GPCR Structure-Based Virtual Screening Approach for CB2 Antagonist Search

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The potential for therapeutic specificity in regulating diseases has made cannabinoid (CB) receptors one of the most important G-protein-coupled receptor (GPCR) targets in search for new drugs. Considering the lack of related 3D experimental structures, we have established a structure-based virtual screening protocol to search for CB2 bioactive antagonists based on the 3D CB2 homology structure model. However, the existing homology-predicted 3D models often deviate from the native structure and therefore may incorrectly bias the in silico design. To overcome this problem, we have developed a 3D testing database query algorithm to examine the constructed 3D CB2 receptor structure model as well as the predicted binding pocket. In the present study, an antagonist-bound CB2 receptor complex model was initially generated using flexible docking simulation and then further optimized by molecular dynamic and mechanical (MD/MM) calculations. The refined 3D structural model of the CB2−ligand complex was then inspected by exploring the interactions between the receptor and ligands in order to predict the potential CB2 binding pocket for its antagonist. The ligand−receptor complex model and the predicted antagonist binding pockets were further processed and validated by FlexX-Pharm docking against a testing compound database that contains known antagonists. Furthermore, a consensus scoring (CScore) function algorithm was established to rank the binding interaction modes of a ligand on the CB2 receptor. Our results indicated that the known antagonists seeded in the testing database can be distinguished from a significant amount of randomly chosen molecules. Our studies demonstrated that the established GPCR structure-based virtual screening approach provided a new strategy with a high potential for in silico identifying novel CB2 antagonist leads based on the homology-generated 3D CB2 structure model.

INTRODUCTION

G-protein coupled receptors (GPCRs), which share a common core structure of seven transmembrane helical regions, are responsible for the majority of cellular recognitions of hormones and neurotransmitters as well as light, odor, and taste sensory messengers, etc.[1] Human genome sequencing has determined that GPCR-encoded genes occupy approximately 3% of the human genome, a fact that has become the driving force for the growing role of GPCRs as drug targets. These findings are expected to provide a pathway to the blockbuster GPCR drugs of tomorrow.[2] However, since GPCRs are membrane proteins, their expression, purification, crystallization, and structure determination present major challenges to the discovery of new drugs. Up to now, only bovine rhodopsin has been mapped with a 3D crystal structure[3] in the GPCR family. Due to this general lack of experimental 3D structures, computer-aided GPCR-targeted drug design has depended upon the application of ligand-based modeling techniques based on the pharmacophore models derived from existing bioactive GPCR ligands.[4−6]

On the other hand, the X-ray crystal structure of bovine rhodopsin[3] can be employed as a structural template of general relevance to generate 3D homology models of other GPCRs for structure-based drug design. In fact, the use of 3D GPCR structural models in drug design and structure-based virtual screening studies has increasingly emerged in recent literature.[6−15] Among these studies, it has been demonstrated that the homology models of dopamine D3, muscarinic M1, vasopressin V1a receptors, and $5HT_{2c}$ were reliable enough to retrieve known antagonists via structure-based virtual screening from several compound databases.[7,14] Rhodopsin-based homology models of the α_{1A} receptor could be used as the structural basis for the lead finding and optimization through the application of a hierarchical virtual screening procedure.[6] In addition, virtual screening has been successfully performed to identify a submicromolar antagonist of the neurokinin-1 receptor based on a ligand-supported homology model.[12] The impressive discovery of novel potent dopamine D3 ligands using a hybrid pharmacophore- and structure-based database searching approach has also been reported.[13] Overall, these few existing virtual screening studies revealed the feasibility of using the homology-

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predicted 3D GPCR structural models for receptor-based in silico drug design, although the virtual screening methods and the hit scoring and ranking processes are still under development.

The CB2 receptor is a subtype of the cannabinoid receptors (CB1 and CB2), which belong to the family I rhodopsin-like GPCRs. It is mainly expressed in the immune system and involved in cannabinoid-mediated immune response. The potential for therapeutic specificity in regulating diseases makes the cannabinoid receptors important GPCR targets for new drug discoveries.[16] So far, at least five structure-diverse sets of cannabinergic ligands have been discovered,[17] including classical cannabinoids, nonclassical cannabinoids, aminoalkylindoles, eicosanoids, and arylpyrazoles. Typically, two arylpyrazole compounds, SR141716A and SR144528 shown in Figure 1, are the first two selective antagonists of the CB1 and CB2 receptors, respectively.[18,19] Since their discovery in the early 1990s, extensive studies have been performed on the chemical modification of arylpyrazoles.[20−25] The advances made in the medicinal chemistry of arylpyrazoles as cannabinergic ligands have been highlighted in recent review articles.[26,27] Structure−activity relationship (SAR) studies were also conducted to gain further insight into quantitative[28,29] and qualitative[30,31] SAR models for the arylpyrazoles affinities on cannabinoid receptors. Furthermore, positive results in the clinical trials of SR141716A have successfully enabled it to become a new drug, named Rimonabant (Acomplia, Sanofi-Synthelabo), for the management of obesity, smoking cessation, and cardimetabolic risk factor[32] in Britain. This significant development in cannabinoid drug research and discovery is the most prominent indication that the cannabinoid receptor antagonists possess medicinal uses and therapeutic potential.

However, most of the known cannabinoids, including the naturally occurring (e.g., Δ^9-THC, anandamide) and synthetic cannabinoid ligands (e.g., CP-55940, WIN55212-2), do not exhibit substantial selectivity for the CB1 or CB2 receptors. Efforts to develop cannabinoid-based medication have involved extensive chemical modifications of cannabinoid structures in order to separate the medicinal properties of these compounds from their undesirable psychotropic effects. The discovery of a potent cannabimimetic compound with novel chemical scaffolds, using computer-aided virtual screening approaches, will enhance the efforts to discover an innovative avenue for providing access to more diverse in cannabinergic lead structures.

