Discovery of Novel CB_2 Receptor Ligands by a Pharmacophore-Based Virtual Screening Workflow

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Cannabinoid receptor 2 (CB_2 receptor) ligands are potential candidates for the therapy of chronic pain, inflammatory disorders, atherosclerosis, and osteoporosis. We describe the development of pharmacophore models for CB_2 receptor ligands, as well as a pharmacophore-based virtual screening workflow, which resulted in 14 hits for experimental follow-up. Seven compounds were identified with K_i values below 25 μM. The CB_2 receptor-selective pyridine tetrahydrocannabinol analogue **8** ($K_i = 1.78$ μM) was identified as a CB_2 partial agonist. Acetamides **12** ($K_i = 1.35$ μM) and **18** ($K_i = 2.1$ μM) represent new scaffolds for CB_2 receptor-selective antagonists and inverse agonists, respectively. Overall, our pharmacophore-based workflow yielded three novel scaffolds for the chemical development of CB_2 receptor ligands.

Introduction

Both cannabinoid receptor 1 (CB_1 receptor) and 2 (CB_2 receptor) belong to the rhodopsin-like family class A of G-protein-coupled receptors (GPCRs). Endocannabinoids are the nonselective arachidonic acid-derived endogenous activators of both receptors. The CB_1 receptor is abundantly expressed in brain tissue such as basal ganglia, cerebellum, hippocampus, and cortex, as well as in peripheral tissues like immune cells, the eye, urinary bladder, ileum, and adipocytes.

CB_1 receptor-selective antagonists are currently used in the therapy of obesity.[1] CB_1 receptor agonists exert analgesic effects, stimulate appetite, and decrease nausea, neurodegeneration, hypermotility, and inflammation.[2,3] However, CB_1 receptor agonists are known to potentially cause side effects in the CNS such as cognitive dysfunction, motor incoordination, and sedation.[4] In contrast, preclinical studies have shown that agonists of the CB_2 receptor, which is primarily expressed in peripheral tissues, reduce inflammatory and neuropathic pain without exerting psychoactive effects.[5] Since potent drugs for the treatment of chronic pain without central side effects are therapeutically more attractive than classical cannabinoids, CB_2 receptor-selective agonists are presently investigated in animal models of pain.[6−8] Although the exact mechanism of CB_2 receptor-mediated inhibition of osteoclast function is unknown, recent data indicate that CB_2 receptor-selective inverse agonists might be promising agents to reduce bone loss.[9] Finally, CB_2 receptor antagonists are used as pharmacological tools to investigate the physiological role of the receptor.[10] Because the 3D structure of the CB_2 receptor has not been determined, current virtual screening approaches for novel CB_2 receptor ligands are mostly focused on protein−ligand docking using rhodopsin-based homology models for the CB_2 receptor.[11−15] However, pharmacophore modeling, a virtual screening technique that not only enables the screening of large compound databases for active ligands but also allows the identification of novel and structurally diverse scaffolds, is several magnitudes faster than protein−ligand docking.[16−18] Here, we describe the development of a 3D pharmacophore-based virtual screening workflow in order to discovery novel CB_2 receptor ligands with focus on CB_2 receptor agonists. The resulting workflow was proved experimentally to be suitable for the discovery of new scaffolds for CB_2 receptor agonists, antagonists, and inverse agonists.

Results

Workflow. Three-dimensional pharmacophore models were used as queries for a virtual screen of chemical databases. The hits were subjected to a physicochemical property filter, analyzed in terms of structural similarity, and inspected visually. Finally, the selected compounds were biologically tested. The virtual screening workflow is summarized in Figure 1.

Pharmacophore Model Generation. The aim of our pharmacophore modeling approach was to retrieve novel scaffolds for CB_2 receptor ligands with focus on CB_2 receptor agonists. Selective models for agonists, antagonists, and inverse agonists were not generated, since all types of CB_2 receptor ligands are interesting for drug research and since several CB_2 receptor-selective agonists and antagonists/inverse agonists, like the CB_2 receptor-selective antagonist **1** (AM630)[19] and the training set agonist **2** (GW405833),[20] are structurally closely related (Chart 1). Figure 2 shows the feature-based alignment of compounds **1** and **2**, indicating that only a highly restrictive pharmacophore model that would include all chemical features of one compound would be able to discriminate between both compounds. Such

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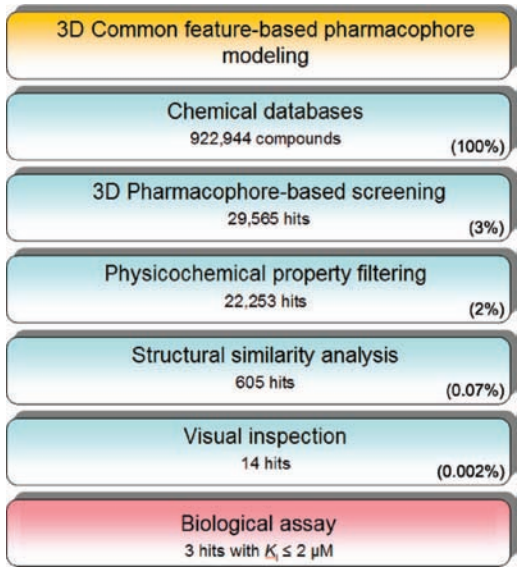
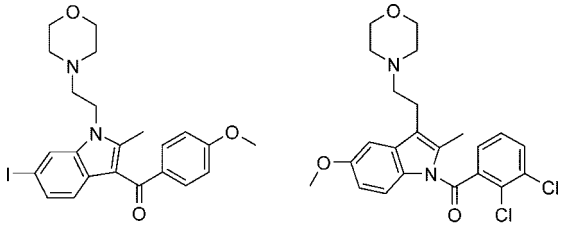


Figure 1. Pharmacophore-based virtual screening workflow. An amount of 3% of the chemical database compounds matched CB_2 receptor ligand model 1 or 2. The following filtering in terms of physicochemical properties of known CB_2 receptor ligands reduced the hits to 2% of all screening compounds. Afterward, analysis of the structural similarity of the remaining compounds resulted in 0.07% hits with unique scaffolds that were analyzed visually in terms of chemical stability and toxicity of the chemical structure. Finally, 0.002% of the chemical database compounds were selected for biological testing.

