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Found PD-1/PD-L1 as CP / CPPS's Target Proteins by Using Peptide T2 as Molecular Probe

Longfei Ding, Jingtao Liu, Xiaomin Xie, Guangpin Pan, Bo Zhao* and Jiang Yuan*

National and Local Joint Engineering Research Center of Biomedical Functional Materials, School of Chemistry and Materials Science, Nanjing Normal University, Nanjing 210023, China

***Corresponding author**: Bo Zhao, National and Local Joint Engineering Research Center of Biomedical Functional Materials, School of Chemistry and Materials Science, Nanjing Normal University, Nanjing 210023, China, E-mail: zhaobo@njnu.edu.cn

Jiang Yuan, National and Local Joint Engineering Research Center of Biomedical Functional Materials, School of Chemistry and Materials Science, Nanjing Normal University, Nanjing 210023, China, E-mail: jyuan@njnu.edu.cn

Abstract

T2 was used as a molecular probe to dock with 18 immune regulation proteins closely associated with CP/CPPS. Through the analysis of the scoring function, the area of action and the binding energy, it was found that T2 and the programmed death receptor-1 (PD-1) Protein has the strongest effect. Therefore, it is speculated that PD-1 may be a potential target protein leading to CP/CPPS. Peptides of T2(T1/2/3/4) were built by Molecular Dynamics in Discovery Studio 2016 and docked on PD-1 using ZDOCK module. Based on the score of the docking result and the distribution of the docking posture and the analysis of the forces on the docking interface, it was found only T2 docking on the active binding area of PD-1/PD-L1. The results of in vitro activity test experiments further verified that T2 can block the combination of PD-1/PD-L1. Therefore, it is inferred that the pathogenesis of CP/CPPS induced by T2 may be because T2 blocks the combination of PD-1/PD-L1. These results have very important clinical significance for the study of the pathogenesis of CP / CPPS, and are an important basis for revealing the pathogenesis, therapeutic approaches and drug development of CP / CPPS.

Keywords: CP / CPPS; TRPM8; Pathogenesis; PD-1; Molecular docking

Introduction

Chronic Prostatitis / Chronic Pelvic Pain Syndrome (CP / CPPS) is an extremely common prostatitis that has a major impact on the quality of life in males [1], its important mechanism is the disruption of the immune regulatory balance. Under normal circumstances, the body maintains immune tolerance, thereby avoiding

excessive immune damage and maintaining a dynamic balance between the immune system and the body itself [2]. Although the exact reason is not clear, recent consensus suggests that autoimmunity contributes to the development of CP / CPPS [3,4]. The body's immune response is mainly mediated by T cells, and T cell activation is a process involving multiple signals. The immune system needs to maintain an optimal balance in preventing autoimmune attacks, and this balance is regulated by a series of receptors / ligands [5]. T cells recognize the antigen as the first signal. However, T cell activation also requires stimulatory receptor / ligand pairs: CD28 / CD86 (CD80), CD40 / CD40L, 0X40 / 0X40L, 4-1BB / 4-1BBL can transmit further activation signals and require immunosuppression receptor / ligand pairs: CTLA-4 / CD86 (CD80), PD-1 / PD-L1 (PD-L2), CD48 / CD224 and TIGIT / CD155 (CD112) can inhibit T cell activation, regulate immune tolerance and the body's immune balance. The eight receptor / ligand pathways mentioned above that involve fine balance regulation include a total of 18 protein molecules that are currently identified as signaling molecules on T cells, which we call immune regulatory sites.

Transient Receptor Potential (TRP) belongs to a class of channel proteins widely distributed in the central and peripheral nerves. Transient Receptor Potential Protein-8 (TRPM8) is a member of the TRP channel family, which can be activated by hypothermia and menthol and belongs to the cold receptor [6,7]. TRPM8 was cloned as a prostate-specific protein in 2001 [8]. It is involved in regulating the body's cold, pain, vasoconstriction and inflammatory response. Studies have found that TRPM8 is significantly expressed in prostate hyperplasia and prostate cancer [9], and is considered to be a prostate-specific antigen [10]. It has been observed that both TRPM8 and the previously discovered Prostate Specific Antigen (PSA) have very strong antigenicity, but the latter is only found in male prostate tissues. However, TRPM8 is not only distributed in the prostate but also in the prostate. Other tissues, such as the Dorsal Root Ganglion (DRG), the brain and other nervous systems [11,12], play an important role in the formation and maintenance of chronic inflammatory pain [13,14]. Recent studies have shown that TRPM8, like PSA, has a strong immunogenicity or immunological relationship [15]. Due to the three characteristics of TRPM8, such as cold sensitivity and distribution in the nervous system and prostate immunogenicity, it is speculated that TRPM8 can connect almost all factors of the pathogenesis of CP / CPPS, such as prostate tissue, immune system, nervous system and pain, and the mechanism of this connection may be directly related to pain and discomfort in patients with CP / CPPS. Therefore, patients with CP / CPPS not only have pain in the prostate region, but also face abnormalities in other organs or tissues, such as the autonomic nervous system closely related to DRG and the brain [16]. In addition, TRPM8 has been studied to successfully induce Experimental Autoimmune Prostatitis (EAP) models [17]. In this study, the antigenic properties of TRPM8 in CP / CPPS were investigated. It turns out that TRPM8 is an autoimmune initiation factor that triggers and maintains autoimmune processes in prostate tissues and the nervous system. Local and systemic symptoms of CP / CPPS that can be promoted clinically, that is, CP / CPPS may not be an independent disease originating from the prostate but a local clinical manifestation of systemic disease caused by a hypersensitivity reaction to the TRPM8 antigen [18,19]. Zhou Xiaohui's group designed several peptide fragments T1 (1033-1052aa), T2 (1074-1094aa), T3 (931-953aa), and T4 (12-27aa) based on the sequence of the TRPM8 protein. It was found through animal experiments that T2 can cause Strong CP / CPPS response in mice. It is speculated that T2 caused CPPS may be associated with immune check points. Through animal experiments, we know that although T2 may cause CP / CPPS in mice, it is unknown which target protein T2 specifically acts on, which hinders its further clinical research. Therefore, it is necessary to develop new methods to clarify the

