

## Quantitative Conformationally Sampled Pharmacophore for $\delta$ Opioid Ligands: Reevaluation of Hydrophobic Moieties Essential for Biological Activity

Denzil Bernard, Andrew Coop, and Alexander D. MacKerell Jr.\*

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Baltimore, Maryland 21201

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Recent studies have indicated several therapeutic applications for  $\delta$  opioid agonists and antagonists. To exploit the therapeutic potential of  $\delta$  opioids developing a structural basis for the activity of ligands at the  $\delta$  opioid receptor is essential. The conformationally sampled pharmacophore (CSP) method (Bernard et al. *J. Am. Chem. Soc.* **2003**, *125*, 3103–3107) is extended here to obtain quantitative models of  $\delta$  opioid ligand efficacy and affinity. Quantification is performed via overlap integrals of the conformational space sampled by ligands with respect to a reference compound. Iterative refinement of the CSP model identified hydrophobic groups other than the traditional phenylalanine residues as important for efficacy and affinity in DSLET and ICI 174 864. The obtained models for a structurally diverse set of peptidic and nonpeptidic  $\delta$  opioid ligands offer good predictions with  $R^2$  values  $>0.9$ , and the predicted efficacy for a set of test compounds was consistent with the experimental values.

### Introduction

Treatments of severe and chronic pain often depend on the use of opioid analgesics, which are generally effective by their action at the  $\mu$  opioid receptor.<sup>1,2</sup> However, the use of  $\mu$  opioids is accompanied by adverse effects including life threatening incidents such as respiratory depression<sup>3</sup> and constipation.<sup>4</sup> In addition, the development of tolerance and dependence<sup>5</sup> complicate the therapeutic use of these drugs. Thus, there is a need for the development of effective medications that lack serious side effects. Since the identification of the enkephalins<sup>6,7</sup> and the  $\delta$  opioid receptors,  $\delta$  opioid ligands have been pursued as analgesic agents and numerous studies have investigated the biological processes involving the  $\delta$  opioid receptor system. This has led to the discovery of several potential therapeutic applications for  $\delta$  opioid ligands such as the treatment of substance abuse and immunosuppression, among others.<sup>8–10</sup> It has also been found that co-administration of a  $\delta$ -antagonist with a  $\mu$ -agonist reduces the development of tolerance and dependence to the  $\mu$ -agonist,<sup>11,12</sup> and a slower development of tolerance has been observed with the administration of a peptide with the dual profile of  $\mu$ -agonism and  $\delta$ -antagonism.<sup>13</sup> Thus, efforts are also being made to develop novel analgesics with this dual profile of  $\mu$ -agonism and  $\delta$ -antagonism.<sup>14</sup>