Chart 1. Structures of the CB_2 Receptor-Selective Antagonist **1** and the CB_2 Receptor Ligand Training Set Compound **2**



1 (AM630) **2** (GW405833)

a restrictive pharmacophore model that is based on the chemical features of only one compound and not on the common features of a set of structurally diverse ligands would only retrieve a very limited number of novel scaffolds. Therefore, CB_2 receptor-selective agonists served as input for the HipHop algorithm[21] included in the software package Catalyst[22] and no antagonist/inverse agonist structure was utilized to make the models selective for agonists and thereby reducing the probability of retrieving novel scaffolds. The two best CB_2 receptor ligand models were based on the CB_2 receptor ligand training set that comprises the five CB_2 receptor-selective agonists **2**, **3** (AM1241),[23] **4** (HU-308),[7] **5** (JWH-133),[24] and **6** (JWH-267)[14] (Chart 2 and Table 1).

A HipHop common feature-based alignment of the training set compounds resulted in 10 pharmacophore models. The two models with the largest number of features were selected. Both models included one hydrogen bond acceptor feature and four hydrophobic features. From each of the five compounds of the CB_2 receptor ligand training set, a Catalyst shape that represents the spatial information of the corresponding compound was derived and combined with both models (Figure 3). Consequently, the ability of the resulting 10 merged models to filter out active compounds from a large chemical database was validated.

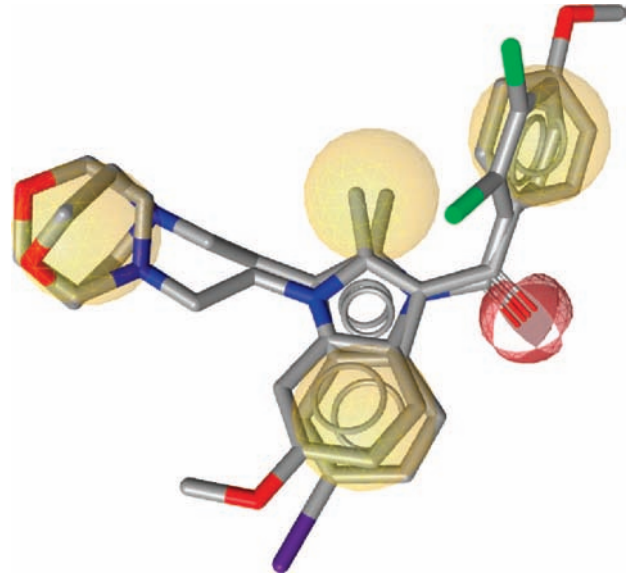


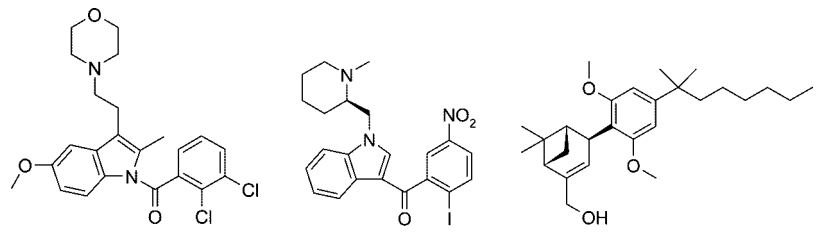
Figure 2. Feature-based alignment of the CB_2 receptor-selective antagonist **1** and the agonist **2** performed by the LigandScout software.[37] The common hydrogen bond acceptor is displayed as a red sphere, whereas the hydrophobic features are shown as yellow spheres. The alignment indicates that the differences in terms of chemical features between some CB_2 receptor agonists and antagonists are subtle. Thus, the discrimination between agonists and antagonists would only be possible with very restrictive pharmacophore models which would not be suitable for a virtual screening workflow that is focused on the discovery of structurally novel scaffolds.

Pharmacophore Model Validation. In order to validate the discriminatory power of the 10 merged models, a validation database that included 15 CB_2 receptor ligand test set structures and 67 046 marketed and developmental drugs from the Derwent World Drug Index 2005 (Derwent WDI),[25] which served as decoys, was screened. The CB_2 receptor ligand enrichment among the validation database compounds was expressed as enrichment factor (EF), which quantifies the number of times the enrichment of ligands improves using pharmacophore models compared to a random-based selection of compounds (see Experimental Section). The best validation results were retrieved for CB_2 receptor ligand model 1, which includes a Catalyst shape derived from **5** (Figure 3a).

Eleven out of 15 test set compounds (73%) and 1539 decoys from the Derwent WDI (2%) were able to match this model. Taking this into consideration, an EF of 32 was calculated for CB_2 receptor ligand model 1. A slightly lower but still excellent enrichment was determined for the second best model, which contains a Catalyst shape based on the 3D structure of **3** and was named CB_2 receptor ligand model 2 (Figure 3b). Ten matching test set structures (67%) and 1659 decoys (3%) retrieved from the Derwent WDI resulted in an EF of 28.

