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Identification of Small-Molecule Inhibitors against Human Leukocyte Antigen-Death Receptor 4 (HLA-DR4) Through a Comprehensive Strategy

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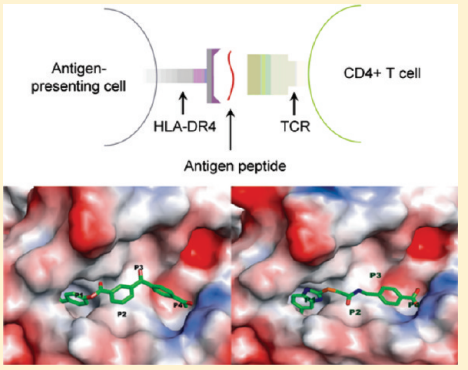
Supporting Information

ABSTRACT: Rheumatoid arthritis (RA) is an autoimmune disease mediated by T-lymphocytes and associated with the human leukocyte antigen-death receptor 4 (HLA-DR4). The HLA-DR4 protein selectively interacts with the antigenic peptides on the cell surface and presents them to the T cell receptor (TCR) on CD4+ T cells. The HLA-DR4-antigen−TCR complex initiates the autoimmune response and eventually causes the chronic inflammation within patients bodies. To inhibit HLA-DR4-restricted T cell activation, an ideal approach is to discover non-T cell stimulating substrates that specifically bind to HLA-DR4. In this paper, a comprehensive structure-based design strategy involved de novo design approach, pharmacophore search, and dock method was presented and applied to "simplify" the known binding peptide ligand of HLA-DR4 and identified specific small-molecule inhibitors for HLA-DR4. The designed three-step strategy successfully identified five nonpeptide ligands with novel scaffolds from a chemical library containing 4×10^6 commercially available compounds within a tolerable computing time. The identified five chemicals, BAS-0219606, T0506-2494, 6436645, 3S-71981, and KM 11073, are all non-T cell stimulators and are able to significantly inhibit HLA-DR4-restricted T cell activation induced by type II collagen (CII) 263-272 peptide. IC_{50} for the best two potentials, BAS-0219606 and T0506-2494, was 31 and 17 μM, respectively, which is equivalent or better than the known peptide ligands. It is hopeful that they can be used as effective therapeutic means for further treatment of RA patients. In addition, the comprehensive strategy presented in this paper exhibited itself to be an effective flow line from peptide ligands to small-molecule inhibitors and will have applications to other targets.



1. INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial joints.[1−4] A variety of leukocytes together with activated synoviocytes from the pannus of proliferative tissue overgrow the articular cartilage. The result of such inflammatory process is always joint deformity and loss of joint function.[5] Previous functional investigations and genetic studies linked RA to human leukocyte antigen, especially its certain subtypes DR1 (HLA-DR1) and DR4 (HLA-DR4).[6−11] It has been shown that disease-related HLA-DR4 antigen selectively binds with antigenic peptides, which comprises the recognition surface for the T cell receptor (TCR) on CD4+ T cells and then triggers the autoimmunity response leading to RA[10−14](Figure 1).

Although the nature of the target antigen in RA is still somewhat not very clear, type II collagen (CII) 263-272 peptide, which is a specific HLA-DR1/4 binding peptide recognized by TCR, has been found as one of the target antigenic peptides for articular cartilage and specific immunity associated with RA.[15−18] The influenza virus hemeagglutinin (HA) 306-318 is another reported high-affinity antigenic peptide that interacts with the same groove of HLA-DR1/4 molecule.[19,20] It has shown that patients previously exposed to influenza A virus (subtype H5N1) exhibited a strong HLA-DR4-restricted T cell response in vivo to an epitope peptide of hemagglutinin from this subtype.[21] Since those two types peptide fragments can both be presented by HLA-DR4 molecule to T cells, which in turn initiate RA-associated autoimmunity responses,[22,23] they are viewed as classical model molecules to help in designing inhibitors that may interfere with the HLA-DR4-restricted T cell proliferation and lead to the effective suppression of autoimmunity.

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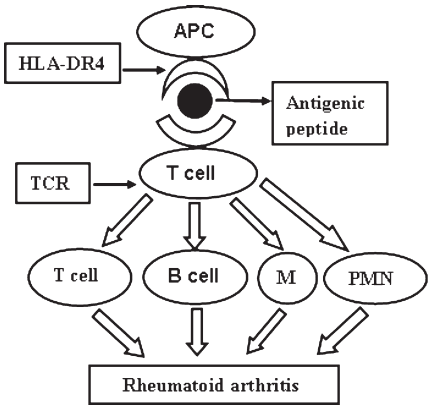

Figure 1. Schematic diagram for the mechanism of RA. We chose to block the HLA-DR4 and T cell receptor binding as the goal of molecular design, selecting small-molecule inhibitors to compete with the original peptide ligands. APC: antigen-presenting cell; TCR: T cell receptor; M: monocyte and macrophage; and PMN: polymorphonuclear neutrophils.