role of T2 and target proteins. Simulation experiments can be performed on a computer according to the actual reaction system [20-22]. Molecular docking is one of the commonly used methods of molecular simulation. Its essence is the identification process between two or more molecules. The molecular docking experiment is mainly aimed at the study of the intermolecular interaction between the receptor and the ligand. It also can calculate the interaction energy between the receptor and the ligand, and then make a preliminary evaluation of its binding. Finally, the best match is selected by a scoring function. In this article, using T2 as a probe molecule, a potential target protein programmed cell death receptor-1 (PD-1) was selected from 18 proteins related to CP / CPPS. PD-1 is an important immunosuppressive molecule. As an immune check point, it plays an important role in down-regulating the immune response and promoting the formation of immune tolerance by suppressing the immune response of T cells [23]. PD-1 / PD-L1 is involved in the development of autoimmune diseases as a negative pathway for immune regulation [24]. After PD-L1 binds to PD-1, T cell activation is inhibited and T cells are in an immune tolerant state. Targeting the PD-1 / PD-L1 pathway, blocking the signal pathway can activate T cells and release their immune tolerance, leading to excessive activation of T cells, leading to autoimmune diseases [25,26]. After taking PD-1 as the research object, the software studied the interaction mechanism of T1, T2, T3, and T4 peptide fragments with PD-1 protein, and found that T2 acts in the PD-1 protein active region and forms with PD-1 strong force, in order to verify the conclusion of molecular docking, we did *in vitro* competition binding experiments, which is consistent with the results of molecular docking.

Materials and Methods

Experimental animals

Thirty male Kunming rats (6-8 weeks, 18-22 g) were purchased from Qinglongshan Animal Breeding Farm in Jiangning district, Nanjing. The mice were kept at room temperature (25℃) with unlimited access to food and water freely for 24 h, and the light and dark periods were 12 h / 12 h. All animals were used in accordance with the experimental animal management regulations promulgated by the National Science and Technology Commission of the People's Republic of China. These 30 male mice were randomly divided into five groups, namely the blank group, the T1 group, the T2 group, the T3 group, and the T4 group, with 6 mice in each group. Then, four peptides (T1-T4) were prepared as immune emulsions and injected into mice. After a period of time, pathological sections of the prostate tissues of the mice were prepared. Finally, the mouse prostates were stained with Hematoxylin-Eosin (HE), and the prostate was observed and photographed under a microscope.

Materials

T1-T4 peptide was synthesized by Nanjing Kingsray Technology Co Ltd, China; Mouse PD-1-his, Mouse PD-L1-mFc and Rabbit Anti-Human IgG1-Fc was purchased from Beijing Solarbio Science & Technology Co Ltd, China; Bovine Serum Albumin (BSA), two-component TMB chromogenic solution, ELISA stop solution, disodium hydrogen phosphate dodecahydrate, sodium chloride, hematoxylin, xylene, absolute ethanol, eosin, formaldehyde solution, diphosphate Potassium hydrogen, potassium chloride, concentrated hydrochloric acid, and sodium hydroxide were purchased from Sinopharm Group Chemical Reagent Co Ltd, China. The experimental water was all double-distilled water.

Calculation simulation experiment

Processing of protein and peptide structures: Accelrys Discovery Studio (DS) 2016 software was used for molecular simulation, and energy minimization and molecular docking experiments were carried out using a

CHARMM force field. Download the three-dimensional structures of 18 immunomodulatory proteins from the RCSB protein data bank **[27],** use DS software to process these protein structures, delete water molecules and small molecule ligand compounds, and finally optimize the protein structure by Prepare protein module of DS software. The amino acid sequence of TRPM8 (Gene ID:79054) **[28]** was collected from the NCBI protein database. Based on the amino acid sequences of T1 (1033-1052aa), T2 (1074-1094aa), T3 (931-953aa) and T4 (12-27aa) isolated from TRPM8, the Builder and Edit Protein module of the DS software were used to construct the initial four peptide ligand's structure. Then the Standard Dynamics Cascade module of DS was used to perform Molecular Dynamics experiments on these 4 fragments to make the 4 fragments reach equilibrium. Accelrys Discovery Studio (DS) 2016 software was used for molecular simulation, and energy minimization and molecular docking experiments were performed using a CHARMm force field. Download the three-dimensional structures of 18 immunomodulatory proteins from the RCSB protein database **[27],** and the structure of these proteins was processed with DS software to remove water molecules and small molecule ligand compounds. Finally, the protein structure was optimized by preparing the protein module of DS software. The amino acid sequence of TRPM8 (Gene ID:79054) **[28]** was collected from the NCBI protein database. Based on the amino acid sequences of T1 (1033-1052aa), T2 (1074-1094aa), T3 (931-953aa) and T4 (12-27aa) isolated from TRPM8, the Builder and Edit Protein module of the DS software were used to construct the initial four peptide ligand's structure. Then the Standard Dynamics Cascade module of DS was used to perform Molecular Dynamics experiments on these 4 fragments. After four stages of minimization, heating, equilibration and production, the 4 fragments reached equilibrium.

