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**BLOKOWANIE ODDZIAŁYWAŃ WYBRANYCH PUNKTÓW
KONTROLNYCH PRZY WYKORZYSTANIU PEPTYDÓW -
INNOWACYJNE PODEJŚCIE DO IMMUNOSUPRESJI**

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HVEM/LIGHT za pomocą peptydów – innowacyjne podejście do immunosupresji”
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1. Wykaz prac wchodzących w skład rozprawy doktorskiej

1. Praca oryginalna

P. Ciura, P. Smardz, M. Spodzieja, A. K. Sieradzan, P. Krupa, „Multilayered Computational Framework for Designing Peptide Inhibitors of HVEM-LIGHT Interaction”, J. Phys. Chem. B 2024, 128, 28, 6770–6785

140 punktów ministerialnych, kwartył 3 (wg. Bazy JCR, stan na rok 2024)

2. Praca oryginalna

P. Ciura, S. Gumpelmair, E. Sikorska, S. Rodziewicz-Motowidło, P. Steinberger, M. Spodzieja, A. K. Sieradzan, „HVEM-derived peptides as inhibitors of HVEM/LIGHT complex formation”, Bioorg., 2025, 166, 109183

100 punktów ministerialnych, kwartył 1 (wg. Bazy JCR, stan na rok 2025)

3. Praca oryginalna

P. Ciura, S. Gumpelmair, S. Rodziewicz-Motowidło, P. Steinberger, M. Spodzieja, A. K. Sieradzan, „Evaluation of LIGHT-derived peptides to disrupt the HVEM/LIGHT immune checkpoint”, Sci Rep, 2025, 15, 40095

140 punktów ministerialnych, kwartył 1 (wg. Bazy JCR, stan na 2025 rok)

2. Wykaz skrótów

AKT – kinaza proteinowa B (ang. *Protein Kinase B*)

APC – komórka prezentująca antygen (ang. *Antigen Presenting Cell*)

ATP – adenosynotrifosforan (ang. *Adenosine Triphosphate*)

BSA – albumina bydlęca (ang. *Bovine Serum Albumine*)

BTLA – białko wyciszające sygnał limfocytów B i T (ang. *B- and T Lymphocyte Attenuator*)

CAR – chimeryczny receptor antygenowy (ang. *Chimeric Antigen Receptor*)

CD – antygen różnicowania komórkowego (ang. *Cluster of Differentiation*)

CD – dichroizm kołowy (ang. *Circular Dichroism*)

CRD – domena bogata w cysteinę (ang. *Cysteine Rich Domain*)

CTLA-4 – antygen 4 związany z limfocytami T cytotoksycznymi (ang. *Cytotoxic T cell Antigen 4*)

DC – komórka dendrytyczna (ang. *Dendritic Cell*)

DcR 3 – receptor przynęta 3 (ang. *Decoy Receptor 3*)

DD – domena śmierci (ang. *Death Domain*)

DIC – N,N'-diizopropylkarbodiimid (ang. *N,N'-Diisopropylcarbodiimide*)

DMF – N,N-Dimetyloformamid (ang. *N,N-Dimethylformamide*)

DR3 – receptor śmierci 3 (ang. *Death Receptor 3*)

eGFP – ulepszone białko zielonej fluorescencji (ang. *enhanced Green Fluorescent Protein*)

ELISA – test immunoenzymatyczny (ang. *Enzyme-Linked Immunosorbent Assay*)

ERK – kinazy regulowane zewnątrzkomórkowym sygnałem (ang. *Extracellular signal-Regulated Kinases*)

FBS – surowica bydlęca (ang. *Fetal Bovine Serum*)

GITR – receptor z rodziny TNF indukowany glikokortykoidami (ang. *Glucocorticoid Induced Tumor Receptor*)

GITRL – ligand dla receptora GITR (ang. *Glucocorticoid-Induced TNF Receptor Ligand*)

GVHD – choroba przeszczep przeciw gospodarzowi (ang. *Graft Versus Host Disease*)

HLA – ludzkie antygeny leukocytarne (ang. *Human Leucocyte Antigens*)

HRP – peroksydaza chrzanowa (ang. *Horseradish Peroxidase*)

HVEM – czynnik wnikania wirusa opryszczki (ang. *Herpesvirus Entry Mediator*)

ICP – punkt kontrolny układu immunologicznego (ang. *Immune Checkpoint Protein*)

IgSF – nadrodzina białek immunoglobulinopodobnych (ang. *Immunoglobulin Superfamily*)

ITIM – hamujący motyw immunoreceptora oparty na tyrozynie (ang. *Immunoreceptor Tyrosine-Based Inhibitory Motif*)

ITSM – przełączający motyw immunoreceptora oparty na tyrozynie (ang. *Immunoreceptor Tyrosine-Based Switch Motif*)

K_d – stała dysocjacji (ang. *Dissociation Constant*)

LC ESI-IT-TOF MS – chromatografia cieczowa sprzężona ze spektrometrią mas z jonizacją typu elektrosprej, pułapką jonową oraz analizatorem czasu przelotu (ang. *Liquid Chromatography–Electrospray Ionization–Ion Trap–Time-of-Flight Mass Spectrometry*).

LIGHT – białko homologiczne z limfotoksyną, wykazujące indukowalną ekspresję i konkurujące z glikoproteiną D wirusa HSV o wiązanie z czynnikiem wnikania wirusa opryszczki (HVEM), receptorem eksponowanym na limfocytach T (ang. *Homologous to Lymphotoxin, Exhibits Inducible Expression and Competes with HSV Glycoprotein D for Binding to Herpesvirus Entry Mediator, a Receptor Expressed on T lymphocytes*)

Limfocyt Tc – limfocyt T cytotoksyczny (ang. *Cytotoxic T Cell*)

LT α – limfotoksyna α (ang. *Lymphotoxin α*)

LT β R – receptor limfotoksyny β (ang. *Lymphotoxin β Receptor*)

MAPK – kinazy białkowe aktywowane mitogenowo (ang. *Mitogen-Activated Protein Kinases*)

MD - dynamika molekularna (ang. *Molecular Dynamics*)

MHC – główny kompleks zgodności tkankowej (ang. *Major Histocompatibility Complex*)

MM-GBSA – mechanika molekularna z modelem uogólnionego Borna i powierzchnią dostępną dla rozpuszczalnika (ang. *Molecular Mechanics Generalized Born Surface Area*)

MST – termoforeza mikrokapilarna (ang. *Microscale Thermophoresis*)

NF- κ B – jądrowy czynnik transkrypcyjny kappa B (ang. *Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells*).

NK – komórka NK (ang. *Natural Killer Cell*)

NKT – limfocyty NKT (ang. *Natural Killer T Cell*)

NMR – magnetyczny rezonans jądrowy (ang. *Nuclear Magnetic Resonance*)

OX40 – receptor nadrodziny czynnika martwicy nowotworu 4 (TNFRSF4, ang. *Tumor Necrosis Factor Receptor Superfamily Member 4*)

OX40L – ligand nadrodziny czynnika martwicy nowotworu 4 (TNFSF4, ang. *Tumor Necrosis Factor Ligand Superfamily Member 4*)

PD-1 – receptor programowanej śmierci komórki 1 (ang. *Programmed Death Receptor 1*)

PD-L1/PD-L2 – ligand 1/2 receptora programowanej śmierci komórki (ang. *Programmed Death-Ligand 1*)

RP-HPLC – wysokosprawna chromatografia cieczowa w układzie faz odwróconych (ang. *Reversed-Phase High-Performance Liquid Chromatography*)

SMD – sterowana dynamika molekularna (ang. *Steered Molecular Dynamics*)

SPR – powierzchniowy rezonans plazmonowy (ang. *Surface Plasmon Resonance*)

SpS – przesunięcie spektralne (ang. *Spectral Shift*)

Tc – limfocyt T cytotoksyczny (ang. *Cytotoxic T lymphocyte*)

TCR – receptor limfocyty T (ang. *T Cell Receptor*)

TCS - stymulatory limfocytów T (ang. *T Cell Stymulator*).

TFA – kwas trifluorooctowy (ang. *Trifluoroacetic Acid*)

Th – limfocyt T pomocniczy (ang. *Helper T lymphocyte*)

THD – domena homologiczna TNF (ang. *TNF Homology Domain*)

TIPSI – triizopropyllosilan (ang. *Triisopropylsilane*)

TL1A – ligand 1A podobny do TNF (ang. *TNF-like ligand 1A*)

TMB – 3,3',5,5'-tetrametylobenzdyna (ang. *3,3',5,5'-Tetramethylbenzidine*)

Tn – limfocyt T dziewiczy, naiwny (ang. *Naïve T Lymphocyte*)

TNFR2 – receptor czynnika martwicy nowotworów typu 2 (ang. *Tumor Necrosis Factor Receptor 2*)

TNFSF – nadrodzina czynnika martwicy nowotworu (ang. *Tumor Necrosis Factor Superfamily*)

TNF α – czynnik martwicy nowotworu (ang. *Tumor Necrosis Factor α*)

TRAF – białka związane z receptorem czynnika martwicy nowotworu (ang. *Tumor Necrosis Factor receptor Associated Factors*)

Treg – limfocyt T regulatorowy (ang. *Regulatory T Lymphocyte*)

3. Streszczenie

Jednym z kluczowych wyzwań współczesnej medycyny jest skuteczna diagnostyka, leczenie oraz zapobieganie chorobom o podłożu autoimmunologicznym. Jedną ze strategii ich leczenia jest immunoterapia ukierunkowana na punkty kontrolne układu immunologicznego (ICP). Częsteczki te regulują intensywność odpowiedzi immunologicznej, poprzez przekazywanie sygnałów aktywujących lub hamujących komórki układu odpornościowego, co umożliwia zachowanie homeostazy organizmu. W niniejszej pracy skoncentrowałem się na białkach: czynniku wnikania wirusa opryszczki (HVEM) oraz jego ligandzie – białku LIGHT, które zaliczane są do stymulujących ICP.

HVEM i LIGHT należą do nadrodziny czynnika martwicy nowotworu, a ich oddziaływanie prowadzi do aktywacji ścieżek sygnałowych odpowiedzialnych m.in. za aktywację limfocytów T, komórek NK oraz dojrzewanie komórek dendrytycznych. Zaburzenie prawidłowego funkcjonowania tego mechanizmu związane jest z nadmierną aktywacją komórek układu odpornościowego, co może prowadzić do powstawania szeregu chorób o podłożu autoimmunologicznym, takich jak nieswoiste zapalenie jelit czy reumatoidalne zapalenie stawów. Zahamowanie tworzenia kompleksu HVEM/LIGHT, przy użyciu inhibitorów może więc stanowić potencjalną strategię terapeutyczną w leczeniu tych chorób. Inhibitory wiązania się białek HVEM/LIGHT mogą znaleźć również zastosowanie u pacjentów po przeszczepach narządów lub tkanek, u których wskazane jest kontrolowanie wyciszenie odpowiedzi immunologicznej, w celu zapobiegania odrzuceniu przeszczepu. Ich zastosowanie może prowadzić do ograniczenia lub zapobiegania występowania powikłań immunologicznych, takich jak choroba przeszczep przeciwko gospodarzowi oraz reakcja gospodarza przeciwko przeszczepowi.

W ciągu ostatnich lat, poczyniono znaczne postępy w chemio-informatyce. Na szczególną uwagę zasługuje projektowanie inhibitorów wspomagane komputerowo, wykonywane w oparciu o szereg wysoko wyspecjalizowanych narzędzi programistycznych.

Pozwalają one w niezwykle szybkim tempie zbadać właściwości wielu proponowanych molekuł, co pozwala na przeprowadzenie wstępnej selekcji związków, a tym samym uniknięcie wielu godzin pracy laboratoryjnej. Połączenie metod projektowania wspomaganego komputerowo z syntezą zaprojektowanych związków oraz ich eksperymentalną walidacją może stanowi efektywną strategię opracowywania skutecznych inhibitorów ukierunkowanych na określony cel molekularny.

W swojej pracy skupiłem się na zaprojektowaniu peptydowych inhibitorów tworzenia kompleksu HVEM/LIGHT, których sekwencja aminokwasowa oraz struktura bazowały na fragmentach białek HVEM oraz LIGHT. W pierwszym etapie moich badań zająłem się analizą kompleksu HVEM/LIGHT. W celu określenia reszt aminokwasowych w białkach HVEM i LIGHT kluczowych dla ich wiązania wykonałem symulacje dynamiki molekularnej (MD). Na ich podstawie zaprojektowałem peptydy – potencjalne inhibitory tworzenia kompleksu HVEM/LIGHT. Dla związków tych również wykonałem symulacje MD, w celu sprawdzenia ich powinowactwa do celów molekularnych. W celu identyfikacji kluczowych oddziaływań stabilizujących kompleks białko/peptyd przeprowadziłem dekompozycję energii wiązania na poziomie pojedynczych reszt aminokwasowych, z wykorzystaniem metody MM-GBSA. Dla peptydów wykazujących najwyższe powinowactwo do białek zaproponowałem substytucje reszt aminokwasowych, mogących potencjalnie negatywnie wpływać na stabilność kompleksu białko/peptyd. Ponadto przeprowadziłem dodatkowe badania, w tym sterowaną dynamikę molekularną oraz ocenę wybranych parametrów termodynamicznych, co umożliwiło dokładniejsze scharakteryzowanie oddziaływań białko/peptyd. Wyselekcjonowane związki następnie zsyntezowałem i poddałem weryfikacji eksperymentalnej.

W moich badaniach eksperymentalnych otrzymałem dwie grupy peptydów – (1) bazujące na sekwencji aminokwasowej białka HVEM oraz (2) bazujące na sekwencji aminokwasowej białka LIGHT. Przeanalizowałem je pod kątem powinowactwa do celów molekularnych metodą termoforezy mikrokapilarnej oraz właściwości hamujących względem kompleksu HVEM/LIGHT, wykorzystując testy ELISA oraz badania na liniach komórkowych.

Badania komórkowe wykonałem podczas odbytego przeze mnie stażu w Instytucie Immunologii Wiedeńskiego Uniwersytetu Medycznego. Wśród peptydów będących fragmentami HVEM największym powinowactwem do LIGHT oraz najwyższą aktywnością hamującą charakteryzowały się CRD2(39–73)e oraz CRD2e_K54E. Natomiast spośród związków zaprojektowanych na podstawie białka LIGHT najlepsze właściwości wykazywały peptyd light3 i jego analog light3_E178L. Uzyskane wyniki wskazują, że cząsteczki te mogą stanowić punkt wyjścia do dalszej optymalizacji, jednak wymagane są dla nich dalsze badania, w celu pełnej oceny ich właściwości immunomodulacyjnych oraz ich potencjału terapeutycznego.

4. Abstrakt

One of the major challenges in contemporary medicine is the accurate diagnosis, effective treatment, and prevention of autoimmune disorders. A promising therapeutic approach involves immunotherapy that targets immune checkpoints (ICPs). These molecules control the strength of the immune response by delivering activating or inhibitory signals to immune cells, thereby ensuring the maintenance of physiological balance.

In this study, I focused on the herpesvirus entry mediator (HVEM) protein and its ligand, LIGHT, both of which belong to the group of immune checkpoint-stimulating molecules. These proteins are members of the tumor necrosis factor superfamily, and their interaction triggers signaling pathways that lead to, among other effects, the activation of T cells, natural killer (NK) cells, and the maturation of dendritic cells.

Disturbances in the HVEM/LIGHT signaling pathway can result in excessive immune activation, contributing to the development of autoimmune diseases such as inflammatory bowel disease and rheumatoid arthritis. Therefore, blocking the interaction between HVEM and LIGHT using specific inhibitors may represent a viable therapeutic strategy. Such inhibitors could also be beneficial in transplantation medicine, where controlled immunosuppression is necessary to prevent graft rejection. Their application may help reduce or prevent complications such as graft-versus-host disease and host-versus-graft responses.

Recent advances in chemoinformatics have significantly accelerated drug discovery processes. In particular, computer-assisted design of inhibitors, using advanced computational tools, enables rapid screening and evaluation of numerous candidate molecules. This approach allows for the preliminary selection of promising compounds, minimizing the need for time-consuming experimental work. Integrating computational design with chemical synthesis and experimental validation represents an efficient strategy for developing targeted inhibitors.

In my research, I concentrated on designing peptide-based inhibitors that disrupt the formation of the HVEM/LIGHT complex. These peptides were derived from specific fragments of the HVEM and LIGHT proteins in terms of both sequence and structure. Initially, I performed a detailed analysis of the HVEM/LIGHT complex using molecular dynamics (MD) simulations to identify key amino acid residues involved in binding.

Based on these findings, I designed a series of peptide candidates and further evaluated their binding affinity through additional MD simulations. To better understand the interactions stabilizing the protein–peptide complexes, I carried out binding energy decomposition at the level of individual residues using the MM-GBSA method. For the most promising peptides, I proposed specific amino acid substitutions that could potentially weaken binding interactions. Additional analyses, including steered molecular dynamics simulations and thermodynamic parameter evaluation, provided further insight into the nature of protein–peptide interactions.

Subsequently, selected peptides were synthesized and subjected to experimental validation. Two main groups of peptides were obtained: (1) those derived from the HVEM protein sequence and (2) those based on the LIGHT protein sequence. Their binding affinity was assessed using microcapillary thermophoresis, while their inhibitory activity against the HVEM/LIGHT interaction was evaluated using ELISA assays and cellular models.

The cellular experiments were conducted during my internship at the Institute of Immunology at the Medical University of Vienna. Among the HVEM-derived peptides, CRD2(39–73)e and CRD2e_K54E showed the strongest binding to LIGHT and the highest inhibitory activity. In the case of LIGHT-based peptides, light3 and its analogue light3_E178L exhibited the most favorable properties.

These findings suggest that the identified peptides may serve as promising starting points for further optimization. However, additional studies are necessary to fully evaluate their immunomodulatory effects and therapeutic applicability.

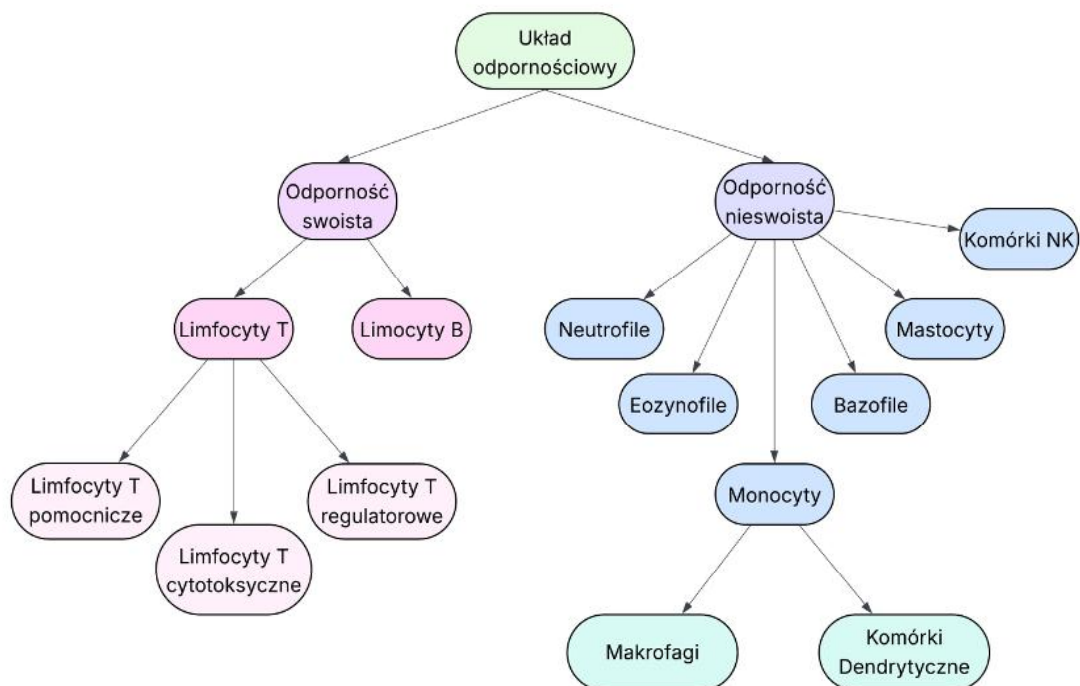
5. Wprowadzenie

5.1. Budowa układu odpornościowego

Układ odpornościowy to zespół wysoko wyspecjalizowanych tkanek, narządów, komórek i białek. Jego zadaniem jest ochrona organizmu przed infekcjami i innymi czynnikami chorobotwórczymi, poprzez identyfikację i eliminację patogenów zagrażających jego prawidłowemu funkcjonowaniu. Składa się on z narządów, do których należą: szpik kostny, grasica oraz śledziona, pełniące kluczowe funkcje w powstawaniu, dojrzewaniu i aktywacji komórek układu odpornościowego¹. Do narządów układu odpornościowego można zaliczyć również węzły chłonne, błony śluzowe i tkanki limfatyczne związane z błonami śluzowymi.

Układ odpornościowy dzieli się na wrodzony (nieswoisty) oraz adaptacyjny (nabyty)². Główne komórki należące do nieswoistej odpowiedzi immunologicznej to neutrofile, monocyty, makrofagi, komórki dendrytyczne (DC, ang. *Dendritic Cell*), eozynofile, bazofile oraz mastocyty. Do ich zadań należy zwalczanie infekcji poprzez bezpośrednią fagocytozę patogenów, indukowanie apoptozy, czy wydzielanie cytokin³. Częścią wrodzonego układu odpornościowego są również komórki NK (NK, ang. *Natural Killer*), które według najnowszej klasyfikacji należą do nieswoistej odpowiedzi immunologicznej i wykazują naturalną cytotoksycznością⁴. Układ ten charakteryzuje się brakiem pamięci immunologicznej⁵.

Układ odpornościowy adaptacyjny odpowiada za specyficzną, ukierunkowaną odpowiedź immunologiczną wobec patogenów i jest aktywowany przy udziale mechanizmów odporności wrodzonej. Mechanizm działania tego układu polega na produkcji swoistych przeciwciał, rozpoznawaniu i niszczeniu komórek eksponujących antygeny oraz na modulowaniu i wzmacnianiu odpowiedzi wrodzonej⁶. Do komórek stanowiących podstawę mechanizmu swoistego można zaliczyć limfocyty B, odpowiedzialne za produkcję przeciwciał i limfocyty T, pełniące funkcje regulacyjne, cytotoksyczne lub pomocnicze^{1,7}. Schemat podziału układu odpornościowego przedstawiłem poniżej (Rys. 1).



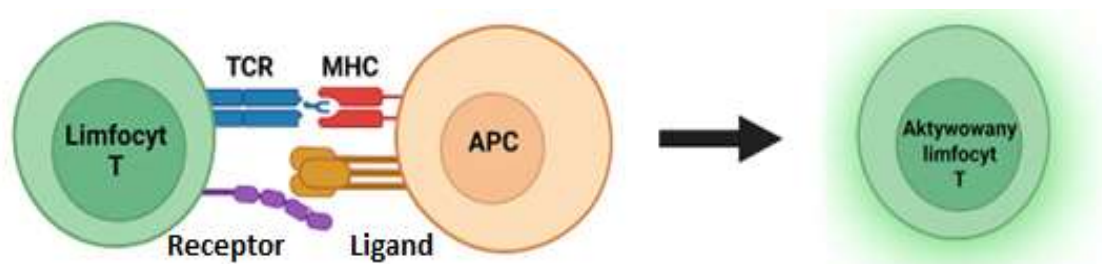
Rysunek 1: Schemat podziału układu odpornościowego na swoisty i nieswoisty wraz z komórkami odpornościowymi charakterystycznymi dla każdego z nich.

5.2. Aktywacja limfocytów T w odpowiedzi immunologicznej organizmu

Aktywacja limfocytów T stanowi kluczowy etap odpowiedzi immunologicznej, warunkujący skuteczne rozpoznanie i eliminację patogenów. Proces ten zachodzi po rozpoznaniu przez limfocyty T antygeny, prezentowanego im przez komórki prezentujące antygen (APC, ang. *Antigen Presenting Cell*). APC na swojej powierzchni prezentują antygeny w postaci peptydów pochodzących z białek endogennych (syntetyzowanych w komórce) oraz egzogennych (pobranych z zewnątrz komórki i rozłożonych w procesie fagocytozy). Peptydy te są prezentowane limfocytom T w kompleksie z białkami głównego układu zgodności tkankowej (MHC, ang. *Major Histocompatibility Complex*), które znajdują się na powierzchni komórek APC⁷.

W wyniku prezentacji antygeny dochodzi do utworzenia kompleksu pomiędzy antygenem prezentowanym przez MHC (na powierzchni komórki APC), a receptorem limfocyty T (TCR, ang. *T Cell Receptor*), zlokalizowanym na powierzchni tej komórki. Stanowi to pierwszy z dwóch sygnałów niezbędnych do aktywacji limfocyty T.

Drugi sygnał pochodzi od interakcji cząsteczek kostymulujących, obecnych na powierzchni limfocytów T (receptory) i komórek APC (ligandy). Cząsteczki te określane są jako punkty kontrolne układu immunologicznego (ICP, ang. *Immune Checkpoint Protein*). Do pełnej aktywacji limfocytów T wymagane jest wystąpienie obu wspomnianych wyżej sygnałów. Brak któregośkolwiek z nich może prowadzić do anergii lub apoptozy komórki T (Rys. 2) ⁸⁻¹⁰.



Rysunek 2: Dwusygnałowy model aktywacji limfocytów T.

W wyniku aktywacji dochodzi do proliferacji oraz funkcjonalnego różnicowania komórek T, co umożliwia dostosowanie odpowiedzi immunologicznej do charakteru zagrożenia. Limfocyty T przed pierwszym kontaktem z antygenem występują w formie komórek dziewiczych (naiwne, T_n, ang. *Naïve T Lymphocyte*), co oznacza, że nie zetknęły się one jeszcze ze swoim swoistym antygenem i pozostają w stanie spoczynkowym, krążąc pomiędzy narządami limfatycznymi. Na skutek aktywacji różnicują się one na poszczególne subpopulacje, takie jak:

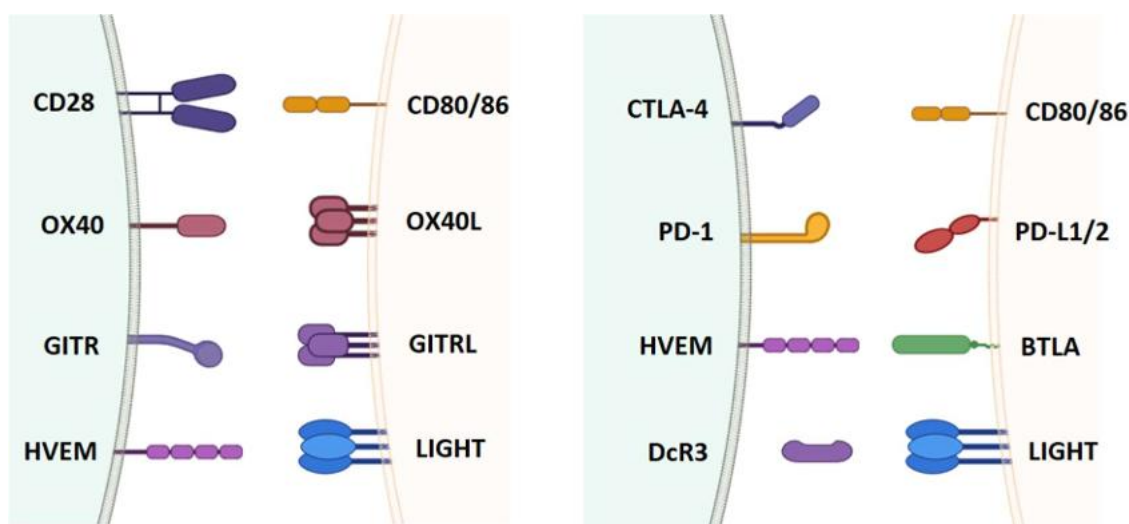
- limfocyty T pomocnicze (T_h, ang. *Helper T Lymphocyte*) – wspomagają odpowiedź immunologiczną poprzez produkcję cytokin oraz aktywację innych komórek układu immunologicznego takich jak limfocyty B, makrofagi i komórki NK;
- limfocyty T cytotoksyczne (T_c, ang. *Cytotoxic T Lymphocyte*) – odpowiedzialne za bezpośrednie zabijanie zakażonych komórek oraz indukcję apoptozy;
- limfocyty T regulatorowe (T_{reg}, ang. *Regulatory T Lymphocyte*), utrzymujące tolerancję immunologiczną i zapobiegającą autoimmunizacji;

- limfocyty T pamięci (ang. *Memory T Lymphocyte*), które w wypadku ponownej ekspozycji na poznany już antygen są w stanie szybko namnożyć się do efektorowych limfocytów T.

Limfocyty T posiadają na swojej powierzchni wspomniane wcześniej receptory limfocytu oraz antygeny różnicowania komórkowego (CD, ang. *Cluster of Differentiation*). Zestaw CD jest zróżnicowany i zależny od subpopulacji komórek T, co umożliwia ich identyfikację i ocenę ich stopnia aktywacji. Tc wyróżniają się obecnością cząsteczek CD8 (dlatego określa się je jako CD8+), natomiast Th wykazują ekspresję białka CD4 (przez co określane są jako CD4+) ³.

5.3. Punkty kontrolne układu odpornościowego i ich funkcja w regulacji odpowiedzi immunologicznej

Punkty kontrolne układu immunologicznego stanowią kluczowy element regulacji aktywności limfocytów T. Można je podzielić na stymulujące, których oddziaływanie prowadzi do aktywacji, proliferacji i różnicowania się komórek T oraz hamujące, które hamują opisane wyżej procesy ¹¹. Do kompleksów o działaniu stymulującym zaliczyć można np. CD28/CD80/CD86 ¹² oraz HVEM/LIGHT ¹³, natomiast do kompleksów hamujących PD-1/PD-L1 ¹⁴, czy CTLA-4/CD80/CD86 ¹². Należy jednak zaznaczyć, że są to jedynie wybrane przykłady, gdyż istnieje wiele innych kompleksów uczestniczących w regulacji odpowiedzi immunologicznej (Rys. 3). Prawidłowe funkcjonowanie układu odpornościowego wymaga precyzyjnej równowagi między aktywacją a wyciszaniem sygnałów. Zaburzenia w obrębie punktów kontrolnych mogą prowadzić zarówno do osłabienia odpowiedzi immunologicznej, jak i jej nadmiernej aktywacji oraz atakowania prawidłowych komórek organizmu ¹⁵.



Rysunek 3: Przykłady kompleksów białek o działaniu stymulującym (po lewej) oraz hamującym (po prawej) odpowiedź immunologiczną; CDX – białko różnicowania komórkowego X (ang. Cluster of Differentiation X), OX40 – receptor nadrodziny czynnika martwicy nowotworu 4 (TNFRSF4, ang. Tumor Necrosis Factor Receptor Superfamily Member 4), OX40L – ligand nadrodziny czynnika martwicy nowotworu 4 (TNFSF4, ang. Tumor Necrosis Factor Ligand Superfamily Member 4), GITR – receptor z rodziny TNF indukowany glikokortykoidami (ang. Glucocorticoid Induced Tumor Receptor), GITRL – ligand dla receptora GITR (ang. Glucocorticoid-Induced TNF Receptor Ligand), HVEM – czynnik wnikania wirusa opryszczki (ang. Herpesvirus Entry Mediator), LIGHT – białko homologiczne z limfotoksyną, wykazujące indukowalną ekspresję i konkurujące z glikoproteiną D wirusa HSV o wiązanie z czynnikiem wnikania wirusa opryszczki (HVEM), receptorem ekspozowanym na limfocytach T (ang. Homologous to Lymphotoxin, Exhibits Inducible Expression and Competes with HSV Glycoprotein D for Binding to Herpesvirus Entry Mediator, a Receptor Expressed on T Lymphocytes), CTLA-4 – antygen 4 związany z limfocytami T cytotoksycznymi (ang. Cytotoxic T Cell Antigen 4), PD-1 – receptor programowanej śmierci komórki 1 (ang. Programmed Death Receptor 1), PD-L1/PD-L2 – ligand 1/2 receptora programowanej śmierci komórki (ang. Programmed Death-Ligand 1/2), BTLA – białko wyciszające sygnał limfocytów B i T (ang. B- and T Lymphocyte Attenuator), DcR3 – receptor przynęta 3 (ang. Decoy Receptor 3).

ICPs należą do dwóch głównych nadrodzin – nadrodziny czynnika martwicy nowotworu (TNFSF, ang. *Tumor Necrosis Factor Superfamily*) oraz nadrodziny białek immunoglobulinopodobnych (IgSF, ang. *Immunoglobulin Superfamily*). W dalszej części pracy skupię się na omówieniu TNFSF, gdyż z nią związane są prowadzone przez mnie badania.

5.4. Nadrodzina czynnika martwicy nowotworu

Receptory i ligandy należące do TNFSF stanowią grupę białek odpowiedzialnych za regulację odpowiedzi immunologicznej, w tym procesów apoptozy oraz proliferacji komórek. Mogą one występować zarówno w formie związanej z błoną komórkową, jak i w postaci rozpuszczalnej. Zalicza się do nich kompleksy tworzone między innymi przez białka: OX40/OX40L, CD30/CD30L, GITR/GITRL, DR3/TL1A, CD27/CD70, TNFR2/TNF α /LT α oraz HVEM/LIGHT/LT α . Ich rolę w aktywacji odpowiedzi immunologicznej przedstawiłem w Tabeli 1.

Ligandy z nadrodziny TNF obejmują grupę 19 molekuł, należących do białek transbłonowych typu II. Ich cechą charakterystyczną jest obecność opartego na strukturach β -kartki motywu domeny homologicznej TNF (THD, ang. *TNF Homology Domain*) oraz tendencja do tworzenia trimerów (zarówno homotrimerów jak i heterotrimerów).

Receptory należące do tej nadrodziny obejmują grupę 29 białek, charakteryzujących się obecnością domen bogatych w cysteinę (CRD, ang. *Cysteine Rich Domain*), stabilizowanych wiązaniami disulfidowymi. Domeny te zaangażowane są w wiązanie się z odpowiednimi ligandami. Zdecydowana większość receptorów z nadrodziny TNF to białka transbłonowe (do wyjątków należy np. białko DcR3). Przekazywanie sygnałów do wnętrza komórki odbywa się za pomocą ścieżek sygnałowych, w których pośredniczą między innymi białka związane z receptorem czynnika martwicy nowotworu (TRAF, ang. *TNF Receptor-Associated Factors*). Niektóre białka zaangażowane w te procesy zawierają w swojej strukturze domenę śmierci (DD, ang. *Death Domain*)¹⁶.

Tabela 1: Wybrane receptory i ich ligandy należące do nadrodziny TNF wraz z miejscem występowania oraz funkcją jaką dany kompleks pełni w organizmie ¹⁷⁻²⁴; LTα – limfotoksyna α (ang. Lymphotoxin α), TNFR2 – receptor czynnika martwicy nowotworów typu 2 (ang. Tumor Necrosis Factor Receptor 2), TNFα – czynnik martwicy nowotworu α (ang. Tumor Necrosis Factor α), DR3 – receptor śmierci 3 (ang. Death Receptor 3), TL1A – ligand 1A podobny do TNF (ang. TNF-like ligand 1A).

Wybrane receptory i ligandy należące do nadrodziny TNF					
Lp.	Receptor	Komórki ekspresjonujące receptor	Ligand	Komórki ekspresjonujące ligand	Funkcja
1	HVEM (TNFRSF14)	limfocyty T i B, komórki dendrytyczne, neurony, komórki śródbłonna i nabłonna	LIGHT (TNFSF14), LTα	LIGHT: aktywowane limfocyty T, komórki NK, DC, komórki mieloidalne; LTα: limfocyty T, B, komórki NK	Złożona rola. W przypadku gdy HVEM jest receptorem – kostymulacja, w przypadku gdy pełni funkcję liganda – koinhibicja
2	OX40 (TNFRSF4)	aktywowane limfocyty T, komórki pamięci T, komórki NK, NKT	OX40L (TNFSF4)	limfocyty B i T, aktywowane makrofagi, DC, komórki limfoidalne, komórki śródbłonna	Proliferacja limfocytów T, szczególnie CD4+
3	4-1BB (TNFRSF9)	aktywowane limfocyty T	4-1BBL (TNFSF9)	DC, makrofagi, limfocyty B	Zwiększenie cytotoksyczności limfocytów T CD8+ i komórek NK
4	CD30 (TNFRSF8)	aktywowane limfocyty T, komórki pamięci T, Treg	CD30L (TNFSF8)	limfocyty B, T, makrofagi, DC	Aktywacja i proliferacja limfocytów T
5	GITR (TNFRSF18)	limfocyty B i T, Treg, NK	GITRL (TNFSF18)	DC, makrofagi, komórki nabłonna, grasicy	Oslabienie działania Treg, promowanie aktywacji limfocytów T
6	TNFR2 (TNFRSF1B)	limfocyty B i T, Treg, DC, makrofagi, komórki nerwowe, śródbłonkowe	TNFα, LTα	TNFα: komórki mieloidalne, limfocyty T i B LTα: limfocyty T, B, komórki NK	Produkcja cytokin, tłumienie odpowiedzi immunologicznej poprzez promowanie proliferacji Treg
7	DR3 (TNFRSF25)	limfocyty B i T, NKT, DC	TL1A (TNFSF15)	aktywowane limfocyty T, makrofagi, DC	Aktywacja limfocytów T
8	CD27 (TNFRSF7)	limfocyty T, B, NKT, komórki NK, DC	CD70	aktywowane limfocyty T, B, DC, komórki NK	Aktywacja i proliferacja limfocytów T, wspomaganie odpowiedzi przeciwwirusowej, regulacja funkcji komórek NK
9	CD40 (TNFRSF5)	DC, makrofagi, limfocyty B, komórki śródbłonna, mięśni gładkich, fibroblasty, komórki nabłonkowe	CD40L (CD154)	aktywowane limfocyty T, komórki NK, komórki śródbłonna	Aktywacja limfocytów B, aktywacja makrofagów

5.5. Terapie ukierunkowane na modulowanie sygnałów punktów kontrolnych układu odpornościowego

Terapie mające na celu blokowanie sygnałów zależnych od punktów kontrolnych są w ostatnich latach przedmiotem wielu badań. Obecnie prowadzone są badania kliniczne m.in. nad przeciwciałem TRX518, będącym agonistą białka GITR, czy przeciwciałami takimi jak: MEDI6469, MEDI0562, MOXR0916, hamującymi powstawanie kompleksu OX40/OX40L. Ich skuteczność dowiedziono w terapiach skojarzonych ^{25,26}. Podobnie projektowane są przeciwciała takie jak BI-1808 oraz BI-1910, będące antagonistami TNFR2, hamującymi wiązanie się receptora obecnego na komórkach Treg z jego ligandem – TNF α . Osłabiają one działanie immunosupresyjne Treg i wzmacniając tym samym odpowiedź przeciwnowotworową ^{27,28}.

Innym podejściem jest zaprojektowanie chimerycznego receptora antygenowego (CAR, ang. *Chimeric Antigen Receptor*) zawierającego w swojej strukturze domenę kostymulacyjną białka 4-1BB, który umożliwia limfocytom T bezpośrednie rozpoznawanie antygeny na komórkach docelowych, z pominięciem prezentacji antygeny w kompleksie MHC. Pozostałe kompleksy, proponowane są jako obiecujące cele molekularne terapii immunomodulacyjnych, zarówno w przypadku chorób autoimmunologicznych, jak i nowotworów ¹⁸.

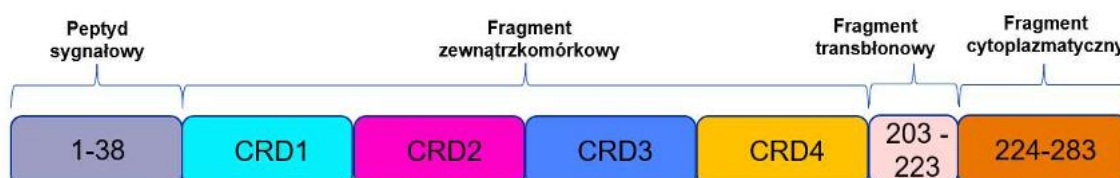
Jednym z punktów kontrolnych odpowiedzialnych za aktywowanie odpowiedzi immunologicznej jest kompleks tworzony przez białka HVEM i LIGHT. W dalszej części pracy skupię się na omówieniu budowy i funkcji tych białek, gdyż to z nimi związane są prowadzone przez mnie badania.

5.6. Białko HVEM

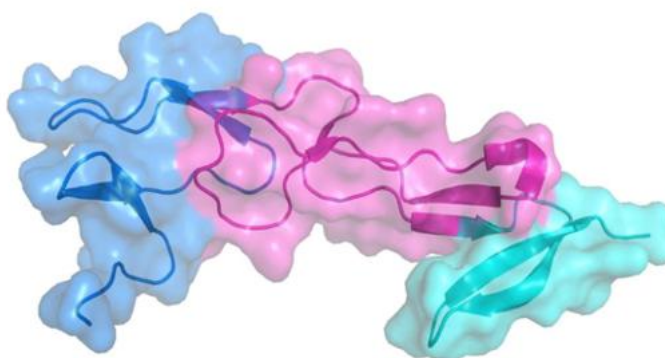
Białko HVEM zbudowane jest z 283 reszt aminokwasowych, które można podzielić na fragmenty: sygnałowy (reszty aminokwasowe: 1-38), zewnątrzkomórkowy (reszty 39-202), transbłonowy (reszty 203-223) oraz wewnątrzkomórkowy (aa 224-283) (Rys. 4A). Fragment zewnątrzkomórkowy białka HVEM składa się z czterech CRD. Pierwsze trzy z nich mają dobrze zdefiniowaną strukturę trzeciorzędową ¹⁶ (Rys. 4B). Domeny CRD złożone są głównie ze struktur β , stabilizowanych mostkami disulfidowymi.

HVEM posiada pojedynczą helisę transbłonową oraz krótki ogon cytoplazmatyczny. W domenie zewnątrzkomórkowej HVEM występują dwa miejsca N-glikozylacji: na resztach aminokwasowych 110 i 173 ²⁹.

A)



B)

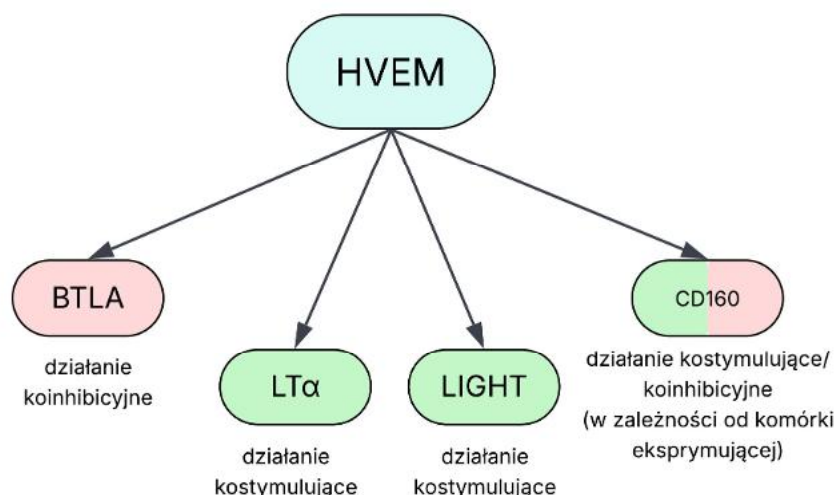


Rysunek 4: A) Schematyczny podział białka HVEM na domeny; B) Struktura krystaliczna domeny zewnątrzkomórkowej białka HVEM (PDB: 4FHQ).

HVEM ulega ekspresji m. in. na limfocytach T, B, komórkach NK oraz komórkach dendrytycznych ³⁰. Białko to pełni w organizmie rolę tzw. przełącznika molekularnego, co oznacza, że może ono być zaangażowane w różne funkcje w zależności od tego na jakiej komórce się znajduje i z jakim receptorem/ligandem oddziałuje. Poza wspomnianymi wcześniej białkami LIGHT i LT α , należącymi do ligandów nadrodziny TNF, może ono również wiązać się z receptorami z IgSF takimi jak BTLA i CD160. W oddziaływanie z LIGHT i LT α zaangażowane są domeny CRD2 i CRD3, natomiast w wiązaniu z BTLA i CD160 uczestniczy domena CRD1 białka HVEM ^{29,31}.

Oddziaływanie pomiędzy białkiem HVEM, znajdującym się na limfocycie T oraz LIGHT lub LT α , obecnym na komórce APC ma charakter stymulujący odpowiedź immunologiczną. W wewnątrzkomórkowej domenie białka HVEM znajduje się motyw umożliwiający wiązanie czynnika 2 związanego z receptorem czynnika martwicy nowotworu (TRAF2, ang. *TNF Receptor-Associated Factor 2*), co prowadzi do aktywacji jądrowego czynnika transkrypcyjnego κ B (NF- κ B, ang. *Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells*). NF- κ B odgrywa kluczową rolę w regulacji ekspresji genów związanych z odpowiedzią zapalną, aktywacją komórek, ich proliferacją oraz przeżyciem lub apoptozą. Dodatkowo aktywacja odpowiedzi immunologicznej z udziałem HVEM może zachodzić również poprzez rekrutację czynnika 3 związanego z receptorem czynnika martwicy nowotworu (TRAF3, ang. *TNF Receptor-Associated Factor 3*) oraz za pośrednictwem szlaku kinazy białkowej B (AKT, ang. *Protein Kinase B*)^{32–35}.

Wiązanie białka HVEM, zlokalizowanego na komórkach APC, z białkiem BTLA obecnym na limfocytach T prowadzi do hamowania aktywacji i proliferacji komórek T. W domenie wewnątrzkomórkowej białka BTLA obecne są dwa motywy: przełączający motyw immunoreceptora oparty na tyrozynie (ITSM, ang. *Immunoreceptor Tyrosine-Based Switch Motif*) i hamujący motyw immunoreceptora oparty na tyrozynie (ITIM, ang. *Immunoreceptor Tyrosine-Based Inhibitory Motif*), które odpowiadają za działanie immunosupresyjne. Jednocześnie w wyniku tworzenia kompleksu BTLA/HVEM do komórek prezentujących HVEM przekazywany jest sygnał kostymulujący, aktywujący ścieżkę sygnałową NF- κ B²⁹. W przypadku interakcji HVEM/CD160 sygnał może mieć dwojaki charakter. Oddziaływanie pomiędzy HVEM obecnym na APC a CD160 obecnym na limfocytach T ma charakter koinhibicyjny, który hamuje produkcję interferonu- γ oraz osłabia proliferację limfocytów T. Natomiast oddziaływanie pomiędzy HVEM obecnym na limfocytach T i CD160 zlokalizowanym na komórkach NK dostarcza sygnał kostymulujący, co skutkuje zwiększeniem produkcji cytokin oraz promuje aktywność lityczną tych ostatnich poprzez fosforylację AKT oraz kinaz regulowanych zewnątrzkomórkowym sygnałem 1 i 2 (ERK, ang. *Extracellular Signal-Regulated Kinases 1 and 2*)^{31,36,37}. Schemat oddziaływań białka HVEM ze swoimi partnerami molekularnymi przedstawiłem poniżej (Rys. 5).



Rysunek 5: Schemat oddziaływań białka HVEM z jego partnerami molekularnymi. Kolorem zielonym oznaczono białka, z którymi HVEM tworzy kompleksy o charakterze kostymulującym, natomiast kolorem różowym zaznaczono białka, które w wyniku oddziaływania z HVEM tworzą kompleksy o charakterze koinhibycyjnym.

5.7. Białko LIGHT

Białko LIGHT to homotrimeryczna cząsteczka należąca do ligandów nadrodziny TNF. Trimer białka LIGHT może występować w organizmie w postaci trzech form, powstających na skutek alternatywnego splicingu bądź enzymatycznego przecięcia³⁸. Pełnej długości monomer białka LIGHT składa się z 240 reszt aminokwasowych i podobnie jak HVEM jest białkiem transbłonowym. Jego sekwencję aminokwasową można podzielić na następujące domeny: cytoplazmatyczną (reszty aminokwasowe 1-37), transbłonową (reszty 38-58) i zewnątrzkomórkową (reszty 59-240) (Rys. 6A). Białko stabilizowane jest przez jedno wiązanie disulfidowe, pomiędzy resztami cysteiny znajdującymi się w pozycjach 154 i 187³⁹. Opisana została również alternatywna forma białka LIGHT, która obecna jest w cytoplazmie aktywowanych limfocytów T. Białko to posiada 204 reszty aminokwasowe i pozbawione jest domeny transbłonowej³⁸.

LIGHT może występować także w formie rozpuszczalnej, która powstaje w wyniku proteolitycznego przecięcia białka przez metaloproteiny pomiędzy resztami Q82 i L83 ⁴⁰. Reszta asparaginy w pozycji 102 w białku ulega modyfikacji posttranslacyjnej w postaci glikozylacji ⁴¹.

W domenie zewnątrzkomórkowej białka LIGHT dominuje motyw określany jako THD. Jest to sekwencja o długości około 150 reszt aminokwasowych, charakterystyczna dla wszystkich ligandów z nadrodziny TNF, wykazująca duże podobieństwo między nimi. Domena THD ma strukturę β -kanapki, na którą składają się dwie ułożone naprzeciwległe β -harmonijki. Każda z nich zbudowana jest z pięciu antyrównoległych nici. β -harmonijka znajdująca się wewnątrz fragmentu zewnątrzkomórkowego, zbudowana z nici A, A', H, C i F odpowiedzialna jest za oddziaływanie z podobną strukturą w sąsiednim monomerze. Oddziaływanie to umożliwia przyjmowanie charakterystycznej formy trimery przez te białka. Zewnętrzna β -harmonijka, zbudowana z nici B, B', D, E i G odpowiedzialna jest natomiast za oddziaływanie z innymi białkami. Struktury te stabilizowane są także przez obecne w monomerze białka wiązanie disulfidowe (Rys. 6B) ¹⁶.

A)

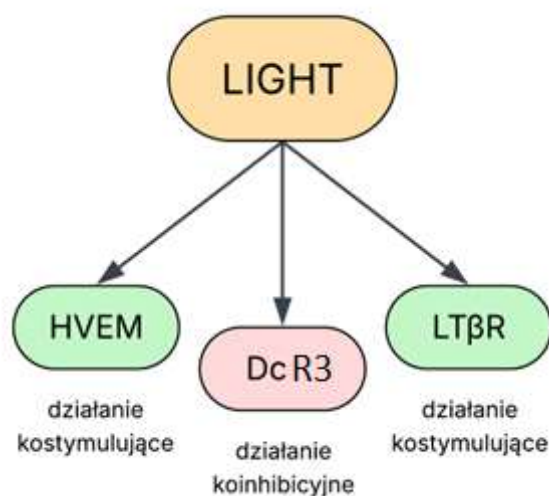


B)



Rysunek 6: A) Schematyczny podział białka LIGHT na domeny; B) Struktura domeny zewnątrzkomórkowej monomeru białka LIGHT ¹⁶ (PDB: 4RSU).

Białko LIGHT jest ekspresjonowane na komórkach dendrytycznych, monocytach, limfocytach T oraz B. Podobnie jak HVEM może ono oddziaływać z różnymi partnerami molekularnymi. Poza omówionym wcześniej białkiem HVEM, LIGHT wiąże się z receptorem limfotoksyny β ($LT\beta R$, ang. *Lymphotoxin β Receptor*)³⁸ oraz z DcR3, białkami należącym do nadrodziny TNF. $LT\beta R$ charakteryzuje się wysoką homologią strukturalną z białkiem HVEM. Utworzenie kompleksu $LT\beta R$ /LIGHT aktywuje ścieżkę sygnałową NF- κB (podobnie jak w przypadku białka HVEM), co skutkuje aktywacją komórek odpornościowych. DcR3 jest rozpuszczalnym receptorem, który wiąże wiele ligandów, w tym LIGHT oraz TL1A. Oddziaływanie DcR3 z tymi molekułami blokuje ich interakcje z receptorami błonowymi, co prowadzi do zahamowania przekazywania sygnału i w konsekwencji osłabienia odpowiedzi immunologicznej^{38,42,43}. Schemat oddziaływań białka LIGHT z jego partnerami przedstawiłem poniżej (Rys. 7).

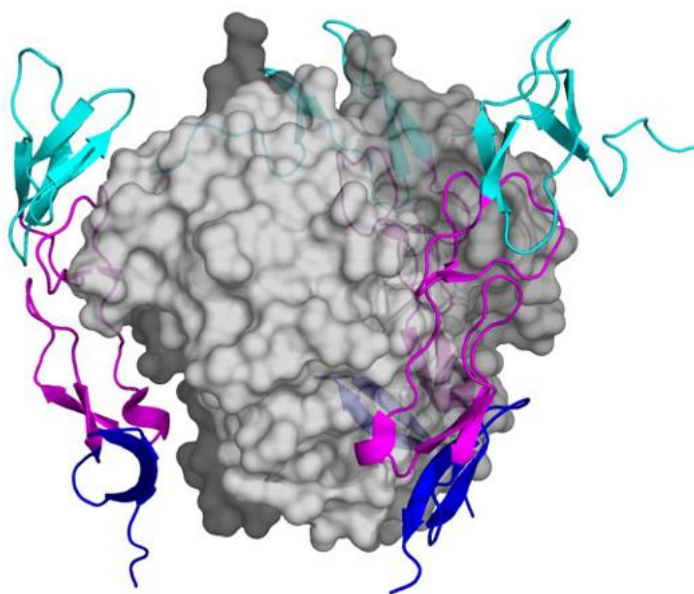


Rysunek 7: Schemat oddziaływań białka LIGHT z jego partnerami molekularnymi.

5.8. Kompleks białek HVEM/LIGHT

Kompleks HVEM/LIGHT charakteryzuje się stechiometrią 3:3, tj. jedna cząsteczka białka LIGHT, złożona z trzech monomerów, oddziałuje z trzema cząsteczkami białka HVEM (Rys. 8). Białko HVEM wiążąc się z homotrimerem LIGHT lokuje się pomiędzy dwoma monomerami LIGHT.

Do najważniejszych oddziaływań, istotnych dla tworzenia kompleksu należą te pomiędzy resztami A47, H48, N50, G51, Q57, Q59, D62 z domeny CRD2 białka HVEM, a resztami Y173 z pętli DE, G100 z pętli AA' i R226, R228 z pętli GH białka LIGHT, a także oddziaływania pomiędzy resztami I90–G94 i A98–R101 z CRD3 białka HVEM, a G151, V152, A159–T161 z pętli CD oraz Q183, R195, V196 i W198 z pętli EF białka LIGHT ⁴⁴.



Rysunek 8: Struktura kompleksu HVEM (różnokolorowa wstążka)/LIGHT (szara powierzchnia) (PDB: 4RSU). Domeny HVEM zaznaczone kolorami odpowiednio: CRD1 – cyjan, CRD2 – magenta, CRD3 – granat. Poszczególne monomery LIGHT oznaczone są różnymi odcieniami szarości.

Utworzenie kompleksu pomiędzy białkami HVEM i LIGHT znajdującymi się na różnych komórkach określone jest jako *trans* i związane jest ze stymulacją nieaktywnych limfocytów T, sekrecją prozapalnych cytokin oraz dojrzewaniem komórek dendrytycznych¹³. Białka te mogą również wiązać się ze sobą gdy ulegają ekspresji na tych samych komórkach i oddziaływanie takie określone jest jako *cis*.

Utworzenie kompleksu *cis* HVEM/LIGHT na aktywowanych limfocytach T utrudnia utworzenie kompleksu BTLA/HVEM, tym samym zmniejszając jego charakter koinhibicyjny. Rozpuszczalna forma LIGHT wiążąc się z HVEM nie dostarcza sygnału kostymulującego, jednakże jest w stanie stabilizować powstający kompleks BTLA/HVEM ¹³.

LIGHT, mimo braku zidentyfikowanych klasycznych motywów sygnałowych w swojej domenie wewnątrzkomórkowej, może uczestniczyć w przekazywaniu sygnałów kostymulujących. Istnieją przesłanki sugerujące, że efekt ten może być związany z aktywacją szlaku kinaz białkowych aktywowanych mitogenami (MAPK, ang. *Mitogen-Activated Protein Kinases*), jednak dostępne dane mają charakter pośredni. Dokładny mechanizm oraz przebieg tej ścieżki sygnałowej nie zostały dotychczas jednoznacznie określone i wymaga dalszych badań ¹³.

Oddziaływanie pomiędzy HVEM a LIGHT było badane pod kątem powinowactwa obu białek, m.in. poprzez wyznaczenie stałej dysocjacji (K_d , ang. *Dissociation Constant*) ich kompleksu. Cheung i wsp. wykazali, że wartość K_d dla interakcji HVEM/LIGHT, określona z wykorzystaniem testów wiązania opartych na cytometrii przepływowej, wynosi odpowiednio 11 nM lub 13 nM, w zależności od zastosowanego układu eksperymentalnego. W pierwszym przypadku analizowano wiązanie HVEM-Fc z LIGHT ekspresjonowanym na komórkach grasicy EL4, natomiast w drugim – oddziaływanie rozpuszczalnej, rekombinowanej formy LIGHT (LIGHTt66) z HVEM ekspresjonowanym na komórkach ⁴⁵. Stała dysocjacji wyznaczona metodą powierzchniowego rezonansu plazmonowego (SPR, ang. *Surface Plasmon Resonance*) również wykazuje zmienność: Morishige i wsp. określili jej wartość na 1,5 nM ⁴⁶, natomiast Liu i wsp. na 18,7 nM ⁴³. W obu przypadkach analizowano interakcję LIGHT z immobilizowaną na powierzchni sensora formą białka HVEM (HVEM-Fc lub HVEM-Ig).

5.9. Zaburzenia odpowiedzi immunologicznej: GVHD i autoimmunizacja

Współczesna medycyna, pomimo znacznego zaawansowania technologicznego i dynamicznego rozwoju nauk biomedycznych, wciąż zмага się z licznymi, często niezwykle złożonymi wyzwaniami klinicznymi i terapeutycznymi. Jednym z przełomowych osiągnięć nowoczesnych nauk biomedycznych jest opracowanie oraz wdrożenie metod transplantacji narządów i tkanek, które umożliwiły ratowanie życia i znaczne wydłużanie jego długości oraz jakości, u pacjentów z niewydolnością wielonarządową.

Transplantologia, mimo że należy do relatywnie młodych dziedzin medycyny, rozwija się w wyjątkowo dynamicznym tempie, obejmując swoim zakresem zarówno przeszczepy narządów litych, jak i komórek krwiotwórczych. Pierwsze udane próby przeszczepów narządów u ludzi miały miejsce na przełomie XIX i XX wieku, co zapoczątkowało erę medycyny rekonstrukcyjnej i immunologii transplantacyjnej, stanowiących dziś fundamenty współczesnej praktyki klinicznej w tej dziedzinie ⁴⁷. Transplantologia jest jedną z najdynamiczniej rozwijających się dziedzin medycyny, z ilością przeszło 170 tys. przeszczepów w 2023 roku, co stanowi wzrost o prawie 10% w stosunku do roku 2022 ⁴⁸. Niestety, równoległe z postępem w tej dziedzinie pojawiają się liczne problemy natury klinicznej i immunologicznej, które wymagają dogłębnego zrozumienia oraz opracowania skutecznych strategii terapeutycznych. Jednym z kluczowych wyzwań jest zjawisko określane mianem choroby „przeszczep przeciwko gospodarzowi” (GVHD, ang. *Graft Versus Host Disease*).

GVHD jest to poważne powikłanie immunologiczne, które występuje głównie po allogenicznych przeszczepieniach komórek krwiotwórczych i polega na tym, że komórki układu odpornościowego, obecne w przeszczepionym materiale (najczęściej limfocyty T dawcy) rozpoznają komórki organizmu biorcy jako obce i inicjują wobec nich odpowiedź cytotoksyczną. W efekcie dochodzi do uszkodzenia tkanek i narządów biorcy, co może prowadzić do ciężkich powikłań wielonarządowych, a w skrajnych przypadkach nawet do śmierci pacjenta. Choroba ta stanowi istotną barierę w dalszym rozwoju skutecznych i bezpiecznych terapii transplantacyjnych, wymagając intensywnych badań nad jej mechanizmami molekularnymi oraz możliwościami immunomodulacyjnymi ⁴⁹.

GVHD dzieli się na ostrą GVHD, gdy występuje w przeciągu pierwszych 100 dni po przeszczepie oraz chroniczną GVHD, występującą między 90 do 600 dniem po przeszczepie ⁵⁰. Ostra GVHD dzielona jest na 4 stopnie ciężkości, gdzie I uznawany jest za najlżejszy, a IV za najcięższy ⁵¹. Szacuje się, że nawet 50% biorców może rozwinąć ostrą formę GVHD ⁵², podczas gdy chroniczna może dotknąć od 6 do 80% biorców ⁵³. Patofizjologia GVHD dotyczy głównie różnic w tzw. ludzkich antygenach leukocytarnych (HLA, ang. *Human Leukocyte Antigen*) kodowanych przez MHC – częstotliwość występowania ostrego GVHD powiązana jest z brakiem zgodności pomiędzy HLA biorcy i dawcy.

Mimo to u 40% biorców o HLA zgodnym z dawcą również rozwija się ostre GVHD ⁵⁴. Objawy GVHD dotyczą głównie zmian skórnych (81% pacjentów), układu pokarmowego (54% pacjentów) oraz wątroby (50% pacjentów). Rokowania w przypadku ostrej formy GVHD są wysoce niekorzystne, długotrwałe przeżycie wynosi około 25% dla stopnia III i zaledwie 5% dla stopnia IV. Objawy chronicznej GVHD są bardziej rozsiane, czyli dotyczą wielu narządów organizmu. Do przykładów należą m.in. depigmentacja skóry, owrzodzenie np. jamy ustnej, narządów płciowych oraz utrata wagi i anemia ⁵¹.

Kolejnym istotnym wyzwaniem, przed którym stoi współczesna medycyna, jest skuteczna diagnoza, leczenie oraz zapobieganie chorobom o podłożu autoimmunologicznym. Są to schorzenia, w przebiegu których układ odpornościowy organizmu ulega patologicznej deregulacji i rozpoczyna reakcję obronną skierowaną przeciwko własnym, prawidłowo funkcjonującym komórkom, tkankom lub narządom. W warunkach fizjologicznych mechanizmy tolerancji immunologicznej zapobiegają takim reakcjom, jednak w przypadku chorób autoimmunologicznych dochodzi do ich zaburzenia, co prowadzi do niekontrolowanej aktywacji limfocytów i produkcji autoprzeciwciał. W efekcie układ odpornościowy nie odróżnia już struktur własnych od obcych i traktuje komórki organizmu jako zagrożenie, inicjując wobec nich procesy zapalne oraz cytotoksyczne. Tego rodzaju zaburzenia immunologiczne stanowią poważny problem zdrowotny o rosnącym znaczeniu społecznym i klinicznym, ponieważ ich leczenie często wymaga długotrwałej immunosupresji oraz interdyscyplinarnego podejścia terapeutycznego ⁵⁵.

Według szacunków, choroby autoimmunologiczne dotyczą od 5 do 10% populacji i odsetek ten ciągle rośnie^{56,57}. Medycyna nie potrafi wskazać dokładnych przyczyn rozwijania się chorób autoimmunologicznych⁵⁸, jednak uważa się, że należą do nich uwarunkowania genetyczne^{59,60}, szeroko pojęte czynniki środowiskowe, takie jak dysbioza układu pokarmowego, podwyższona przepuszczalność błon jelit, czy ogólnie podwyższona reaktywność immunologiczna⁶¹, jak również niektóre infekcje⁶².

5.10. Rola kompleksu HVEM/LIGHT w chorobach autoimmunologicznych i odrzucaniu przeszczepów

Kompleks białek HVEM/LIGHT, który odgrywa istotną rolę w aktywacji odpowiedzi immunologicznej jest wspólnym elementem pełniącym istotną rolę zarówno w chorobach autoimmunologicznych jak i w odrzucaniu przeszczepów⁶³. Ekspresja białka LIGHT jest ściśle kontrolowana i zmienna w czasie. Stężenie białka LIGHT jest mierzalne 4 godziny po aktywacji limfocytu T, osiąga szczyt po 24-48 godzinach i zanika po ok. 5 dniach od aktywacji tej komórki³⁸. Fakt indukowalności ekspresji LIGHT na komórkach APC (jak np. komórki dendrytyczne) w wyniku wystawienia na działanie bodźców stresowych koreluje ze zmiennością ekspresji białka LIGHT w czasie⁶⁴. Zmienność w czasie jest kluczowa dla aktywacji limfocytów T (poprzez tworzenie kompleksu HVEM/LIGHT), a co za tym idzie, odpowiedzi immunologicznej. Zaburzenie tej właściwości, np. przez czynniki wspomniane we wcześniejszym fragmencie, związane może być z występowaniem szeregu chorób o podłożu autoimmunologicznym. Udowodniono, że podwyższone poziomy białka LIGHT są wykrywane w przypadku chorób autoimmunologicznych⁴⁵, czy cukrzycy typu 2⁶⁵. U myszy transgenicznych, o ciągłej ekspresji LIGHT, rozwijał się stan ciągłego zapalenia płuc, który wykazywał podobieństwo do astmy⁶⁶, stan zapalny skojarzony z nieswoistym zapaleniem jelit⁶⁷⁻⁶⁹, jak również ogólny stan zapalny całego organizmu⁷⁰. Ponadto jak wielokrotnie wykazano, LIGHT może pełnić istotną rolę w GVHD i ogólnym odrzuceniu przeszczepu. Zablokowanie białka LIGHT pozwala na złagodzenie GVHD i zwiększenie przeżywalności w modelu mysim poprzez znaczne ograniczenie odpowiedzi limfocytów T cytotoksycznych⁷¹.

5.11. Zastosowanie inhibitorów HVEM/LIGHT w immunosupresji

Dotychczas proponowane bądź prowadzone terapie, ukierunkowane na hamowanie tworzenia kompleksu HVEM/LIGHT, opierały się na wykorzystaniu przeciwciał, bądź białek. Badania Tamady i wsp. wykazały, że blokowanie białka LIGHT, przy użyciu rozpuszczalnych form LT β R-Ig i HVEM-Ig, skutkowało znacznym zmniejszeniem intensywności allogenicznej odpowiedzi limfocytów T w modelu *in vitro*⁷².