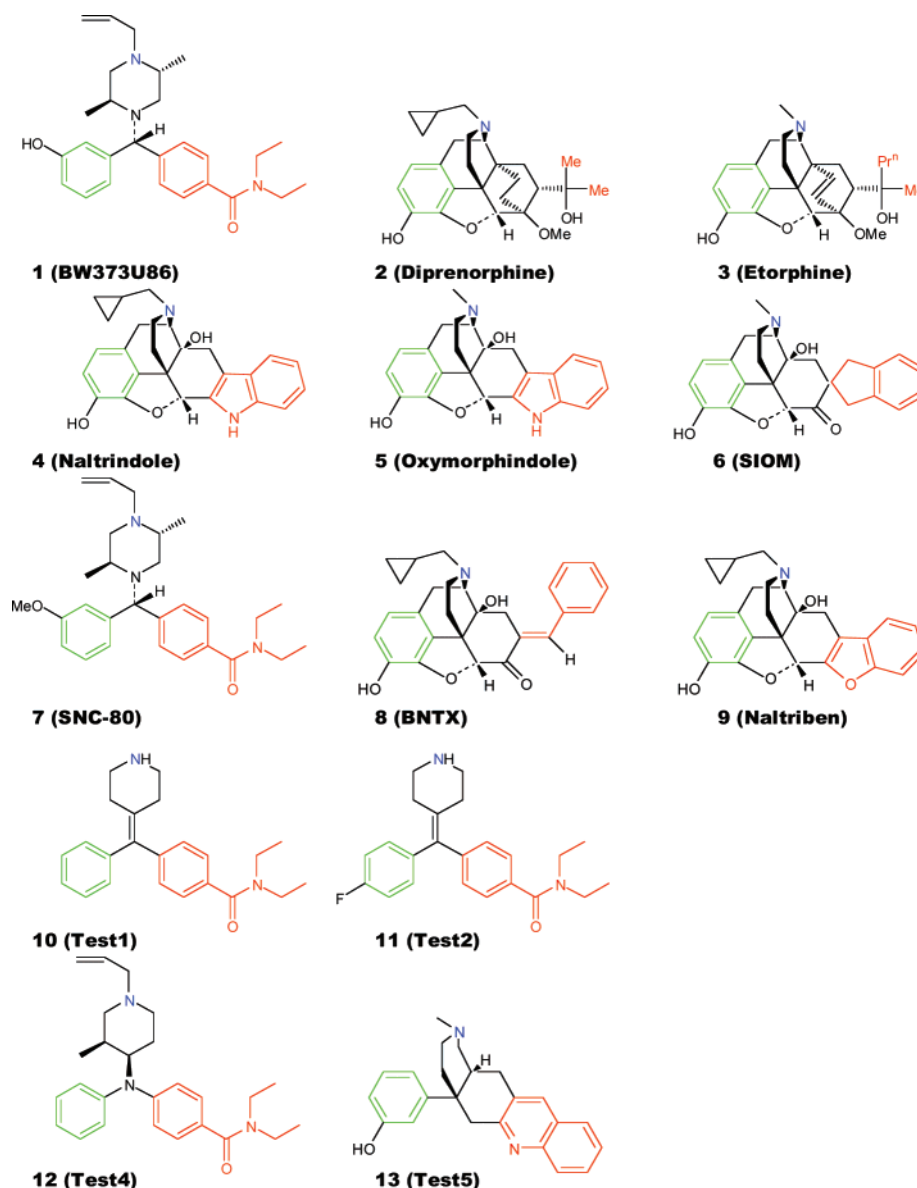
The rational design of drugs for a specific target is greatly aided by structural information of the receptor. However, in the absence of an experimentally obtained three-dimensional (3D) structure of the receptor, drug development methods must rely on information obtainable from known ligands of the receptor. This is the case with the G-protein coupled  $\delta$  opioid receptor<sup>15,16</sup> for which no experimental structure is available. While computational 3D models of the  $\delta$  opioid receptor have been developed,<sup>17–23</sup> a large number of studies have focused on developing structure–activity relationships (SAR) based on the study of known  $\delta$  opioid ligands<sup>24</sup> via use of both pharmacophore<sup>25–35</sup> and QSAR<sup>36–41</sup> models. The former method typically involves identification of low-energy conformations of the ligands of interest, followed by structural alignment and

identification of common features that are predictive of biological activity. While these studies have advanced our understanding of  $\delta$  opioid SARs, the information is typically restricted to a congeneric series of compounds and omit consideration of conformational changes that may occur upon binding of ligands to their target receptor.<sup>36–41</sup>

To overcome the limitations of traditional methods of pharmacophore development, an approach involving extensive conformational sampling of ligands followed by the use of all sampled conformers in the pharmacophore model was developed.<sup>27,28</sup> The method, which is referred to as a *conformationally sampled pharmacophore (CSP)*, was applied to the study of nonpeptidic  $\delta$  opioid ligands<sup>27</sup> and peptidic  $\delta$  opioid ligands<sup>28</sup> resulting in models distinguishing  $\delta$  opioid agonists from antagonists. The inclusion of all sampled conformers of the ligands in the model accounts for the inherent dynamic nature of molecules and the nature of their interaction with biomolecules, as molecules at room temperature possess kinetic energy sampling a variety of conformations other than just the lowest energy conformation(s).<sup>42</sup> More importantly, the favorable interaction with the receptor may enable a molecule to overcome the conformational strain associated with assuming a higher energy conformation, and hence, the bound conformation of a molecule need not be among the low energy conformers of the unbound molecule.<sup>43</sup> Accordingly the inclusion of all conformers increases the probability of including the bioactive conformer in the model. The importance of conformational sampling and the use of higher energy conformers in addition to the low energy ones has gained increasing attention,<sup>44,45</sup> and the utility of the CSP method is emphasized by its consideration in studies by workers in the field<sup>46–52</sup> including its application for pharmacophore development of compstatin analogues.<sup>53</sup>

Here we describe an extension of the CSP method into quantitative models predicting the efficacy and the affinity of  $\delta$  opioid ligands. Subtypes of the  $\delta$  opioid receptor (i.e.,  $\delta_1$  and  $\delta_2$ ) have been proposed but are not invoked in the current model, as their existence remains a matter of debate.<sup>10</sup> The overlap in conformational space, as defined by various combinations of geometric parameters for different ligands with respect to a reference ligand, are quantified and then used as parameters

\* To whom correspondence should be addressed. Room 629, HSF II, 20 Penn Street, Baltimore, Maryland 21201. Tel.: 410-706-7442. Fax: 410-706-5017. E-mail: amackere@rx.umaryland.edu.