Molecular docking: To investigate the interaction between receptor protein and 4 peptide ligands, molecular docking study was performed with ZDOCK module in Discovery Studio 2016, ZDOCK is an initial-stage docking algorithm that utilizes a Fast Fourier Transform (FFT) search algorithm **[29],** it involves various physical and chemical properties of ligands and receptors, including electrostatic energy due to Van der Waals potential energy, complementary shapes, and changes in free energy of dissolution, etc. Finally, the ZDOCK score of each structural conformation is obtained. While, based on the CHARMM polar H force field, RDOCK is used to optimize and reorder the energy minimization algorithm of ZDOCK results [30]. The steps include: (1) removing collisions based on analysis of Van der Waals force and internal energy; (2) minimizing overall energy on the basis of limiting non-hydrogen atoms, while maintaining the charged side chain to remain neutral; (3) overall energy minimize. Finally, the collision structure is removed and the conformation structure is re-ranked. Set the Euler angle step size of the rotation direction of the ligand sample to 6, "RMSD Cutoff" to 6.0, and "Interface Cutoff" It is 9.0, the "Maximum Number of Clusters" is 60, and the combination mode "Top Poses" is 2000. The other parameters are calculated using the default values.

***In vitro* experiments: ELISA:** The effect of four peptides (T1-T4) on PD-1 and the blocking concentration IC50 of PD-1 and PD-Ll were determined by competitive ELISA. The experimental method was as follows: the coated protein PD-1 was added at the same time with the tested sample (peptide T1-T4) and the enzyme-labeled secondary antibody (PD-Ll protein), and finally the substrate was added for color development. If the peptide can compete with PD-Ll to bind to PD-1, the absorbance value will change significantly. The absorbance value was used to observe whether the peptide could inhibit PD-1 / PD-Ll binding.

Results and Discussion

Analysis of animal experiment results

According to the pathological section results of HE staining, we found that the prostate epithelial cells in the T2 group were atrophic, and there were more inflammatory cells in the interstitium. Compared with the control group, T1, T3 and T4 groups did not observe more extensive infiltration, while in the blank control group, the prostate structure was intact without collapse, and there was no obvious inflammatory cell infiltration in the stroma and around the blood vessels **(Figure 1).**

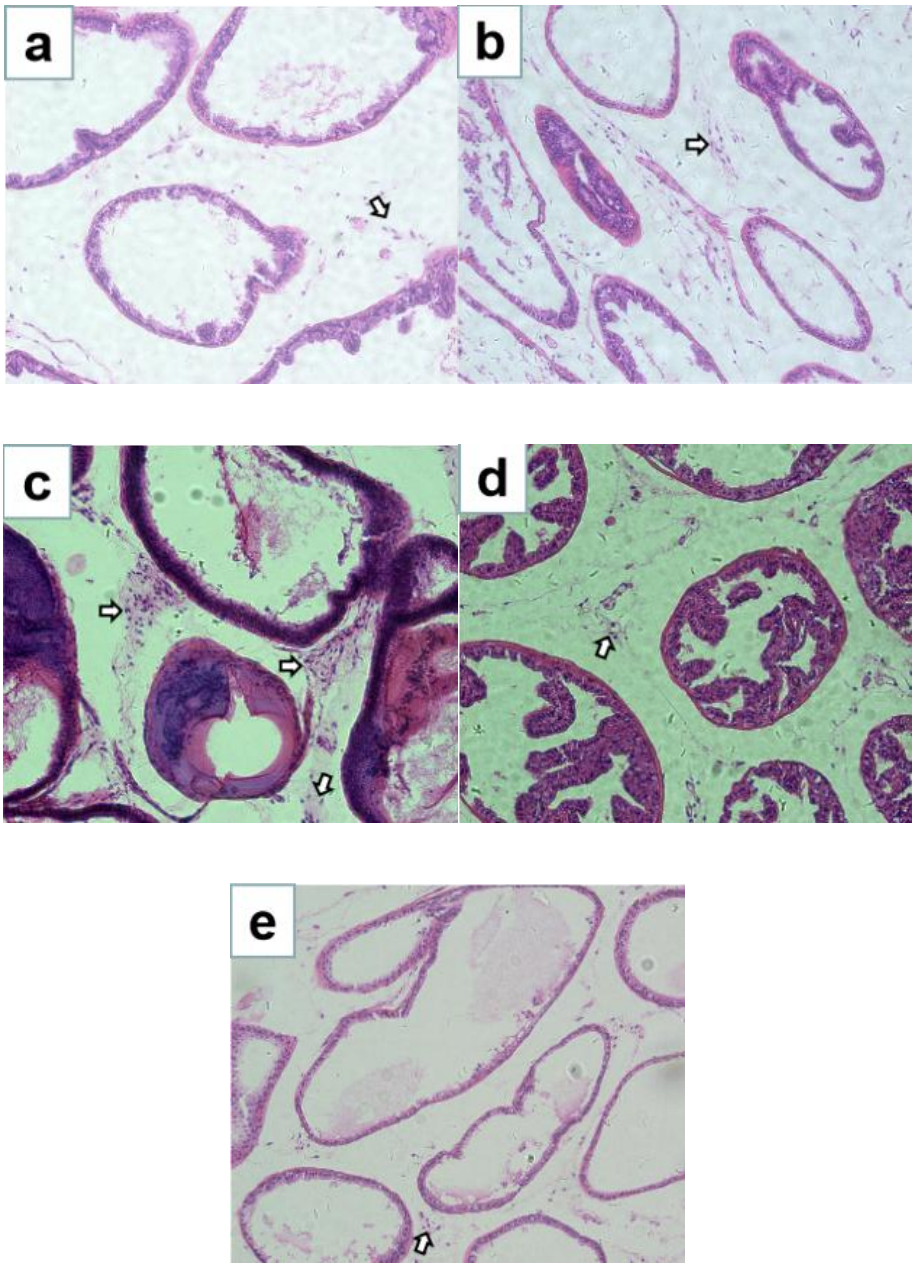

Figure 1: Prostate tissue pathological sections and HE staining results (magnification 200X) in each group: (a) blank group; (b) T1 group; (c) T2 group; (d) T3 group; (e) T4 group.

The three-dimensional structure of the four peptides

Based on the amino acid sequences of T1 (1033-1052aa) CKEKNMESSVCCFKNEDNET, T2 (1074-1094aa) CSEEMRHRFRQLDTKLNDLKG, T3 (931-953aa) FTGNESKPLCVELDEHNLPRFPE and T4 (12-27aa) NRRNDTLDSTRTLYSS, the original four peptides (T1-T4) were constructed using DS software structure. And the three-dimensional structure of these peptide ligands output after kinetic optimization is shown in Figure 2.