For this purpose, an ideal approach is to use non-T cell stimulating molecules that specifically bind to HLA-DR4 but will not be recognized by TCR. The molecule may competitively bind to the binding groove of HLA-DR4 and occupy the antigenic peptide (such as CII) binding sites, thereby suppressing HLA-DR4 specific T cell responses. In the past decade, several studies focused on the alteration of peptide substrates and on examining the impacts on T cell proliferation and differentiation.[24−26] Altered peptide ligands (APLs) carrying amino acid substitutions of the TCR-contact residues on the peptides can function as antagonists, which are able to induce T cell unresponsiveness[27−29] or changes in profile of cytokines production and prevent T cell proliferation in response to stimulating peptides.[30−32] However, these peptides are not feasible due to the difficulty to synthesize. In addition, the internal environment of human body would weaken the peptides' actual efficacies in vivo. Furthermore, exogenous peptides are easily degraded by inborn enzymes, therefore, cannot fulfill their proposed functions. All of these shortcomings associated with known peptide antagonists prevent the successful progression to clinic trails. Therefore, it is more attractive to find small-molecule inhibitors with equivalent or better inhibitory activities as opposed to altered peptide ligands.

Here in this paper, based upon the structures of known antigenic peptide, an approach combining several structure-based design strategies has been proposed to "simplify" the peptide ligands to small chemical molecule. Specifically, an "ideal" inhibitor was generated by de novo design program Ligbuilder1.2,[33] and a pharmacophore model was created by Unity4.4.1 program.[34,35] Then, a virtual screening protocol combining both pharmacophore search and dock method was applied to identify novel HLA-DR4 inhibitors from a chemical library containing about 4×10^6 chemical structures. The "hits" were tested on HLA-DR4-restricted T cell proliferation assay.

2. METHODS AND MATERIALS

2.1. Computational Schema. A virtual screening protocol combining both pharmacophore search and dock method is depicted in Figure 2.

The schema is divided in three steps: (1) De novo build and refine the "ideal" ligand inside the binding groove of HLA-DR4. The ideal ligand is expected to cover as many features as possible that are important for binding interactions. The parameters were set to maximize the chemical diversities while still giving the highest priority to chemical interaction; (2) create and screen commercially available chemical compound libraries using the pharmacophore model derived from the ideal ligands. Step 2 was designed to solve the synthesizable problem of peptide inhibitors by identifying small molecule inhibitors from commercially available compound libraries. A set of structural filters were applied, and a pharmacophore search was used as an efficient filter to perform the screen procedure within a reasonable time; and (3) validate search hits by inspecting binding affinities and binding modes. All the outcomes from pharmacophore search were checked again with a dock method for both binding affinity and binding mode. The docking conformation of these compounds should be capable of superimposing onto the pharmacophore, as indicated by low root mean square deviation (rmsd) values. The final compounds to be purchased were derived from the hit list and inspected by experts in the lab manually.

The computational tasks were carried out with the Redhat Linux 9 operating system on a Xeon workstation. The binding affinities of compounds with the HLA-DR4 protein were computed by the Gibbs free energy (ΔG) obtained from the Dock4.06 program,[36] and the values of pK_i (K_i represents dissociation constant) calculated by the AutoDock3.5 program.[37,38]

2.1.1. Screen Model and Binding Pocket Analyses. A cocrystal structure of human T cell receptor, influenza HA peptide, and HLA class II histocompatibility antigen[39] (PDB: 1J8H) was chosen as the starting point of the structure-based de novo design work (Figure 3A).

Y308, which locates in the deepest hydrophobic pocket (P1), is a key residue directing the peptide anchored in the groove appropriately. HA showed little binding activity toward HLA-DR4 after Y308 was mutated to a nonaromatic amino acid.[41−43] Another key residue is Q311. Its side chain is buried in a relative shallow pocket (P4), but the oxygen atom at the end of side chain is surrounded by three nitrogen atoms with partially or fully positive charges. It establishes strong electrostatic interactions between HA and HLA-DR4 protein. In addition, the residue T313 also contributes to the binding interaction with the P6 pocket, although the distance between O (T313) and N (His) is slightly longer than a normal hydrogen bond (H-bond). The calculated pK_i value for the wild-type HA308−317 peptide was 8.94, which was used as the binding affinity cutoff value for the following "hits" selection.

2.1.2. De novo Building and Refinement. LigBuilder1.2, a de novo ligand design program,[33] was used to generate theoretically the best ligands. Based on a "seed" structure database, a desired ideal molecule can be built within the binding pocket. LigBuild1.2 uses a genetic algorithm to grow ligands and link building blocks for ligand generation. Within this research, we expanded the building blocks library of the LigBuilder1.2 program to cover as many as scaffolds appearing in the popular drugs.

Here, the benzene ring of Y308 was selected as initial "seed" structure, and two para-hydrogens were selected as "growing" points for de novo ligand building. One hydrogen locates at the position previously connected to the peptide chain, and the other hydrogen substitutes the hydroxyl group. Since pocket 1 is deeper than where it can reach, and no obvious H-bond is formed around, we tried to find a better group that could take the place of the hydroxyl group. The growing result was modified and checked by docking the molecule achieved back to the binding groove.