Natomiast del Rio i wsp. pokazali, że przeciwciało anty-LIGHT (klon 10F12) osłabiło cytotoksyczną odpowiedź limfocytów T wobec komórek allogenicznych *in vivo*⁷³. Ponadto zastosowanie przeciwciała anty-LIGHT (klon 3D11), blokującego tworzenie kompleksów HVEM/LIGHT i LT β R/LIGHT, prowadziło do zahamowania cytotoksycznej aktywności limfocytów T, skierowanej przeciwko tkankom gospodarza w mysim modelu GVHD. Efektem tego było zwiększenie przeżywalności zwierząt⁷⁴. Fan i wsp. wykazali, że równoczesna inhibicja kostymulujących szlaków CD28/CD80/CD86 oraz HVEM/LIGHT, uzyskana poprzez skojarzone zastosowanie CTLA4-Ig i LT β R-Ig, sprzyjała długotrwałemu utrzymaniu przeszczepów wysp trzustkowych poprzez indukcję tolerancji allospecyficznej w mysim modelu doświadczalnym⁷⁵. Z kolei zwiększoną przeżywalność oraz łagodniejsze GVHD zaobserwowano w przypadku zaaplikowania myszom komórek pozbawionych ekspresji LIGHT⁷⁶. Xu i wsp., w celu zablokowania wiązania HVEM/LIGHT zastosowali przeciwciało anty-HVEM (klon LBH1) i wykazali, że jego podanie łagodziło przebieg GVHD⁷⁶. Perlman i wsp. przeprowadzili badania kliniczne z użyciem przeciwciała anty-LIGHT oznaczonego jako CERC-002, u pacjentów z zapaleniem płuc oraz zespołem ostrej niewydolności oddechowej związanymi z COVID-19, co skutkowało zmniejszeniem śmiertelności w grupie badanej, w porównaniu z grupą kontrolną⁷⁷. Przeciwciało anty-LIGHT, określane jako CBS001, znajduje się obecnie na etapie badań klinicznych w leczeniu schorzeń takich jak nieswoiste zapalenie jelit oraz włóknienie płuc⁷⁸.

Przedstawione dane wskazują, że blokowanie kompleksu HVEM/LIGHT może stanowić potencjalnie skuteczną strategię terapeutyczną w leczeniu chorób autoimmunologicznych, a także może znaleźć zastosowanie jako element terapii immunosupresyjnej w transplantologii.

5.12. Peptydowe inhibitory punktów kontrolnych jako nowoczesna metoda immunomodulacji

Większość z wprowadzonych do użytku leków, oddziałujących z rodziną TNF, oparte jest na przeciwciałach, co związane jest m.in. z ich wysokim powinowactwem do celu molekularnego. Jednakże przeciwciała mają również szereg wad, do których zaliczyć można ich niską trwałość poza organizmem oraz odwrotnie, wysoką trwałość w organizmie. Może to prowadzić do ich agregacji w trakcie trwania terapii oraz licznych skutków ubocznych, np. stanów zapalnych ⁷⁹. Alternatywne terapie oparte m.in. na wykorzystaniu peptydów jako inhibitorów ICP są coraz bardziej atrakcyjne do zastosowania w leczeniu ⁸⁰. Peptydy jako leki charakteryzują się szeregiem pozytywnych właściwości, do których zaliczyć można wysoką selektywność, wysoką tolerancję organizmu na te związki, jak również łatwy do przewidzenia metabolizm. Ich mniejsza w stosunku do przeciwciał masa cząsteczkowa oraz rozmiar sprawiają, że mogą one łatwiej penetrować tkanki i skuteczniej wiązać się z ze swoimi celami molekularnymi. Dodatkowo, są one stosunkowo proste w syntezie czy oczyszczaniu, a także możliwe do modyfikacji w warunkach laboratoryjnych, co ułatwia prowadzenie badań nad nimi ⁸¹. Peptydy charakteryzują się także szeregiem wad, do których zaliczyć można np. niską biodostępność, szczególnie przy podaniu doustnym, krótki okres półtrwania *in vivo*, czy niższe powinowactwo do celów molekularnych w porównaniu do przeciwciał. Wady te mogą być jednak w znacznym stopniu niwelowane poprzez odpowiednie modyfikacje chemiczne oraz strukturalne, takie jak np. cyklizacja czy wprowadzanie nienaturalnych aminokwasów, co pozwala na poprawę ich stabilności i właściwości farmakokinetycznych ⁸².

6. Cel pracy

Celem niniejszej rozprawy doktorskiej było opracowanie peptydowych inhibitorów tworzenia kompleksu HVEM/LIGHT poprzez ich zaprojektowanie, optymalizację strukturalną oraz weryfikację ich właściwości wiążących i inhibicyjnych.

Cel ten postanowiłem osiągnąć poprzez:

1. przeprowadzenie analiz strukturalnych i energetycznych kompleksu HVEM/LIGHT z wykorzystaniem metod obliczeniowych, w celu identyfikacji kluczowych oddziaływań odpowiedzialnych za stabilność kompleksu;
2. zaprojektowanie oraz optymalizację sekwencji peptydowych zdolnych do zaburzania interakcji HVEM/LIGHT;
3. syntezę i oczyszczenie wyselekcjonowanych peptydów;
4. eksperymentalną ocenę powinowactwa zaprojektowanych związków do odpowiedniego białka oraz określenie ich właściwości inhibicyjnych względem kompleksu HVEM/LIGHT;
5. analizę stabilności otrzymanych peptydów oraz ocenę ich cytotoksyczności w modelach komórkowych.

7. Materiały i metody

7.1. Dynamika molekularna i analizy MM-GBSA

Analizy dynamiki molekularnej wykonałem w oparciu o strukturę krystaliczną kompleksu białkowego HVEM/LIGHT z bazy PDB (kod 4RSU). Brakujące fragmenty sekwencji uzupełniłem przy użyciu programu modeller 9.16⁸³. Pliki potrzebne do symulacji przygotowałem przy użyciu narzędzia tleap, stosując pole siłowe ff19SB wraz z czteropunktowym modelem wody OPC^{84–86}. Symulacje przeprowadziłem za pomocą modułu pmemd.cuda z pakietu AMBER24⁸⁷. Każdy z badanych układów otoczyłem warstwą wody o grubości od 17,5 do 20,0 Å, w zależności od symulowanego układu, o geometrii ściętego ośmiościanu. Całkowity ładunek układu zneutralizowałem poprzez dodanie jonów Na⁺ lub Cl⁻.

Każdy system poddałem dwustopniowej minimalizacji energii: w pierwszej etapie wykonałem 1000 kroków minimalizacji metodą największego spadku, nakładając więzy o stałej siłowej wynoszącej $100 \frac{\text{kcal}}{\text{mol} \times \text{\AA}^2}$ na wszystkie atomy poza rozpuszczalnikiem; w drugim etapie wykonałem 2000 kroków minimalizacji metodą gradientu sprzężonego z więzami nałożonymi na wszystkie atomy ciężkie łańcucha głównego oraz węgiel C β . Następnie przeprowadziłem trzyetapowy proces równowagowania układu. Najpierw układ stopniowo podgrzałem od 10 K do 300 K w trakcie 10^4 kroków dynamiki molekularnej (MD, ang. *Molecular Dynamics*), nakładając więzy na wszystkie atomy ciężkie łańcucha głównego oraz węgiel C β . W drugim etapie, trwającym 10^5 kroków, prowadziłem symulacje przy stałym ciśnieniu (NPT), w celu osiągnięcia docelowej gęstości układu, stosując więzy na wszystkie atomy ciężkie łańcucha głównego oraz węgiel C β . W ostatnim etapie wykonałem 4×10^5 kroków w warunkach NPT z użyciem termostatu Langevina oraz więzami na atomach Ca i C β . We wszystkich trzech etapach stała siłowa była taka sama, tj. $100 \frac{\text{kcal}}{\text{mol} \times \text{\AA}^2}$. Na podstawie zrównowagowanych układów przeprowadziłem trzy niezależne symulacje dynamiki molekularnej, z których każda trwała 200 ns, co odpowiada 10^8 krokom czasowym o długości 2 fs. Analizie poddałem ostatnie 20 ns każdej z trajektorii. Dla wybranych układów wykonałem również dłuższe symulacje.

W ich przypadku procedury minimalizacji i równowagowania były identyczne, natomiast właściwe obliczenia obejmowały 5×10^8 kroków, co dawało 1 μ s czasu symulacji przy kroku 2 fs. W dłuższych symulacjach analizie poddałem ostatnie 500 ns trajektorii. Symulacje przeprowadziłem bez więzów oraz przy stałej objętości (NVT).

Energię swobodną tworzenia kompleksów obliczyłem metodą mechaniki molekularnej z modelem uogólnionego Borna i powierzchnią dostępną dla rozpuszczalnika (MM-GBSA, *ang. Molecular mechanics – Generalized Born Surface Area*), stosując model GB-Neck2 oraz dostosowując parametry promieni atomowych do tej metody^{88–91}. Wkład entropowy dla peptydów wyznaczyłem używając metody analizy modułów normalnych, bazując na 10 konformacjach próbkowanych w równych odstępach czasowych, z końcowych 20 ns symulacji trwających 200 ns. W przypadku dłuższych przebiegów symulacji analizowałem 20 konformacji z ostatnich 200 ns. Zmianę energii wewnętrznej peptydu w kompleksie i w stanie niezwiązanym obliczyłem na podstawie różnicy wartości uzyskanych metodą MM-GBSA.

Na podstawie przeprowadzonych symulacji wybrałem struktury reprezentatywne używając analizy skupień. Analizę skupień wykonałem metodą DBSCAN⁹² ustawiając parametr odcięcia (ϵ) 10 Å i maksymalną ilość klastrów wynoszącą 5.

7.2. Sterowana dynamika molekularna

Dla wybranych układów wykonałem symulacje MD obejmujące 20 trajektorii, z których każda trwała 80 ns. Pliki wejściowe (topologie i koordynaty) przygotowałem na podstawie reprezentatywnej struktury każdego symulowanego kompleksu, uzyskanej w wyniku analizy skupień ostatnich 20 ns trzech wcześniej uzyskanych trajektorii MD.⁹² Układy otoczyłem warstwą wody o grubości 70 Å. Następnie przeprowadziłem standardowy proces minimalizacji i równowagowania opisany w poprzedniej części. Środek masy atomów Ca peptydu odsuwano od środka masy atomów Ca białka HVEM.

Odciąganie prowadzono przy stałej siłowej równej $10 \frac{\text{kcal}}{\text{mol} \times \text{\AA}^2}$ i prędkości ciągnięcia 0,05 m/s, aż do uzyskania odległości 40 Å między środkami masy obu cząsteczek. Struktury odpowiadające rozciągnięciu przy maksymalnej wartości siły ($\pm 2 \text{\AA}$) wyodrębniłem z symulacji, po czym wykonałem analizę skupień jak opisano wcześniej przy użyciu oprogramowania cpptraj⁸⁴.

7.3. Materiały i uwagi ogólne

- Wszystkie wykorzystywane w trakcie eksperymentów białka zakupione zostały w firmie ACRO Biosystems. Sekwencje aminokwasowe białek HVEM, LIGHT oraz BTLA obejmowały odpowiednio reszty aminokwasowe: 39-202, 74-240 oraz 31-150. Wszystkie białka były ekspresjonowane w komórkach HEK293.
- Do oczyszczania oraz analizy peptydów używałem faz ruchomych: A – 0,1% roztwór kwasu trifluoroctowego (*TFA*, ang. *Trifluoroacetic Acid*) w wodzie oraz B – 0,1% roztwór TFA w mieszaninie acetonitryl:woda w stosunku objętościowym 4:1.
- W eksperymentach stosowałem bufor PBS oraz PBS-T. Pierwszy z nich składał się z 0,01 M buforu fosforanowego z dodatkiem 0,0027 M KCl oraz 0,437 M NaCl. W drugim przypadku do buforu PBS dodawałem 0,05% Tween-20.
- Do przygotowywania buforów, faz ruchomych itp. wykorzystywałem wodę MiliQ.

7.4. Synteza i oczyszczanie peptydów

Peptydy zsyntezowałem na nośniku stałym, wykorzystując syntezaor Liberty Blue wspomagany mikrofalowo oraz strategię Fmoc/tBu. Do syntezy wykorzystałem żywicę Rink Amide Pro Tide (LL) o stopniu osadzenia 0,18 mmol/g. Używałem 0,2 M roztworów *N*-chronionych aminokwasów rozpuszczonych w dimetyloformamidzie (*DMF*, ang. *N,N-Dimethylformamide*). Każdy aminokwas był przyłączany do żywicy w dwóch cyklach sprzęgania. Do usunięcia grupy ochronnej Fmoc każdego z aminokwasów używałem 20% roztworu piperidyny w DMF.

Jako odczynników sprzęgających używałem 0,5 M roztwór diizopropylkarbodiimidu (DIC, ang. *N,N* - *Diisopropylcarbodiimide*) wraz z 1 M roztworem estru etylowego kwasu cyjano(hydroksyimino)octowego (Oxyma) w DMF. Po każdym cyklu deprotekcji i sprzęgania, żywica przemywana była za pomocą DMF, w celu usunięcia nadmiaru reagentów. Po syntezie, *N*-końcową grupę aminową peptydu poddawałem reakcji acetylowania. Do peptydy żywicy dodałem *N*-acetyloimidazol w ilości 1,1 g na 1 g żywicy i pozostawiałem w temperaturze pokojowej na 24 h. Reszty metioniny w sekwencjach aminokwasowych zaprojektowanych peptydów zastępowałem norleucyną (Nle), natomiast reszty cysteiny, niezaangażowane w wiązania disulfidowe, zastępowałem kwasem 2-aminobutanowym (Abu). W celu selektywnego utworzenia mostków disulfidowych w peptydach zawierających dwa wiązania disulfidowe, grupy sulfhydrylowe jednej pary cystein chronione były grupą tritylową (Trt), natomiast grupy tiolowe drugiej pary cystein zabezpieczano grupą acetamidometylową (Acm).

Odszczepienie peptydu od nośnika stałego wykonywałem przy pomocy mieszaniny składającej się z TFA, fenolu, wody, triizopropylsilanu (*TIPSi*, ang. *Triisopropylsilane*) w stosunku objętościowym 88:5:5:2 w ilości 10 ml na 1 g żywicy. Mieszaninę umieściłem na wytrząsarce i mieszałem przez 3 godziny. Po tym czasie peptyd odsączyłem od żywicy i strąciłem za pomocą zimnego eteru etylowego, a następnie odwirowałem. Otrzymany po dekantacji peptyd rozpuściłem w wodzie i poddałem liofilizacji.

Otrzymane peptydy oczyściłem techniką wysokosprawnej chromatografii cieczowej w układzie faz odwróconych (RP-HPLC, ang. *Reversed-Phase High-Performance Liquid Chromatography*), na kolumnie Luna C8(2) o wymiarach 250 × 4,6 mm, 5 μm, 100 Å. Przed wprowadzeniem na kolumnę, do wodnego roztworu peptydu, w sekwencji którego obecne były reszty cysteiny z wolnymi grupami tiolowymi, dodałem 10-krotny nadmiar ditiotreitolu (DTT), względem ilości wolnych grup -SH i poddawałem sonifikacji w temperaturze 60°C. Do oczyszczania peptydów użyłem: roztworu A (woda dejonizowana zawierająca 0,1% kwas trifluorooctowy) oraz roztworu B (80% acetonitryl w wodzie z 0,1% TFA). Rozdział prowadziłem w liniowym gradiencie od 5% do 50% roztworu B w roztworze A przez 120 minut, monitorując absorbancję przy długościach fali UV 222 nm i 254 nm.

Masę cząsteczkową peptydów wyznaczyłem przy użyciu chromatografii cieczowej sprzężonej ze spektrometrią mas z jonizacją typu elektrosprej, pułapką jonową oraz analizatorem czasu przelotu (LC ESI-IT-TOF MS, ang. *Liquid Chromatography–Electrospray Ionization–Ion Trap–Time-of-Flight Mass Spectrometry*). Czystość peptydów potwierdziłam przy użyciu ultrawysokosprawnej chromatografii cieczowej w odwróconym układzie faz (RP-UHPLC), na chromatografie wyposażonym w detektor z matrycą fotodiodową (PDA) oraz detektor rozpraszania światła powstałego w wyniku odparowania (ELSD), na kolumnie Kinetex C8 o wymiarach 100 × 2,1 mm, 2,6 μm, 100 Å, w gradiencie od 5% do 100% B w A, w czasie 15 minut.

7.5. Tworzenie mostków disulfidowych

W celu utworzenia wiązania disulfidowego peptydy rozpuściłem w mieszaninie wody i metanolu (1:9, v:v), w taki sposób, aby ich stężenie końcowe nie było większe niż 40 mg/l. Następnie pH roztworu doprowadziłem do wartości 8–9, przy użyciu 25% roztworu amoniaku. Roztwór peptydu poddałem ciągłemu mieszaniu przez 7 dni, jednocześnie wtłaczając do mieszaniny sprężone powietrze. Przebieg reakcji kontrolowałem za pomocą RP-UHPLC, a po jej zakończeniu metanol odparowałem, peptydy rozpuściłem w wodzie i poddałem liofilizacji. Następnie każdy z peptydów ponownie oczyściłem, wykorzystując procedurę analogiczną do tej opisaną powyżej.

Peptydy, w których obecne były dwa wiązania disulfidowe poddałem drugiemu utlenianiu (bez ponownego oczyszczania opisanego powyżej). Najpierw rozpuściłem je w mieszaninie kwasu octowego, wody i metanolu (1:1:9, v:v:v), tak aby ich stężenie w mieszaninie nie było większe niż 40 mg/l. Następnie do roztworu dodałem 25–50-krotny nadmiar jodu w metanolu i mieszaninę reakcyjną pozostawiłem w temperaturze pokojowej przez tydzień, mieszając ją na mieszadle magnetycznym. Po tym czasie mieszaninę przefiltrowywałem przez żywicę jonowymienną Dowex, w celu usunięcia nadmiaru jodu. Następnie odparowałem rozpuszczalniki, a peptyd rozpuściłem w wodzie i poddałem liofilizacji. Peptydy oczyściłem zgodnie z procedurą opisaną wcześniej.

7.6. Charakterystyka peptydów

Otrzymane przez mnie peptydy scharakteryzowałem za pomocą spektrometrii mas oraz wysokosprawnej chromatografii cieczowej. Wyznaczone wartości masy cząsteczkowej, wraz z masą teoretyczną, a także czas retencji peptydów w piętnastominutowym gradiencie 5-100% układu B w A przedstawiłem poniżej (Tabela 2).

Tabela 2: Charakterystyka peptydów, fragmentów białka HVEM oraz LIGHT otrzymanych w toku pracy doktorskiej.

Nazwa peptydu	Masa teoretyczna (Da)	Masa obserwowana (Da)	Czas retencji (min)	% układu B
CRD2d	4600,5	4600,5	7,6	48,1
CRD2e	4600,5	4600,3	7,5	47,6
CRD2f	4600,5	4600,5	5,4	34,0
CRD2de	4633,5	4633,3	7,7	48,6
CRD2df	4633,5	4633,6	7,1	44,7
CRD2ef	4633,5	4633,2	5,8	36,9
CRD3g	2385,2	2385,5	5,4	34,1
CRD3h	2385,2	2385,9	6,0	37,7
CRD3gh	2420,7	2421,0	5,5	35,0
CRD2e_K54E	4600,4	4600,5	7,9	49,8
CRD2de_K54E	4634,4	4634,7	7,6	47,9
CRD2(39-73)e	3755,7	3755,2	8,0	50,4
light1	3571,1	3570,9	4,2	26,6
light2	3615,1	3615,5	5,6	35,8
light3	2963,5	2963,4	6,6	41,7
light4	2976,0	2976,4	5,4	34,5
light3_E178L	2947,5	2946,5	7,6	48,0

7.7. Termoforeza mikrokapilarna

W celu określenia powinowactwa peptydów do białek HVEM lub LIGHT przeprowadziłem analizy termoforezy mikrokapilarnej (MST, ang. *Microscale Thermophoresis*), wyznaczając przesunięcie spektralne (*SpS*, ang. *Spectral Shift*). Eksperymenty wykonałem z użyciem urządzenia Monolith X. Znakowanie białek przeprowadziłem przy użyciu zestawu His-Tag Labeling Kit RED-tris-NTA drugiej generacji. W celu otrzymania roztworu barwnika fluorescencyjnego o stężeniu 0,1 μM , 2 μl roztworu podstawowego barwnika o stężeniu 5 μM rozcieńczyłem 98 μl wody dejonizowanej. Następnie 90 μl tak sporządzonego roztworu zmieszałem z 90 μl białka HVEM-His o stężeniu 200 nM lub białka LIGHT-His o stężeniu 100 nM, rozpuszczonego w buforze PBS-T. Całość inkubowałem przez 30 minut w temperaturze pokojowej.

W następnym kroku 10 μl znakowanego białka zmieszałem z 10 μl roztworów peptydów o stężeniach od 2 μM do 6,1 pM (dla peptydów bazujących na sekwencji białka HVEM) lub od 4 μM do 12,2 pM (dla peptydów bazujących na sekwencji białka LIGHT). Mierzyłem sygnał fluorescencji (670/650 nm) przy 40% mocy wzbudzenia i średniej mocy lasera IR, w temperaturze 25°C. Analizę danych przeprowadziłem przy użyciu oprogramowania MO.Control 2. Każdy pomiar wykonałem w trzech powtórzeniach, a wyznaczona stała odpowiada średniej wartości uzyskanej z trzech pomiarów.

7.8. Test immunoenzymatyczny

Testy immunoenzymatyczne (ELISA, ang. *Enzyme-Linked Immunosorbent Assay*) wykonałem na 96-dółkowych, przezroczystych, polistyrenowych płytkach. W zależności od wariantu eksperymentu dołki pokrywałem odpowiednio białkiem LIGHT-His rozpuszczonym w PBS o stężeniu 2 $\mu\text{g/ml}$ (100 μl na dołek) lub HVEM-His o stężeniu 6 $\mu\text{g/ml}$ (100 μl na dołek). W celu opłaszczania płytki inkubowałem z białkiem przez noc w temperaturze 4°C. Miejsca w dołkach, w których nie związało się białko blokowałem przy użyciu 5% roztworu albuminy bydlęcej (*BSA*, ang. *Bovine Serum Albumine*) w PBS-T (200 μl na dołek).

Następnie do każdego dołka dodawałem peptydy rozpuszczone w PBS-T w jednym z trzech stężeń: 50 μM , 12,5 μM lub 3,125 μM (200 μl na dołek). W kolejnym etapie do dołków dodawałem białka: HVEM-Fc o stężeniu 6 $\mu\text{g/ml}$ (100 μl na dołek) – w przypadku płytek z immobilizowanym LIGHT-His lub LIGHT-Fc o stężeniu 2 $\mu\text{g/ml}$ (100 μl na dołek) – w przypadku płytek z immobilizowanym HVEM-His. Następnie do dołków wprowadziłem kozie przeciwciało antyludzkie IgG sprzężone z peroksydazą chrzanową (HRP, ang. *Horseradish Peroxidase*), rozcieńczone w PBS-T w stosunku 1:3000 (v:v, 100 μl na dołek). Po każdym etapie testu (opłaszczanie, blokowanie, dodawanie peptydów, dodawanie białek fuzyjnych i przeciwciała) wykonałem pięciokrotne płukanie płytki, stosując za każdym razem 200 μl PBS-T na dołek. Wszystkie inkubacje prowadziłem w temperaturze 37°C przez 2 godziny, z wyjątkiem przeciwciała, które inkubowałem przez 1 godzinę. W ostatnim etapie do dołków dodałem 200 μl substratu 3,3',5,5'-tetrametylobenzydyny (TMB, ang. *3,3',5,5'-Tetramethylbenzidine*). Po 15 minutach inkubacji w ciemności zmierzyłem absorbancję przy długościach fali 492 nm oraz 650 nm za pomocą czytnika Infinite M200 Pro.

Test ELISA dla kompleksu LT β R/LIGHT wykonałem analogicznie jak opisałem powyżej, z tą różnicą, że na płytkę z immobilizowanym białkiem LIGHT zamiast białka HVEM-Fc dodałem białko LT β R-Fc w stężeniu 2 $\mu\text{g/ml}$ (100 μl na dołek). Kontrolę negatywną w trakcie eksperymentów stanowił PBS. Wszystkie testy wykonałem przynajmniej trzykrotnie, a wyniki przedstawiłem jako średnią z trzech powtórzeń

7.9. Hodowle komórkowe

W badaniach komórkowych wykorzystywałem następujące linie komórkowe:

- Jurkat E6.1 – linia komórkowa wykorzystywana do oznaczania cytotoksyczności peptydów;
- J-NF- κ B::eGFP HVEM – modyfikowana linia komórkowa Jurkat E6.1 eksponująca białko HVEM, w której na skutek aktywacji ścieżki sygnałowej NF- κ B dochodzi do ekspresji białka eGFP; wykorzystywana do oznaczania właściwości hamujących peptydów;
- BW5147 – modyfikowane mysie linie komórkowe, oznaczane, w zależności od eksponowanych białek, kodem TCS Ctrl, TCS-CD86, TCS-LIGHT, TCS-BTLA).

Linia komórkowa Jurkat E6.1 zakupiona została z firmy Cell Line Service GmbH. Komórki reporterowe oraz TCS są własnością laboratorium prof. Petera Steinbergera z Instytutu Immunologii Uniwersytetu Medycznego w Wiedniu, Austria.

Wszystkie linie komórkowe hodowałem w medium RPMI1640 (Roswell Park Memorial Institute 1640) zawierającym 10% surowicy bydlęcej (FBS, ang. *Fetal Bovine Serum*) oraz 1% mieszaniny penicyliny i streptomycyny. Hodowle prowadziłem w inkubatorze w temperaturze 37°C, w atmosferze zawierającej 5% CO₂. Medium hodowlane wymieniałem co 2–3 dni.

7.10. Cytotoksyczność peptydów

Na białe, sterylne, 96-dołkowe płytki, przeznaczone do hodowli komórkowych, naniostałem 1×10^4 komórek TCS-Ctrl lub 2×10^4 komórek Jurkat E6.1 na dołek, zawieszonych w 40 μ l medium hodowlanego. Następnie, przygotowałem seryjne rozcieńczenia peptydów w tym samym medium, w zakresie od 100 μ M do 6,25 μ M. Do każdego dołka z komórkami dodałem 40 μ l roztworu peptydu, co dało objętość końcową 80 μ l i stężenia końcowe peptydów mieszczące się w przedziale 50–3,125 μ M. Komórki inkubowałem z peptydami przez 24 godziny w temperaturze 37°C i atmosferze 5% CO₂. Po zakończeniu inkubacji płytki pozostawiłem na 10 minut w temperaturze pokojowej, po czym do każdego dołka dodałem 80 μ l odczynnika CellTiter-Glo.

Po 15 minutach zmierzyłam luminescencję w każdym dołku przy użyciu czytnika płytek CLARIOstar Plus. Komórki, do których zamiast peptydu dodałam 40 µl medium hodowlanego służyły jako kontrola pozytywna. Żywotność komórek wyraziłam procentowo, na podstawie porównania żywotności komórek inkubowanych z peptydem oraz komórek kontrolnych.

7.11. Stabilność peptydów

Ocenę stabilności peptydów przeprowadziłam, wykorzystując supernatant uzyskany z hodowli komórek Jurkat E6.1. Supernatant pobierałam po 24 godzinach inkubacji 1×10^6 komórek w 10 ml medium, hodowanych w warunkach opisanych powyżej. Zawiesinę komórek odwirowywałam w probówkach typu Falcon przy obrotach $3000 \times g$ przez 3 minuty, po czym supernatant przenieśli do nowych naczyń. Następnie do 200 µl supernatantu dodawałam 50 µl roztworu peptydu rozpuszczonego w wodzie, tak aby uzyskać stężenie końcowe 1,5 mg/ml. Całość inkubowałam w temperaturze 37°C, w warunkach ciągłego mieszania. Próbkę do analizy pobierałam zaraz po zmieszaniu peptydu z supernatantem oraz po 24 godzinach inkubacji, a następnie zamrażałam do czasu wykonania analiz. Przed pomiarami próbki rozmrażałam na lodzie (w temperaturze 0°C).

Do próbek dodawałam czterokrotną objętość bezwodnego etanolu (v:v), w celu strącenia białek znajdujących się w próbkach, po czym wirowałam je przy obrotach $24270 \times g$ przez 20 minut w temperaturze 4°C. Uzyskany supernatant przenosiłam do świeżych probówek i odparowywałam.

Do tak przygotowanych próbek dodawałam 120 µl wody zawierającej 0,1% TFA (v:v) i analizowałam przy wykorzystaniu RP-HPLC, stosując kolumnę analityczną Kromasil C8 o wymiarach $250 \times 2,1$ mm, 5 µm oraz liniowy gradient od 5% do 100% roztworu B w A, w czasie 60 minut. Ilość peptydu pozostałego w próbce obliczyłam poprzez porównanie pola powierzchni pod pikiem próbki pobranej w danym czasie z polem powierzchni pod pikiem peptydu rozpuszczonego w wodzie, bez inkubacji. Każdy pomiar wykonałam w trzech powtórzeniach, a podane stężenie stanowi wartość średnią z tych pomiarów.

7.12. Testy komórkowe

Linie komórkowe wykorzystywane w trakcie eksperymentów hodowałem w warunkach opisanych powyżej. W zależności od badanej puli peptydów (bazujące na fragmentach białka HVEM lub LIGHT) metodologia przygotowywania eksperymentu różniła się.

W pierwszym przypadku 25 μ l peptydów (fragmentów białka HVEM) o trzech stężeniach: 200, 50 i 12,5 μ M, poddałem wstępnej inkubacji w probówkach z 25 μ l komórek TCS-LIGHT (2×10^4 komórek na probówkę) przez 1 godzinę w temperaturze 37°C. Po tym czasie próbki odwirowałem przy obrotach 500 $\times$ g przez 3 minuty, a medium hodowlane z niezwiązanymi peptydami usunąłem. Następnie do próbek dodałem 50 μ l świeżej pożywki, a komórki ze związanymi peptydami przenieśliem na 96-dotkową, przezroczystą płytkę do hodowli komórkowych. W kolejnym kroku dodałem 50 μ l komórek J-NF- κ B::eGFP HVEM (50×10^4 komórek na dotek). W drugim przypadku peptydy (fragmenty białka LIGHT) w trzech stężeniach (200, 50 i 12,5 μ M) mieszałem z 50 μ l komórek J-NF- κ B::eGFP HVEM (50×10^4 komórek na probówkę). Po godzinnej inkubacji w temperaturze 37°C próbki wirowałem przy obrotach 500 $\times$ g przez 3 minuty, a pożywkę z niezwiązanymi peptydami usunąłem, dodając jednocześnie taką samą ilość świeżej pożywki. Całość przenieśliem na płytki i dodałem do nich 25 μ l komórek TCS-LIGHT (2×10^4 komórek na dotek). W obu przypadkach płytki inkubowałem w temperaturze 37°C i 5% CO₂ przez 24 godziny. Po tym czasie płytki odwirowałem przy obrotach 800 $\times$ g przez 3 minuty, a do każdego dotka płytki dodałem 6 μ l przeciwciała monoklonalnego mCD45 sprzężonego z alofikocyjaniną i inkubowałem przez 20 minut w temperaturze 4°C, w celu identyfikacji populacji komórek TCS w analizowanej próbce i odrzucenia jej w analizie wykonanej przy pomocy cytometru. Każdy z dotków przemyłem 100 μ l buforu FACS (PBS, 5-10% FBS, 0,1% NaN₃), a następnie do każdego dotka dodałem 50 μ l świeżego buforu FACS. Ekspresję eGFP w obecności i nieobecności peptydów (kontrola) mierzyłem następnie za pomocą cytometru przepływowego CytoFLEX i analizowałem za pomocą oprogramowania FlowJo v10.10.

Dla wybranych peptydów przeprowadziłem również analizy, w których oceniłem ich wpływ na tworzenie kompleksu BTLA/HVEM. Badania te wykonałem w celu określenia specyficzności peptydów wobec kompleksu HVEM/LIGHT. Testy te przeprowadziłem analogicznie jak dla peptydów – fragmentów białka HVEM, z tą różnicą że zamiast komórek TCS-LIGHT wykorzystywałem komórki TCS-BTLA. Równolegle w ramach kontroli eksperymentu przeprowadziłem również eksperymenty z użyciem komórek bez ekspresji białek LIGHT bądź BTLA (TCS CD86 Ctrl i TCS Ctrl), aby wykluczyć niespecyficzny wpływ peptydów na te komórki. Wszystkie testy wykonałem przynajmniej trzykrotnie, a wyniki przedstawiłem jako średnią z trzech powtórzeń.

7.13. Widma dichroizmu kołowego

Widma dichroizmu kołowego (CD, ang. *Circular Dichroism*) zarejestrowano w Laboratorium Dichroizmu Kołowego na Wydziale Chemii Uniwersytetu Gdańskiego. Pomiary wykonano przy użyciu spektrometru CD J-815 firmy Jasco. Do analiz wykorzystano roztwory peptydów o stężeniu 0,15 mg/ml, a rejestrację widm przeprowadzono w wodzie w temperaturze 25°C.

7.14. Analiza statystyczna danych

Wyniki testów ELISA, badań komórkowych oraz cytotoksyczności przedstawiłem jako średnie wartości wraz z odchyleniem standardowym (SD). Analizę statystyczną przeprowadziłem z wykorzystaniem jednoczynnikowej analizy wariancji (ANOVA), a następnie zastosowałem test post-hoc Dunnetta, w celu porównania poszczególnych grup z grupą kontrolną. Za statystycznie istotne uznałem różnice przy poziomach istotności: *** $p < 0,001$, ** $p < 0,01$ oraz * $p < 0,05$. Analizy statystyczne oraz wizualizację danych w postaci wykresów wykonałem z wykorzystaniem oprogramowania GraphPad Prism 6.

8. Omówienie publikacji wchodzących w skład pracy doktorskiej

Na rozprawę doktorską składają się trzy oryginalne publikacje naukowe. W ich toku przeprowadziłem dogłębne analizy kompleksu HVEM/LIGHT, przy wykorzystaniu metod komputerowych. Na ich podstawie zaprojektowałem szereg peptydów, bazujących na sekwencji aminokwasowej i strukturze białek HVEM oraz LIGHT. Zaprojektowanie związków, potencjalnych inhibitorów wiązania się białek HVEM/LIGHT, wymagało przeanalizowania wielu ich potencjalnych cech strukturalnych, takich jak konformacja przestrzenna i łączący się z tym wpływ przestrzennego ułożenia poszczególnych reszt aminokwasowych na ich zdolność do wiązania się z celami molekularnymi. Jak udowodniono, nawet substytucja pojedynczej reszty aminokwasowej może mieć znaczący wpływ na powinowactwo związku do białka ⁴⁴. Interakcje zaprojektowanych peptydów z celem molekularnym przeanalizowałem za pomocą metod komputerowych, takich jak analiza MM-GBSA, bazująca na dynamikach molekularnych różnej długości, jak również analiza SMD. Pozwoliło to na wyznaczenie wkładu energetycznego poszczególnych reszt na tworzenie się kompleksu białko/peptyd. Analizy komputerowe umożliwiły mi również zbadanie płaszczyzny interakcji białko/peptyd.

Część zaprojektowanych peptydów została przez mnie zsyntezowana i oczyszczona, a następnie wykonałem dla nich liczne badania mające na celu sprawdzenie ich powinowactwa do celu molekularnego przy wykorzystaniu SpS oraz właściwości hamujących względem kompleksu HVEM/LIGHT przy wykorzystaniu testów ELISA i badań komórkowych. Badania komórkowe wykonałem podczas 4-miesięcznego stażu w Instytucie Immunologii Wiedeńskiego Uniwersytetu Medycznego. Dodatkowo sprawdziłem również stabilność peptydów w nadsączu komórkowym oraz ich cytotoksyczność wobec wybranych linii komórkowych. Zbadałem również potencjał hamujący wybranych peptydów względem kompleksów LTβR/LIGHT oraz BTLA/HVEM. Dodatkowo zarejestrowano dla nich widma magnetycznego rezonans jądrowy (NMR, ang. *Nuclear Magnetic Resonance*) oraz CD.

8.1. Publikacja 1 - Multilayered Computational Framework for Designing Peptide Inhibitors of HVEM-LIGHT Interaction

P. Ciura, P. Smardz, M. Spodzieja, A. K. Sieradzan, P. Krupa, „Multilayered Computational Framework for Designing Peptide Inhibitors of HVEM-LIGHT Interaction”, J. Phys. Chem. B 2024, 128, 28, 6770–6785

<https://pubs.acs.org/doi/10.1021/acs.jpcb.4c02255> (załącznik 1)

140 punktów ministerialnych, Q3 (wg. Bazy JCR, stan na rok 2024)

W pierwszej publikacji skupiłem się na białku HVEM. Przeanalizowałem wpływ ilości oraz rozmieszczenia wiązań disulfidowych, natywnie obecnych w strukturze tego białka, na jego zdolność do tworzenia kompleksu z białkiem LIGHT. Następnie zaprojektowałem szereg peptydów, bazujących na domenach CRD1, CRD2 oraz CRD3 białka HVEM, wyznaczonych na podstawie struktury krystalicznej o kodzie PDB 4RSU. Analizy prowadziłem dla związków bez wiązań disulfidowych (nazwa domeny z dopiskiem „no”), z obecnym jednym, bądź dwoma wiązaniami (litery od a do h i ich odpowiednie permutacje, np. ab, ac), jak również dla peptydów mających zachowane wszystkie trzy wiązania disulfidowe (nazwa domeny z dopiskiem „all”). W pierwszym etapie prowadzonych badań określiłem efektywną energię wiązania białka HVEM oraz zaprojektowanych peptydów z białkiem LIGHT. Wartości te wyznaczyłem na podstawie analizy MM-GBSA wykonanej dla 200 ns MD. Takim analizom poddałem 65 zaprojektowanych układów białko/białko i białko/peptyd. Dla wybranych kompleksów przeprowadziłem również dłuższe dynamiki (1000 ns), które pozwoliły na bardziej dogłębne poznanie interakcji peptydów z białkiem LIGHT oraz analizę MM-GBSA, dzięki którym określiłem właściwości termodynamiczne, takie jak zmiana energii swobodnej Gibbsa, czy entropii.

Z wstępnych analiz powinowactwa symulowanych związków wynikało, że centrum interakcji HVEM z LIGHT zorientowane jest wokół domeny CRD2 HVEM. Z tego powodu większość zaprojektowanych peptydów oparłem o strukturę tej części białka. Największym powinowactwem do białka LIGHT charakteryzowały się peptydy CRD2no i CRD2de.

Ponadto analiza wkładów energetycznych poszczególnych reszt aminokwasowych w tworzenie kompleksu LIGHT/peptyd wykazała, że negatywny wpływ na jego stabilność ma reszta K54, znajdująca się w domenie CRD2 HVEM. Ze względu na fakt, iż głównym celem mojej pracy doktorskiej oraz prowadzonych analiz komputerowych było zaprojektowanie związków – inhibitorów tworzenia kompleksu HVEM/LIGHT, zaproponowałem serię analogów peptydów z lizyną zastąpioną: seryną, leucyną, izoleucyną, kwasem glutaminowym, kwasem asparaginowym, tyrozyną oraz waliną. Modyfikacje te wprowadziłem do peptydów CRD2no oraz CRD2de, ze względu na ich najwyższe wcześniej wyznaczone powinowactwo do LIGHT, a także do peptydu CRD2e, który tworzył stabilny kompleks z białkiem LIGHT. W toku przeprowadzonych analiz wykazałem, że w przypadku peptydu CRD2no, żadna z zastosowanych substytucji nie prowadziła do zwiększenia powinowactwa do LIGHT w porównaniu z peptydem wyjściowym. W przypadku peptydu CRD2de jedynie zastąpienie reszty lizyny kwasem glutaminowym skutkowało zwiększeniem powinowactwa do LIGHT. Największy wzrost powinowactwa do LIGHT uzyskałem natomiast w wyniku substytucji K54 kwasem glutaminowym w cząsteczce CRD2e.

Zaprojektowałem również analogi podwójne, w których oprócz substytucji wspomnianej wcześniej K54, zastąpiłem również resztę kwasu asparaginowego w pozycji 62 następującymi aminokwasami: alaniną, leucyną, lizyną lub seryną. Wybór ten wynikał z faktu, że w związkach CRD2e_K54E oraz CRD2de_K54E reszta ta wykazywała negatywny wpływ na wiązanie się peptydu do białka LIGHT. Żaden z zaprojektowanych peptydów nie wykazywał jednak zwiększonego powinowactwa do LIGHT, w porównaniu ze związkami wyjściowymi.

Dodatkowo, na podstawie dekompozycji energii na wkłady pochodzące od poszczególnych reszt dla kompleksu HVEM/LIGHT zaprojektowałem dwa peptydy bazujące na fragmencie domeny CRD1 białka HVEM – (CRD1(16-38)bc oraz CRD1(16-38)no) oraz związki oparte na fragmencie domeny CRD2 – (CRD2(39-73)no, CRD2(39-73)d, CRD2(39-73)e oraz CRD2(39-73)de). Posiadają one skróconą sekwencję aminokwasową w stosunku do całych domen białka HVEM i obejmują tylko te reszty aminokwasowe, które w toku wcześniejszych analiz wyznaczyłem jako kluczowe dla tworzenia kompleksu białko/peptyd.

Energia wiązania peptydu CRD2(39-73)e z białkiem LIGHT była najniższa spośród wszystkich przebadanych związków. Na podstawie otrzymanych wyników zaprojektowałem również analogi tego peptydu, w których P39, K54, C61 oraz N72 zastąpiłem innymi resztami aminokwasowymi. Wybór tych reszt wynikał z ich potencjalnego negatywnego wpływu na stabilność kompleksu białko/peptyd. Żaden z zaprojektowanych w ten sposób peptydów nie wykazywał wyższego powinowactwa do białka LIGHT. Zaprojektowałem również dwa peptydy będące połączeniem domen CRD1 oraz CRD2 białka HVEM – CRD1-2(16-73)bce oraz jego analog CRD1-2(16-73)bce_K54E, jednak ich powinowactwo do białka LIGHT było niższe niż CRD2e_K54E oraz CRD2(39-73)e.

Dla wybranych peptydów oraz białka LIGHT określiłem również wybrane właściwości termodynamiczne, takie jak zmiana energii swobodnej Gibbsa (ΔG), czy entropii (ΔS). W przypadku peptydów CRD2e_K54E oraz CRD2(39-73)e, które we wcześniejszych analizach charakteryzowały się największym powinowactwem do LIGHT, energia swobodna przyjmują wartość ujemną. Praca wewnętrzna (ΔW) pierwszego z wymienionych związków nie zmienia się drastycznie w toku symulacji, natomiast ΔG oraz ΔH wzrosły. Świadczy to o spadającej stabilności kompleksu CRD2e_K54E/LIGHT w trakcie trwania symulacji. W przypadku CRD2(39-73)e można wyciągnąć dwa ważne wnioski – po pierwsze w miarę trwania symulacji ΔW maleje, co świadczy o tendencji do stabilizowania się kompleksu. Ponadto, mimo iż zmiana entropii przyjmuje wyraźnie większą wartość w porównaniu do innych peptydów, to czynnik ten jest minimalizowany przez wyższą wartość zmiany entalpii.

Dla wybranych peptydów przeprowadziłem symulacje widm CD na podstawie dynamik molekularnych, które następnie porównałem z widmami otrzymanymi eksperymentalnie. W obu przypadkach dla wszystkich zbadanych peptydów dominującą strukturą był nieuporządkowany kłębek statystyczny.

Podsumowanie publikacji 1

Wyniki uzyskane w trakcie przeprowadzonych badań wskazują, że kluczowym obszarem oddziaływania pomiędzy białkami HVEM i LIGHT jest domena CRD2 białka HVEM, co pozostaje w zgodzie z danymi wynikającymi ze struktury krystalicznej kompleksu tych białek. Przeprowadzone przeze mnie symulacje dynamiki molekularnej, zarówno dla kompleksu HVEM/LIGHT, jak i dla peptydów obejmujących odpowiednie domeny białka HVEM, umożliwiły zaprojektowanie serii analogów peptydowych, charakteryzujących się niższą energią oddziaływania z LIGHT niż natywne fragmenty analizowanych domen. Spośród 65 przeanalizowanych przez mnie peptydów, fragmentów białka HVEM, największe powinowactwo oraz najbardziej korzystne właściwości termodynamiczne wykazywały dwa zaprojektowane przeze mnie analogi: CRD2e_K54E oraz CRD2(39–73)e. Uzyskane wyniki stanowią podstawę do dalszych badań eksperymentalnych, których rezultaty przedstawiłem w publikacji 2.

8.2. Publikacja 2 - HVEM-derived peptides as inhibitors of HVEM/LIGHT complex formation

P. Ciura, S. Gumpelmair, E. Sikorska, S. Rodziewicz-Motowidło, P. Steinberger, M. Spodzieja, A. K. Sieradzan, „HVEM-derived peptides as inhibitors of HVEM/LIGHT complex formation”, *Bioorg.*, 2025, 166, 109183,
<https://doi.org/10.1016/j.bioorg.2025.109183> (załącznik 2)
100 punktów ministerialnych, Q1 (wg. Bazy JCR, stan na rok 2025)

Druga publikacja stanowi eksperymentalną kontynuację prac opisanych w publikacji pierwszej. W ramach prowadzonych przez mnie badań otrzymałem dwanaście peptydów, które podzielić można na trzy grupy. Pierwsza grupa obejmowała sześć peptydów, które bazowały na domenie CRD2 białka HVEM i różniły się ilością i rozmieszczeniem wiązań disulfidowych (3 peptydy z jednym wiązaniem disulfidowym oraz trzy z dwoma wiązaniami disulfidowymi w różnych pozycjach). Drugą grupę stanowiły trzy peptydy pokrywające domenę CRD3 białka HVEM (dwa z jednym wiązaniem disulfidowym oraz jeden z dwoma wiązaniami disulfidowymi). Do trzeciej grupy należały natomiast trzy analogii, określane jako: CRD2e_K54E, CRD2de_K54E oraz CRD2(39-73)e, które w analizach komputerowych wykazywały największe powinowactwo do białka LIGHT. Związki te wraz z ich sekwencjami aminokwasowymi przedstawiłem w tabeli 3.

Tabela 3: Sekwencja aminokwasowa zaprojektowanych peptydów, fragmentów domen CRD2 i CRD3 białka HVEM; X_1 – kwas 2-aminobutanowy, X_2 – norleucyna, & – położenie wiązania disulfidowego, numeracja reszt aminokwasowych HVEM nie obejmuje peptydu sygnałowego.

Nr	Oznaczenie peptydu	Sekwencja aminokwasowa peptydu
1	CRD2d	Ac-PC(& ₁)P ⁴¹ PGTYIAHLNG ⁵¹ LSKC(& ₁)LQX ₁ QX ₂ X ₁ ⁶¹ DPAX ₂ GLRASR ⁷¹ NX ₁ SRTENAVX ₁ ⁸¹ G-NH ₂
2	CRD2e	Ac-PX ₁ P ⁴¹ PGTYIAHLNG ⁵¹ LSKX ₁ LQC(& ₁)QX ₂ X ₁ ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(& ₁)SRTENAVX ₁ ⁸¹ G-NH ₂
3	CRD2f	Ac-PX ₁ P ⁴¹ PGTYIAHLNG ⁵¹ LSKX ₁ LQX ₁ QX ₂ C(& ₁) ⁶¹ DPAX ₂ GLRASR ⁷¹ NX ₁ SRTENAVC(& ₁) ⁸¹ G-NH ₂
4	CRD2de	Ac-PC(& ₁)P ⁴¹ PGTYIAHLNG ⁵¹ LSKC(& ₁)LQC(& ₂)QX ₂ X ₁ ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(& ₂)SRTENAVX ₁ ⁸¹ G-NH ₂
5	CRD2df	Ac-PC(& ₁)P ⁴¹ PGTYIAHLNG ⁵¹ LSKC(& ₁)LQX ₁ QX ₂ C(& ₂) ⁶¹ DPAX ₂ GLRASR ⁷¹ NX ₁ SRTENAVC(& ₂) ⁸¹ G-NH ₂
6	CRD2ef	Ac-PX ₁ P ⁴¹ PGTYIAHLNG ⁵¹ LSKX ₁ LQC(& ₁)QX ₂ C(& ₂) ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(& ₁)SRTENAVC(& ₂) ⁸¹ G-NH ₂
7	CRD3g	Ac-GC(& ₁)SPGHFX ₁ IV ⁹¹ QDGDHX ₁ AAC(& ₁)R ¹⁰¹ AYAG-NH ₂
8	CRD3h	Ac-GX ₁ SPGHFC(& ₁)IV ⁹¹ QDGDHC(& ₁)AAX ₁ R ¹⁰¹ AYAG-NH ₂
9	CRD3gh	Ac-GC(& ₁)SPGHFC(& ₂)IV ⁹¹ QDGDHC(& ₂)AAC(& ₁)R ¹⁰¹ AYAG-NH ₂
10	CRD2e_K54E	Ac-PX ₁ P ⁴¹ PGTYIHLNG ⁵¹ LSEX ₁ LQC(& ₁)QX ₂ X ₁ ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(& ₁)SRTENAVX ₁ ⁸¹ G-NH ₂
11	CRD2de_K54E	Ac-PC(& ₁)P ⁴¹ PGTYIHLNG ⁵¹ LSEC(& ₁)LQC(& ₂)QX ₂ X ₁ ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(& ₂)SRTENAVX ₁ ⁸¹ G-NH ₂
12	CRD2(39-73)e	Ac-PX ₁ P ⁴¹ PGTYIHLNG ⁵¹ LSKX ₁ LQC(& ₁)QX ₂ X ₁ ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(& ₁)G-NH ₂

Powinowactwo otrzymanych peptydów do białka LIGHT określiłem z wykorzystaniem techniki MST poprzez wyznaczenie stałej dysocjacji K_d na podstawie zmian sygnału fluorescencyjnego podczas tworzenia kompleksu białko/peptyd. Wszystkie zaprojektowane i otrzymane przez mnie związki wiązały się z celem molekularnym. W przypadku peptydów bazujących na domenie CRD2 białka HVEM, wyznaczone wartości K_d mieściły się w zakresie od 2,08 μM dla peptydu CRD2de do 8,50 μM dla związku CRD2f. K_d zmierzone dla peptydów bazujących na domenie CRD3 były wyższe i wynosiły od 7,62 μM dla CRD3h do 15,32 μM dla CRD3g, co świadczy o ich słabszym oddziaływaniu z białkiem LIGHT. Analogii CRD2e_K54E, CRD2de_K54E oraz CRD2(39-73)e charakteryzowały się największym powinowactwem do białka LIGHT (najniższa wartość K_d) a wyznaczone dla nich wartość K_d wynosiły odpowiednio 0,46, 0,76 i 0,06 μM .

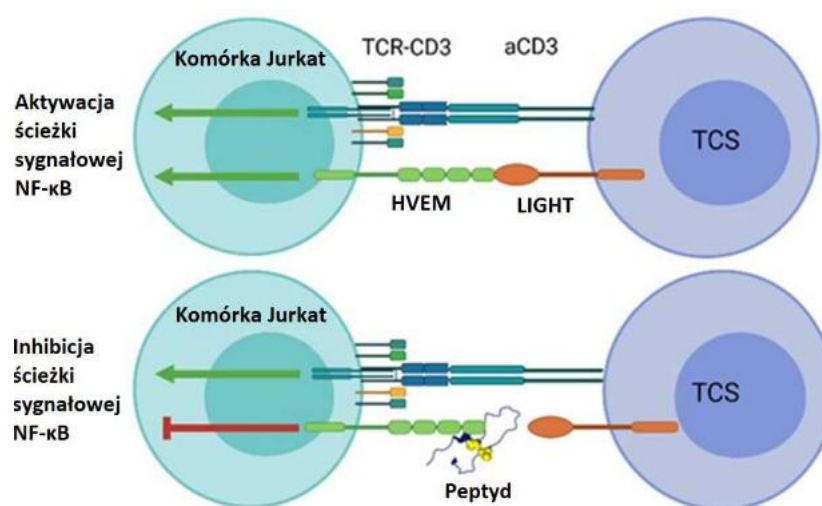
Zmierzona przez mnie stała dysocjacji dla kompleksu HVEM/LIGHT wynosiła 0,92 μM , co sugeruje, że tych warunkach eksperymentalnych zaprojektowane przez mnie analogii wiążą się z LIGHT silniej niż białko HVEM.

Właściwości inhibicyjne peptydów względem kompleksu HVEM/LIGHT określiłem za pomocą testów ELISA. Wszystkie otrzymane peptydy wykazywały zdolność hamowania oddziaływania pomiędzy badanymi białkami, przy czym obserwowany poziom inhibicji mieścił się w zakresie od 18% dla peptydu CRD3h do 31% dla peptydu CRD2e. Najlepsze właściwości hamujące wykazywały dwa analogii: CRD2e_K54E i CRD2(39-73)e, które we wcześniejszych analizach charakteryzowały się również największym powinowactwem do białka LIGHT. Hamowały one tworzenie kompleksu HVEM/LIGHT odpowiednio w 38 i 41%. W toku prowadzonych badań otrzymałem również peptyd o losowej sekwencji (tzw. scrambled peptide), w którym reszty aminokwasowe peptydu CRD2(39-73)e zostały pomieszczone (Ac-HX₁STGIPLQPYX₁KGLLNPC(&)AX₂LX₁DGNQPRARX₂SC(&)G-NH₂). Peptyd ten wykorzystałem jako kontrolę, a wyniki otrzymane z testu ELISA wykazały, że nie hamuje on tworzenia kompleksu HVEM/LIGHT. Potwierdza to specyficzność działania badanych analogów.

Właściwości hamujące peptydów zbadałem również na liniach komórkowych, a eksperymenty te wykonałem w Instytucie Immunologii Wiedeńskiego Uniwersytetu Medycznego. Grupa badawcza pod kierownictwem Prof. Petera Steinbergera opracowała platformy komórkowe, które składają się z modyfikowanych komórek Jurkat E6.1 z ekspresją białka HVEM, określanych mianem komórek reporterowych (JE6.1-NF- κ B::eGFP HVEM) oraz modyfikowanych komórek BW5147 z ekspresją białka LIGHT, określanych jako stymulatory limfocytów T (TCS, ang. *T Cell Stimulator*). Do aktywacji komórek reporterowych wykorzystywałem komórki TCS ekspresjonujące błonowo zakotwiczony fragment pojedynczego łańcucha przeciwciała anty-ludzkiego CD3, który specyficznie aktywuje kompleks CD3-TCR, obecny na komórkach reporterowych.

Oddziaływanie pomiędzy LIGHT ekspresjonowanych na komórkach TCS a białkiem HVEM na komórkach reporterowych prowadzi do aktywacji czynnika transkrypcyjnego NF- κ B, co można ocenić na podstawie poziomu ekspresji ulepszanego białka zielonej fluorescencji (eGFP, ang. *Enhanced Green Fluorescent Protein*). Aktywację NF- κ B w komórkach reporterowych oceniałem poprzez pomiar fluorescencji eGFP za pomocą cytometru przepływowego. Schemat przeprowadzonego eksperymentu przedstawiłem poniżej (Rys. 9).

Peptydy CRD2e_K54E oraz CRD2(39-73)e charakteryzowały się inhibicją odpowiednio 21 oraz 26%. Wyniki te potwierdzają wszystkie wcześniejsze przeprowadzone analizy.



Rysunek 9: Schemat oznaczania inhibicji kompleksu HVEM/LIGHT przez peptyd na liniach komórkowych.

Przed przystąpieniem do testów na liniach komórkowych niezbędne było określenie stabilności peptydów w nadsączy komórkowym oraz ich cytotoksyczności względem linii komórkowych, wykorzystywanych w prowadzonych eksperymentach. Stabilność peptydów oceniałem za pomocą techniki RP-HPLC. Powierzchnię pod pikami chromatograficznymi peptydów po 0 i 24 godzinach inkubacji w nadsączy pobranym znad komórek porównałem z powierzchnią pod pikami odpowiednich peptydów rozpuszczonych w wodzie. Po 24 godzinach inkubacji, stabilność peptydów, określona jako procent peptydu pozostającego w próbce, wahała się od 34% dla CRD2e_K54E do 77% dla CRD2de.

W analizach chromatograficznych nie zaobserwowałem obecności dodatkowych sygnałów na chromatogramach, świadczących o ich degradacji. Może to wskazywać, że peptydy te wiążą się ze składnikami nadsącza komórkowego.

Do oceny liczby żywych komórek zastosowałem test luminescencyjny CellTiter-Glo®, oparty na ilościowym oznaczaniu adenozynotrifosforanu (ATP), którego poziom koreluje z aktywnością metaboliczną komórek i jest proporcjonalny do liczby komórek żywych. Mechanizm testu opiera się na reakcji lucyferaza–lucyferyna, w której substratami są ATP, tlen cząsteczkowy oraz jony Mg^{2+} pochodzących z pożywki hodowlanej. W wyniku reakcji dochodzi do emisji światła o maksimum intensywności przy 560 nm. Analizy przeprowadziłem na liniach komórkowych Jurkat oraz TCS Ctrl. Żaden z badanych peptydów nie wykazywał istotnej cytotoksyczności w stężeniu 50 μM i niższym. W wyższych stężeniach 150 i 300 μM peptydy wykazywały cytotoksyczność wobec badanych komórek.

Przebadłem również wpływ dwóch najlepszych peptydów (CRD2e_K54E oraz CRD2(39–73)e) na tworzenie kompleksów BTLA/HVEM, z wykorzystaniem testu ELISA oraz badań na liniach komórkowych, a także na tworzenie kompleksu LT β R/LIGHT w teście ELISA. Analizy te przeprowadziłem, w celu wstępnej oceny specyficzności działania badanych związków. Oba peptydy nie hamowały tworzenia kompleksu BTLA/HVEM w żadnym z zastosowanych testów, co jest zgodne z doniesieniami literaturowymi, zgodnie z którymi za interakcję z BTLA odpowiada domena CRD1 białka HVEM⁴⁴. Peptyd CRD2e_K54E blokował wiązanie LT β R i LIGHT w teście ELISA z siłą porównywalną do inhibicji kompleksu HVEM/LIGHT, natomiast CRD2(39–73)e wykazywał jedynie słabe właściwości hamujące w stosunku do układu LT β R/LIGHT. Uzyskane wyniki sugerują, że jednoczesne blokowanie kompleksów HVEM/LIGHT oraz LT β R/LIGHT, przy braku wpływu na kompleks BTLA/HVEM, mogłoby umożliwić selektywne hamowanie sygnalizacji kostymulacyjnej zależnej od białka LIGHT.

Dla trzech peptydów – CRD2e, CRD2e_K54E, CRD2(39-73)e zarejestrowane zostały widma NMR przez dr hab. inż. Emilię Sikorską. Ze względu na nakładanie się sygnałów na otrzymanym widmie jedynie dla ostatniego z nich udało się przyporządkować sygnały do odpowiednich atomów. Z analizy widma wynika, że peptyd przyjmuje konformację nieuporządkowanego kłęбка statystycznego.

Podsumowanie publikacji 2

W ramach prowadzonych przez mnie badań zsyntezowałem i oczyściłem 12 peptydów, fragmentów białka HVEM. Ich wiązanie się z białkiem LIGHT potwierdziłem za pomocą techniki MST, a ich właściwości hamujące względem kompleksu HVEM/LIGHT zbadałem za pomocą testów ELISA i badań komórkowych. Dwa zaprojektowane przez mnie analogi CRD2e_K54E oraz CRD2(39–73)e wiązały się z białkiem LIGHT silniej niż HVEM i hamowały tworzenie kompleksu HVEM/LIGHT. Konieczne są jednak dalsze badania w celu oceny bezpieczeństwa tych związków a także ich potencjalnego działania immunomodulacyjnego w kontekście chorób autoimmunologicznych i odrzucania przeszczepów.

8.3. Publikacja 3 - Evaluation of LIGHT-derived peptides to disrupt the HVEM/LIGHT immune checkpoint

P. Ciura, S. Gumpelmair, S. Rodziewicz-Motowidło, P. Steinberger, M. Spodzieja, A. K. Sieradzan, „Evaluation of LIGHT-derived peptides to disrupt the HVEM/LIGHT immune checkpoint”, Sci Rep, 2025, 15, 40095.

<https://doi.org/10.1038/s41598-025-23946-4> (Załącznik 3)

140 punktów ministerialnych, Q1 (wg. Bazy JCR, stan na 2025 rok)

Ostatnia z publikacji wchodzących w skład rozprawy doktorskiej dotyczyła analiz teoretycznych i eksperymentalnych, które przeprowadziłem dla peptydów będących fragmentami białka LIGHT. W celu zaprojektowania peptydowych inhibitorów opartych na tych fragmentach przeprowadziłem symulacje dynamiki molekularnej kompleksu HVEM/LIGHT, na podstawie których zidentyfikowałem kilka centrów interakcji w białku LIGHT, odpowiedzialnych za wiązanie z HVEM. HVEM oddziałuje z dwoma monomerami LIGHT, wiążąc się z resztami 171–177 pierwszego monomeru LIGHT oraz z pięcioma regionami drugiego monomeru: T99–L105, E115–F122, Q148–V152, S192–W198 i R226–F235. Analiza MM-GBSA wskazała, że najniższe wartości energii wyznaczone zostały dla fragmentu R226–R228 oraz reszty R172 w LIGHT, co wskazuje, że aminokwasy te są kluczowe dla interakcji z HVEM. Na podstawie otrzymanych wyników zaprojektowałem pięć peptydów – light1 oraz light2, bazujących na fragmencie R226–F235 białka LIGHT oraz light3, light4 i light5, pokrywających fragment P171–L177 liganda. Sekwencje zaprojektowanych peptydów przedstawiałem w tabeli 4 (peptydy nr 1-5). Ponieważ w natywnym białku LIGHT omawiane fragmenty tworzą struktury β -spinki, do zaprojektowanych peptydów wprowadziłem mostki disulfidowe, w celu stabilizacji ich struktury drugorzędowej. Wybrane reszty aminokwasowe, leżące naprzeciw siebie w sekwencji aminokwasowej peptydów zastąpiłem resztami cysteiny, które następnie utleniłem.

Tabela 4: Sekwencja aminokwasowa zaprojektowanych peptydów, fragmentów wiążących białka LIGHT; & – położenie wiązania disulfidowego. Reszta metioniny w syntetyzowanych peptydach została zastąpiona norleucyną.

Nr	Oznaczenie peptydu	Sekwencja aminokwasowa peptydu
1	light1	Ac-E ²¹⁰ AGEEVC(&)RVL ²²⁰ DERLVRLRDG ²³⁰ TRSYC(&)GAFMV ²⁴⁰ -NH ₂
2	light2	Ac-E ²¹⁰ C(&)GEEVVRVL ²²⁰ DERLVRLRDG ²³⁰ TRSYFGAFC(&)V ²⁴⁰ -NH ₂
3	light3	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEELELL ¹⁸⁰ VSQC(&)S-NH ₂
4	light4	Ac-TIC(&)HGLYKRT ¹⁷⁰ PRYPEELELL ¹⁸⁰ VSC(&)QS-NH ₂
5	light5	Ac-TITC(&)GLYKRT ¹⁷⁰ PRYPEELELL ¹⁸⁰ VC(&)QQS-NH ₂
6	light3_E176K	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEKLELL ¹⁸⁰ VSQC(&)S-NH ₂
7	light3_E176L	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPELELL ¹⁸⁰ VSQC(&)S-NH ₂
8	light3_E176Q	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEQLELL ¹⁸⁰ VSQC(&)S-NH ₂
9	light3_E178K	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEELKLL ¹⁸⁰ VSQC(&)S-NH ₂
10	light3_E178L	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEELLLL ¹⁸⁰ VSQC(&)S-NH ₂
11	light3_E178Q	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEQLLL ¹⁸⁰ VSQC(&)S-NH ₂
12	light3_E176K_E178K	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEKLL ¹⁸⁰ VSQC(&)S-NH ₂
13	light3_E176L_E178L	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPELLLL ¹⁸⁰ VSQC(&)S-NH ₂
14	light3_E176Q_E178Q	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEQLLL ¹⁸⁰ VSQC(&)S-NH ₂

Dla zaprojektowanych peptydów (Tabela 4, peptydy nr 1-5) i białka HVEM przeprowadziłem symulacje dynamiki molekularnej o długości 200ns. Dla każdego układu wykonałem trzy niezależne trajektorie. Analizy wykazały, że dla kompleksu light5/HVEM energia interakcji oscyluje koło zera, co świadczy o oddysocjowaniu peptydu od celu molekularnego, w związku z tym peptyd ten odrzuciłam z dalszych badań. Największym powinowactwem do białka HVEM (najniższą energią kompleksu), jak również najniższym odchyleniem standardowym pomiędzy poszczególnymi trajektoriami charakteryzował się peptyd light3. Wyniki te wskazują, że spośród analizowanych związków tworzy on najbardziej stabilny kompleks z białkiem HVEM, dlatego też na nim skupiłem swoje dalsze badania.

Dla analiz MM-GBSA kompleksu peptydu light3 z białkiem HVEM przeprowadziłem analizę wkładów poszczególnych reszt aminokwasowych do całkowitej energii oddziaływania białko/peptyd. Pierwsza z nich pozwala na określenie energii interakcji danej reszty aminokwasowej z każdą inną resztą obecną w układzie, natomiast druga określa wkład energetyczny danej reszty w całkowitą energię wiązania⁹³.