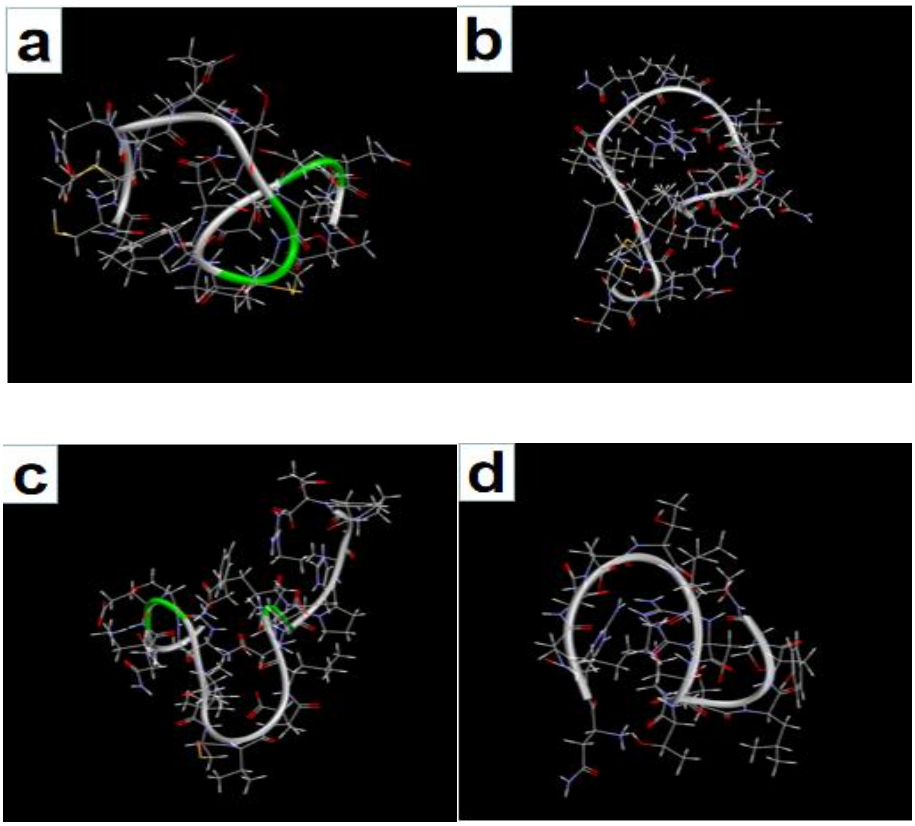

Figure 2: Three-dimensional structure of peptide ligands: (a) the three-dimensional structure of peptide T1; (b) the three-dimensional structure of peptide T2; (c) the three-dimensional structure of peptide T3; (d) the three-dimensional structure of peptide T4. Peptides are shown in a secondary structure, and amino acid residues are shown in a line.

Simulation of CP / CPPS Potential Target Molecules

Statistical analysis and evaluation of the docking effect of T2 and immune regulation points: Peptide T2 was used as a molecular probe, and the Peptide T2 fragment was molecularly docked with 18 immunoregulatory proteins in the ZDOCK module of DS software to screen potential target molecules. **Table 1** shows ZDOCK scores and ZRANK scores after docking Peptide T2 with 18 immunoregulatory proteins. Through analysis, it was found that the four proteins PD-1, CD40, CTLA-4, and OX40 all had ZDOCK scores greater than 10, and the ZRANK scores were lower than others protein molecules. For the docking of proteins to proteins or p eptides, a higher ZDOCK score indicates better binding, and a lower ZRANK score indicates tighter binding. These four proteins may be potential target molecules for CP / CPPS through preliminary screening.

Table 1: T2 and immune check point docking score and energy parameters.

Immune check point	PDB ID	ZDOCK Score	ZRANK Score	ZRANK E_elec1	ZRANK E_vdw2	ZRANK E_ elec1	ZRANK E_vdw2
PD-1	3BIK	10.74	-74.88	-48.79	-3.91	-51.66	-7.42
CD40	6PE9	10.28	-65.13	-46.56	0.31	-49.32	-2.73
CTLA-4	5E56	10.08	-62.52	-45.53	1.39	-52.78	-2.36
OX40	2HEV	10.02	-63.80	-46.55	1.73	-46.66	-2.18
PD-L1	3SBW	9.36	-60.21	-34.94	-1.26	-42.27	-0.75
CD80	2HEW	9.22	-61.01	-41.14	-0.51	-43.57	-0.73
PD-L2	3BP5	9.18	-65.13	-37.02	-0.24	-37.50	-0.55
CD86	1NCN	8.98	-67.41	-32.37	-0.255	-49.73	-0.49
CD28	4R0L	8.94	-55.18	-31.16	-1.76	-42.16	-0.43
CD40L	3QD6	8.52	-50.69	-36.16	-4.28	-43.37	-9.65
4-1BB	6A3W	8.24	-58.41	-38.73	-2.53	-43.06	5.36
0X40L	2HEV	7.44	-48.34	-33.13	-1.70	-52.24	-2.84
4-1BBL	6FIB	7.38	-51.23	-36.86	2.15	-41.43	1.64
CD48	2DRU	7.21	-34.63	-36.78	0.21	-39.29	2.25
TIGIT	5V52	7.03	-41.68	-29.76	-2.10	-51.58	-6.40
CD224	2DRU	6.93	-37.81	-24.93	-0.41	-32.28	-5.22
CD155	6ARQ	6.91	-36.68	-17.07	-4.06	-4.20	-4.87
CD112	4DFH	6.78	-27.68	-11.29	-1.52	-36.38	1.76

Prediction of the binding region of T2 and immune check points: The RDOCK module of DS software was used to further optimize the ZDOCK docking results of the four proteins PD-1, CD40, CTLA-4 and OX40, therefore we selected the conformation with the lowest RDOCK score for each protein for analysis. The docking results are as follows **Figure 3.** According to analysis, T2 binds to the active regions of two proteins, PD-1 and CTLA-4. The effect of the peptide on the active region of the protein will affect the binding between the protein

and its receptor or ligand, thereby affecting the transmission of it's signaling pathway and further affecting the immune response of the body. Therefore, based on the docking results, we screened two potential target proteins with high possibility, namely PD-1 and CTLA-4.

