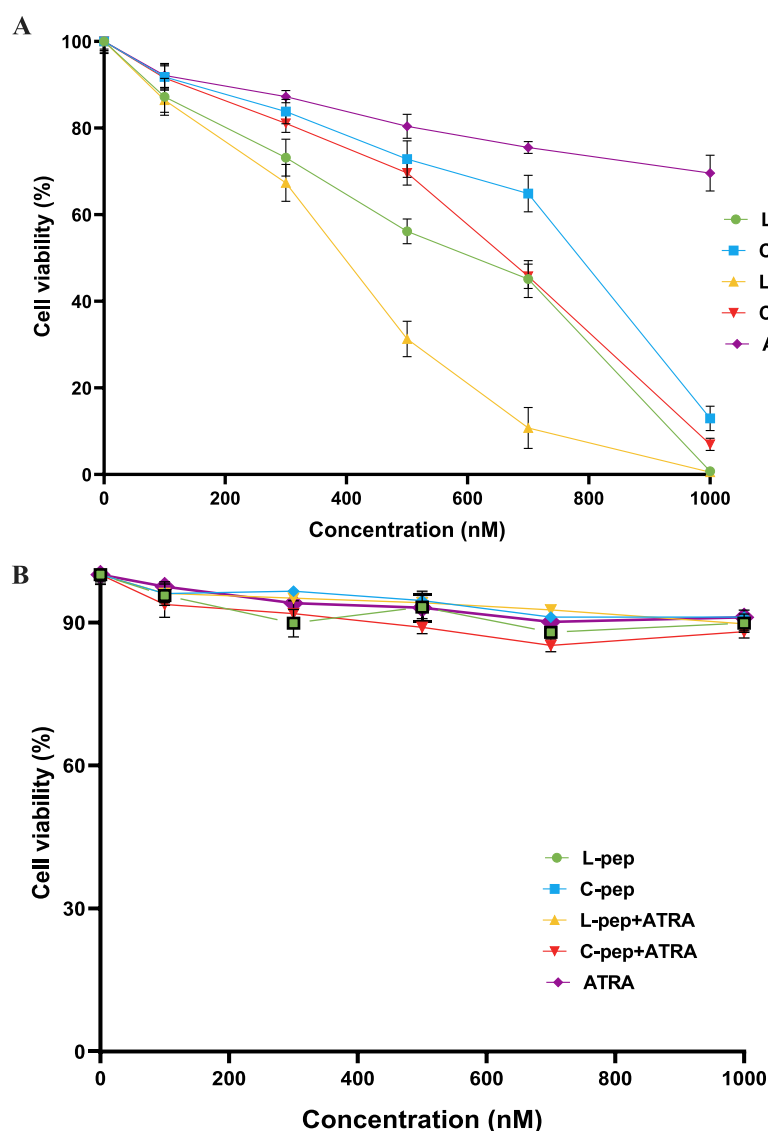


Fig. 10. Evaluation of anti-proliferative effects of the designed peptides, ATRA, and their combination together on the A) HL60 and B) SH-SY5Y cells for 48 h using MTT assay. The P value for all groups was achieved as $P < 0.0001$ for HL60 cell line, while it was not significant (ns) for SH-SY5Y cell line.

the CD38 in hippocampus cells. Therefore, CD38 may be a viable treatment option for neurological illnesses like epilepsy in addition to being a key target for hematologic malignancies [45].

Multiple myeloma treatment has changed dramatically with the advent of anti-CD38 monoclonal antibodies such as isatuximab and daratumumab [46]. Nevertheless, these antibodies are costly and not accessible everywhere. Therefore, we set out to design anti-CD38 peptides derived from the isatuximab, and MU523 and MU1067 nanobodies and assessed the binding and function of the more appropriate one. In this line, only one study has developed a peptide derived from isatuximab targeting CD38 with the K_d of 1 μ M. Using the isatuximab-derived peptide, Omstead D. et al. investigated the delivery of therapeutic drugs in multiple myeloma cells using liposomal nanoparticles that target CD38. Therefore, the suppression of CD38 was not the primary emphasis [7].