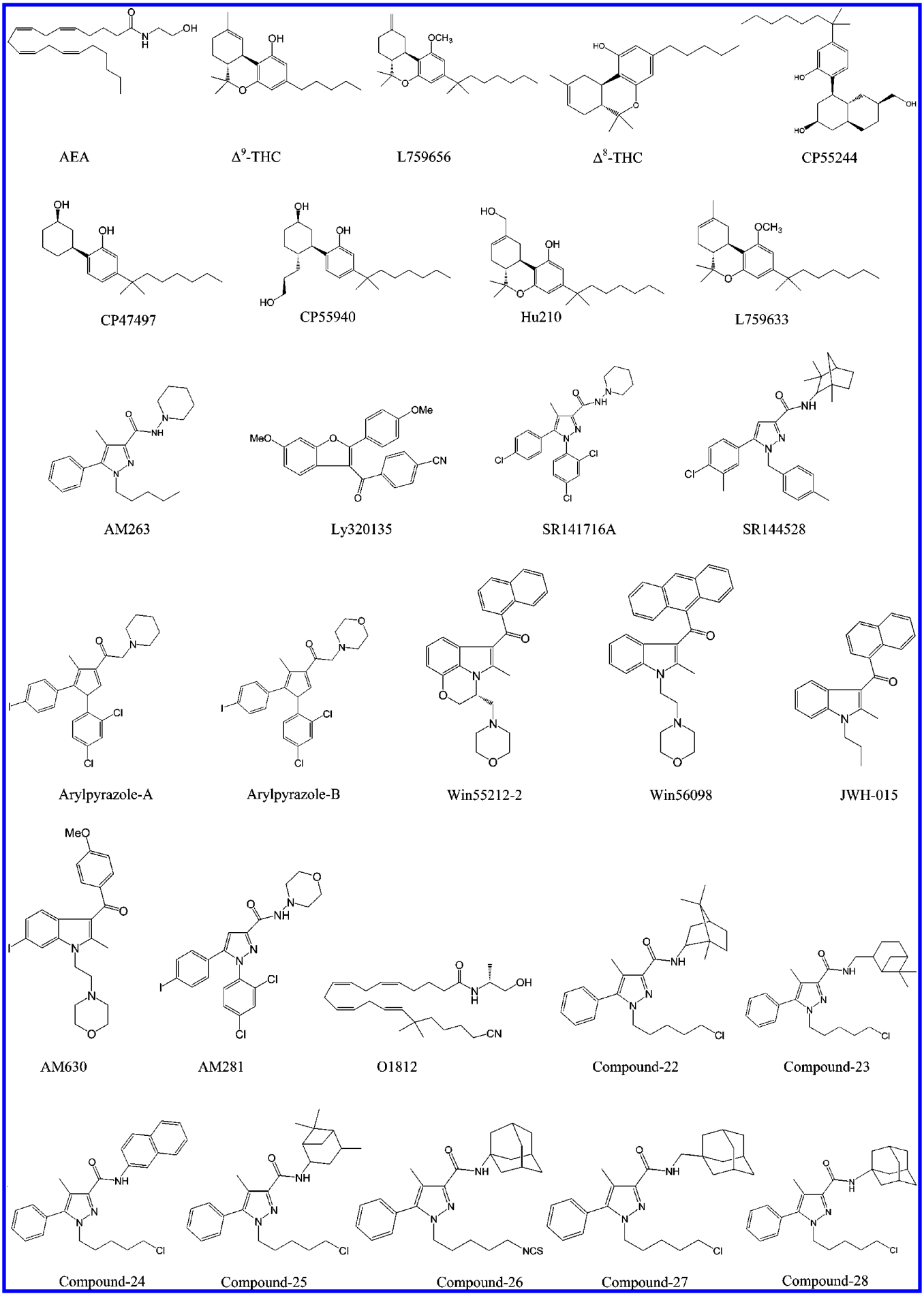
In the present manuscript, we report our recent research in the development of a structure-based virtual screening protocol for CB2-selective antagonist discovery. The protocol, based on the 3D homology structural model of the human CB2 receptor, was generated using the pharmacophore-constrained FlexX docking method. Structure-based virtual screening was conducted previously in order to discover the structure-diverse agonists for the CB2 receptor.[15] However, biochemical studies indicated that the agonist and antagonist of the cannabinoid receptors might bind at the different active sites of the CB2 receptor.[33,34] Therefore, our present CB2 structure-based antagonist virtual screening studies will allow us to establish an alternative avenue for the novel lead discovery of a CB2 antagonist. In our studies, the SR144528-bound CB2 receptor structure model was first constructed through flexible docking and molecular dynamic/mechanic (MD/MM) simulation on the basis of important binding residues derived from site mutagenesis data. The generated CB2-SR144528 complex model was then used to examine the potential binding pocket for the CB2-selective antagonist. The predicted binding pocket was further evaluated in terms of its ability to identify known cannabinoid antagonists seeded in a testing compound database. Subsequently, the CB2 structure-based virtual screening protocol was established using the FlexX-Pharm docking algorithm. In this developed protocol, the consensus scoring (CScore) procedure, in association with the five scoring functions of FlexX,[35] PMF,[36] ChemScore,[37] D_Score,[38] and G_Score,[39] was used to rescore binding energies of the hits screened from the testing compound database by the FlexX docking in the CB2 homology model. The enrichment factor was calculated to evaluate the performance of structure-based virtual screening protocols under the different CScore functions for the CB2 antagonists. The established in silico CB2 antagonist screening method and the known testing database demonstrated that the homology-generated CB2 receptor structure, with a predicted binding pocket, is a promising model for structure-based virtual screening for CB2 antagonist lead discovery.

METHODS

Preparing the 3D Compound Training Database. In order to establish a reliable protocol for the in silico screening of bioactive CB2 lead compounds, a training compound database was constructed by mixing the selected known bioactive cannabinoid ligands with a larger set of random compounds that are hypothetically considered inactive. Such a small compound library was used as the testing database to develop, refine, and evaluate the pharmacophore queries and structure-based virtual screening protocol.

In preparation for the training compound database, we compiled a test set of compounds randomly selected from the National Cancer Institute compound repository (NCI version 2000: ~250K small molecules). In order to do so, the NCI2000 was filtered to extract unwanted compounds such as molecular mixtures, metal organic compounds, and molecules with unsuitable molecular weights (lower than 250, higher than 600) by using the Tripos Selector program.[40] The remaining 135K molecules were then imported into a Sybyl Molecular SpreadSheet (MSS). Using a random row-selection algorithm coded in a written Sybyl-SPL script, 967 compounds were arbitrarily chosen from the spreadsheet to compose a subset of "inactive" compounds in a training database.

The training database also contained some bioactive ligands. In order to span the broadest chemically diverse range of selected compounds for cannabinoid receptors, various known bioactive cannabinoids were chosen to maximize structural-variation. As shown in Figure 1, 30 reference compounds were selected from five traditional major classes of cannabinoid ligands, including CB1 or CB2 bioactive agonists and antagonists. In addition, three compounds, JTE907, tricyclicpyrazole compound-32, and sulfonamide compound-33, were also included in the training database to maximize the structural diversity of known CB2 antagonists. The 3D structure of each known cannabinoid ligand was generated using the Tripos Sketch module and


AEA
Δ9-THC
L759656
Δ8-THC
CP55244
CP47497
CP55940
Hu210
L759633
AM263
Ly320135
SR141716A
SR144528
Arylpyrazole-A
Arylpyrazole-B
Win55212-2
Win56098
JWH-015
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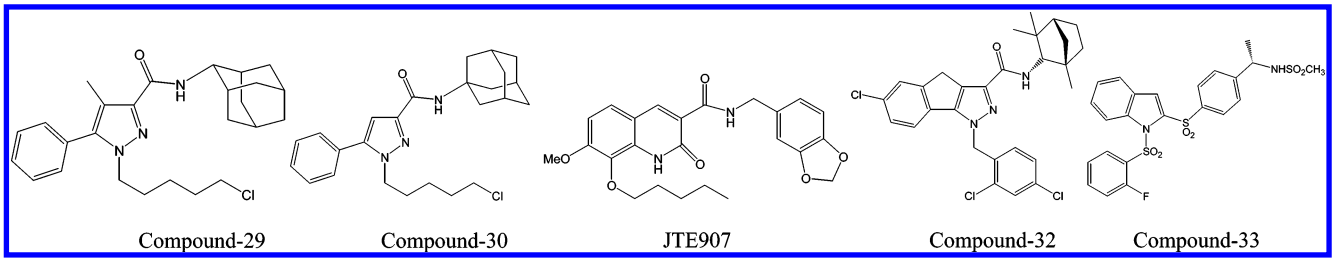

Figure 1. Structures of cannabinoid ligands used in the training database. Among them, AM263,[28] AM630,[26] SR144528, JTE907, and the compounds 22−30,[24] 32, and 33[27,53] were reported to be bioactive CB2 antagonists.

minimized roughly with a Tripos force field and Gasteiger−Hückel atomic charges.[40] The 33 cannabinoid ligands with their computer-generated 3D structures were then added to the training database to create the final *in-house* training compound library (a total of 1000 compounds) allowing us to examine the structure-based virtual screening protocol based on the 3D CB2 homology model as the following.