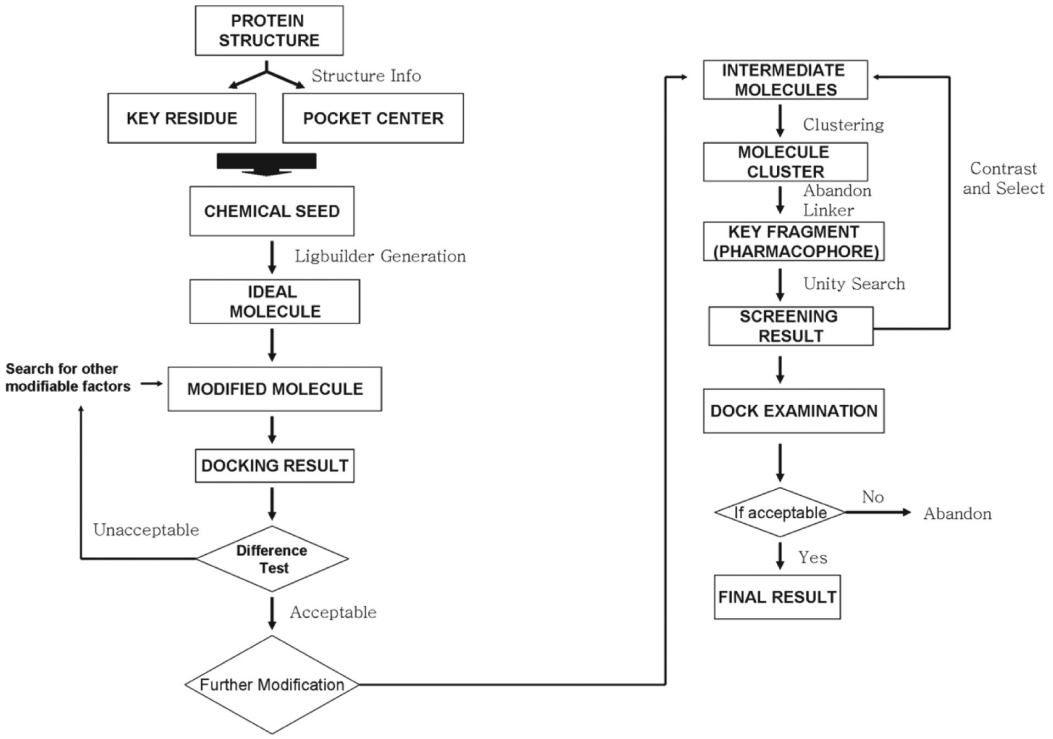

Figure 2. Flowchart illustrating the comprehensive design strategy.

2.1.3. Pharmacophore-Based Search for Hits. Following the first designing step, the key fragments of the "ideal" inhibitor were defined as the pharmacophore query for Unity search. Specifically, the ring structures bound to P1 and P4 were viewed as key fragments, and their orientations and relative distance were also included as pharmacophore features. The pharmacophore model used for Unity queries is depicted in Figure 4.

Unity 4.4.1 was then used to search matching compounds in a huge chemical library collection that covered six commercially available chemical databases, including MDL Screening Compounds Directory[44](formerly Available Chemicals Directory-Screening Compounds Database, ACD-SC), MDL Drug Data Report[45](MDDR), National Cancer Institute compound database[46] (NCI), Tripos LeadQuest database,[47] VS-3D (VS-3D contains more 6000 drug-like chemicals used for HTS) and the Bio-Active Molecular Database (BAMD contains about 1960 drugs and endogenous ligands). VS-3D and BAMD were comprised by our lab. The six libraries contain about 4×10^6 virtual chemical structures. The maximum acceptable computed log P value for each chemical was set to 7, and the molecular weight was limited to less than 800 Da.

The flexible search mode of Unity4.4.1 was chosen to generate possible conformations for each molecule, and the hits were the conformers fitting the pharmacophore query best. The hits from the pharmacophore search were manually inspected and then docked back into the binding pocket. The chemicals that passed through the docking filter were kept for further validations. The hits with nondrug-like features, such as containing more than five side chains, or distorted structures, etc., were discarded. Twenty-three chemicals were eventually selected to purchase after the virtual screening campaign (see Table S1, Supporting Information), and five of them were finally purchased to test their in vivo effects on T cell activation.

2.2. Cell-Based Assays. *2.2.1. Cells and Reagents.* Antigen presenting cell (APC) and HLA-DR4 specific T cell hybridoma for CII used in this study were Priess cells (human B cells transfected with HLA-DRB1*0401) and HLA-DR4-restricted 3838 clone. The Priess cells were cultured in Roswell Park Memorial Institute medium (RPMI) containing 10% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. The CII specific cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 50 μM 2-mercaptoethanol.

2.2.2. T cell Proliferation Assay. T cell proliferation experiments were assessed by the methyl thiazol tetrazolium bromide (MTT) assay, as described previously. The experiments were performed in 96-well microtiter plates with a total volume of 200 μL. Irradiated (3000 rad) Priess cells (2.0×10^4 cell/well) were incubated with various concentrations (0.1, 1, 10, or 100 μg/mL) of wild-type CII 263−272 or selected molecule or medium alone for 2 h at 37 °C with 5% CO_2.