Na ich podstawie określiłem, że E176 oraz E178 w light3 mają dodatni wkład energetyczny, tym samym negatywnie wpływają na wiązanie się peptydu do białka HVEM. W celu wyeliminowania tego wpływu zaprojektowałem serię analogów peptydu light3, w których reszty te zastąpiłem: lizyną, leucyną oraz glutaminą. Analogi te można podzielić na trzy grupy – pierwsza, w której wymieniałem kwas glutaminowy w pozycji 176 (Tabela 4, peptydy nr 6-8), druga, w której modyfikowałem kwas glutaminowy w pozycji 178 (Tabela 4, peptydy nr 9-11) oraz trzecia, w której substytucji podlegały obie reszty kwasu glutaminowego w położeniu 176 oraz 178 (Tabela 4, peptydy nr 12-14). W wyniku przeprowadzonych analiz wykazałem, że najlepszymi powinowactwem do HVEM charakteryzuje się peptyd light3_E178L.

Dla wybranych peptydów przeprowadziłem również sterowane dynamiki molekularne, na podstawie których określiłem wartość siły (ΔF) oraz pracy (ΔW), wymaganej do odciągnięcia peptydu od białka HVEM. W trakcie analiz centrum masy peptydu odciągano od centrum masy białka HVEM. Analizy te przeprowadzałem dla wyjściowych peptydów wykazujących powinowactwo do białka HVEM (light1, 2, 3, 4 i 5), jak również dla najlepszego analogu peptydu light3 (light3_E178L). Dla peptydów light3 i light3_E178L zaobserwowałem wyraźne maksimum siły oraz skok całkowitej pracy przy odległości między centrami mas wynoszącej 19 Å. Świadczy to o wysokim powinowactwie tych związków do białka HVEM, potwierdzając jednocześnie wyniki uzyskane z analizy MM-GBSA. W przypadku pozostałych analizowanych w badaniu peptydów nie obserwowałem podobnych efektów. Zbadałem również właściwości termodynamiczne kompleksów białko/peptyd. Dla peptydów light1, light2 i light4 obserwowałem wyższą wartość czynnika entropowego ($T\Delta S$) nad entalpowym (ΔH), co skutkuje dodatnią wartością ΔG , wyjaśniając tym samym słabe powinowactwo tych związków do celu molekularnego. Odmienną sytuację zaobserwowałem dla peptydów light3 i light3_E178L – w tym przypadku dominującą rolę odgrywa ΔH , a uzyskane wartości ΔG są ujemne bądź bliskie zeru, co świadczy o silniejszym wiązaniu z HVEM.

Zsumowana wartość $\Delta G + \Delta W$ pozwala dodatkowo ocenić właściwości peptydów zarówno w stanie wolnym, jak i po utworzeniu kompleksu. Dla peptydów light1 i light2 wartość ta potwierdza ich niskie powinowactwo do HVEM. W przypadku light4 suma ta przyjmuje wartości ujemne, co oznacza, że mimo iż samo ΔG wskazuje na słabe wiązanie, to znacząca redukcja pracy wewnętrznej wykonywanej przez peptyd po związaniu w dużym stopniu kompensuje ten efekt. Dla peptydu light3 wartość ΔW jest dodatnia, a tym samym dodatnie staje się także $\Delta G + \Delta W$. Obniżenie tej wartości może zwiększyć powinowactwo do HVEM, jednak – jak pokazuje przykład wariantu light3_E178L – zmniejszenie niekorzystnego wpływu ΔW na skutek podstawienia reszty kwasu glutaminowego równoważone jest przez wzrost ΔG .

Wyniki uzyskane w analizach teoretycznych postanowiłem zweryfikować eksperymentalnie. W tym celu otrzymałem peptydy wyjściowe (Tabela 4, light 1-4) oraz analog peptydu light3, o najwyższym powinowactwie do HVEM (Tabela 4, light3_E178L). Wiązanie się tych związków z białkiem HVEM zbadałem za pomocą techniki MST wyznaczając SpS. Analizy wykazały, że peptydy light3 i jego analog light3_E178L charakteryzują się najwyższym powinowactwem do białka HVEM, a pozorne stałe dysocjacji K_d wyznaczone dla kompleksów białko/peptyd wynoszą odpowiednio 0,58 i 0,71 μM .

Właściwości inhibicyjne peptydów wobec kompleksu HVEM/LIGHT określiłem podobnie jak dla fragmentów białka HVEM, za pomocą testów ELISA oraz badań komórkowych. Peptydy light3 oraz light3_E178L, przy najwyższym badanym stężeniu (50 μM), wykazywały odpowiednio 22 i 19% inhibicji w testach ELISA oraz 22 i 26% w badaniach komórkowych. Pozostałe zaprojektowane i analizowane przez mnie związki, fragmenty białka LIGHT, charakteryzowały się słabszym potencjałem hamującym wobec kompleksu HVEM/LIGHT.

Dla związków tych przeprowadziłem również analizy stabilności i cytotoksyczności, analogicznie jak dla fragmentów białka HVEM. Najniższą zawartość peptydu w próbce, po 24 godzinach inkubacji w nadsączy komórkowym, obserwowałem dla light3_E178L (29,41%), natomiast na chromatogramie nie były obecne dodatkowe sygnały świadczące o degradacji peptydu.

Podobnie jak w przypadku fragmentów białka HVEM może to świadczyć o wiązaniu się peptydu ze składnikami pożywki. Efekt taki był już obserwowany we wcześniejszych badaniach ⁹⁴. W przypadku pozostałych związków obserwowałem mniejsze zmiany intensywności odpowiadającego im sygnału przed i po inkubacji w nadsączu komórkowym. Dla peptydów przeprowadziłem również testy cytotoksyczności na liniach komórkowych TCS Ctrl oraz Jurkat E6.1. W najwyższym testowanym stężeniu (50 μ M) istotny spadek żywotność komórek Jurkat E6.1 zaobserwowałem dla peptydu light4. Pozostałe związki nie miały znaczącego wpływu na przeżywalność obu linii komórkowych.

Podsumowanie publikacji 3

W trzeciej publikacji wchodzącej w skład mojej rozprawy doktorskiej zaprojektowałem peptydy, potencjalne inhibitory tworzenia kompleksu HVEM/LIGHT, których sekwencja aminokwasowa oraz struktura bazowały na białku LIGHT. Wstępnie badania dynamiki molekularnej wykonałem dla pięciu peptydów. Cztery z nich formowały stabilny kompleks z białkiem HVEM w trakcie symulacji, a największym powinowactwem do HVEM charakteryzował się peptyd określany jako light3. Na podstawie dekompozycji energii wykonanej dla light3, zaprojektowałem serię jego analogów. Związek light3_E178L, w którym kwas glutaminowy został zastąpiony leucyną tworzył z białkiem HVEM najbardziej stabilny kompleks. Najlepszym powinowactwem oraz właściwościami inhibicyjnymi charakteryzowały się dwa peptydy light3 oraz light3_E178L. Uzyskane przez mnie związki mogą pełnić rolę struktur wiodących w projektowaniu nowych cząsteczek, skuteczniej hamujących tworzenie kompleksu HVEM/LIGHT.

9. Podsumowanie

Postępujące zapotrzebowanie na nowe i bardziej skuteczne terapie immunosupresyjne tworzy przestrzeń do nowych badań naukowych w dziedzinie immunomodulacji. Dotychczas stosowane terapie, w tym te oparte na przeciwciałach monoklonalnych, wiążą się z ryzykiem akumulacji przeciwciał w organizmie oraz zwiększonym prawdopodobieństwem wystąpienia działań niepożądanych, takich jak przewlekłe stany zapalne czy reakcje autoimmunologiczne. Alternatywą dla przeciwciał mogą być peptydy i peptydomimetyki, które charakteryzują się krótszym okresem półtrwania, wysoką specyficznością wobec wybranych celi molekularnych, niską immunogennością, łatwością modyfikacji oraz lepszą penetracją tkanek. Te cechy czynią je obiecującymi kandydatami do opracowywania nowych terapii immunomodulacyjnych.

Celem molekularnym terapii immunosupresyjnej może być kompleks HVEM/LIGHT, należący do kostymulujących punktów kontrolnych układu immunologicznego. Wykazano, że blokowanie wiązania się białek HVEM i LIGHT prowadzi do osłabienia odpowiedzi immunologicznej poprzez zahamowanie sygnałów kostymulujących niezbędnych do pełnej aktywacji limfocytów T a także ograniczenie ich proliferacji i produkcji cytokin prozapalnych. Opisany mechanizm działania może być szczególnie korzystny zarówno w leczeniu chorób o podłożu autoimmunologicznym, jak i w terapii po przeszczepieniach narządów.

Celem badań prowadzonych w mojej rozprawie doktorskiej była analiza kompleksu HVEM/LIGHT oraz zaprojektowanie na jej podstawie inhibitorów wiązania się tych białek. W trakcie przeprowadzonych badań wykonałem dynamiki molekularne kompleksu HVEM/LIGHT i kompleksów białko/peptyd. Dla wybranych układów przeprowadziłem również sterowane dynamiki molekularne oraz analizy MM-GBSA. Na ich podstawie określiłem centra interakcji obu białek, odpowiedzialne za ich oddziaływanie oraz określiłem wpływ poszczególnych reszt aminokwasowych na tworzenie kompleksu HVEM/LIGHT.

Następnie, zaprojektowałem peptydy – potencjalne inhibitory wiązania się tych białek, bazujące na fragmentach wiążących białek HVEM (65 związków) i LIGHT (14 związków).

Wybrane peptydy zsyntezowałem, oczyściłem a ich powinowactwo do odpowiednich białek wyznaczyłem za pomocą MST. Ich potencjał inhibicyjny względem kompleksu LIGHT/HVEM zbadałem za pomocą testów ELISA i badań komórkowych. Określiłem również stabilność tych związków w nadsącu komórkowym, a także ich cytotoksyczność względem linii komórkowych wykorzystywanych w badaniach. W przypadku peptydów bazujących na białku HVEM najlepszym powinowactwem do LIGHT oraz najwyższą inhibicją charakteryzował się peptyd CRD2(39-73)e. Wiązał się on z LIGHT ze stałą K_d wynoszącą 0,06 μM a jego potencjał hamujący wynosił odpowiednio 41 oraz 26% w testach ELISA oraz badaniach komórkowych. W przypadku związków zaprojektowanych na podstawie sekwencji aminokwasowej białka LIGHT największy potencjał wykazywał peptyd light3_E178L. Wiązał się on z białkiem HVEM z wartością K_d wynoszącą 0,71 μM oraz hamował tworzenie kompleksu HVEM/LIGHT w testach ELISA oraz testach komórkowych odpowiednio w 19% oraz 26%.

Uzyskane przez mnie wyniki badań eksperymentalnych wykazują wysoką korelację z danymi obliczeniowymi, co potwierdza zasadność przyjętej metody postępowania w przypadku projektowania podobnych związków w przyszłości. Tym samym analizowane peptydy mogą stanowić punkt wyjścia do projektowania cząsteczek o większym powinowactwie do białek HVEM i LIGHT oraz o wyższym potencjale hamującym względem kompleksu HVEM/LIGHT. Jednocześnie otrzymane związki wymagają dalszych, pogłębionych badań, w celu oceny ich potencjału immunomodulacyjnego oraz optymalizacji ich właściwości.

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12. Oświadczenia współautorów

Piotr Ciura, Pamela Smardz, Marta Spodzieja, Adam K. Sieradzan, Pawel Krupa
“Multilayered Computational Framework for Designing Peptide Inhibitors of HVEM-LIGHT Interaction”, *The Journal of Physical Chemistry B*, **128 (28)**, 6770-6785, (2024)
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my contribution included the design and execution of molecular dynamics simulations of individual inhibitors, analysis of structural properties with a particular emphasis on disulfide bond behavior, running enhanced-sampling simulations, visualization of results, and co-writing the main text of the manuscript.

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Niniejszym oświadczam, że w publikacji pt. *Multilayered computational framework for designing peptide inhibitors of HVEM-LIGHT interaction*, Piotr Ciura, Pamela Smardz, Marta Spodzieja, Adam K. Sieradzan, Paweł Krupa, *The Journal of Physical Chemistry B*, 2024, 128 (28), 6770–6785, moja rola obejmowała konsultacje naukowe oraz conceptualizację prowadzonych badań.

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My contribution involved supervising the project from the disulfide-bond analysis perspective, including assistance with conducting enhanced-sampling simulations and their subsequent analysis.

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Piotr Ciura, Simon Gumpelmair, Emilia Sikorska, Sylwia Rodziewicz-Motowidło, Peter Steinberger, Marta Spodzieja, Adam K. Sieradzan, “HVEM-derived peptides as inhibitors of HVEM/LIGHT complex formation”, *Bioorganic Chemistry*, **166**, 109183, (2025). <https://doi.org/10.1016/j.bioorg.2025.109183>.

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Marta Spodzieja



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Multilayered Computational Framework for Designing Peptide Inhibitors of HVEM-LIGHT Interaction

Piotr Ciura, Pamela Smardz, Marta Spodzieja, Adam K. Sieradzan,* and Pawel Krupa



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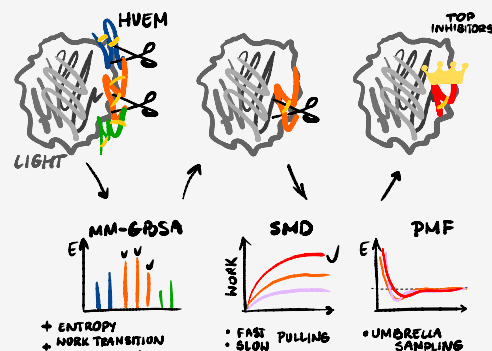


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Supporting Information

ABSTRACT: The herpesvirus entry mediator (HVEM) and its ligand LIGHT play crucial roles in immune system regulation, including T-cell proliferation, B-cell differentiation, and immunoglobulin secretion. However, excessive T-cell activation can lead to chronic inflammation and autoimmune diseases. Thus, inhibiting the HVEM-LIGHT interaction emerges as a promising therapeutic strategy for these conditions and in preventing adverse reactions in organ transplantation. This study focused on designing peptide inhibitors, targeting the HVEM-LIGHT interaction, using molecular dynamics (MD) simulations of 65 peptides derived from HVEM. These peptides varied in length and disulfide-bond configurations, crucial for their interaction with the LIGHT trimer. By simulating 31 HVEM domain variants, including the full-length protein, we assessed conformational changes upon LIGHT binding to understand the influence of HVEM segments and disulfide bonds on the binding mechanism. Employing multitrajectory microsecond-scale, all-atom MD simulations and molecular mechanics with generalized Born and surface area (MM-GBSA) binding energy estimation, we identified promising CRD2 domain variants with high LIGHT affinity. Notably, point mutations in these variants led to a peptide with a single disulfide bond (C58–C73) and a K54E substitution, exhibiting the highest binding affinity. The importance of the CRD2 domain and Cys58–Cys73 disulfide bond for interrupting HVEM-LIGHT interaction was further supported by analyzing truncated CRD2 variants, demonstrating similar binding strengths and mechanisms. Further investigations into the binding mechanism utilized steered MD simulations at various pulling speeds and umbrella sampling to estimate the energy profile of HVEM-based inhibitors with LIGHT. These comprehensive analyses revealed key interactions and different binding mechanisms, highlighting the increased binding affinity of selected peptide variants. Experimental circular dichroism techniques confirmed the structural properties of these variants. This study not only advances our understanding of the molecular basis of HVEM-LIGHT interactions but also provides a foundation for developing novel therapeutic strategies for immune-related disorders. Furthermore, it sets a gold standard for peptide inhibitor design in drug development due to its systematic approach.



1. INTRODUCTION

The herpesvirus entry mediator (HVEM) and its ligand LIGHT are members of the tumor necrosis factor superfamily (TNFSF).^{1,2} These proteins play multiple roles in the immune system by regulating diverse processes and T-cell responses. In particular, HVEM and LIGHT function as costimulatory molecules, and their interaction results in the enhancement of T-cell growth, differentiation, and cytokine secretion.^{3,4}

HVEM is a type I transmembrane glycoprotein composed of 283 amino-acid residues, divided into a signaling peptide (–37–0) and extracellular (1–164), transmembrane (165–185), and cytoplasmic (186–245) parts. The extracellular fragment of HVEM contains four cysteine-rich domains (CRD1:1–38, CRD2:39–81, CRD3:82–124, and CRD4:125–164), and the disulfide bonds within these domains are critical for establishing the tertiary structure of this receptor.⁵ HVEM provides a stimulatory signal by interacting with the LIGHT and LT α ligands via the CRD2 and CRD3 domains^{2,6} (Figure 1), and an inhibitory signal

when it binds to the BTLA⁷ or CD160⁸ receptors via the CRD1 domain. On the other hand, neither the CRD4 structure nor its affinity to other molecules is known.⁹ This binding pattern of HVEM offers multiple targetable molecules for modulating immunological responses.

LIGHT, also known as TNFSF14, is a homotrimeric type II transmembrane protein expressed on activated T-cells and immature dendritic cells.^{2,4} It is involved in various biological processes, including inflammation, immune regulation, and apoptosis, and plays a critical role in the pathogenesis of various autoimmune diseases and cancer,¹⁰ as well as COVID-

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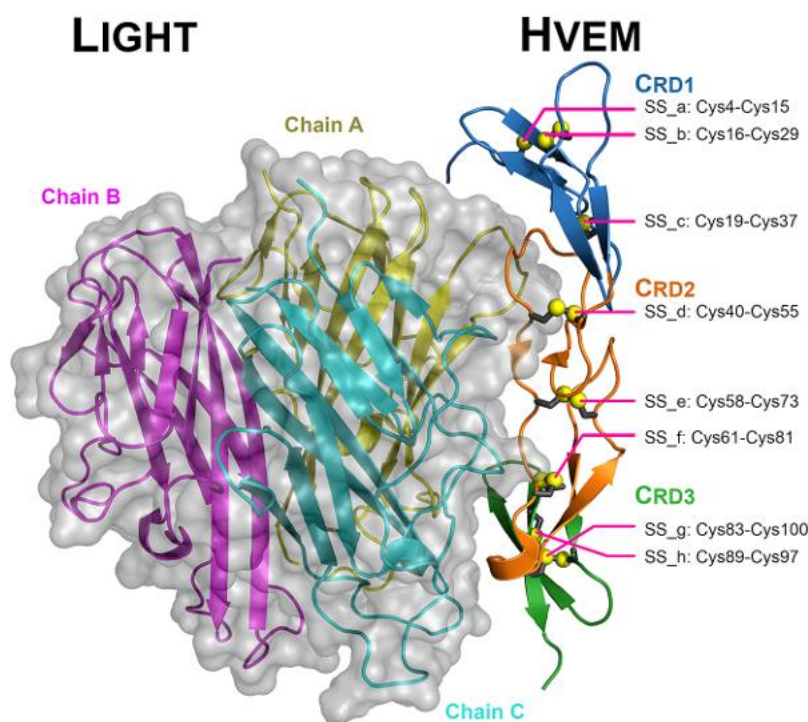


Figure 1. Cartoon representation of the LIGHT-HVEM complex with the semitransparent surface representation of the LIGHT trimer (PDB code: 4RSU). CRD's1–3 are indicated as blue, orange, and green colors, respectively, while chains A–C from the LIGHT trimer are colored yellow, magenta, and cyan, respectively. HVEM disulfide bonds are shown as gray sticks and yellow spheres (a–h).

19-induced pneumonia and inflammation.¹¹ Therefore, understanding the molecular mechanisms of LIGHT signaling is crucial for developing new therapies for these diseases.

The HVEM-LIGHT complex could be involved in various inflammatory processes, such as inflammatory bowel disease¹² and rheumatoid arthritis (RA),^{13,14} due to the modulation of T-cell proliferation. Furthermore, studies suggest that the interaction between these proteins may play a major role in transplantation. For example, targeting LIGHT protein using HVEM-Ig and LT β R-Ig fusion proteins significantly reduces allogeneic T-cell immune responses, including proliferation and cytotoxic T-cell activity.⁴ Similarly, the use of HVEM mAbs has shown a similar effect.¹⁵ All these data highlight that the HVEM-LIGHT complex could be a key factor in controlling T-cell immune responses and may be a candidate to target in different immune-mediated diseases.

As short peptides usually have lower toxicity, drug–drug interaction, and production cost,¹⁶ the aim of the present study is to systematically design short peptide inhibitors of the HVEM-LIGHT interaction based on HVEM-binding fragments. Another reason why we conduct research on peptides is the fact that they are easily degraded in the body.¹⁷ This fact, at first glance negative, in therapeutic applications may have a positive effect. Application of peptides may avoid severe side effects present in immunotherapy using antibodies,¹⁸ which are related to their long half-life in the body. To achieve this, we designed peptides that are fragments of the HVEM molecule, which can potentially block the formation of the HVEM-LIGHT complex and determined their affinity to the trimeric LIGHT protein. In our work, we used standard L-amino acids. Due to the fact that our work is based on structures occurring in nature, the use of other compounds, e.g., D-amino acids, could significantly affect the final structure of the designed compound, which would affect their affinity to the LIGHT

protein. At the same time, since the pharmaceutical and laboratory costs of introducing disulfide bonds in proteins are high, we strive to minimize their presence to only those disulfide bonds which are essential. The presence and importance of the disulfide bonds in the HVEM molecule, used as a template for the design of the ligands, is one of the reasons why typical machine learning tools, such as AlphaFold 3,¹⁹ cannot be effectively used to examine various disulfide-bond variants.

A common, yet effective, computational approach for estimating binding affinities involves docking the ligand molecule to the receptor, followed by the estimation of binding free energy using methods such as molecular mechanics with generalized Born or Poisson–Boltzmann surface area solvation (MM-GBSA, MM-PBSA) on the MD trajectories of the obtained complex models.²⁰ In this work, we performed detailed analyses of binding free energies, dynamics, and stability of the obtained complexes based on extensive molecular dynamics (MD) simulations in a state-of-the-art force field and water model. However, this approach assumes rigid docking, where the conformation of the ligand does not change upon binding.²¹ This assumption is unlikely to hold in the case of HVEM-based fragments, especially when not all disulfide bonds are present. Therefore, we studied the influence of disulfide bonds on HVEM domain stability. We also determined amino-acid residues and disulfide bonds that have a crucial influence on the formation and stability of the HVEM-LIGHT complex. Moreover, we performed a comparison between trajectories of free and bound HVEM variants, incorporating this information into the binding free energy estimations. To further elucidate the binding mechanism of the designed peptides to LIGHT, we conducted steered MD (SMD) simulations to pull the most promising HVEM-based inhibitors from the LIGHT trimer at various pulling speeds

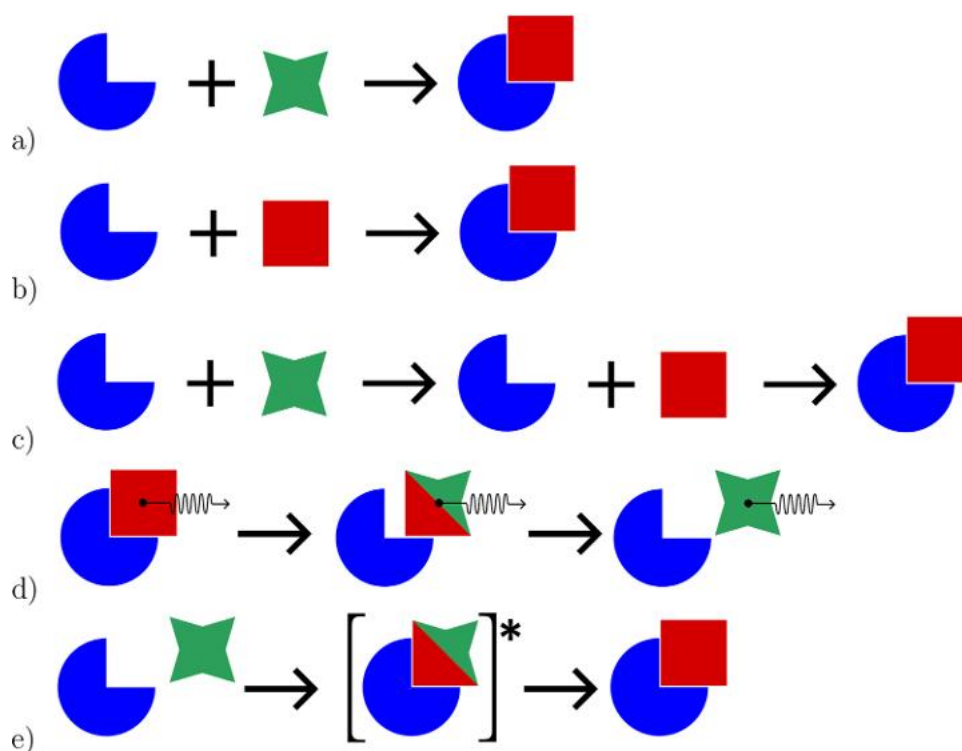


Figure 2. Diagram presents a simplified representation of receptor (blue; in this study, the LIGHT trimer) and ligand (red and green, with colors indicating bound and free conformational states, respectively; in this study, HVEM-based inhibitors) binding-unbinding pathways and mechanisms: (a) physiological, (b) MM-GBSA/MM-PBSA approach, (c) MM-GBSA/MM-PBSA approach with an additional calculation of the internal work connected to the transition between free and bound states of the ligand, (d) SMD approach to pull the ligand from the receptor at various pulling speeds, allowing for various levels of the bound-free state transition of the ligand due to the nonequilibrium character of the SMD simulations and limited time for conformational changes depending on the pulling speed, and (e) PMF calculation based on a series of US trajectories capturing the transition between bound and free states of the ligand depending on a distance between molecules.

and then calculated the potential of mean force (PMF) using the umbrella sampling method. This allowed us to obtain a single-residue mutant of the CRD2 domain (CRD2e_K54E) with a single disulfide bond (C58–C73) that binds most strongly to LIGHT out of all the examined variants based on whole domains. Additionally, we identified a truncated variant of the CRD2 domain (CRD2(39–73)e) with the same disulfide bond present, which is characterized by similar binding strength despite being shorter in length; however, it exhibits a slightly different binding mechanism.

2. METHODS

2.1. Binding Free Energy Estimation Approaches. One of the most important properties taken into account during drug design is the binding free energy of the complex formation, which can be presented as

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

where ΔG is the binding free energy, ΔH is the enthalpy of binding, T is temperature, and ΔS is the entropy change of the system upon complex formation.

It is directly related to the binding affinity/equilibrium dissociation constant $K_D = \exp(\Delta G_{\text{bind}}/RT)$. When designing peptides, one should take into consideration their flexibility and conformational changes upon binding (Figure 2A), as peptides bind either by an induced fit mechanism (where the ligand changes conformation upon binding)²² or by a conformational selection mechanism (where only a few

conformations appropriate for binding are selected from the conformation space available to the ligand).²³

In theoretical approaches, the most commonly used method to determine ΔG is MM-GBSA²⁴ (Figure 2B). In this approach, a conventional MD simulation in explicit (more often) or implicit (less often) water is performed, followed by the selection of a certain number of frames from a given trajectory and computation of the binding energy as given by the eq 2:²⁵

$$\Delta G_{\text{bind}} = \langle G^{\text{complex}}(i) - G^{\text{receptor}}(i) - G^{\text{ligand}}(i) \rangle_i \quad (2)$$

where $\langle \dots \rangle_i$ denotes an average over i snapshots extracted from the MD trajectory. ΔG represents the free energy of a given system. It should be noted that this is not literally the free energy, as this term contains only solvent entropy when simulations are run with the explicit solvent model. The entropy contribution in the MM-GBSA method could be further extended to the difference between bound and unbound systems in a given conformation. This is usually obtained via normal-mode analysis or quasi-harmonic approximation.²⁵ In this manner, one should, in theory, obtain binding free energies that are closer to experimental ones. However, this method is computationally expensive and may even lead to a worsening of the correlation between theoretically and experimentally obtained binding free energies.²⁶

MM-GBSA and MM-PBSA methods, despite being extremely fast and commonly used, have several important downsides:²¹ (a) It considers the same conformation of the

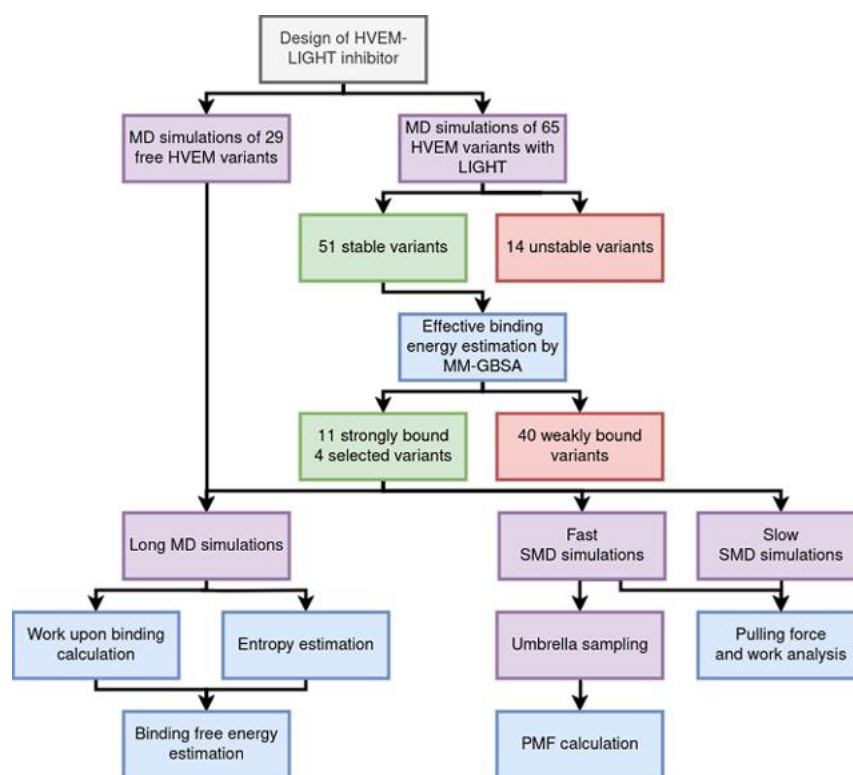


Figure 3. Flowchart illustrates the framework for designing highly effective HVEM-LIGHT inhibitors based on the HVEM molecule (gray box) using MD, SMD, and US simulations (purple boxes), along with their analyses (blue boxes), while arrows denote the general order of operations. The number of contacts between inhibitors and LIGHT during MD simulations was used as exclusion criteria for weakly bound inhibitors to reduce the computational costs of further studies.

unbound and bound states, similar to rigid docking. (b) Entropy is only incorporated as solvent entropy, making it a very rough approximation of the whole system entropy. (c) Entropy can be obtained via normal-mode analysis or quasi-harmonic approximation. However, this method is computationally expensive, often requiring hours to days of calculation for a single snapshot depending on the system size, and may only provide a rough approximation of the entropic contribution. (d) It ignores solvent/counterion-mediated interactions.

MM-GBSA calculation could be, in principle, improved by simulating the unbound ligand (Figure 2C). In this way, the internal work associated with the conformational changes can be computed with the use of eq:

$$\Delta G_{\text{conf}}^* = \langle H^{\text{bound}}(i) \rangle_i - \langle H^{\text{unbound}}(i) \rangle_i \quad (3)$$

where ΔG_{conf}^* is the free energy associated with conformational changes with the restriction that it contains only solvent entropic contributions. This energy describes the difference between the average internal (conformational) energy of a ligand in a bound ensemble state and the average internal energy of the ensemble in an unbound state.

In this way, one can take into consideration conformational changes upon binding; however, the peptide conformational enthalpy difference might be burdened with a large error as (i) it comes from separate simulations, (ii) may be sensitive to ligand conformation changes, especially in the case of poorly structured molecules, like most peptides, and therefore should be handled with extreme care. Moreover, this method does not overcome the solvent/counterions-mediated interaction problem.

Another technique that allows the computation of free energy is SMD. In this method, the ligand is pulled away from the receptor (Figure 2D), and the free energy can be computed from Jarzynski equality:²⁷ $\exp(-\Delta G/RT) = \langle \exp(-W/RT) \rangle$, where W is the work of a system. This method takes into consideration entropy in a direct manner and conformational changes during unbinding, as well as solvent effects; however, it is prone to other issues:

(a) requires a large number of trajectories and many starting points coming from equilibrium states and therefore may have problems with convergence, (b) for practical reasons, the Jarzynski equality is simplified to infinitely slow pulling limit ($\Delta G \sim W$), however, as the work depends on the velocity of pulling and is always greater than the free energy of binding ($\Delta G \leq W$).

In the case of many peptides, only one binding mode is dominant, and therefore, in many studies, a single starting point is used; therefore, this starting point should be handled with extreme care.

Finally, binding free energy can be computed from the PMF using eq 4:

$$\Delta G = -RT \ln \frac{\int_{r_0}^{r_1} \exp(-\text{PMF}(x)/RT) 4\pi r^2 dr}{\frac{4}{3}\pi r_1^3} \quad (4)$$

where x is the coordinate in which PMF is derived, r_0 and r_1 are the boundaries determined for the bound state, R is the gas constant, and T is the simulation temperature. This technique has to be performed based on the data from enhanced sampling algorithms (Figure 2E), such as replica exchange MD,²⁸ but even then it still can lead to nonuniform sampling

of the energy landscape. Therefore, the safest way is to perform umbrella sampling MD,²⁹ which can be computationally demanding as it requires running multiple separate trajectories, from which all have to reach equilibration. Another downside of this method is the choice of the coordinate of the derivation of PMF (x), as the PMF may depend on this choice.³⁰ In some cases, one can face with convergence problem as the umbrella sampling depends on the force constant and bin size.

There are also other techniques that allow estimating binding free energies.^{31–33} Overall, there is no perfect technique to determine the binding free energy, and each technique has its pros and cons. Therefore, in this work, we performed a rigorous analysis using all techniques showing their differences and similarities when applied to peptide drug design.

2.2. Simulated Systems. Our studies involved MD simulations of 65 molecules (Figure 3), based on the HVEM protein, including various lengths, disulfide-bond combinations, and amino-acid substitutions interacting with the LIGHT trimer (Table S1), while selected 29 variants were simulated also alone, in bulk water, without the presence of LIGHT protein, for sake of the comparison (Table S2). To simplify the naming, each disulfide bond was assigned a letter from a to h (Figure 1), based on the position of the first cysteine residue involved in a bond (detailed explanation is provided in Table S1).

2.3. All-Atom MD Simulations. The first set of MD simulations was used to determine the stability of the HVEM protein and its variants in an aquatic environment, while the second set was used to determine the binding affinity of proposed inhibitors to LIGHT and the change of structural properties upon binding. All MD simulations adapted the experimental model of HVEM-LIGHT (PDB: 4RSU,⁹) as the initial conformation, with or without disulfide-bond modifications, amino-acid substitutions, and truncations (Table S1).

Subsequently, we focused primarily on the amino-acid residues that exhibited the most repulsive or least attractive tendency toward the LIGHT trimer, as testing all possible combinations was infeasible. The mutations were selected based on chemical differences, change of charge, hydrophobicity/hydrophilicity nature, and large/small side-chain. If a single mutant demonstrated improved binding affinities compared to the wild-type molecule, we attempted to replace other residues showing the most repulsive or least attractive interactions. For every simulated variant, we modified it by manually removing the side-chain of the original residue and changing the backbone residue name to a given mutation in the text editor. Then, the side-chain was reconstructed during the preparation of the coordinates and topology files in tLeap. Minimization, equilibration, and production runs were then performed.

All systems were prepared using the tleap program, part of AmberTools23,³⁴ with the ff19SB force field³⁵ and four-point OPC water model,³⁶ while the simulations were run in pmemd.cuda, part of Amber22.³⁷ The HVEM/LIGHT complex and its equivalents were surrounded by a minimum 17.5 (20 for CRD1) Å layer of water in the shape of a truncated octahedron. To avoid the introduction of additional entropy from the ions, the system's charge was only neutralized, and no constant salt concentration was used with the use of Na⁺ or Cl[−] counterions. Each system underwent 2 minimization procedures: first with 1000

minimization steps (400 of steepest descent followed by 600 steps of conjugate gradient) and restraints placed on all solute atoms with constant $k = 100$ kcal/mol/Å², and second with 2000 minimization steps (800 of steepest descent followed by 1200 steps of conjugate gradient) and restraints placed on all heavy atoms with constant $k = 100$ kcal/mol/Å². Subsequently, each system underwent 3 equilibration cycles: first to set up the proper temperature of the system (heating up from 10 to 300 K over 10,000 MD steps in the NVT ensemble, each of 1 fs with restraints placed on heavy atoms), second to reach a proper density (100,000 MD steps in the NPT ensemble, each of 1 fs with restraints placed on heavy atoms), and the third to equilibrate the system (400,000 MD steps in the NPT ensemble, each of 2 fs with restraints placed on C α and C β atoms). Subsequently, three independent MD trajectories were carried out for 100×10^6 steps, each of 2 fs, providing 600 ns in total for each system. Four systems (CRD2e, CRD2def, CRD2e_K54E, and CRD2(39–73)e) were simulated for a longer time 500×10^6 steps, each of 2 fs, providing a total of 3000 ns per system. All production simulations were carried out in the NVT ensemble and with no restraints, with the Langevin thermostat set to 300 K. In all explicit solvent simulations, the Particle Mesh Ewald method with a cutoff for nonbonded interactions set at 9 Å was used to speed up calculations.

In addition to classical MD simulations, a series of SMD simulations (25 trajectories per system, each of at least 80 ns) was run to establish a binding-unbinding mechanism for CRD2e, CRD2def, CRD2e_K54E, and CRD2(39–73)e (Figure 5) representative models. Each of them started from the representative model of the above-mentioned HVEM variants obtained by the clustering of the last parts of the trajectories. To ensure that no interperiodic contacts were formed during the simulation, a larger number of water molecules was added to form a layer of 30 Å, giving a truncated octahedron box dimensions of approximately $122 \times 122 \times 122$ Å. Then, a standard minimization and equilibration procedure was performed as for the regular MD simulations, followed by a 10 ns run to make sure that the water molecules oriented properly around the complex. For those systems, 25 independent SMD trajectories were run with restraints placed on the centers of mass of LIGHT and CRD2 molecules with a force constant of 10 kcal/mol and a pulling speed of 0.05 m/s to achieve full dissociation of the system (minimal distance of any heavy atoms from the proteins above 6 Å), which translates to extensions above 40 Å.

To investigate the influence of pulling speed on the observed force and work response, an additional SMD run was performed with a pulling speed five times slower at 0.01 m/s. However, to expedite calculations, a smaller water box of 20 Å was used, and simulations were conducted only until most, but not all, trajectories were dissociated (to 25 Å of extension). This adjustment should not significantly impact the results but allows for some computational time savings. Given the slower pulling speed, five times more data points were collected for averaging. Consequently, we opted to run only 10 independent trajectories, each lasting 250 ns (totaling 2500 ns per system), as opposed to 25 trajectories, each lasting approximately 80 ns (totaling 2000 ns per system), for the faster pulling.

After the SMD simulations, PMFs were computed using the distance between the centers of mass of the proteins as a PMF variable. To set up umbrella sampling simulations, the strongest interacting conformation from SMD at a given

distance was used as a starting structure for the umbrella sampling simulation. For each system, at least 44 trajectories were run (with the distance of centers of mass from -5 to $+37$ Å with 1 Å interval compared to the distance from the initial position from classical MD simulations), each of 100 ns with a spring constant $k = 5$ kcal/mol/Å². Distances were collected every 1000 steps, and the last 95 ns of the trajectories (47,500 snapshots) were used for the weighted histogram analysis method (WHAM)³⁸ to calculate the PMF. From PMFs, association constants were computed (eq 4).

2.4. Analysis. Binding free energy ΔG and effective free energy analyses were performed on the converged parts of the trajectories (three for each of the systems): the last 20 out of 200 ns (last 200 out of 2000 snapshots) and the last 500 out of 1000 ns (5000 out of 10,000 snapshots) for simulations of HVEM variants with and without LIGHT and averaged out over three independent trajectories. The free energy change between the bounded and free states of the receptor–ligand complexes was estimated using the MM-GBSA method³⁹ with the recommended GB-Neck2 estimation method with the corresponding radii values.⁴⁰ In our research, we used it as a method of MD analysis because it allows us to obtain a satisfactory estimate of the receptor–ligand interaction energy at a relatively low time and computational cost. The use of other methods (such as free energy perturbation, FEP), given the number of systems analyzed in our work, could significantly increase the computational cost of the research without increasing the accuracy of calculations that would have an impact on further research.⁴¹ For most of the systems, the effective free energy⁴² was analyzed, which should provide good qualitative results for similar compounds.⁴³ Energy decomposition was performed on a per-residue and pairwise per-residue basis, with the latter summarized for each residue in the HVEM variant of its interactions only with LIGHT residues, to show the difference in how each residue influences overall binding stability and the influence on the interaction partner. The entropy contribution was calculated using the normal-mode analysis method for 200 ns of designed systems with the highest binding affinity and 1000 ns simulations of CRD2e, CRD2def, CRD2e_K54E, and CRD2(39–73)e systems. In the first case, calculations were performed for 10 frames extracted evenly from the last 20 ns of 200 ns simulations, while for the longer simulations, 20 frames from the last 200 ns were used for entropy calculation due to the large computational cost with the maximum number of iterations to calculate entropy set up to 1000. Such a combination of force field, water model, and MM-GBSA method is the modern standard for the binding affinity prediction.⁴⁴ To even further refine this approach, internal work associated with the change between bound and unbound compounds was calculated by an additional run of the MM-GBSA analysis for selected unbound HVEM variants (in bulk water) to obtain their enthalpy values and compared them with the one from bound molecules. The correlation between the values determined on the basis of MD, SMD, and PMF was confirmed on the basis of Pearson and Spearman correlation coefficients.

Analyses of: $C\alpha$ root-mean-square deviation (RMSD); root-mean-square fluctuations (RMSF); radius of gyration (R_g); maximum distance of any heavy atom to center of mass of the protein ($R_{g,max}$); end-to-end distance ($e2e$); solvent accessible surface area (SASA) (using the LCPO algorithm⁴⁵); contacts based on distance cutoffs of 8 and 6 Å for determining

dissociation of the complex in MD and SMD, respectively, (contact is defined as 0 if there are no heavy atoms from the complex partner within the range, and as 1 if there is at least one such heavy atom). During our work, we wanted to obtain highly effective inhibitors, for which it is important to maintain a structure that allows the formation of a stable complex with the receptor. Values aforementioned above can be used to determine the stability of compounds in a relatively simple way during the simulations and to visualize any changes in the conformation of simulated compounds that occur during the studies. The fraction of secondary structure was analyzed with the DSSP algorithm⁴⁶ implemented in AmberTools2023 cpptraj.³⁴ For comparison, the fraction of secondary structure was analyzed with STRIDE⁴⁷ and KAKSI.⁴⁸ LRMSD, calculated only for the backbone of the ligand after superposition on the receptor, was determined using DockQ.⁴⁹ As STRIDE, KAKSI, and DockQ can process only a single structure at the time, an in-house script was used to process equilibrated parts of trajectories. Representative models were generated by hierarchical clustering of the converged parts of the simulation. All figures were prepared with the use of Python 3.7 and gnuplot5.2, while structures were visualized with PyMOL 2.5.0.⁵⁰

2.5. Selectivity Check. To check if the designed peptides characterized by the strongest effective binding energy are binding selectively to the LIGHT binding groove, docking studies with use of HDOCK⁵¹ were performed. We also checked if the molecules could effectively bind to other proteins, which in physiological conditions bind the HVEM protein, such as BTLA.

Additionally, the UNRES-dock⁵² procedure was performed as in our previous paper.²⁰ The dominant structure of the peptide from the all-atom simulation was used as the starting structure. The peptide was randomly oriented with respect to LIGHT, and weak restraints were imposed on each chain. Note that no peptide-LIGHT restraints were imposed. Afterward, multiplexed-replica exchange MD⁵³ with the NEWCT-9P force field was applied,⁵⁴ with temperatures ranging from 250 to 400 K and 10,000,000 steps performed. After the simulation, the bin-less weighted histogram analysis method⁵⁵ along with clustering⁵⁶ at 280 K was used to obtain 10 clusters for each of the systems.

2.6. Peptide Synthesis. Selected peptides were synthesized by the solid phase peptide synthesis method on LibertyBlue synthesizer using the Fmoc/tBu strategy. The resin used was Rink Amide Pro Tide (LL) with a capacity of 0.18 mmol/g. In order to selectively create disulfide bridges, two cysteine derivatives were used: Fmoc-Cys(Acm)–OH and Fmoc-Cys(Trt)–OH. Cysteine residues present in the amino-acid sequence of HVEM and not involved in the disulfide bond were replaced with α -aminobutyric acid (Abu), while methionine residues were replaced with norleucine (Nle) in order to avoid the oxidation of sulfur in side-chain. The reaction was carried out at room temperature for 24 h. Then the peptides were cleaved from the resin using a mixture consisting of 88% TFA, 5% phenol, 5% deionized water, and 2% triisopropylsilane. 10 mL of the mixture was used per 1 g of resin, and all were stirred for 4 h at room temperature. Then resin was filtered off, and Et₂O was added to the remaining mixture to precipitate the peptide. The mixture was centrifuged three times at 4000 rpm for 15 min at 4 °C. Then the peptide was dissolved in deionized water and freeze-dried.

2.7. Peptide Purification. The purification of the peptides was carried out on a reverse-phase high-performance liquid chromatography (RP-HPLC) on a Luna 5 μm C8(2) 100 Å column. A 10-fold excess of DTT relative to free sulfhydryl groups was added to the aqueous solution of the purified peptide. The mixture was subjected to ultrasound at a temperature of 40 °C before being applied to the column. Two solutions were used during the purification: (A) deionized water with 0.1% TFA (v/v), and (B) 80% solution of acetonitrile in water with 0.08% TFA (v/v). A linear concentration gradient from 5 to 50% B in A was applied over 120 min. The purification process was monitored using a UV detector measuring the absorption at 222 and 254 nm. Peptide purity was verified using RP-UHPLC with PDA and ELSD-LT detectors (SHIMADZU, Kyoto, Japan) Kromasil C8 analytical column Kinetex C8 (100 $\times$ 2.1 mm; 2.6 μm ; 100 Å) with using a 5–100% gradient of solution B in 15 min.

2.8. Disulfide Bond Formation. The first disulfide bond was formed between the cysteine residues with the sulfhydryl group protected by a trityl group, which was removed when the peptide was pulled from the resin. After purification, the peptide was dissolved in a mixture of water and methanol (1:9, v/v) at a concentration of 40 mg/L. The pH of the mixture was adjusted to between 8 and 9 using ammonia–water. The mixture was stirred for 7 days, and compressed air ran through the solution. The progress of the reaction was monitored by RP-HPLC. After this time, the methanol was evaporated, and the remaining aqueous solution of the peptide was freeze-dried. The peptides with one disulfide bond were purified according to the procedure given earlier, while the peptides with two disulfides were subjected to the second oxidation. The second disulfide bond was created according to the following procedure: The peptide was dissolved in a mixture of acetic acid, water, and methanol (1:1:9, v/v/v) at a concentration of 40 mg/L. A 25–50 fold excess of iodine dissolved in methanol was then added to the mixture. The mixed solution was left for a week. After this time, the mixture was filtered through a Dowex ion exchange bed to remove excess iodine. The solvent was evaporated, and the obtained peptide was dissolved in water and lyophilized. Then, the peptide was purified according to the procedure given earlier.

Circular dichroism (CD) spectra were recorded at the Circular Dichroism Laboratory, Faculty of Chemistry, University of Gdańsk. The analysis was carried out using a CD J-815 circular dichroism spectrometer by Jasco. Peptide solutions with concentrations of 0.15 mg/mL were used for the tests, and all CD spectra were taken in water at 298 K as recommended.⁵⁷ The results are presented in the form of the dependence of the molar ellipticity on the wavelength.

3. RESULTS

3.1. Structural Properties of Unbound HVEM Protein and Its Disulfide-Bond Variants. For all of the structural fragments in which cysteine residues forming disulfide bonds are present, reduction of those bonds causes spatial separation of these elements (Figure S1). When the full HVEM structure is present, reduced cysteines tend to maintain native contacts between them, particularly noticeable in the case of the second domain and one disulfide bridge in the first domain. However, this effect is not observed in the specific HVEM domains when all cysteine residues are reduced (Figure S1). The introduction of any single disulfide bond consistently enhances structural stability. However, the strengthening of this effect does not

always occur with the addition of another disulfide bond. Furthermore, an interdomain stabilizing effect can be observed. This phenomenon is particularly pronounced in the case of CRD2, which exhibits remarkable stability even in the absence of any disulfide bonds when surrounded by CRD1 and CRD3.

In CRD2, which is the most important domain for binding LIGHT, the disulfide bond between Cys58–Cys73 (e) is the only one that significantly increases structural stability when only one disulfide bond is present on its own (reducing RMSD from 10.52 ± 0.28 to 4.93 ± 0.65 Å; Figure S2) and can be compared to the stability of the two-disulfide-bond variants CRD2df and CRD2ef. When the (e) disulfide bond is combined with Cys40–Cys55 (d), CRD2 domain stability slightly increases (CRDde: 3.84 ± 0.79 Å), approaching the case when all three bonds are present (CRD2def: 2.60 ± 0.18 Å).

The radius of gyration (RG) and RGmax analyses show that the compactness of CRD2 is not significantly affected by the presence or absence of disulfide bonds, except for the CRD2no and CRD2d (Figure S2) cases, which exhibited a more relaxed or loose structure.

The details of the influence of all disulfide bonds on CRD stability (Figure S1) are described in the Supporting Information section Influence of disulfide bonds on CRD stability.

The content of both α -helices and β -sheets in HVEM domains is heavily impacted by the presence (or absence) of given disulfide bonds and the presence (or absence) of the interdomain interactions (Table S3). While the full HVEM sequence has a strong tendency to form β -sheets over α -helices, which is understandable, as this secondary structure element requires distant parts of the protein to obtain complete stabilization, domains usually prefer more disordered and α -helical structures. The largest differences are observed for variants with all and none of the disulfide bonds present; however, a truncation of the CRD2 further modifies its behavior. Despite that, the predominant type of secondary structure in all of the HVEM variants is always disordered (coil) and turn, which is closely followed by β -sheets, while α -helix content is always low (<8%). The addition of disulfide bonds has varying degrees of impact but predominantly draws the secondary structure content close to the all-disulfide-bond variants and the complete HVEM chain with all disulfide bonds present.

The CD spectra generated with a Web server (PDB2CD)⁵⁸ reveal some helical (Figure S3B) content which is not present in the analysis with other methods and is not observed in the experimental CD spectra (Figure S3A). Despite the fact that CD is a very low-resolution method of secondary structure determination, especially for flexible structures,⁵⁹ for all peptides, the coil structure is the dominant one as the value of molar ellipticity is below 0 for 200 nm, which is in agreement with all secondary structure determination tools used for these simulations. Therefore, it can be concluded that peptides do not form significant amounts of stable secondary structures and are mainly unstructured.

3.2. Changes of the Structural Properties of HVEM and Its Variants Upon Binding LIGHT Trimer. HVEM variant structures become more rigid upon LIGHT binding, which is particularly evident in variants without any disulfide bonds present (Figure S2). This effect is even more pronounced when the complex exhibits high binding affinity. However, upon binding, the structure of CRD2 variants

becomes slightly more expanded. This is evident in the relatively small increase in the RG and a larger increase in RGmax, with the only exception being CRD2no. In the case of CRD2no, the combination of high flexibility, change in SASA, and high binding affinity makes it more strongly impacted by the binding of LIGHT.

Interestingly, the theoretical SASA of HVEM variants (except for CRD2no), calculated without the presence of any other molecules, remains similar for both free and bound HVEM variants with only a slight increase (Figure S4 and Table S4). In most of the cases, the difference in SASA upon binding arising from hydrophobic residue is relatively small. This observation suggests that hydrophobic interactions are not the primary contributors, in most cases, to HVEM-LIGHT interactions.

In general, the binding of HVEMall to LIGHT does not have a significant impact on the fluctuations (RMSF; Figure S5) and secondary structure of the molecule (Figure S6). Moreover, it is noteworthy that the most structurally stable region upon binding to LIGHT is CRD2. The most stable HVEM variant overall is CRD2all, whose secondary structure remains unchanged if all disulfide bonds are present, similarly to the complete HVEM chain with all disulfide bonds present. The most drastic changes in the secondary structure upon LIGHT binding are observed for CRD3no, which shifts strongly into β -sheets.

3.3. Effective Binding Energy to Select the Best Candidates for LIGHT Inhibition. The effective binding energy of HVEM and its variants to the LIGHT trimer was determined using the MM-GBSA method, while the contribution of particular amino-acid residues to the total effective binding energy was calculated using per-residue and pairwise energy decomposition, however, only for the complexes which did not dissociate during MD simulations (Figure S7). Our analysis revealed that, among the three HVEM domains, CRD2 emerged as the primary and the most robust interacting entity with the LIGHT trimer (Figures 4, S8–9); therefore, our further efforts to design the strongest binding peptide were focused mostly on this HVEM fragment. It should be noted that disulfide bonds have tremendous influence on the binding affinities, i.e., CRD2no (CRD2 domain without disulfide bonds) demonstrated significantly

higher affinity to LIGHT compared to CRD2all (with all disulfide bonds).

In the next step, we established that Lys54 has the most unfavorable energetic effect in most of the CRD2 variants; therefore, it was our first candidate for point mutations in order to attempt to increase binding affinity to the LIGHT trimer. Serine, leucine, isoleucine, glutamic acid, aspartic acid, tyrosine, and valine were selected as candidates for replacements to scan amino-acid residues of the opposite charge and properties to lysine. We selected CRD2 variants with different disulfide bond patterns which exhibited the best affinity: CRD2no and CRD2de, and the worst, but most stable in solution, one: CRD2e.

Unfortunately, no point mutations in CRD2no significantly improved the binding energy (Figure S8), while the mutation to serine dramatically reduced the binding affinity (-21.46 ± 3.90 kcal/mol). In the case of CRD2de, only the mutation to glutamic acid improved the effective binding energy (-54.92 ± 10.52 kcal/mol). Surprisingly, the highest effective binding energy was observed for the same substitution, but in the CRD2e variant, which is characterized by the lowest binding affinity among all CRD2 variants, but with the single-point mutation, it becomes the variant with the highest affinity (CRD2e_K54E: -58.55 ± 9.45 kcal/mol). Smaller improvement was observed for CRD2e with a substitution of Lys54 to valine (-41.17 ± 7.20 kcal/mol).

We also designed a series of double-point mutants to mitigate the unfavorable energy effect from Asp62, which was emphasized in the designed mutants. This residue was replaced with alanine, leucine, lysine, and serine. Unfortunately, none of the designed double mutants exhibited a more favorable effective binding energy than the original variant (Figure S8).

Based on the binding energy decomposition (Figure S9), we determined that not all amino-acid residues are involved in the formation of a complex with the LIGHT trimer. Therefore, we designed four peptides accordingly: two based on the CRD1 domain, namely CRD1(16–38)bc and CRD1(16–38)no, and two on the CRD2 domain, CRD2(39–73)e and CRD2(39–73)no, which included only amino-acid residues with sufficient affinity for the LIGHT trimer. It should be noted that, in general, peptides based on the CRD1 domain have much lower affinity for LIGHT than those based on the CRD2 domain, and the truncated variant showed even lower affinity (-18.89 ± 6.41 and -44.35 ± 6.06 kcal/mol, respectively). However, truncation of the CRD2 amino-acid residues resulted in a significant increase in effective binding energy, with the CRD2(39–73)e peptide being the strongest binding peptide (-61.43 ± 4.63 kcal/mol), despite its shorter polypeptide chain. Conversely, CRD2(39–73)no exhibited lower affinity than CRD2no. Remarkably, the removal of the 8 C-terminal residues from CRD2e resulted in a CRD2(39–73)e conformation that barely extends beyond the binding site of the LIGHT trimer (Figure 5).

In an effort to further improve the affinity of CRD2(39–73)e, we designed a series of mutants in which the Pro39, Lys54, Cys61, and Asn72 residues were substituted by other amino-acid residues. Unfortunately, these mutants displayed lower affinity for the LIGHT trimer than the parent peptide. To check the shortened configuration with all natively present disulfide bonds in this fragment (d and de disulfide bonds), we designed CRD2(39–73)d and CRD2(39–73)de molecules. These peptides, although showing improvement in affinity in comparison to their counterparts, CRD2d and CRD2de,

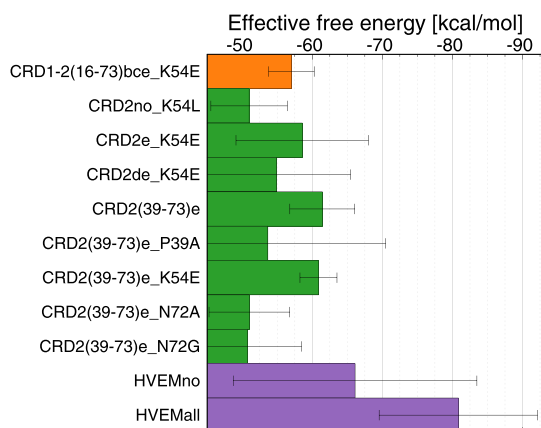


Figure 4. Bar plot of the effective binding free energy for HVEM variants with LIGHT with the highest binding affinity (< -50 kcal/mol), calculated as the average over three independent trajectories, with error bars representing standard deviations.

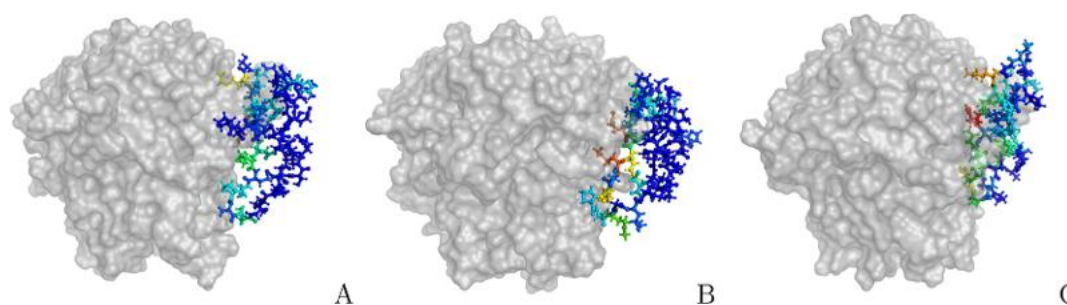


Figure 5. Representative models of selected HVEM-based variants, obtained by clustering three independent trajectories, colored by the pairwise effective binding energy (rainbow colors from blue to red, where blue indicates the lowest binding energy and red is the highest), in ball-and-stick representation for: (A) CRD2e, (B) CRD2e_K54E, and (C) CRD2(39–73)e, interacting with the LIGHT trimer (gray surface).

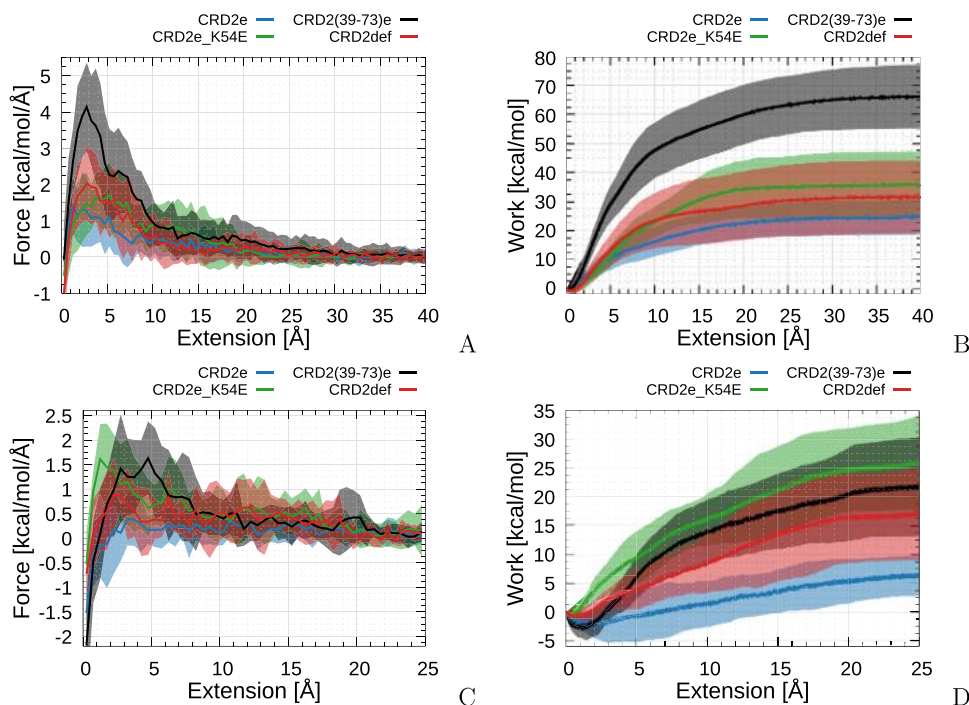


Figure 6. Plots of force (A, C) and work (B, D) averaged over 25 and 10 SMD trajectories (solid lines) with the corresponding standard deviation (SD) values (shown as semitransparent areas) for HVEM variants interacting with the LIGHT trimer run with 0.05 m/s (A, B) and 0.01 m/s (C, D) pulling speeds, respectively.

present significantly higher energy than CRD2(39–73)e (-45.17 ± 17.72 and -49.70 ± 17.19 for CRD2(39–73)d and CRD2(39–73)e, respectively).

Our final attempt was to design a peptide combining the best CRD1 and CRD2 variants, namely CRD1(16–38)bc and CRD2(39–73)e peptides, resulting in CRD1–2(16–73)bce. Its affinity for the LIGHT protein, although higher than the peptide based on the whole second domain (-49.53 ± 3.04 kcal/mol compared to CRD2e -35.26 ± 1.94 kcal/mol), does not show improvement compared to CRD2(39–73)e and CRD2e_K54E. Therefore, we decided to try the K54E mutation, which was determined to improve affinity in the case of CRD2 peptides. A mutant designed this way, CRD1–2(16–73)bce:K54E, exhibited an effective binding free energy only slightly higher than the CRD2e_K54E peptide. Due to our desire to design the shortest possible peptides, further research on this compound was abandoned.

3.4. Binding Energy and Process Captured by the SMD Simulations. In order to gain a comprehensive understanding of the interactions influencing the binding

between HVEM variants and LIGHT trimer, a series of 25 SMD trajectories for representative conformations of the two most promising systems and two reference systems were performed, namely CRD2e_K54E, CRD2(39–73)e, CRD2e, and CRD2def, in which the complex components were extended from each other using a spring constant on all $C\alpha$ atoms to reach a full dissociation. Observed initial distances of the centers of mass were equal to 28.70, 28.21, 30.25, and 24.28 Å for CRD2e, CRD2def, CRD2e_K54E, and CRD2(39–73)e, respectively, indicating that the truncated variant formed a much more compact complex than the complete CRD2 molecules. It also suggests that the truncated amino-acid residues do not stick to the LIGHT or even prohibit other amino-acid residues from forming tight contacts. Although the CRD2e_K54E variant shows that larger work is needed for the dissociation, increased stability, compared to the CRD2e, is observed only after about 3 Å extension, and $F_{\max}$ values of both variants are comparable (Figure 6), whereas CRD2e_K54E reveals a smaller $F_{\max}$ than CRD2def. The situation is completely different when CRD2(39–73)e is taken

into consideration—it shows not only a significantly larger work needed for complete dissociation than both untruncated variants (24.68 ± 6.16 , 31.75 ± 12.23 , 35.70 ± 11.43 , and 66.18 ± 11.07 kcal/mol, for CRD2e, CRD2def, CRD2e_K54E, and CRD2(39–73)e, respectively) but also a much greater F_{max} value is observed (1.41 ± 0.80 , 2.04 ± 1.05 , 1.70 ± 0.90 , and 4.14 ± 1.22 kcal/mol/Å, respectively for CRD2e, CRD2def, CRD2e_K54E, and CRD2(39–73)e). Moreover, truncation did not diminish the long-range interactions of the molecules; therefore, it should not have a negative impact on the recognition and early stages of the HVEM-fragment-LIGHT binding (Figure S10).

In CRD2e, amino-acid residues 42–44, 50–52, and 54–63 play the most important role in interacting with the LIGHT trimer when bound, with 50–54 being responsible for long-range interactions, persisting even after the extension of proteins to about 8 Å. The CRD2def exhibits a similar contact map pattern to CRD2e, with the key residues showing tighter binding. This is particularly evident for residues 132–141 of LIGHT chain B, which remained in contact even after a 12 Å extension.

Mutation of residue 54 in CRD2e_K54E impacts the binding mode to the LIGHT trimer by decreasing contacts of residues 50–52 and 54–56 of ligand. However, it allows for the strengthening of interactions by residues 57–63 and 40–44 of the ligand, enabling stabilization after a large extension to about 12 Å (compared to 8 Å in the case of nonmutated CRD2e).

For the truncated variant, namely CRD2(39–73)e, almost all amino-acid residues from CRD2(39–73)e form contacts with LIGHT without extending the molecules, especially regions 41–49, 54–68, and 71. This is also visible in Figure 5, where CRD2(39–73)e is inserted deeper into the LIGHT trimeric binding groove and no residues are sticking out outside of the complex. In addition to amino-acid residues 45–49, residues 56 and 68 are mostly responsible for the long-range interactions of CRD2(39–73)e with LIGHT. It should be noted that CRD2(39–73)e is the only variant in which almost all amino-acid residues can form contacts with LIGHT even after an extension of 22 Å, and multiple contacts are still present even if the extension exceeds 30 Å.

From the point of view of the LIGHT trimer, residues involved in the binding of all four CRD2 variants are similar, with the truncated variant forming interactions with most of the LIGHT amino-acid residues among the studied variants. While upon extension, CRD2e interacts mostly with amino-acid residues 76–93, with the help of regions around residues 160, 180, and 290, the K54E substitution pronounces the interaction with LIGHT residues 160–180 and 280–295. The binding pattern of the truncated variant, on the other hand, is much more similar to the CRD2e, forming way more interactions that are more stable during extending proteins. Overall, this suggests that point mutation K54E changes the binding mechanism, while truncation does not change the binding mechanism in the initial stages (large extension) but makes the tighter binding feasible.