Modeling the SR144528-Bound CB2 Receptor Structure. Since the CB2 receptor does not have an experimental 3D structure available yet, a 3D CB2 homology structural model has been constructed based on the crystal structure of bovine rhodopsin and further refined by MD/MM simulations in a previous study.[41] Based on this model, the initial docking position of CB2-selective antagonist SR144528 was subsequently characterized on the basis of the site-directed mutagenesis data[33] and the molecular modeling results of the interaction between SR144528 and the CB2 receptor. For this purpose, MOLCAD[40] analysis was performed on the constructed 3D homology structure of the CB2 receptor[41] to find a solvent-accessible cavity around the two key residues Ser161 and Ser165.[33] The compound SR144528 was then placed inside the MOLCAD-created solvent-accessible cavity, and the nitrogen atom of pyrazole ring and the oxygen atom of carboxyamide of the ligand were positioned in the vicinity of the residues Ser161 and Ser165 of the CB2 receptor. No H-bonding constraints were added for the following docking simulations.

Furthermore, the receptor−ligand binding geometry was optimized using a flexible docking method with the Tripos FlexiDock program.[40] In this docking simulation, a CB2 binding pocket was first defined to cover all residues within 4 Å of the ligand in the initial CB2-SR144528 complex. During flexible docking by the FlexiDock module, all of the single bonds of residue side chains inside the defined CB2 binding pocket were regarded as rotatable or flexible bonds, and the ligand was allowed to rotate on all single bonds and move flexibly within the tentative binding pocket. The atomic charges were recalculated using the Kollman all-atom approach for the protein and the Gasteiger−Hückel approach for the ligand. The H-bonding sites were marked for suitable atoms, of both SR144528 and CB2 residues within the defined CB2 active site region, that were able to act as H-bond donors or acceptors. The binding interaction energy was calculated to include van der Waals, electrostatic, and torsional energy terms defined in the Tripos force field.[40] The structure optimization was performed for 20 000-generations using a genetic algorithm, and the 20 best-scoring ligand−protein complexes were kept for further analyses. The Flexidock simulation indicated that the obtained 20 best-scoring SR144528-CB2 complex models have very similar 3D structures with little different energies ranging from −111.32 kcal/mol to −111.50 kcal/mol. Thus, only the lowest energy SR144528-CB2 complex model was selected for further MD/MM simulations as described in the next step.

In order to obtain more consistent antagonist-CB2 interaction modes, further MD/MM computations were carried out on the FlexiDock-simulated lowest-energy SR144528-CB2 complex using the InsightII Discover program.[42] Before the optimization, a subset of the complex was first defined using InsightII Subset[42] to include the ligand and receptor residues within 8.0 Å of the ligand in the SR144528-CB2 complex. In the computations, the AMBER95 force field was applied to optimize the intermediate ligand-bound CB2 receptor models with a 15 Å cutoff distance for the nonbonded interactions, and a distance-dependent dielectric function ($\epsilon = 5r$) was used to simulate the transmembrane environment around the receptor.[41] The detailed MD/MM protocol was described in our previous publication[41] and is briefly restated here: (i) The minimization was initially run for 500 iterations of steepest descents, followed by a conjugate gradients optimization until the maximum derivative of energy became less than 0.1 kcal·mol^{-1}·Å^{-1}. (ii) MD simulations were then performed at a constant temperature of 1000 K with a time step of 1 fs for a total of 50 ps. Initially, atomic constraints were applied to retain the backbone atoms in the seven transmembrane (7-TM) helical domains and all side chain atoms of the residues outside the defined subset of the CB2 receptor. (iii) Fifty representative SR144528-CB2 receptor complexes were retrieved from the molecular dynamic simulations. Each of these complexes was further minimized with 500 iterations of steepest descent without the constraints defined in the MD simulation and subsequently minimized using a conjugate gradient method until the maximum derivative of the total energy was less than 0.1 kcal·mol^{-1}·Å^{-1}. The obtained 50 MD/MM simulated SR144528-CB2 complex models were filtered to remove the complexes with high conformational strains of CB2 residues side chains or SR144528 and the complexes lack of H-bond interactions between Ser161/Ser165 and SR144528. Finally, among the remaining MD/MM simulated conformers, the lowest-energy 3D structural model of the SR144528-bound CB2 receptor was chosen to define the binding site for later structure-based virtual screening with the FlexX docking method.

Virtual Screening with the FlexX-Pharm/CScore. The virtual screening protocol was based upon the FlexX-Pharm method with the Tripos force field.[40] First, a CB2 receptor description file (RDF file) was generated to contain the information about the protein, its amino acids, the active site, non-amino acid residues, and specific torsion angles for the FlexX-Pharm calculations. In this file, the CB2 binding pocket for its antagonist was defined as a set of residues within a radius of 8 Å around the ligand in the SR144528-

bound CB2 receptor model, whereas no torsion angle was defined for the specific residues' interaction with the ligand. After the RDF file was created, a constraint description file (CDF file), defined for the virtual screening of CB2 antagonist by FlexX-Pharm, was generated to contain information about the pharmacophore constraints for the interaction between the receptor and docked ligand according to a computer-simulated SR144528-CB2 complex model. All pharmacophore constraints were identified to enclose the hydroxyl groups of Ser161 and Ser165 as H-bond donors and the indole ring of Trp158 as hydrophobic center on the basis of the simulated CB2-SR144528 complex by flexible docking and MD/MM studies above. The default value, maximum of 30 docking solutions, for each docked molecule was set to be scored and saved for further analysis. Last, other default parameters were chosen for the virtual screening in the FlexX-Pharm program,[40] including the undefinition of the stereochemistry mode, the default FlexX configuration file from Tripos, the parallel execution of CScore mode, etc.