CB_2 receptor ligand models 1 and 2 complement one another, since two compounds of the CB_2 receptor ligand test set fitted perfectly to CB_2 receptor ligand model 2 but were missing in the virtual screening hit list for CB_2 receptor ligand model 1. For the remaining eight merged models, significantly lower EFs were determined than for CB_2 receptor ligand models 1 and 2. Thus, these models were discarded. When the validation results for CB_2 receptor ligand models 1 and 2 were merged, 13 out of 15 test set compounds (87%) and 2712 decoys (4%) were

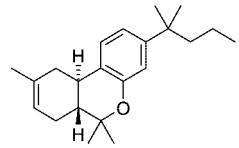
Chart 2. Structures of the CB_2 Receptor Ligand Training Set Compounds That Were Used for Common Feature-Based Model Generation



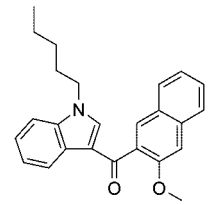
2 (GW405833)

3 (AM1241)

4 (HU-308)



5 (JWH-133)



6 (JWH-267)

Table 1. CB_1 and CB_2 Receptor Binding Affinities for the Compounds of the CB_2 Receptor Ligand Training Set

compd	CB_1 K_i (nM)	CB_2 K_i (nM)	(CB_1 K_i)/(CB_2 K_i)	ref
2	1917	12	160	27
3	5000	15.1	331	23
4	10000	22.7	441	7
5	677	3.4	199	24
6	381	7.2	53	14

retrieved. The resulting EF was 21. Thus, a virtual screening approach based on only one of the two best models would retrieve fewer decoys than a chemical database search using CB_2 receptor ligand models 1 and 2. However, the aim of our study was not to discover as many CB_2 receptor ligands as possible but to discover structurally novel scaffolds. On that account, we applied both pharmacophore models as queries for virtual screening in order to increase structural diversity of hits.

Agreement of the Pharmacophore Models with the Results of Previous CB_2 Receptor Homology Studies. Tuccinardi et al.[26] performed homology studies for CB_1 and CB_2 receptors. The results of these studies indicate that a hydrogen bond between the ligand and Ser112 and a hydrophobic contact to Phe197 are essential for CB_2 receptor selectivity. Furthermore, their studies showed that one of our CB_2 receptor ligand test set compounds, the potent and selective CB_2 receptor agonist **7** (182880-44-0),[27] forms a hydrogen bond between the morpholine oxygen of the compound and residue Ser112 of the CB_2 receptor and a hydrophobic interaction between the 2,3-dichlorobenzoyl group and Phe197 (Chart 3). Therefore, we mapped the test set compound **7** to CB_2 receptor ligand models 1 and 2 in order to see if the pharmacophore features were placed on the CB_2 receptor agonist in accordance with the ligand−protein interactions predicted by Tuccinardi et al. As displayed in Figure 4, the hydrogen bond acceptor of both models was placed on the morpholine moiety of the compound.

Moreover, one of the hydrophobic features was fitted to the 2,3-dichlorobenzoyl group of compound **7**. Thus, both models include pharmacophore features that are suggested by CB_1/CB_2 receptor homology studies to be essential for CB_2 receptor selectivity. Taking into account this fact, as well as the similar discriminatory power of the models and the retrieval of different test set compounds, both models were selected for the virtual screening of chemical databases.

Virtual Screening. Six chemical databases containing 922 944 compounds in total were screened utilizing CB_2 receptor ligand models 1 and 2 as queries (see Experimental Section), and 29 565 unique structures (3% of all virtually screened compounds) were able to match the two pharmacophore models. Subsequently, these virtual screening hits were filtered by a Pipeline Pilot[28] script, rejecting compounds of physicochemical properties that differ significantly from that of known CB_2 receptor-selective ligands. The tolerant criteria for this filter were selected carefully in order to not reject any scaffolds that may be of interest for binding to CB_2 receptor. Compounds not passing the filter were shown to have unfavorable properties that make them unsuitable as candidates for novel CB_2 agents (e.g., pharmacologically unfavorable degrees of lipophilicity). More detail is provided in the Experimental Section. The application of this physicochemical property filter resulted in 22 253 hits (2%). When a Pipeline Pilot script was used to cluster the rejected compounds in terms of structural similarity (see Experimental Section), we found that only a moderate number of scaffolds (52), which all represented not very druglike structures, have been excluded by the physicochemical property filtering. Applying the same script for the structural similarity analysis of the remaining compounds yielded a far higher number of clusters (605).

The 605 cluster centers (0.07%) were selected and inspected visually with respect to chemical stability and toxicity. For example, compounds that could be cleaved in aqueous solution because of a hydrolyzable ester moiety located in the middle of their scaffold, as well as structures containing electrophilic warheads such as hydrazones that are known for haptenization, were discarded.[29] Overall, 14 compounds (0.002%) fulfilled all filter criteria and were available for purchase at this time (Chart 4).

Finally, in order to determine the structural distance of the 14 compounds to known CB receptor ligands, these compounds were used as queries for a SciFinder database[30] similarity search. A Tanimoto score of ≥ 70 was applied as cutoff. For compound **8** only, analogues were retrieved that have been reported as CB receptor ligands. In particular, Marriott et al.[39] described CB_2 receptor-selective cannabinoids that are structurally related to compound **8**. However, to our knowledge, to date, no CB_2 receptor-selective pyridine tetrahydrocannabinol analogue has

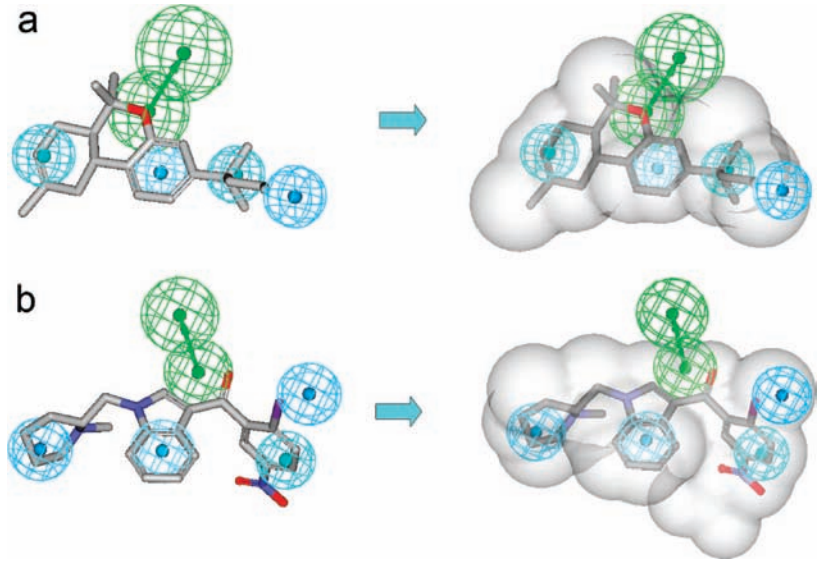
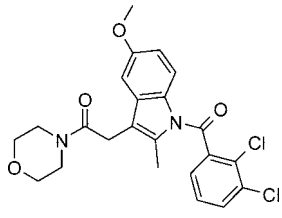


Figure 3. Generation of CB2 receptor ligand model 1 (a) and model 2 (b). For refinement, the two pharmacophore models were merged with a Catalyst shape derived from the 3D structure of the training set compounds **5** (a) and **3** (b). Screenshots were taken from Discovery Studio, which uses the following feature code: hydrogen bond acceptor (green spheres), hydrophobic feature (turquoise sphere), hydrophobic aliphatic feature (dark blue sphere), and hydrophobic aromatic feature (light blue sphere). The Catalyst shape is displayed as a gray cloud.