We bioinformatically generated three peptides against CD38 as part of a thorough investigation, and for the first time, we experimentally assessed the binding and functionality of the best one. Although all of the designed peptides (CDR3a23, CDR3a67, and Isa) revealed favorable binding to the CD38, but based on the docking and MD results, the CDR3a23 (L-pep) peptide indicated somewhat more appropriate peptide

against the CD38. Following synthesis, the L-pep binding to CD38 was experimentally assessed in both the linear and circular forms ($K_d = 630$ nm for the L-pep). Furthermore, we demonstrated that ATRA dramatically increases the expression of CD38 in HL-60 cells, which is connected to the differentiation process. Thus, CD38 serves as a modulator of myeloid differentiation in addition to being a marker [47].

Similar to Zainab N. et al. study, the increased amount of the receptor was similar together at the different incubation times of the HL60 cells treated with 1 μ M ATRA [48]. Additionally, in line with the previous investigation, ATRA only caused differentiation in SH-SY5Y cells and had no influence on the number of extracellular CD38 receptors [49]. Because the binding qualities of the peptides depended on the amount of extracellular CD38 receptors, the binding data significantly confirmed the bioinformatic results. However, compared to the L-pep, the C-pep displayed a lower affinity for the CD38. This finding first suggests that any alteration to the peptide sequence may impact its binding or function, and second, the peptides' specificity is demonstrated by their reliance on the quantity of extracellular CD38 receptors.

Investigating the peptides' activity and impact on HL60 and SH-SY5Y cells further validated their reliance on the extracellular CD38. In this line, four experiments were conducted. Trypan blue assay, MTT