Interestingly, analysis of the slower pulling speed (0.01 m/s) compared to the faster one (0.05 m/s) indicates that the CRD2(39–73)e variant exhibits much tighter binding, making it more susceptible to hydrodynamic effects upon mechanical unbinding compared to the other HVEM variants. This is evident in the larger drops in force and work observed for this variant compared to the others (Figure 6). It should also be

noted that almost no F_{max} drop is observed for CRD2e_K54E, indicating that only a few residues are mainly responsible for binding and no hydrodynamic effect is involved upon unbinding. During the slow pulling, the order of work needed to unbind is changed, with the CRD2e_K54E requiring the most, followed by CRD2(39–73)e. It should be noted that both the force and the work required for the CRD2e variant to dissociate become very small during slow pulling SMD, indicating that this molecule can easily dissociate, as observed in some of the longer (1000 ns) conventional MD trajectories performed for this molecule.

It should also be noted that pulling speeds used in this work (0.05 and 0.01 m/s) are significantly slower than in most computational works, which proved that SMD can predict well binding affinities of small molecules to proteins and used a pulling speed of 0.5 to 5 m/s.^{60–62}

3.5. Equilibrium Binding Energy Presented by PMF Calculations. In contrast to SMD trajectories, which depict the nonequilibrium properties of the system, PMF determination is based on conformations from the stretching trajectories but represents equilibrium phenomena. PMF results show that CRD2e_K54E is the strongest binding variant, surpassing even CRD2(39–73)e, in terms of energy difference between bound and free forms (Figure 7). It should

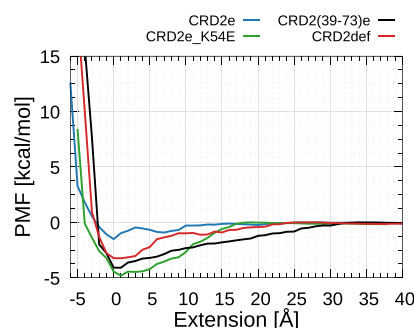


Figure 7. Plot of the potential of mean force (PMF) calculated based on approximately 44 trajectories for each of the HVEM variants interacting with LIGHT, obtained from umbrella sampling simulations. The extension 0 is taken as an approximation of the initial positions from conventional MD trajectories, which are used as starting points for SMD. Similarly, for PMF, it is equal to 29, 30, 24, and 28 for the centers of mass between CRD2e, CRD2e_K54E, CRD2(39–73)e, and CRD2def variants of HVEM and LIGHT, respectively.

be noted that CRD2(39–73)e exhibits a plethora of favorable positions in wider ranges of distances from the LIGHT trimer, which is most likely caused by its tightest binding.

Binding free energy computed from the PMF using eq 4 confirms raw PMF data analysis that CRD2e_K54E is the strongest binding moiety, surpassing the CRD2(39–73)e variant (computed ΔG is 0.37, -0.88 , -1.58 , and -2.44 kcal/mol for CRD2e, CRD2def, CRD2(39–73)e, and CRD2e_K54E, respectively). This trend is understandable, as it is confirmed by the same observation when a slower pulling speed was applied in the SMD approach, resulting in a drop in F_{max} and W_{total} values compared to the faster pulling speed trajectories. This change is equivalent to the transition from nonequilibrium to equilibrium simulations.

3.6. Changes in Binding Free Energy, Internal Work, Enthalpy, and Entropy Due to Complex Formation. To evaluate the relationship between different techniques in

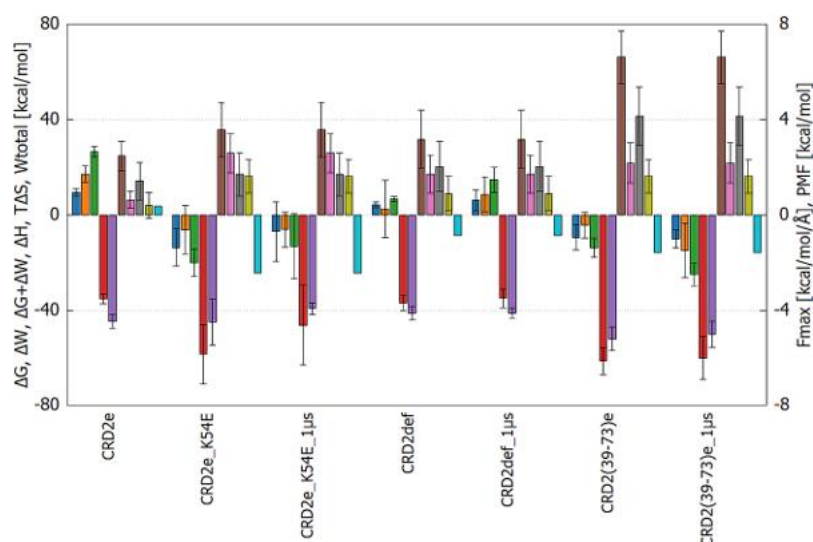


Figure 8. Change of: Binding free energy (ΔG , blue), internal work (ΔW , orange), summary of Gibbs free energy and internal work ($\Delta G + \Delta W$, green), enthalpy (ΔH , red), entropy ($T\Delta S$, violet) in 200 and 1000 ns long simulations ("1 μs " postscript), total work (W_{total} , pulling speed 0.05 m/s brown, 0.01 m/s pink), force max (F_{max} , pulling speed 0.05 m/s gray, 0.01 m/s yellowish green), and potential mean force (PMF, cyan) calculated on SMD basis of four selected systems. CRD2e_1 μs , due to dissociating, is not shown.

predicting binding energy, we compared their results and found that fast pulling SMD aligns well with MM-GBSA, while slow SMD aligns well with PMFs (Figures 8 and 9). For

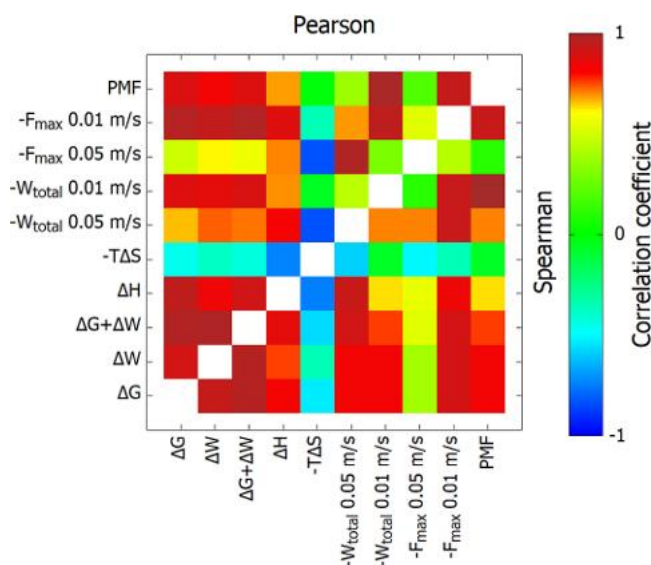


Figure 9. Pearson and Spearman correlation coefficients of the properties computed by various methods to calculate energetic components contributing to the binding affinity of the complexes determined for the systems included in Figure S8.

comparison, we included all the results for strong binding peptides in the Supporting Information (Figure S11). We found that CRD2e is the least stable among the closely studied peptides. In the case of the CRD2def molecule, defined as a reference structure, binding free energy (ΔG) and work transition between free and bound states of the ligand (ΔW) are also positive, while for CRD2e_K54E and CRD(39–73)e, they are both negative. Moreover, these values tend to increase during simulations (as indicated in CRD2def_1 μs).

In the case of the CRD2e peptide, the stability of its complex with LIGHT is even weaker. In the best mutant,

CRD2e_K54E, ΔW tends to remain at the same level during longer simulations, but ΔG and enthalpic difference (ΔH) increase, indicating that the complex is losing its stability over the course of the simulation. The increased affinity to LIGHT, marked by a lower ΔH value, may originate from new interactions, but they have little impact on the overall stability of the complex.

In the shortened molecule, CRD2(39–73)e, a different pattern is observed: ΔW decreases in longer simulations, indicating further stabilization of the molecule structure as a result of complex formation. The entropic contribution ($T\Delta S$) is significantly larger (more negative) than other counterparts, but this effect is compensated by enthalpy, and therefore, $\Delta G + \Delta W$ is more negative than other studied variants. The maximum force peak (F_{max}) and total work needed for dissociation (W_{total}) values are similar in CRD2e, CRD2def, and CRD2e_K54E, but in CRD2(39–73)e, they are visibly more favorable, further suggesting the highest affinity for the receptor. However, this trend is no longer visible in the case of PMFs and slower SMD simulations.

This indicates that MM-GBSA overemphasizes the solvent component, while fast SMD depends on the hydrodynamic properties as water cannot accommodate overly rapid pulling. When PMF or slow SMD results are analyzed, CRD2e_K54E emerges as the strongest binding peptide; however, PMF and SMD seem to overemphasize a single strong interaction. Therefore, both CRD2e_K54E and CRD2(39–73)e are great HVEM-LIGHT inhibitor candidates, albeit through different properties.

Strong positive correlation between ΔG , ΔW , $\Delta G + \Delta W$, and ΔH with $-W_{total}$, $-F_{max}$, and PMF implies that all methods used provide reliable estimation of binding energy. Those values are related due to the fact that with the decrease of the Gibbs free energy and enthalpy, the strength of the peptide binding to the protein increases, which is expressed by the increase in the work and force needed to detach the peptide from the protein marked by SMD. PMF correlates very well with both W_{total} and F_{max} from the slow pulling SMD simulations. This suggests that slow SMD simulations capture

Table 1. Top 5 Amino-Acid Residues from CRD2e, CRD2e_K54E, and CRD2(39-73)e and LIGHT Protein in Complexes, Ranked Based on Their Effective Binding Free Energy Decomposition [kcal/mol] Determined by MM-GBSA Pairwise Analysis Averaged Over Three Independent Trajectories with Standard Deviation^a

CRD2e	CRD2e_K54E	CRD2def	CRD2(39-73)e
Gln57 -9.04 ± 3.67	Met60 -11.76 ± 2.94	Gln57 -8.07 ± 3.13	Gln57 -13.90 ± 2.70
Lys54 -8.09 ± 4.20	Gln57 -11.34 ± 3.99	Lys54 -4.70 ± 4.34	Lys54 -10.21 ± 8.46
Met60 -4.74 ± 2.15	Asp62 -8.48 ± 3.60	Met60 -4.34 ± 1.75	Met65 -8.01 ± 3.62
Met65 -3.87 ± 3.11	Gln59 -7.94 ± 2.34	Pro63 -2.61 ± 2.00	Arg68 -6.03 ± 5.10
Gln59 -2.95 ± 1.86	Met65 -6.28 ± 2.03	Ala64 -2.25 ± 1.46	Gln59 -5.87 ± 2.86
LIGHT	LIGHT	LIGHT	LIGHT
Tyr173(A) -7.79 ± 3.84	Asn102(B) -11.02 ± 3.37	Asn102(B) -6.34 ± 1.64	Glu178(A) -10.23 ± 5.61
Asn102(B) -6.65 ± 3.40	Arg228(B) -10.56 ± 3.06	Leu118(B) -3.99 ± 1.48	Leu118(B) -8.07 ± 2.38
Arg228(B) -6.21 ± 2.71	Arg226(B) -9.48 ± 2.91	Arg228(B) -2.81 ± 1.77	Gly119(B) -7.66 ± 2.65
Leu118(B) -5.58 ± 2.89	Ser103(B) -6.78 ± 2.39	Ser104(B) -2.17 ± 0.99	Arg228(B) -6.93 ± 1.67
Glu175(A) -4.66 ± 2.67	Arg172(A) -6.47 ± 5.82	Glu115(B) -2.10 ± 1.07	Arg172(A) -5.72 ± 4.90

^aAs LIGHT is a homotrimer and HVEM variants interact with two of its chains, a chain letter (A–C) is presented in brackets.

the equilibrium properties of the system better than fast SMD, aligning well with the PMF results.

Anticorrelation of ΔG , ΔW , $\Delta G + \Delta W$, and ΔH with a negative $T\Delta S$ value should be noted, which could be interpreted as the more strongly the ligand binds to the receptor, the more negative the $T\Delta S$ term becomes, meaning the structure becomes more rigid upon binding.

The weakest correlation was observed for $-F_{\max}$ for fast pulling; therefore, it is not recommended to rely solely on this property when assessing binding affinities using SMD simulations. This is in agreement with previous observations that total work is more accurate estimation of the binding affinity than the main force peak.⁶³

Comparing the Pearson and Spearman correlation coefficients, we observe that they are generally in good agreement. However, in some cases, the Spearman coefficient is slightly higher, suggesting a stronger monotonic relationship between the variables, even if the linear correlation (captured by Pearson) is not as strong. This highlights the robustness of the Spearman coefficient in capturing nonlinear relationships between the binding energy estimates from different methods.

Notably, the correlation coefficients between PMF and W_{total} for slow pulling are 0.94 (Pearson) and 0.90 (Spearman), indicating a very strong agreement between these two methods. In contrast, the correlation coefficients between PMF and W_{total} for fast pulling are lower, with values of 0.77 (Pearson) and 0.80 (Spearman). This further supports the notion that slow SMD simulations better capture the equilibrium properties of the system compared to fast SMD simulations.

3.7. Designed Truncated Peptide Is Highly Selective to the LIGHT Binding Groove. As the LIGHT trimer consists of three identical binding sites, the analysis of the HDock results reveals that CRD2(39-73)e is docked to these three positions in its top 10 binding modes, with one of these modes ranking as 'top1'. This finding suggests that CRD2(39-73)e and HVEM can effectively compete for the same binding site on the LIGHT trimer, therefore inhibiting the HVEM-LIGHT complex formation. This, along with the docking score of -342.41 and confidence score of 0.9791, shows a strong preference of the CRD2(39-73)e peptide to bind to the LIGHT trimer.

As this study primarily focuses on silencing the stimulatory signal with LIGHT as opposed to the inhibitory signal with BTLA, we also docked the best molecule candidates to BTLA.

Docking of the CRD2(39-73)e peptide to the BTLA dimer shows low specificity, as the molecules in the top 10 binding modes are located in the interface between the molecules with the best docking score of -239.82 and confidence score of 0.8577, which are significantly worse than for the binding with the LIGHT trimer. CRD2e_K54E and CRD2e show worse docking (-252.88 and -213.45, respectively) and confidence (0.8867 and 0.7806, respectively) scores when binding to LIGHT, as well as lower selectivity compared to binding to the BTLA molecule, making CRD2(39-73)e the best drug candidate. Moreover, calculated IRMSD values, representing the position of CRD2e_K54E and CRD2(39-73)e in the LIGHT binding groove, were the lowest (most stable) among all tested variants (Figure S12), further indicating high selectivity. Additionally, we confirmed the binding sites using the coarse-grained UNRES force field and the UNRES-Dock procedure. The results showed that CRD2e, CRD2e_K54E, CRD2(39-73)e, and CRD2def reveal similar binding sites as observed in the all-atom simulations (Figure S14).

4. DISCUSSION

The crystal structure of the HVEM/LIGHT complex (PDB: 4RSU)⁹ shows a 3:3 stoichiometry. Crystallographic studies confirmed that CRD2, CRD3, and a small part of CRD1 of HVEM are involved in the interactions with LIGHT. CRD1 and CRD2 of HVEM interact with the loop regions G100, G151, T170-E175, L177, V255, and R226-G230 of LIGHT, while CRD3 of HVEM binds to loop regions: G151-V152, A159-T161, Q183, R195-V196, and W198 from LIGHT. It should be noted that HVEM is located between two LIGHT proteins (Figure 1). In our study, residues from both binding loop regions (T170-E175 and R226-G230) are in the top 5 amino-acid residues for the most promising variants (Table 1).

During the last several years, many single and double mutants of HVEM proteins were designed, and their interactions with LIGHT were tested. Shrestha et al. computationally redesigned the HVEM recognition interfaces using a residue-specific pharmacophore approach and postulated that single mutations H48I, D62R, M65K, and double mutations H48I/M65K or D62R/M65K in CRD2 of HVEM significantly reduced binding to LIGHT or resulted in a loss of interactions. According to the structural modeling, the substitution of methionine 65 for lysine in HVEM delivers unfavorable electrostatic interactions with R226 in LIGHT, while the mutation H48I disrupts favorable polar interactions

between H48 and E175 and R226 in LIGHT. The other single mutations in HVEM, such as D7F, E14R, E14K, S20L, S20K, S20Q, E31R, L32D, L49F, L40W, G51F, and V91K, and double mutations S20Q_L32D and E31R_G51F, do not have significant effects on the binding to LIGHT.⁶⁴ Cheung et al. pointed out that Y9F, S20A, Y23F, R24A, K26A, E27A, and E38A in CRD1 of HVEM and R75A in CRD2 do not affect binding to LIGHT.⁶⁵

The performed effective binding energy decomposition (Figure S9) explains why experimental mutations of the selected amino-acid residues Y9, S20, Y23, R24, K26, E27, E38, R75,⁶⁵ and D7, E14, S20, E31, and L32⁶⁴ do not affect the binding affinity to LIGHT, due to their near-zero contribution to LIGHT binding and presence in regions that are not directly involved in binding. We found that L52 and M60 play an important role in the binding interface for the complete HVEM molecule (Figure S9, Table 1), while other experimentally determined amino-acid residues,⁹ such as H48 and L56, are in their vicinity and may play a role in stabilizing the local structure of HVEM rather than playing a role in the formation of direct interactions. This is further confirmed by the observation of Liu et al. that only pairwise mutagenesis of H48, L52, and L56 has an observable effect on LIGHT binding.

Shrestha et al. showed that the G51F mutant of HVEM binds to LIGHT comparably to wt HVEM. Residue S1 of HVEM forms contact with R172 of LIGHT through their backbone atoms (as mentioned above), and therefore, this substitution is not so important for protein binding.⁶⁶ This work is a good example that not all mutations of the amino-acid residues involved in the interaction with the partner molecule have a significant influence on the total binding affinity. In our study, we observed similar behavior for some of the substitutions of K54, C55, C61, D62, and N72, while some other amino-acid residues, such as P39, are much more sensitive to substitution. Overall, this proves that MM-GBSA with pairwise and per-residue decomposition can be a useful tool that explains the key interactions and mutation effects.

5. CONCLUSIONS

In this study, we conducted a comprehensive investigation into the stability of the HVEM molecule, its domains, and domain fragments, as well as their interactions with the LIGHT trimer, to identify potential strategies for modulating LIGHT activity. Our extensive MD simulations, totaling over 175 μ s of production runs, provided valuable insights into the role of disulfide bonds in domain stability and their varying stabilizing properties. While the addition of disulfide bonds generally increases protein stability, it is important to note that this is not always the case, as exemplified by peroxiredoxin enzymes, where disulfide bond formation can introduce structural frustration and increased dynamics.⁶⁷

Our simulations confirmed CRD2 as a key fragment for interactions with LIGHT, in agreement with experimental observations. The presence of disulfide bonds emerged as a critical determinant of HVEM domain stability, with specific combinations exerting varying impacts on structural integrity. Interestingly, we found that in some cases, such as CRD2no, the lack of a disulfide bond allows the structure to open up and adjust during binding to LIGHT, resulting in increased flexibility and binding strength compared to CRD2all (Figures S4, S5, S7).

Through MM-GBSA analyses, we successfully identified promising peptide inhibitors targeting the LIGHT trimer, with the CRD2 domain serving as the key binding site. Mutational and truncational studies of the CRD2e peptide provided valuable insights into enhancing binding affinity, albeit with intriguing complexities that warrant further investigation. Steered MD simulations at various pulling speeds revealed dynamic insights into the binding and dissociation events between HVEM fragments and LIGHT, highlighting the influence of specific amino-acid residue truncations on complex stabilization without impacting molecular recognition.

Our study advances the understanding of the molecular basis of HVEM-LIGHT interactions and HVEM domain stability, contributing to the development of targeted therapeutic interventions for immune-related disorders. We propose a truncated variant, CRD2(39–73)e, and a mutant, CRD2e_K54E, as the most promising compounds for selective interaction with the LIGHT trimer. Our results can be used for further experimental verification, as demonstrated in our previous work.²⁰

Importantly, our studies revealed that each computational technique has its own limitations in predicting binding affinities. MM-GBSA tends to overemphasize the solvent component, while fast SMD simulations are heavily influenced by hydrodynamic properties, as water molecules cannot effectively enter the cavity formed during protein dissociation, leading to an overestimation of binding affinity for more tightly bound complexes. This effect can be mitigated by using slower pulling speeds, albeit at the cost of increased computational resources. Conversely, PMF and SMD, when used to study binding affinities, may overemphasize the contribution of single strong interactions. Therefore, we recommend using these techniques in a complementary manner to gain a comprehensive understanding of the system. By combining insights from multiple methods, one can obtain a more complete picture of binding affinities and the underlying mechanisms that give rise to the differences observed between various systems. This multifaceted approach ensures a thorough analysis of the protein–protein interactions under investigation.

In conclusion, our multistep study of structural properties, binding affinity, selectivity, and mechanisms sets a new standard for the computational design of peptide drugs, while the insights gained from this work pave the way for the development of novel therapeutic strategies targeting the HVEM-LIGHT interaction with potential applications in the treatment of immune-related disorders.

■ ASSOCIATED CONTENT

Data Availability Statement

Three PDB models of the LIGHT inhibitors: PM0084527, PM0084528, and PM0084592.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcb.4c02255>.

Table with names and amino-acid sequences of designed peptides and simulation details; estimated secondary structure content; solvent accessible surface area (SASA) and hydrophobic SASA; plots of distance between sulfur atoms in disulfide bonds; structural properties comparisons; experimental and simulated CD spectra; heatmaps for SASA and RMSF; change in secondary structures

heatmap; native contacts between HVEM variants and LIGHT trimer; effective free energies bar plot; MM-GBSA binding energy decomposition results; contact heatmaps for SMD simulations; plot of binding free energies; structural properties bar plots; and structural comparison between all-atom and UNRES-dock structures (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information for "Multi-Layered Computational Framework for Designing Peptide Inhibitors of HVEM-LIGHT Interaction"

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1 Influence of disulfide bonds on CRD stability

The HVEM domains demonstrate a very different role of particular disulfide bonds on their stability. In CRD1, out of all simulations with only one bond, Cys16-Cys29 (marked as b) lowered RMSD to 7.47Å from 11.20Å in simulations without bonds. Yet, only when coupled with Cys19-Cys29 (c), the RMSD lowers to 3.27Å, which is close to the result of simulations with all bonds present (2.89Å). Both the combinations "ab" and "ac" have results comparable to the one bond "b". It can be concluded that "a" has the smallest importance in the overall stability of CRD1.

In CRD3, which has up to two disulfide bonds, the presence of Cys83-Cys100 (g) has a greater effect on stability (3.89 Å) than the presence of Cys89-Cys97 (h). The measured end-to-end distance for CRD3h is comparable to that without bonds, and for CRD3g, it is comparable to all bonds present. The RMSF of residues in both the C- and N-terminal in simulations without (g) are much higher compared to those with (h) present (Fig. S5).

Further increase in the stability of the domain was observed when the rest of the HVEM was present, indicating that these are rather protein fragments than structurally independent moieties.

The radius of gyration (RG) and RGmax analyses provide additional confirmation that, for CRD1, the most crucial bond is (b). This is evident from the similarity in values between simulations where it is present and those where all bonds are present, indicating a consistent level of compactness. In contrast, the compactness of CRD2 was not significantly affected by the presence or absence of disulfide bonds, except for CRD2no and CRD2d (Fig. S2), which exhibited a more relaxed or loose structure, in comparison to the structures with disulfide bonds in position (e) and (f). Regarding CRD3, disulfide bond (g) appears to contribute more significantly to structural stabilization than (h). It is worth noting that for molecules without disulfide bonds the rigidify occurs upon binding. The higher the bonding affinity, the more structure rigidity is observed (Fig S2, S3). The SASA of bound and unbound HVEM variants (Fig. S4) are similar (the variants bound to LIGHT trimer have slightly larger SASA), this indicate that hydrophobic interaction are not the key players in HVEM-LIGHT interactions.

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Table S1: Names and amino acid sequences of the designed peptides

name	disulfide bonds	residues
CRD1no	without	L ¹ PSCKEDEYPV ¹¹ GSECCPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1a	C ⁴ -C ¹⁵	L ¹ PSCKEDEYPV ¹¹ GSECCPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1b	C ¹⁶ -C ²⁹	L ¹ PSCKEDEYPV ¹¹ GSECCPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1c	C ¹⁹ -C ³⁷	L ¹ PSCKEDEYPV ¹¹ GSECCPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1ab	C ⁴ -C ¹⁵ ;C ¹⁶ -C ²⁹	L ¹ PSCKEDEYPV ¹¹ GSECCPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1ac	C ⁴ -C ¹⁵ ;C ¹⁹ -C ³⁷	L ¹ PSCKEDEYPV ¹¹ GSECCPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1bc	C ¹⁶ -C ²⁹ ;C ¹⁹ -C ³⁷	L ¹ PSCKEDEYPV ¹¹ GSECCPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1abc	C ⁴ -C ¹⁵ ;C ¹⁶ -C ²⁹ ;C ¹⁹ -C ³⁷	L ¹ PSCKEDEYPV ¹¹ GSECCPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1(16-38)no	without	CPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1(16-38)bc	C ¹⁶ -C ²⁹ ;C ¹⁹ -C ³⁷	CPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCE
CRD1-2(16-73)bce	C ¹⁶ -C ²⁹ ;C ¹⁹ -C ³⁷ ;C ⁵⁸ -C ⁷³	CPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCEPCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD1-2(16-73)bce_K54E	C ¹⁶ -C ²⁹ ;C ¹⁹ -C ³⁷ ;C ⁵⁸ -C ⁷³	CPKCSP ²¹ GYRVKEACG ³¹ ELTGTVCEPCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2no	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2no_K54D	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2no_K54E	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2no_K54I	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2no_K54L	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2no_K54S	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2no_K54V	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2no_K54Y	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2d	C ⁴⁰ -C ⁵⁵	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54D	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54E	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54I	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54L	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54S	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54V	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54Y	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54E_D62A	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54E_D62K	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54E_D62L	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2e_K54E_D62S	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2f	C ⁶¹ -C ⁸¹	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹

Table S1: Continued: Names and amino acid sequences of the designed peptides (x denotes point deletion)

CRD2de	C ⁴⁰ -C ⁵⁵ ;C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2de_K54D	C ⁴⁰ -C ⁵⁵ ;C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LS ~~D~~ CLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2de_K54E	C ⁴⁰ -C ⁵⁵ ;C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LS ~~E~~ CLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2de_K54I	C ⁴⁰ -C ⁵⁵ ;C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LS ~~I~~ CLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2de_K54L	C ⁴⁰ -C ⁵⁵ ;C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LS ~~L~~ CLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2de_K54S	C ⁴⁰ -C ⁵⁵ ;C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LS ~~S~~ CLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2df	C ⁴⁰ -C ⁵⁵ ;C ⁶¹ -C ⁸¹	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2ef	C ⁵⁸ -C ⁷³ ;C ⁶¹ -C ⁸¹	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2def	C ⁴⁰ -C ⁵⁵ ;C ⁵⁸ -C ⁷³ ;C ⁶¹ -C ⁸¹	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NCSRTENAVC ⁸¹
CRD2(39-73)d	C ⁴⁰ -C ⁵⁵	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_P39A	C ⁵⁸ -C ⁷³	~~A~~ CP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_P39L	C ⁵⁸ -C ⁷³	~~L~~ CP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_P39I	C ⁵⁸ -C ⁷³	~~I~~ CP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_P39V	C ⁵⁸ -C ⁷³	~~V~~ CP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_P39W	C ⁵⁸ -C ⁷³	~~W~~ CP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_P39x	C ⁵⁷ -C ⁷²	CP ⁴⁰ PGTYIHLNG ⁵⁰ LSKCLQCQCMC ⁶⁰ DPAMGLRASR ⁷⁰ NC ⁷²
CRD2(39-73)e_K54E	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LS ~~E~~ CLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_C55A	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LS ~~A~~ LQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_C55V	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LS ~~V~~ LQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_C61S	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCM ~~S~~ ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)e_N72A	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ ~~A~~ C ⁷³
CRD2(39-73)e_N72G	C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ ~~G~~ C ⁷³
CRD2(39-73)de	C ⁴⁰ -C ⁵⁵ ;C ⁵⁸ -C ⁷³	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQCMC ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD2(39-73)no	without	PCP ⁴¹ PGTYIHLNG ⁵¹ LSKCLQCQMS ⁶¹ DPAMGLRASR ⁷¹ NC ⁷³
CRD3no	without	GCSPQHFCIV ⁹¹ QDGDHCAACR ¹⁰¹ AYA
CRD3g	C ⁸³ -C ¹⁰⁰	GCSPQHFCIV ⁹¹ QDGDHCAACR ¹⁰¹ AYA
CRD3h	C ⁸⁹ -C ⁹⁷	GCSPQHFCIV ⁹¹ QDGDHCAACR ¹⁰¹ AYA
CRD3gh	C ⁸³ -C ¹⁰⁰ ;C ⁸⁹ -C ⁹⁷	GCSPQHFCIV ⁹¹ QDGDHCAACR ¹⁰¹ AYA
CRD3(89-97)h	C ⁸⁹ -C ⁹⁷	CIV ⁹¹ QDGDHC
CRD3(89-97)no	without	CIV ⁹¹ QDGDHC

Table S2: Details of the simulations performed in the project. cMD stands for conventional Molecular Dynamics simulations, SMD for Steered Molecular Dynamics simulations with 0.05 and 0.01 m/s for fast and slow pulling, respectively, and US for Umbrella Sampling. All designed variants of HVEM-based molecules are treated as possible inhibitors, while complete HVEM simulations were run for comparison only.

Description	No. of Molecules	Number and Length of Trajectories	Total Time [μs]
cMD of inhibitors with LIGHT	65	3×200 ns	37.8
cMD of HVEM-LIGHT complex	2	3×200 ns	1.2
cMD of top inhibitors with LIGHT	4	3×800 ns (continuation from 200ns)	9.6
cMD of unbound inhibitors	29	3×1000 ns	87
cMD of complete unbound HVEM	2	3×1000 ns	6
SMD _{fast} of inhibitors with LIGHT	4	25×80 ns	8
SMD _{slow} of inhibitors with LIGHT	4	10×250 ns	10
US of inhibitors with LIGHT	4	44×100 ns	17.6
			177.2

Table S4: Solvent accessible surfaces area (SASA) [$\text{\AA}^2$] obtained for last 20 ns averaged over three trajectories and their standard deviation between trajectories. Δ Sasa hydrophobic indicates the difference in SASA when only hydrophobic residues (GLY,ALA,VAL,LEU,ILE,PRO,PHE,MET,TRP) are taken into consideration

Name	SASA of complex	Sum of SASA of components	Ratio	Δ SASA hydrophobic
CRD1no	21793 $\pm$ 123	22566 $\pm$ 259	0.966 $\pm$ 0.006	121.89 $\pm$ 65.17
CRD1bc	25262 $\pm$ 2090	25763 $\pm$ 1755	0.980 $\pm$ 0.015	85.53 $\pm$ 67.89
CRD1(16-38)no	23833 $\pm$ 1703	24237 $\pm$ 2002	0.984 $\pm$ 0.012	16.26 $\pm$ 26.36
CRD1-2(16-73)bce	25231 $\pm$ 1528	25737 $\pm$ 1404	0.980 $\pm$ 0.016	108.92 $\pm$ 104.12
CRD1-2(16-73)bce.K54E	24095 $\pm$ 578	25235 $\pm$ 578	0.955 $\pm$ 0.024	328.86 $\pm$ 192.27
CRD2no	25123 $\pm$ 2189	25582 $\pm$ 1986	0.982 $\pm$ 0.023	56.50 $\pm$ 63.87
CRD2no_K54D	24743 $\pm$ 1492	25361 $\pm$ 1314	0.975 $\pm$ 0.018	155.94 $\pm$ 200.26
CRD2no.K54I	24971 $\pm$ 2016	25456 $\pm$ 1592	0.980 $\pm$ 0.028	122.83 $\pm$ 206.07
CRD2no_K54L	21883 $\pm$ 403	23306 $\pm$ 445	0.939 $\pm$ 0.002	416.64 $\pm$ 35.44
CRD2no_K54S	24718 $\pm$ 2741	25081 $\pm$ 2421	0.984 $\pm$ 0.015	80.03 $\pm$ 95.60
CRD2no_K54V	24767 $\pm$ 2205	25474 $\pm$ 1805	0.971 $\pm$ 0.021	260.70 $\pm$ 223.09
CRD2no_K54Y	26345 $\pm$ 2527	26944 $\pm$ 2218	0.977 $\pm$ 0.016	133.45 $\pm$ 89.89
CRD2def	24550 $\pm$ 1650	25751 $\pm$ 1548	0.953 $\pm$ 0.007	317.82 $\pm$ 94.98
CRD2d	23337 $\pm$ 1531	24674 $\pm$ 1702	0.946 $\pm$ 0.004	319.52 $\pm$ 134.31
CRD2e	24237 $\pm$ 2004	24922 $\pm$ 1860	0.972 $\pm$ 0.024	128.37 $\pm$ 6.76
CRD2e_K54D	23486 $\pm$ 324	23842 $\pm$ 439	0.985 $\pm$ 0.007	83.56 $\pm$ 46.29
CRD2e_K54E	24103 $\pm$ 1751	24662 $\pm$ 1499	0.977 $\pm$ 0.025	307.50 $\pm$ 216.24
CRD2e_K54I	25385 $\pm$ 1477	26133 $\pm$ 1708	0.972 $\pm$ 0.007	320.30 $\pm$ 75.40
CRD2e_K54L	22874 $\pm$ 1091	24023 $\pm$ 554	0.952 $\pm$ 0.037	262.49 $\pm$ 229.68
CRD2e_K54S	24145 $\pm$ 1782	24429 $\pm$ 1542	0.988 $\pm$ 0.017	85.41 $\pm$ 147.93
CRD2e_K54V	23837 $\pm$ 1628	24581 $\pm$ 1306	0.969 $\pm$ 0.016	201.54 $\pm$ 121.90
CRD2e_K54Y	23456 $\pm$ 3045	24879 $\pm$ 2996	0.942 $\pm$ 0.011	368.41 $\pm$ 90.04
CRD2e_K54E_D62A	22354 $\pm$ 595	23329 $\pm$ 557	0.959 $\pm$ 0.030	229.90 $\pm$ 199.63
CRD2e_K54E_D62K	25340 $\pm$ 2252	25662 $\pm$ 2122	0.987 $\pm$ 0.012	66.02 $\pm$ 83.04
CRD2e_K54E_D62L	23559 $\pm$ 608	24065 $\pm$ 750	0.979 $\pm$ 0.019	94.17 $\pm$ 101.94
CRD2f	24639 $\pm$ 1542	25361 $\pm$ 1181	0.971 $\pm$ 0.021	207.58 $\pm$ 179.94
CRD2de	23300 $\pm$ 1938	24183 $\pm$ 1870	0.963 $\pm$ 0.019	210.06 $\pm$ 183.06
CRD2de_K54D	22298 $\pm$ 949	22956 $\pm$ 719	0.971 $\pm$ 0.012	148.10 $\pm$ 94.97
CRD2de_K54S	21582 $\pm$ 744	22400 $\pm$ 501	0.963 $\pm$ 0.018	164.07 $\pm$ 53.50
CRD2de_K54E	22901 $\pm$ 464	24198 $\pm$ 594	0.947 $\pm$ 0.013	341.12 $\pm$ 74.60
CRD2de_K54I	22798 $\pm$ 376	23902 $\pm$ 88	0.954 $\pm$ 0.018	313.70 $\pm$ 131.19
CRD2df	21272 $\pm$ 165	22639 $\pm$ 254	0.940 $\pm$ 0.004	232.40 $\pm$ 154.92
CRD2ef	24146 $\pm$ 1967	25097 $\pm$ 2028	0.962 $\pm$ 0.017	235.08 $\pm$ 230.81
CRD2(39-73)no	22343 $\pm$ 209	23489 $\pm$ 492	0.951 $\pm$ 0.012	215.44 $\pm$ 39.36
CRD2(39-73)d	26290 $\pm$ 1109	26524 $\pm$ 981	0.991 $\pm$ 0.011	42.95 $\pm$ 62.62
CRD2(39-73)e	23765 $\pm$ 2148	24621 $\pm$ 1688	0.964 $\pm$ 0.034	447.85 $\pm$ 87.44
CRD2(39-73)e_P39A	23400 $\pm$ 2209	23953 $\pm$ 2368	0.977 $\pm$ 0.017	103.85 $\pm$ 92.87
CRD2(39-73)e_P39I	24892 $\pm$ 1753	25883 $\pm$ 1624	0.961 $\pm$ 0.011	183.16 $\pm$ 92.87
CRD2(39-73)e_P39L	22699 $\pm$ 133	23398 $\pm$ 57	0.970 $\pm$ 0.004	137.40 $\pm$ 30.19
CRD2(39-73)e_P39V	25384 $\pm$ 1813	25852 $\pm$ 2016	0.983 $\pm$ 0.015	74.50 $\pm$ 64.61
CRD2(39-73)e_P39W	23523 $\pm$ 1889	24169 $\pm$ 2042	0.974 $\pm$ 0.019	95.89 $\pm$ 83.10
CRD2(39-73)e_P39x	24127 $\pm$ 1826	24842 $\pm$ 1282	0.970 $\pm$ 0.024	216.31 $\pm$ 261.48
CRD2(39-73)e_C55A	24474 $\pm$ 1943	25498 $\pm$ 1605	0.959 $\pm$ 0.022	268.11 $\pm$ 239.23
CRD2(39-73)e_C55V	23251 $\pm$ 2839	24533 $\pm$ 2756	0.947 $\pm$ 0.009	359.40 $\pm$ 45.79
CRD2(39-73)e_C61S	23123 $\pm$ 365	24232 $\pm$ 557	0.954 $\pm$ 0.015	326.65 $\pm$ 145.24
CRD2(39-73)e_N72A	23896 $\pm$ 2472	24779 $\pm$ 2119	0.963 $\pm$ 0.033	234.76 $\pm$ 227.51
CRD2(39-73)e_N72G	22300 $\pm$ 454	23785 $\pm$ 470	0.938 $\pm$ 0.006	374.15 $\pm$ 48.42
CRD2(39-73)de	21193 $\pm$ 347	22760 $\pm$ 453	0.931 $\pm$ 0.012	425.29 $\pm$ 104.52
CRD3no	24141 $\pm$ 849	24272 $\pm$ 857	0.995 $\pm$ 0.005	34.82 $\pm$ 41.84
CRD3(89-97)no	22657 $\pm$ 1922	22871 $\pm$ 1645	0.990 $\pm$ 0.017	0.45 $\pm$ 0.54
HVEMno	26750 $\pm$ 223	28726 $\pm$ 229	0.931 $\pm$ 0.011	505.543 $\pm$ 166.06
HVEMall	25892 $\pm$ 2028	27787 $\pm$ 1332	0.931 $\pm$ 0.030	496.38 $\pm$ 349.50

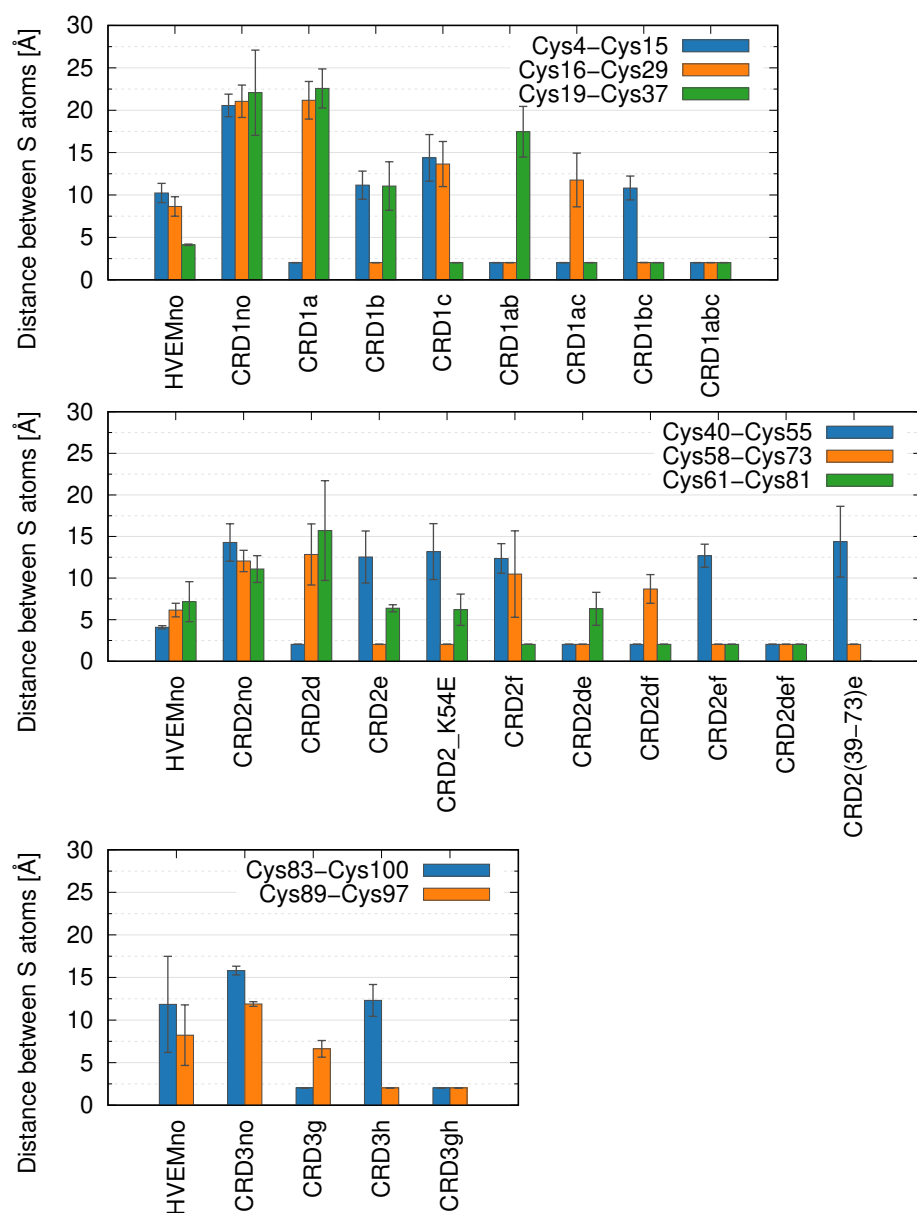


Figure S1: Distance between S atoms in disulfide bonds averaged for 3 trajectories for the second halves of the simulations (500-1000ns) for CRD1, CRD2 and CRD3 without the LIGHT trimer.

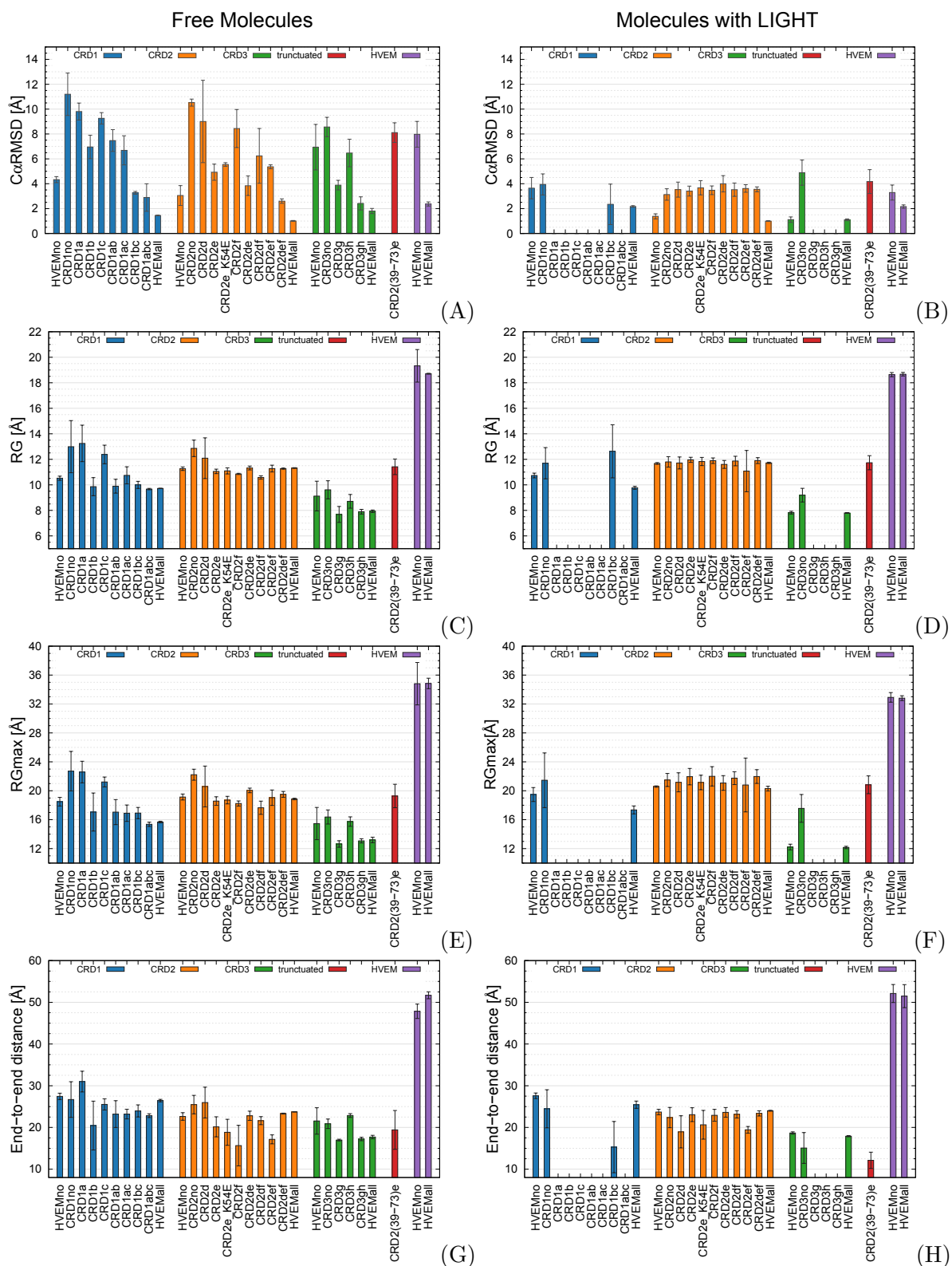


Figure S2: (A) RMSD, (C) RG, (E) RGmax, (G) End-to-end distance calculated from simulations of free domains and (B) RMSD, (D) RG, (F) RGmax and (H) End-to-end distance values calculated from simulations with LIGHT, averaged over the second halves of the 3 trajectories.

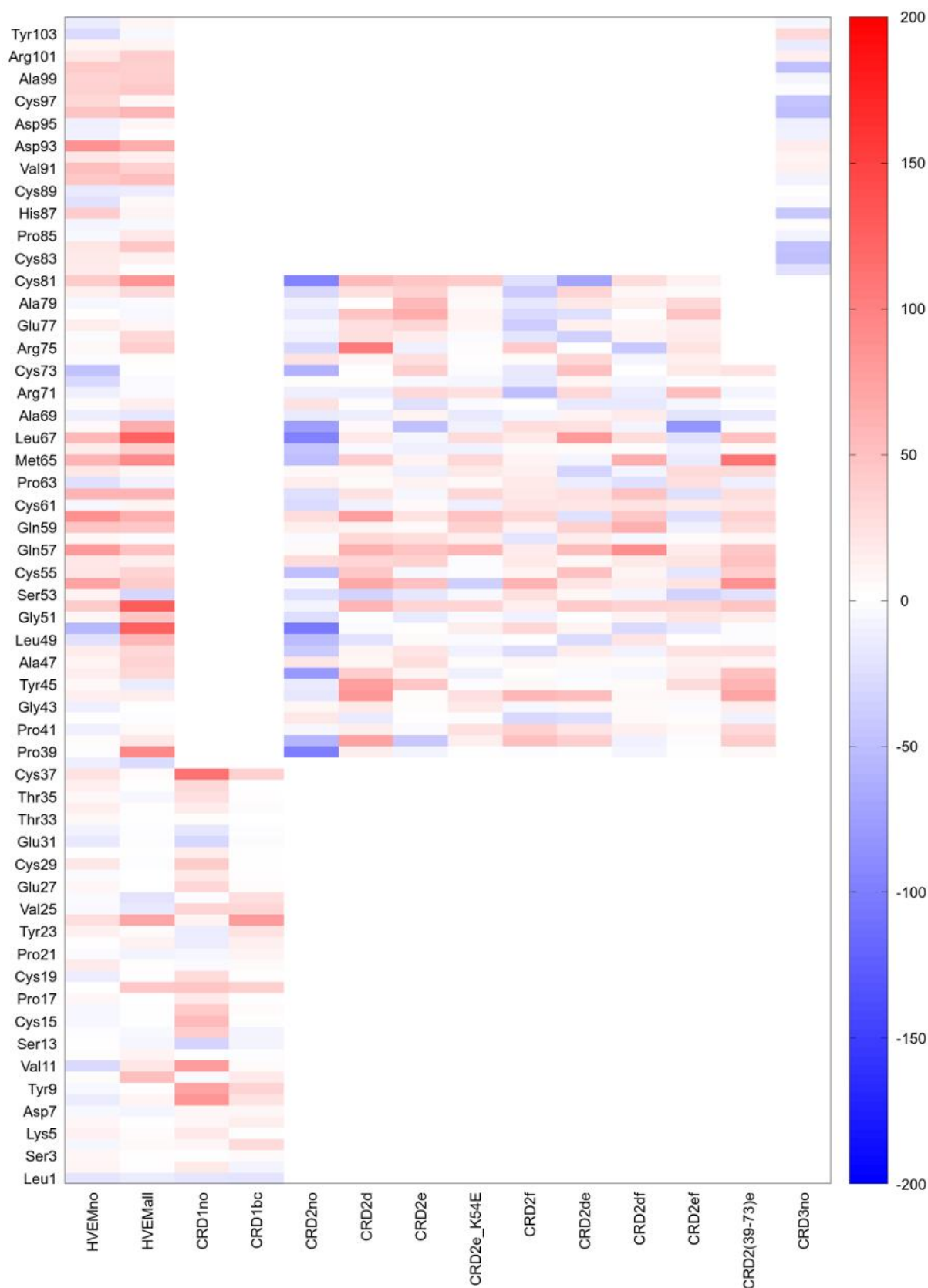


Figure S4: Heatmap of per-residue $\Delta SASA = SASA_{\text{free}} - SASA_{\text{complex}}$ (right-hand bar [$\text{\AA}^2$]) calculated for all combinations of disulfide bonds present and absent in the simulations for the full HVEM molecule, its domains (CRD1-3) and its variant, and CRD2e.K54E, and fragment (CRD(39-73)e), averaged over the second halves of the 3 trajectories.

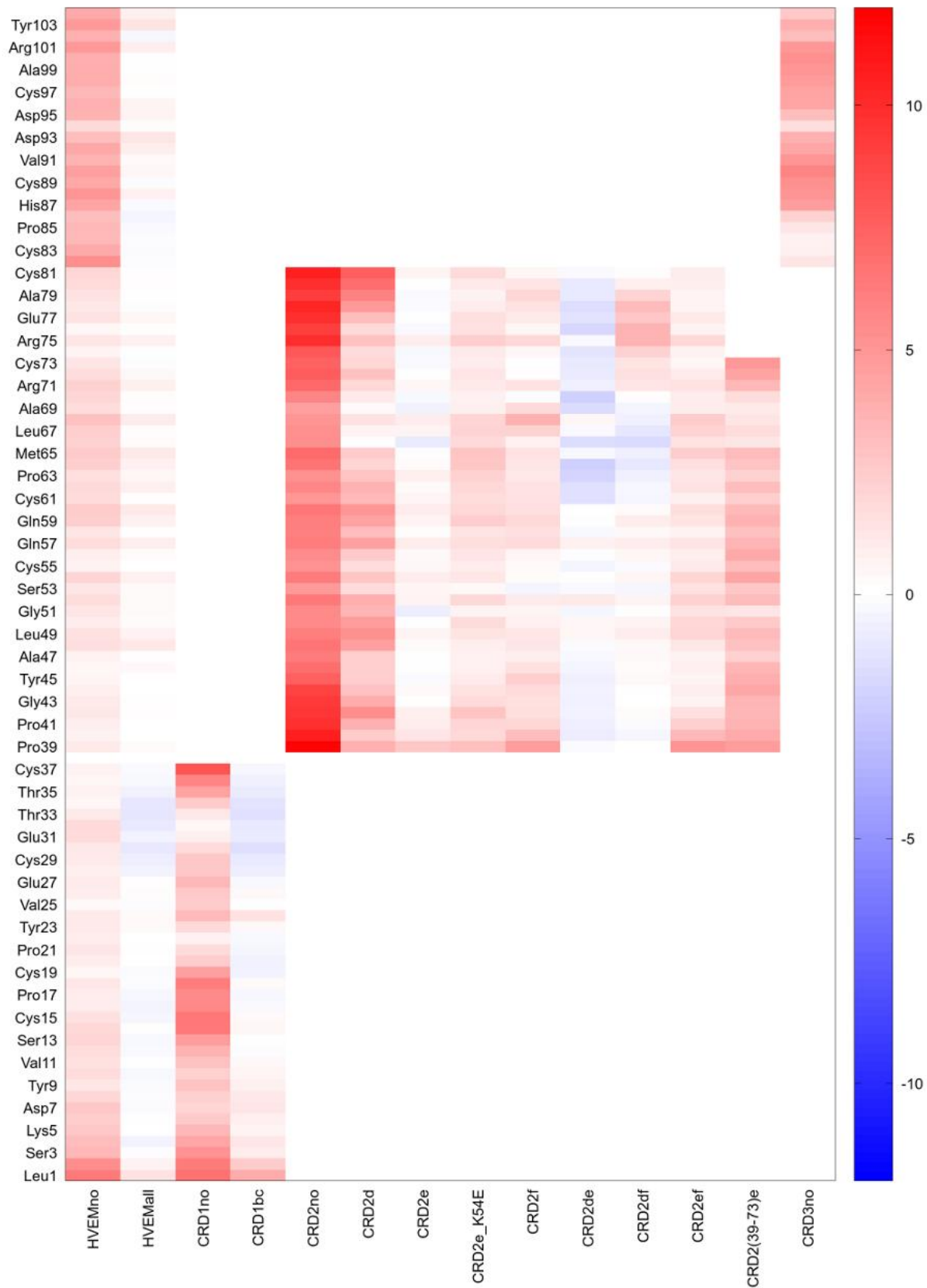


Figure S5: Heatmap of per-residue $\Delta RMSF = RMSF_{\text{free}} - RMSF_{\text{complex}}$ (right-hand bar [Å]) calculated for HVEM with and without disulfide bonds, and its selected variants forming stable complexes with LIGHT, averaged over the second halves of the 3 trajectories.

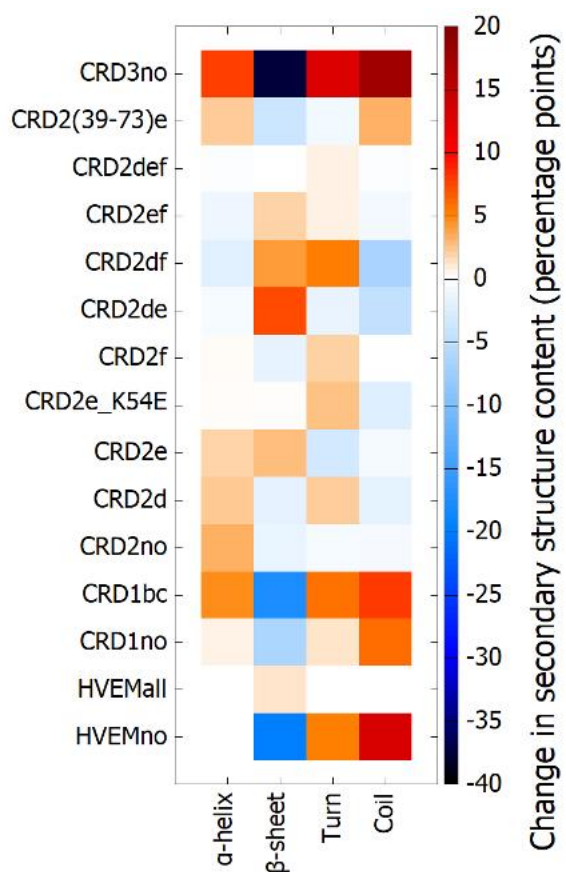


Figure S6: Heatmap of the change in the content of secondary structures expressed in percentage points calculated for HVEM with and without disulfide bonds, and its selected variants forming stable complexes with LIGHT, averaged over the second halves of the 3 trajectories

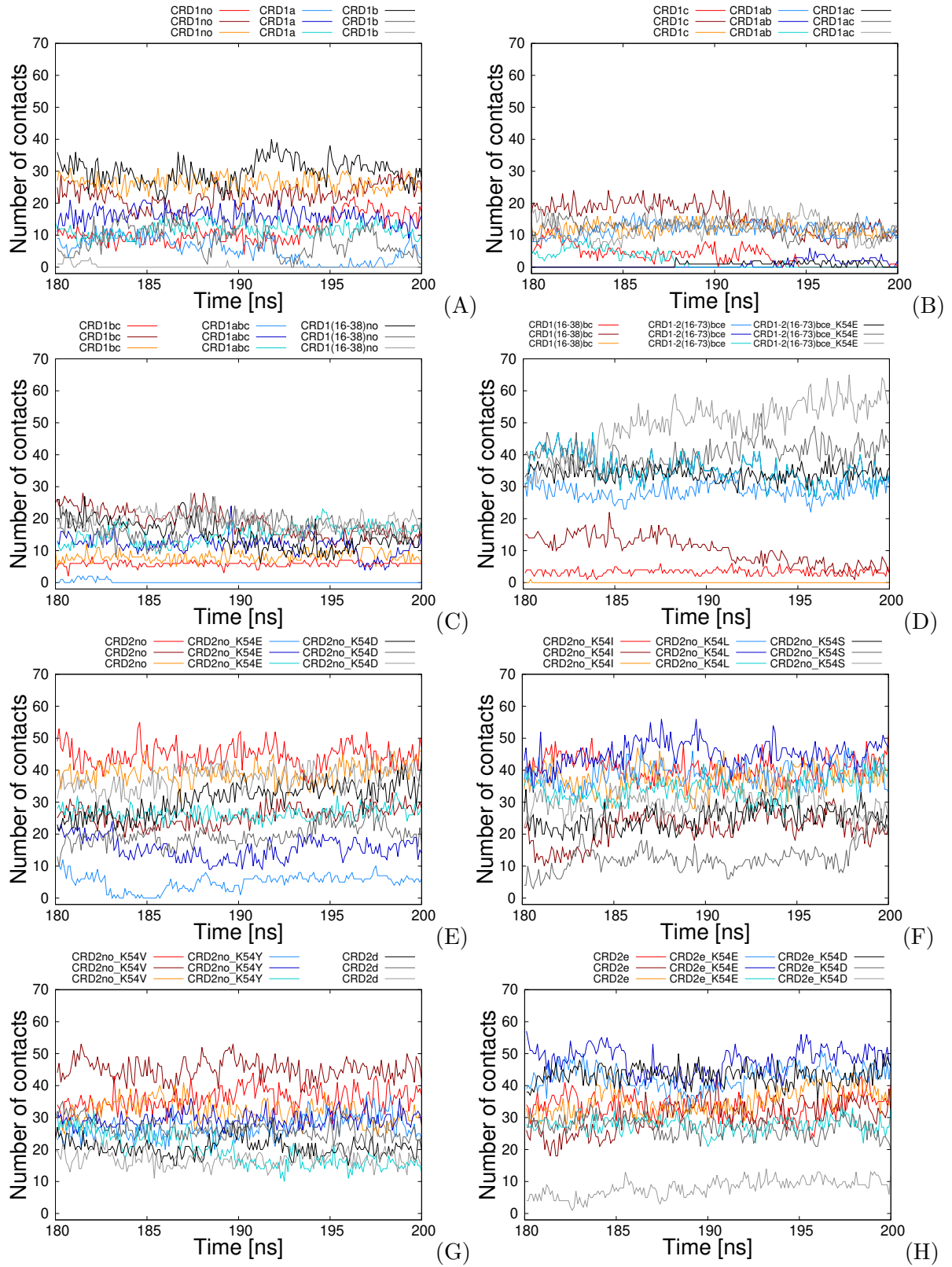


Figure S7: Number of native contacts between HVEM variant and LIGHT trimer as a function of time for last 20ns for (A): CRD1no (red), CRD1a (blue), and CRD1b (grey); (B): CRD1c (red), CRD1ab (blue), and CRD1ac (grey); (C): CRD1bc (red), CRD1abc (blue), and CRD1(16-38)no (grey); (D): CRD1(16-38)bc (red), CRD1-2(16-73)bce (blue), and CRD1-2(16-73)bce_K54E (grey); (E): CRD2no (red), CRD2no_K54E (blue), CRD2no_K54D (grey); (F): CRD2no_K54I (red), CRD2no_K54L (blue), and CRD2no_K54S (grey); (G): CRD2no_K54V (red), CRD2no_K54Y (blue), and CRD2d (grey); (H): CRD2e (red), CRD2e_K54E (blue), and CRD2e_K54D (grey). Different trajectories are depicted as various color tone.

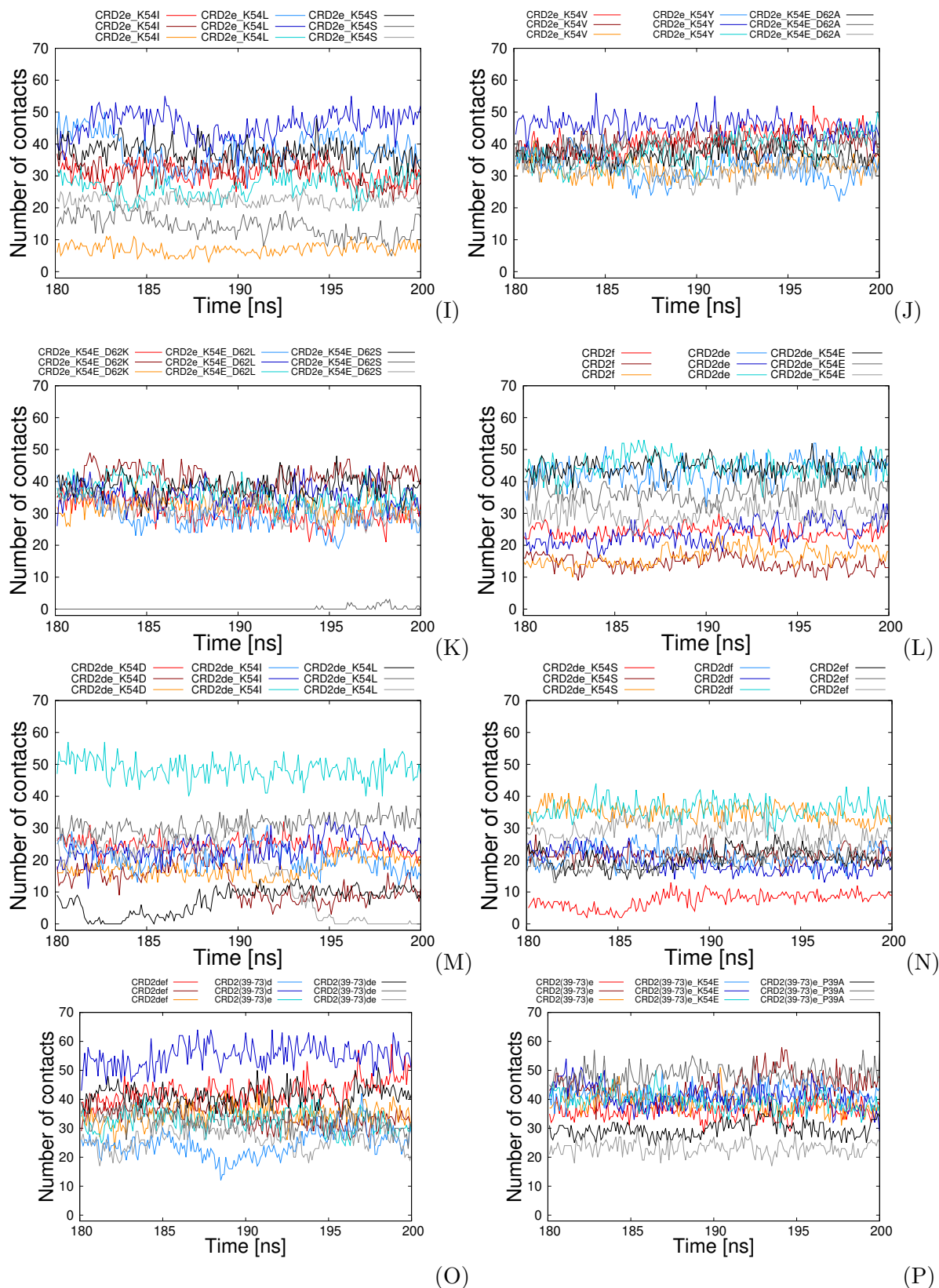


Figure S7: continued: (I): CRD2e_K54I (red), CRD2e_K54L (blue), and CRD2e_K54S (grey); (J) CRD2e_K54V (red), CRD2e_K54Y (blue), and CRD2e_K54E_D62A (grey); (K): CRD2e_K54E_D62K (red), CRD2e_K54E_D62L (blue), and CRD2e_K54E_D62S (grey); (L): CRD2f (red) and CRD2de_K54E (grey); (M): CRD2de_K54D (red), CRD2de_K54I (blue), and CRD2de_K54L (grey); (N): CRD2de_K54S (red), CRD2df (blue), and CRD2ef (grey); (O): CRD2def (red), CRD2(39-73)d (blue), and CRD2(39-73)de (grey); (P): CRD2(39-73)e (red), CRD2(39-73)e_K54E (blue), and CRD2(39-73)e_P39A (grey). Different trajectories are depicted as various color tone.