In competitive studies, irradiated (3000 rad) Priess cells (2.0×10^4/well) were incubated with the antigenic peptide CII263−272 (10 μg/mL) and various concentrations of compounds as indicated for 2 h at 37 °C with 5% CO_2. T cells (2×10^4/well) were then added and coincubated at 37 °C with 5% CO_2. After 48 h, 10 μL of methyl thiazol tetrazolium bromide (MTT) solution (5 mg/mL) was added to each well, and the incubation was continued for 4 h. Next, all the medium were removed by inverting and tapping the plates, the formazan crystals formed were dissolved in 100 μL dimethyl sulfoxide (DMSO) at 37 °C for 30 min in the dark. The absorbance was recorded at the wavelength of 595 nm in a microplate reader (BIO-RAD model 3550). Viable cell numbers were correlated directly to the concentration of formazan. The ratio of inhibiting

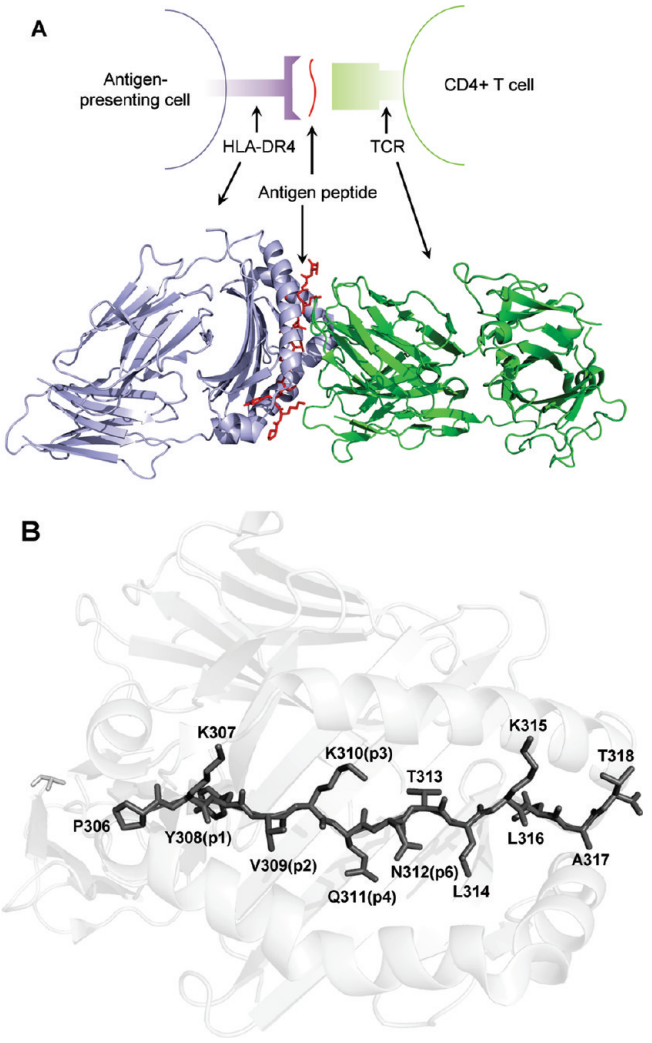

Figure 3. Illustration of binding model derived from cocrystal structure. (A) Schematic representation and cocrystal structure of human T cell receptor, influenza HA peptide, and HLA class II histocompatibility antigen, DR4 (PDB ID: 1J8H). (B) The binding interaction between HA 306-318 peptide and HLA-DR4. The HA peptide is buried within the groove formed by two α helices of HLA-DR4. The details of formed hydrogen bonds can be found in Table 1. The residues Y308, V309, Q311, and N312 (labeled as p1, p2, p4, and p6 as opposed to the binding pocket P1, P2, P4, and P6 on HLA-DR4 protein) are critical residues that anchor the peptide within the groove. The figure was generated by using the PyMOL program.[40]

was represented as the ratio of the CII-treated/compound-treated/CII-treated sample calculated from the absorbance values. IC_{50} value (50% inhibiting concentrations) was calculated by regression analysis. All experiments were repeated at least three times.