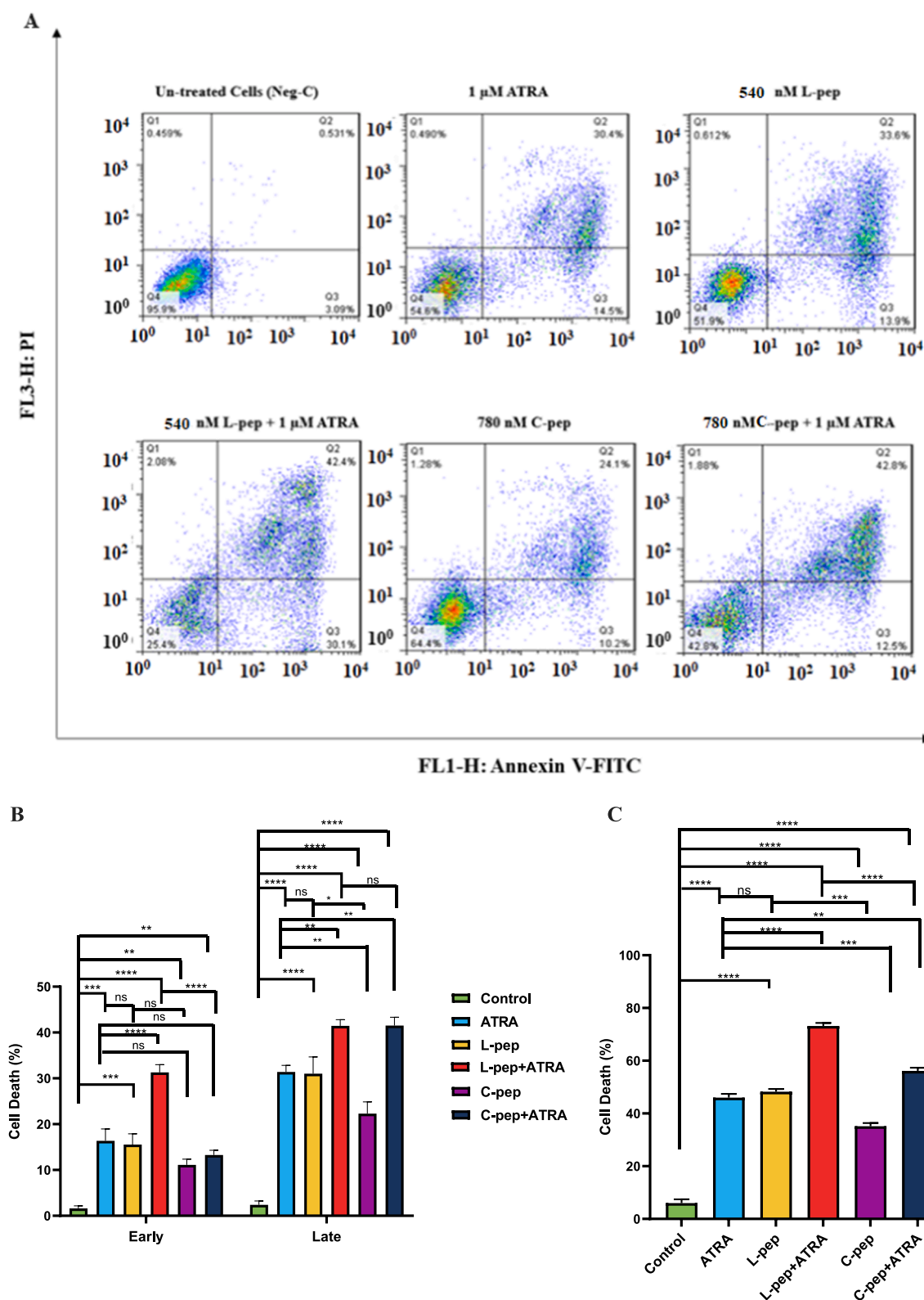


Fig. 11. Evaluation of the apoptotic effect of the peptides, ATRA, and their combinations at their IC_{50} concentrations for 48 h in the HL60 cells. A) Flow cytometry plots: Q1: necrosis %, Q2: late apoptosis %, Q3: early apoptosis %, and Q4: viable cells %. B) Chart comparing the percentage of early and late apoptosis and C) Comparison of total apoptosis (early and late) among the mentioned treatment conditions. ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

assay, apoptosis, and cell cycle that all showed the peptides' effect increased when HL60 was treated with 1 μ M ATRA; however, SH-SY5Y cells showed no effect. Both of the peptides indicated anti-proliferative effects on HL60 dose-dependently and time-dependently according to

the trypan blue and MTT assays, respectively. However, the L-pep effect was greater than the C-pep effect on either untreated or treated HL60 cells with 1 μ M ATRA, which is consistent with the binding data. These findings suggest that, first, the function of peptides depends on their