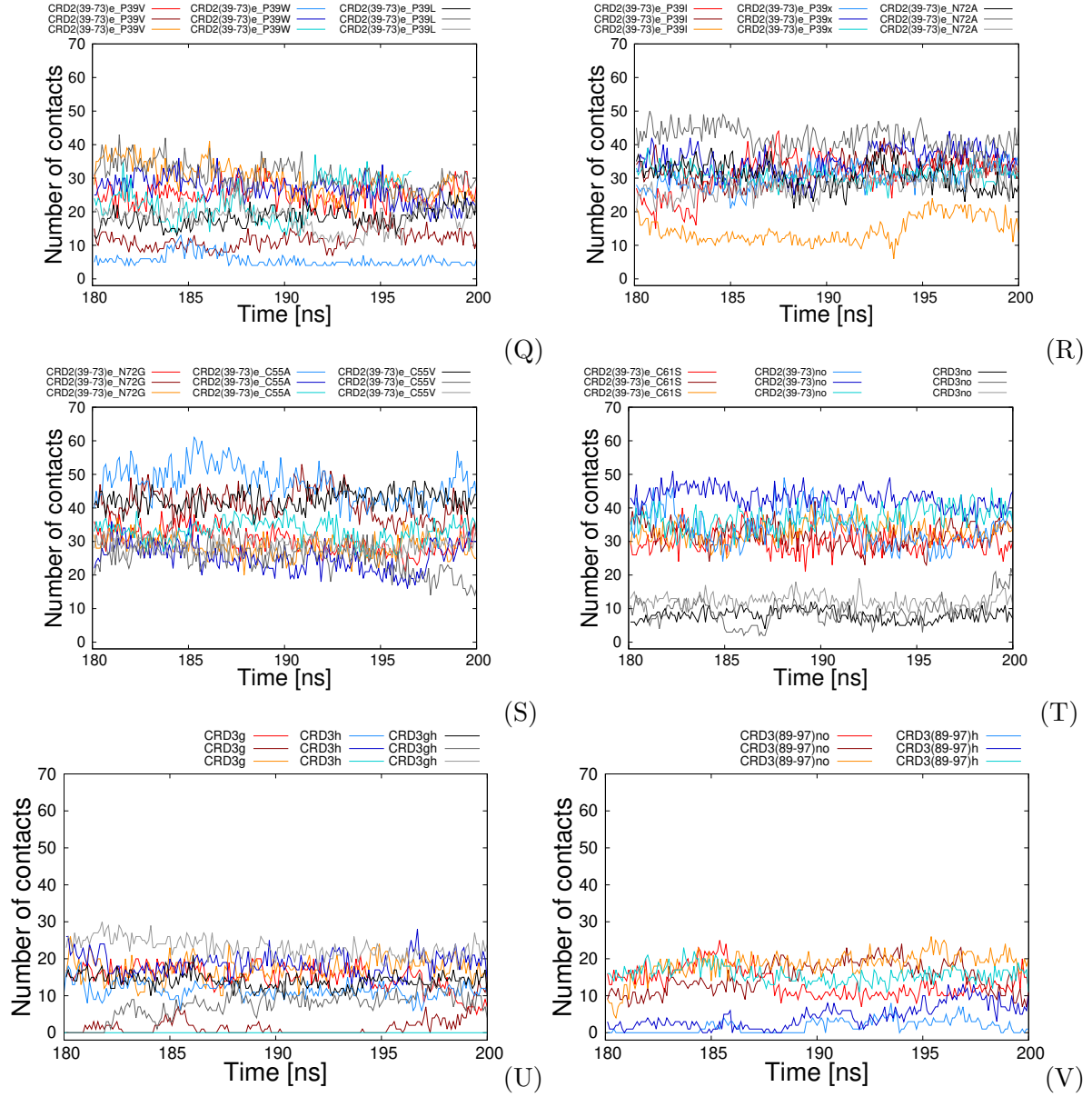


Figure S7: continued (Q): CRD2(39-73)e_P39V(red), CRD2(39-73)e_P39W (blue), and CRD2(39-73)e_P39L (grey); (R): CRD2(39-73)e_P39I (red), CRD2(39-73)e_P39x(blue), and CRD2(39-73)e_N72A (grey); (S): CRD2(39-73)e_N72G(red), CRD2(39-73)e_C55A (blue), and CRD2(39-73)e_C55V (grey); (T): CRD2(39-73)e_C61S (red), CRD2(39-73)no (blue) and CRD3no (grey); (U): CRD3g (red), CRD3h (blue) and CRD3gh(grey); (V): CRD3(89-97)no (red), and CRD3(89-97)h (blue) . Different trajectories are depicted as various color tone.

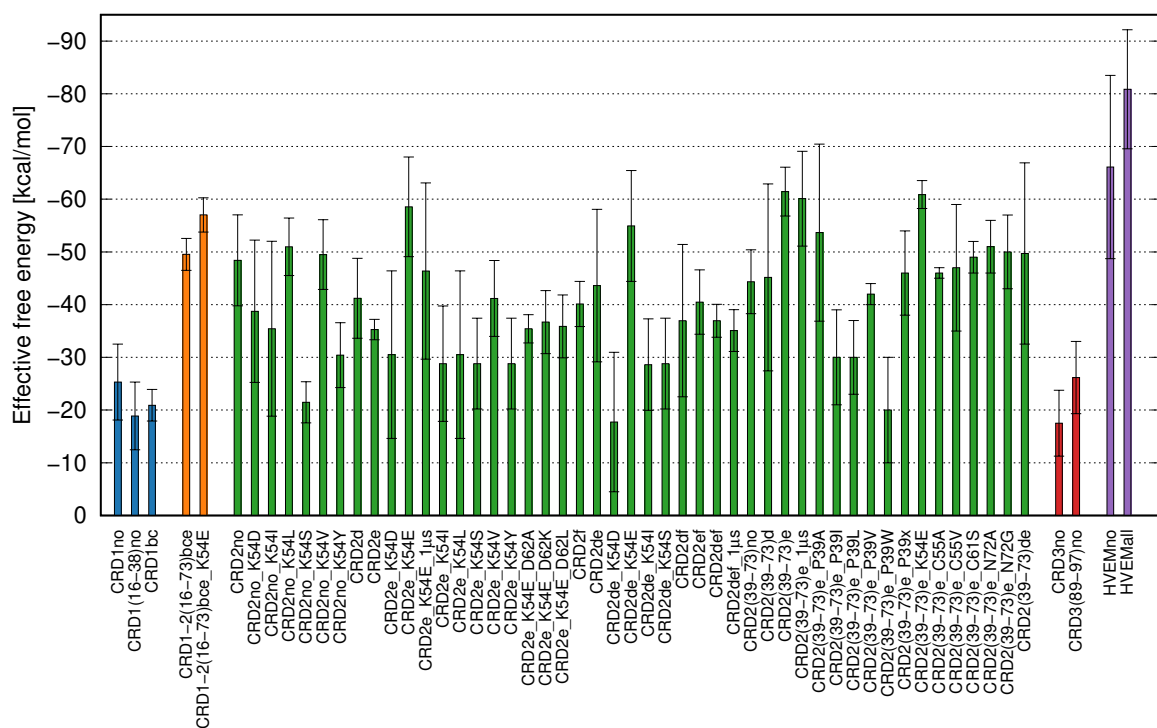
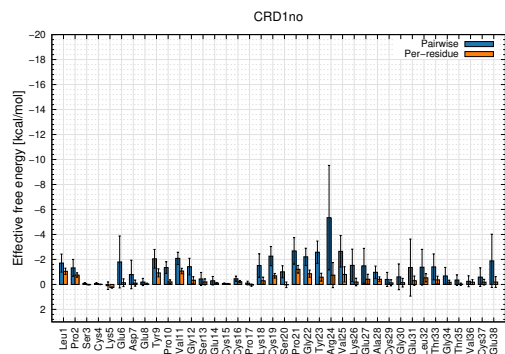
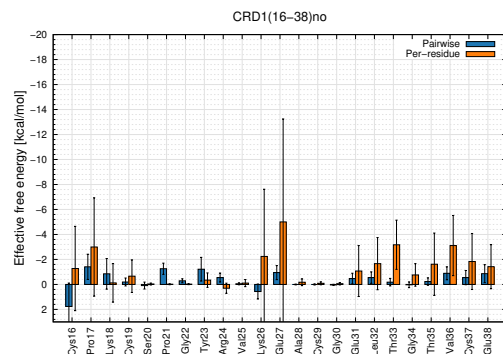


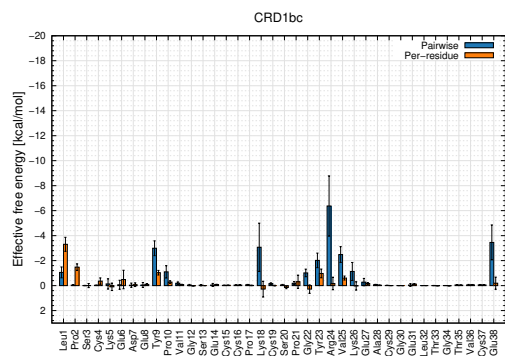
Figure S8: Bar plot of the effective free energies of the various stable variants of the HVEM molecule interacting with the LIGHT trimer.



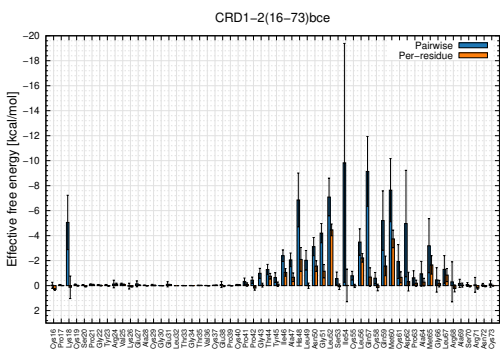
(A)



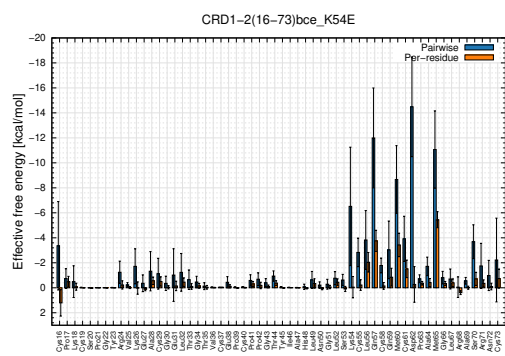
(B)



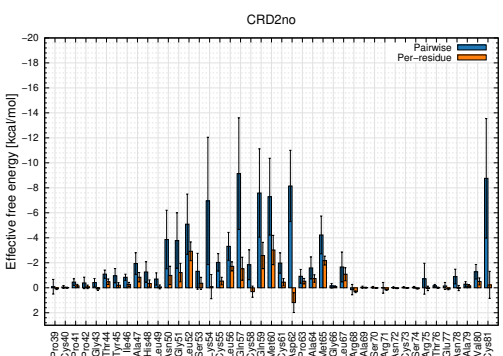
(C)



(D)

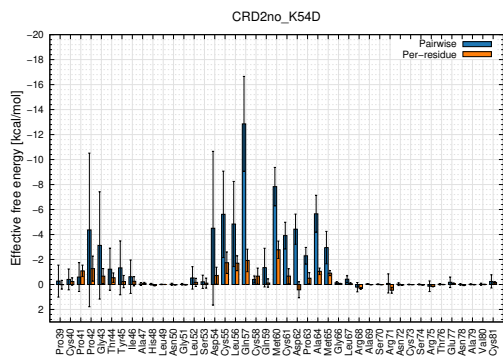


(E)

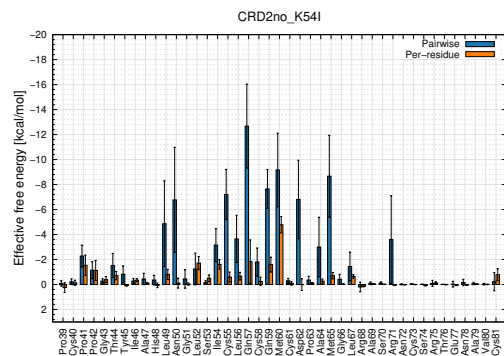


(F)

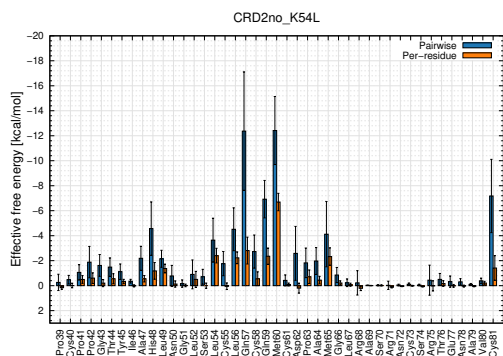
Figure S9: MM-GBSA effective binding energy decomposition results for (A) CRD1no, (B) CRD1(16-38)no, (C) CRD1bc, (D) CRD1-2(16-73)bce, (E) CRD1-2(16-73)bce_K54E, (F) CRD2no variants



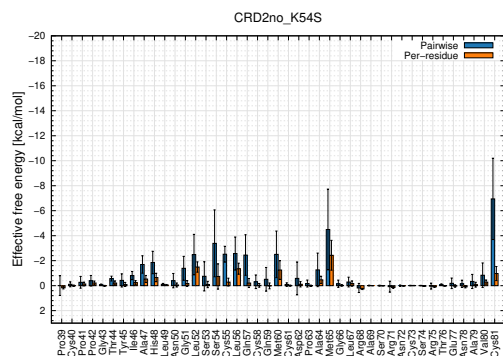
(G)



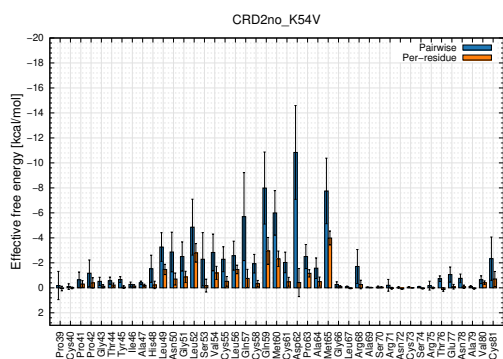
(H)



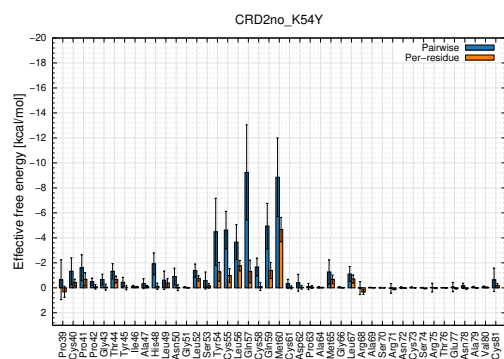
(I)



(J)

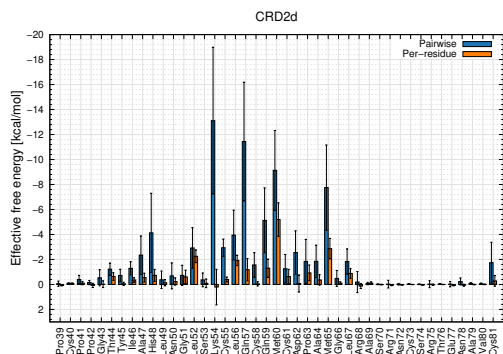


(K)

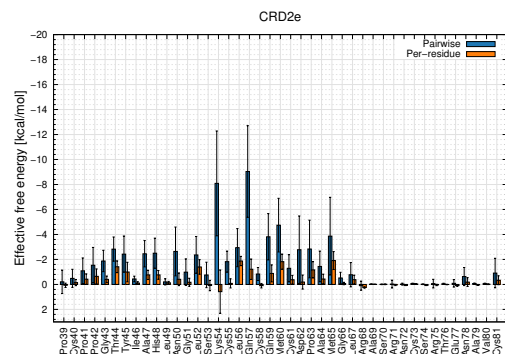


(L)

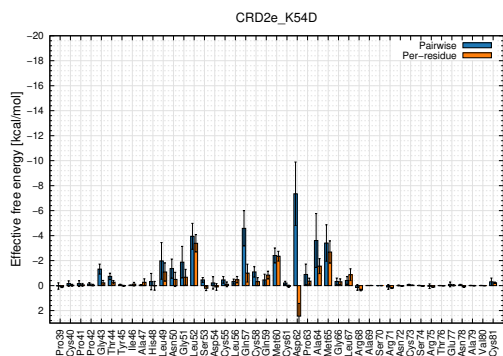
Figure S9: continued: MM-GBSA effective binding energy decomposition results for (G) CRD2no_K54D, (H) CRD2no_K54I, (I) CRD2no_K54L, (J) CRD2no_K54S, (K) CRD2no_K54V, (L) CRD2no_K54Y variants



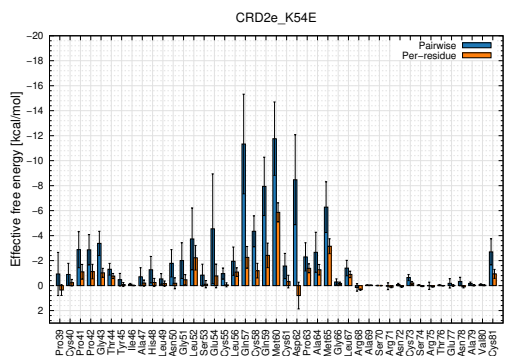
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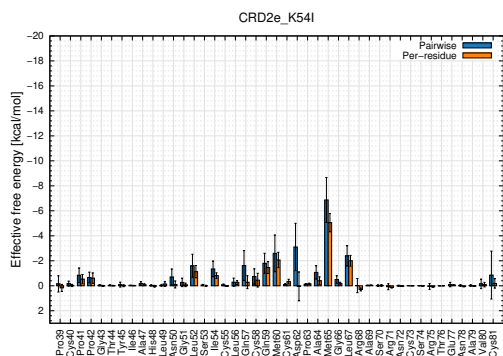
(N)



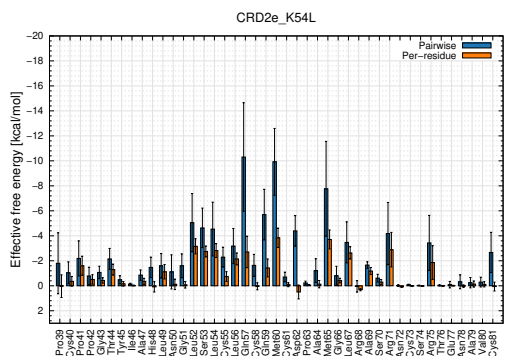
(O)



(P)

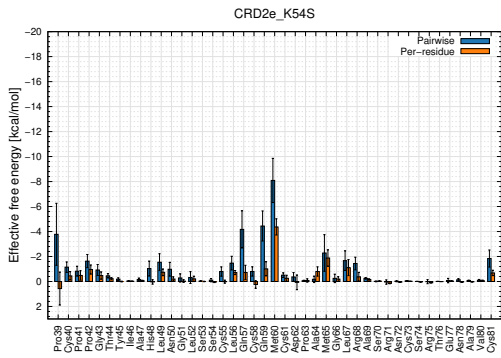


(Q)

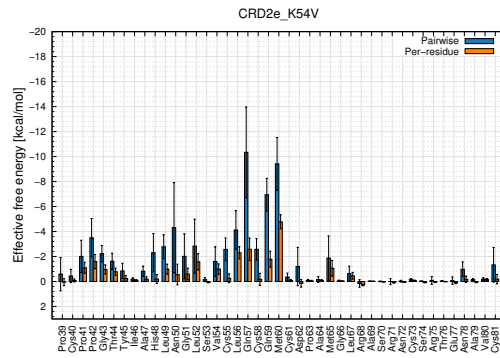


(R)

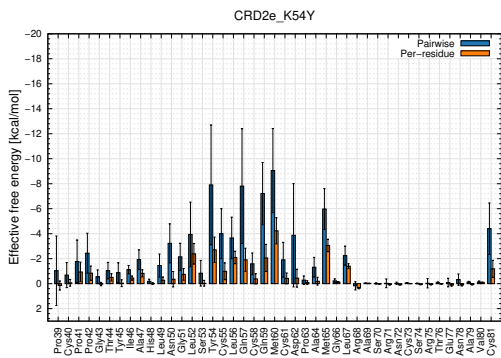
Figure S9: continued: MM-GBSA effective binding energy decomposition results for: (M) CRD2d, (N) CRD2e, (O) CRD2e_K54D, (P) CRD2e_K54E, (Q) CRD2e_K54I, (R) CRD2e_K54L variants



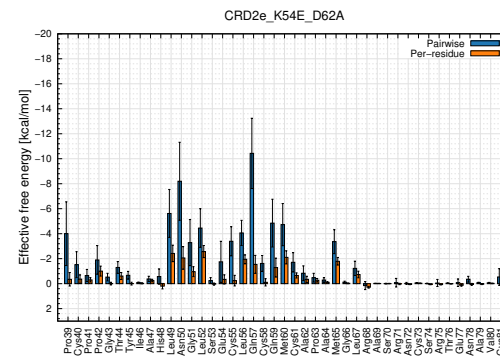
(S)



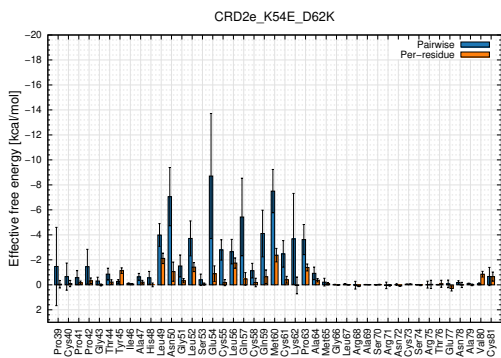
(T)



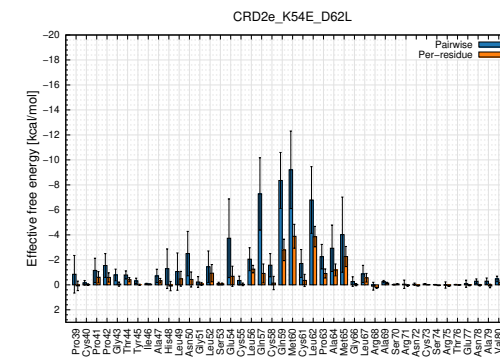
(U)



(V)

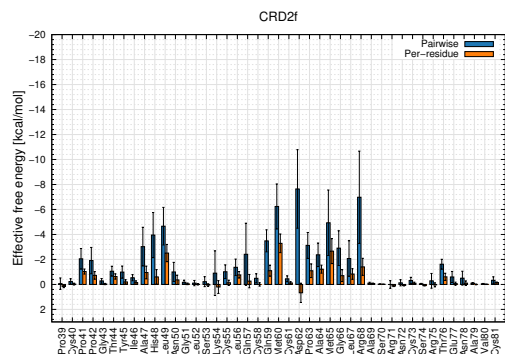


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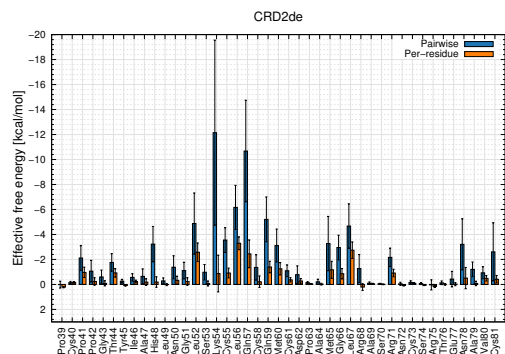


(X)

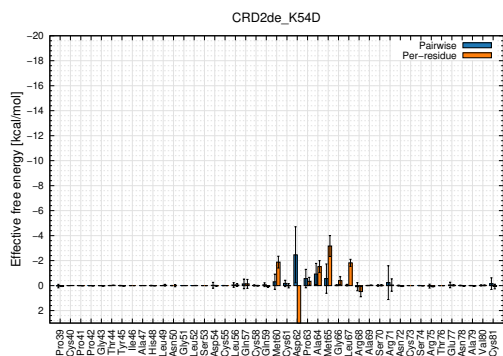
Figure S9: continued: MM-GBSA effective binding energy decomposition results for: (S) CRD2e_K54S, (T) CRD2e_K54V, (U) CRD2e_K54Y, (V) CRD2e_K54E_D62A, (W) CRD2e_K54E_D62K, (X) CRD2e_K54E_D62L variants



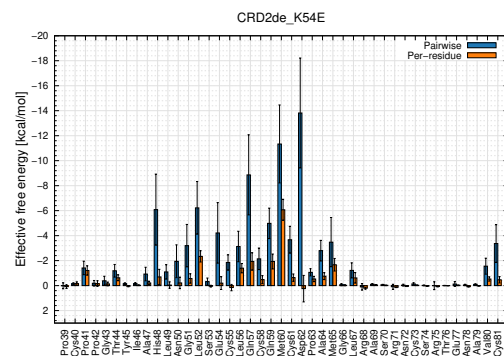
(Y)



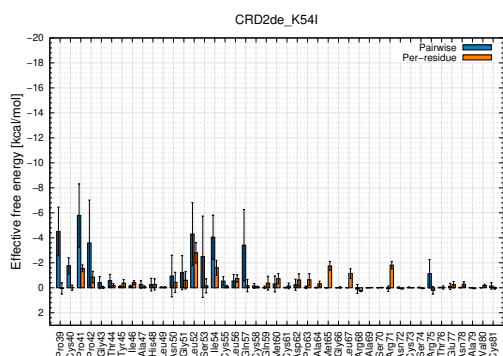
(Z)



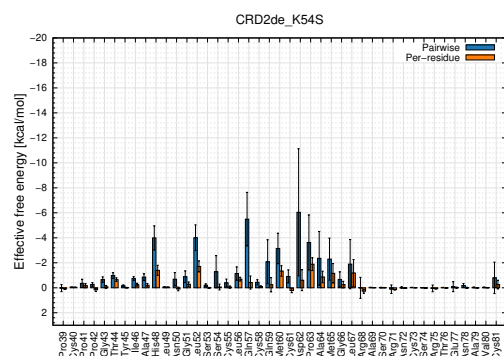
(AA)



(AB)

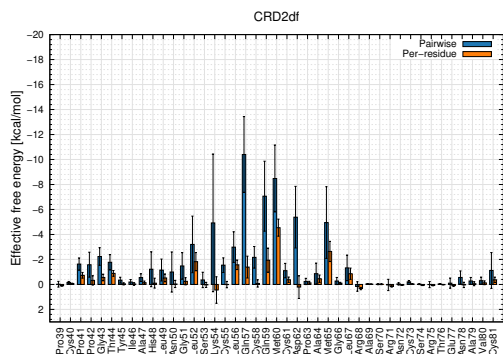


(AC)

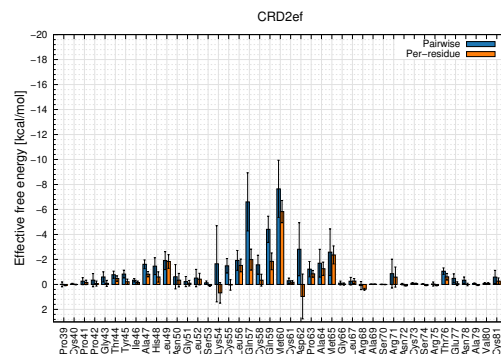


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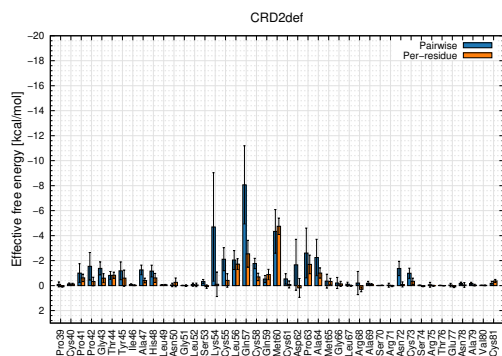
Figure S9: continued: MM-GBSA effective binding energy decomposition results for: (Y) CRD2f, (Z) CRD2de, (AA) CRD2de_K54D, (AB) CRD2de_K54E, (AC) CRD2de_K54I, (AD) CRD2de_K54S variants



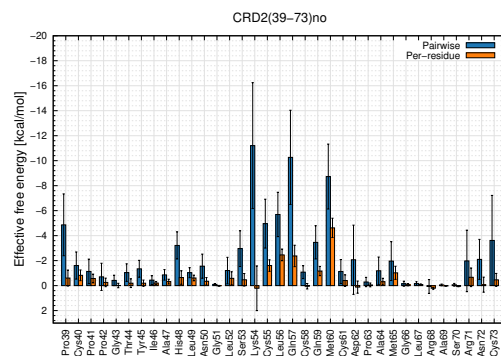
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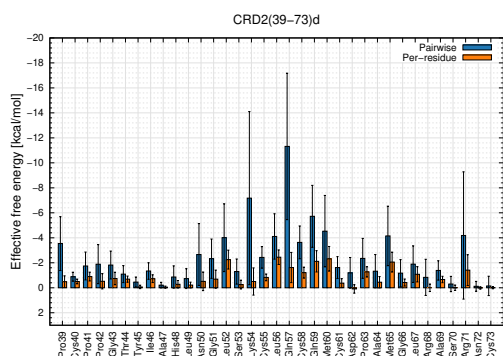
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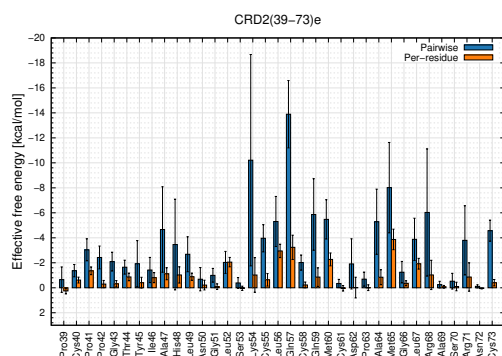
(AG)



(AH)

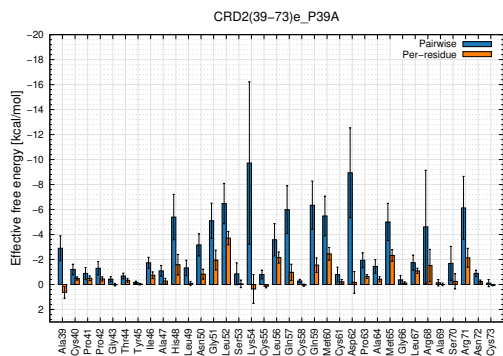


(AI)

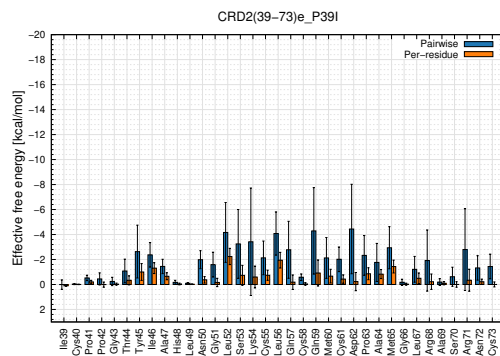


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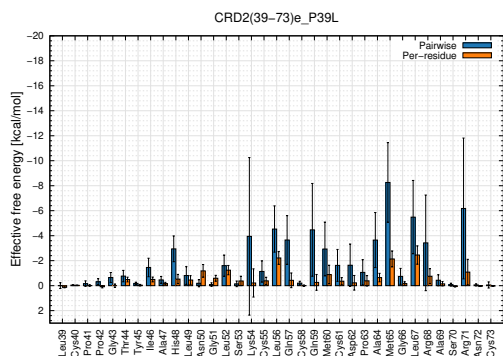
Figure S9: continued: MM-GBSA effective binding energy decomposition results for: (AE) CRD2df, (AF) CRD2ef, (AG) CRD2def, (AH) CRD2(39-73)no, (AI) CRD2(39-73)d, (AJ) CRD2(39-73)e variants



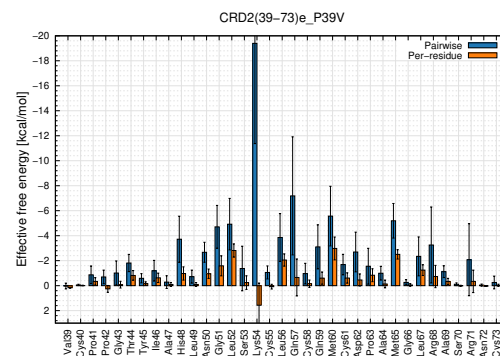
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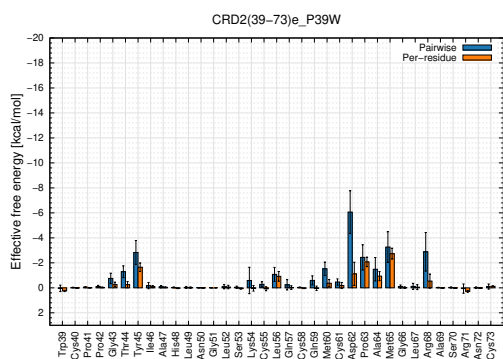
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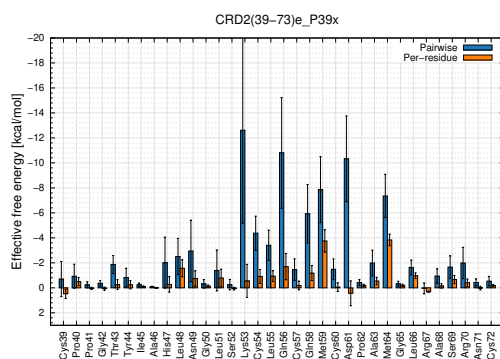
(AM)



(AN)

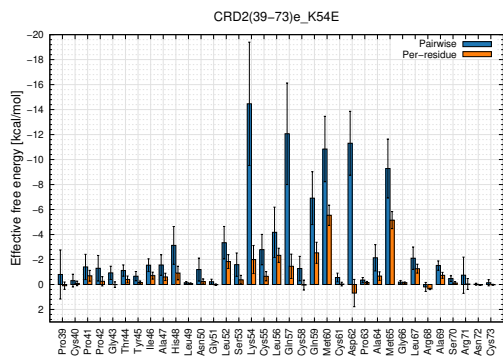


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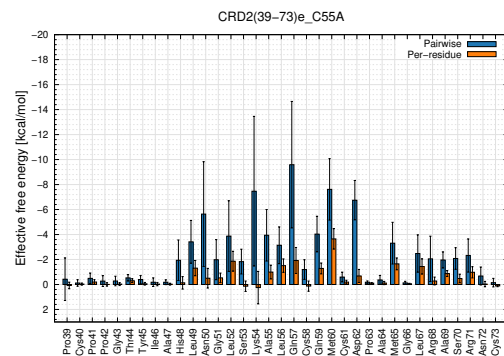


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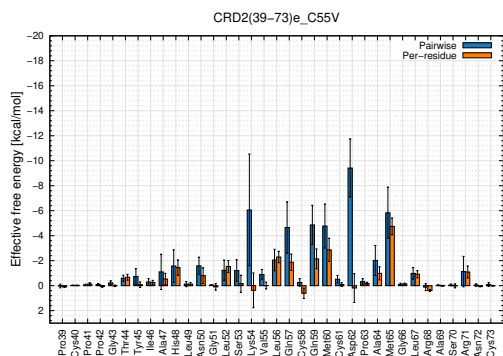
Figure S9: continued: MM-GBSA effective binding energy decomposition results for: (AK) CRD2(39-73)e_P39A, (AL) CRD2(39-73)e_P39I, (AM) CRD2(39-73)e_P39L, (AN) CRD2(39-73)e_P39V, (AO) CRD2(39-73)e_P39W, (AP) CRD2(39-73)e_P39x variants



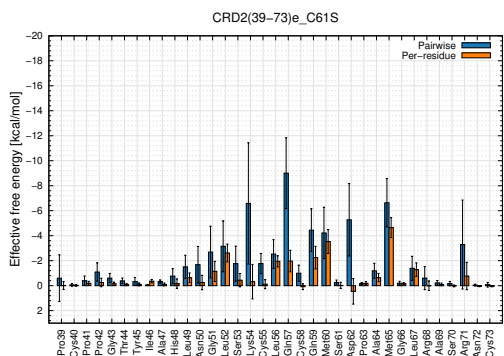
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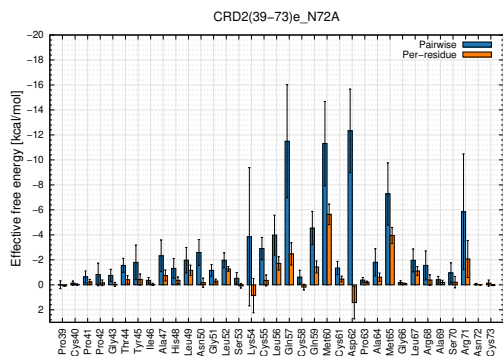
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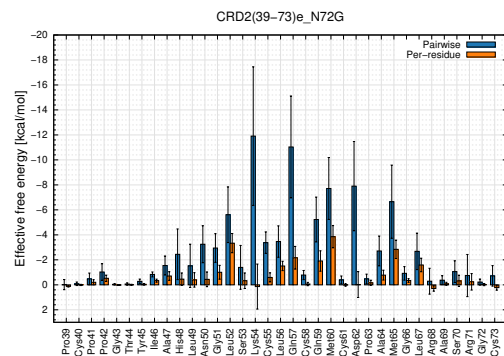
(AS)



(AT)

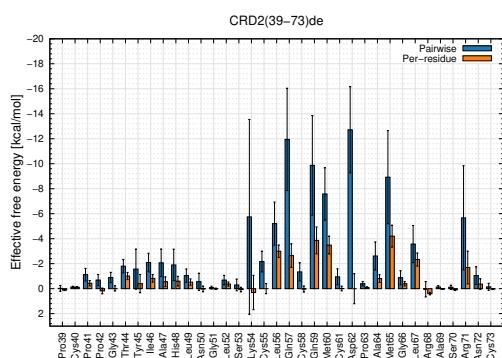


(AU)

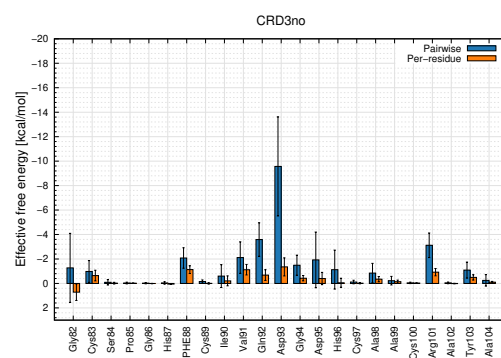


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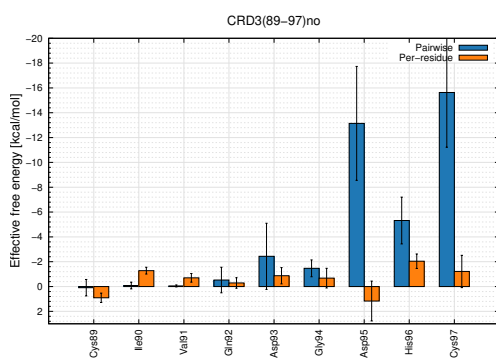
Figure S9: continued: MM-GBSA effective binding energy decomposition results for: (AQ) CRD2(39-73)e_K54E, (AR) CRD2(39-73)e_C55A, (AS) CRD2(39-73)e_C55V, (AT) CRD2(39-73)e_C61S, (AU) CRD2(39-73)e_N72A, (AV) CRD2(39-73)e_N72G variants



(AW)

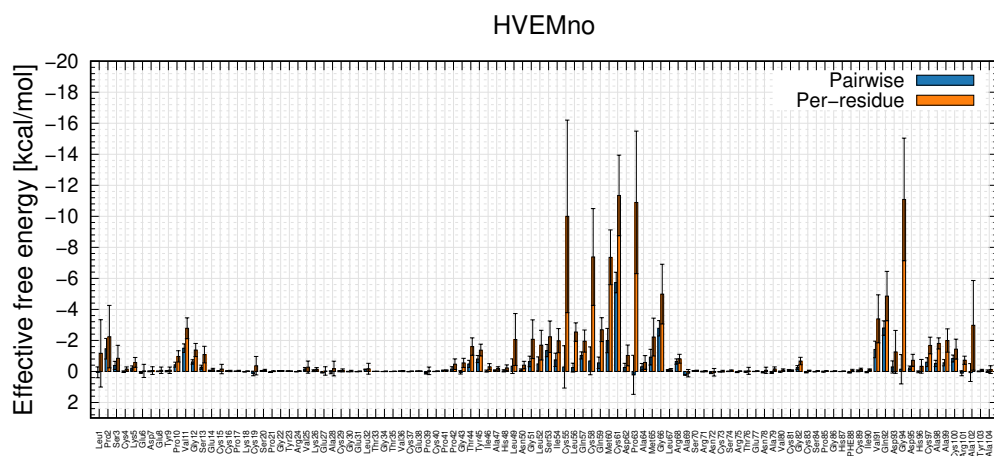


(AX)

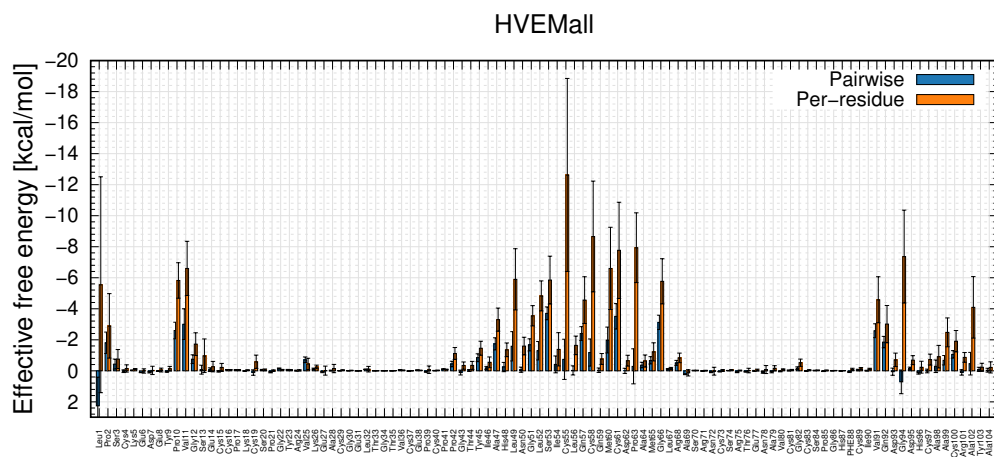


(AY)

Figure S9: continued: MM-GBSA effective binding energy decomposition results for: (AW) CRD2(39-73)de, (AX) CRD3no, (AY) CRD3(39-73)no



(AZ)



(AAA)

Figure S9: continued: MM-GBSA effective binding energy decomposition results for: (AZ) HVEMno variant, (AAA) HVEMall

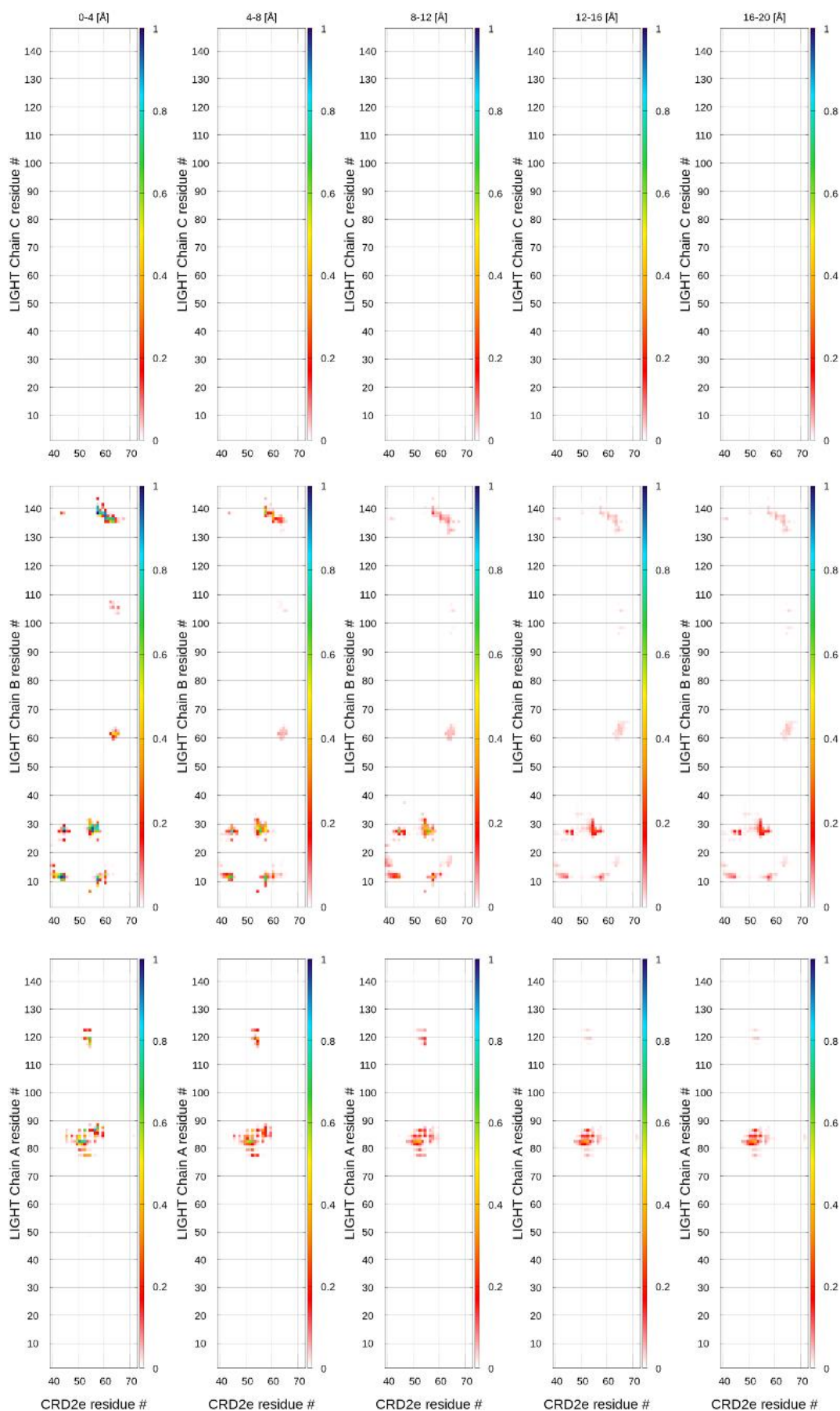


Figure S10: Contact heatmap averaged over 25 SMD simulations for HVEM variant CRD2e with the LIGHT trimer. The values are normalized in the range of increasing extension (relative difference of the distance between centers of mass of HVEM variant and LIGHT trimer) averaged for 4 Å.

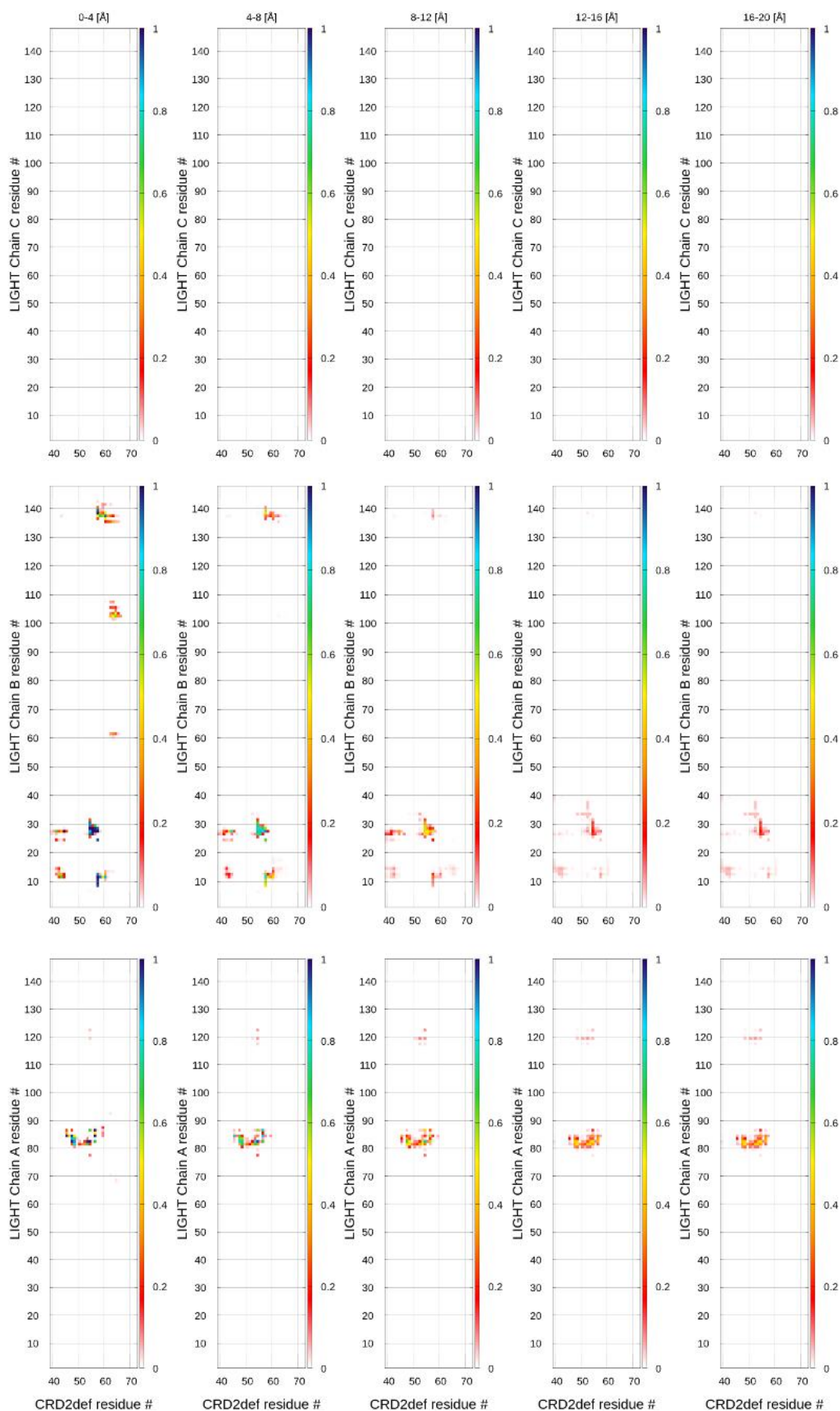


Figure S10: continued: Contact heatmap averaged over 25 SMD simulations for HVEM variant CRD2def with the LIGHT trimer. The values are normalized in the range of increasing extension (relative difference of the distance between centers of mass of HVEM variant and LIGHT trimer) averaged for 4 Å.

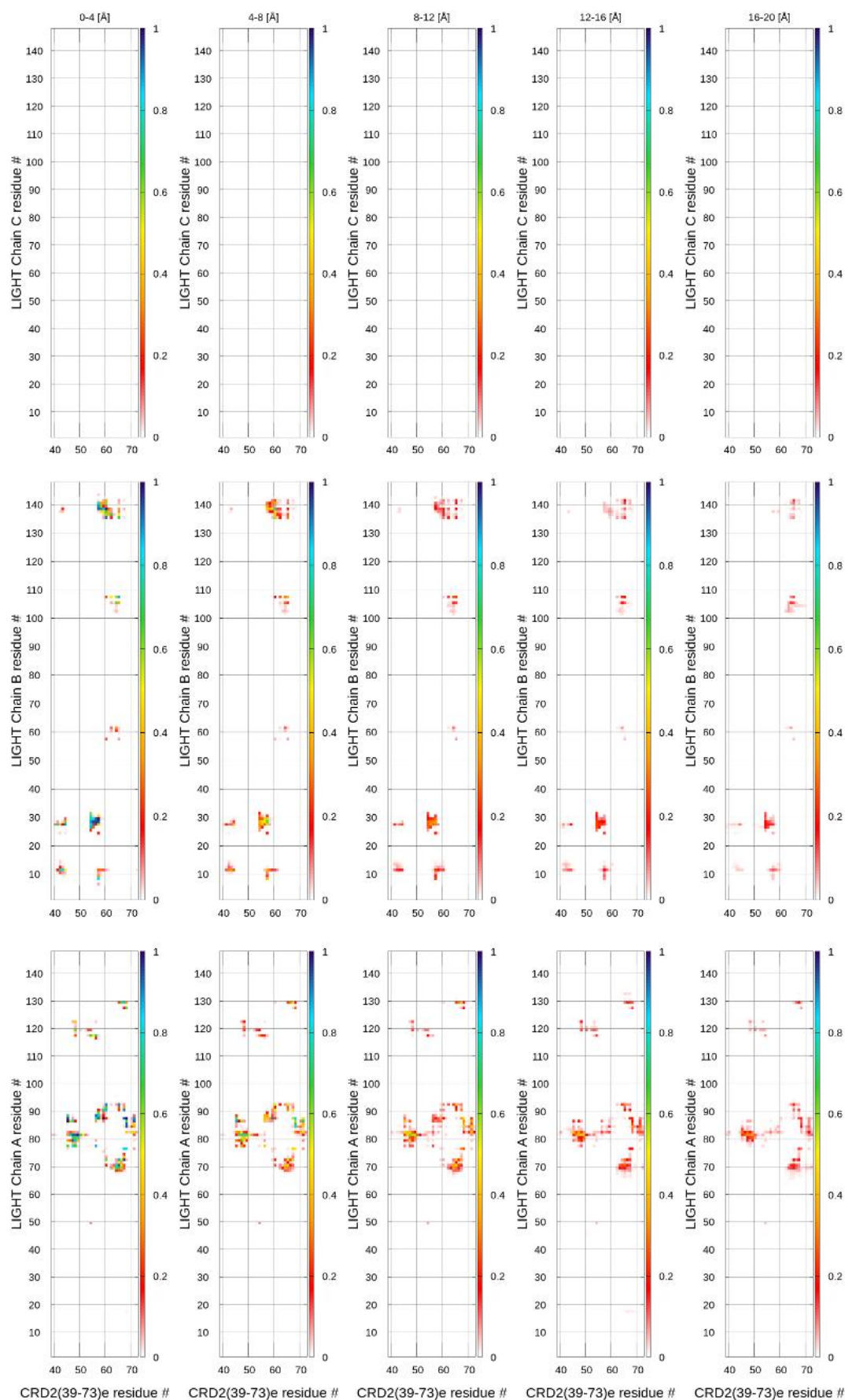


Figure S10: continued: Contact heatmap averaged over 25 SMD simulations for HVEM variant CRD2(39-73)e with the LIGHT trimer. The values are normalized in the range of increasing extension (relative difference of the distance between centers of mass of HVEM variant and LIGHT trimer) averaged for 4 Å.

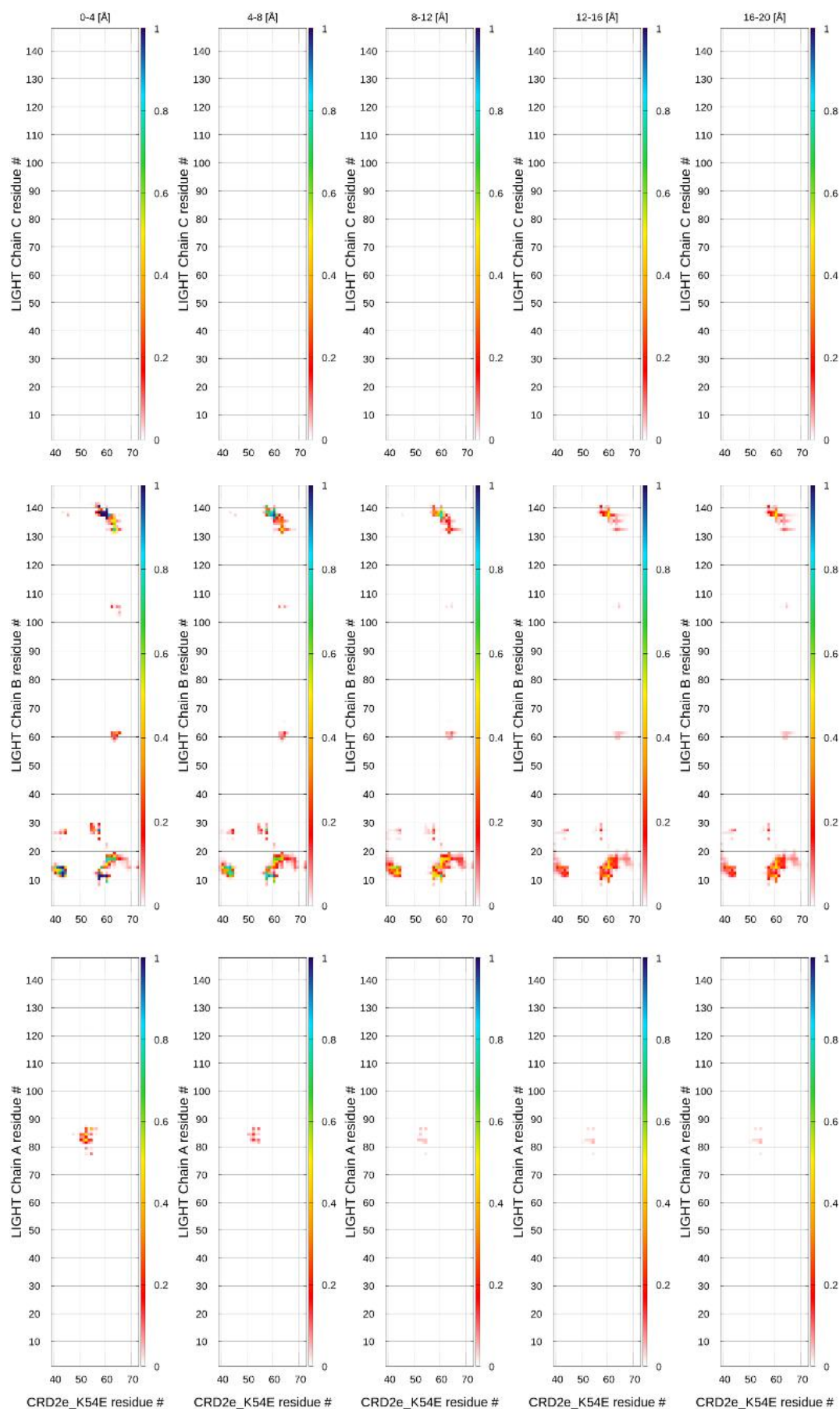


Figure S10: continued: Contact heatmap averaged over 25 SMD simulations for HVEM variant CRD2e_K54E with the LIGHT trimer. The values are normalized in the range of increasing extension (relative difference of the distance between centers of mass of HVEM variant and LIGHT trimer) averaged for 4 Å.

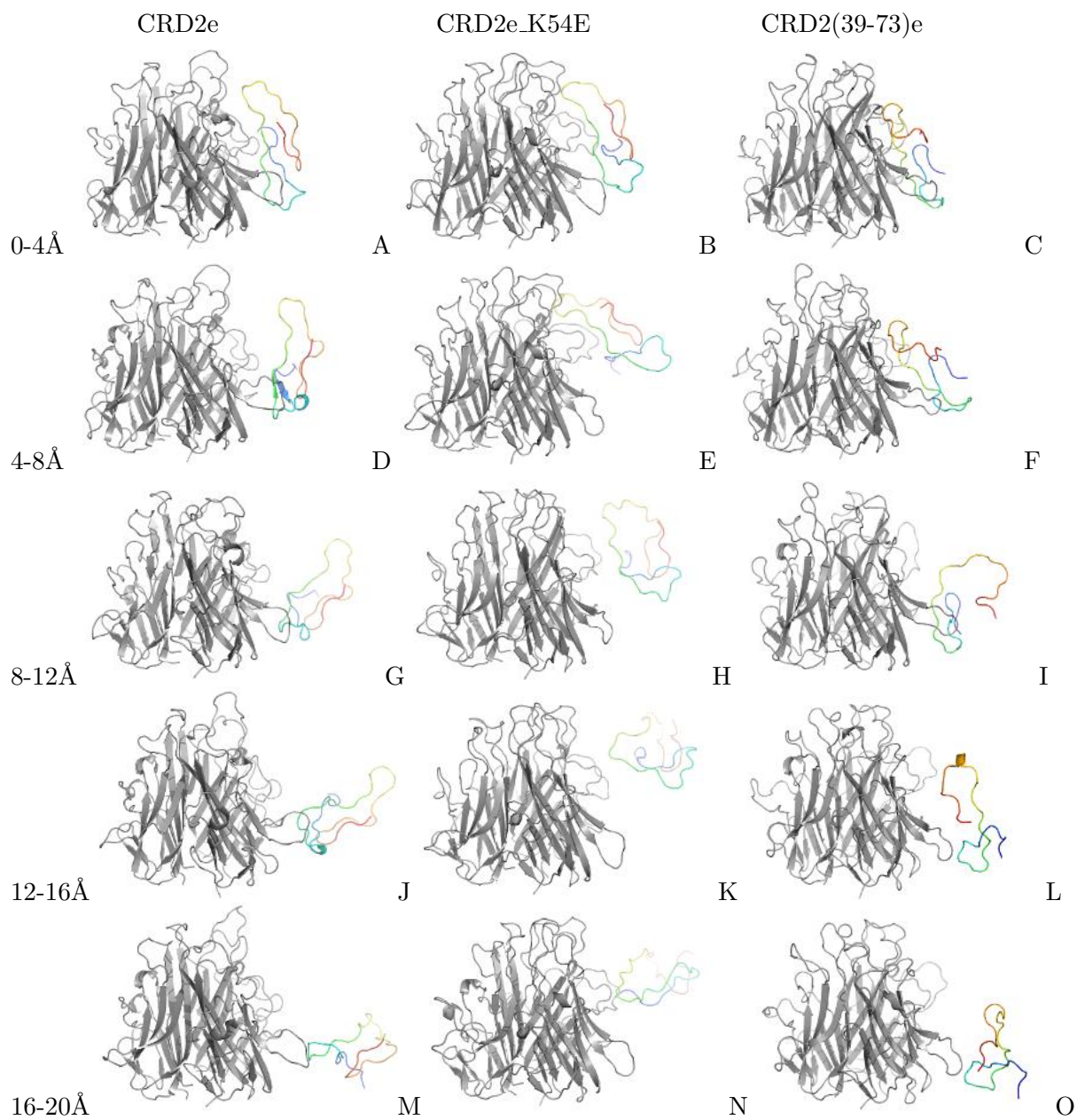


Figure S10: Representative structures upon extending (relative difference of the distance between centers of mass of HVEM variant and LIGHT trimer) averaged for 4 Å.

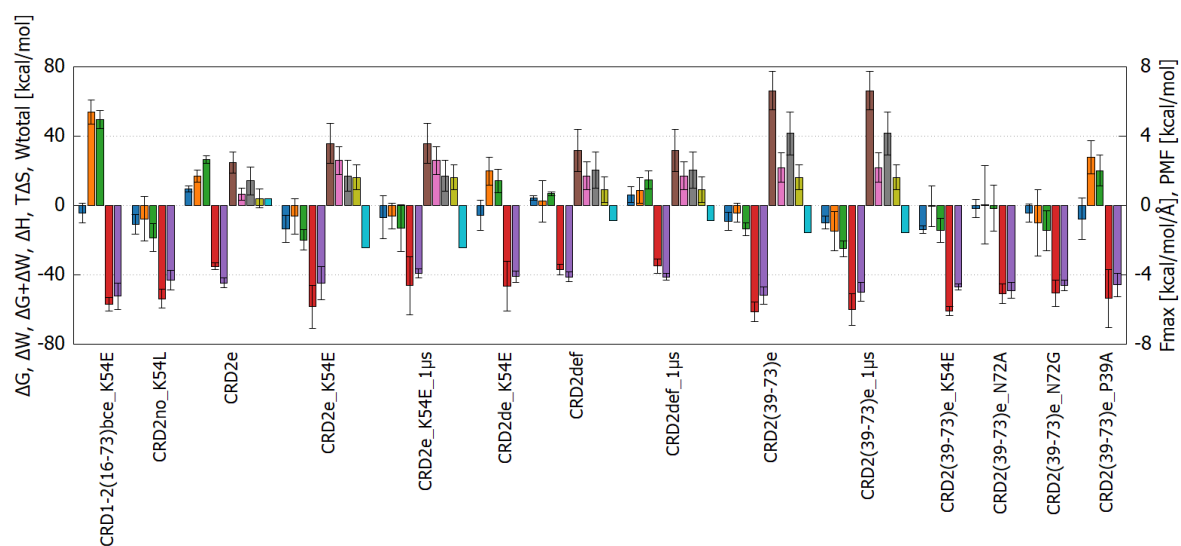


Figure S11: Change of: Binding free energy (ΔG , blue), internal work (ΔW , orange), summary of Gibbs free energy and internal work ($\Delta G + \Delta W$, green), enthalpy (ΔH , red), entropy (ΔS , violet) in 200 ns and 1000 ns long simulations ("1 μ s" postscript), total work (W_{total} , pulling speed 0.05 m/s brown, 0.01 m/s pink), force max (F_{max} , pulling speed 0.05 m/s gray, 0.01 m/s yellowish green) and potential mean force (PMF, cyan) calculated on SMD basis of eleven selected systems with highest affinity to LIGHT. CRD2e_1 μ s, due to dissociating, is not shown.