Docking Hits Selection. The scoring function is another important factor in the docking approach to calculate interaction energies between receptor and ligands in structure-based virtual screening. For hit ranking and evaluation, we adopted the concept of consensus scoring functions introduced by Charifson et al.[43] Consensus score, or CScore, combines information from different score functions to account for errors and possibly improve the chances of identifying "true" ligands. In this way, consensus scoring is technically feasible for large library screening because tens to thousands of protein−ligand complexes can be scored, with scoring functions, for interactions per minute.[40] In the current in silico screening, a scoring protocol was established to evaluate the binding poses of FlexX-docked compound at the CB2 receptor using the Tripos CScore module,[40] which comprises five different scoring functions, including the FlexX score,[35] G_Score,[39,44] PMF score,[36] D_Score,[45] and ChemScore.[37] PMF[36] is derived from a knowledge-based approach, and the others are based on force fields or derived from empirical principles. The Tripos default CScore parameter file[40] was selected with proper adjustments as needed in the CScore calculation of the binding interactions between the docked compound and the CB2 receptor.

Having rescored the docked poses with each of the five scoring functions, followed by the "consensus scoring" (or CScoring) computation, we selected the top 10% or 15% of the individual ranking lists as hits for the calculation of the enrichment factors in order to evaluate the effectiveness of the scoring functions ability and to assign high ranks to CB2 antagonists. The resultant top hits were then subjected to further CB2 receptor-directed database searches, using our in-house programmed SPL script, to find the poses in which key H-bonds between the docked compound and the receptor were present. For each compound, if at least one pose was found to possess the desired hydrogen bonds with residue Ser161 or Ser165, it was considered a real hit.

RESULTS AND DISCUSSION

Analysis of the CB2 Potential Binding Pockets. In our previous study,[41] a CB2 3D structural model was constructed using a comparative protein structure prediction method on the basis of the crystal structure of bovine rhodopsin.[3] Such a computational approach has been widely used to study the structural characteristics of experimental-structure-unavailable proteins, including cannabinoid receptors.[41,46,47] The defined homology model was then employed to analyze the CB2 structural characterization of the 7-TM helical bundle for its helix tilt angles, interhelix hydrophobic interaction, interhelix H-bonding network, conserved residues and motifs, and a possible disulfide bond between residues Cys174 and Cys179.[41] The structures of the associated amphipathic cytoplasmic helix domain VIII in both CB1 and CB2 receptors were verified and compared using a NMR method to study the solution structures of relative peptides in membrane mimetic dodecylphosphocholine (DPC) micelles.[48] Our data enabled us to better understand the CB2 structural model from the calculated 3D CB2 structural model.

Furthermore, on the basis of the homology-constructed CB2 structural model, Connolly solvent-accessible protein channel analysis[40,41] was carried out to explore and predict the possible CB2 receptor binding cavity using the Tripos MOLCAD program.[40] Results showed that solvent-accessible surface calculations identified two potential binding domains as shown in Figure 2A. One is located in a cavity among the helices III, V, VI, and VII on the extracellular side of the 7TM bundles. This cavity consists of a hydrophilic center pointed toward the extracellular loop 2 (e2 loop) interface and framed by the polar residues Asn188, Asp189, Tyr190, and Gln276 and a large hydrophobic cleft (brown) surrounded by hydrophobic aromatic residues Phe117, Phe197, and Trp258. The distance between the hydrophilic and hydrophobic centers of the identified cavity is estimated to be 9−11 Å, which is approximately the typical size of a CB2 agonist, such as the WIN55212-2 molecule. The defined amphipathic binding pocket is also congruent with the biochemical studies and shows the residues (such as the e2 loop, Tyr190 and Phe197) around the identified cavity. This is critical to the binding affinity of CB2 ligands including CP55940 and Win55212-2 as summarized in a recent review article.[27] Therefore, this site was speculated to be the CB2 agonist binding pocket. The defined CB2 agonist binding pocket shows certain differences with the model reported by Salo et al.[15] The correspondent structure-based database search results for novel CB2 agonist leads will be reported elsewhere, whereas the current study is mainly focused on developing a virtual screening protocol for a CB2 antagonist search.

As illustrated in Figure 2A, another calculated solvent-accessible surface is located in a cavity surrounded by the helices II, III, and IV. The second cavity also shows amphipathic characteristics. Figure 2B shows that the size of this predicted cavity perfectly matches the conformation of the CB2 antagonist SR144528. The two key residues Ser161 and Ser165, which have been demonstrated to be critical to the binding of SR144528 at the CB2 receptor according to the biochemical studies reported by Gouldson et al.,[33] also reside within this domain. Such a pocket would be hypothesized as a CB2 antagonist binding site and would serve as a good starting point for mapping the binding site of SR144528 at the CB2 receptor for the establishment of a reliable structure-based virtual screening protocol for CB2-selective antagonist leads. The relative results will be discussed in detail later. Therefore, our homology structures of the cannabinoid receptors are helpful in elucidating their

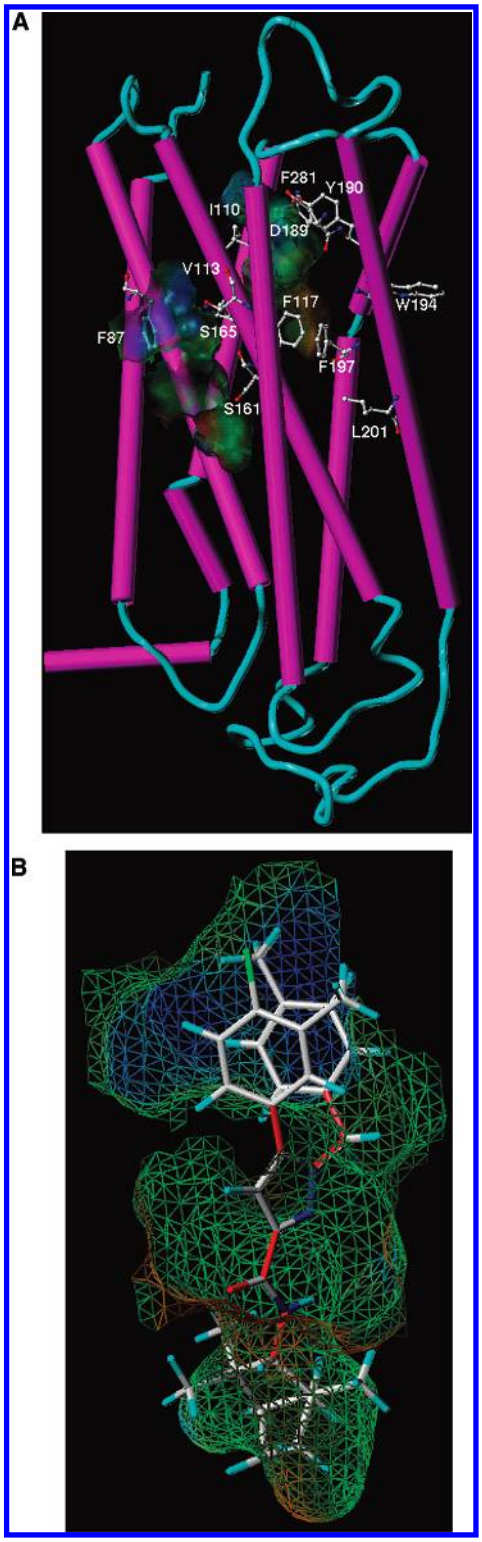

Figure 2. (A) Graphic representations of the putative CB2 binding pockets for agonists (top right) and antagonists (lower left) that were predicted on the basis of 3D CB2 structure model constructed using the homology and multiple sequence alignment method. The CB2 agonist pocket is located inside of the CB2 receptor helix bundle surrounded by transmembrane helices III, V, and VI and close to the extracellular side. The antagonist site is predicted to be located on the side of the CB2 receptor − a grove leaned on transmembrane helices II, III, IV, V. The important mutagenesis-determined binding residues are represented using the mode of ball and stick. Helical portions of the protein, including the seven transmembrane helices and cytoplasmic helix, are shown as violet cylinders. Loop regions are shown as blue ribbons. (B) SR144528 placed inside the MOLCAD-created solvent-accessible cavity.