Chart 3. Structure of the Test Set Compound **7** That Was Aligned to CB_2 Receptor Ligand Models 1 and 2 in Order To Compare the Mapping of the Pharmacophore Model Features on CB_2 Receptor Agonist **7** with the CB_2 Ligand-Receptor Interactions for the Same Compound Predicted by Homology Studies[a]



7 (182880-44-0)[#]

[a] The number indicated by "#" is the CAS registration number.

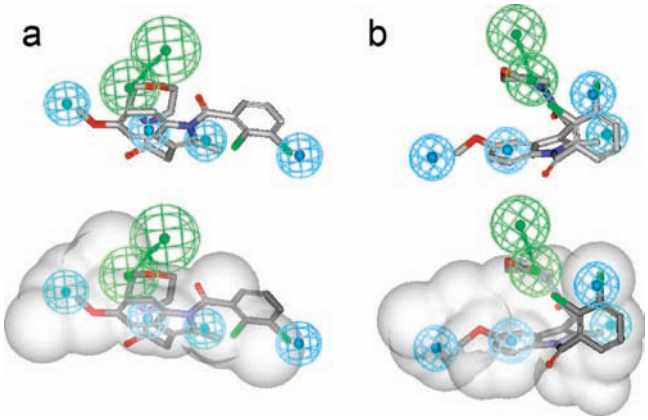


Figure 4. Compound **7** mapped to CB_2 receptor ligand model 1 (a) and model 2 (b).

been published. The CB_1 receptor-selective antagonist **9** (AM281)[31] and the CB_1 and CB_2 receptor antagonists patented by Makriyannis et al. (e.g., the CB_2 receptor-selective antagonist **10** (335196-79-7)[32]) are based on a pyrazole scaffold (Chart 5). Compounds **11**, **13**, and **18** contain a triazole moiety. However, these pyrazole compounds were not retrieved when compounds **11**, **13**, and **18** were subjected to the SciFinder similarity search, indicating that the substitution patterns of the patented compounds are dissimilar to those of compounds **11**, **13**, and **18**. Therefore, all 14 compounds were selected for biological testing.

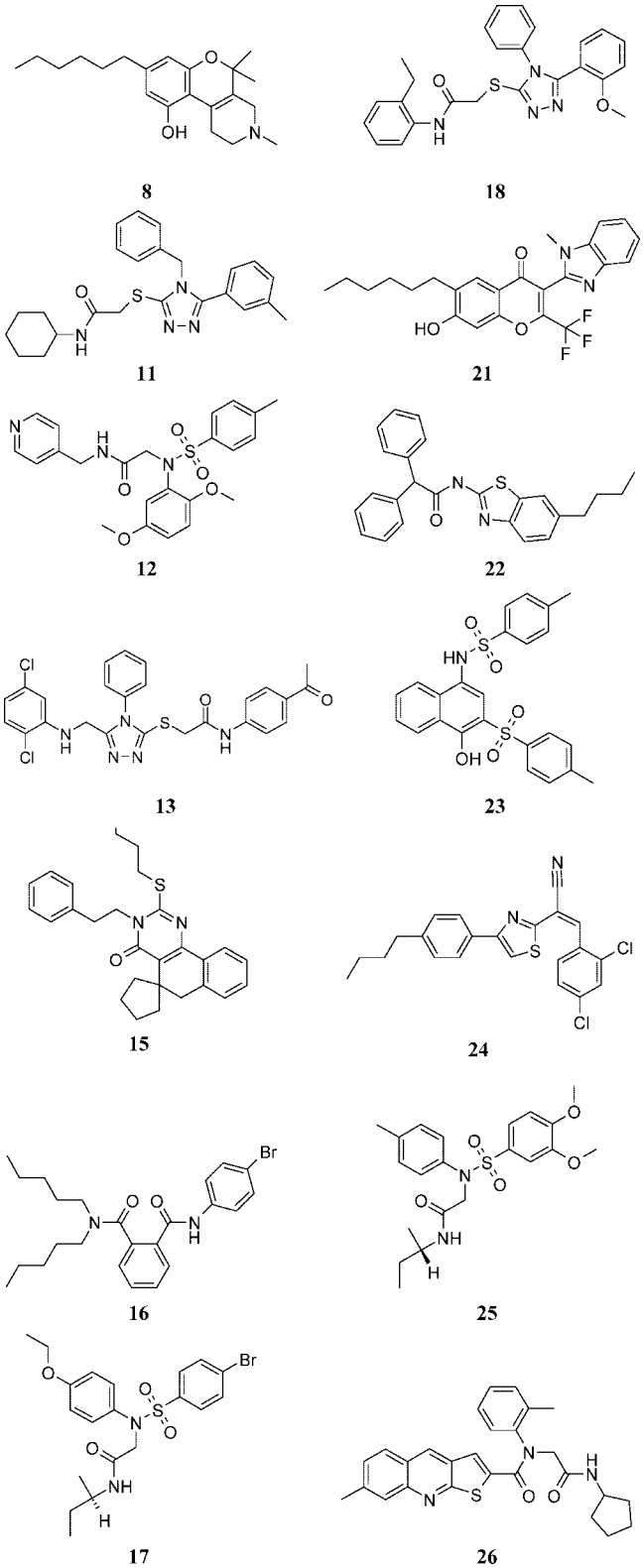
Receptor Pharmacology. The displacement of radioligand **14** ([^{3}H]CP-55,940, Chart 6)[33] by the 14 selected compounds was measured to determine their K_i values (see Experimental Section, Figure 5).