2.2.3. Statistical Analysis. Student's t-tests were used for the comparisons of optical density (OD). All analysis was performed using SPSS[48] (Version 11.5). P values less than 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Virtual Screening. *3.1.1. Building and Refining of Ideal Ligand.* Figure 5A showed an "ideal" ligand generated by Ligbuilder1.2. A methyl group instead of hydroxyl group was

Table 1. Interrelated H-bond Pairs in HA/HLA-DR4 Complex

HA peptide			HLA-DR4 protein			
name	ID	atom	name	ID	atom	distance (nm)
TYR	308	N	(α)SER	53	O	0.292
VAL	309	N	(β)ASN	82	O	0.287
VAL	309	O	(β)ASN	82	N	0.289
LYS	310	N	(α)ASN	62	O	0.286
GLN	311	N	(α)GLN	9	O	0.270
GLN	311	O	(α)GLN	9	N	0.317
GLN	311	O[a]	(α)ASN	62	N	0.286
GLN	311	O[a]	(β)GLN	70	N	0.308
GLN	311	O[a]	(β)LYS	71	N	0.277
ASN	312	O	(β)LYS	71	N	0.317
ASN	312	O	(β)LYS	71	N	0.297
THR	313	O[a]	(α)GLU	11	O	0.294
THR	313	O[a]	(β)HIS	13	N	0.315
LEU	314	N	(β)TYR	30	O	0.296
LEU	314	O	(α)ASN	69	N	0.280
LYS	315	O	(β)TRP	61	N	0.307
LEU	316	N	(α)ASN	69	O	0.270
ALA	317	N	(β)ASP	57	O	0.289
ALA	317	O	(α)ARG	76	N	0.300

[a] Atoms located in the side chains of HA peptide.

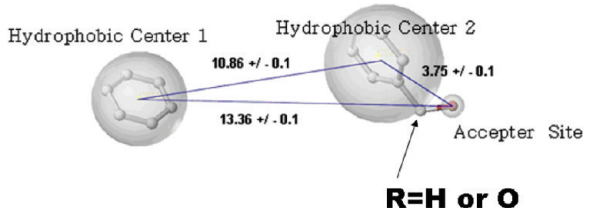

Figure 4. Triangular pharmacophore model used as Unity queries for virtual screening.

picked for the benzene ring. The qinoline ring appeared at the P4 position, which was a little surprising because there seemed to be not enough space around this area to adopt a conjugated ring structure. The fact that the qinoline ring protruded from the P4 position was consistent with this speculation, which prompted us to consider the possible substitution of this qinoline ring.

Another interesting issue happening next to P4 was the appearance of an anticipated "donor" group. The sulfonyl group at site 8 of the qinoline ring formed a tight H-bond with HLA-DR4 protein which helped HLA-DR4 to better recognize the inhibitor molecule, although a carbonyl or carboxyl group may be a better choice due to easier synthesis.

The generated molecule, although theoretically the best compound, needs to be modified since it is not easy to synthesize or purchase. We need to know which fragment can be altered without interfering the binding. However, the LigBuilder1.2 software cannot give us such information. To solve this problem, we used a dock method as a complement. The Dock4.06 program was employed to generate possible conformations of submitted ligands in the substrate environment and then ranks them according to force field energies. It was assumed that the structure manipulation was acceptable only if the changes of the binding score were negligible and the docking conformation of the modified

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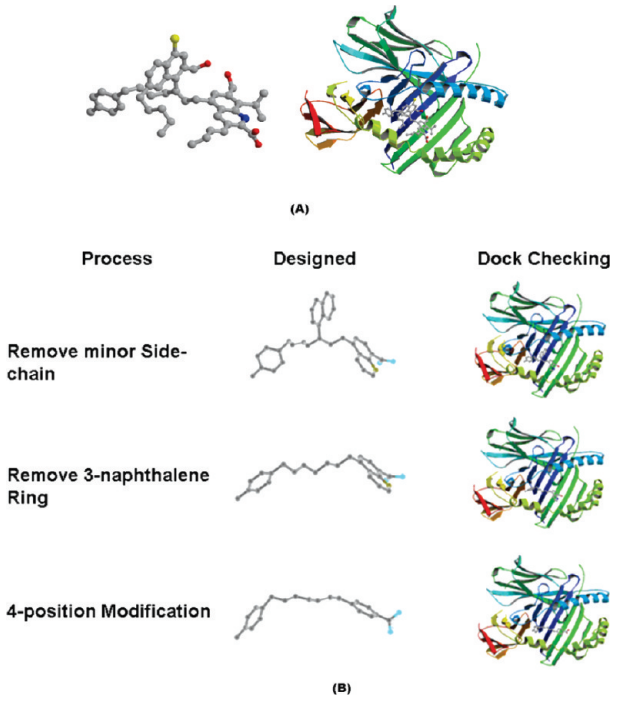

Figure 5. The "ideal" inhibitor generated by Ligbuilder1.2 and the following modification steps. (A) The docking conformation of the de novo designed inhibitor built by LigBuilder1.2 shown without or with the binding pocket of HLA-DR4. (B) The evolution of the de novo designed ligand. From top to bottom, the complex represents docking conformation of the growing scaffold within the side chain, docking conformation of the scaffold without naphthalene ring above P3, and docking conformation of the modified scaffold with a monocyclic structure at P4, respectively. To better exhibit the binding mode, high-resolution pictures of these docking complexes are included in Figures S1−S4, Supporting Information.

compound paralleled well with the original compound. As seen in Figure 5 B, a three-step refinement of the ligand was carried out without sacrificing the original binding conformation.

First, the de novo ligand was refined twice by removing the excess side chain. The bone sketch without the side chains fit more suitable in P1 and P4 pockets. There was a little difference in P3 with the orientation of naphthalene ring reversed. The dissimilarity was neglected because it was better that the naphthalene ring has more freedom within the open space above P3.