structural features and interaction patterns with cannabinoid ligands. The results from the homology structures are consistent with biochemical mutation studies on cannabinoid receptors.[49]

Generation and Refinement of the Antagonist-Bound CB2 Structural Model. Since the ligand binding pocket of a receptor is first defined to simulate the interaction between a docked compound and the receptor in a docking method, it is a critical factor to be identified for structure-based virtual screening. Such a pocket for a receptor that has no experimental structure might be predicted and explored by applying computer modeling approaches, in correspondence with key amino acid residues around the binding pocket identified by site-directed mutagenesis data. We performed flexible docking and MD/MM simulations to hypothesize the CB2 active pocket for its antagonist based on the result of previous MOLCAD analysis of the generated homology model.

The homology models of several GPCRs, generated using the X-ray structure of bovine rhodopsin[3] as a template, have been reported for structure-based drug design.[6−15] However, the relatively low global sequence identity between the structure-unknown GPCRs and bovine rhodopsin is generally considered to be insufficient for reliable homology modeling.[6] The different conformation states of GPCRs could also be induced by various types of bound ligand. In addition, it was shown previously that, depending on the composition of the binding pocket to be modeled, considerable deviations from the native structure may be obtained.[6] Therefore, unlike the experimentally determined structure of other kinds of ligand-bound protein, the homology models of GPCRs could not be directly applied to structure-based virtual screening with the FlexX docking method. In general, the homology-constructed GPCR 3D structural model would be refined by flexible docking and MD/MM simulations of the ligand−receptor complex to achieve more relevant geometries of protein binding sites.

The reported CB2 receptor computation and site-directed mutagenesis studies, carried out by Goldson et al.,[33] revealed that two key residues of Ser161 and Ser165 are important for SR144528 binding at the CB2 receptor. On the other hand, their reported structural model of the CB2 receptor was generated on the crystal structure of bacteriorhodopsin that does not belong to the GPCR family. Therefore, before conducting virtual screening based on the CB2 structural model using the FlexX-Pharm method, we performed flexible docking with the Tripos FlexiDock program[40] and MD/MM simulations with the InsightII Discover program[42] to generate 3D coordinates for the antagonist-bound structural model of the CB2 receptor based on crystal structure of bovine rhodopsin. For this purpose, the compound SR144528, the first potent and selective antagonist of the CB2 receptor, was chosen to be docked into the initial CB2 homology model with the support of its mutagenesis data.

As shown in Figure 2A, the potential binding cavity of the receptor was identified for ligand binding through a MOLCAD[40] simulation. A preliminary topographical interaction model was derived by considering additional mutagenesis studies and comparative affinity determinations based on SR144528 binding at the CB2 receptor.[33] Combining our homology model[41] and this mutation data,[33] it is further hypothesized that the antagonist binding pocket of the CB2

receptor is embraced by the transmembrane (TM) domains of helices II, III, and IV. Next, SR144528 was manually docked into the hypothetical binding pocket of an ensemble of the CB2 homology model. Then, an automated flexible docking procedure (FlexiDock)[40] was carried out to determine the most energetically favorable binding location and orientation for the ligand to interact with the CB2 receptor. FlexiDock[40] considers both the selected side chains of protein residues in the defined binding pocket and the docked ligand to be flexible. Subsequently, the most favorable ligand−receptor complex models were selected by ranking the binding interactions between SR144528 and the CB2 receptor model. The FlexiDock-simulated results showed that the best score was −111.50 kcal/mol for the CB2-SR144528 interaction, incorporating the sum of the van der Waals, electrostatic, and torsional energy terms in the Tripos force field. Since the FlexiDock calculation is only a rough molecular modeling process, the FlexiDock-generated models of the SR144528-CB2 complex were subjected to further MD/MM simulation. Finally, SR144528-CB2 complex models were obtained by combining the best ranked side-chain conformers from a set of different models followed by molecular dynamic and mechanic simulations of the entire complex using the InsightII Discover program[42] with the Amber force field.[50]

The simulated SR144528-bound CB2 structural model is depicted in Figures 3 and 4, showing hydrophobic and hydrophilic interactions between the ligand and CB2. Large hydrophobic pockets consisting mostly of residues in the TMs II, III, IV, and V embrace the big bulk fenchyl group of SR144528. Hydrophobic amino acids, which participate in hydrophobic interaction with the fenchyl group of SR144528, may include Leu82, Ala83, Val86, Leu108, Ile110, Pro168, Leu169, and the aromatic ring of Tyr166. Such results are consistent with the results of our previous 3D-QSAR studies.[28] The CoMFA-generated 3D-QSAR model revealed the presence of a significant hydrophobic region in the CB2 binding site that is capable of accommodating a large hydrophobic group, such as the bulky fenchyl group of SR144528. In addition, the aromatic stack interaction between the indole ring of Trp158 and aromatic rings of SR144528 also serves to increase the thermal stability of the complex. Important hydrophilic interactions include two H-bonds. One H-bond is formed between the oxygen atom in the carboxyamide functional group of SR144528 and the hydroxyl group of Ser165 of the CB2 receptor with a H-bond length of 2.02 Å and an angle of 153.7°. Another one is formed between the nitrogen atom in the pyrazole ring of SR144528 and the hydroxyl group of Ser161 of the CB2 receptor with a H-bond length of 2.45 Å and an angle of 156.9°. The two H-bond interactions demonstrated that the residues Ser161 and Ser165 play critical roles in the binding of SR144528 at the CB2 receptor. Such a docking result is consistent with previously reported biochemical data, which indicate that the mutants of the CB2 receptor (S161A and S165A) lose the high-affinity binding of SR144528 at the receptor.[33]

FlexX-Pharm Database Virtual Screening. The FlexX program[35] is a flexible docking algorithm that takes into account ligand flexibility while keeping the protein rigid. It allows for fast docking of small molecules into protein active sites for the performance of 3D database searches. In the present research, the CB2 structure-based virtual screening experiments were carried out using the FlexX-Pharm[51] method, which is an extended version of FlexX. FlexX-Pharm allows the information regarding important characteristics of protein−ligand binding modes to be included into the docking calculation.[51] FlexX-Pharm outperforms FlexX alone in most cases, and it has been verified to be more reliable in predicting accurate poses of ligand binding at the receptor.[52] Many structure-based drug design studies have demonstrated that the introduction of pharmacophore constraints for FlexX-Pharm docking might improve enrichment factors significantly.[52] Such effects could be attributed to the efficient filtering of inactive molecules and a decrease in the number of false positives.[52] Therefore, FlexX-Pharm was used to establish our CB2 receptor-based virtual screening protocol.