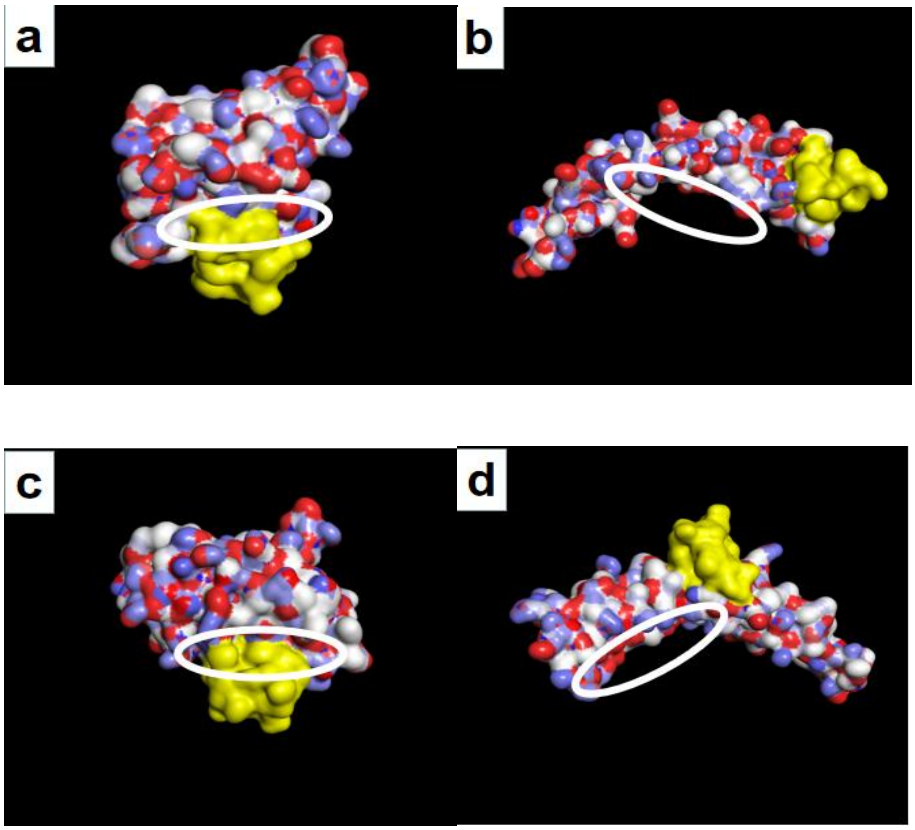

Figure 3: T2 binding sites on different proteins: (a) PD-1 protein; (b) CD40 protein; (c) CTLA-4 protein; (d) OX40 protein. Protein is expressed as a solid surface.

Based on the above scoring function and binding region, we calculated the binding energy of the PD-1 and CTLA-4 proteins to T2. The results are listed in **Table 2.** The lower the binding energy value, the tighter the binding. Analysis shows that the binding energy value of Peptide T2 and PD-1 protein is -42.72 kcal / mol, which is much lower than the binding energy value of Peptide T2 and CTLA-4 protein. Therefore, from the perspective of binding energy, PD-1 as the potential target protein had the greatest possibility.

Table 2: Statistics of interfacial interaction energy between T2 and protein molecules.

Protein	Total Interaction Energy / (kcal/mol)	Total VDW Interaction Energy / (kcal/mol)	Total Electrostatic Interaction Energy / (kcal/mol)
PD-1	-42.72	-11.75	-30.97
CTLA-4	95.72	134.33	-38.61

Pathogenesis of CP / CPPS based on the mechanism of PD-1 / PD-L1

Introduction of Crystal Structure of PD-1 / PD-L1 Complex: Analysis of the interaction surface of the PD-1 / PD-L1 complex, as shown in **Figure 4(a),** the yellow-labeled protein is PD-L1, and the left is PD-1. A groove is formed when the surface of PD-1 is combined with PD-L1. Compared with PD-L1, the PD-1 binding surface is more suitable as a receptor in molecular docking. The grooved surface formed by the combination of PD-L1 on PD-1 is also the active area of PD-1 in the yellow circle in **Figure 4(b).**

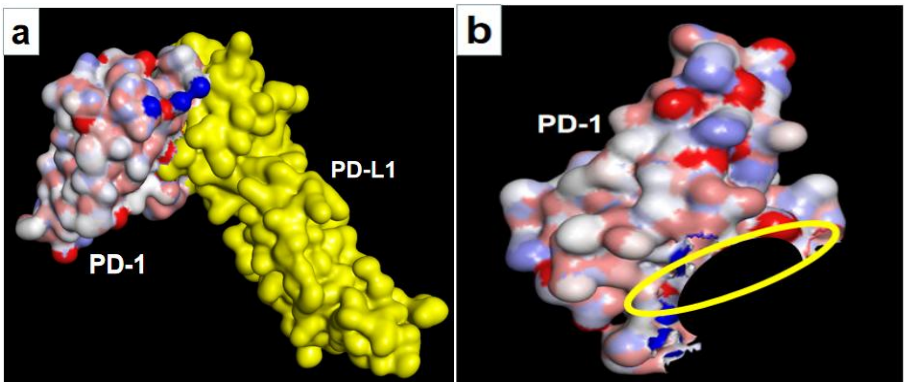

Figure 4: (a) Crystal structure of PD-1 / PD-L1 complex; (b) active surface on PD-1 / PD-L1 complex crystal PD-1. PD-1 and PD-L1 proteins are shown on a solid surface.