Seven compounds showed binding affinity for the CB_2 receptor. Compounds **11**, **15**, **16**, and **17** were determined as weak CB_2 receptor ligands with binding affinities lower than 25 μM, whereas low micromolar hCB_2 K_i values were measured for the more potent compounds **8** (K_i = 1.78 μM), **12** (K_i = 1.35 μM), and **18** (K_i = 2.1 μM), which possess a 27-fold, 37-fold, and 23-fold selectivity for the CB_2 receptor, respectively. To determine the relative potency of our compounds compared to well-known CB_2 receptor ligands, the CB_2 receptor agonist **5** was retested using our radioligand displacement assay. A hCB_1 K_i value of 0.797 μM and a hCB_2 K_i value of 0.023 μM was observed that corresponds to a 35-fold selectivity for the CB_2 subtype over the CB_1 receptor (Table 2).

A Dixon plot analysis identified compounds **8** and **18** as competitively binding ligands, whereas compound **12** appears to be a noncompetitive ligand (Figure 5). Moreover, a forskolin-stimulated cAMP assay was performed in CB_2 receptor-transfected CHO cells to determine the agonistic/antagonistic character of these three compounds (see Experimental Section). While at a concentration of 20 μM compound **12** was able to partially block the concentration-dependent inhibitory effect of **19** (WIN55,212−2, Chart 6)[34] on cAMP production and compound **18** significantly induced cAMP, compound **8** showed an additive effect to that of **19** and reduced cAMP production (see Supporting Information, Figure S1).

With respect to the results of Dixon plot analysis and the cAMP assay, compound **8**, which partially inhibited forskolin-stimulated cAMP, is a CB_2 receptor-selective partial agonist, whereas compound **12**, which does not appear to share the same

Chart 4. The Pharmacophore-Based Virtual Screening Workflow Resulted in 14 Compounds That Were Biologically Tested



binding site as **20** (CP-55,940, Chart 6),[35] acts as a silent CB_2 receptor-selective antagonist and compound **18** was identified as a competitive CB_2 receptor-selective inverse agonist (Table 2).

Putative Essential Structural Features of Novel CB_2 Receptor-Selective Ligands. In order to determine the structural features of compounds **8**, **12**, and **18**, which were predicted by our pharmacophore models to be essential for CB_2 receptor binding, the novel CB_2 receptor-selective ligands were aligned to the pharmacophore model that matched them during the virtual screening and visualized (Figure 6). (i) The ether oxygen of compound **8** matches the hydrogen bond acceptor of CB_2 receptor ligand model 1, whereas the hydrophobic features are placed on the tricyclic scaffold and the hexyl side chain of the compound. (ii) One of the two sulfonamide oxygens of compound **12** is fitted to the hydrogen bond acceptor of CB_2 receptor ligand model 2, and the four hydrophobic features of the model are mapped on the *p*-tolyl, the pyridine, and the 2,5-dimethoxyphenyl moiety. (iii) The amide oxygen of compound **18** is aligned to the hydrogen bond acceptor of CB_2 receptor ligand model 2, and the three phenyl moieties map the four hydrophobic features.

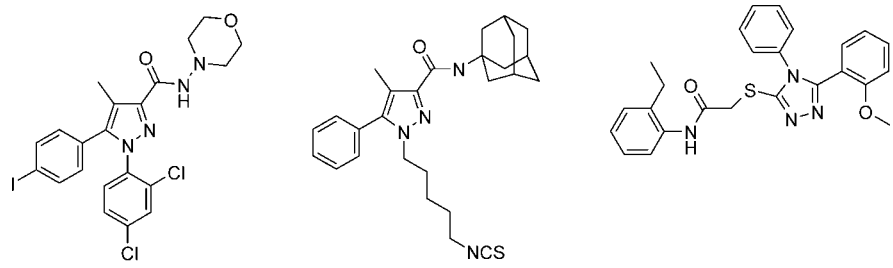
Discussion

We have generated common feature-based pharmacophore models for CB_2 receptor ligands. With respect to their EF values, CB_2 receptor ligand models 1 and 2 were determined as our best pharmacophore models and therefore used as queries for a virtual screen of 922 944 compounds obtained from six chemical databases. The hits were subjected to physicochemical property filtering and structural similarity analysis. The remaining hits were inspected visually in terms of chemical stability and toxicity. Fourteen compounds matched all these filter criteria. Finally, a SciFinder database similarity search was performed to determine the structural distance of the 14 compounds to CB receptor ligands reported in literature. Structurally similar ligands for CB receptor have been described for compound **8**, which is based on the tetrahydrocannabinol scaffold. Marriott et al. reported CB_2 receptor-selective hexahydrocannabinols that are structurally related to compound **8**. Therefore, compound **8** does not strictly represent a novel scaffold for CB_2 receptor ligands, but to our knowledge no pyridine tetrahydrocannabinol analogue has been biologically tested for CB_2 receptor activity. Compounds **11**, **13**, and **18** are based on a triazole scaffold. The CB_1 receptor antagonist **9** and the CB_1 and CB_2 receptor antagonists patented by Makriyannis et al.[32] contain a pyrazole scaffold. However, **9** and the patented CB receptor antagonists were not retrieved during the SciFinder database similarity search. On that account, a considerable structural distance between the scaffolds of compounds **11**, **13**, and **18** and these CB receptor antagonists exists. Thus, the binding affinities of all 14 compounds to the CB_1 and CB_2 receptor were determined using a radioligand displacement assay. For seven compounds an hCB_2 K_i value below 25 μM was detected. Compounds **8**, **12**, and **18**, showed low micromolar hCB_2 K_i values (1.78, 1.35, and 2.1 μM, respectively; 0.023 μM for retested **5**) and CB_2 receptor selectivity (>27-fold, >37-fold, and >23-fold, respectively; 35-fold for retested **5**). With respect to Dixon plot analysis and cAMP assay results, compound **12** acts as a noncompetitive CB_2 receptor-selective silent antagonist, whereas compound **18** is a competitive CB_2 receptor-selective inverse agonist and compound **8** was identified as a competitive CB_2 receptor-selective partial agonist. To sum up, the biological results demonstrate that our virtual screening workflow, which was created to obtain new scaffolds for CB_2 receptor ligands with focus on agonists, represents a valuable tool for the discovery of structurally novel CB_2 receptor agonists, antagonists, and inverse agonists.