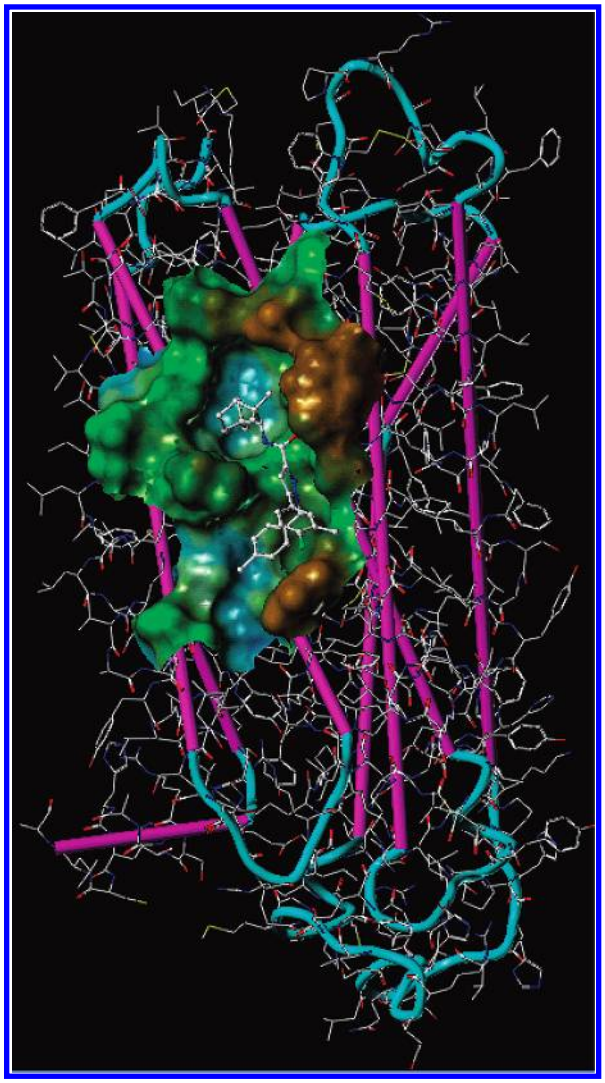

Figure 3. The MOLCAD-predicted CB2 antagonist binding pocket, showing SR144528 is situated in the predicted CB2 binding cavity. The CB2 antagonist SR-144528 was docked by the FlexiDock program and was further refined by using MM/MD simulations.

The flexible docking and MD/MM simulation results above implicate the important hypothetic pharmacophore queries for CB2 structure-based virtual screening by using the FlexX-Pharm method. As shown in Figure 5, the capped stick model in green represents the conformation of SR144528 in the simulated CB2-SR144528 complex. This was used to define the antagonist binding site of the CB2 receptor while

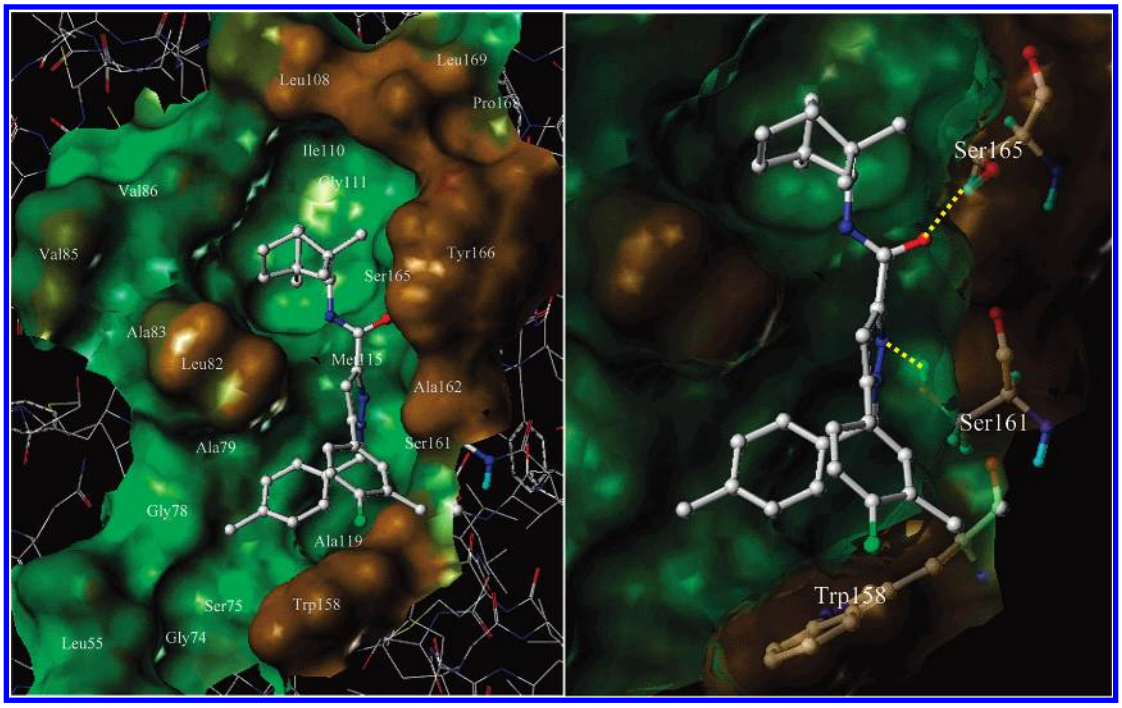

Figure 4. Close views of the CB2 antagonist binding pocket revealed in Figure 3, depicting the SR144528-CB2 binding model resulting from FlexiDocking and MM/MD simulation. The left picture shows the CB2 receptor residues located around the binding pocket. The right one reveals the two important residues Ser 161 and Ser 165 have H-bonding interactions (2.51 and 1.90 Å) with the N atom of pyrazole ring and the O atom of carboxy group of SR144528, respectively. The binding pocket was rendered from the molecular surface created using the Sybyl MOLCAD module and was color-coded by hydrophobicity (brown to blue: a scale of hydrophobic to hydrophilic properties). The refined 3D model was used as the predicted binding mode for virtual screening of CB2 antagonist by FlexX-Pharm/CScore docking method.