**Figure 1.** Nonpeptidic  $\delta$  opioid ligands used in the development of the quantitative efficacy and affinity models. The pharmacophore groups are A in green, B in red, and N in blue. Compounds **8** and **9** were included only for affinity modeling and compounds **10**–**13** were used as external tests for efficacy prediction.

for the prediction of ligand efficacies and affinities using multiple regression models. The utility of the approach is shown in the identification of novel functional groups on selected peptides that are essential for biological activity and ligand affinity.

### Computational Methods

Conformational sampling of peptidic and nonpeptidic  $\delta$  opioid ligands was achieved by utilizing molecular dynamics (MD) simulations<sup>54</sup> using the program CHARMM.<sup>55,56</sup> Initially, the  $\delta$  opioid ligands (Figure 1 and Table 1) were modeled using Sybyl 6.2<sup>57</sup> and energy minimized to a gradient of 0.05 kcal/mol·Å using the Tripos force field. Each molecule was then subjected to 200 steps of Adopted Basis Newton Raphson minimization in CHARMM using the Merck Molecular Force Field (MMFF)<sup>58,59</sup> prior to MD simulations. Conformational sampling for the nonpeptidic ligands (Figure 1) was carried out with 10 ns MD simulations at 300 K, with snapshots saved every 100 integration time steps for analysis. Sampling for the peptidic ligands (Table 1) was carried out using replica exchange MD simulations<sup>60</sup> that involved 10 ns simulations with four replicas between 300 and 400 K using an exponential

**Table 1.** Peptidic  $\delta$  Opioid Ligands Used in the Development of CSP<sup>a</sup>

peptide	sequence
(14) deltorphin II	<b>Tyr</b> -D-Ala- <i>Phe</i> -Glu-Val-Val-GlyNH <sub>2</sub>
(15) DPDPE	<b>Tyr</b> -c[D-Pen-Gly- <i>Phe</i> -D-Pen]
(16) pCl-DPDPE	<b>Tyr</b> -c[D-Pen-Gly- <i>Phe</i> (pCl)-D-Pen]
(17) DSLET	<b>Tyr</b> -D-Ser-Gly- <i>Phe</i> -Leu-Thr
(18) ICI 174,864	(H <sub>2</sub> C=CCH <sub>2</sub> ) <sub>2</sub> - <b>Tyr</b> -Aib-Aib- <i>Phe</i> -Leu

<sup>a</sup> The pharmacophore points are the protonated nitrogen (N) on Tyr1, the centroid of the phenolic group (A) on Tyr1 (bold) and the centroid of the hydrophobic group (B; italic). For **17** and **18**, the various groups considered as the hydrophobic moiety are shown in italic.

scale; 300, 330, 363, and 400 K. For each replica, simulations were carried out for 100 integration time steps, following which the coordinates were saved for analysis and an exchange of replicas was attempted. Conformations from all four replicas were used in the analysis. For all MD simulations, Langevin dynamics<sup>61</sup> were performed with an integration time step of 0.002 ps, including SHAKE of all covalent bonds involving hydrogens,<sup>62</sup> and aqueous solvation was treated via the Generalized Born Continuum Solvent Model.<sup>63,64</sup> The physiologically relevant protonation states of the ligands were used in the study, which in the case of some of the

nonpeptidic ligands involved multiple configurations of the proton on the basic nitrogen, as previously performed<sup>27</sup>.