Prediction of the binding region between T2 and immune check points: After screening a potential target protein PD-1 related to CP / CPPS with T2 as a probe, another three of the four peptides (T1-T4) were constructed and optimized using software, and their relationship with the potential target protein PD- 1 mechanism of action. From **Figure 5(a),** the 20 pose distributions of the combination of T1 and PD-1 show that the T1 fragment mainly acts on the N-terminus of PD-1, the β-sheet of PD-1, and the intermediate positions of the N-terminus and C-terminus of PD-1. From **Figure 5(b),** the 20 pose distributions of T2 and PD-1 binding show that the T2 fragment mainly acts on the extracellular domain of PD-1 (the binding region of PD-1 / PD-L1), that is, the active region and PD-1. The N-terminus and C-terminus are two regions, and the top 5 poses are distributed in the extracellular domain of PD-1. From **Figure 5(c),** the 20 pose distributions of the combination of T3 and PD-1 show that the T3 fragment mainly acts on the N-terminus of PD-1, the C-terminus of PD-1, the middle position between N-terminus and C-terminus of PD-1, and PD-1 is in the active region, but only one of the top 5 poses is distributed in the active region. From the distribution of the 20 poses in which T4 and PD-1 are combined in **Figure 5(d),** it can be seen that the T4 fragment mainly acts on the middle portion of the N- and C-terminus of PD-1 and the β-sheet of PD-1. From the above, we know that T2 has the highest probability of acting on the PD-1 / PD-L1 binding region, that is, T2 is most likely to affect the PD-1 / PD-L1 pathway.

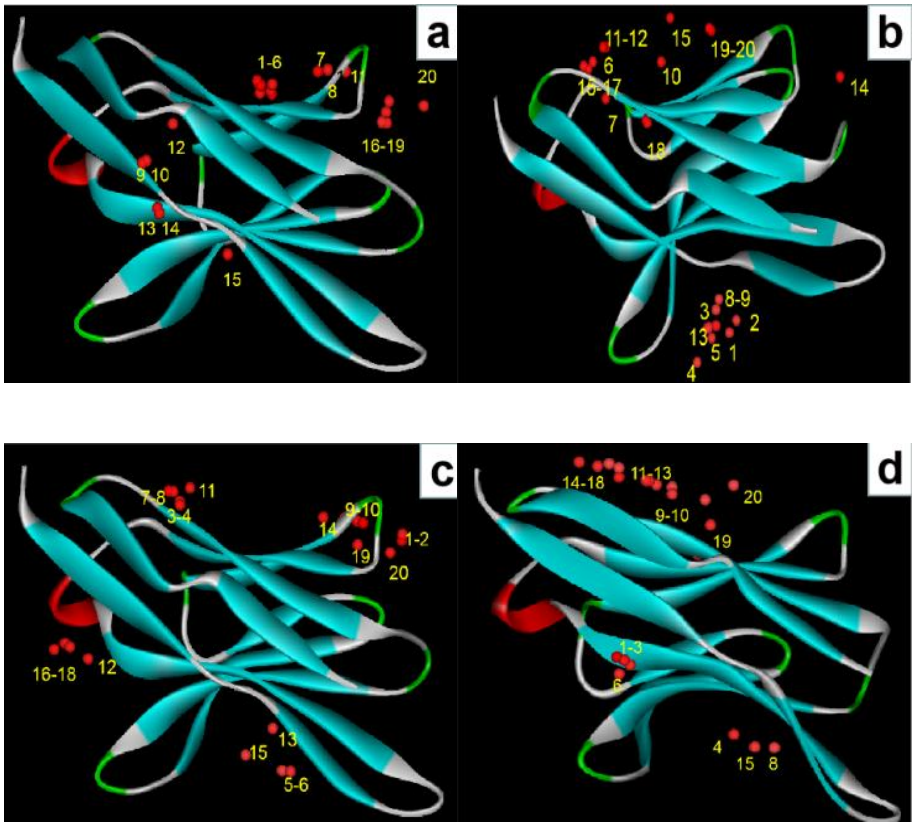

Figure 5: Optimal top 20 posture distributions of different peptides bound to PD-1: (a) Peptide T1; (b) Peptide T2; (c) Peptide T3; (d) Peptide T4. The PD-1 protein is shown as a band, and the position of the peptide distribution is indicated by a red dot.

Analysis of the mechanism of T2 and PD-1

Hydrophobic interaction analysis: The semi-empirical method quantitatively describes the hydrophobicity between PD1 and T-2 (Equation 1): The formed hydrophobicity between PD-1 and T2 is 48.67%. The hydrophobic interaction between PD-1 and T2 promotes the binding between the two. **Figure 6** is a distribution diagram of hydrophobic amino acid residues in a complex formed by PD-1 and T2.

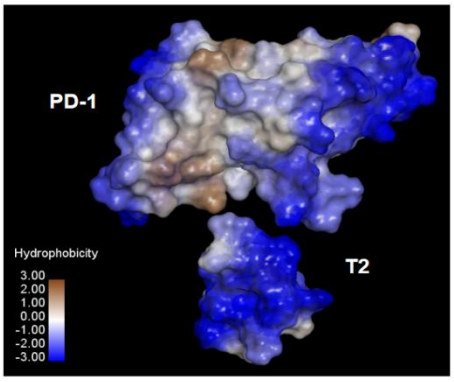

Figure 6: Hydrophobic distribution at the binding interface between PD-1 and T2. Blue represents hydrophilic amino acid residues and brown represents hydrophobic amino acid residues. The depth of color is positively related to the size of hydrophilicity and hydrophobicity, and the range from hydrophilic to hydrophobic is -3.0 to 3.0.

$$Hydrophobicity(\%) = \frac{NON_{SAS1} + NON_{SAS2}}{NON_{SAS1} + POL_{SAS1} + NON_{SAS2} + POL_{SAS2}} \quad (1)$$

Note: Among them, POLSAS and NONSAS respectively represent the total surface area of the solvents of the polar group and non-polar group on the interface of PD-1 and T2, and the subscripts 1 and 2 represent the PD-1 protein and Peptide T2, respectively.