Conclusions

In this work, we describe the development and validation of common feature-based pharmacophore models for CB_2 receptor

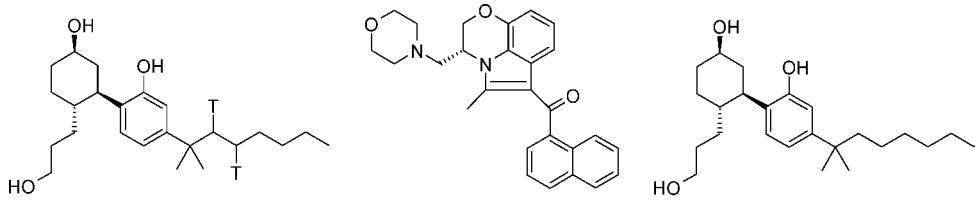
Chart 5. Structures of the CB1 Receptor-Selective Antagonist **9**, the CB2 Receptor-Selective Antagonist **10**, and Compound **18**[a]



9 (AM281) **10** (335196-79-7)[#] **18**

[a] The number indicated by "#" is the CAS registration number.

Chart 6. Structures of the CB_2 Receptor Ligands Used for the Radioligand Displacement Assay and cAMP Assay



14 ([^{3}H]CP-55,940) **19** (WIN55,212-2) **20** (CP-55,940)

ligands. Furthermore, we report the virtual screening of chemical databases using our two best pharmacophore models, as well as physicochemical property filtering, structural similarity analysis, and visual inspection. This virtual screening workflow resulted in the selection of 14 compounds for biological testing. For seven of these compounds, CB_2 $K_i \leq 25$ μM was determined. Compounds **8**, **12**, and **18** were identified as a competitive CB_2 receptor-selective partial agonist, a noncompetitive antagonist, and a competitive inverse agonist, respectively. Compounds **12** and **18** represent novel classes of CB_2 receptor-selective ligands. The three novel scaffolds for CB_2 receptor ligands can be subjected to medicinal chemistry optimization in order to obtain new leads for subsequent pharmacological evaluation.

Experimental Section

Hardware Specifications. Molecular modeling studies were carried out on an Intel Pentium Core 2 Duo 6400 equipped with 1 GB RAM running Linux Fedora Core 6.

Software Specifications. The following software programs were used for this study: Catalyst 4.11 for the generation of ligand-based pharmacophore models and pharmacophore-based virtual screening, Discovery Studio 2.0[36] for visualization of Catalyst pharmacophore models, LigandScout 2.0[37] for visualization of compound alignments, MOE 2007.09[38] for physicochemical property analysis, and Pipeline Pilot 5.0.1.100 for physicochemical property filtering and structural clustering.

Compilation of Compound Sets. For the compilation of the CB_2 receptor ligand training set and the CB_2 receptor ligand test set, CB_2 receptor-selective agonists found either in the literature or in the Derwent WDI, which contains 67 050 marketed drugs and developmental agents, were built and energetically minimized within the Catalyst software package. With respect to their CB_2 receptor selectivity, 18 potent agonists described in literature and two out of four CB_2 receptor-selective agonists included in the Derwent WDI were selected. Subsequently, a Pipeline Pilot script was applied to cluster the 20 compounds in terms of structural diversity. The script describes the compounds as extended connectivity fingerprints (ECFP) and uses the setting ECFP_6, as implemented in Pipeline Pilot. The structural similarity between the compounds was expressed as Tanimoto coefficient. All compounds with a Tanimoto dissimilarity score equal or lower than 0.7 formed one cluster, whereas structurally more diverse compounds with a score higher than 0.7 were assigned to a new cluster. This structural analysis resulted in five clusters from which five compounds were assigned to the CB_2 receptor ligand training set (Chart 2), whereas the remaining 15 compounds formed the CB_2 receptor ligand test set (see Supporting Information). Conformational models for all compounds were generated using the conformer generator catConf[22] with the following settings: maximum number of conformers = 250, generation type = best quality, and energy range = 20 kcal/mol above the calculated lowest energy conformation.

Pharmacophore Modeling. We applied the HipHop algorithm of Catalyst to generate qualitative common feature-based pharmacophore models for CB_2 receptor ligands. The qualitative approach was preferred over quantitative pharmacophore modeling because there was not enough structure−activity data available, including compounds with a spread of activity of several orders of magnitude, to create significant models for activity prediction of the virtual screening hits. The discriminatory power of the resulting ligand-based pharmacophore models was evaluated by screening a validation database that included the 15 structures from the CB_2 receptor ligand test set and all decoys from the Derwent WDI. As mentioned above, four Derwent WDI compounds have been reported as CB_2 receptor-selective agonists. Thus, the remaining 67 046 Derwent WDI structures were categorized as decoys and used for model validation. As a measure of the discriminatory power of the models, the EF was calculated using the following equation:[39,40]

$$\mathrm{EF} = \frac{\mathrm{TP}/n}{A/N}$$

where EF is the enrichment factor, TP is the number of CB_2 receptor-selective agonists matched by the model, n is the number of CB_2 receptor-selective agonists and decoys matched by the model, A is the number of CB_2 receptor-selective agonists in the validation database, and N is the number of all compounds in the validation database.