Angles and distances between the three pharmacophoric points (Figure 1 and Table 1) were measured for all conformations of the  $\delta$  opioid ligands obtained from the MD simulations. Geometric data from all configurations for the nonpeptidic ligands with multiple protonation states were combined for analysis. All possible combinations of distances with angles were then utilized to obtain 2D probability distributions of the pharmacophoric parameters for each ligand. These probability distributions were obtained with a bin size of 0.1 Å and 1° for the distances and angles, respectively. Overlap coefficients (OCs<sup>a</sup>), for the 2D pharmacophoric parameters were calculated using eq 1

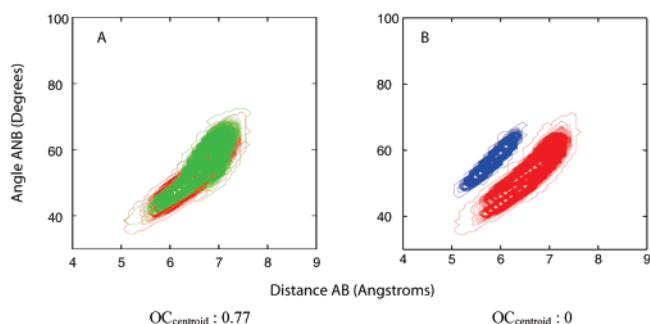
$$OC = \frac{\sum_{ij} P_{ij}^k \cdot P_{ij}^l}{\sqrt{\sum_{ij} (P_{ij}^k)^2 \cdot \sum_{ij} (P_{ij}^l)^2}} \quad (1)$$

where P represents the normalized probability at pixel *ij* from the 2D distributions for compounds *k* (i.e., the reference compound) and *l*.

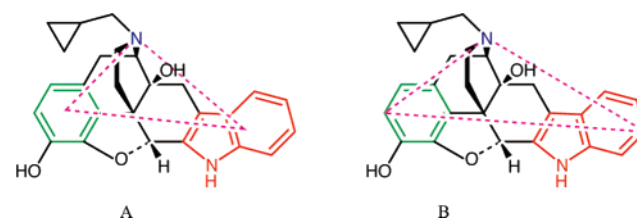
Overlap coefficients were then utilized as independent variables in regression analysis with respect to the reported biological activity and affinity (i.e., the dependent variables, Table S.1, Supporting Information) using the program Excel. For model development, compounds **1–9** in Figure 1 and **14–18** in Table 1, were used as the training set; compounds **10–13** were used as test molecules. Compounds **1** and **7** are structurally almost identical, with the only difference being the methoxy substituent in **7** as opposed to the hydroxyl substituent in **1** on the aromatic ring (Figure 1). This similarity in structure results in very high overlaps in the pharmacophoric parameters and preliminary regression analysis indicated a bias in the models due to **7** dominating the regression fits. This compound was therefore excluded during the initial phase of development of the quantitative model; however, once preliminary models were developed **7** was reintroduced into the analysis and is included in the final models. The OC were calculated for all nine distance-angle pairs for each compound with respect to the reference compound. Combinations of the different distance-angle OCs for all compounds were then used in multiple regression analyses to fit the experimental efficacies for the ligands. From these regression analysis sets of OC values that yielded the highest correlation coefficients, ( $R^2 > 0.9$ ), with suitable *P*-values (<0.05) for the coefficients were determined and subjected to further analysis.

## Results and Discussions

To generate a quantitative CSP model involving the 2D pharmacophoric parameters, a set of  $\delta$  opioid ligands with efficacies and affinities determined under identical experimental conditions were selected for the training set.<sup>65</sup> The selected data set involved the binding and G-protein activation by a set of peptidic and nonpeptidic  $\delta$  opioid ligands in C6 glioma cell lines stably transfected with the  $\delta$  opioid receptor from rat for efficacy measurements, and the displacement of radiolabeled **4** for determination of ligand affinities. Table S.1 of the Supporting Information presents the list of ligands and the experimental data extracted from Table 1 of Clarke et al.<sup>65</sup> Because the reported ligand efficacies were obtained based on the maximum stimulation of [<sup>35</sup>S]GTP- $\gamma$ S binding with respect to that by the  $\delta$  opioid ligand **1**, evaluation of the OC pharmacophoric



**Figure 2.** 2D probability distributions and calculated OC for (A) **1** (red) and **7** (green) and (B) **1** (red) and **4** (blue).



**Figure 3.** Examples of centroid and maximum distance based calculations of pharmacophoric parameters shown for **4**. The pharmacophore groups are A in green, B in red, and N in blue. The lines in magenta indicate the type of measurement: (A) centroid and (B) maximum A–B distance (MaxD).

parameters were carried out using **1** as the reference compound. In addition, the influence of the identity of the reference compound was studied by individually using **7** and **6** as the reference compounds.