For further "simplification", the ligand was docked again without naphthalene ring above P3. Only a slight variety occurred after the deletion. Additionally, the Dock4.06 energy score had only a very small change (from −40.04 to −37.35 KJ/mol), which indicated that the naphthalene ring made a rather limited contribution to the binding process.

Last of all, it was even thought that the qinoline ring was not a suitable fragment for P4, which extruded from the shallow pocket. Other monocyclic groups might fit as well as or even better than the qinoline ring did. The protruded ring was removed, and the decrease of dock energy score was limited to a satisfactory extent as 5.05 KJ/mol (from −40.04 to −34.99 KJ/mol) demonstrated that the mutations were acceptable.

3.1.2. Pharmacophore Building-Up and Virtual Screening. As indicated in Figure 4, we targeted the functional groups at P1 and P4 sites as the pharmacophore query for library screening.

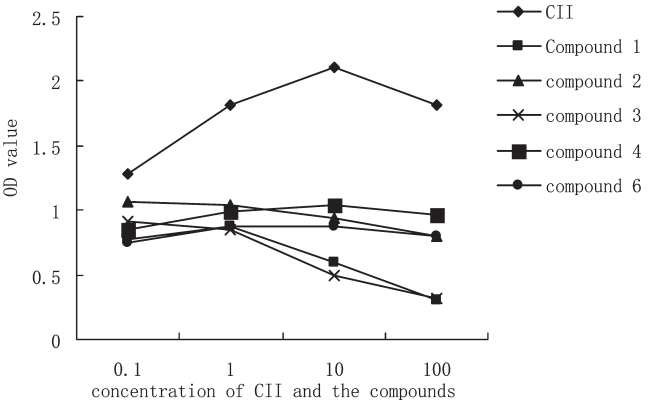

Figure 6. T cell proliferation in response to five selected compounds or CII263-272 at various concentrations (μg/mL). Compared to the medium alone, T cell proliferation was significantly increased by CII263-272. T cell proliferation was not affected by selected compounds and was significantly lower than wild-type CII263-272 at concentrations from 0.1 to 100 μg/mL.

The functional groups came from the modified "ideal" ligand of the last modification step, not from the original ligands generated. Another issue is that the orientation of the two groups with the pharmacophore were not simply copied from the best docking conformation of the modified ligand but from the average of the best conformation clusters provided by Dock4.06 program. A two-step examination was applied on docking results to make sure that the binding mode was "similar" to the pharmacophore. The first step was to directly compare the rmsd value between the dock yielded conformations and the queried pharmacophore. The cutoff value was set to 0.35 nm, which means that compared to the pharmacophore, compounds with a docking conformation larger than a 0.35 nm rmsd value would be abandoned. The second step was to do a manual inspection. The reason is screening results from Unity4.4.1 program only indicated the theoretical possibility of the molecule that may form the configuration coincident with the pharmacophore query. The configuration is generated without considering the features of receptor binding site and space constrains. Most of the selected chemicals are unfeasible because they would like to bump into the receptor when forming the "hit" configuration. The manual inspection was performed here to "filter" most of the false-positive hits of the pharmacophore search. Only compounds sharing the same docking conformation with the pharmacophore query were kept as genuine hits. That was why the final hit list contained only 23 compounds, which is much less than the regular yield of a traditional virtual screening.

3.1.3. Combined Structure-Based Design Strategy. LigBuilder1.2 is an effective de novo design tool and was applied to generate the hypothetic ideal ligands in order to increase the knowledge of the binding mode. The fragment library used here with LigBuilder1.2 program contained as many as possible fragments in known drugs, which was thought to construct a clear and proper description of the binding environment. Although the growing scaffold is theoretically ideal, it is often peculiarly constructed and hard for synthesis or purchase. Therefore, the de novo design work was only used for binding pocket analysis as a starting point for further design.

For the dock method, it exerts force field energies to rank the generated configurations of submitted complex and picks out the best matching. It can be used to distinguish the best candidate

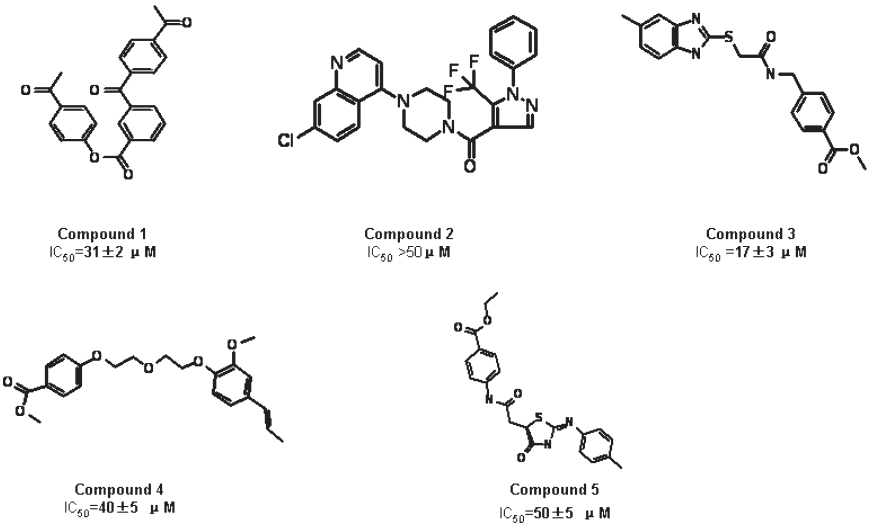

Figure 7. Inhibitory effects of selected compounds on T cell proliferation (MTT methods).