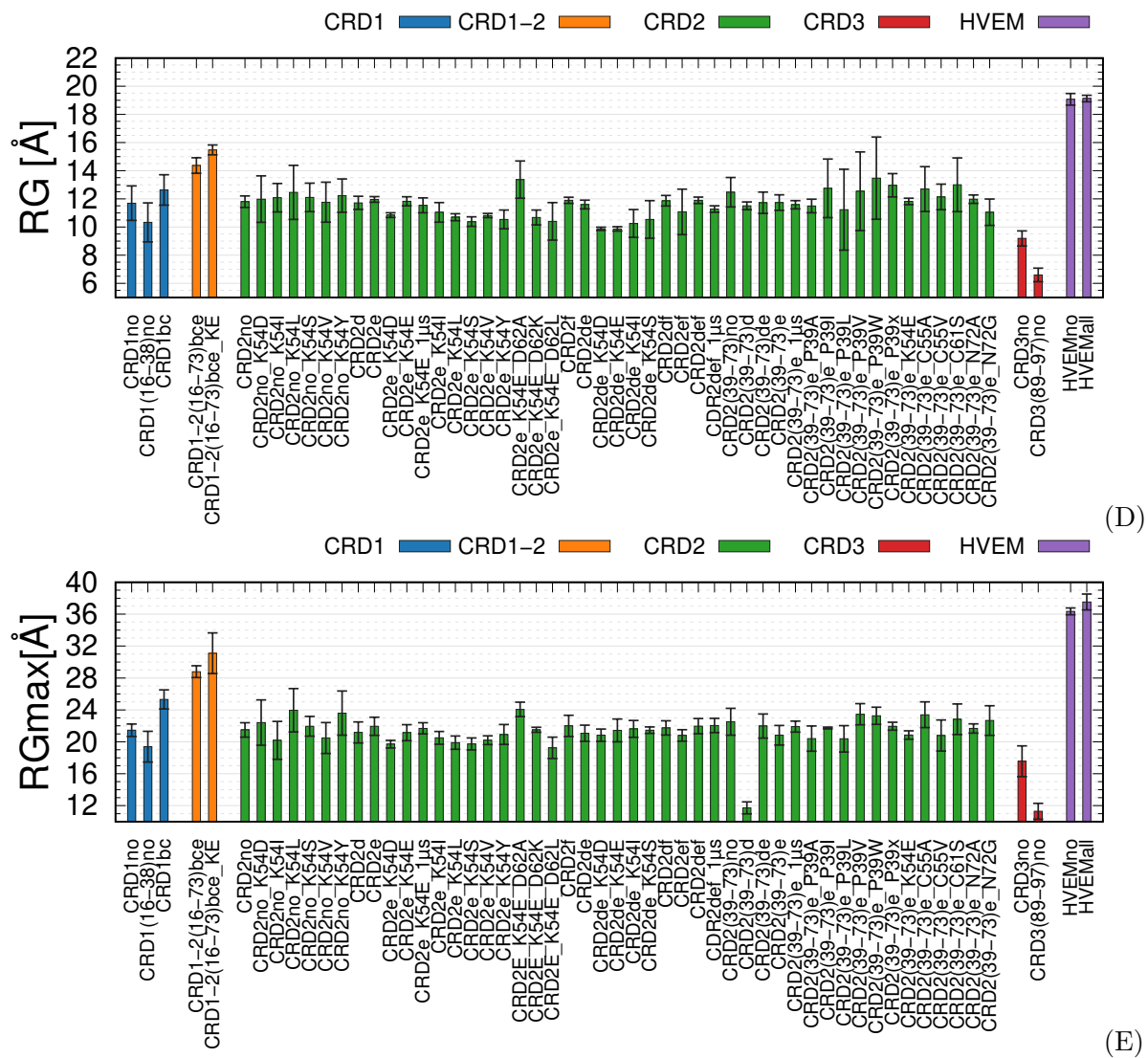


Figure S12: continued: Bar plots of the stable complexes of the structural properties: (D) RG and (E) RGmax values averaged for 3 trajectories for the last 20 ns of simulation. For the RMSD calculation, an initial conformation adapted from the PDB file was used as a reference.

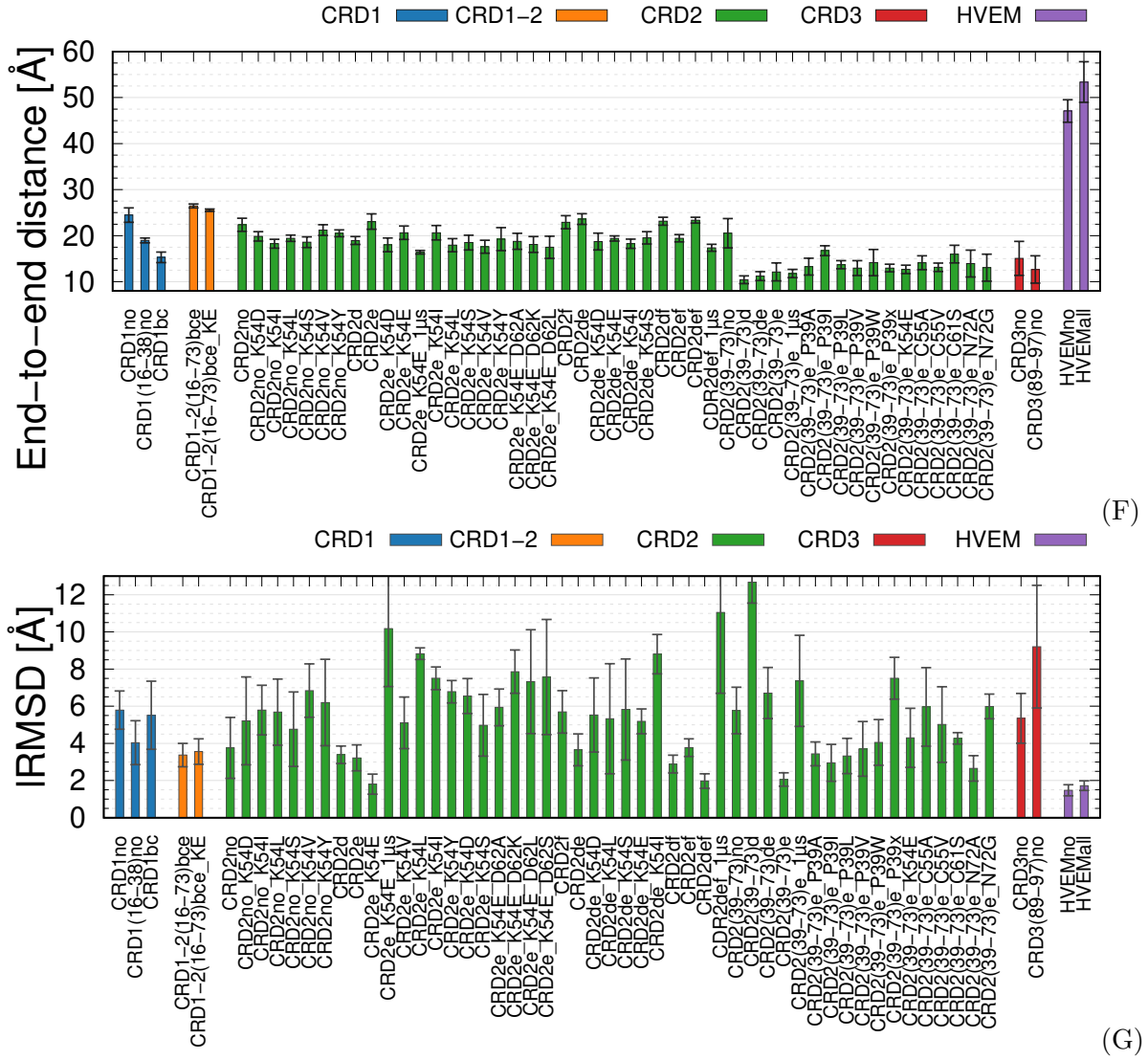


Figure S12: continued: Bar plots of the stable complexes of the structural properties: (F) End-to-end distance and (G) IRMSD values averaged for 3 trajectories for the last 20 ns of simulation. For the RMSD calculation, an initial conformation adapted from the PDB file was used as a reference.

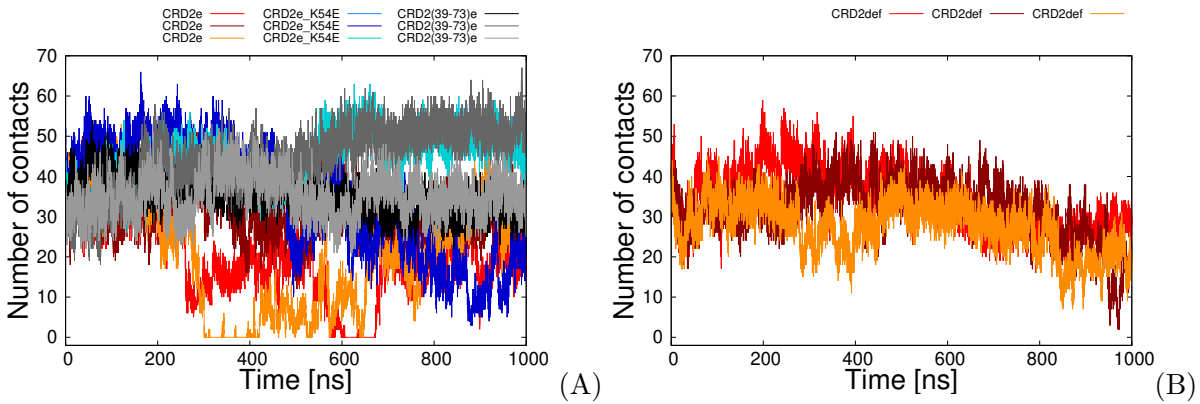
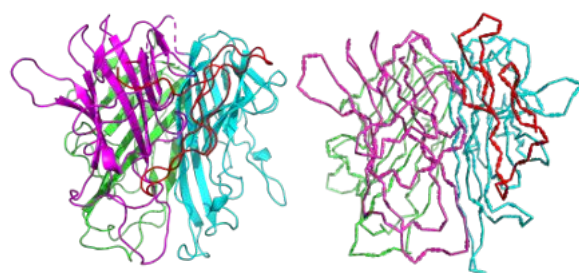
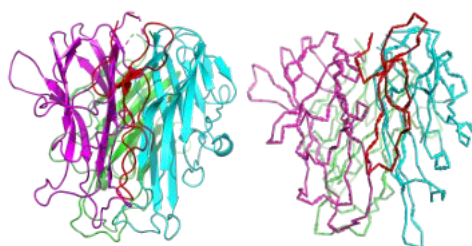


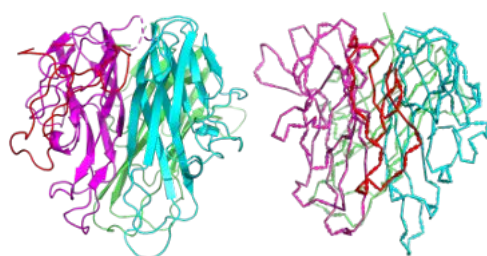
Figure S13: Number of native contacts between HVEM variant and LIGHT trimer as a function of time for 1 μ s simulations (A): CRD2e (red), CRD2e.K54E (blue), and CRD2(39-73)e (grey); (B): CRD2def (red). Different trajectories are depicted as various color tone.



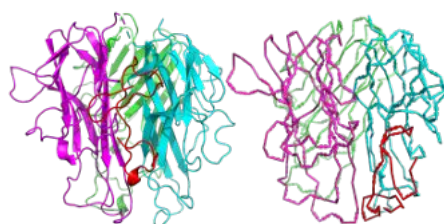
(A)



(B)



(C)



(D)

Figure S14: Comparison between the all-atom dominant structure (left, cartoon representation) with the UNRES-dock cluster with lowest RMSD to respective all-atom structure (right, ribbon representation). Each LIGHT chain is colored green, cyan, magenta and peptide is colored in red for: A) CRD2e (RMSD 4.80Å), B) CRD2def (RMSD 4.39Å), C) CRD2_K54E (RMSD 6.30Å) and D) CRD2(39-73)e (RMSD 4.95Å).



HVEM-derived peptides as inhibitors of HVEM/LIGHT complex formation

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ABSTRACT

T cells are important targets for therapeutic intervention in many diseases. Modifying T cell activation via immune checkpoints plays a central role in such approaches. The HVEM/LIGHT complex is one of the stimulatory immune checkpoints involved in T cell activation, and binding of these proteins results in proliferation and development of effector functions of T cells. In patients suffering from autoimmune diseases and in transplant recipients it is desirable to suppress immune responses. This could be achieved by blocking the HVEM and LIGHT interactions.

Our studies concern blockage of the formation of the HVEM/LIGHT complex using peptides. To design the inhibitors of this interactions, we relied on the amino acid sequence and the structure of the LIGHT-binding fragments of HVEM. We measured the affinity of designed peptides to LIGHT using SpS technique and tested their ability to inhibit the formation of the HVEM/LIGHT complex using ELISA and cellular studies. That led to the identification of two peptides, namely CRD2e_K54E and CRD2(39–73)e that strongly bind to LIGHT and possess blocking capacities towards HVEM/LIGHT complex formation. Obtained data indicate that HVEM-derived peptides could form the basis for future therapeutics, highlighting the need for further exploration of their immunomodulatory potential.

1. Introduction

The receptor – herpes virus entry mediator (HVEM) and its ligand, called LIGHT, are transmembrane proteins that belong to the tumor necrosis factor superfamily (TNFSF). The HVEM/LIGHT complex acts as a stimulatory immune checkpoint (ICP), and the interaction between these proteins activates the immune response [1]. Blocking of the HVEM

and LIGHT binding may be important in the treatment of various types of diseases in which the immune system is overactivated or suppression of the immune response is desirable.

The HVEM is a 283 amino acid protein that consists of a signal peptide and an extracellular, a transmembrane, and an intracellular portion. The extracellular fragment of HVEM is divided into four cysteine-rich domains (CRDs), determined by the presence of disulfide

Abbreviations: AcM, Acetamidomethyl; ANOVA, Analysis of variance; APC, Antigen presenting cells; BSA, Bovine serum albumin; BTLA, B- and T-lymphocyte attenuator; CD160, Cluster of differentiation 160; CRD, Cysteine-rich domain; CTLA-4, Cytotoxic T lymphocyte antigen-4; DC, Dendritic cell; DcR3, Decoy receptor 3; DTT, Dithiothreitol; eGFP, Enhanced green fluorescent protein; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; Fc, Fragment crystallisable; gD, Glycoprotein D; HRP, Horseradish peroxidase; HSV-1/HSV-2, Herpes simplex virus 1/2; HVEM, Herpes virus entry mediator; GVHD, Graft-versus-host disease; ICP, Immune checkpoint; IgSF, Immunoglobulin superfamily; K_d , Dissociation constant; K_D , Equilibrium dissociation constant; LIGHT, Homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes; LT β R, Lymphotoxin β receptor; LT α , Lymphotoxin α ; MD, Molecular dynamics; MHC, Major histocompatibility complex; MM-GBSA, Molecular mechanics generalised born and surface area continuum solvation; NF- κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells; NMR, Nuclear magnetic resonance; RP-HPLC, Reverse phase-high performance liquid chromatography; RP-UHPLC, Reverse-phase ultra-high-performance liquid chromatography; SA, Simulated annealing; SASA, Solvent accessible surface area; SD, Standard deviation; SMD, Steered molecular dynamics; SPPS, Solid phase peptide synthesis; SPR, Surface plasmon resonance; SpS, Spectral shift; TCR, T cell receptor; TMB, 3,3',5,5'-tetramethylbenzidine; TNFSF, Tumor necrosis factor superfamily; Trt, Triphenylmethyl.

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bonds, of which only the first three have a well-known and defined structure [2]. CRDs are involved in the interaction of HVEM with various ligands and receptors. HVEM binds to members of the immunoglobulin superfamily (IgSF), such as the B and T-lymphocyte attenuator (BTLA) [3,4], cluster of differentiation 160 (CD160) [5], and glycoprotein D (gD) of herpes simplex virus 1/2 (HSV-1/2) [6,7], and the members of TNFSF such as lymphotoxin α (LT α) and LIGHT [8,9]. HVEM is expressed on T cells, B cells, natural killer cells, dendritic cells (DCs), and myeloid cells [10]. The structure of the HVEM protein and its expression on different cells determine its function in the body. The HVEM present on T cells interacts with the LIGHT or LT α through the CRD2 and CRD3 domains and promotes stimulatory signaling pathways, including activation and proliferation of T cells. However, when HVEM is present on antigen presenting cells (APCs) and binds to BTLA or CD160, mainly through CRD1, the inhibitory signal is transmitted and the immune response is inhibited [3,5,8,11,12]. HVEM can therefore act as a molecular switch, maintaining homeostasis in the body.

The LIGHT protein consists of 240 amino acids divided into a cytoplasmic, a transmembrane, and an extracellular domain, but could also occur in soluble form (sLIGHT), which is created by enzymatically cleaving the transmembrane protein by metalloproteinases [13]. LIGHT is present naturally in the body as a homotrimer [2]. It binds to three different members of TNFSF, such as mentioned above HVEM, decoy receptor 3 (DcR3), and lymphotoxin β receptor (LT β R). The interaction of LIGHT with HVEM or LT β R results in stimulation of the immune response and pro-inflammatory activity, while binding to DcR3 blocks the possibility of LIGHT to form a complex with other receptors, resulting in a lack of immune response [14,15]. LIGHT is expressed on immature and mature DCs, monocytes, T cells, B cells, and myeloid cells [15].

In the body, the HVEM/LIGHT complex activates the immune system in case of threats [16]. The HVEM/LIGHT complex has a 3:3 stoichiometry and the LIGHT trimer interacts with the three molecules of HVEM. The CRD1 and CRD2 of HVEM bind to the DE, AA', and GH loops of LIGHT, while the CRD3 domain interacts with the CD and EF loops of LIGHT. Among the important residues are A47, H48, N50, G51, Q57, Q59, D62 from CRD2 of HVEM and residues I90–G94 and A98–R101 from CRD3 of HVEM (the amino acid residue numbering in the HVEM protein does not include the signal peptide) [16]. As HVEM is expressed on naïve T cells and LIGHT is expressed on immature DCs, this indicates a role of this complex in the T cell activation dependent on ICPs. The interaction of these proteins leads to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the production of interferon gamma and other cytokines [17–19]. It should be noted that T cell-LIGHT expression is stage-activation dependent. In the case of naïve T, its expression is almost undetectable. Upon activation, LIGHT expression increases to a maximum and then decreases to a minimum in resting/memory T cells. Disruption of this process, i.e., continuous expression of LIGHT on T cells, can lead to overactivation of these cells and be associated with a number of autoimmune diseases such as rheumatoid arthritis [20] or inflammatory bowel disease such as Crohn's disease [21–23]. Moreover, LIGHT plays an important role in the case of graft tolerance [24–26]. Studies on transgenic mice with impaired LIGHT expression revealed that allogeneic transplant tolerance was higher in those mice. All of these reports make the HVEM/LIGHT complex an interesting molecular target, and the studies presented here focus on blocking this protein complex formation using peptides. The proposed strategy could be used in the treatment of autoimmune diseases and in the case of transplant rejection.

The aim of our research was to determine the ability of the peptides to block the HVEM/LIGHT complex formation. Potential peptide-based inhibitors of HVEM/LIGHT binding, were designed based on the crystal structure of the protein complex (PDB:4RSU). The design of the peptides analyzed here was described in our previous publication and their amino acid sequences were based on the LIGHT-binding fragments of HVEM [27]. In our previous studies, we checked the importance of individual

amino acid residues in peptides for interaction with LIGHT and determined the affinity of compounds to LIGHT using computer based methods such as solvent accessible surface area (SASA), molecular mechanics – generalised Born and surface area continuum solvation (MM-GBSA) based on molecular dynamics (MD), and steered molecular dynamics (SMD). In this work, we study the activity of peptides designed in this way using experimental methods. We checked their affinity to LIGHT using spectral shift (SpS) technique and then investigated their inhibitory properties using enzyme-linked immunosorbent assays (ELISA), and cellular-based studies. Moreover, we evaluated their stability in the medium and their cytotoxicity towards the cells used in cellular studies. The results obtained showed that two peptides, namely CRD2e_K54E and CRD2(39–73)e have the ability to block the HVEM/LIGHT interaction. They both cover the CRD2 domain of the HVEM and possess one disulfide bond. All presented data indicate that they are good candidates for further studies to evaluate their immunomodulatory potential *in vivo*.

2. Results

2.1. Design and synthesis of peptides

The peptides designated in the course of our research are HVEM binding fragments to LIGHT covering CRD2 (peptides 1–6) and CRD3 (peptides 7–9) (Fig. 1). For the designed peptides, the MD simulations were performed first, which allowed the selection of compounds that showed the strongest binding to the LIGHT. It was determined that two peptides namely CRD2e and CRD2de had the best affinity for LIGHT. These compounds cover the same fragment of the HVEM protein, but the former has one disulfide bond, while the latter has two. In the next step, the structure of these peptides was optimised and three analogues, namely CRD2e_K54E, CRD2de_K54E and CRD2(39–73)e (peptides 10–12) were designed. In the first two, the lysine at position 54 is replaced by glutamic acid, while the CRD2(39–73)e is a truncated version of CRD2e shortened at the C-terminus by 8 amino acids. They also differ in the number of disulfide bonds; CRD2e_K54E and CRD2(39–73)e have one disulfide bond, while CRD2de_K54E has two. The rationale for these changes and their direction have been clarified in our

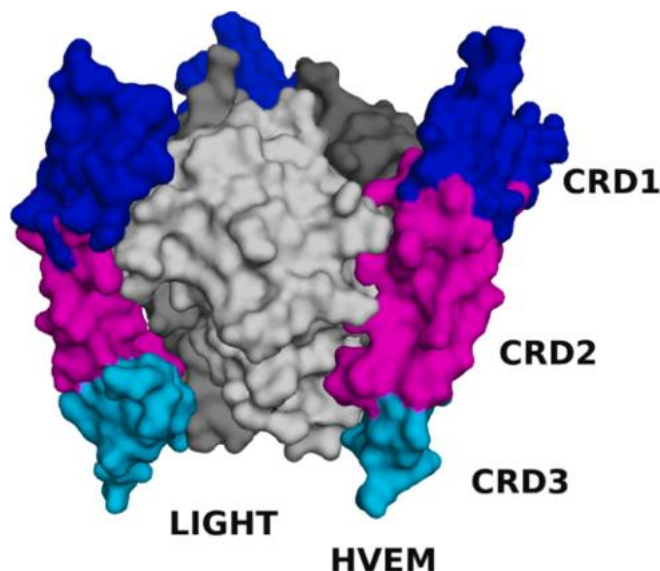


Fig. 1. Trimer of LIGHT interacting with three molecules of HVEM (PDB:4RSU). HVEM is divided into CRD1 (blue), CRD2 (magenta), and CRD3 (cyan); CRD4 is not present in the crystal structure of the protein complex, LIGHT is presented as a grey surface. The designed peptides are the fragments of CRD2 and CRD3 of HVEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previous research [27], and here we have checked if the results obtained by computational methods are consistent with experimental studies. CRD2 and CRD3 in HVEM possess three disulfide bonds, while in peptides only one or two of them are present. Compounds with three disulfide bonds were not studied because their yields were very low and also because theoretical studies predicted them to be weaker inhibitors [27]. The amino acid sequences of the peptides are present in Table 1.

Cysteine residues not involved in the formation of disulfide bonds were replaced by 2-aminobutanoic acid (X_1) while methionine residues present in the amino acid sequences of all designed peptides were replaced by norleucine (X_2) in order to avoid their oxidation in subsequent test steps.

2.2. Peptides affinity to LIGHT measured by SpS technique

In order to assess the affinity of the peptides for LIGHT and determine the dissociation constant (K_d) of the protein/peptide complex, the SpS analyses were performed. In the SpS measurements, the LIGHT with the His tag was labelled with a RED-tris-NTA 2nd Generation dye. The labelled protein was then added to the peptides at 16 different concentrations and incubated to allow complex formation. The mixtures were transferred to the capillaries and the ratio of fluorescence intensity at two emission wavelengths as a function of the peptide concentration was measured. All studied peptides bound to LIGHT (Fig. S1). However, the binding curves did not reach saturation, which was attributed to peptide aggregation at higher concentrations. Therefore, the determined dissociation constants are not absolute but rather apparent values, which can be used only to compare the relative binding affinities between the peptides and the LIGHT. The apparent K_d values determined for the HVEM-derived peptides covering CRD2 (peptides 1–6) were in the range of 2.08 to 8.50 μ M, while the compounds covering CRD3 of HVEM (peptides 7–9) had the apparent K_d in the range from 7.62 to 15.32 μ M (Table 2). The strongest binding to LIGHT was observed for CRD2e and CRD2de, which is consistent with the results obtained from computational methods [27]. Similar analyses were also performed for the designed analogues (peptides 10–12) and the apparent K_d values were 0.46, 0.76 and 0.06 μ M for CRD2e_K54E, CRD2de_K54E, and CRD2(39–73)e respectively, indicating that the introduced modifications significantly improved the binding of the peptides to LIGHT. The apparent K_d measured for the HVEM/LIGHT complex in the same

Table 1

The amino acid sequence of the designed peptides, fragments of CRD2 and CRD3 of HVEM; X_1 –2-aminobutanoic acid, X_2 – norleucine, & – position of disulfide bond, numeration of HVEM amino acid residues does not include the signal peptide.

No.	Peptide name	Peptide amino acid sequence
1	CRD2d	Ac-PC(&1)P ⁴¹ PGTYIHLNG ⁵¹ LSKC(&1) LQX ₁ QX ₂ X ⁶¹ DPAX ₂ GLRASR ⁷¹ NX ₁ SRTEAVX ⁸¹ G-NH ₂
2	CRD2e	Ac-PX ₁ P ⁴¹ PGTYIHLNG ⁵¹ LSKX ₁ LQC(&1) QX ₂ X ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(&1)SRTEAVX ⁸¹ G-NH ₂
3	CRD2f	Ac-PX ₁ P ⁴¹ PGTYIHLNG ⁵¹ LSKX ₁ LQX ₁ QX ₂ C (&1) ⁶¹ DPAX ₂ GLRASR ⁷¹ NX ₁ SRTEAVC(&1) ⁸¹ G-NH ₂
4	CRD2de	Ac-PC(&1)P ⁴¹ PGTYIHLNG ⁵¹ LSKC(&1)LQC(&2) QX ₂ X ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(&2)SRTEAVX ⁸¹ G-NH ₂
5	CRD2df	Ac-PC(&1)P ⁴¹ PGTYIHLNG ⁵¹ LSKC(&1)LQX ₁ QX ₂ C (&2) ⁶¹ DPAX ₂ GLRASR ⁷¹ NX ₁ SRTEAVC(&2) ⁸¹ G-NH ₂
6	CRD2ef	Ac-PX ₁ P ⁴¹ PGTYIHLNG ⁵¹ LSKX ₁ LQC(&1)QX ₂ C (&2) ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(&1)SRTEAVC(&2) ⁸¹ G-NH ₂
7	CRD3g	Ac-GC(&1)SPGHFC(&1)IV ⁹¹ QDGDHC(&1)R ¹⁰¹ AYAG-NH ₂
8	CRD3h	Ac-GX ₁ SPGHFC(&1)IV ⁹¹ QDGDHC(&1)AAX ₁ R ¹⁰¹ AYAG-NH ₂
9	CRD3gh	Ac-GC(&1)SPGHFC(&2)IV ⁹¹ QDGDHC(&2)AAC(&1) R ¹⁰¹ AYAG-NH ₂
10	CRD2e_K54E	Ac-PX ₁ P ⁴¹ PGTYIHLNG ⁵¹ LSEX ₁ LQC(&1) QX ₂ X ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(&1)SRTEAVX ⁸¹ G-NH ₂
11	CRD2de_K54E	Ac-PC(&1)P ⁴¹ PGTYIHLNG ⁵¹ LSEC(&1)LQC(&2) QX ₂ X ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(&2)SRTEAVX ⁸¹ G-NH ₂
12	CRD2(39–73)e	Ac-PX ₁ P ⁴¹ PGTYIHLNG ⁵¹ LSKX ₁ LQC(&1) QX ₂ X ⁶¹ DPAX ₂ GLRASR ⁷¹ NC(&1)G-NH ₂

Table 2

Affinity of the peptides to LIGHT determined by Monolith X. Results are shown for experiments performed in triplicate. Data are presented as mean $\pm$ standard deviation (SD).

No.	Peptide name	Apparent K_d (M)	SD
1	CRD2d	5.64×10^{-6}	1.39×10^{-6}
2	CRD2e	2.71×10^{-6}	1.88×10^{-6}
3	CRD2f	8.50×10^{-6}	6.46×10^{-6}
4	CRD2de	2.08×10^{-6}	0.67×10^{-6}
5	CRD2df	4.29×10^{-6}	3.91×10^{-6}
6	CRD2ef	5.29×10^{-6}	2.52×10^{-6}
7	CRD3g	15.32×10^{-6}	9.74×10^{-6}
8	CRD3h	7.62×10^{-6}	5.63×10^{-6}
9	CRD3gh	14.53×10^{-6}	8.30×10^{-6}
10	CRD2e_K54E	0.46×10^{-6}	0.27×10^{-6}
11	CRD2de_K54E	0.76×10^{-6}	0.15×10^{-6}
12	CRD2(39–73)e	0.06×10^{-6}	0.04×10^{-6}
	HVEM	0.92×10^{-6}	0.13×10^{-6}

experimental conditions was 0.92 μ M, which indicates that the analogues bind more strongly to LIGHT than HVEM. The dose-response curves of the spectral shift for the interactions between LIGHT and HVEM as well as the peptides with the highest binding affinities are presented in Fig. 2, while the remaining curves are presented in Fig. S2.

2.3. The inhibitory properties of the peptides towards HVEM/LIGHT complex formation determined by ELISA

To evaluate the ability of the peptides to inhibit HVEM/LIGHT complex formation, an ELISA assay was performed. LIGHT was immobilized on a 96-well plate, and incubated with peptides at three different concentrations. Subsequently, HVEM with a fragment crystallisable (HVEM-Fc) was added, and its binding was detected using horseradish peroxidase (HRP) – conjugated anti-human IgG and 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The inhibitory properties of the peptides were determined relative to those of the control, in which LIGHT was incubated with buffer instead of peptides.

The obtained results showed that the peptides covering the CRD2 of HVEM have very similar inhibitory properties and block the HVEM/LIGHT binding in the highest used concentration in the range from 26 % to 31 %. The peptides, which are the fragment of CRD3 of HVEM, also disrupt the HVEM/LIGHT binding but much weaker and for them the inhibition was from 18 % to 21 % at concentration 50 μ M. The strongest blocking capacities were observed for CRD2e_K54E and CRD2(39–73)e, which achieved about 38 %, and 41 % inhibition, respectively at the highest concentration used (Fig. 3). This is in line with the SpS results that indicated the highest affinity of these peptides for LIGHT. Additionally, we synthesized a scrambled peptide based on CRD2(39–73)e, in which the amino acid residues were randomly rearranged, while the position of the disulfide bond were preserved (Table S1). This peptide did not show any significant inhibitory activity towards HVEM/LIGHT complex formation (Fig. S3).

2.4. Cytotoxicity and stability studies of the peptides

As the peptides revealed inhibitory potential in ELISA, therefore, in the next step, we decided to check whether similar results would be obtained in cell-based assays. It was, however, necessary to verify the cytotoxicity of the peptides towards the studied cells and their stability in the medium used in the cultures, before performing tests.

To determine the range of concentrations that could be used in cell-based assays, the peptides were incubated with two cell lines: Jurkat E6.1 and modified BW5147 (hereinafter referred to as TCS Ctrl) for 24 h. No toxicity was observed for any of the peptides at the concentration used in the ELISA in either of the tested cell lines (Fig. S4). The procedure of the assay and the cytotoxicity results are presented in the Supplementary Materials.

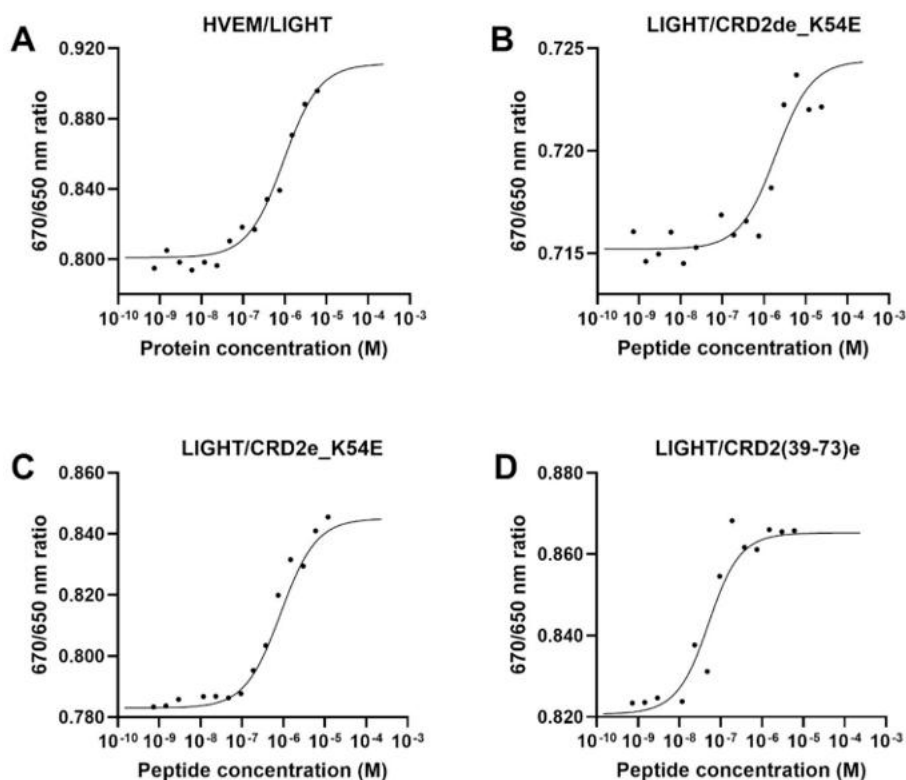


Fig. 2. The spectral shift dose-response curves for the interactions between LIGHT and: A) HVEM, B) CRD2de_K54E, C) CRD2e_K54E, D) CRD2(39–73)e.

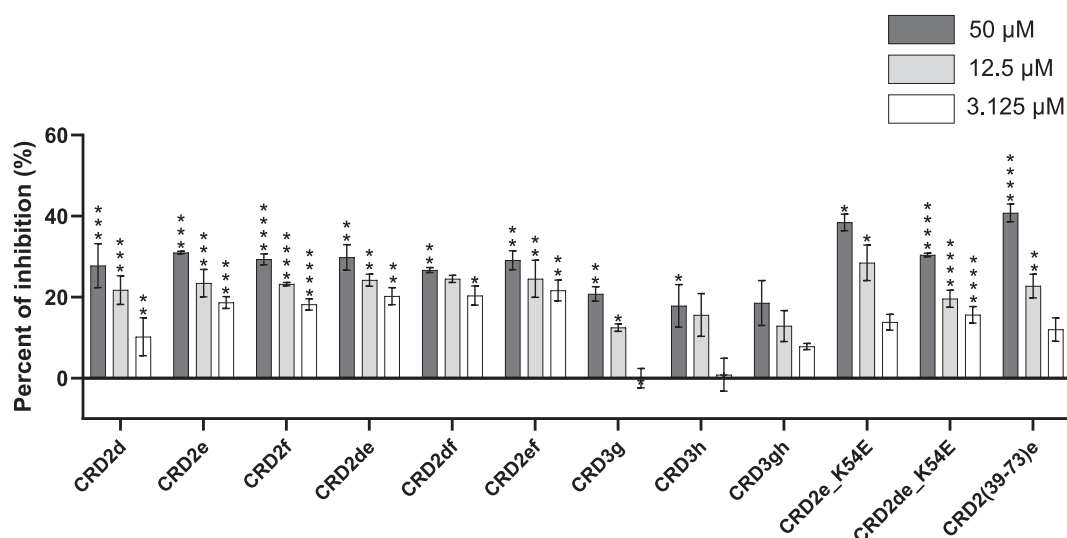


Fig. 3. The inhibitory properties of HVEM-derived peptides towards HVEM/LIGHT complex determined by ELISA. The results are shown for two experiments performed independently in triplicate. Data are depicted as mean with SD (mean $\pm$ SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

In the next step, we determined the stability of the designed peptides using reverse phase-high performance liquid chromatography (RP-HPLC) in cell culture medium in time 0 h and after 24 h of incubation. The area under the peak corresponding to the peptide after incubation time was compared with the area under the peak obtained for the control (peptide dissolved in H_2O without incubation). The analyses indicate that all peptides are relatively stable at time 0 h (between 77.5 and 100 %). After 24 h of incubation, the amount of peptide remaining in the sample decreases and is in the range of 34.1 % for CRD2e_K54E and 77.2 % for CRD2de. Generally, no additional peaks indicating degradation

were observed on the chromatogram, suggesting that they bind to the components of the medium such as fetal bovine serum (FBS) and are removed from the sample during the preparation process. We already observed a similar effect when we studied the stability of peptides, that were potential inhibitors of BTLA/HVEM binding [28–30]. The results are presented in Fig. 4 and the chromatograms obtained for the selected peptides are presented in Fig. S5.

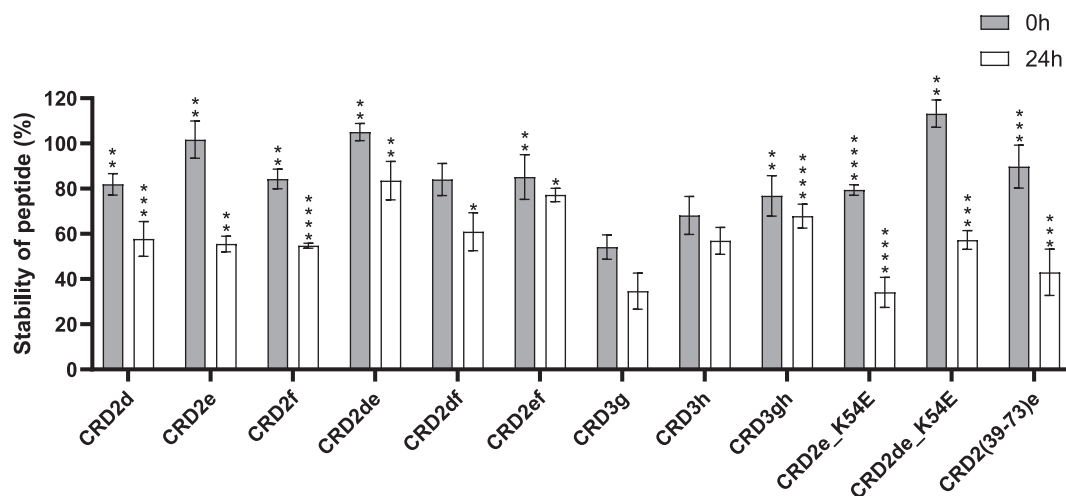


Fig. 4. Stability of peptides in Jurkat E6.1 cell supernatant at 0 and 24 h of incubation compared to the control (peptide dissolved in H₂O). The results are shown for three independent experiments. Data are depicted as mean with SD (mean $\pm$ SD). Statistical analysis was performed using one-way ANOVA followed by the Dunnett's post hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

2.5. The inhibitory properties of the peptides towards HVEM/LIGHT complex formation determined by cellular-based assay

In order to verify the inhibitory properties of the peptides the cellular-based assays were performed. The cell platform used in this studies is similar, in principle, to that described in [31]. In summary, reporter cells expressing the HVEM protein (JE6.1-NF- κ B::eGFP HVEM), were co-cultured with T cell stimulators expressing LIGHT (TCS LIGHT) in the presence of HVEM-derived peptides at different concentrations. The reporter cells are based on Jurkat E6.1 and possess on surface T cell receptors (TCR) and CD3, while TCSs are based on the murine thymoma cell line (BW5417) and express membrane bound anti-human CD3 single chain antibody fragments (mb aCD3). The interactions between TCR-CD3/aCD3 and HVEM/LIGHT stimulate the activation of reporter cells and the expression of NF- κ B, which is associated with the enhanced green fluorescent protein (eGFP). However, if the formation of the HVEM/LIGHT complex is disrupted, the level of both NF- κ B and eGFP decreases. The activation of the reporter cells in the presence of TCS

LIGHT and control peptide was set as 100 %, and it is inversely proportional to the inhibitory properties of the tested compounds.

The experiments showed that the peptides based on the CRD2 and CRD3 of HVEM possess either very weak or no inhibitory properties. However, two designed analogues of the CRD2e peptide, namely CRD2e_K54E and CRD2(39-73)e, demonstrated stronger blocking effects than the other tested peptides inhibiting the HVEM/LIGHT complex formation by 21 % and 26 %, respectively, at the highest concentration used (Fig. 5). This is consistent with their performance in the ELISA and SpS, which indicate that these compounds exhibit the strongest blocking capacity and the highest affinity among all the tested peptides.

2.6. The inhibitory properties of the peptides, the best inhibitors of HVEM/LIGHT towards BTLA/HVEM and LT β R/LIGHT complexes formation

All the results we have described above are consistent and show that

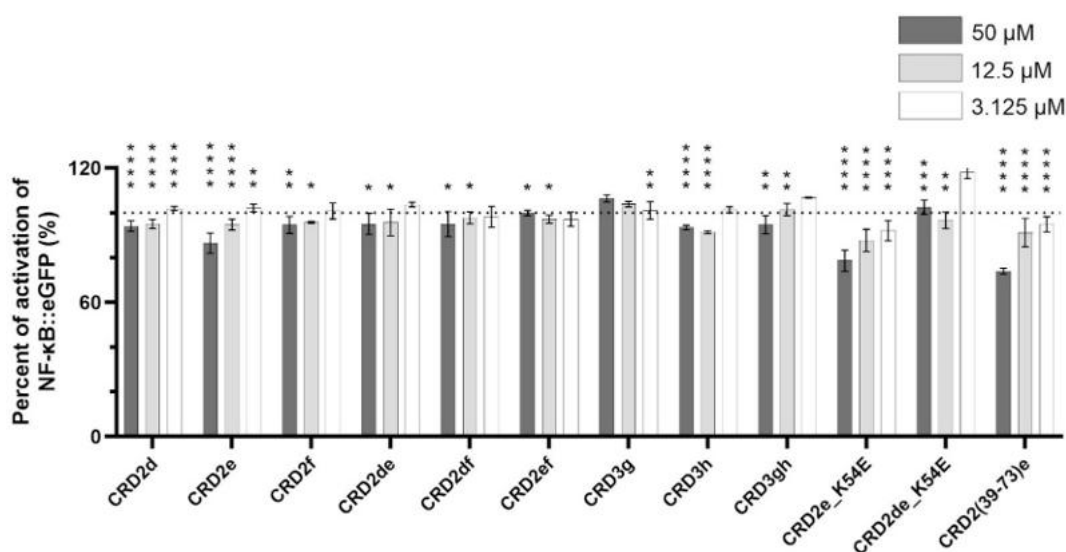


Fig. 5. Activation of HVEM reporter cells stimulated by TCS LIGHT in the presence of peptides at different concentrations. The percentage of activation was measured by eGFP expression using flow cytometry. The results are shown for three experiments performed independently in triplicate. Data are depicted as mean with SD (mean $\pm$ SD). Statistical analysis was performed using one-way ANOVA followed by the Dunnett's post hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

the best inhibitors of the HVEM/LIGHT complex formation are two peptides, namely CRD2e_K54E and CRD2(39–73)e. As these peptides are based on the amino acid sequence of the HVEM protein, we decided to verify their specificity towards HVEM/LIGHT by checking their inhibitory properties towards BTLA/HVEM complex. Moreover, as LT β R protein binds to the LIGHT protein in a pattern similar to that of the HVEM protein, we evaluated their inhibitory properties towards LT β R/LIGHT complex formation. For this purpose, the ELISA was used, where either BTLA or LIGHT was immobilized on a plate. In the next step, the proteins were incubated with the selected peptides, and then HVEM-Fc or LT β R-Fc was applied. The remaining steps of the test were the same as described above for the HVEM/LIGHT complex. The peptides did not significantly interfere with BTLA/HVEM binding (Fig. 6A) while they showed considerable blocking capacities towards LT β R and LIGHT (Fig. 6B). The CRD2(39–73)e peptide inhibits LIGHT/LT β R binding definitely weaker than HVEM/LIGHT while the inhibitory properties of CRD2e_K54E are comparable for both complexes. The blocking effect of peptides towards the BTLA/HVEM complex was also studied using cell-based assays, and these data also confirmed that they have a minor impact on the BTLA/HVEM interaction (Fig. 6C).

2.7. Conformation studies of peptides using NMR

Nuclear magnetic resonance (NMR) spectra were obtained for three peptides: CRD2e, the reference peptide used for analogues design; CRD2e_K54E, and CRD2(39–73)e, which exhibited the highest potential as an inhibitor of HVEM/LIGHT complex formation. Unfortunately, for the 43-amino acid peptides CRD2e and CRD2e_K54E, the 2D NMR techniques were insufficient for conformational studies due to poor spectra quality and significant signal overlap (Fig. S6). For shorter 36-

amino acid peptide, CRD2(39–73)e, the vast majority of resonance lines in the NMR spectra were assigned. However, poor spectral resolution ultimately yielded low quality of the calculated peptide structures. In particular, a comparison of the NMR spectra of CRD2(39–73)e with its longer analogues, CRD2e and CRD2e_K54E (Fig. S6), revealed significant similarity, suggesting a comparable conformation of the 1–36 fragment. The general data obtained during these studies reveal that the peptide adopts a flexible statistical coil conformation, which correlates well with our theoretical studies [27]. More detailed data on peptide conformation is presented in the Supplementary Materials.

3. Discussion and conclusions

The activation of the T cell-dependent immune response requires two signals. The first of them is provided by molecules of the major histocompatibility complex (MHC) in APCs, which present the antigens to TCRs on T cells, while the second signal is derived from the interaction of costimulatory immune checkpoints. The second signal plays an important role in numerous immune responses, and its absence induces T cell anergy [32–34]. The best-known costimulatory ICP is a complex created by the CD28 receptor and its ligands, CD80/CD86 [35]. It was reported that mice treated with a soluble cytotoxic T lymphocyte-associated antigen 4-Ig (CTLA-4Ig), the protein that block the CD28/CD80/CD86 binding, induced antigen-specific tolerance that prevented the progression of autoimmune diseases and organ graft rejection [36]. Furthermore, inhibition of CD28/CD80/CD86 signaling pathway blocked xenograft rejection, while allograft rejection was significantly delayed. CTLA-4Ig known as Belatacept is currently used to suppress the immune response in transplant recipients [37]. These results as well as many other studies [38,39] initiated the search for drugs that, by

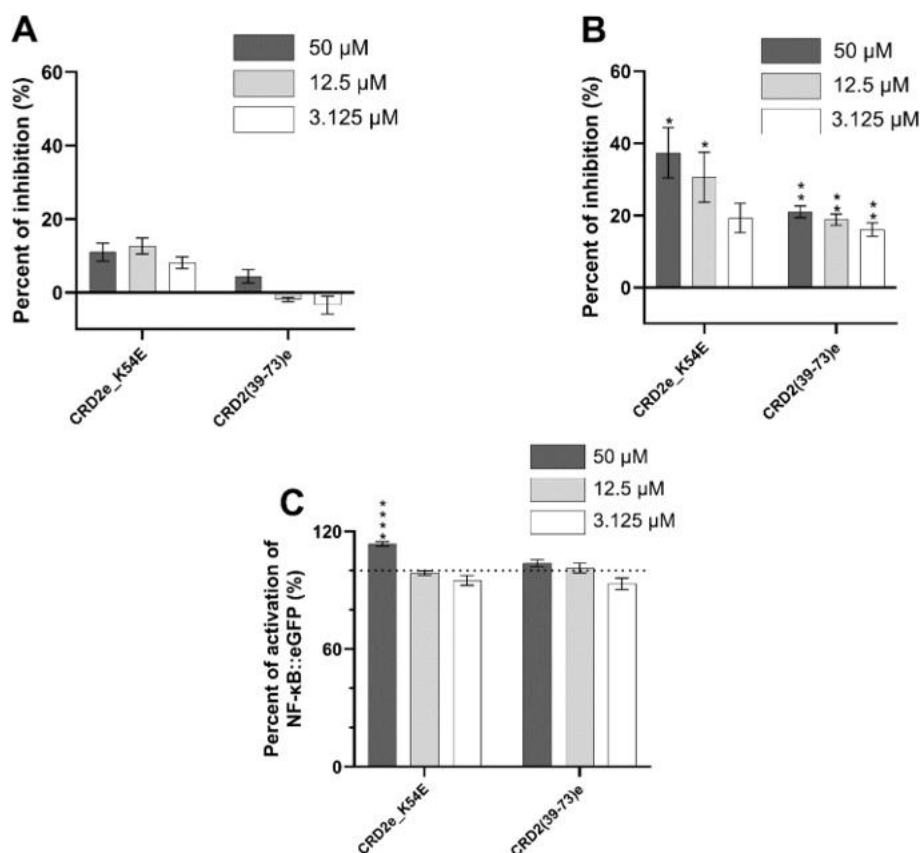


Fig. 6. The inhibitory properties of HVEM-derived peptides towards A) BTLA/HVEM complex formation determined by ELISA, B) LT β R/LIGHT complex formation determined by ELISA, C) BTLA/HVEM complex formation determined by cellular assays. The results are shown for two experiments performed independently in triplicate. Data are depicted as mean with SD (mean $\pm$ SD). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

blocking costimulatory molecules, attenuate or modify T cell-dependent responses to treat autoimmune diseases and prevent graft rejection.

The HVEM and LIGHT that are the subject of this study belong to the costimulatory ICPs. HVEM/LIGHT interaction activates T cells, enhances their proliferation, and promotes the production of cytokines independently of the CD28/CD80/CD86 pathway [40]. Tamada et al. showed that blocking LIGHT by soluble LT β R-Ig and HVEM-Ig significantly reduced allogeneic T cell immune responses *in vitro* [41], while del Rio et al. demonstrated that the anti-LIGHT antibody (clone 10F12) attenuates *in vivo* cytotoxic allogeneic responses [42]. Furthermore, del Rio et al. demonstrated that anti-LIGHT antibody (clone 3D11) blocking the interaction of LIGHT with its receptors HVEM and LT β R inhibits anti-host cytotoxic T cell responses in a murine graft-versus-host disease (GVHD) model, resulting in improved survival rates [43]. Fan et al. showed that double blocking of CD80/CD80/CD86 and HVEM/LIGHT stimulatory pathways by combined treatment of soluble CTLA4-Ig and LT β R-Ig mediate long-term survival of islet grafts by induction of allo-specific tolerance in a mouse model [24]. Xu et al. used an HVEM antibody to block HVEM/LIGHT binding and showed that administration of this mAb profoundly ameliorated GVHD [25]. Perlin et al. conducted clinical trials of an anti-LIGHT antibody, namely CERC-002 in patients with COVID-19 related pneumonia and acute respiratory distress syndrome. By blocking LIGHT using antibodies, they have received promising results in reducing the mortality rate due to respiratory failure compared to the control group [44]. Another anti-LIGHT antibody, called CBS001, is in clinical trial, and its safety is currently tested on healthy volunteers. It was developed to treat long-term (chronic) conditions involving inflammation, such as inflammatory bowel disease, and pulmonary fibrosis [45]. These facts support the assumption that blocking the HVEM/LIGHT signaling pathway can be used in immunotherapy, in which suppression of the immune response is desired. Considering the results of these studies, we designed HVEM-derived peptides to block the formation of the HVEM/LIGHT complex.

Our group was the first to design, synthesize, and study peptides as inhibitors of the formation of the HVEM/LIGHT complex. We chose the peptides due to the relatively low production cost, the simplicity of synthesis, and further modifications compared to antibodies [46,47]. The peptides are also less stable and are much more rapidly enzymatically degraded than antibodies, which would prevent their accumulation and the occurrence of potential negative effects on the patient's health [48]. However, the peptides designed in this study are relatively long (from 24 to 43 residues) and contain non-natural amino acids. Therefore, they may exhibit immunogenicity, which should be carefully evaluated in future studies. The peptides studied here were designed based on HVEM binding fragments to LIGHT, and all details on peptide design are described in our previous publication [27]. Here we focused on investigating their interaction with LIGHT and determining their inhibitory properties towards the formation of the HVEM/LIGHT complex. Peptides 1–6 cover the CRD2 of HVEM, peptides 7–9 are fragments of the CRD3 of HVEM, and compounds 10–12 were designed using molecular modelling methods and based on previous calculations for the compounds 1–9.

The interaction of peptides with LIGHT was studied using SpS. This technique allows the monitoring changes in the emission spectrum of a fluorescently labelled target, which could be the result of peptide binding and may induce conformational alterations in the protein [49]. The data obtained showed that all designed peptides interact with the LIGHT protein. However, for these peptides, the maximum binding plateau could not be reached due to aggregation at higher concentrations. As a result, the calculated K_d values should be treated as apparent and interpreted qualitatively rather than quantitatively. The peptides covering CRD2 and CRD3 of HVEM (peptide 1–9) have a similar affinity for LIGHT, and the strongest interaction was observed for CRD2e and CRD2de, which correlates with our previous computational studies [27]. The proposed modifications in the amino acid sequence of these peptides increased their affinity, and the three obtained analogues (peptides

10–12) interact significantly more strongly with LIGHT than the unmodified peptides.

The apparent K_d determined by us for HVEM/LIGHT using SpS is 0.92 μ M when LIGHT protein was labelled. In contrast, when HVEM protein was labelled, the K_d value was 8.79 nM (data not shown), which aligns with values reported in the literature. The binding of protein has also been studied by other research groups. Cheung et al. reported that the K_d determined for HVEM/LIGHT using flow cytometry-based binding assays is 11 nM or 13 nM, depending on the proteins used in experiments. In the first, the binding of HVEM-Fc to LIGHT expressed in EL4 thymoma cells was measured, while in the second, the interaction of the soluble form of recombinant LIGHT (LIGHTt66) with cell-expressed HVEM was determined [50]. The equilibrium dissociation constant (K_D) determined for the HVEM/LIGHT complex by Morishige et al. was 1.5 nM [51], while by Liu et al. it was 18.7 nM [14]. Both groups used a surface plasmon resonance (SPR) technique and studied the interaction between HVEM-Fc/HVEM-Ig and LIGHT. In both of these studies, HVEM protein was immobilized on the surface of the sensor. The determined dissociation constants for the HVEM/LIGHT complex can differ significantly depending on the method and the proteins used in the experiment. Nevertheless, the value evaluated by the same technique can be used to compare the strength of a molecule's binding. Our data suggest that designed analogues interact stronger with LIGHT than HVEM.

The results obtained by ELISA and cellular-based assays showed that inhibitory properties possess two peptides CRD2e_K54E and CRD2(39–73)e, which blocked protein binding in a dose-dependent manner in both assays. They are the first peptides targeting the HVEM/LIGHT interaction possessing the inhibitory potential. In ELISA, some inhibitory properties were observed for all peptides. However, cell-based experiments demonstrated that only two of them were capable of modulating intracellular signaling pathway dependent on HVEM/LIGHT interaction. It is important to emphasize that, in ELISA, one protein is immobilized on the plate while the other is present in solution. In contrast, in cellular assays, both interacting proteins are membrane-bound. These differences may significantly influence the inhibitory potential of the peptides and underscore the importance of employing multiple experimental approaches to reliably evaluate the inhibitory capacity of the tested compounds.

The cellular signaling mediated by HVEM and LIGHT is very complex because both proteins interact with numerous molecules in the immune system, which have different effects in the final stage of the immune response [15,52,53]. For example, HVEM binds also to BTLA, and this interaction inhibits the immune response, which plays a vital role in the protection against autoimmunity [3]. The studied peptides do not block the formation of the BTLA/HVEM complex in both ELISA and cellular assays. They are the fragment of CRD2 of HVEM, while for binding to BTLA, mainly CRD1 is crucial [12]. These data indicate that CRD2e_K54E and CRD2(39–73)e are selective inhibitors of HVEM/LIGHT, blocking the HVEM dependent stimulatory signaling pathway while the BTLA-dependent inhibitory effect is preserved. This is essential in the treatment of diseases in which the T cell-dependent immune response should be suppressed.

We also studied the effect of peptides on LT β R/LIGHT complex formation. The LT β R is the second receptor for LIGHT that stimulates the immune response. It is expressed in follicular DCs, DCs, macrophages, stromal cells, and high endothelial venules but not on lymphocytes [54]. It was also shown that blockade of LT β R/LIGHT can contribute to prolonging allograft survival and improve liver fibrosis [55]. Furthermore, blocking the interactions of LIGHT with both of its receptors could improve the clinical symptoms of fibrosis in the lung and skin [56,57]. LT β R is highly homologous to the HVEM protein, so it should bind to similar fragments of the LIGHT. Therefore, it can be assumed that peptides designed based on HVEM binding sites to LIGHT can also be used to block the LT β R/LIGHT formation. Our data indicate that CRD2e_K54E blocks LT β R and LIGHT binding in ELISA with similar strength as HVEM/LIGHT, while CRD2(39–73)e exhibits very weak inhibitory

properties towards LT β R/LIGHT. CRD2(39–73)e differs from CRD2e_K54E in the lack of the eight C-terminal amino acids, and in the second of them, lysine 54 was substituted by glutamic acid. The crystal structure of LT β R/LIGHT is not available, but the results obtained could suggest that the LIGHT binding sites of HVEM and LT β R are not identical or that the peptides interact not with the exactly same site of LIGHT.

In the final stage of this study, we attempted to determine the structures of three selected peptides using NMR. However, due to insufficient spectral resolution, the vast majority resonance line was assigned only for the CRD2(39–73)e peptide. In the HVEM protein, the fragment 39–73 forms a β -strands and is stabilized by two disulfide bonds, whereas in the CRD2(39–73)e peptide, it adopts a flexible statistical coil conformation, stabilized by only one disulfide bond. Our previous research performed for PD-1/PD-L1 [58] and BTLA/HVEM [29,30] complexes demonstrated that β structures are essential for the inhibitory properties of peptides. On the other hand, the peptides covering a similar HVEM fragment as CRD2(39–73)e but stabilizing by two disulfide bonds (CRD2de, CRD2df, and CRD2ef) do not exhibit stronger blocking capacities and bind to LIGHT weakly. It could suggest that the higher flexibility of CRD2(39–73)e may allow it to better adapt to the LIGHT protein, thereby enhancing its inhibitory properties.

In summary, all data indicate that two peptides, namely CRD2e_K54E and CRD2(39–73)e, interact with LIGHT and significantly block the formation of the HVEM/LIGHT complex in ELISA and cellular assays. Further studies are necessary to evaluate the safety of these compounds, including cytotoxicity, immunogenicity, metabolic stability, and in vivo toxicity, as well as their potential immunomodulatory effects in the context of autoimmune diseases and transplant rejection.

4. Materials and methods

4.1. Sources of proteins

Recombinant human HVEM-Fc (#HVM-H5258), LIGHT-His (#LIT-H5242), LT β R-Fc (#LTR-H5259), and BTLA-His (#BTA-H52H3) proteins were purchased from ACRO Biosystems, Newark, DE, USA.

4.2. Peptide synthesis and purification

The peptides were synthesized using the solid phase peptide synthesis (SPPS) method on a Liberty Blue synthesiser (CEM Corporation, Matthews, USA), following the Fmoc/tBu strategy. Rink Amide Pro Tide (LL) resin (CEM Corporation, Matthews, USA), with a loading capacity of 0.18 mmol/g, was used for the synthesis. To selectively form disulfide bonds, two cysteine derivatives were incorporated: Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH. For peptides containing a single disulfide bond, cysteine residues were protected with Trt groups, which were removed during the cleavage of the peptide from the resin. In the case of peptides possessing two disulfide bonds, orthogonal protection with Trt and Acm groups was employed. Following the formation of the first disulfide bond, the Acm groups were removed using iodine in methanol. This strategy allowed for the selective and controlled formation of disulfide bonds. The cysteine residues in the HVEM sequence that were not involved in disulfide bond formation were substituted with 2-aminobutanoic acid (X₁), while the methionine residues were replaced with norleucine (X₂). Peptide synthesis was performed according to the methodology presented in the previous work [59].

The peptides were purified using RP-HPLC on a Luna C8(2) column (125 × 4.6 mm, 5 μ m, 100 Å), (Phenomenex, CA, USA). A 10-fold molar excess of dithiothreitol (DTT), relative to the free sulfhydryl groups of cysteines, was added to the aqueous solution of the purified peptide. The mixture was sonicated at 40 °C before being loaded onto the column. Two solutions were used for the purification: A – consisted of deionized water with 0.1 % TFA (v/v) and B – contained 80 % acetonitrile in water with 0.1 % TFA (v/v). A linear gradient ranging from 5 % to 50 % of B in A was applied over a 120-min period. UV absorption at 222 nm and 254

nm was used to monitor the purification process.

The molecular weight and purity of the peptides were confirmed using liquid chromatography coupled with electrospray ionisation, ion trap, and time-of-flight mass spectrometry (LC ESI-IT-TOF MS) (Shimadzu, Kyoto, Japan). Peptide purity was also confirmed by reverse phase ultra-high performance liquid chromatography (RP-UHPLC) equipped with a photodiode array (PDA) and an evaporative light scattering detector (ELSD-LT) (Shimadzu, Kyoto, Japan) on a Kinetex C8 column (100 × 2.1 mm, 2.6 μ m, 100 Å), (Phenomenex, CA, USA), using a gradient from 5 to 100 % B in A over 15 min.

4.3. Disulfide bonds formation

To create the first disulfide bond, the peptides were dissolved in a mixture of water and methanol (1:9, v/v) at a concentration of 40 mg/L. The pH of the solution was adjusted to a range of 8 to 9 using 25 % ammonia water, and the mixture was stirred for 7 days while compressed air was bubbled through it. The reaction progress was monitored via RP-UHPLC. Then, the methanol was evaporated, and the remaining aqueous solution was freeze-dried. Peptides with a single disulfide bond were purified as previously described, while those with two disulfide bonds were subjected to a second oxidation process.

To form the second disulfide bond, the peptides were dissolved in a mixture of acetic acid, water and methanol (1:1:9, v/v/v) at a concentration of 40 mg/L. A 25–50-fold excess of iodine dissolved in methanol was then added to the solution. The magnetic-stirred mixture was left to react for a week at room temperature. Afterward, the mixture was filtered through a Dowex ion-exchange resin to remove the excess iodine. The solvents were then evaporated, and the peptide was dissolved in water and freeze dried. Finally, the peptides were purified according to the procedure described earlier.

4.4. SpS measurements

SpS experiments were performed using Monolith X (Nanotemper, Munich, Germany). Analyses were performed using the His-Tag Labeling Kit RED-tris-NTA 2nd Generation (Nanotemper, Munich, Germany). A fluorescent dye solution used in the experiment was prepared by dissolving 2 μ L of the original 5 μ M original dye solution in 98 μ L of deionized water to the final concentration of 0.1 μ M. Then 90 μ L of dye solution was added to 90 μ L of 100 nM LIGHT His protein dissolved in PBS-T (phosphate buffered saline with 0.05 % Tween-20 and 0.3 M NaCl, pH 7.4). The mixture was incubated at room temperature for 30 min. After this time, 10 μ L of solution was mixed in tubes with 10 μ L of peptides dissolved in PBS-T. For each peptide 16 concentrations ranging from 48 μ M to 1.464 nM were prepared, resulting in a final concentration of labelled protein at 25 nM and analyzed peptides from 24 μ M to 0.732 nM. The mixtures were loaded into standard Monolith X compatible capillaries. The fluorescence ratio of 670/650 nm was measured with an excitation power of 40 % and a medium IR laser power. The data was processed with MO.Control 2 software v2.5.4 (NanoTemper, Munich, Germany). Each experiment was repeated in triplicate.

4.5. ELISA

ELISA was prepared using immunograde transparent polystyrene 96-well plates (Brand, Wertheim, Germany). LIGHT-His protein, dissolved in PBS-T for a concentration of 2 μ g/mL, was added to each well in a volume of 100 μ L per well, and the plate was incubated at 4 °C overnight. After this time, unoccupied by LIGHT sites in wells were blocked using 5 % BSA in PBS-T, 200 μ L per well and incubated. Then, peptides dissolved in PBS-T in three concentrations 50 μ M, 12.5 μ M, and 3.125 μ M were added to the wells in a volume of 200 μ L per well and incubated. In the next step, 100 μ L of HVEM-Fc in PBS-T was added to each well in a concentration of 6 μ g/mL and incubated. Next step consisted of

adding 100 μL goat anti-human IgG antibody conjugated with HRP (Bio-Rad, Hercules, CA, USA) diluted in PBS-T in a ratio of 1:3000. After each of the above steps, the washing procedure was carried out, which consisted of adding 200 μL of PBS-T buffer to each well, and this process was repeated five times. The incubation processes described in this procedure were performed at 37 °C for 2 h, with the exception of the antibody, which was incubated for 1 h. In the final step, 200 μL of TMB (Thermo Fisher Scientific Inc., Waltham, USA) was added to each well and after 15 min of incubation in the dark, the absorbance was measured at 492 and 650 nm using an Infinite M200 Pro reader (Tecan, Männedorf, Switzerland). Wells in which PBS-T was added instead of the peptides were used as a control. The experiments were performed in at least in duplicate. The data are presented as mean with SD. The results were analyzed by one-way ANOVA followed by Dunnett's post hoc test using GraphPad Prism 8 software (San Diego, CA, USA).

4.6. Stability of peptides

Peptide stability tests were performed on supernatant obtained from Jurkat E6.1 cells. The supernatant was collected after 24 h of incubation of the cells (1 million cells per 10 mL of medium) under conditions previously described. The cells suspended in medium were transferred to a falcon and centrifuged in $300 \times g$ for 3 min, and then the supernatant was transferred to a separate vessel. 200 μL of supernatant was added to 50 μL of peptide solution in water at a final concentration of 1.5 mg/mL. Then the mixture was incubated at 37 °C with constant stirring. Samples were collected at times 0 h and 24 h of incubation and then frozen until the end of the experiment. Before analysis, the samples were thawed on ice. The 4-fold excess of absolute ethanol (v/v) was added to the samples and they were centrifuged at $24270 \times g$ for 20 min at 4 °C. The supernatant was collected, transferred to new tubes and evaporated. Next, the supernatant was resuspended in 120 μL water with 0.1 % TFA (v/v). The samples were analyzed by analytical RP-HPLC using Kromasil C8 analytical column (250×2.1 mm, 5 μm) with a linear gradient of 5 % to 100 % B solution in 60 min. The amount of peptide remaining in the sample was calculated by comparing the peak area of the sample collected at the corresponding time with the area of the peak of the peptide sample dissolved in water without incubation. The tests for each peptide were performed in triplicate.

4.7. Cellular-based assay

Cell lines used in experiments (JE6.1-NF- κB ::eGFP HVEM, TCS LIGHT, TCS Ctrl, TCS BTLA, TCS CD86 Ctrl) were derived from in-house stocks and have been previously described [31,60]. Cell lines was maintained in RPMI 1640 (Sigma Life Sciences, Burlington, USA) supplemented with 1 % penicillin and streptomycin with the addition of 10 % heat-inactivated FBS (Sigma Life Sciences, Burlington, USA).