The CSP method is based on relating the regions of conformational space sampled by the ligands to their biological activity, with the conformational space defined as the distributions of the distances and angles between the pharmacophoric points (Figure 1 and Table 1) obtained from all accessible conformations of the respective ligands. Shown in Figure 2 are selected 2D conformational distributions for the nonpeptidic ligands **1**, **4**, and **7**. As may be seen, the overlap of the distributions of **1** and **7** (Figure 2A), both agonists, is high, while that of **1** and an antagonist **4** (Figure 2B) is low. This is the basis for the qualitative 2D CSP that discriminates  $\delta$  opioid agonists from antagonists.<sup>27,28</sup> To extend the approach to a quantitative method, the extent of overlap of the distributions between compounds may be obtained using eq 1. In the case of the distributions shown in Figure 2A,B, the computed OC values are 0.77 and 0.00, respectively. By obtaining the OC values for all compounds in a training set with respect to a reference compound, those values may be regressed against the biological data, yielding a quantitative model.

**Development of  $\delta$  Opioid Ligand Efficacy Model.** Development of the quantitative CSP used the following pharmacophore points (Figure 1 and Table 1): the basic nitrogen (N), and centroids of the aromatic ring (A) and hydrophobic (B) moieties in the  $\delta$  opioid ligands (centroid; Figure 3A), as previously performed.<sup>27,28</sup> This yields three distances and three angles, from which nine possible 2D pharmacophore parameters are obtained. In lieu of the centroids for pharmacophore points A and B, the atoms that were the maximum distance apart between the A and B groups were also used to define the respective pharmacophore points (MaxD; Figure 3B). This was achieved by computing the maximum (AB) distance between all nonhydrogen atoms of the aromatic group, A, and of the hydrophobic group, B, for all saved conformers of a compound and then selecting the two relevant atoms for the computation

<sup>a</sup> Abbreviations: OC, overlap coefficient; centroid, pharmacophore parameters calculated using centroids of groups of atoms; MaxD, pharmacophore parameters calculated using maximum distance between groups of atoms; centroid=, pharmacophore parameters calculated using centroids of groups of atoms with equal weighting of all conformers; MaxD=, pharmacophore parameters calculated using maximum distance between groups of atoms with equal weighting of all conformers.

of the remaining distances, AN and BN, and angles ANB, NAB, and NBA, (Figure 3B). Additionally, in the calculation of the OC values, overlaps were also measured by equal weighting (centroid= or MaxD=) of all points in conformational space. This was performed by using a binary measure of occupancy for the summation of points occupied by a compound and the reference compound (i.e., 1 if sampled, 0 if never sampled), normalized by the total number of points occupied. Development of the quantitative efficacy model involved compounds **1–7** and **14–18**. The appropriate definition of the B group in  $\delta$  opioid ligands with multiple hydrophobic residues or groups, such as **17** and **18**, were obtained as a result of refinement of the predictive models as discussed below, with the successive inclusion of these ligands in the quantitative model. Initially, **17** was included in model development with Phe as the hydrophobic B group. Further refinement of the model involved consideration of the Leu residue in **17** as the B group followed by inclusion of **18** in the training set, where the N-terminal allyl substituents were also considered as the B group. The OC values for the nine 2D pharmacophoric parameters used in the initial model for the various ligands are presented in Tables S.2 and S.3 of the Supporting Information. In the majority of cases, finite OC values are obtained; however, with some agonists, such as **2**, **3**, **5**, and **17** and the antagonist **4**, zero OC values were obtained.