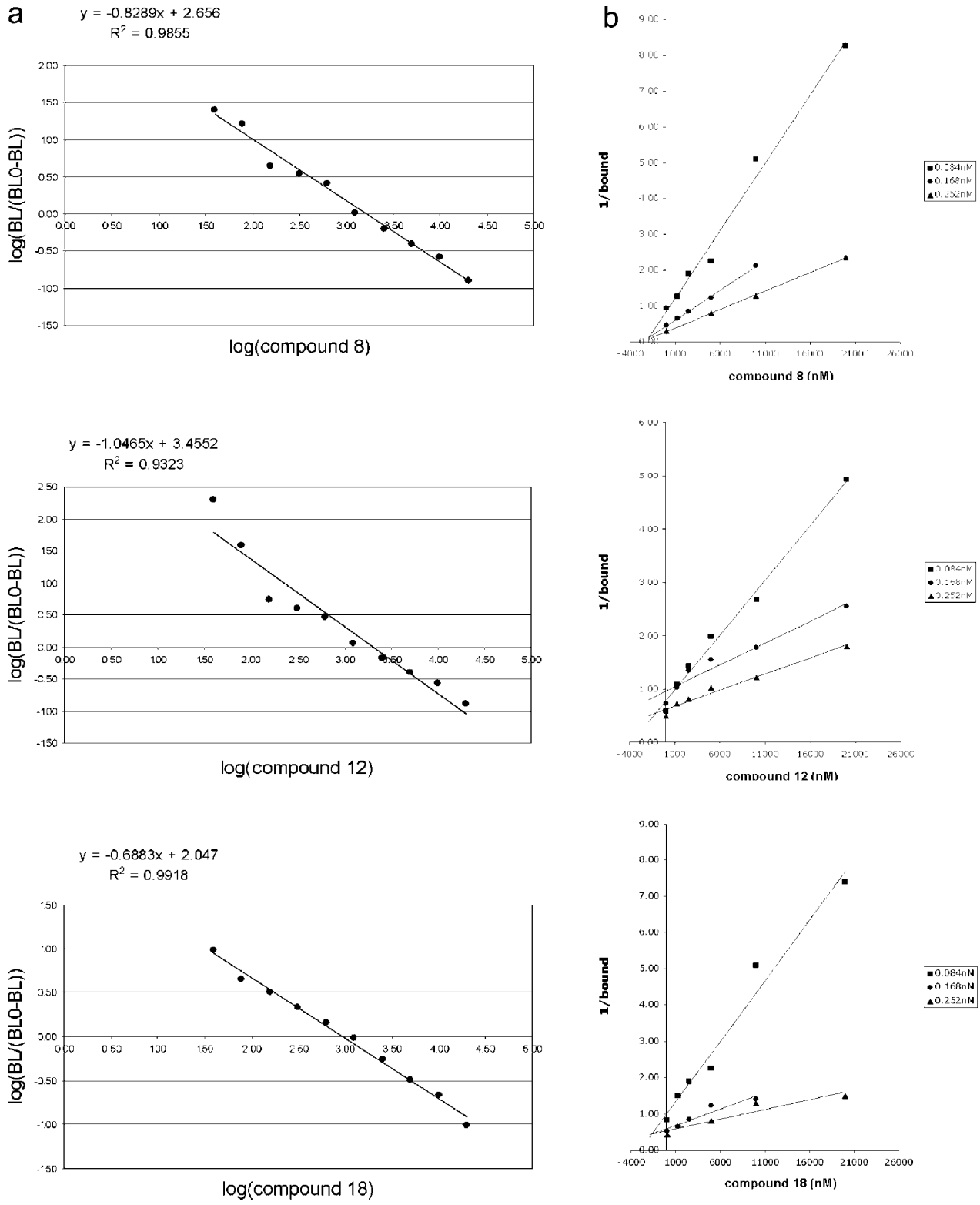


Figure 5. Radioligand displacement assay results: (a) Hill plot derived from linearization of displacement curves obtained in the radioligand displacement assays; (b) Dixon plot analysis showing competitive displacement of compounds **8** and **18** vs apparent noncompetitive binding of compound **12**. For a competitive inhibitor, the lines converge above the *x* axis, and the value of compound concentration where they intersect is $-K_i$. For a noncompetitive inhibitor, the lines converge on the *x* axis.

Chemical Databases. For the discovery of novel scaffolds for CB_2 receptor ligands, the following six chemical databases were screened against our best CB_2 receptor ligand models: the Asinex Gold database,[41] the Asinex Platinum database,[42] the Maybridge Screening Collection,[43] the Chemical Block Screening Library,[44] the Specs Screening Compounds database,[45] and the Vitas-M STK database.[46]

Virtual Screening. The virtual screening of the CB_2 receptor test set, the Derwent WDI, and the six chemical databases was performed within Catalyst applying the Best Flexible Search algorithm.[22] Only compounds that matched all the features of the pharmacophore model, which was used as virtual screening query, were retrieved as hits.

Table 2. Experimental Binding Affinities (K_i Values) Measured for the Seven Novel CB_2 Receptor-Selective Ligands Obtained from a Pharmacophore-Based Virtual Screening Workflow and for the Well-Known CB_2 Receptor Agonist **5**

compd	hCB_1 K_i (μM)[a]	hCB_2 K_i (μM)	(hCB_1 K_i)/(hCB_2 K_i)	binding behavior	receptor activity
5	0.797 ± 0.018	0.023 ± 0.004	35		
8	>50	1.78 ± 0.09	>27	competitive	partial agonist
11	>50	<25	>2		
12	>50	1.35 ± 0.17	>37	noncompetitive	antagonist
15	>50	<25	>2		
16	>50	<25	>2		
17	>50	<25	>2		
18	>50	2.1 ± 0.11	>23	competitive	inverse agonist

[a] K_i values are the mean values of three separate experiments performed in triplicate. The standard deviations for all K_i determinations are less than 15%.