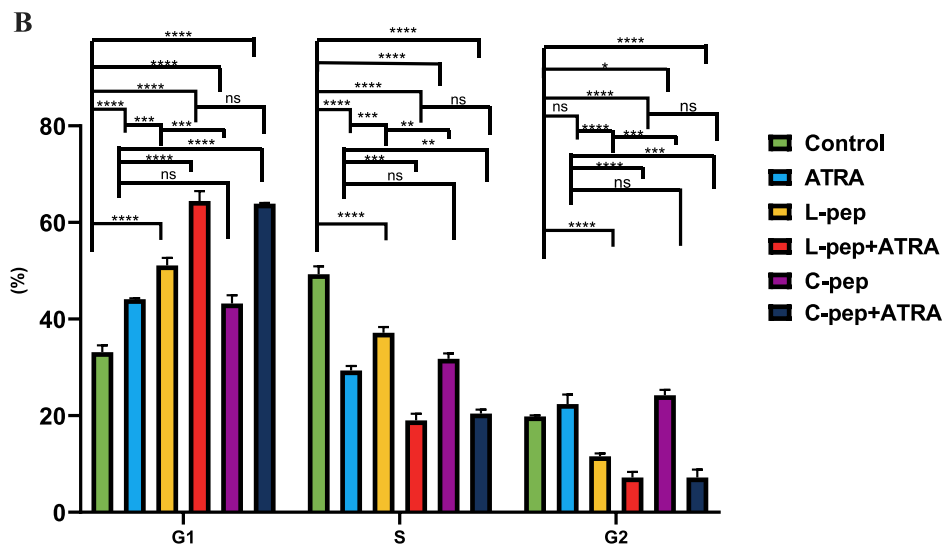
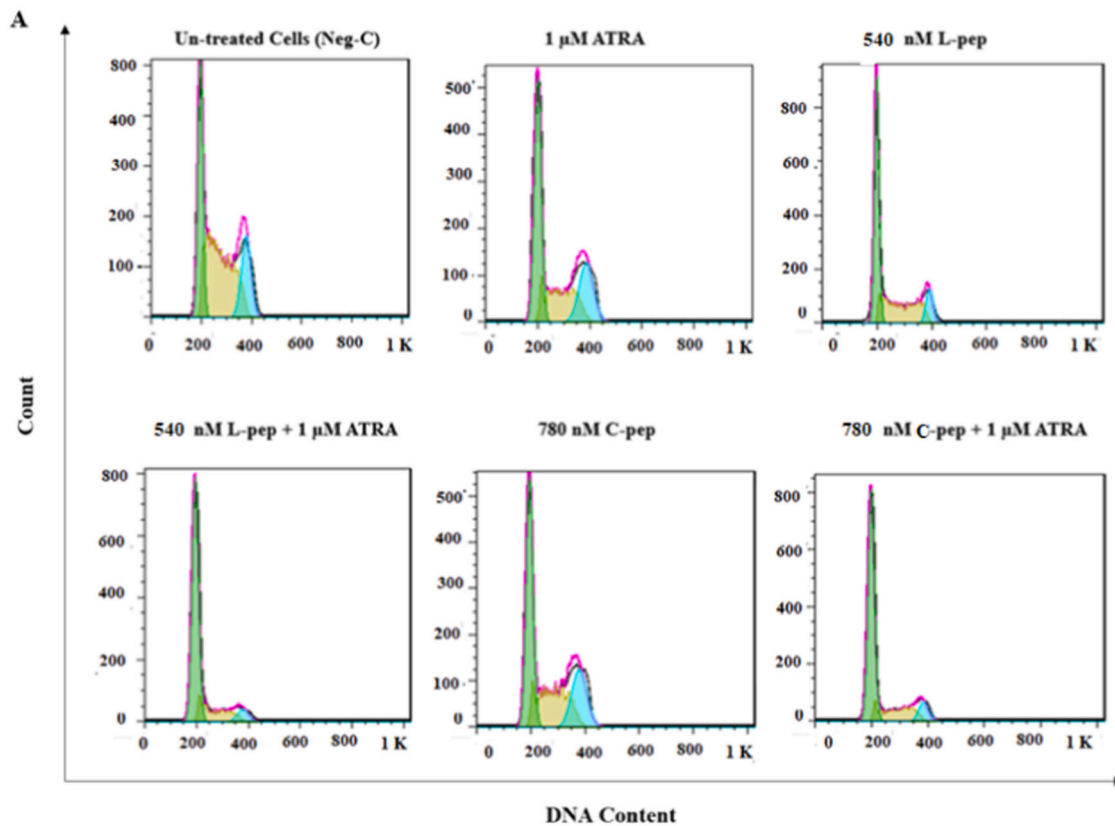


Fig. 12. A) Evaluation of the cell cycle of HL60 cells after treatment with the designed peptides, ATRA, and their combinations at their IC_{50} concentrations for 48 h. B) Quantitative evaluation of the cell cycle of HL60 cells after treatment under the mentioned conditions. ns: not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

ability to bind to their target; second, the peptides only act on cells that express a high level of the CD38; and third, in hematologic cancers, the combination of ATRA and peptides can be used in place of ATRA plus chemotherapy or anti-CD38 antibodies. Similarly, Nijhof I et al., for example, examined the effects of daratumumab both by itself and in conjunction with ATRA on multiple myeloma (MM) cells obtained from MM patients. They demonstrated that daratumumab had no effect on cells when used alone, but that when combined with ATRA, it had a discernible effect by raising the extracellular CD38 [50]. Congleton J. et al. looked into the combination of F-araNAD⁺ and ATRA, and their findings were appropriate and distinct from those of the earlier study.