The nine 2D OC parameters based on the different definitions of the pharmacophore points, centroid and MaxD, with and without equal weighting of conformers, were then used in multiple regression analysis with respect to the efficacy data. Initial analysis selected those models with high  $R^2$  values (Table S.4 Supporting Information), showing the best models to include pharmacophoric parameters based on equal weighting of all conformers for both the centroid (centroid=) and maximum A to B distance (MaxD=) measurements. Further evaluation of these models was performed using  $P$ -values to estimate the significance of the individual OC parameters in these models (Table S.5, Supporting Information). It was seen that the centroid= combination of the NA–NBA and BN–NAB 2D pharmacophoric parameters resulted in a  $R^2$  value of 0.898 with  $P$ -values  $<0.05$  for both OC parameters. However, **2** and **5**, which have low but significant experimental activity (Table S.1), show no overlap in either of these parameters with respect to **1** (Table S.2ii), suggesting that these parameters may not be indicative of the requirements for activity at the  $\delta$  opioid receptor. Alternatively, the AB–NBA + NA–NBA MaxD= combination gives an  $R^2$  value of 0.936 with  $P$ -values  $<0.001$  for both parameters (Table S.5ii). However, detailed analysis of the selected OC parameters revealed that for the highly active  $\delta$  opioid ligand **17**, both of the selected OC parameters were zero with respect to **1** (Table S.3ii). While this may indicate limitations in the model, with **17** there is a second functional group on the peptide that may act as the hydrophobic B group, leucine, rather than the phenylalanine side chain that is traditionally accepted as the hydrophobic group. Thus, this group was tested as the hydrophobic B group, with the resultant OC values giving reasonable overlaps for all nine parameters (Table S.6, Supporting Information).

The recalculated OC values for **17** were utilized in additional regression analysis (Table S.7, Supporting Information). The best correlations were seen with the parameters obtained using the maximum A to B distance (MaxD), with probability based OC values giving  $R^2$  values  $>0.92$  and with the AB–NBA and NA–NBA MaxD distance angle combination yielding the best  $R^2$ . Importantly, AB–NBA and NA–NBA MaxD combination

**Table 2.** Model Properties, Selected Pharmacophore Parameters, and a Comparison of Observed and Predicted Relative Efficacies of  $\delta$  Opioid Ligands with **1** as the Reference Molecule<sup>a</sup>

model				
$R^2 = 0.978$	Y-intercept	AB–NBA	NA–NBA	
coefficients	0.072	3.375	11.026	
$P$ values	0.0431	0.0004	0.0007	

cmpd	biological activity		OC values	
	experimental	predicted	AB–NBA	NA–NBA
<b>2</b>	0.08	0.1	0.007492	0
<b>3</b>	0.36	0.27	0.057046	0.000529
<b>4</b>	0	0.08	0.000996	0
<b>5</b>	0.12	0.08	0.001019	0
<b>6</b>	0.18	0.22	0.044116	0.000063
<b>14</b>	0.59	0.56	0.074764	0.021763
<b>15</b>	0.59	0.59	0.070327	0.025072
<b>16</b>	0.8	0.8	0.084515	0.040306
<b>17</b>	0.9	0.93	0.180618	0.022434

<sup>a</sup> Model obtained using multiple regression with the maximum A to B distance based calculation of OC.

had significant coefficients ( $p < 0.05$ ) supporting the validity of the model (Table 2). Thus, the application of the quantitative 2D CSP method predicts that the hydrophobic moiety responsible for  $\delta$  activity in **17** is the side chain of the Leu moiety rather than the traditional Phe side chain.

Detailed analysis of the AB–NBA and NA–NBA MaxD based model is presented in Table 2. In accord with the high  $R^2$ , 0.978, and significant  $P$  values, the model nicely predicts the activity of all the compounds in the training set. Examination of the 2D parameters AB–NBA and NA–NBA shows that all compounds have overlap with reference **1** for the AB–NBA distance angle parameter. On the other hand, the low efficacy compounds, **2** and **5**, and the antagonist **4** have no overlap for the NA–NBA parameter. It is also seen that the AB–NBA parameter is most directly correlated with the efficacies with compounds having higher overlaps being more active, indicating that the structural features associated with this parameter are probably the most significant determinants of  $\delta$  opioid activity. Notable is the ability of the model to predict the difference between the peptidic ligands **15** and **16**. These ligands only differ by the presence of the chlorine atom on Phe<sup>3</sup> suggesting that the present approach is capable of predicting changes in activity associated with subtle structural changes in a ligand. The ligand for which the model makes the poorest prediction is **4**, which is a full antagonist (i.e., no discernible biological activity in the applied experimental assay), although the model predicts it to have a low, but nonzero efficacy. The structural similarity between **4** and **5** (Figure 1) results in almost identical OC values for the two compounds and seems to be the cause for the predicted activity for **4**. However, *N*-cyclopropylmethyl substituents as in **4** tend to give lower opioid efficacy than *N*-methyl substituents,<sup>8</sup> an effect not accounted for in the present model; future models will incorporate this substituent to further fine-tune the model.