Hydrogen bond analysis: In view of the important role of hydrogen bonding, we conducted an in-depth analysis. The nature of hydrogen bonding is determined by the bond length and bond angle. Generally, when the bond length is less than 2Å, it is considered to be a strong hydrogen bond. Through analysis, it was found that peptide T2 and the amino acid residues in the active site of PD-1 formed a hydrogen bond interaction **(Figure 7).** **Table 3** lists these hydrogen bond parameters. The hydrogen bond formed between T2 and PD-1 promotes the bonding between T2 and PD-1. Then calculate the interaction energy of PD-1 and T2 under the CHARMm force field. The calculation results show that the total interaction energy of PD-1 and T2 is -42.72 kcal / mol.

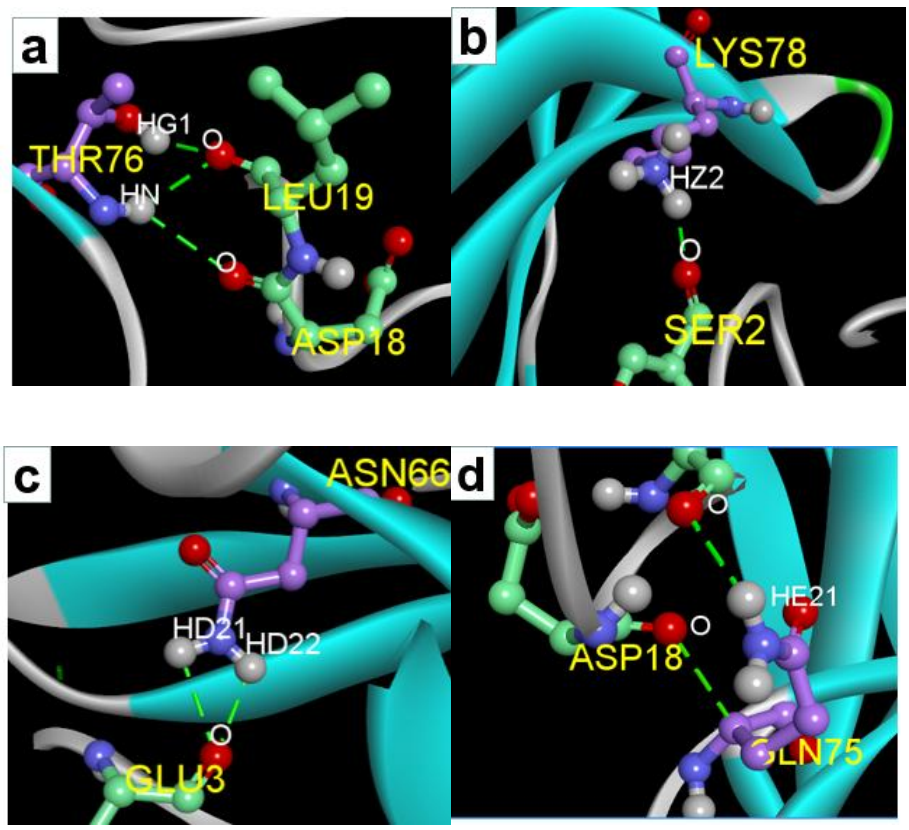

Figure 7: Diagram of the main hydrogen bonds formed in the experimental area at the interface. The active amino acids are shown in bright yellow. The green dotted line shows hydrogen bonding. Amino acid residues are shown in a club shape.

Table 3: Bond length and bond angle of the hydrogen bond formed in the interaction region between PD-1 and T2.

X─H…Y	d(X─H)	d(H…Y)	d(X…Y)	(XHY)

PD-1:THR76:OG1 –HG1… O:LEU19:T2	1.00	1.95	2.82	152.08
PD-1:THR76:N–NH… O:ASP18:T2	0.99	2.93	3.71	135.18
PD-1:THR76:N–NH… O:LEU19:T2	0.99	2.46	3.24	134.87
PD-1:LYS78:NZ–HZ2… O:SER2:T2	1.00	1.82	2.79	152.98
PD-1:ASN66:ND2–HD21…O:GLU3:T2	1.02	2.62	2.82	90.77
PD-1:ASN66:ND2–HD22…O:GLU3:T2	1.02	2.29	2.82	111.38
PD-1:GLN75:NE2–HE21…O:LYS20:T2	1.01	2.57	3.37	137.40

According to the analysis of hydrogen bonds, the three terminal oxygen atoms of the amino acids ASP18 and LEU19 on T2 and the hydrogen and nitrogen atoms on the amino group of THR76 on PD-1 protein respectively form three hydrogen bonds, of which one is a strong hydrogen bond **(Figure 7a).** Strong hydrogen bonding plays an important role in the interaction of T2 and PD-1. The amino acid SER2 on T2 and LYS78 on PD-1 protein also formed a strong hydrogen bond **(Figure 7b).** The existence of hydrogen bonds strengthened the role of amino acids on T2 and PD-1. The amino acid GLU3 on T2 has two hydrogen bonds with ASN66 on PD-1 protein **(Figure 7c),** and the amino acids ASP18, LYS20 on T2 and GLN75 on PD-1 protein also have two hydrogen bonds **(Figure 7d).** The existence of these hydrogen bonds is important for studying the interaction between T2 and PD-1 in biological experimental regions.