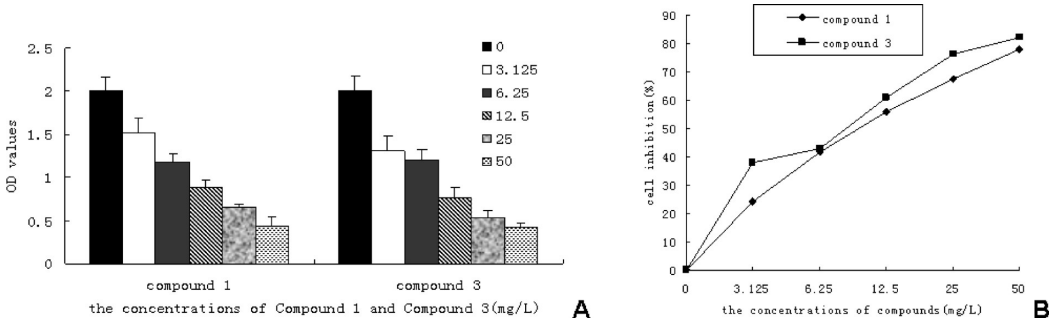

Figure 8. Inhibition effects of selected compounds on T cell proliferation. T cell proliferation induced by CII263-272 was significantly inhibited by selected compounds dose dependently (*P < 0.05 and **P < 0.01). (A) Concentration-dependent data for compounds 1 and 3 used for IC_{50} determination. (B) Inhibiting curve of HLA-DR4 induced T cell proliferation by the two compounds. All measurements were repeated at least three times.

from a large pool of small molecules with appropriate binding conformation. The Dock4.06 program was used in the refine step to gain the knowledge of replaceable fragments without destroying the binding mode. Although Dock4.06 program ignores the entropy change and is unsatisfactory due to its behavior of high false positive, it is well accepted as an effective tool and is suitable for the primary screening with a large chemical library. Other software, such as the AutoDock3.05 program, may provide more accurate results by consuming significantly more computing time, which is inappropriate for a screening large database.

Unity4.4.1 searches and analyzes chemicals based on a given model. It can quickly identify molecule that fits well with the pharmacophore, but quite a few selected molecules always twist to sort of unfavorable conformation to fit the query. The step of rmsd checking and manual inspection were applied to avoid this issue.

Compared to using Ligbuilder1.2, Dock4.06, and Unity4.4.1 programs solely, the cross-application method can make full use of their strong points and avoid their shortcomings respectively.

3.2. Cell Biological Section. *3.2.1. Effects of Selected Compounds on T cell Proliferation.* To evaluate T cell stimulating ability of the selected compounds, T cell proliferation was investigated following T cell stimulation by five selected compounds. The wild-type CII263-272 was used as a positive control in T proliferation. The results in Figure 6 showed that wild-type antigenic CII263-272 induced T cell proliferation dose dependently, and the most effective stimulating concentration was 10 μg/mL of CII. In contrast, five selected compounds had only slight effects on T cell proliferation. Especially, compounds 1 (BAS-0219606) and 3 (T0506−2494) treatment resulted in lower proliferative abilities at the concentrations from 10 to 100 μg/mL compared with the other three compounds. These results suggested minimum effects on T cell activation by the selected compounds

In addition, the toxic effects of all the compounds were tested before biological assays. The compound 1 exhibited appreciable toxicity at the concentration of 100 μM but almost none at 10 μM. The compound 3 did not kill cells at any concentration up to 100 μM.

3.2.2. Inhibition of HLA-DR4 Restricted T cell Proliferation by Selected Compounds. The inhibitory effects of selected compounds on T cell proliferation were measured by cotreating cells with CII 263-272 peptide, and these five compounds and the IC_{50} values for those chemicals are shown in Figure 7.