Structurally, both distance AB and angle NBA involve the hydrophobic pharmacophoric moiety, B, indicating the relevance of this group for  $\delta$  opioid activity. The fact that the maximum distance based criterion is the best predictor of the ligand efficacies also indicates that the spatial extent of the B group influences the activity, as does its orientation with respect to the other pharmacophoric groups. This is consistent with previous observations by us<sup>27,28</sup> and others.<sup>35,66,67</sup> In addition, it was seen that for the two parameter combinations of AB–NBA with the remaining 2D parameters involving either the

AB or BN distance with any angle gave  $R^2$  values  $>0.9$ , further indicating the significance of the hydrophobic group, B (Table S.7, Supporting Information).

**Impact of Reference Compound.** Compound **1** is a full  $\delta$  agonist, and because experimental efficacy data were available with reference to this compound, the initial development of a quantitative model used it as the reference compound. To test if the selection of the reference compound would impact the resulting model, the quantitative 2D CSP was performed using two alternate compounds as the reference. Compound **7** was selected as it is structurally similar to **1**. The second, **6**, was selected as it is a weak agonist with low efficacy and is also structurally different from **1**.

OC values based on **7** as the reference differ significantly from those with respect to **1** (data not shown). This difference is an outcome of using all conformations of the ligands with the CSP method, which accounts for the inherent dynamics of molecules, whereby though **7** and **1** are almost structurally identical, the difference in conformations sampled becomes evident. While this may complicate the refinement of the pharmacophore, it indicates that the CSP method goes beyond the biases associated with structural alignment techniques used typically in pharmacophore development by not limiting the conformations considered to only those that satisfy predetermined scaffolds.

With **7** as the reference, regression analyses were performed for various combinations of 2D pharmacophoric parameter OC, as above, yielding a number of combinations with good correlation. The best predictions of efficacy with  $R^2 > 0.9$  were seen with the MaxD parameters (Table S.8, Supporting Information) similar to that seen with **1**, and the use of the probability based weighting again provided better predictions. Several of these models had  $R^2$  values  $>0.9$ , although the best model, (AB-NBA + NA-NBA,  $R^2 = 0.946$ ) had a large  $P$  value for the AB-NBA term. Of the remaining AB-NBA models with  $R^2 > 0.9$  (Table S.8), the  $P$  values were all  $<0.05$ , indicating good reliability in the model. Thus, with **7** as the reference compound, predictive models are obtained, with the AB-NBA parameter being the most relevant for biological activity. However, the second parameter differed from that of the model with **1** as the reference, indicating that subtle differences in the models are obtained with different reference compounds.

With **6** as the reference compound, the OC values were very different from those using **1** (data not shown), as expected due to the structural differences in the two compounds. On performing multiple regression analyses, reasonable  $R^2$  values (i.e.,  $>0.9$ ) were obtained only with combinations involving three or more 2D pharmacophoric parameters (Table S.9, Supporting Information). Interestingly,  $R^2$  values  $>0.9$  were obtained only for the MaxD= based parameter, where, unlike for **1** or **7** as reference compounds, equal weighting of the conformers gave better predictions. Of the top models, the two best with  $R^2 > 0.96$ , NA-NBA, BN-NAB, NA-ANB and NA-NBA, NA-NAB, BN-NBA, were also the only two with  $P$  values  $<0.05$  for all three parameters.

For the **6** based model, the two significant differences with respect to the models based on **1** and **7** was the lack of the term AB-NBA and the use of equal weighting of the MaxD term in the best models. In the use of all sampled conformations of a molecule in pharmacophore development, one may expect a highly active compound to populate regions of conformational space that are relevant to receptor activation to a greater extent than a compound with low activity. As a result, for a low-efficacy reference compound, both the regions of overlap

relevant to activity and the population of those regions may be much smaller, such that the ability of the compound to sample that region of conformational space is more relevant rather than details of the extent and probability of sampling active conformations. This difference may lead to equal weighting yielding more predictive models when using a low-efficacy compound as the reference. Concerning the lack of inclusion of the AB-NBA term in the best models, this term was included in one model with  $R^2 > 0.9$  (Table S.9), and because the other best models contain the NA, BN, and various angle terms, the AB-NBA related information is implicitly in the model. Thus, the use of a low-efficacy compound as reference does allow for the development of a predictive model. However, because of the probable limitations in sampling conformational regions related to receptor activation in low efficacy compounds, it is suggested that higher efficacy compounds serve as better reference compounds.