25 μL of peptides at three different concentrations 200, 50 and 12.5 μM were preincubated in tubes with 25 μL of TCS LIGHT (2×10^4 cells per tube) for 1 h in 37 °C. After this time, the tubes were centrifuged at $500 \times g$ for 3 min, and the medium with unbound peptides was removed. Then the fresh medium was added and the cells with bound peptides were transferred to a 96-well culture-treated transparent plate (Sarstedt, Nümbrecht, Germany). In the next step 50 μL of JE6.1-NF- κB ::eGFP HVEM (50×10^4 cells per well) was added to each well. The plate was incubated at 37 °C 5 % CO_2 for 24 h. After this time, the plate was centrifuged in $800 \times g$ for 3 min, and 6 μL of allophycocyanin (APC)-conjugated mCD45 mAb was added to each well and incubated for 20 min at 4 °C to exclude TCS from analysis. The plate was washed with 100 μL of FACS buffer (PBS, 5–10 % FBS, 0.1 % NaN_3 sodium azide), which was later removed from the wells, and 50 μL of fresh FACS buffer was added to each well. The expression of eGFP in presence and absence (control) of peptides was then measured using a CytoFLEX flow cytometer (Beckman Coulter, Brea, USA) and analyzed using the FlowJo v10.10 software (FlowJo LLC, Ashland, USA). Data were analyzed using

GraphPad Prism 9 software (San Diego, CA, USA). Statistical analysis was performed using one-way ANOVA followed by the Dunnett's post hoc test. In parallel, experiments were also performed using cells without expression of the LIGHT protein (TCS CD86 Ctrl and TCS Ctrl), to exclude nonspecific effects of the peptides on these cells. The experiments were performed at least in duplicate. The same procedure was applied to test the inhibitory properties of the peptides towards the BTLA/HVEM complex, but TCS BTLA was used instead of TCS LIGHT. The inhibitory properties of the peptides were calculated by comparison with a control peptide that does not bind to the LIGHT protein and does not exhibit any inhibitory effect on the HVEM/LIGHT complex.

4.8. NMR measurements

NMR spectra were recorded at 25 °C on a Bruker 500 MHz spectrometer (^1H frequency 500.13 Hz, 11.7 T) and a Bruker 700 MHz spectrometer (^1H frequency 700.55 MHz, 16.4 T). The initial sample concentration was 2 mM in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1, v/v). The following NMR experiments were performed: 1D ^1H , 80 ms TOCSY, 100 and 200 ms NOESY and ^1H – ^{13}C HSQC. The ^1H – ^{13}C HSQC spectrum was recorded on the natural abundance of the ^{13}C isotope. All chemical changes were referenced to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) using $\Xi = 0.251449530$ ratio to indirectly referencing ^{13}C resonances [61].

4.9. Molecular modelling

Molecular dynamics simulations were performed with the ff19SB force field in the AMBER 24 package [62]. The structures of the peptide were computed using a simulated annealing (SA) algorithm with interproton distance restraints. The interproton distances were calculated on the basis of NOEs intensities by the CALIBA algorithm of the CYANA 2.1 program [63]. SA started from a random conformation. The interproton distances were restrained with the force constants of $f = 50$ kcal/(mol $\times \text{\AA}^2$). The geometry of the peptide groups (all *trans*) was kept fixed according to the NMR data ($f = 50$ kcal/(mol $\times \text{rad}^2$)). 300 SA cycles were carried out, where the n^{th} conformation initiated the $n + 1$ cycle. Each SA cycle consisted of 30,000 MD steps (30 ps). The system was heated for 1 ps to 1200 K, annealed at 1200 K for 2 ps, and finally cooled to 0 K over 27 ps. To approximate the solvent interaction, the generalised Born model was used [64]. Deviations from the experimental restraints were calculated using the SANDER module in AMBER. The conformations with a total distance penalty not exceeding 0.2 kcal/mol were minimised without any constraints. Afterward, the set of conformations was clustered into conformational families with the hierarchical agglomerative algorithm implemented in the CPPTRAJ module of the AMBER package. Principal moments of inertia (PMI) around inertial axes: I1, I2, and I3 were obtained using standard tool of GROMACS [65]. The molecular shape was represented by normalised PMI ratios (I1/I3 and I2/I3) for each conformation. Visualization of the structures was achieved with the help of VMD program [66]. Ramachandran plots were generated using the MOLMOL 2 K.2 program [67].

CRediT authorship contribution statement

Piotr Ciura: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Simon Gumpelmair:** Investigation. **Emilia Sikorska:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation. **Sylwia Rodziewicz-Motowidlo:** Writing – review & editing, Conceptualization. **Peter Steinberger:** Writing – review & editing, Supervision, Methodology. **Marta Spodzieja:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Adam K. Sieradzan:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2025.109183>.

Data availability

Data will be made available on request.

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Supplementary materials

HVEM-derived peptides as inhibitors of HVEM/LIGHT complex formation.

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Spectral shift

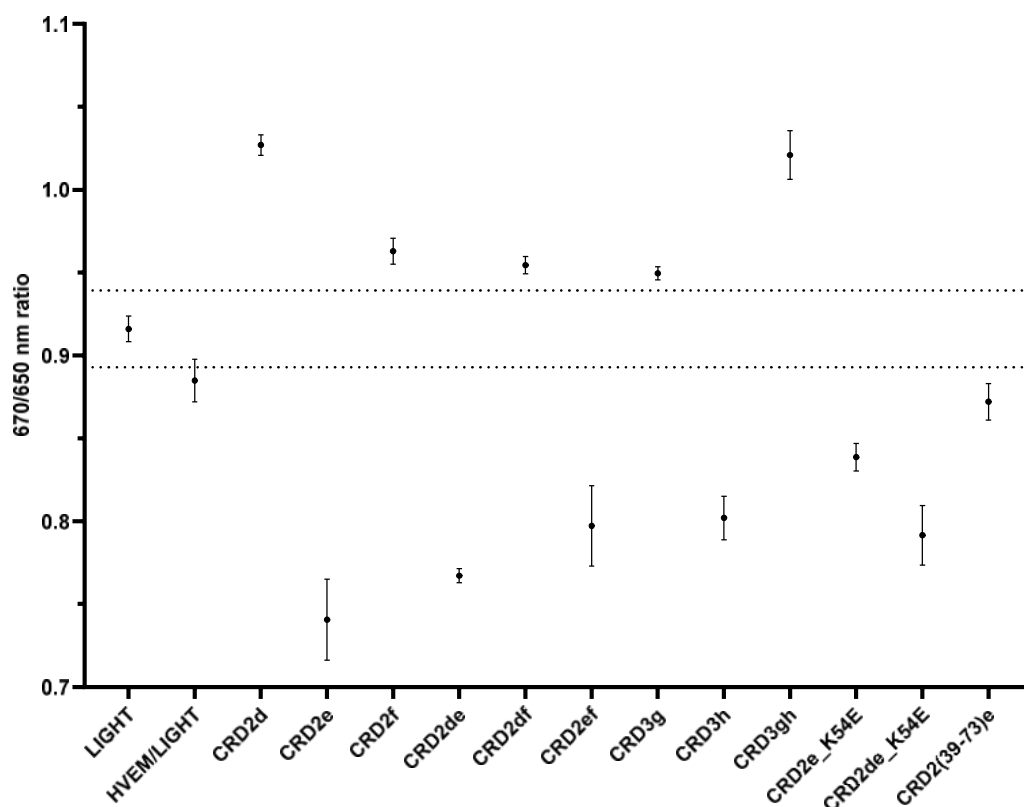


Figure S1. SpS binding check experiments of all designed compounds compared to control (LIGHT protein without any ligand). Dotted line represents three times the standard deviation range. The graph shows results from 3 independent experiments. Results are presented as mean with SD.

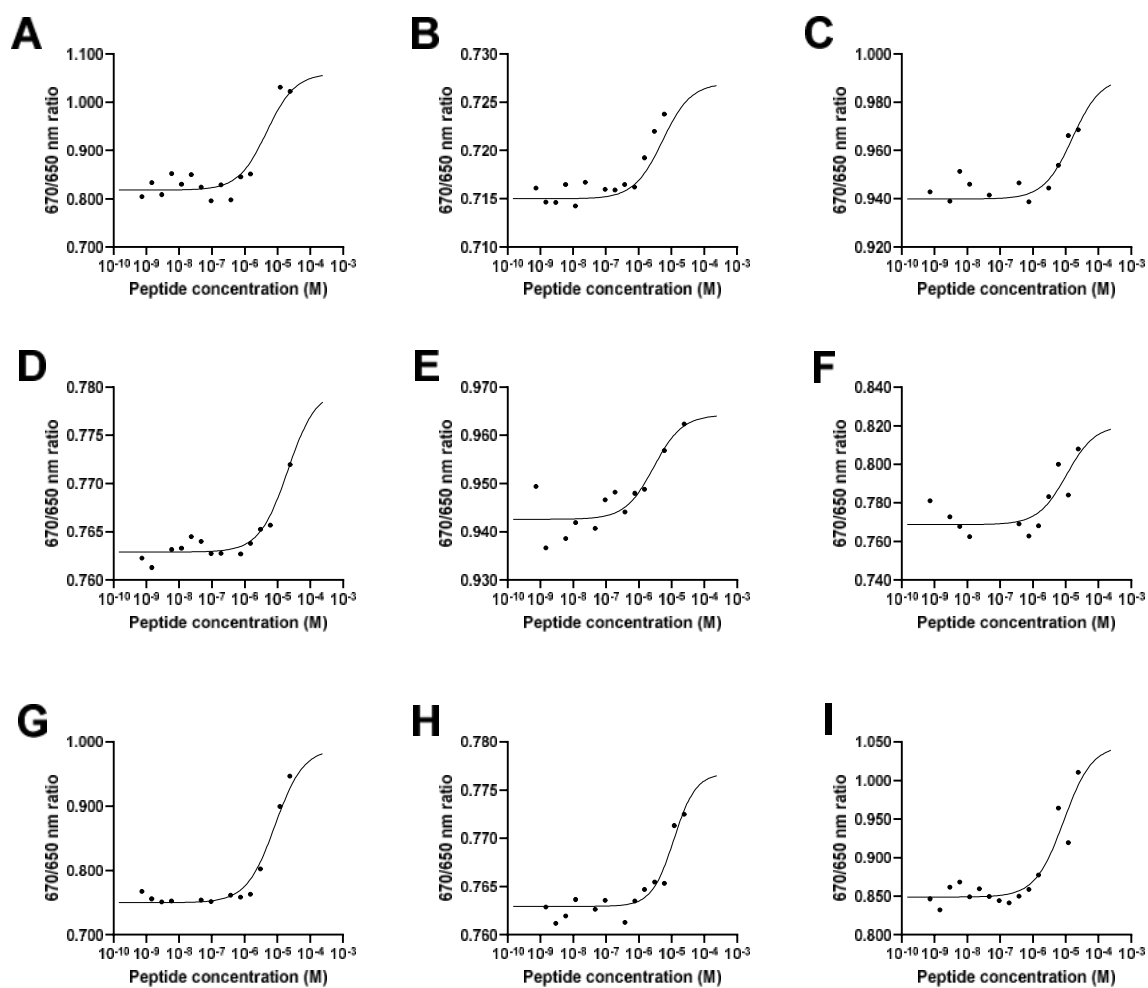


Figure S2. The spectral shift dose-response curves for the interactions between LIGHT and: A) CRD2d, B) CRD2e, C) CRD2f, D) CRD2de, E) CRD2df, F) CRD2ef, G) CRD3g, H) CRD3h, I) CRD3gh.

ELISA

Table 1. The amino acid sequence of CRD2(39-73)e and scrambled peptide; X_1 – 2-aminobutanoic acid, X_2 – norleucine, & – position of disulfide bond.

Peptide name	Peptide amino acid sequence
CRD2(39-73)e	Ac-PX ₁ PPGTYIHLNGLSKX ₁ LQC(&)QX ₂ X ₁ DPAX ₂ GLRASRNC(&)G-NH ₂
Scrambled peptide	Ac-HX ₁ STGIPLQPYX ₁ KGLLNPC(&)AX ₂ LX ₁ DGNQPRARX ₂ SC(&)G-NH ₂

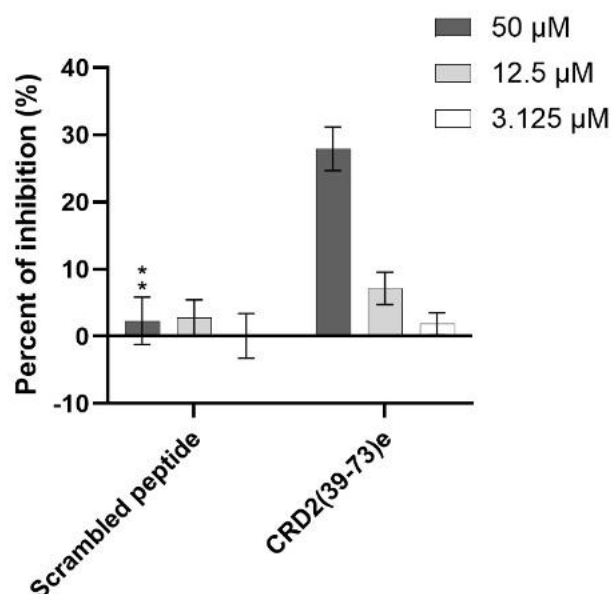


Figure S3. Comparison of the inhibitory properties of the CRD2(39–73)e peptide and the scrambled peptide toward HVEM/LIGHT complex formation, as determined by ELISA. The results represent a single experiment performed with nine replicates. Data are presented as the mean SD (mean $\pm$ SD). The ELISA tests were conducted under the same conditions as described in the manuscript, except that a different plate was used for protein immobilization: Nunc ImmunoPlates MaxiSorp (Thermo Fisher Scientific), instead of the ImmunoGrade (Brand) described in the main text. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

Cytotoxicity of peptides

Cytotoxicity of the compounds was designated using the commercially available CellTiter-Glo® Luminescent Cell Viability Assay. This test allows to determine the amount of adenosine triphosphate (ATP), which is an indicator of metabolically active cells. It is based on the enzyme luciferase, which in the presence of ATP (produced by living cells) is converted to oxyluciferin. The beforehand mentioned reaction generates a "glow-type" luminescent signal, which is then measured as luminescence. The percentage of viable cells was determined by comparing the luminescence measured for samples with and without peptide (control).

The cytotoxicity of the peptides was assessed for two cell lines – Jurkat E6.1 and TCS-Ctrl (modified BW5147 cells). Jurkat E6.1 cell line was purchased from Cell Line Service GmbH, and the TCS-Ctrl cell line is property of the laboratory of Prof. Peter Steinberger, Institute of Immunology, the Medical University of Vienna, Vienna, Austria. Cell lines prior to the experiments were maintained in RPMI1640 medium (Sigma-Aldrich, Missouri, USA) with

the addition of 1% of the antibiotics penicillin and streptomycin (Sigma-Aldrich, Missouri, USA) and 10% heat-inactivated FBS (Sigma-Aldrich, Missouri, USA) and incubated in a humidified incubator at 37°C in 5% CO₂ atmosphere. The culture medium was replaced every 2 to 3 days.

In white sterile cell culture intended 96-well plates (Thermo Fisher Scientific, Waltham, USA), 40 µL of cells containing 1×10^4 TCS/well and 2×10^4 Jurkat E6.1/well in culture medium were seeded. At the same time, serial dilutions of peptides in culture media ranging from 100 µM to 6.25 µM were prepared, and then 40 µL of peptides were added to each well with cells. The final volume in the well was 80 µL, and the final concentrations of the peptides ranged from 50 µM to 3.125 µM. Cells were incubated with peptides at 37°C in an atmosphere of 5% CO₂ for 24 h. After this time, the plates were equilibrated at room temperature for 10 min and CellTiter-Glo (Promega Corporation, Madison, USA) was added to each well in a volume of 80 µL per well. After 15 min luminescence was measured on a CLARIOstar plus plate reader (BMG Labtech, Ortenberg, Germany). As a control, cells without the peptides were used and the percentage of cell viability was calculated by comparing the viability of cells incubated with and without the peptide. The results are shown for three experiments performed independently in triplicate. Data were analysed using GraphPad Prism 9 software (San Diego, CA, USA). Statistical analysis was performed using one-way ANOVA followed by the Dunnet post hoc test.

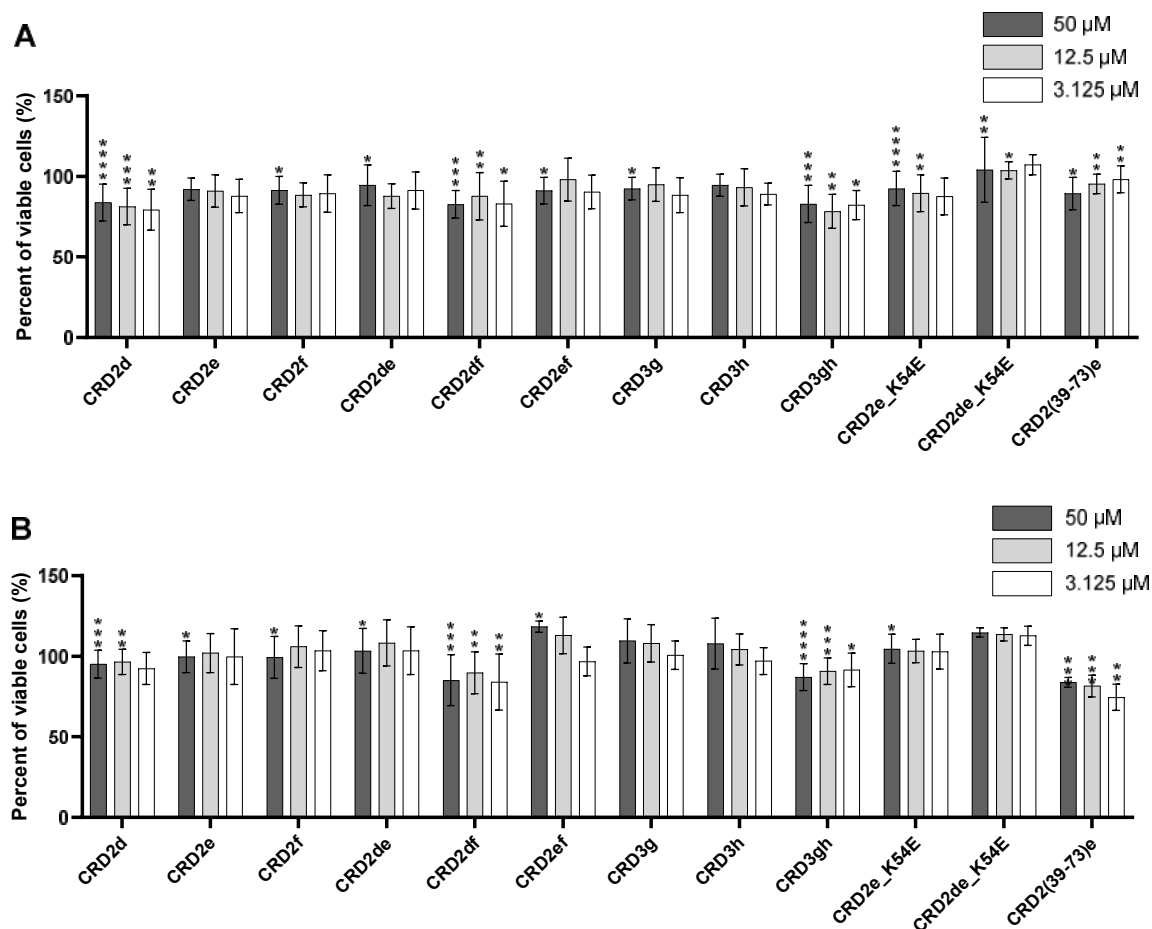


Figure S4. Cell viability assay used to define the influence of HVEM-derived peptides on A) Jurkat E6.1, B) TCS Ctrl cell lines after incubation for 24 hours. The results are shown for three experiments performed independently in triplicate. Data are depicted as mean with SD (mean $\pm$ SD). Statistical analysis was performed using one-way ANOVA followed by the Dunnett's post hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

Stability of peptides

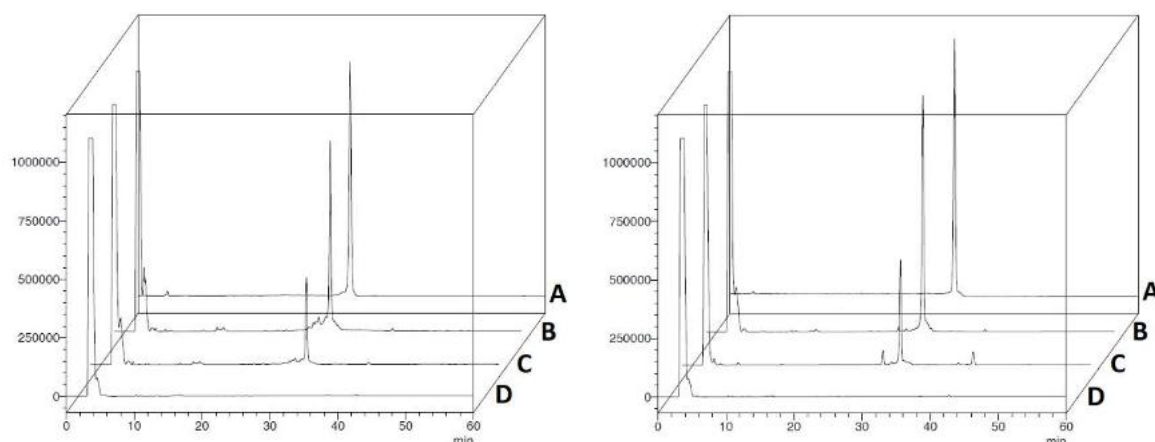


Figure S5. Chromatograms obtained for the peptides – the best inhibitors of HVEM/LIGHT complex formation: CRD2e_K54E (left) and CRD2(39-73)e (right):
A) peptide in H₂O at t=0 h, B) peptide in supernatant at t=0 h, C) peptide in supernatant after 24 h of incubation, D) supernatant at t=24 h.

NMR

Assignments of the proton resonances of CRD2(39-73)e were accomplished using standard procedures based on 2D TOCSY and 2D NOESY datasets (Table S2). The homonuclear NMR spectra were complemented by a heteronuclear 2D ¹H-¹³C HSQC experiment. Sequence-specific assignments were carried out in a standard manner, utilizing the H α -HN and HN-HN regions from the 2D ¹H-¹H NOESY spectra. Unfortunately, the resolution of the NMR spectra was insufficient for a complete assignment of the resonances. Increasing the temperature to 35°C resolved some of the resonances that were overlapping at room temperature. However, the assignment remained incomplete and, for instance, none of the norleucine residues were identified. All backbone amide protons were in the narrow range from 7.7 to 8.8 ppm, demonstrating that CRD2(39-73)e is unstructured. Moreover, the lack of medium- and long-range interproton distance constraints, along with H α chemical shift analysis, confirmed the absence of a well-defined secondary structure (Figure S6). Sequential connectivities between H α and H δ (X-Pro) indicated the presence of all *trans* X-Pro peptide bonds. However, additional cross-peaks observed in the NMR spectra suggested the presence of another isomer, likely arising from *cis/trans* isomerization at one of the X-Pro peptide bonds.

The upper interproton distances inferred from the NOE intensity were incorporated into molecular dynamics simulations using a simulated annealing protocol to model the three-dimensional structure of CRD2(39-73)e. However, the overlapping NOE cross-peaks, whose

intensities reflected contributions from multiple interproton distances, were largely excluded from the structural calculations. Ultimately, a total of 103 ^1H - ^1H distance constraints (67 intraresidual and 36 sequential) were employed. From an ensemble of 300 structures obtained for CRD2(39-73)e, 103 structures, each with a total distance penalty not exceeding 0.2 kcal/mol, were selected, minimized, and clustered into conformational families. The root mean square deviation (RMSD) of the backbone atoms in the cyclic part of the peptide was used as the distance metric. This process yielded 10 conformational families, with two dominant families collectively accounting for 52% of the structures (Figure S7). Despite this clustering, the peptide adopts a flexible statistical-coil conformation, with no discernible differences between the structures of the two dominant conformational families. The RMSD values for the backbone atoms from residues 20 to 35 were 2.38 ± 0.35 Å and 2.20 ± 0.37 Å relative to the average conformation for families 1 and 2, respectively. These high RMSD values indicated significant variability of the conformations within each conformational family. The average radii of gyration for conformations in the families 1 and 2 were 7.60 ± 0.45 and 7.23 ± 0.38 Å, respectively. The molecular shape, as defined by the normalized principal moments of inertia and visualized in 2D triangular graphs (Figure S9), revealed a distribution of molecular shapes rather than a single, well-defined point. Overall, the conformations adopted relatively rodlike shapes, though some structures within the largest conformational family (family 1) showed a slight tendency toward a more spherical geometry.

Table S2. Proton chemical shifts for CRD2(39-73)e at 25°C.

Residue	Proton chemical shifts /ppm			
	HN	H α	H β	Others
P1	-	4.39	1.89, 2.26	$\underline{\text{CH}_3}\text{CO}$ 2.08, H γ 1.97, H δ 3.62
X12	8.26	4.5	1.65, 1.78	γ -CH $_3$ 0.95
P3	-	4.7	1.86, 2.36	H γ 2.00, 2.03, H δ 3.59, 3.86
P4	-	4.42	1.94, 2.33	H γ 2.06, H δ 3.66, 3.85
G5	8.54	3.99, 3.92		
T6	7.86	4.25	4.08	γ -CH $_3$ 1.12
Y7	8.30	4.55	2.97	H δ 7.07, H ϵ 6.78
I8	7.92	3.97	1.72	H γ 1.11, 1.39, γ -CH $_3$ 0.78; δ -CH $_3$ 0.85
A9	8.19	4.16		β -CH $_3$ 1.31
H10	8.37	4.67	3.17, 3.26	H ϵ 1 8.60, H δ 2 7.27
L11	8.28	4.32	1.55, 1.64	H γ 1.60, δ -CH $_3$ 0.84, 0.89
N12	8.52	4.67	2.79, 2.85	δ -NH $_2$ 6.94, 7.61
G13	8.35	3.94		
L14	8.06	4.38	1.61, 1.66	H γ 1.60, δ -CH $_3$ 0.86, 0.91
S15	8.24	4.4	3.86, 3.91	
K16	8.35	4.27	1.75, 1.85	H γ 1.41, 1.45, H δ 1.67, H ϵ 2.98, ζ -NH $_2$ 7.53

X ₁ 17	8.09	4.15	1.71, 1.77	γ -CH ₃ 0.80
L18	8.12	4.31	1.57, 1.64	H γ 1.60, δ -CH ₃ 0.86, 0.91
Q19	8.32	4.29	2.01, 2.11	H γ 2.37, ϵ -NH ₂ 6.77, 7.43
C20	8.39	4.64	3.08, 3.18	
Q21	8.41	4.35	2.02, 2.10	H γ 2.36, ϵ -NH ₂ 6.77, 7.38
X ₂ 22	Not determined			
X ₁ 23	8.10	4.13	1.71, 1.77	γ -CH ₃ 0.91
D24	8.29	4.94	2.75, 2.93	
P25	-	4.34	2.30	H γ 2.01, H δ 3.82
A26	8.1	4.26		β -CH ₃ 1.40
X ₂ 27	Not determined			
G28	8.45	3.91		
L29	7.98	4.32	1.59, 1.65	H γ 1.60, δ -CH ₃ 0.86, 0.91
R30	8.31	4.28	1.78, 1.88	H γ 1.61, 1.66, H δ 3.19, ϵ -NH ₂ 7.20
A31	8.19	4.32		
S32	8.22	4.44	3.82, 3.92	
R33	8.32	4.35	1.77, 1.88	H γ 1.62, 1.65, H δ 3.20, ϵ -NH ₂ 7.18
N34	8.44	4.73	2.76, 2.86	δ -NH ₂ 6.92, 7.59
C35	8.37	4.71	3.02, 3.27	
G36	8.44	3.92		CO-NH ₂ 7.01, 7.45

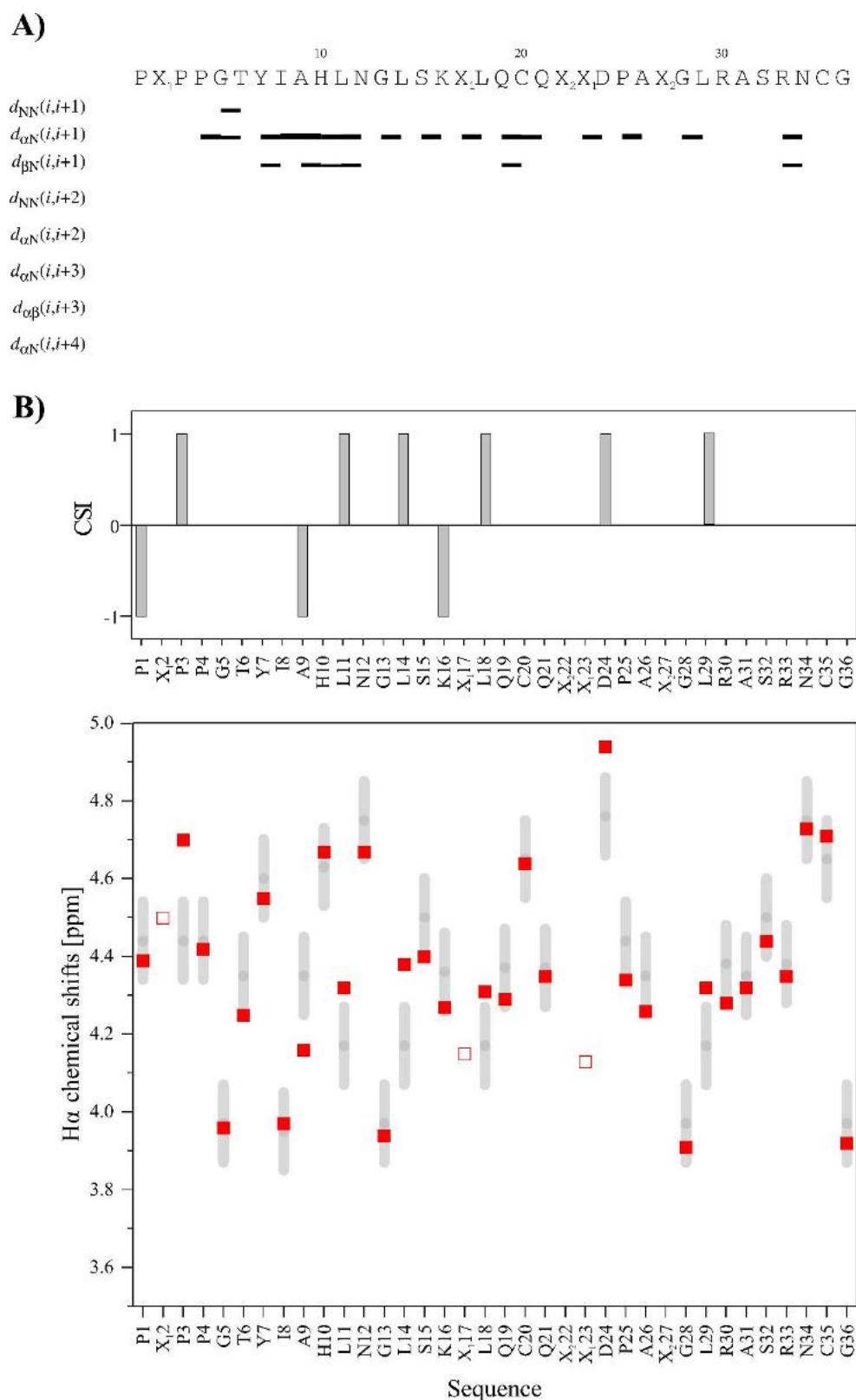


Figure S7. (A) Sequence plots of NOESY distance constraints for CRD2(39-73)e. (B) H_{α} chemical shift index (top) and a more detailed analysis of H_{α} chemical shifts (bottom). Red squares correspond to the H_{α} chemical shifts of the CRD2(39-73)e residues at 25°C, while gray bars indicate the random coil H_{α} chemical shift range only for naturally occurring amino acids. The H_{α} chemical shifts of X_1 (2-aminobutanoic acid) residues are marked with empty squares. The chemical shifts of X_2 (norleucine) residues were not determined.

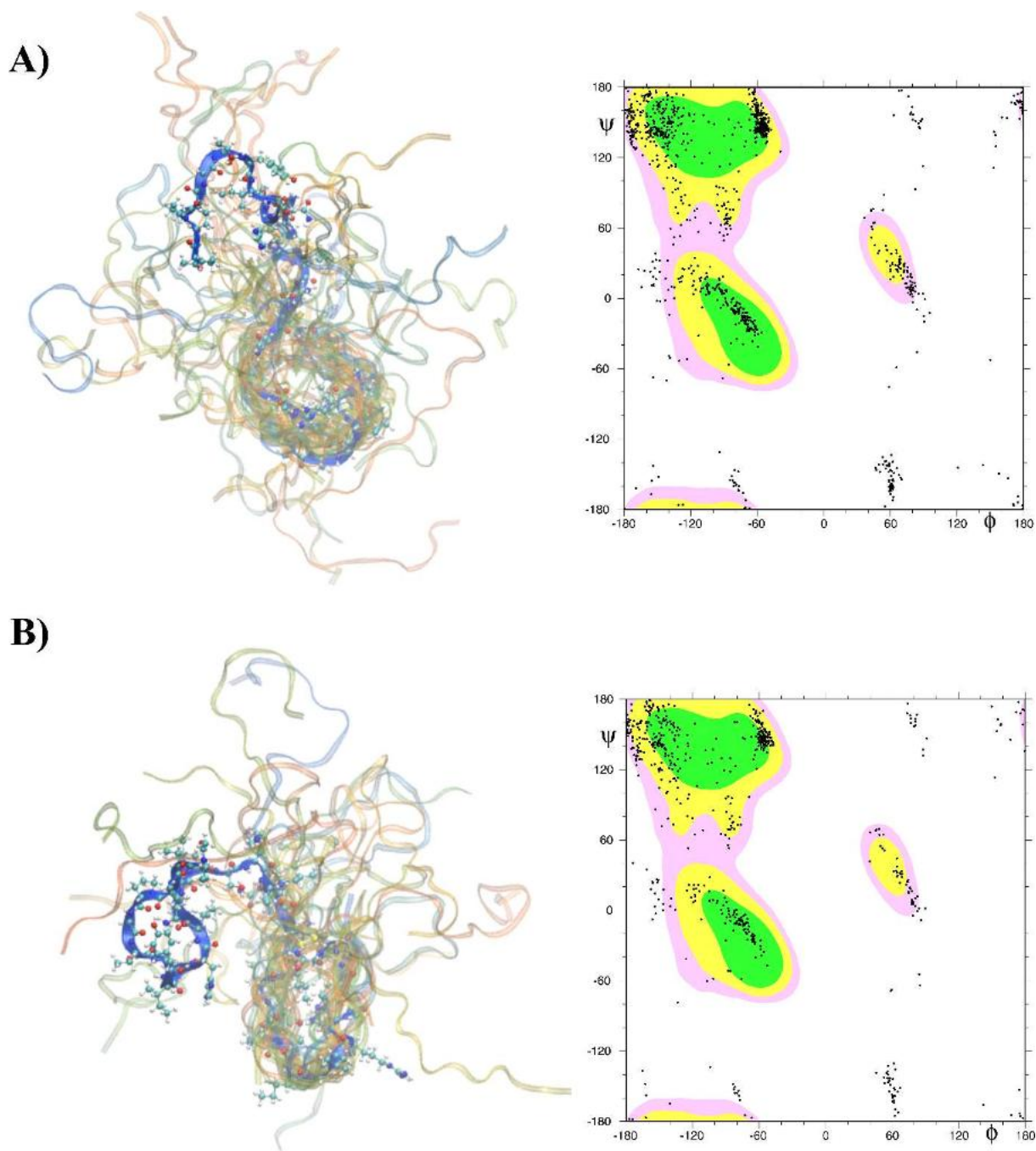


Figure S8. Superimposed conformations and Ramachandran plots of two dominant conformational families of CRD2(39-73)e. (A) Family 1 consisted of 33 structures. RMSD for the backbone atoms from residues 20 to 35 was 3.48 ± 0.56 Å relative to the first conformation and 2.38 ± 0.35 Å relative to the average conformation, (B) Family 2 consisted of 21 structures. RMSD for the backbone atoms from residues 20 to 35 was 2.77 ± 0.49 Å relative to the first conformation and 2.20 ± 0.37 Å relative to the average conformation.

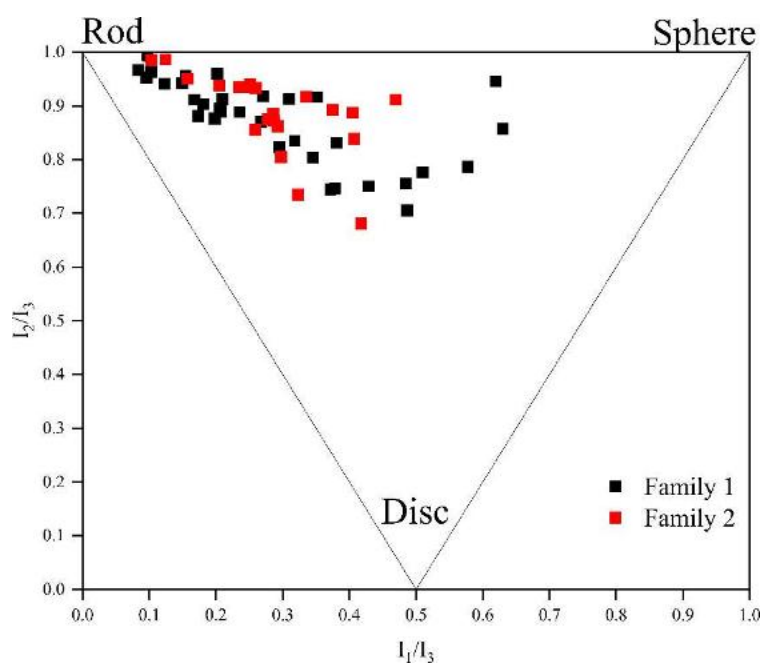


Figure S9. Principal moment of inertia (PMI) plot illustrating the three-dimensional shape diversity of the conformations of CRD2(39-73)e from two dominant conformational families.



OPEN Evaluation of LIGHT-derived peptides to disrupt the HVEM/LIGHT immune checkpoint

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Immune checkpoints are critical regulators of immune homeostasis and have become prominent targets in the treatment of various malignancies and autoimmune disorders. While monoclonal antibodies currently dominate checkpoint-targeted therapies, there is a growing interest in alternative approaches, which may offer advantages including smaller size, greater specificity, ease of manufacturing, or a reduced risk of immune-related adverse events. In this study, we focused on the HVEM and its ligand LIGHT, a receptor-ligand pair involved in modulating T cell responses. Overstimulation of HVEM/LIGHT pathway is associated with the pathogenesis of autoimmune diseases and can lead to graft rejection. Therefore, targeting this interaction may offer novel therapeutic strategies for inducing immunosuppression and preventing graft rejection. Utilizing computational approaches, we designed a series of LIGHT-derived peptides. Various simulation techniques, such as molecular dynamics with MMGBSA analyses (with and without NMA), work associated with conformational change and SMD were employed to predict their binding affinities to HVEM. Selected peptides were synthesized and subjected to experimental validation to assess their binding capabilities to HVEM, determined using the spectral shift technique, and inhibitory effects on the HVEM/LIGHT complex formation, evaluated through immunoenzymatic assays and cellular studies. Among these, two peptides demonstrated inhibitory potential, suggesting that they might have utility as scaffold for further optimization. These findings underscore the potential of peptide-based inhibitors in modulating immune checkpoints and pave the way for novel immunotherapeutic strategies.

Keywords Disulfide-linked peptide, ELISA, Cellular assay, Spectral shift, MMGBSA

The immune response is precisely regulated through a complex network of co-stimulatory and co-inhibitory signals orchestrated by immune checkpoints (ICPs), which play a crucial role in maintaining immunological balance^{1,2}. Herpes virus entry mediator (HVEM) and its ligand – homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes (LIGHT) are proteins belonging to stimulatory checkpoints, and their interaction leads to the stimulation of the immune system, resulting in activation and proliferation of naïve T cells, and the secretion of pro-inflammatory cytokines. The immune system utilizes this complex to combat threats, but its dysregulation can contribute to immune pathologies, making it a valuable target for therapeutic intervention^{1–3}. Previous studies have shown that blockade of the HVEM/LIGHT interaction with the use of antibodies or soluble receptors can reduce allogeneic immune responses, improve graft survival, and mitigate graft-versus-host disease (GVHD)^{4,5}. Nevertheless, a need for novel compounds with different pharmacokinetic properties capable of disrupting HVEM/LIGHT interaction and inducing effective immunosuppression remains.

LIGHT is a ligand of the tumour necrosis factor superfamily (TNFSF). The protein is a cell-surface homotrimer. Each monomer is made up of 240 amino acids and divided into three domains: the cytoplasmic (residues 1–37), the transmembrane (residues 38–58), and the extracellular (residues 59–240)⁶. The monomer of the LIGHT is stabilized by one disulfide bond between cysteines at positions 154 and 187. A distinctive feature of LIGHT is the presence of a TNF homology domain (THD) at the C-terminal part of the ectodomain, which enables the protein trimerization and is responsible for the interaction of LIGHT with its receptors⁵. This

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protein can also exist in a soluble form generated through alternative mRNA splicing or proteolytic cleavage of its extracellular domain⁷. The LIGHT interacts with several receptors from TNFSF: the previously mentioned HVEM, decoy receptor 3 (DcR3)⁸ and lymphotoxin β receptor (LT β R)⁹. The binding of the LIGHT with the HVEM and LT β R results in the activation of signalling pathways responsible for the stimulation of the immune system¹⁰, while its interaction with the DcR3 inhibits immune activation via LIGHT by preventing the formation of the HVEM/LIGHT and LT β R/LIGHT complexes¹¹. LIGHT is expressed on dendritic cells (DCs), monocytes, T cells, B cells, and myeloid cells³. Its expression on immature DCs implies that LIGHT may have a significant role in the DC-mediated co-stimulation of T cells⁵.

HVEM belongs to the TNFSF receptor, and it is a 283 amino acid protein composed of a signal peptide (residues 1–38), extracellular (39–202), transmembrane (203–223), and intracellular (224–283) parts. The extracellular region of HVEM contains four cysteine-rich domains (CRDs) linked by disulfide bonds^{12–14}. Structural information is well-defined for the first three CRDs of HVEM, which are involved in binding receptors from the immunoglobulin superfamily (IgSF), such as B and T-lymphocyte attenuator (BTLA)^{15,16} and cluster of differentiation 160 (CD160)¹⁷, as well as ligands from TNFSF, such as LIGHT and lymphotoxin α (LT α)^{18,19}. HVEM exhibits bifunctional immunomodulatory properties. It can engage with inhibitory receptors such as BTLA and CD160 to suppress T cell activation or function as a receptor for LIGHT and LT α , thereby delivering co-stimulatory signals that enhance T cell activation^{20,21}. HVEM is expressed on T cells, B cells, natural killer (NK) cells, DCs, and myeloid cells^{13,22}.

The HVEM/LIGHT complex has a 3:3 stoichiometry, in which a trimer of LIGHT binds three molecules of HVEM. CRD2 and CRD3 of HVEM are mainly involved in specific interactions with LIGHT; however, some residues from CRD1 are also engaged in protein binding. The crystal structure of the HVEM/LIGHT complex (PDB code 4RSU) shows that Y173 from DE loop, G100 from AA' loop, and R226, R228 from GH loop of LIGHT interact with CRD1 and CRD2 of HVEM, while G151, V152, A159–T161 from CD loop, and Q183, R195, V196 and W198 from EF loop of LIGHT bind to CRD3 of HVEM²³. The interaction of HVEM present on T cells and LIGHT on immature DCs mediates the co-stimulation of T cells, resulting in the activation of several signalling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which is crucial for regulating immune responses, cell survival, and inflammation²⁴. Under certain conditions, such as autoimmune diseases characterized by excessive immune activation or in transplant patients it is necessary to dampen immune responses. One promising therapeutic approach involves inhibiting the formation of the HVEM/LIGHT complex, thereby attenuating downstream signalling pathways involved in immune activation. Many studies confirmed that blocking the interaction of LIGHT with its receptors HVEM and LT β R using monoclonal antibodies or soluble form of receptors (LT β R-Ig and HVEM-Ig) reduced allogeneic T cell immune responses *in vitro* and *in vivo*^{25–27}.

Our research is one of the first to focus on the design of peptide inhibitors based on the LIGHT protein. Agostino et al. also performed the computational analysis and designed number of peptides based on LIGHT protein-binding fragments to HVEM and LT β R, where they showed that those peptides have a potential as therapeutics for the metabolic syndrome, but their affinity to receptors and mechanism of action were not investigated²⁸. Our research focused on designing peptides to block HVEM/LIGHT interactions. The crystal structure of the HVEM/LIGHT complex was used as a template to design five compounds for which molecular dynamics (MD) and molecular mechanics generalised Born and surface area continuum solvation (MMGBSA) analyses were performed. The amino acid sequences of these peptides were based on two fragments of LIGHT, the first covering the residues from 210 to 240 (peptides light1 and light2) and the second from 171 to 195 (peptides light3, light4, and light5). The binding of compounds to LIGHT was, at first studied, using computational methods – MMGBSA and steered molecular dynamics (SMD). The highest theoretical affinity and the most stable complex with HVEM was created by light3, and therefore nine analogues of this peptide were designed. In the next step, the selected peptides were studied in more detail: enthalpy, entropy, binding free energy, internal work, total work, and force max for the peptide/HVEM complexes were determined. These peptides were also synthesized and their affinity for the HVEM by spectral shift (SpS) technique, and their inhibition properties by enzyme-linked immunosorbent assay (ELISA) and cellular assays were evaluated. We designed overall 14 peptides based on LIGHT structure, which were termed with lower case “light” and number and mutation (if occurring). The best results were obtained for peptide light3 and its analogue light3_E178L. These compounds are promising candidates for further exploration as immunomodulatory agents.

Results and discussion

Peptide design based on various computational approaches

The first step of this study was to design peptides – fragments of the LIGHT protein that can bind to HVEM and thus inhibit the formation of the HVEM/LIGHT complex. For this purpose, MMGBSA analysis performed for the crystal structure of the HVEM/LIGHT complex (PDB code 4RSU, chains A–D) were used to determine key amino acids within the LIGHT that are responsible for binding to HVEM (Figure S1). The analysis was carried out for a complex consisting of a LIGHT trimer and a HVEM monomer. This method allows for a relatively quick and low-cost estimation of the energy of the interaction between the target and the ligand^{29,30}. Per-residue and pairwise per-residue energy decomposition were also performed to determine the interaction energy of individual residues and their contribution to the interaction with HVEM.

Analyses of the HVEM/LIGHT complex, carried out, revealed that HVEM interacts with two monomers of LIGHT, binding to residues 171–177 of the first LIGHT molecule and five regions: T99–L105, E115–F122, Q148–V152, S192–W198 and R226–F235, of the second LIGHT molecule. Per-residue and pairwise per-residue energy decomposition indicate that the lowest energy values were observed for the fragment R226–R228 and residue R172 in LIGHT, confirming that these amino acids are essential for interaction with the receptor (Figure S1). The crystal structure of the HVEM/LIGHT complex, including the binding sites that served as the basis for

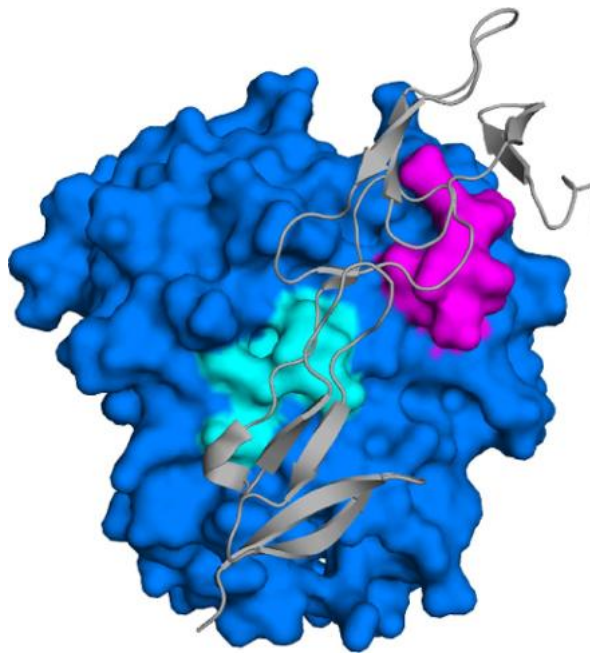


Fig. 1. Structure of the HVEM (grey ribbon)/LIGHT (blue surface) complex with two binding regions of the LIGHT highlighted: P171–L177 (magenta) used for the design of light3, light4, and light5 peptides, and R226–F235 (cyan), used for the development of light1 and light2.

No.	Peptide designation	Amino acid sequence
1	light1	Ac-E ²¹⁰ AGEEVVC(&)RVL ²²⁰ DERLVRRLRDG ²³⁰ TRSYC(&)GAFMV ²⁴⁰ -NH ₂
2	light2	Ac-E ²¹⁰ C(&)GEEVVVRVL ²²⁰ DERLVRRLRDG ²³⁰ TRSYFGAFC(&)V ²⁴⁰ -NH ₂
3	light3	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEELELL ¹⁸⁰ VSQC(&)S-NH ₂
4	light4	Ac-TIC(&)HGLYKRT ¹⁷⁰ PRYPEELELL ¹⁸⁰ VSC(&)QS-NH ₂
5	light5	Ac-TITC(&)GLYKRT ¹⁷⁰ PRYPEELELL ¹⁸⁰ VC(&)QQS-NH ₂
6	light3_E176K	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEKLELL ¹⁸⁰ VSQC(&)S-NH ₂
7	light3_E176L	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPELELL ¹⁸⁰ VSQC(&)S-NH ₂
8	light3_E176Q	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEQLELL ¹⁸⁰ VSQC(&)S-NH ₂
9	light3_E178K	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEELKLL ¹⁸⁰ VSQC(&)S-NH ₂
10	light3_E178L	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEELLLL ¹⁸⁰ VSQC(&)S-NH ₂
11	light3_E178Q	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEELQLL ¹⁸⁰ VSQC(&)S-NH ₂
12	light3_E176K_E178K	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEKLKLL ¹⁸⁰ VSQC(&)S-NH ₂
13	light3_E176L_E178L	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPELLLLL ¹⁸⁰ VSQC(&)S-NH ₂
14	light3_E176Q_E178Q	Ac-TC(&)THGLYKRT ¹⁷⁰ PRYPEQLQLL ¹⁸⁰ VSQC(&)S-NH ₂

Table 1. The amino acid sequence of the designed peptides, fragments of LIGHT protein; & – position of disulfide bond. Methionine in synthesized peptides has been replaced with norleucine.

peptide design, is shown in Fig. 1. Based on these results, five peptides were initially designated namely: light1 and light2, covering the fragment 210–240 of LIGHT, and light3, light4, and light5, based on the residues of 161–185 of LIGHT.

The fragments of LIGHT involved in the interaction with HVEM adopt a β -harpin conformation. To stabilize the structure of the designed peptides the appropriate amino acid sequences were elongated and disulfide bonds were introduced by substituting selected residues with cysteines. This strategy, previously employed by us and others, has demonstrated that both the presence and the position of a disulfide bond within a peptide can be critical for its interaction with a molecular target^{31–36}. The amino acid sequences of the peptides are shown in Table 1 (Peptide 1–5).

Short molecular dynamics simulations and their analyses reveal most promising inhibitors

Next, short molecular dynamics simulation (200 ns) were conducted to estimate the binding affinity of all of the designed peptides to HVEM. The RMSD analysis confirmed that peptides reach equilibrium within 200 ns (Figure S2). In addition, longer simulations (1000 ns) were carried out for a subset of selected complexes to

gain deeper insights into the nature of the peptide/HVEM interactions. Based on results obtained from short simulations, light5 was excluded from future studies, because it did not form a stable complex with receptor (Fig. 2A) whereas light2 and light4 exhibited very weak binding to HVEM. A similar pattern was observed in case of long simulations (Figure S3). The strongest interaction with the HVEM was obtained for the light3 (Fig. 2A and S3); therefore, the effective theoretical binding energy decomposition (per-residue and pairwise per-residue) for the light3/HVEM complex was performed to identify key amino acids contributing to binding with HVEM.

Determination of substitution directions to improve affinity based on per-residue and pairwise decomposition

The energy values obtained from both, per-residue and pairwise per-residue decomposition indicated that R172, Y173, and P174 in light3 are crucial for the interaction with HVEM, whereas the remaining residues in the peptide have no significant effect on the binding to the protein. This was also confirmed by LigPlot +³⁷ analysis (Figure S4). The spatial analysis of representative structure of light3 reveals that those residues bind to HVEM-formed groove. This groove is formed by residues L1, Y9–V11 and I71–L77 from HVEM (Figure S5). A potentially minor destabilizing effect of amino acids E176 and E178 on the protein/peptide complex was observed (Fig. 3). Therefore, in the next step, these residues were substituted by lysine, leucine and glutamine (Table 1, Peptides 6–16). The negatively charged Glu178 of Light3, in conjunction with Asp97 and Asp99 of HVEM, electrostatically disfavors the formation of an extended interaction surface (Figure S6). These amino acid substitutions were chosen to change the chemical nature of these residues – aimed to alter the properties associated with the side chain of glutamic acid – opposite charge, hydrophobic residue with similar size and neutral charge with the same size, respectively, were selected). Structural analysis of light3 allowed to conclude that these residues are not located in close proximity to the HVEM protein and therefore do not have an impact on interaction with HVEM. Rather they seem to have a destabilizing effect on the peptide itself as both negatively charged residues are in close proximity. Their electrostatic repulsion may contribute to an unfavourable entropic effect on the peptide structure; therefore, substituting these residues could potentially enhance the peptide/HVEM interactions. The best result was obtained for the peptide light3_E178L, in which glutamic acid in position 178 was replaced by leucine (Fig. 2B).

It should be noted that regions of LIGHT established in our work are similar to previously established by Agostino et al.²⁸. Their computational analysis lead to designing of several peptides based on LIGHT fragments that interact with HVEM and LTβR, covering residues 98–117, 166–180, and 19–22^{28,29}. Their in vitro studies indicated that peptide 2 composed of the amino acid from 166 to 180 promotes insulin sensitivity and its sequence was optimized using computational methods. The authors developed six analogues of this peptide, each containing a disulfide bond to stabilize the β-hairpin conformation (similarly as in our case) and conducted a series of in vitro and in vivo experiments. Their results suggest that peptides derived from LIGHT could be a novel class of therapeutics for obesity and type 2 diabetes by improving glucose and lipid metabolism.

In our previous studies, using molecular dynamics simulations and MMGBSA analysis, we designed several peptides covering the CRD2 and CRD3 of the HVEM, responsible for interacting with LIGHT. Next we demonstrated that two peptides (CRD2(39–73) and CRD2_K54E) have the highest inhibitory potential to disrupt HVEM/LIGHT complex formation in molecular dynamics simulations³⁸, which was also confirmed by

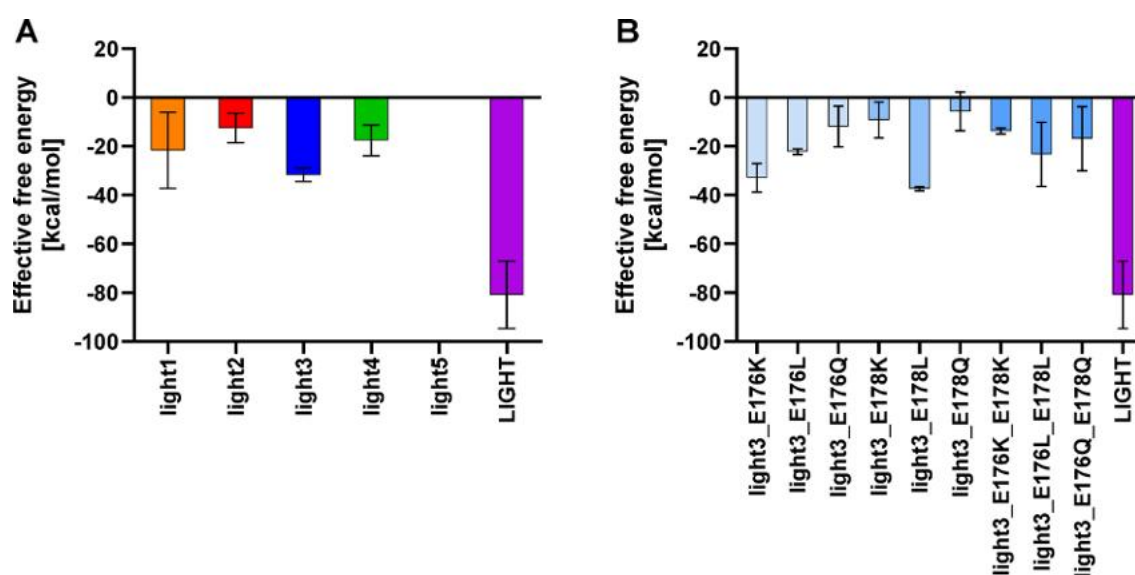


Fig. 2. Diagram of MMGBSA – derived effective binding free energy for 200 ns simulations for initial peptides (A) and light3 peptide analogues (B). The results are presented as mean ± SD based on analyses from three independent trajectories.

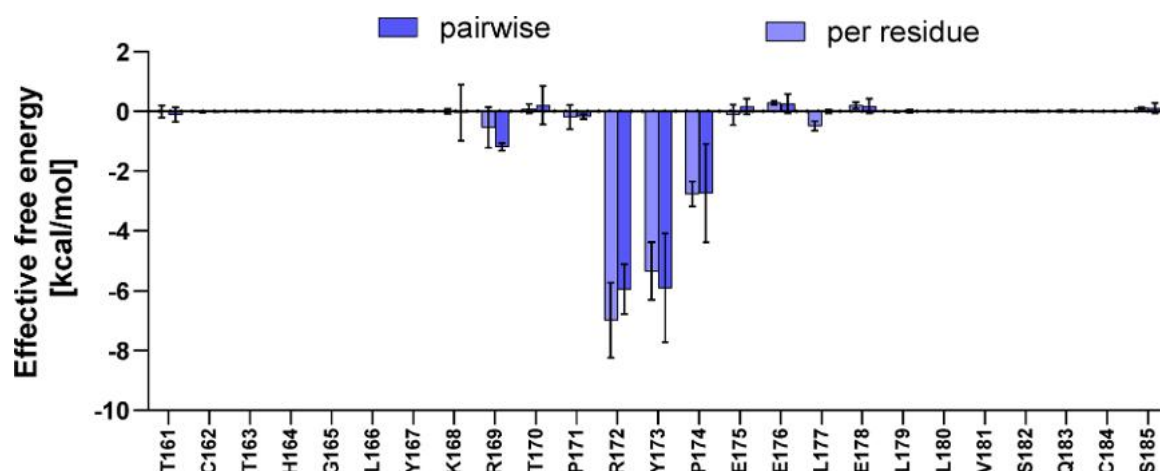


Fig. 3. MMGBSA effective theoretical binding energy decomposition (per-residue and pairwise) calculated for light3 peptide. Results are shown for 3 independent trajectories as mean $\pm$ SD.

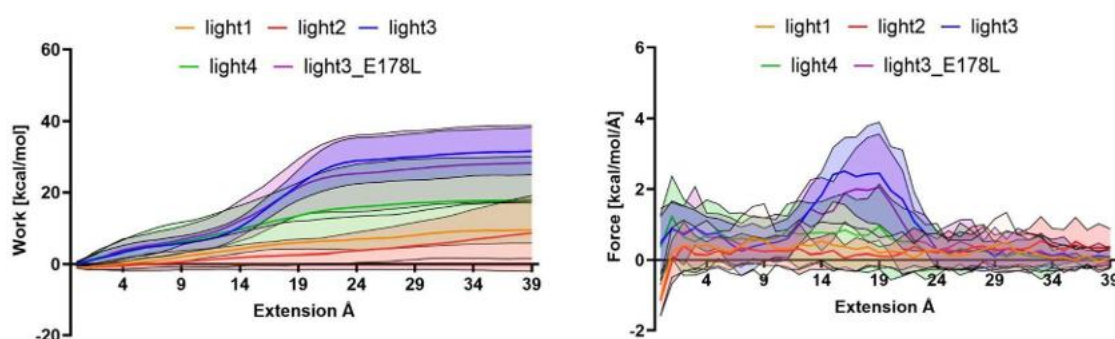


Fig. 4. Diagram of total work (A) and maximum force (B) necessary for dissociation of the peptide/HVEM complex. The results are presented as mean $\pm$ SD.

experimental studies (Ciura et al. submitted). Taken together, these results highlight the utility of computational methods in both the design and affinity prediction of target-specific compounds.

The study of peptide/HVEM binding using SMD

As MMGBSA does not always reveal all important aspects of binding properties³⁸, therefore, for subsequent analyses, peptides light1–4 and the most promising of light3 analogue, light3_E178L, were selected for SMD simulations. This method, which involves the application of a mechanical force to gradually displace the peptide from the binding site on HVEM, provides valuable quantitative data regarding the strength and stability of peptide/protein interactions. The binding strength was evaluated based on the force required for unbinding and the work performed during the pulling process^{39,40}.

Among the tested peptides, light3 and light3_E178L required the highest work to dissociate from the HVEM protein, indicating the strongest theoretical binding affinity (Fig. 4A). Additionally, detachment of light3 and light3_E178L, is associated with significant energy barriers, which is revealed as a pronounced force peak at around 19 Å between centres of mass (Fig. 4B). In contrast, no such force spikes were observed for the other peptides, whose dissociation occurred in a more gradual and unresisted manner, indicative of weaker interactions with the molecular target (Fig. 4A and B). These findings further support the conclusion that light3 and light3_E178L exhibit the highest theoretical binding affinity toward HVEM and are in correlation with previous findings from both long and short MD simulations.

Representative structures of the light3 and light3_E178L peptides were obtained by clustering the conformations at extension 19 ± 0.5 Å, corresponding to the largest force and work peaks identified in the SMD simulations (Fig. 5). Structural analysis of these, revealed notable differences in the interaction interfaces between the light3/HVEM and its analogue, light3_E178L/HVEM. The light3 peptide engages the HVEM receptor through a localized interaction hotspot, primarily involving residues R172 and Y173. In contrast, the light3_E178L establishes contacts with a broader set of HVEM residues; in the analysis of the starting structure interactions in SMD appear to be more dispersed and potentially weaker (Fig. 5), suggesting a different binding mode. Similar properties were noted in our earlier work when the shortening of the peptide, modified to improve affinity for LIGHT, also altered the character of the interaction plane³⁸.

Analysis of changes in thermodynamic values of peptides in complex with HVEM

To gain deeper insights into the thermodynamic properties of the peptide/HVEM complexes and allow for the future sequence optimisation, we analysed several energetic parameters such as Gibbs free energy (ΔG), internal work (ΔW), the sum of these two values ($\Delta G + \Delta W$), free enthalpy of complex formation (ΔH), and entropy ($T\Delta S$). In addition, we also analyzed the values of $F_{\max}$ (maximum force determined during dissociation) and W_{total} (work determined at maximum dissociation). Together these values allow for a deeper understanding of the nature of the peptide/protein interaction, which was proven in our previous work³⁸. The results of these analyses were summarised in Fig. 6. The entropic contribution ($T\Delta S$, magenta) reveals the unfavourable effect of reduced conformational freedom following binding. These parameters were analysed for both short and, for comparison, long (Figure S3) simulation timescales. W_{total} and $F_{\max}$ obtained from SMD simulations were also included in the Fig. 6 to provide a deeper understanding of the interactions between peptides and the HVEM and to achieve a more comprehensive analysis. Similar analyses in our previous study allowed for detailed analyses of peptide/protein interactions and confirmed peptide affinity for the molecular target, as determined by independent methods³⁸.

The analyses indicate that for peptides light1, light2 and light4, the $T\Delta S$ contribution dominates over the ΔH , resulting in positive ΔG value, which suggest a low affinity of these peptides to the molecular target. In contrast, peptides light3 and light3_E178L exhibit a dominant or similar ΔH contribution over $T\Delta S$, and ΔG is negative or close to zero, indicating strong binding to the molecular target.

The $\Delta G + \Delta W$ value allows us to take a broader view of the properties characterizing the peptide in the complex as well as the unbound one. This value for light1 and 2 peptides further confirms their weak affinity to HVEM. In the case of light4, summing these values yields a negative value. Although this peptide does not bind HVEM strongly according to ΔG , forming the complex significantly reduces the internal work performed by the peptide. This decrease in internal work largely compensates for the low affinity, as confirmed by the SMD results. In the case of light3, we can observe a positive ΔW and the resulting positive $\Delta G + \Delta W$. A decrease in this property could potentially have a positive effect on the peptide's affinity for HVEM, but as we observe in the case of its analogue light3_E178L, the attenuation of the negative effect of ΔW as a result of glutamic acid substitution is also compensated by an increase in ΔG . Similar conclusions can be drawn on the basis of the values of $F_{\max}$ and W_{total} , where the peptides light3 and light3_E178L are significantly higher than those for the other three. This property indicates that a high force is required to detach these two peptides from the molecular target, which indicates their high affinity for HVEM and thus potentially high inhibitory properties.