The concentration-dependent inhibitions of compounds 1(BAS-0219606) and 3 (T0506-2494) with lower proliferation are shown in Figure 8. The T cell proliferation induced by CII263-272 was significantly inhibited by compounds 1 and 3 in a dose-dependent manner from 3.125 to 50 μg/mL. The IC_{50} values of compounds 1 and 3 were 21.12 and 18.59 μg/mL,

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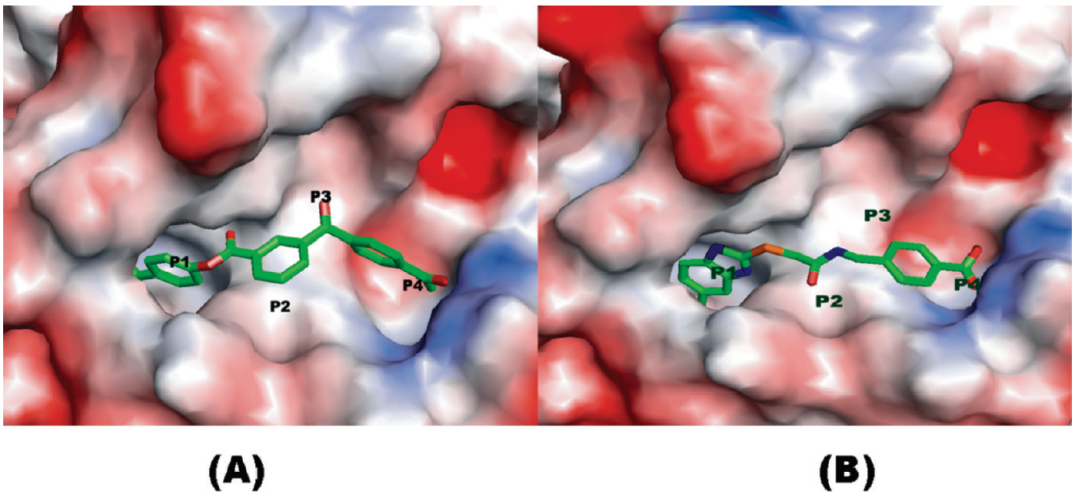

Figure 9. Docking conformation of: (A) compound 1 (BAS-0219606) or (B) compound 3 (T0506-2494) in the pocket of HLA-DR4 protein.

respectively. Both of them were functioned equivalently or better as inhibitors compared with those known altered peptide ligands, which have the IC_{50} values no less than 50 μg/mL (20−30 μM).

3.3. Binding Modes Analysis. To further understand the inhibitory effect of compounds 1 and 3, we docked these two molecules back into the binding pocket of HLA-DR4 protein with the AutoDock3.05 program, and the complexes are shown in Figure 9. Take compound 1 as an example, the benzene ring at the P1 site fit nicely into the binding site. As discussed before, this deeper pocket at the P1 site functions as an "anchor" for the binding interaction. We realized that the acetyl group from compound 1 reached further into the P1 pocket than the hydroxyl group on the tyrosine from the peptide, which was probably the reason that compound 1 bound better compared with others. The benzene ring at P2 also contributes to the binding by providing favorable hydrophobic interaction around this area, and the length of the connection "bridge" spanning P1 and P4 fit very well with the center piece of compound 1. The interaction also suggested possible sites for modification. For example, the P3 pocket is unoccupied with compound 1 binding. We assume that either adding a hydrophobic group at P3 or changing the linker benzene ring to a bicyclic structure may increase the binding affinity of compound 1.

For compound 3, the benzoimidazole ring buried into P1 position, reaching even deeper than compound 1. Given the critical role of P1 pocket on positioning the binding molecule, the better fit of compound 3 in this region may be the major reason that it has a lower IC_{50} value. The linker connecting p1 and p4 sites within this compound has the appropriate length, although the middle amide bond does not fit perfectly with the surrounding hydrophobic area (P2 and P3). Therefore, it is quite logic to predict that switching to a more hydrophobic linker would increase the binding affinity.

In addition to compounds 1 and 3, the other three tested compounds also exhibited decent inhibitory effects, although not as good as compounds 1 and 3. The possible reasons may vary. Compound 2 has many rigid ring structures, which may lead to a higher energy barrier when the molecule tries to span across the P1 and P4 pocket. Compound 4 has two negative charges that may impair the ability to cross the cell membrane. The compound 6 has a polar and more rigid linker that may cause the similar problem with compound 2.

4. CONCLUSIONS

The impact of ligands on T cell activation has attracted high attention in recent years. Studies have demonstrated that ligands with high affinity for HLA-DR4 were able to competitively bind to HLA-DR4 with antigenic peptides and block T cell activation.[22−27] Those ligands have shown promising effects in several animal models of human autoimmune diseases and already been used as an immunotherapy for patients with multiple sclerosis and myasthenia gravis.[28−32]

Here we applied a three-step designing strategy to identify small-molecule ligands that target the HLA-DR4 protein. The potential compounds discovered in the present study can bind to HLA-DR4 with a similar manner as CII and HA antigen peptides. It was proved that the identified chemicals have the ability not only to bind to HLA-DR1/4 molecule but also to block the HLA-DR4-restricted T cell responses. It is hopeful that they can be used as effective therapeutic tools for further treatment of patients with rheumatoid arthritis. However, further studies are needed to investigate the precise mechanism of their inhibitory effects and whether the chemicals are effective in inhibiting T cell proliferation in vivo, such as in HLA-DR4 transgenic animal models. The identification work of chemicals itself was proved to be a successful strategy to design nonpeptide inhibitors by "simplifying" known peptide ligands.

■ ASSOCIATED CONTENT

Supporting Information. The information of twenty-three molecules identified by virtual screening campaign and the high resolution pictures of Figure 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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