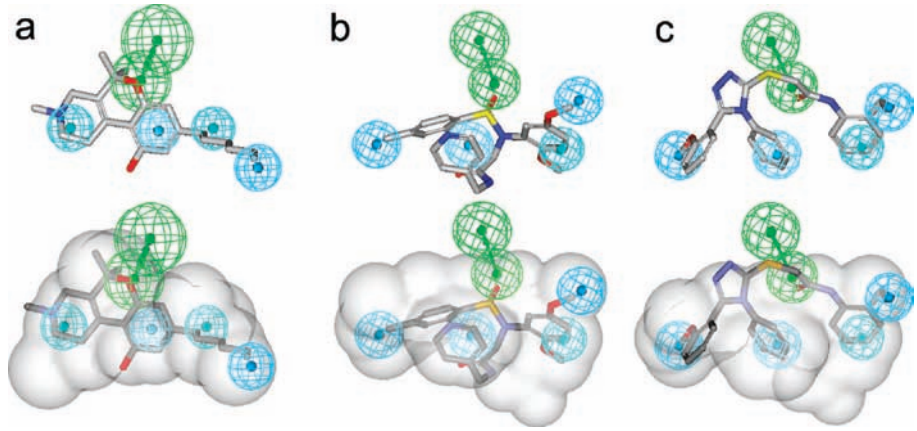


Figure 6. Alignment of the three most potent compounds to the pharmacophore models. Compound **8** appeared in the virtual screening hit list obtained for CB_2 receptor ligand model 1, whereas compounds **12** and **18** resulted as hits from the virtual screening of the six chemical databases against CB_2 receptor ligand model 2. The alignment of compound **8** (a) to CB_2 receptor ligand model 1 and the mapping of compounds **12** (b) and **18** (c) to CB_2 receptor ligand model 2 are displayed.

Physicochemical Filtering. The hits retrieved from the chemical databases were filtered using a physicochemical property filter. To determine the physicochemical filter criteria, the 20 compounds from the CB_2 receptor ligand training and test set were analyzed within the software package MOE. An analysis of the physicochemical property distribution resulted in the following filter criteria: number of heavy atoms, ≤35; molecular weight, ≤500; number of hydrogen bond donors, ≤2; number of hydrogen bond acceptors, ≤6; AlogP ≤ 8. Finally, a Pipeline Pilot script was created to automatically filter the hits according to these filter criteria.

Structural Similarity Analysis. The structural similarity analysis of the remaining virtual screening hits was performed by the Pipeline Pilot script which was also used for the compilation of the CB_2 receptor ligand training and test set and is mentioned above.

Test Compounds. The 14 candidate compounds were purchased from the following companies: Compounds **15**, **18**, and **21** were obtained from Specs (Delft, NL). Compounds **16**, **17**, and **22** were supplied by Vitas-M Laboratory Ltd. (Moscow, Russia), and compounds **8**, **11**−**13**, and **23**−**26** were purchased from Asinex Ltd. (Moscow, Russia).

Radioligand Displacement Assays on CB_1 and CB_2 Receptors. Experiments were performed as described in ref 47. For the CB_1 receptor, binding experiments were performed in the presence of 0.39 nM radioligand **14** at 30 °C in siliconized glass vials together with 7.16 μg of membrane recombinantly overexpressing CB_1 receptor (RBHCB1M, PerkinElmer Life Sciences), which was resuspended in 0.2 mL (final volume) of binding buffer (50 mM Tris-HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 0.5 mg/mL fatty acid free bovine serum albumin, pH 7.4). CB_1 receptor concentration (B_{max}) was 2.5 pmol/mg protein. Test compounds were present at varying concentrations, and the nonspecific binding of the radioligand was determined in the presence of 10 μM **20**. After 90 min of incubation, the suspension was rapidly filtered through 0.05% polyethyleneimine presoaked GF/C glass fiber filters on a 96-well cell harvester and washed nine times with 0.5 mL of ice-cold washing buffer (50 mM Tris-HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 2% bovine serum albumin, pH 7.4). Radioactivity on filters was measured with a Beckman LS 6500 scintillation counter in 3 mL of Ultima Gold scintillation liquid. Data collected from three independent experiments performed in triplicate were normalized between 100% and 0% specific binding for **14**. These data were fitted in a sigmoidal curve and graphically linearized by projecting Hill plots, which for both cases allowed the calculation of IC_{50} values. Derived from the dissociation constant (K_D) of **14** (0.18 nM for CB_1 receptor and 0.39 nM for CB_2 receptor) (vide infra) and the concentration-dependent displacement (IC_{50} value), inhibition constants (K_i) of competitor compounds were calculated using the Cheng−Prusoff equation ($K_i = IC_{50}/(1 + L/K_D)$). For CB_2 receptor binding studies, 3.8 μg of membrane recombinantly overexpressing CB_2 receptor (RBXCB2M, PerkinElmer Life Sciences) was resuspended in 0.6 mL of binding buffer (see above) together with 0.11 nM radioligand **14**. The CB_2 receptor radioligand binding assay was conducted in the same manner as for the CB_1 receptor. CB_2 receptor concentration (B_{max}) was 4.7 pmol/mg protein. B_{max} and K_D values of **14** were determined by PerkinElmer, Life and Analytical Sciences, Boston, MA.

cAMP Assay. Measurements of cAMP were carried out as described in ref 47. Human CB_2 receptor expressing CHO-K1 cells were plated in 96-well plates at a density of 3×10^5 cells/mL and incubated overnight. After the media were aspirated, the cells were chilled for 10 min at room temperature in RPMI 1640 (without supplements) containing 500 μM 3-isobutyl-1-methylxanthine. Cells (approximately 5×10^5 cells/mL) were then treated with different concentrations of test compounds and incubated for 30 min at 37 °C in a total volume of 100 μL. After another 30 min of incubation at 37 °C with 20 μM forskolin, intracellular cAMP levels were detected by HitHunter for adherent cells EFC chemiluminescent detection assay (catalog no. 90000302, Amersham) according to the manufacturer's instructions and measured on a Microlumat Plus Microplate

Luminometer LB 96V (EG&G Berthold). The high-affinity CB receptor ligand **19** was used as positive control.

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Supporting Information Available: Results of the cAMP assay, as well as structures of the 15 CB_2 receptor ligand test set compounds with the corresponding CB_1 and CB_2 receptor binding affinities. This material is available free of charge via the Internet at http://pubs.acs.org.

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