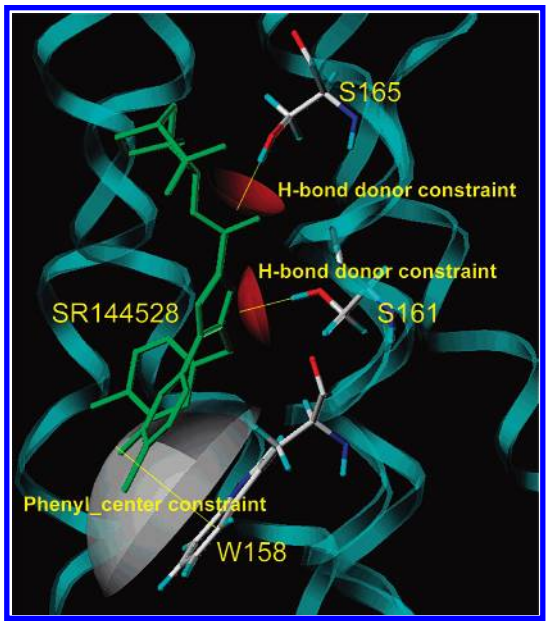

Figure 5. A set of three pharmacophore constraints identified in the active site of CB2 receptor for SR144528 was used in virtual screening using the FlexX-Pharm program. Constraints include (1) an essential H-bonding donor at the OH group of S161 (red surface), (2) an essential H-bonding donor at the OH group of S165 (red surface), and (3) a phenyl center at the indole ring of W158 (gray surface).

generating the CB2 receptor description file for FlexX-Pharm searching. In addition, the OH groups of the residues Ser161 and Ser165 were defined as the essential H-bond donor constraints, represented by red surfaces, for interaction with the H-bond acceptor atom of the docked ligand. The indole ring of the residue Trp158 was defined as an essential phenyl center constraint, represented in white, for interaction with phenyl-ring, CH_3-Phe, or amide on the ligand.[40]

The 33 cannabinoid ligands, including CB1 and CB2 agonists and antagonists, were enclosed in the training database. Among these cannabinoids, the ligands AM263,[28] SR144528, and compounds 22−30[24] were reported to be CB2 antagonists. As reviewed by Huffman,[26] the indole compound AM630 is different from the traditional cannabinoid agonist Win55212-2. It was identified instead as a CB2 selective inverse agonist or antagonist. In addition, another three compounds, including JTE907, tricyclicpyrazole compound-32, and sulfonamide compound-33, were also identified as CB2 antagonists.[27,53] Therefore, these 15 compounds were considered as bioactive CB2 antagonists in the training database for the generation of the CB2 structure-based virtual screening search protocol. All of the randomly selected compounds from the NCI2000 compound database and the other cannabinoids were considered inactive ligands in the training database. Normally, a top percentage set of the compounds, resulting from FlexX-Pharm database searches and CScoring evaluations, was chosen as "true" hits to calculate the enrichment factor for the evaluation of our structure-based virtual screening protocol.

The evaluation and ranking of predicted ligand binding conformations at a receptor is a crucial aspect of structure-based virtual screening. Although the accurate free-energy simulation techniques have been currently developed for scoring the binding interaction between ligand and protein, they are too complicated for the structure-based virtual screening of the large compound library. For practical purposes, several approximate scoring functions were developed with various assumptions and simplification in the

Table 1. Numbers of Bioactive CB2 Antagonists Shown up in the Top 10% or 15% of Hits Selected by Single Docking Scores and the Consensus Scores of the Various Combinations of the Five Scoring Functions for the Training Database Searches

	single scoring functions[a]				
	S1	S2	S3	S4	S5
the number of CB2 antagonists retrieved in 10% hits	2	10	2	11	10
the number of CB2 antagonists retrieved in 15% hits	4	12	2	14	11
	consensus scoring functions[b]				
	C12	C13	C14	C15	C23
the number of CB2 antagonists retrieved in 10% hits	6	3	9	3	3
the number of CB2 antagonists retrieved in 15% hits	9	5	11	7	4
	consensus scoring functions[b]				
	C24	C25	C34	C35	C45
the number of CB2 antagonists retrieved in 10% hits	11	10	5	3	11
the number of CB2 antagonists retrieved in 15% hits	13	13	6	5	13
	consensus scoring functions[b]				
	C123	C124	C125	C134	C135
the number of CB2 antagonists retrieved in 10% hits	5	10	6	6	4
the number of CB2 antagonists retrieved in 15% hits	6	12	9	9	5
	consensus scoring functions[b]				
	C145	C234	C235	C345	C1234
the number of CB2 antagonists retrieved in 10% hits	9	7	6	7	7
the number of CB2 antagonists retrieved in 15% hits	11	8	7	8	11
	consensus scoring functions[b]				
	C1235	C2345	C12345		
the number of CB2 antagonists retrieved in 10% hits	5	7	7		
the number of CB2 antagonists retrieved in 15% hits	6	10	11		

[a] Single scoring function: S1 representing FlexX Score, S2 representing D_Score, S3 representing PMF_Score, S4 representing G_Score, and S5 representing ChemScore. [b] Consensus scoring functions correspondent to the different combinations of five scoring functions, for example, C12 representing a combination of FlexX and D_Score, C123 representing a combination of FlexX, D_Score, and PMF_Score, etc.

evaluation of binding energies for computer-simulated ligand−protein complexes.[54] Typically, the scoring functions utilized in the present consensus scoring scheme were representatives of the three main classes of scoring functions, namely the empirical-based scoring functions, the force-field-based scoring functions, and the knowledge-based scoring functions, including ChemScore, G_Score, D_Score, and PMF, described in the review.[54] The combination of different scoring functions, so-called consensus scoring (CScoring),[43] have been developed to balance errors in single scores and improve the probability of identifying 'true' ligands.[54]

Among the retained 30 conformations of each FlexX-docked compound, FlexX-Pharm only considers the interaction energy between bound compound and receptor but not its intraenergy. In other words, some binding conformers of certain docked compounds interact well with the CB2 receptor, but their conformational energy is probably so high that the conformation or binding pose is very bad for this compound. The binding poses of hits are filtered out to eliminate those with high internal energy. Therefore, in the hits scoring scheme, the ligand-binding pose extraction was handled separately from the ranking. Both pose extraction and ranking were executed by five scoring functions, including FlexX, G_Score, PMF, D_Score, and ChemScore.[36] The best binding pose of each hit was first picked with its relatively low conformational energy. Then, the scores were ranked in 28 different combinations of five scoring functions, as shown in Table 1. As defined in the *Methods* section, only the top 10% or 15% of the individual ranking lists were regarded as the "true" hit lists from the structure-based virtual screening protocol. Table 1 summarizes the number of bioactive cannabinoid antagonist hits that were calculated with the different combinations of consensus scoring functions.

Furthermore, comparative enrichment factor calculations were performed to study the efficiency of FlexX-Pharm database searches using the computer-stimulated antagonist-bound CB2 structural model. The goal of these computations was to examine the enrichment effectiveness of our structural-based virtual screening protocol for bioactive CB2 antagonists using different combinations of the five scoring functions included in the Tripos CScore module.[40] In order to achieve greater effectiveness in structure-based virtual screening protocols with various settings, the enrichment factor[6,52] was calculated to judge the quality of the rankings using the following equation

$$\mathrm{EF} = \frac{\mathrm{Hits}_{\mathrm{active}}/\mathrm{Hits}_{\mathrm{sampled}}}{N_{\mathrm{active}}/N_{\mathrm{total}}}$$

where EF is the enrichment factor, $\mathrm{Hits}_{\mathrm{active}}$ is the number of bioactive CB2 antagonists in the "true" hit list, $\mathrm{Hits}_{\mathrm{sampled}}$ is the number of compounds in the "true" hit list, N_{active} is the number of CB2 bioactive antagonists in the training database, and N_{total} is the number of compounds in the training database. Based on the definition of the enrichment