3.5 Evaluation of in vitro experiments

The optimal concentration of each factor variable in the ELISA experiment is shown in the supplementary material. **Figure 8** records the relationship between the different concentration logarithms and absorbance values for each of T1, T2, T3, and T4. It is found from the figure that the absorbance values of T1, T3 and T4 with different concentrations are all around 1.0 and above, but the absorbance values of T2 with different concentrations are different. As the T2 concentration increases, the absorbance value gradually decreases. When these fragments are not added under the same conditions, the absorbance of PD-1 and PD-L1 binding is about 1.0. Therefore, the blocking rate of PD-1 / PD-L1 by T1, T3, and T4 is almost 0, that is, PD-1 / PD-L1 is not blocked, while T2 can block PD-1 / PD-L1. **Figure 9** plots the logarithm of the concentration of T2 as the abscissa, and plots the OD ratio before and after T2, that is, the blocking rate on the ordinate, to obtain a linear curve of ΔB / B0 and lgC_{T2}. From the figure, the T2 concentration in the range of 0.1-200 μg / mL shows a good linear relationship: Y = 0.364X + 0.213, correlation coefficient R^2 = 0.944, T2 mass concentration at the 50% blocking rate is IC_{50}, IC_{50} is 20.04 μg / mL.

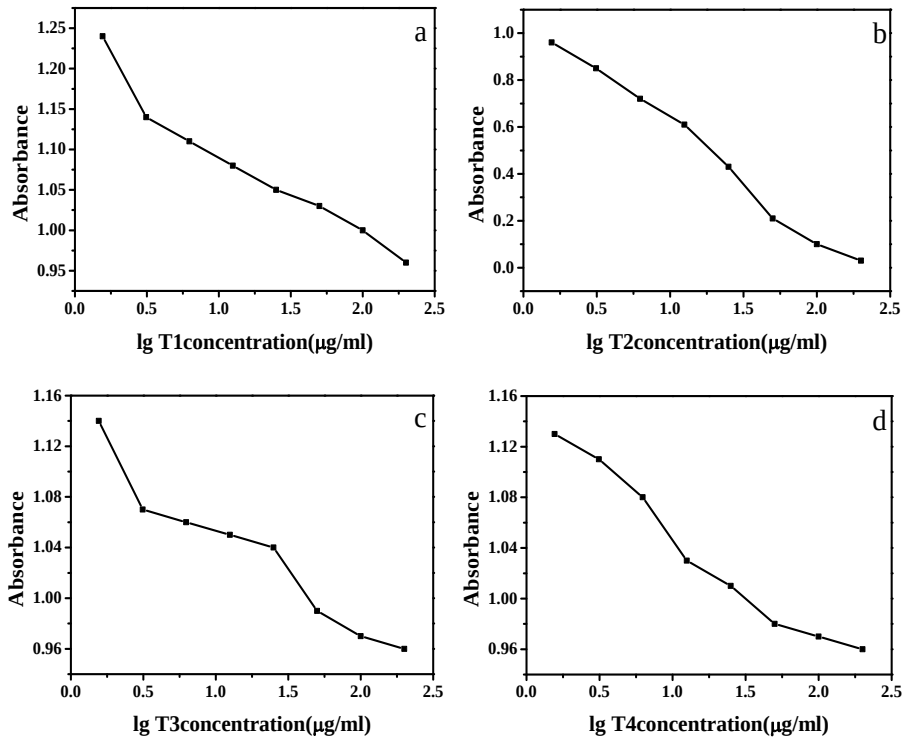

Figure 8: Relationship between peptides and absorbance at different concentrations: (a) the relationship between T1 at different concentrations and absorbance; (b) the relationship between T2 at different concentrations and absorbance; (c) the relationship between T3 at different concentrations and absorbance; (d) the relationship between different concentrations of T4 and absorbance value.

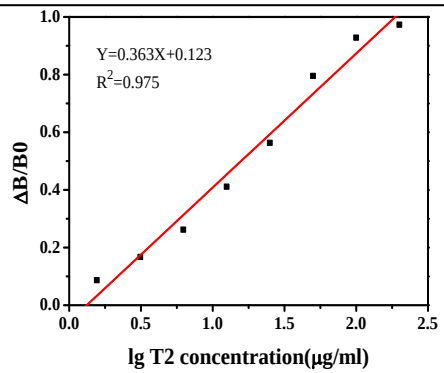

Figure 9: Standard curve of ELISA for peptide molecule competition.

Conclusion

In this study, several peptide fragments T1 (1033-1052aa), T2 (1074-1094aa), T3 (931-953aa) and T4 (12-27aa) were designed based on the sequence of the prostate-specific antigen TRPM8 protein. The murine CP / CPPS model found that T2 can induce a strong CPPS response in mice. Based on this, the docking of 4 peptide ligands with PD-1 protein was compared using DS software. In addition, the blocking effect ranking of four peptide ligands, possible binding sites and postures were obtained, and combined with ZDOCK and ZRANK comprehensive scores, it was found that T2 most likely blocked PD-1 and PD-L1. Subsequently, based on the

CHARMM polar H force field, the RDOCK algorithm was used to optimize the docking results of PD-1 and T2. It was found that T2 not only acts on the active area of PD-1, but also forms a tight combination with PD-1 through hydrophobicity, hydrogen bonding, and static electricity. And it has a large binding energy with PD-1, that is, T2 can bind with PD-1, and the binding of T2 and PD-1 may block PD-1 / PD-L1. ELISA verification results show that T2 can compete with PD-L1 to bind PD-1. In vitro experiments have further verified the molecular docking results. Therefore, it is verified that the conjecture of the article may be due to the combination of T2 and PD-1, which may block the PD-1 / PD-L1 signaling pathway, activate T cells, and cause excessive activation of T cells, which may lead to autoimmune diseases CP / CPPS. While much of the previous literature has focused on T2 inducing a strong CP/CPPS response in mice, there is few studies which investigate the specific target protein of T2. Therefore, this article explores the possible pathogenesis of CPPS on the basis of molecular simulation methods, and verifies it through in vitro experiments, which is extremely scope for further research the etiology, pathogenesis and clear treatment of CP / CPPS.

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