On the basis of the comparison of both short and long simulations, we can see that in the case of long simulations, the standard deviations between the three dynamics are many times higher than in short simulations, which indicates the loss of stability of the complex in the course of the simulation progress. In addition, the main conclusion from the comparison of thermodynamic properties determined on the basis of short and long

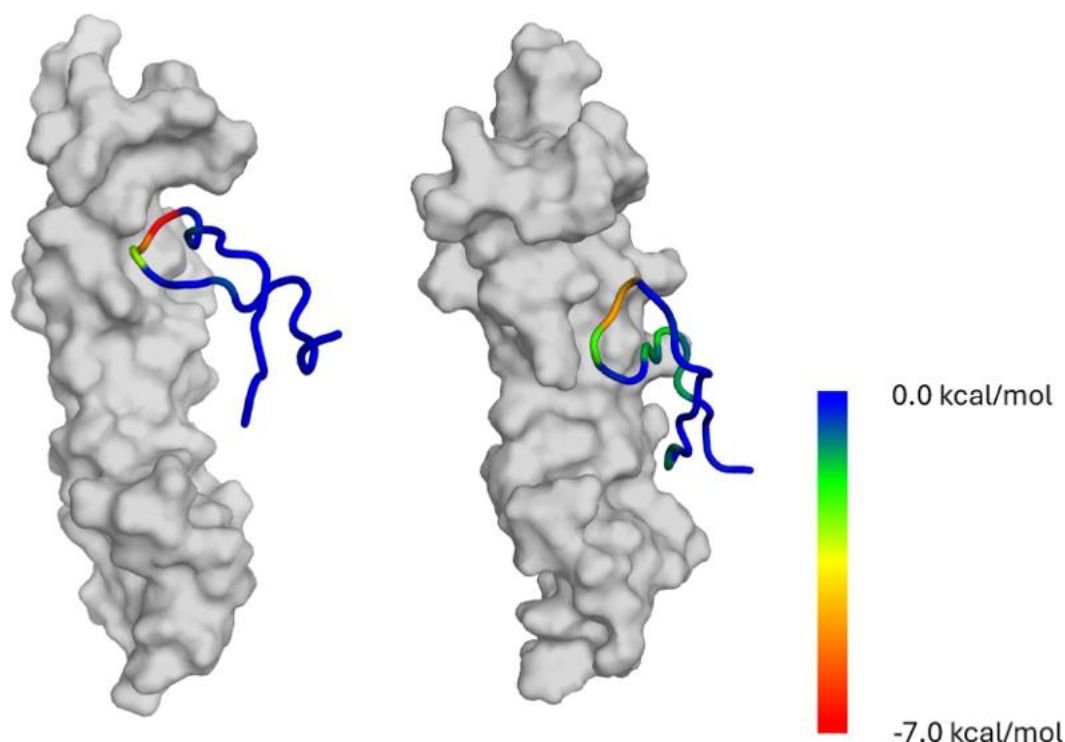


Fig. 5. Representative models of light3 (left) and light3_E178L (right), obtained by clustering three independent trajectories, coloured by the per-residue effective theoretical binding energy. The influence of each residue to total binding energy was marked as color in rainbow style ranging from 0 (blue) to -7.0 kcal/mol (red), with HVEM protein presented as grey surface.

simulations is the fact that, in the course of simulation, the change in internal work increases significantly in the case of light1 and 4 peptides. This indicates a decreasing difference in the internal work performed by the peptide bound in the complex and the free peptide, which may be related to the increasing lability of compounds bound during the simulation. Comparing the values obtained during these studies to the values obtained in our previous studies, we can observe a similar trend, according to which peptides in which the enthalpic contribution dominating over the entropic contribution, and with the highest W_{total} and F_{max} values showed the highest theoretical affinity for the molecular target determined by experimental methods. This correlation confirms the high efficiency of the analyses and their correlation with experimental results. Short molecular dynamics simulations, followed by MMGBSA calculations, revealed that light5 exhibited energy very close to zero, which indicate that during simulations it dissociate from the protein, which exclude it from further analysis. Among the remaining four peptides, light3 exhibited the most promising binding characteristics, warranting further investigation. MMGBSA decomposition indicated that E176 and E178 might have negatively impact for the stability of the HVEM/light3 complex. In order to improve the stability of peptide/protein complex they were substituted by lysine, leucine and glutamine in various configurations, which enabled us to design 9 analogues of the light3. Light3_E178L demonstrated an enhanced affinity for its molecular target. A comprehensive set of theoretical analyses performed for peptides indicated that both light3 and its analogue, light3_E178L, had the highest theoretical affinity for HVEM. The significant amount of work and force required to separate these peptides from HVEM suggested strong interactions between peptides and protein. In contrast, light1 and light2 did not exhibit significant force or work leap during SMD, implying weaker affinity toward HVEM. It should also be noted that the peptide light3, which is most strongly bound to HVEM, and light5, which does not form a stable complex with HVEM, differ only in the position of the disulfide bond. The amino acids substituted by cysteines in both tested peptides are not crucial for the interaction with HVEM, what strongly confirmed our previous observation, that the position of disulfide bond is crucial for the interaction of molecular target. The W_{total} and ΔS strongly suggest that the sequence could be further optimised by introduction of additional rigidity, for example by change of disulfide bond position.

The affinity of the selected peptides to HVEM determined by SpS

Based on the computer analyses conducted for the designed peptides in complex with the HVEM, five compounds – peptides light1, light2, light3, light4, and light3_E178L were selected for experimental validation and their affinity to HVEM was evaluated using SpS. Fluorescence intensity was measured at 670 and 650 nm (Figure S4). The apparent dissociation constants (K_d) determined for the peptide/HVEM complexes ranged from 1.72 μ M for light4 to 0.58 μ M for light3 (Table 2). Corresponding dose-response spectral shifts are presented in Fig. 7. Due to the fact that peptides at higher concentrations aggregated, it was not possible to fully determine the K_d . Therefore, only the apparent K_d values were determined and were used for HVEM binding strength comparison. Note that apparent K_d is only approximate value and should be used only for qualitative comparison. Among the tested peptides light3 and its analogue light3_E178L exhibited the highest binding affinities toward HVEM, even stronger than the LIGHT protein. These results strongly correlate with the data obtained from computational analyses.

Despite the fact that the light3 and light3_E178L reveal very high binding affinity, the HVEM-derived fragments previously designed and synthesized, targeting the HVEM/LIGHT complex, exhibited even lower dissociation constants compared to the corresponding LIGHT fragments, indicating stronger binding to the molecular target (Ciura et al. submitted). It is also noteworthy that K_d values for the HVEM/LIGHT complex differ significantly depending on the proteins used in experiments and the measurement techniques applied. The K_d previously determined by us using the SpS technique indicated that LIGHT binds to the HVEM protein with a K_d of 0.92 μ M. However, other research groups have also investigated this interaction, reporting K_d values ranging from 18.7 nM¹¹ to 1.5 nM⁴¹.

Peptides inhibitory properties assessed by competitive ELISA

To assess the inhibitory properties of the peptides the ELISA was conducted. Based on the obtained results, it can be concluded that the inhibitory properties of the peptides are strongly correlated with their affinity to HVEM as determined by theoretical methods, as well as with those measured by SpS. The weakest inhibitory effects were observed for light4 with inhibition remaining below 10% across all tested concentrations. In contrast, the peptides light3 and its analogue light3_E178L exhibited the most significant ability to disrupt HVEM/LIGHT complex formation, with inhibition reaching 22% and 19%, respectively, at the highest concentration tested (Fig. 8).

No.	Compound name	Apparent K_d (M)	SD
	LIGHT	0.92×10^{-6}	0.13×10^{-6}
1	light1	1.68×10^{-6}	1.01×10^{-6}
2	light2	1.38×10^{-6}	0.39×10^{-6}
3	light3	0.58×10^{-6}	0.17×10^{-6}
4	light4	1.72×10^{-6}	0.30×10^{-6}
5	light3_E178L	0.71×10^{-6}	0.15×10^{-6}

Table 2. Affinity of the selected LIGHT-derived peptides to HVEM designated by SpS. Results are shown for experiments performed in triplicate. Data are presented as mean $\pm$ SD.

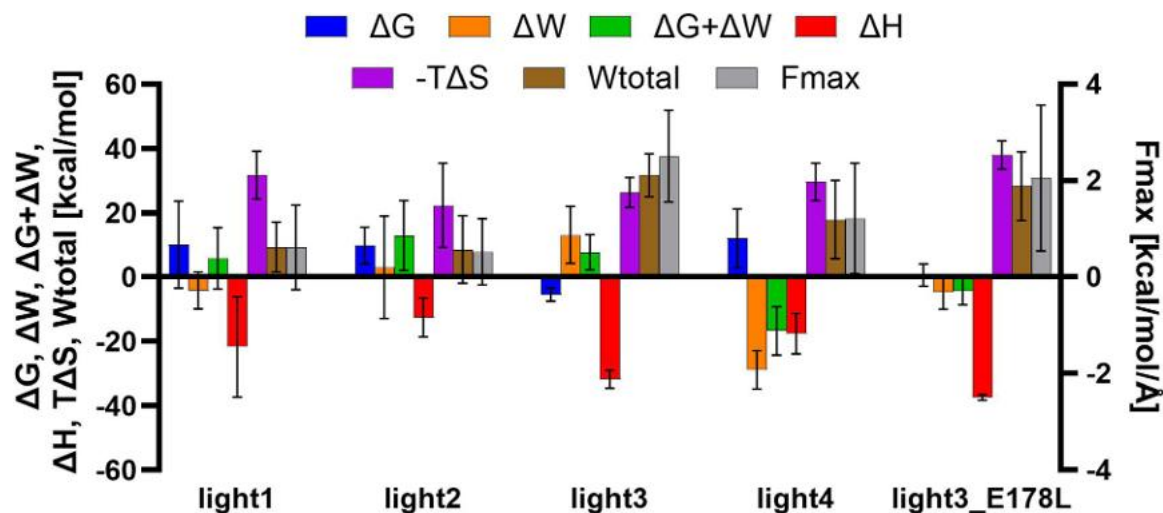


Fig. 6. Diagram presenting change of: Gibbs free energy, internal work, sum of Gibbs free energy and internal work, free enthalpy, and entropy for the four determined for peptides and the light3 peptide analogue.

However, the obtained peptides exhibited significantly weaker inhibitory properties compared to the HVEM-derived fragments we previously studied. The highest blocking activity was observed for CRD2e_K54E and CRD2(39–73)e, which inhibited the HVEM/LIGHT interaction by approximately 38% and 41%, respectively, at the highest concentration tested (Ciura et al. submitted). In our earlier studies, we also demonstrated that commercially available anti-HVEM antibodies inhibit HVEM/LIGHT binding by approximately 75% at a concentration of 1 $\mu\text{g}/\text{well}$ (10 ng/mL)³⁴. The obtained results highlight the need for further structural optimization of LIGHT-derived peptides to enhance their inhibitory properties.

Peptides inhibitory properties measured by cellular assay

The inhibitory effects of the peptides were also evaluated using cell line-based platform, but before performing this tests, the stability of peptides in medium used in co-culture and cytotoxicity towards cell lines used in the experiments had to be determined. After 24 h of incubation (the time when the peptides are incubated with cells), there is a clear difference in stability between peptides based on one of the interaction centres (light1 and 2 – stability is 88% and 77% respectively) and between those based on the other centre (light3, 4 and 3_E178L – stability ranging from 49% to 29%). However, all peptides, despite some degradation, are present in the sample during the experiment (Figure S5). The cytotoxicity studies indicate that the analyzed peptides do not have significant effect on cell viability up to a concentration 50 μM (Figure S6) and this concentration was chosen as the highest in the cellular studies.

Reporter cells expressing the HVEM protein (JE6.1-NF- κ B::eGFP HVEM) and T cell stimulators expressing LIGHT (TCS LIGHT) were utilized in cellular assays and the principle of this cell-based assay has been described in detail in previous publication²¹. In short, when the peptides inhibited the HVEM/LIGHT interaction, a reduction in NF- κ B activation was observed, accompanied by a corresponding decrease in enhanced green fluorescent protein (eGFP) levels. The percentage of reporter cell activation was calculated relative to a control condition, which was reporter cell co-cultured with TCS without peptides.

A slight reduction in HVEM/LIGHT binding was observed in the presence of all tested peptides (Fig. 9). Light3 and light3_E178L peptides exhibited the best inhibitory effects, leading to a decrease in reporter cell activation levels to 78% and 74%, respectively, at the highest tested concentration. These findings are in agreement with previous results obtained from ELISA, which further confirmed the HVEM/LIGHT-complex-formation-disruptive properties of these peptides.

It should, however, be noted that the inhibitory properties of peptides based on the LIGHT protein are notably weaker than those of the HVEM fragments studied by us previously (Ciura et al. submitted). Two peptides, namely CRD2e_K54E and CRD2(39–73)e, have significantly higher blocking effects than the LIGHT-derived peptides, and inhibit the HVEM/LIGHT complex formation in 41% and 46%, respectively, at the highest concentration used. This may suggest that targeting the LIGHT protein is a more effective method of blocking the HVEM/LIGHT complex than targeting HVEM. All theoretical and experimental data suggest, that designing peptides when two molecules (two LIGHT monomers) are involved in complex formation is more challenging than when only single molecule (HVEM) is involved. Moreover, it should be noted that the LIGHT-derived peptides are prone to degradation, which may also affect their observed ability to inhibit protein binding in cell-based assays. To address this issue, structural modifications are necessary to enhance their resistance to degradation, such as introducing non-natural amino acids into the sequence or substituting L-amino acids with their D-enantiomers. The LIGHT-derived peptides are linear, whereas our previously studied and described immune checkpoint inhibitors³⁸ were primarily stabilized by disulfide bonds, which, as confirmed by literature data⁴², improve peptide stability. Therefore, peptide stability represents a significant limitation of the present study.

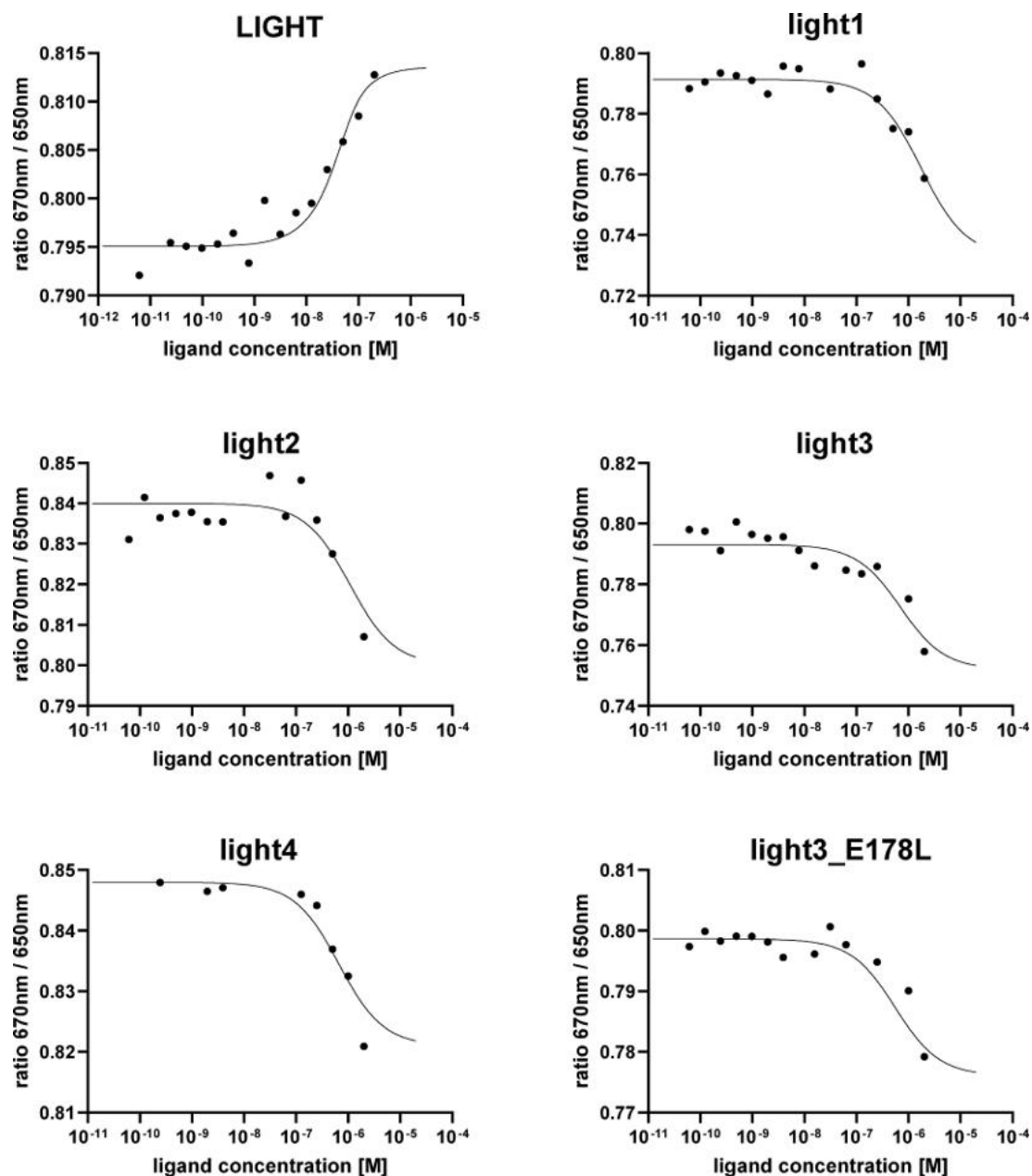


Fig. 7. The spectral shift dose-response curves for the interactions between HVEM and LIGHT protein and synthesized peptides.

Conclusion

Our study contributes to the growing field of immune checkpoint modulation and peptide-based therapeutics by identifying and characterizing LIGHT-derived peptides as potential inhibitors of the HVEM/LIGHT complex. During our research the peptides light3 and its analog light3_E178L exhibited favorable binding characteristics and promising inhibitory properties. All our in silico and experimental studies indicate promising binding properties of light3 and light3_E178L. Theoretical studies suggest that light3 sequence could be further improved by altering position of disulfide bond, which is our plan for the next studies. All these results provide a solid foundation for further structural optimization and preclinical evaluation, supporting their future development as novel immune-modulating agents.

Nevertheless, several limitations of the present study should be acknowledged. In particular, the employed in vitro assays, such as SpS, ELISA, and cell-based tests, do not fully capture the complexity of in vivo biological systems. Additionally, peptides often exhibit limited stability and bioavailability, which may affect their therapeutic potential. Comprehensive evaluation of the immunomodulatory potential of the identified peptides requires further investigation. This includes in vitro assays to assess their effects on T cell activation, proliferation, differentiation, and cytokine production, as well as in vivo experiments employing advanced models, such as

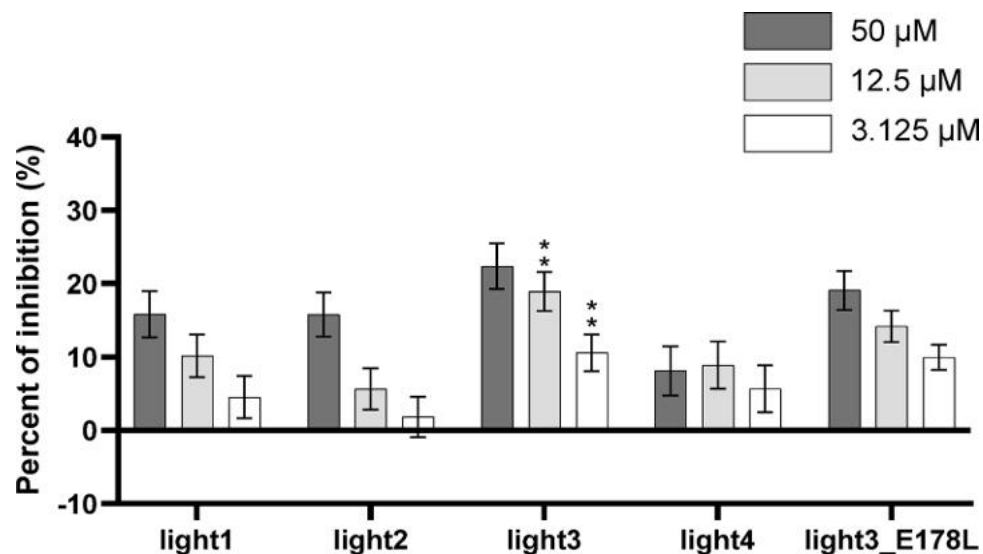


Fig. 8. The inhibitory properties of LIGHT-derived peptides toward the formation of the HVEM/LIGHT complex determined by ELISA. The results are shown for two experiments performed independently in triplicate. Data are depicted as mean with SD (mean $\pm$ SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

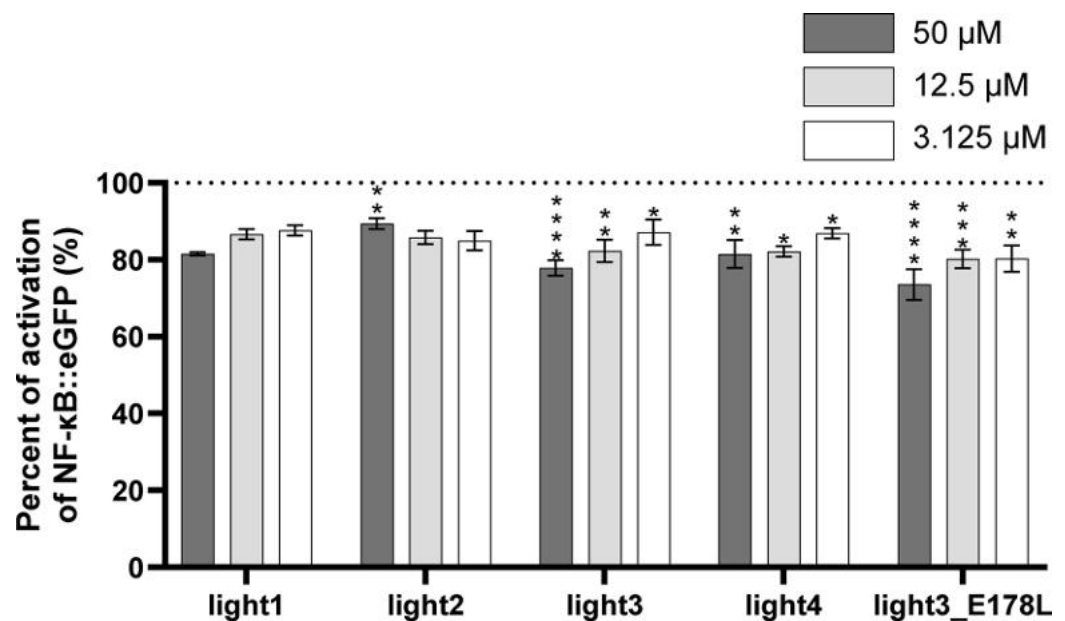


Fig. 9. The percentage of activation of the NF-κB::eGFP signal pathway, which is correlated with HVEM/LIGHT complex formation in the presence of LIGHT-derived peptides determined by a cellular-based assay. The HVEM reporter cells were stimulated with TCS LIGHT in the absence or presence of the peptides. HVEM/LIGHT stimulation in the absence of peptides was set to 100% activation. The results are shown for three experiments performed independently in triplicate. Data are depicted as mean with SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

mice with induced autoimmune diseases. Such approaches are essential to fully elucidate the therapeutic efficacy and clinical relevance of these peptides.

Materials and methods

The overall workflow of this study is depicted in Figure S11. The analysis commenced with the structural characterization of the HVEM/LIGHT complex. Subsequently, the designed peptides were subjected to MD simulations, and only conformationally stable complexes were retained for in-depth theoretical investigations. Computational peptide optimization was performed prior to advancing to the experimental validation stage.

Molecular dynamics and MMGBSA analyses and thermodynamic values assessment

Analyses were conducted based on the crystal structure of the HVEM/LIGHT protein complex (PDB code 4RSU). The missing residues were added using modeller9.16⁴³. The files necessary for the simulation were prepared on the basis of the tleap program using the ff19SB force field with a four-site OPC water model^{44–46}. The simulations were run using pmemd.cuda from the AMBER24⁴⁷ package. The simulated systems were enclosed within a 17.5 Å layer of water, shaped as a truncated octahedron, and their total charge was neutralized by adding either Na⁺ or Cl[−] ions. Each system underwent two stages of energy minimization: the first minimization (steepest descent) consisted of 1000 steps with restraints applied to all non-solvent atoms, while the second minimization (conjugated gradient) involved 2000 steps with restraints on all heavy atoms. This was followed by a three-phase equilibration process. Initially, the system was gradually heated from 10 K to 300 K over 10⁴ MD steps. The second phase, consisting of 10⁵ steps, was used to establish the target density. Finally, a third equilibration consisting of 4 × 10⁵ steps npt with Langevin thermostat and weak restraints on Ca and Cβ atoms was performed to ensure system stability. Based on these equilibrated setups, three independent molecular dynamics simulations were conducted, each lasting 200 ns, corresponding to 10⁸ steps with a 2 fs time step. For the selected systems longer molecular dynamics were also carried out. In this case equilibration and minimisation steps were conducted the same way, but the overall simulations were longer – 5 × 10⁸ steps of simulations (2 fs each) resulting in 1 μs of simulation. In this case we have analysed last 500 ns of these simulations. For analysis, we focused on the final 20 ns of each trajectory to minimize any influence from initial simulation convergence. The binding free energy was estimated using the MMGBSA approach, specifically employing the GB-Neck2 model with its associated atomic radii parameters^{48–51}. The entropic input was also used to calculate Gibbs free energy with normal mode analysis methods for 10 frames drawn uniformly from the last 20 ns of 200 ns simulations. For longer analyses, 20 frames from the last 200 ns of the simulation were analyzed. Change of internal work was calculated by subtracting the energy calculated from the MMGBSA of the peptide not bound in the complex from the energy of the peptide in the complex.

Steered molecular dynamics

For each of the selected peptides, simulations consisting of 20 trajectories of 80 ns each were carried out. The output files were prepared on the basis of a structure representative of each complex based on clustering. The systems were surrounded by a 70 Å layer of water. This was followed by the standard course of minimization and equilibration described in the previous paragraph. The mass center of the Ca carbons of the peptide was pulled away from the mass center of the Ca carbons of the HVEM protein. Extraction with a force constant of 10 kcal/mol/Å² and pulling speed of 0.05 m/s was continued until a distance of 40 Å between the mass centres was reached. Structures near force peak were extracted from simulation and DBSCAN clustering algorithm⁵² was performed with use of ccpptraj⁴⁴.

Peptides synthesis and purification

The peptides were synthesized using solid-phase peptide synthesis (SPPS) on a Liberty Blue synthesizer (CEM Corporation, Matthews, USA) following the Fmoc/tBu strategy. Rink Amide Pro Tide (LL) resin (CEM Corporation, Matthews, USA) with a loading capacity of 0.18 mmol/g was used. Methionine residues in synthesized peptides were replaced with norleucine to prevent their oxidation during experiments, as in our previous studies. The synthesis protocol was based on a previously described method³⁸. Purification was conducted using reverse-phase high-performance liquid chromatography (RP-HPLC) on a Luna C8(2) column (250 × 4.6 mm, 5 μm, 100Å, Phenomenex, CA, USA). Prior to loading, the peptide solution was treated with a 10-fold excess of dithiothreitol (DTT) relative to the free thiol groups of cysteine residues and sonicated at 60 °C. Two solvents were used for purification: solvent A (deionized water with 0.1% TFA) and solvent B (80% acetonitrile in water with 0.1% TFA). A linear gradient from 5% to 50% of solvent B in A over 120 min was applied, with UV detection at 222 nm and 254 nm. Peptide molecular weight and purity were verified using liquid chromatography coupled with electrospray ionization, ion trap, and time-of-flight mass spectrometry (LC ESI-IT-TOF MS, Shimadzu, Kyoto, Japan). Additional purity confirmation was performed using reverse-phase ultra-high-performance liquid chromatography (RP-UHPLC) with a photodiode array detector (PDA) and an evaporative light scattering detector (ELSD-LT) (Shimadzu, Kyoto, Japan) on a Kinetex C8 column (100 × 2.1 mm, 2.6 μm, 100Å, Phenomenex, CA, USA). A gradient from 5% to 100% solvent B in A over 15 min was used.

Formation of disulfide bond

To create the disulfide bond, peptides were dissolved in a water-methanol mixture (1:9, v/v) at concentration 40 mg/L. The pH was adjusted to 8–9 using 25% ammonia, and the solution was stirred for 7 days while simultaneously introducing compressed air to promote oxidation. Reaction progress was monitored by RP-UHPLC. Next, the methanol was evaporated, and the remaining solution was freeze-dried. The peptides were purified again using the same protocol as previously described.

Spectral shift

SpS experiments were conducted using the Monolith X instrument (Nanotemper, Munich, Germany). Protein labelling was performed using the His-Tag Labeling Kit RED-tris-NTA 2nd Generation (Nanotemper, Munich,

Germany). To prepare the fluorescent dye solution at concentration 0.1 μM , 2 μL of a 5 μM dye stock was diluted in 98 μL of deionized water. Next 90 μL of this solution was mixed with 90 μL of 100 nM HVEM-His protein (ACROBiosystems, Newark, USA) dissolved in PBS-T (phosphate buffer with 0.05% Tween-20 and 0.3 M NaCl, pH 7.4) and incubated for 30 min at room temperature. Next, 10 μL of the labelled protein was mixed with 10 μL of peptide solutions at concentrations ranging from 4 μM to 12.2 pM. This resulted in a final protein concentration of 50 nM and peptide concentrations ranging from 2 μM to 6.1 pM. The mixtures were loaded into Monolith X-compatible capillary tubes, and fluorescence ratios (670/650 nm) were measured at 40% excitation power and average IR laser power at a temperature of 25 °C. Data were analyzed using MO.Control 2 software (version 2.5.4, NanoTemper, Munich, Germany). Each experiment was performed in triplicate.

Enzyme-linked immunosorbent assay

ELISA was conducted using transparent polystyrene 96-well plates (Brand, Wertheim, Germany). Wells were coated with HVEM-His protein at concentration 6 $\mu\text{g}/\text{mL}$ dissolved in PBS-T (100 μL per well) and incubated overnight at 4 °C. Unbound sites on the wells by HVEM were blocked with 5% bovine serum albumin (BSA) in PBS-T (200 μL per well). Peptides at three concentrations 50 μM , 12.5 μM , and 3.125 μM dissolved in PBS-T were added and incubated (200 μL per well). These peptide concentrations were selected because they did not exhibit cytotoxicity toward the cells used in subsequent experiments. Subsequently, LIGHT-Fc (ACROBiosystems, Newark, USA) at concentration 2 $\mu\text{g}/\text{mL}$ (100 μL per well) was introduced and incubated. In the next step horseradish peroxidase (HRP)-conjugated goat antibody (Bio-Rad, Hercules, USA) at dilution 1:3000 in PBS-T (100 μL per well) was applied. After each step, wells were washed five times with 200 μL of PBS-T. Incubations were carried out at 37 °C for 2 h, except for the antibody, which was incubated for 1 h. Finally, 200 μL of TMB substrate (Thermo Fisher Scientific, Waltham, MA, USA) was added, and after 15 min of incubation in the dark, absorbance was measured at 492 nm and 650 nm using an Infinite M200 Pro reader (Tecan, Switzerland). The negative control consisted of PBS-T buffer without peptides, while the positive control was mouse anti-human HVEM antibodies (1 $\mu\text{g}/\text{well}$, Bio-Rad, Hercules, CA, USA; #MCA6072GA). Each experiment was performed in three biological replicates, each consisting of three technical replicates and data were analyzed using one-way ANOVA with Dunnett's post-hoc test in GraphPad Prism (San Diego, USA).

Cellular assays

The experiments utilized the JE6.1-NF- κB ::eGFP HVEM (modified Jurkat), TCS LIGHT, TCS Ctrl and TCS CD86 Ctrl cell lines (modified BW5147) which have been described in earlier studies^{57,58}. Jurkat E6.1 cell line (describe in Supplementary Materials) was purchased from Cell Line Service GmbH, while the JE6.1-NF- κB ::eGFP HVEM, TCS LIGHT, TCS Ctrl and TCS CD86 Ctrl cell lines are property of the laboratory of Prof. Peter Steinberger, Institute of Immunology, the Medical University of Vienna, Vienna, Austria. Cell lines were cultured in RPMI 1640 medium (Sigma Life Sciences, USA) supplemented with 1% penicillin and streptomycin with the addition of 10% heat-inactivated fetal bovine serum (FBS, Sigma Life Sciences, USA). For the cellular assays, 25 μL of peptide solutions at three concentrations (200, 50, and 12.5 μM) were incubated with 50 μL of JE6.1-NF- κB ::eGFP HVEM cell suspension (50×10^4 cells per tube) for 1 h at 37 °C. The tubes were then centrifuged ($500 \times g$, 3 min), the supernatant containing unbound peptides was removed, and 75 μL of fresh medium was added. The peptide-bound reporter cells were transferred to a 96-well plate (Sarstedt, Germany) and 25 μL of TCS LIGHT cell suspension (2×10^4 cells per well) was added to each well. The plates were incubated for 24 h at 37 °C and 5% CO_2 . After incubation, the plates were centrifuged ($800 \times g$, 3 min), and 6 μL of APC-mCD45 antibody was added to each well to exclude TCS cells from the analysis. The plates were incubated for 20 min at 4 °C, washed with FACS buffer (PBS, 5–10% FBS, 0.1% NaN_3), and resuspended in 50 μL of fresh FACS buffer. eGFP expression was measured using a CytoFLEX flow cytometer (Beckman Coulter, USA). Data were analyzed using FlowJo v10.10 (FlowJo LLC, USA) and GraphPad Prism (San Diego, USA) with univariate ANOVA and Dunnett's post-hoc test. Control experiments were performed using cells not expressing the LIGHT protein (TCS CD86 Ctrl and TCS Ctrl) to ensure the specificity of the peptide effects. The results were represented as an average. Each analysis was performed in three biological replicates, each consisting of three technical replicates.

Figure preparation

Figures 1 and 5 were prepared with Pymol version 2.6, Figs. 2, 3, 4, 6, 7, 8 and 9 were prepared with GraphPad Prism version 10.4.

Data availability

The datasets generated and/or analysed during the current study are available in the University of Gdańsk: (<https://repozytorium.bg.ug.edu.pl/info/researchdata/UOG8e9011f720ef48bbbd342089280d3168>) DOI:10.71853/5dj5-sp15.

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Author contributions

P.C. data acquisition, figure preparation, first draft of manuscript; S.G. cellular assay supervision, review the manuscript; S.R.M. scientific consultation, review the manuscript; P.S. cellular assay supervision, review the manuscript; M.S. provided the original concept, experimental studies supervision, review the manuscript; A.S. theoretical studies supervision, review the manuscript, funding acquisition, research idea.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Supporting Information for:
Evaluation of LIGHT-Derived Peptides to Disrupt the HVEM/LIGHT Immune Checkpoint.

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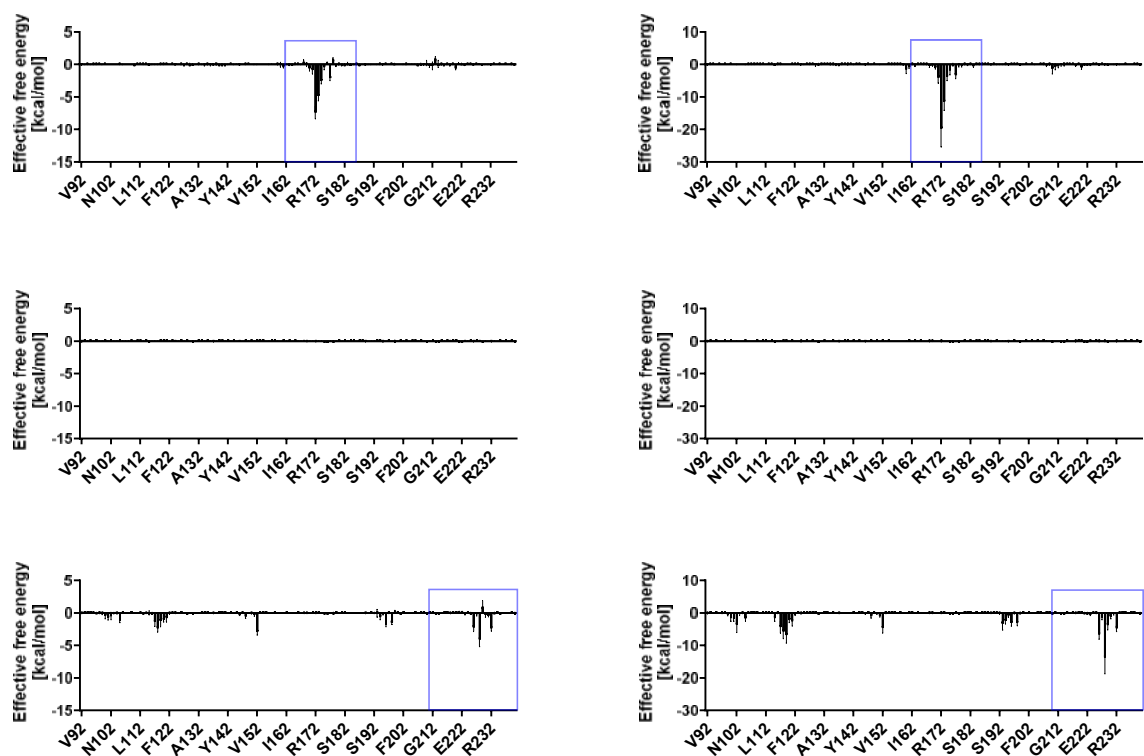


Figure S1. MM-GBSA per residue (left) and pairwise (right) effective binding energy decomposition results for three LIGHT protein monomers. The fragments on the basis of which the peptides were designed are marked with a blue rectangle. Results are shown for 3 independent trajectories as mean $\pm$ SD.

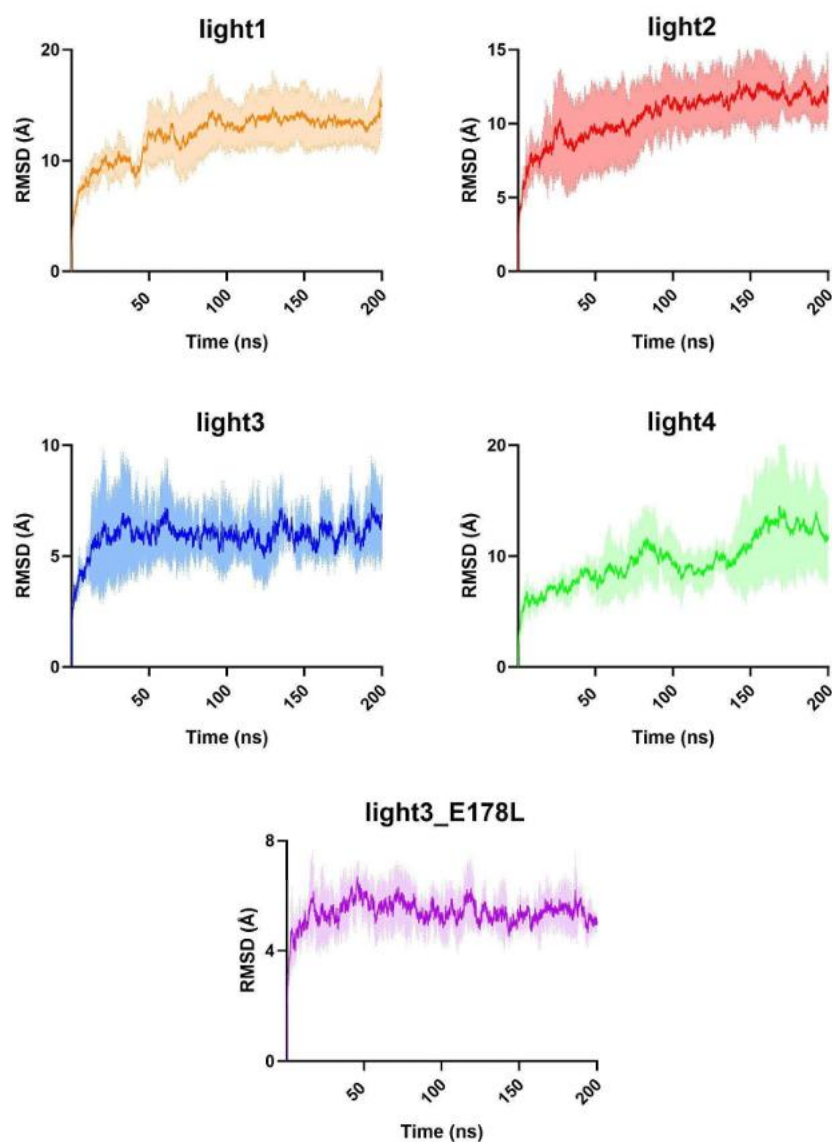


Figure S2. Root-Mean-Square Deviation plot of light1-4 peptides and the light3_E178L analogue based on three 200 ns trajectories. Results are presented as mean (bold line) $\pm$ standard deviation (shaded area). The flattening of the curves in the last stage of the simulation indicates that the system has reached equilibrium.

Table S1. Top 10 amino acid residues of the LIGHT protein with the highest summarised energy contribution for the formation of the HVEM/LIGHT complex, determined on the basis of pairwise decomposition of MM-GBSA analysis together with HVEM protein residues constituting the main interaction partner of a given residue. The letters A and B after residue number distinguish between the individual monomers of the LIGHT protein. Results are shown for 3 independent trajectories as mean $\pm$ SD.

No.	LIGHT residue	HVEM residue	Theoretical interaction energy [kcal/mol]
1	R 172 A	P 48	-19.81 $\pm$ 5.53
2	R 228 B	D 100	-13.71 $\pm$ 4.97
3	Y 173 A	H 86	-11.52 $\pm$ 2.61
4	G 119 B	K 92	-6.75 $\pm$ 2.46
5	R 226 B	D 100	-6.62 $\pm$ 1.42
6	L 118 B	Q 95	-5.68 $\pm$ 2.18
7	R 232 B	D 131	-4.67 $\pm$ 1.06
8	V 152 B	M 103	-4.63 $\pm$ 1.53
9	Q 117 B	K 92	-4.34 $\pm$ 1.89
10	P 171 A	P 48	-4.04 $\pm$ 1.76

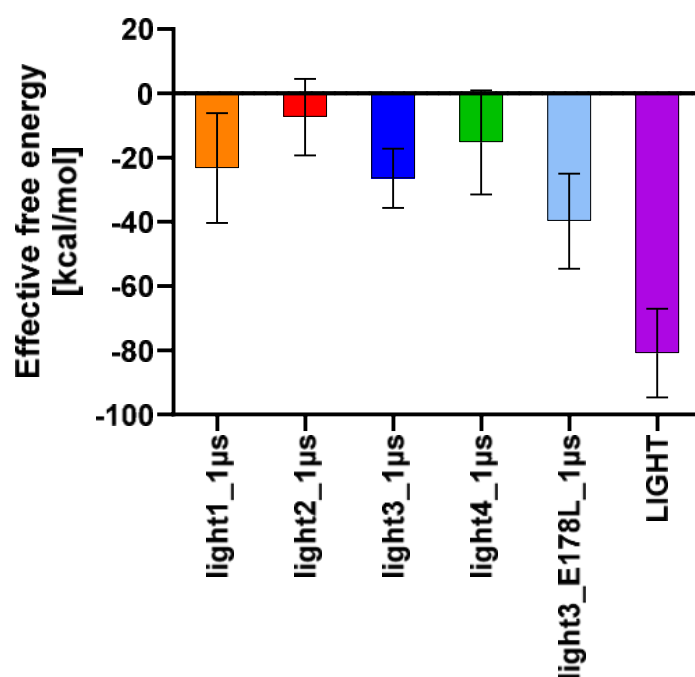
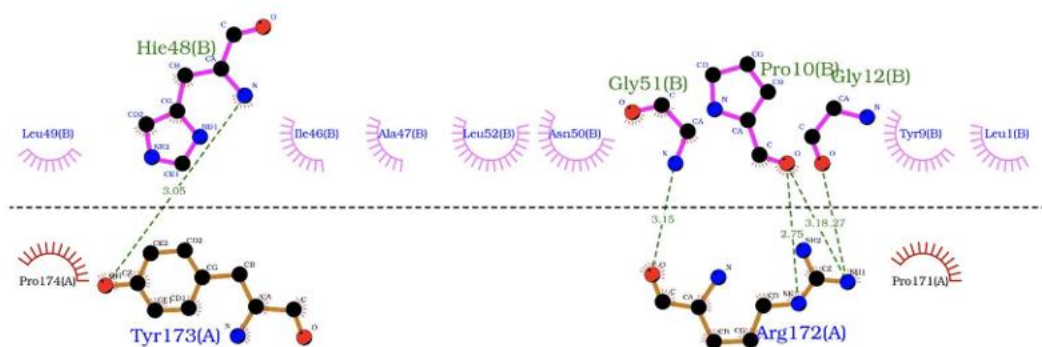
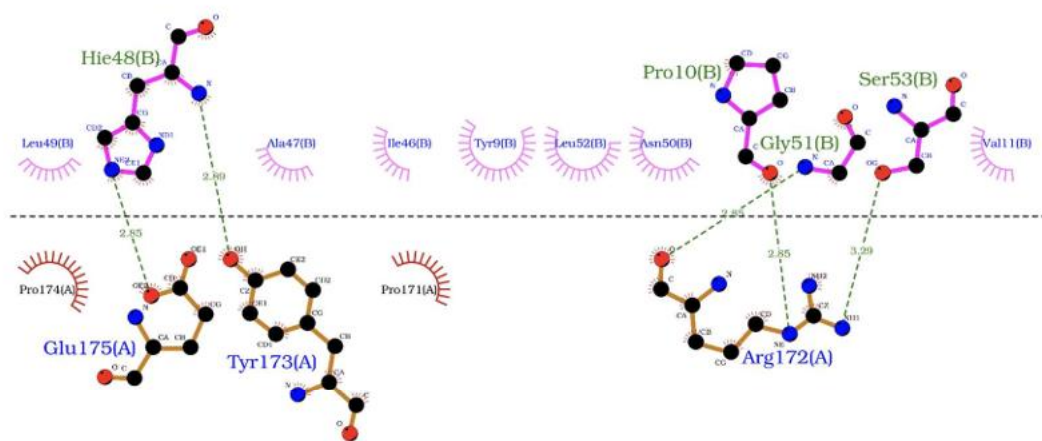


Figure S3. Diagram of effective binding free energy for 1000 ns calculations based on MM-GBSA of initial peptide structures and the light3 peptide analogue. The results are presented as mean $\pm$ SD based on analyses from three independent trajectories.



light3



light3_E178L

Figure S4. Analysis of interaction performed with LigPlus for light (top) and light3_E178L.

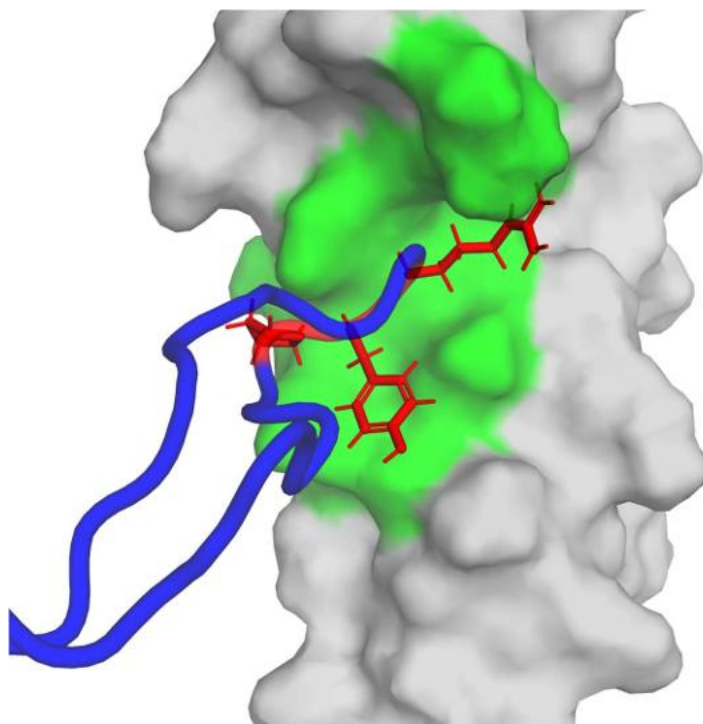


Figure S5. Representative structure of the light3 peptide (blue cartoon) and the HVEM protein (gray area) complex. Residues R172, Y173, and P174 (red) which were determined during the analysis to have a significant energy contribution to the formation of the peptide/protein complex interact with the groove formed by L1, Y9-V11, and I71-L77 of the HVEM protein (green area).

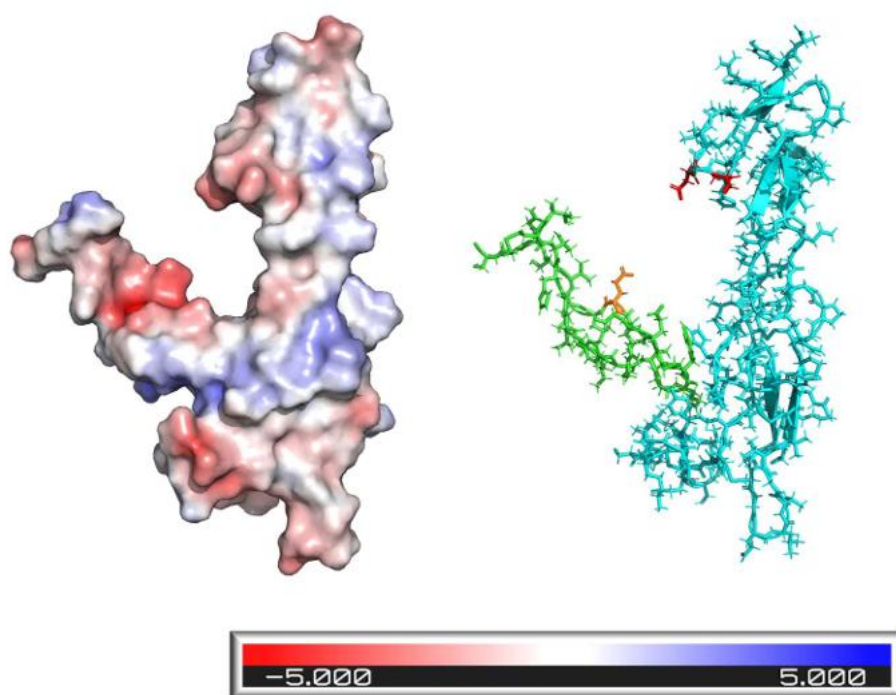


Figure S6. Structural representation of the light3–HVEM complex. The left panel shows the electrostatic surface potential of the complex, calculated using APBS, with the potential isosurface color scale displayed at the bottom. The right panel depicts a cartoon and stick representation, with light3 in green and HVEM in cyan. The side chains of light3 Glu17 (orange), and HVEM Asp97, and Asp99 (red) are highlighted.

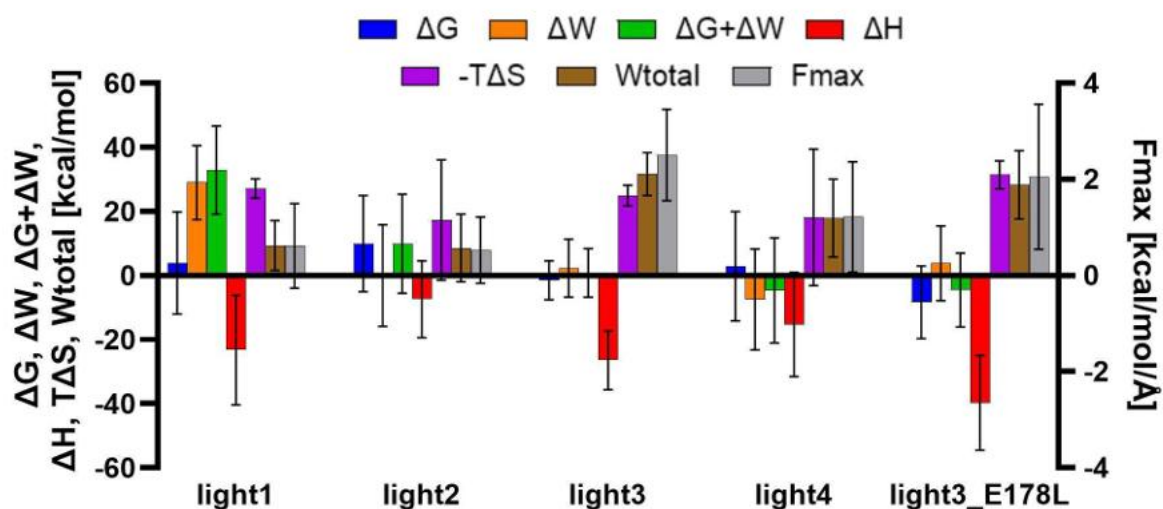


Figure S7. Diagram presenting change of: Gibbs free enthalpy, internal work, sum of Gibbs free enthalpy and internal work, free enthalpy, entropy, total work and maximum force (right Y axis) for the four starting peptides and the light3 peptide analogue for long (1 μ s) simulations.

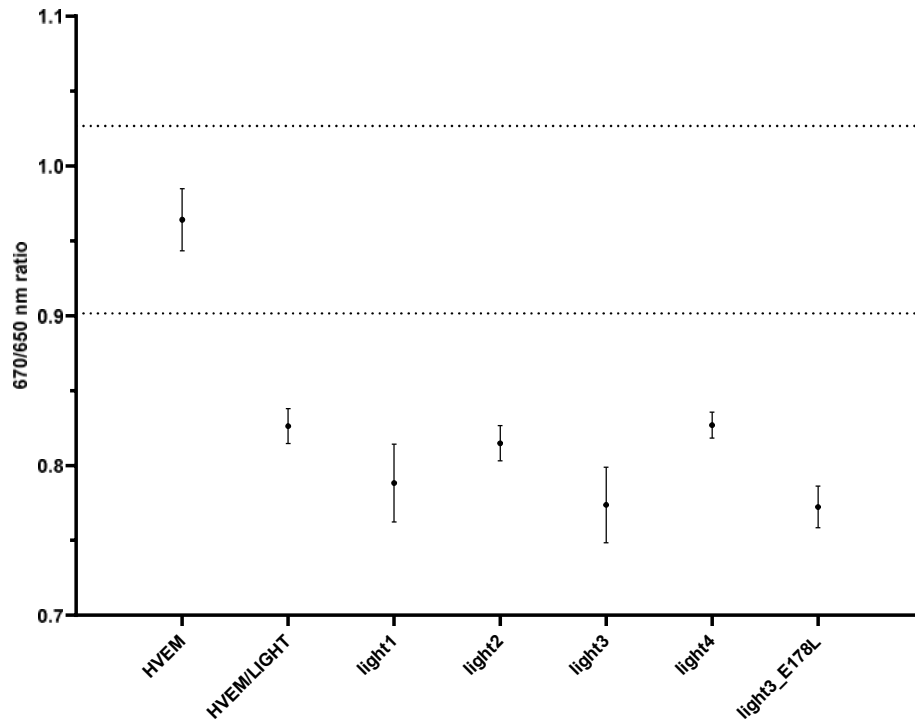


Figure S8. *SpS binding check experiments for selected LIGHT-derived peptides and HVEM compared to negative control (HVEM protein without any ligand). Dotted line represents three times the standard deviation range. The graph shows results from 3 independent experiments. Results are presented as mean with SD.*

Peptides stability in medium

Peptide stability was assessed using the supernatant from the Jurkat E6.1 cell line. The supernatant was collected after 24 hours of cell incubation (1 million cells in 10 mL of medium) under standard culture conditions. The cells were centrifuged (3000×g, 3 minutes), and the supernatant was transferred to a clean container. A 50 µL aliquot of peptide solution in water was added to 200 µL of supernatant, achieving a final peptide concentration of 1.5 mg/mL. The mixture was incubated at 37°C with continuous stirring. Samples were taken at 0 and 24 hours, frozen, and stored until analysis. Prior to analysis, the samples were thawed on ice, mixed with a 4-fold excess of ethanol (v/v), and centrifuged (24,270×g, 20 minutes, 4°C). The supernatant was evaporated and reconstituted in 120 µL of a solution containing 13% acetonitrile in water with 0.1% TFA (v/v). The samples were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Kromasil C8 column (250 nm × 4.6 mm, 5 µm) using a gradient of 5% to 100% solvent B over 60 minutes. Peptide amounts were quantified by

comparing the peak areas of the peptides in the appropriate samples to those of peptide dissolved in water at time 0. All experiments were conducted in triplicate.

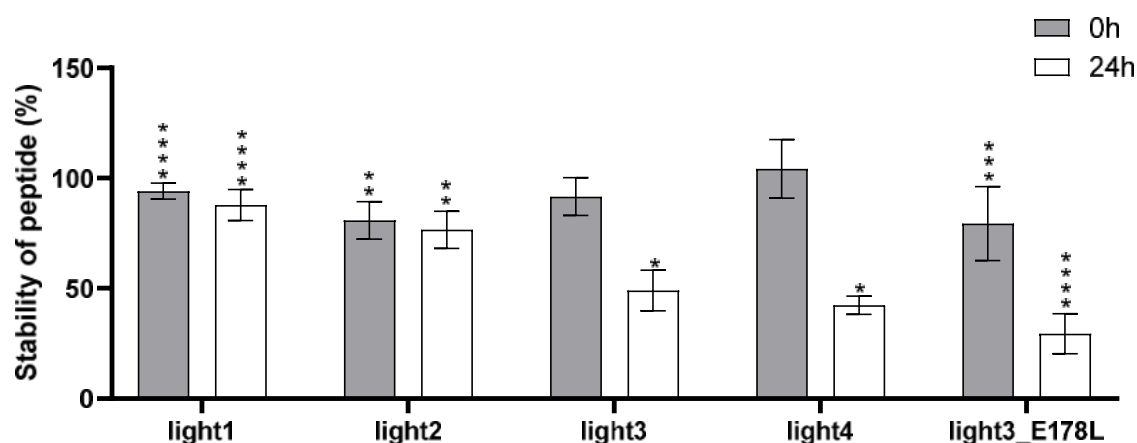


Figure S9. Stability of peptides in Jurkat E6.1 cell supernatant at 0 and 24 h of incubation compared to the control (peptide dissolved in H₂O at time 0). The results are shown for three independent experiments. Data are depicted as mean with SD (mean $\pm$ SD). Statistical analysis was performed using one-way ANOVA followed by the Dunnet post hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

Peptides cytotoxicity

The cytotoxic effects of the peptides were evaluated on two cell lines: Jurkat E6.1 and TCS-Ctrl (a modified BW5147 cell line). The Jurkat E6.1 cells were obtained from Cell Line Service GmbH, while the TCS-Ctrl line was provided by Prof. Peter Steinberger's laboratory at the Medical University of Vienna. Both cell lines were maintained in RPMI1640 medium (Sigma-Aldrich, USA) supplemented with 1% antibiotics (penicillin and streptomycin) and 10% fetal bovine serum (FBS), and cultured at 37°C in a 5% CO₂ atmosphere. For the cytotoxicity assay, white 96-well plates (Thermo Fisher Scientific, USA) were seeded with 40 μ L of cell suspension (1×10^4 TCS cells/well or 2×10^4 Jurkat E6.1 cells/well) in culture medium. Peptide solutions were prepared in the medium at concentrations ranging from 100 μ M to 6.25 μ M, and 40 μ L of each solution was added to the wells, resulting in final peptide concentrations of 50 μ M to 3.125 μ M in a total volume of 80 μ L per well. The cells were incubated with the peptides for 24 hours at 37°C and 5% CO₂. After incubation, the plates were allowed to cool to room temperature for 10 minutes, followed by the addition of 80 μ L of CellTiter-Glo reagent

(Promega, USA) to each well. Luminescence was measured after 15 minutes using a CLARIOstar plus reader (BMG Labtech, Germany). Cell viability was calculated as a percentage relative to peptide-free control cells which consisted of cells incubated under the same conditions but without the addition of peptides. Cell viability in this control group was defined as 100%, and the effects of the peptides on cell viability were assessed relative to this baseline. The results represent the average of three independent experiments, each performed in triplicate. Data were analyzed using GraphPad Prism 9 (San Diego, USA) with univariate ANOVA and Dunnett's post-hoc test.

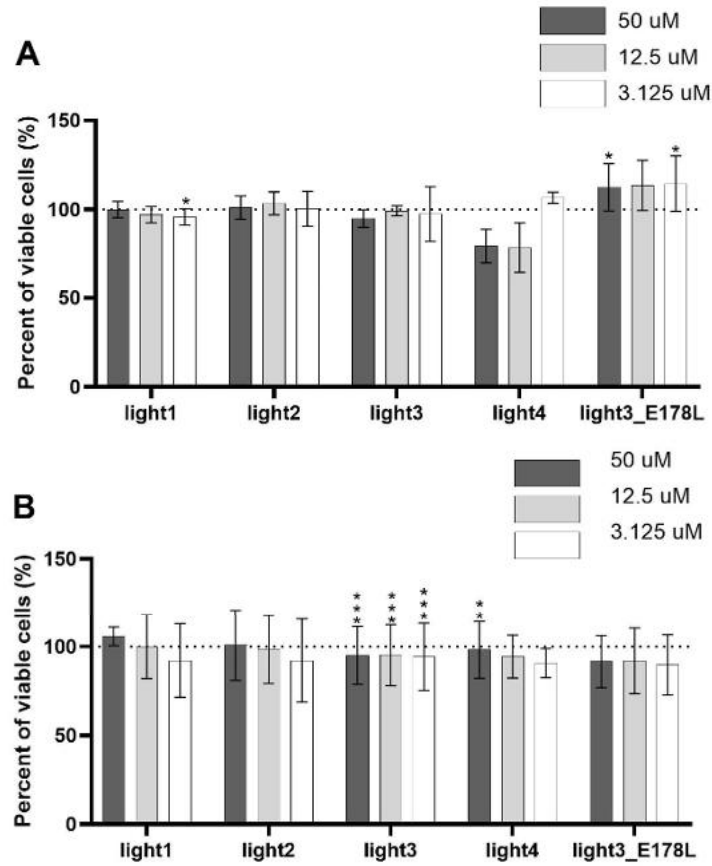


Figure S10. Cell viability assay used to define the influence of LIGHT-derived peptides on A) Jurkat E6.1, B) TCS Ctrl cell lines after incubation for 24 hours. The results are shown for three experiments performed independently in triplicate. The dashed line indicates the value for the control (incubation of cells without the presence of peptide). Data are depicted as mean with SD (mean $\pm$ SD). Statistical analysis was performed using one-way ANOVA followed by the Dunnet post hoc test. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

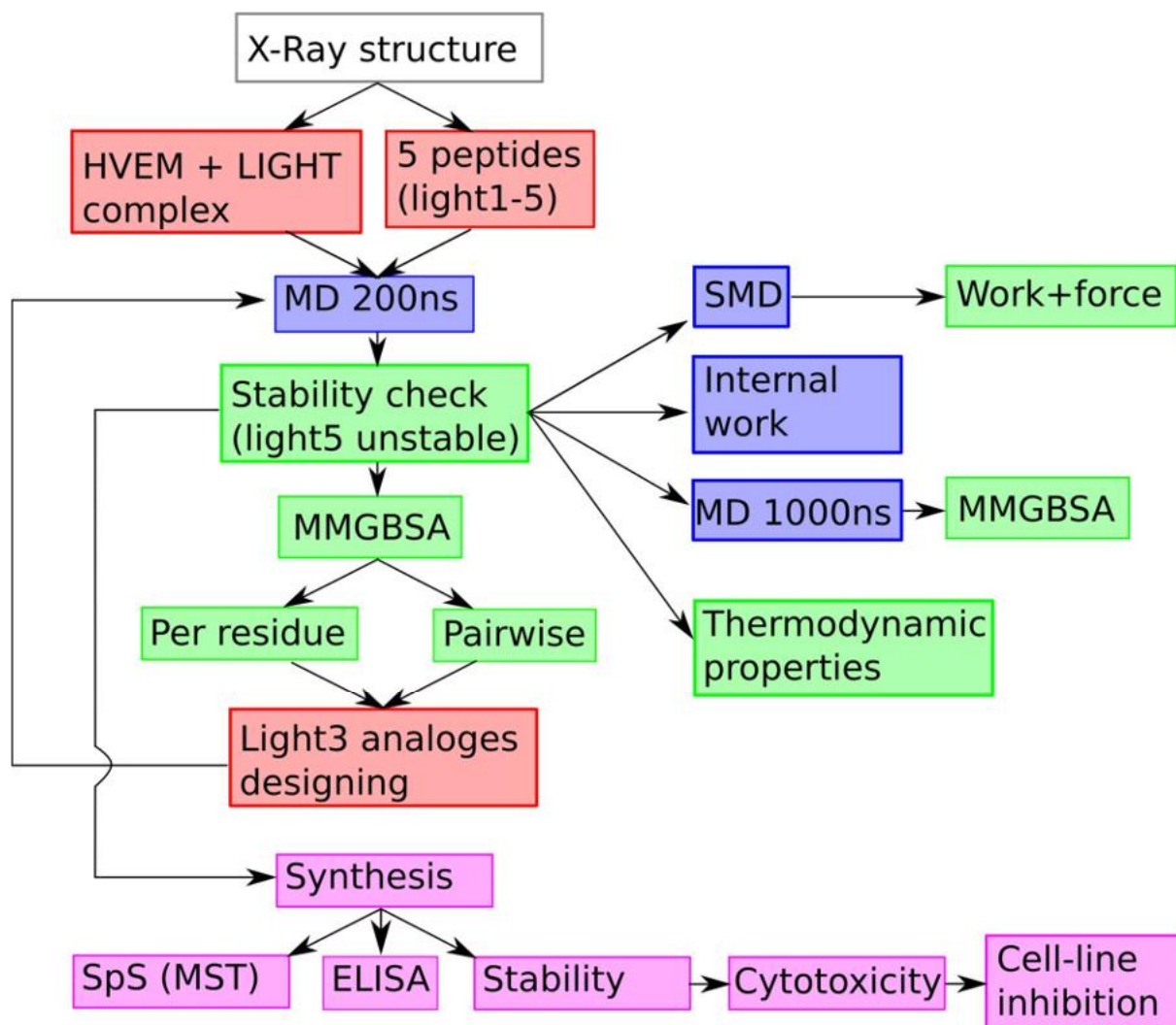


Figure S11. Schematic overview of the integrated workflow. Processes involving manual analysis are delineated in red, theoretical simulations in blue, theoretical analyses in green, and experimental approaches in purple.