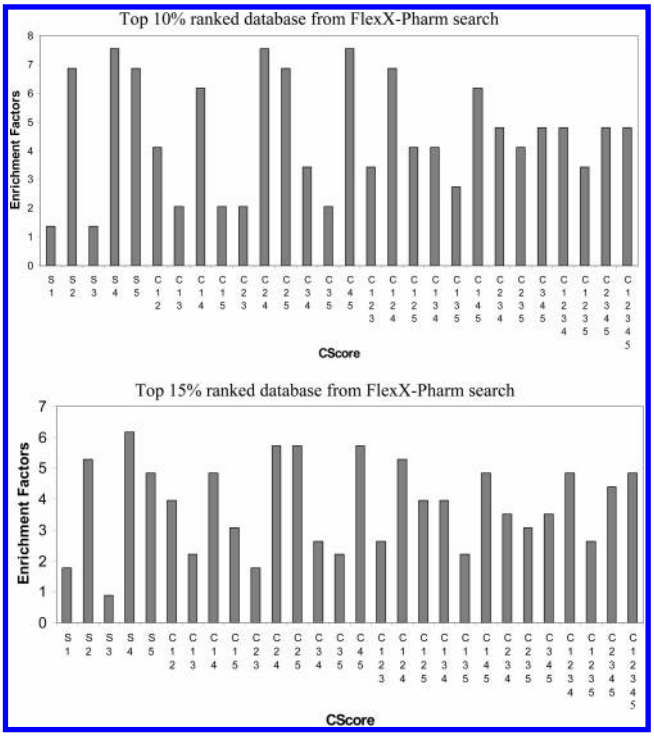

Figure 6. The calculated enrichment factors for the structure-based CB2 antagonists virtual screening with the rankings from 28 different combinations of five scoring function, including FlexX, ChemScore, D-Score, ChemScore, and PMF.

factor, EF depends on the total number of compounds in the "true" hit lists, which are composed of both bioactive and inactive compounds. Thus, the enrichment factor was used to estimate the ability of consensus scoring functions in the docking/scoring protocol studies to assign a high ranking to bioactive CB2 antagonists.

In this study, multiple scoring functions have been utilized to rescore the ligand poses binding at the receptor. Such a consensus scoring approach provides a popular strategy for postprocessing the results generated from virtual screening. However, it is more prudent to identify the combination of scoring functions that together optimize the specific 3D database search, since it is possible that the standard combination of several scoring functions in consensus may not give the best enrichment factor. Figure 6 describes the enrichment factors for the different combinations of five scoring functions on the basis of validated hits, defined as the top 10% or 15% of the ranked database search in our virtual screening. The applied data set illustrated that the highest enrichment factor was obtained when G_Score (S4) was used as a scoring function. As shown in Table 1 and Figure 6, G_Score can retrieve 11 CB2 antagonists in the top 10% of ranked hits (a total of 16 compounds) or 14 CB2 antagonists in the top 15% of ranked hits (a total of 25 compounds). These 14 bioactive compounds consist of the highly structure-diverse known CB2 antagonists, including biarylpyrazoles, AM630, JTE907, and tricyclicpyrazole compound 32. Conversely, the PMF scoring function (S3) provided the lowest enrichment factors. In the 15% compounds of the ranked database, only two known bioactive CB2 antagonists were retrieved with this scoring function.

In addition, our data shows that the enrichment factors are influenced when five scoring functions are used together to rescore the hits with different combinations. In various combinations of the five scoring functions, the consensus scoring functions C24 (the combination of D_Score and G_Score), C25 (the combination of D_Score and ChemScore), and C45 (the combination of G_Score and ChemScore) provided the second-highest enrichment factor for the top 10% or 15% of the ranked database (Figure 6). In the top 15% of the ranked hit lists, these three CScore functions can retrieve 13 of 15 bioactive CB2 ligands, from the testing database which contained 1000 compounds, including almost all kinds of CB2 antagonists, such as arylpyrazoles, tricyclicpyrazole compound-32, indole derivative AM630, and 2-oxoquinoline derivative JTE907. Among other CScore functions, S2 (D_Score), S5 (ChemScore), and C124 (the combination of FlexX, D_Score and G_Score) also offered high enrichment factors for the top 10% of the ranked database, and S2 (D_Score), C25 (the combination of D_Score and ChemScore), and C124 (the combination of FlexX, D_Score, and G_Score) generated high enrichment factors and brought a total 12 of 15 bioactive CB2 antagonists from the testing database for the top 15% of the ranked database. The data presented in Figure 6 illustrated that the identification of true-positive hit compounds were dependent on retaining a large number of docked poses and rescoring these on the basis of the consensus scoring with different combinations of the five scoring functions. For our ongoing CB2 structure-based antagonist virtual screening of actual compound databases, these CScore functions are currently applied to rank the FlexX-docking hits to be selected later for experimental bioassay validation.

The results of the present CB2 homology model-based virtual screening also showed that the CB2 bioactive sulfonamide compound-33 was included in the ranked database. Some consensus scoring functions, for example C123 (the combination of FlexX Score, D_Score, and PMF_score) and C12345 (the combination of FlexX_Score, D_Score, PMF-_Score, G_Score, and ChemScore), ranked compound-33 in the top 15% of the screening hits. Therefore, our in silico screening protocol not only regained pyrazole CB2 bioactive antagonist but also recovered other kinds of CB2 bioactive antagonist. Furthermore, there were no cannabinoid agonists screened with our structure-based CB2 antagonist virtual screening protocol. This ensured the reliability of our established method. Therefore, the generated CB2 homology model-based virtual screening protocol can be applied to efficiently retrieve all kinds of CB2-active antagonists in the testing database.

Finally, the top 10% or 15% of the screening hits also included some compounds that were randomly selected from NCI2000 in the training database. Although these compounds were hypothetically regarded as inactive CB2 ligands in the development of our virtual screening protocol, they have H-bond interactions with the key residues, Ser161 and Ser165, of the CB2 receptors and hydrophobic interaction with the CB2 residue Tyr158. However, they have totally different structures in comparison with known CB2 antagonists. This result suggests that the highly diverse structural compounds can be in silico screened from the compound library for the discovery of new leading CB2 antagonists using our generated virtual screening protocol. On the other hand, such a hypothesis can only be demonstrated by bioassay testing of certain virtual screening hits in the future.

CONCLUSION

A virtual screening protocol using the 3D CB2 homology model generated from the crystal structure of bovine rhodopsin was developed using combined flexible docking, MD/MM simulations, and FlexX-Pharm docking approaches as well as the CScore ranking method. The generated CB2 structure-based virtual screening protocol was successful in identifying all known bioactive CB2 antagonists from our training database with good enrichment effectiveness. In addition, the present study also demonstrated that our refined CB2 receptor homology model provides a relevant structural basis for rationalizing the CB2 receptor binding. The established in silico receptor-based screening approach and consensus scoring functions as well as the 3D CB2 structure model and predicted binding pocket are currently applied in our 3D database searches for new chemical scaffold CB2-selective antagonist lead discovery.

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