According to the study, the small molecule inhibited the enzymatic activity of CD38, which had not anti-proliferative effect against the HL60 either alone or in combination with ATRA [51]. On the other hand, 78c is a strong and specific inhibitor of the CD38 enzyme that can lower the growth of CLL cells [52,53]. This compound is characterized as a reversible and uncompetitive inhibitor for the CD38. The different reports highlight the importance of the inhibitor binding site of on the CD38. Therefore, the binding locations of daratumumab and our designed peptides with ATRA (the back of the receptor, epitopes 3 and 2, respectively) may be the source of their synergistic effects, whereas small compounds bind into the receptor's active site. This hypothesis is

based on Congleton J. and colleagues' findings. They showed that ATRA-induced differentiation is dependent on the membrane receptor function rather than the CD38 ectoenzyme function. These findings might be compatible with impairing the activity of key enzymes, such as the Src family kinases, required for membrane-expressed CD38 signal transmission [51]. This finding demonstrates that certain substances may interact with the CD38 either directly or indirectly and that daratumumab and our designed peptides may interfere with these essential connections for signal transduction. For instance, non-substrate ligands such as CD31 and hyaluronic acid, which control cell-cell and cell-matrix interactions and enhance chemotaxis and neoplastic B cell proliferation, interact with CD38 [54]. Furthermore, a study showed that CD38 is a key modulator of CD45 activity through CD43 and galectin-1, which in turn promotes the growth of CLL. The protein tyrosine phosphatase CD45 plays a crucial role in controlling B cell receptor (BCR) signaling thresholds by removing an inhibitory phosphate group from the Src family kinases, hence increasing their kinase activity and cell proliferation [52].

However, another study found that isatuximab can cause multiple myeloma cells to undergo caspase-dependent apoptosis. This outcome further supports the possibility that certain variables interact with the CD38. On the other hand, isatuximab prevents CD38's ADP ribosylcyclase ectoenzymatic activities. It's interesting to note that myeloma progression is connected with adenosine produced in the bone marrow niche through a CD38-mediated pathway. So, the two ways that CD38 fulfills its functions are through 1) CD38 catalytic activity and 2) CD38 receptor function. However, future research is necessary. This outcome further validates our cell cycle and apoptosis findings [55]. Our findings showed that the designed peptides strongly cause the HL60 cells to arrest at the G1 phase and then undergo apoptosis, especially when combined with ATRA. However, the mechanism of the peptides should be investigated to understand whether unknown factors in interaction with the CD38 are playing a significant role in this line or not.

5. Conclusions and future perspectives

In this study, we introduced potential peptides against CD38 that can be utilized to either diagnose disorders associated with CD38 over-expression or treat them. Because of their low toxicity, high specificity, and affordability, the peptides can be used in place of authorized antibodies like daratumumab and isatuximab, as well as in conjunction with ATRA for patients. Nevertheless, there are drawbacks including limited bioavailability and short half-life that require further study to overcome.

Ethical Approval and Consent to Participate

Not applicable.

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CRediT authorship contribution statement

Yaghoob Fathollahi: Methodology. **Sorour Ramezanzpour:** Methodology. **Khosro Khajeh:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Conceptualization. **Mohsen Nabi-Afjadi:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Data curation. **S. Mohsen Asghari:** Writing – review & editing, Validation, Investigation. **Bahareh Dabirmanesh:** Writing – review & editing, Writing – original draft, Validation, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Consent for Publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2025.118347](https://doi.org/10.1016/j.biopha.2025.118347).

Availability of data and materials

All data and materials are within the paper.

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