**Inclusion of 18 in the Training Set.** As mentioned above, the peptide antagonist **18** possesses multiple hydrophobic groups, including the Phe and Leu residues and the allyl substituents on the amino group, which may influence the nature of the interaction of this peptide with the  $\delta$  opioid receptor. In addition, this peptide is different from the other peptidic ligands in that it has a tertiary amino group that forms one of the pharmacophoric points, which could also be a cause for the antagonistic nature of this peptide. Due to the structural differences in this ligand and the different hydrophobic groups that could serve as the pharmacophoric B group, it was not included in the initial training set. As mentioned above, the assumption of the Phe residue as the hydrophobic group in **17** was found to be inconsistent and, therefore, model development was extended to include **18** with Leu<sup>5</sup> as well as the allyl substituents on the N, in addition to Phe<sup>4</sup> being considered as the required hydrophobic B group.

Overlap calculations for each of the possible groups in **18** were undertaken with respect to **1**, with the resulting OC values reported in Table S.10 of the Supporting Information. The different B group definitions offered variable degrees of overlap for the pharmacophoric parameters, with the B1 (Phe<sup>4</sup>) and B4 (Leu<sup>5</sup>) definitions providing overlaps for all parameters. The B2 and B3 (allyl substituents) definitions gave finite overlaps in only a few parameters, including the AB-NBA and the NA-NBA parameters and, in some instances, the NA-ANB parameter. In addition, due to the symmetry of the allyl groups, they were considered as one group and the OC values were calculated. Regression analyses were then performed with **18** in the training set using each set of pharmacophoric parameters for the different B group definitions individually. Only in the case of the MaxD based parameters without equal weighting were regression models with  $R^2$  values  $>0.9$  obtained, (Table S.11, Supporting Information). This observation is consistent with the results without **18** (see above). Interestingly it was observed that reasonable fits ( $R^2 > 0.85$ ) could be obtained with each of the B group definitions for **18**, although the best models were obtained with the allylic substituents as the B group. Once again, all models with  $R^2$  value  $>0.9$  involved AB-NBA, and the combination with BN-NBA provided the best  $R^2$  value of 0.95, with acceptable  $P$ -values for the regression parameters. Thus, the quantitative CSP model can be extended to include **18**, with the model suggesting that the hydrophobic B moieties may be the allyl substituents on the tertiary amino group, which also acts as the essential basic N on the compound.

**Inclusion of 7 and 18 in the Final Model.** Compound **7** is structurally very similar to the reference compound, **1**, which

**Table 3.** Statistical Analysis of the Three Best Regression Models for Different Definitions of the Hydrophobic B Group in **18** Obtained with MaxD Based OC and the Inclusion of **7** in the Training Set<sup>a</sup>

2D pharmacophoric parameter	R <sup>2</sup>	coefficient 1	coefficient 2	coefficient 3
ICI-B1				
AB-NAB + AB-NBA + BN-NAB	0.945	6.8053	5.4185	-11.6207
AB-NAB + AB-NBA + BN-ANB	0.944	9.1739	5.2474	-13.4950
AB-ANB + AB-NAB + AB-NBA	0.939	-8.1779	3.9389	5.2830
ICI-B2-3				
AB-ANB + AB-NBA + BN-NAB	0.973	-20.4463	5.0678	17.0941
AB-ANB + AB-NAB + AB-NBA	0.963	-8.2981	4.0780	5.2779
AB-ANB + AB-NBA + BN-ANB	0.957	-14.4864	5.2995	10.3428
ICI-B4				
AB-ANB + AB-NBA + BN-NAB	0.973	-20.4520	5.0703	17.0959
AB-ANB + AB-NAB + AB-NBA	0.961	-8.2618	4.0408	5.2812
AB-NBA + BN-NBA + NA-NBA	0.960	3.3760	-21.5598	17.4879

<sup>a</sup> Multiple regression was performed without a constant. The values for the regression coefficients and the corresponding *P* values are listed for the different 2D pharmacophoric parameter OC combinations.

resulted in very high OC values, such that it dominated the regression fits during model development, leading to its exclusion from the initial fitting step. With the change in definitions of the hydrophobic B group for **17** and the multiple hydrophobic groups in **18** and the identification of the MaxD OC parameters as the best predictor of activity, an attempt was made to extend the model to include both **7** and **18** using **1** as the reference compound. For these analyses, the hydrophobic groups identified for **18**, namely, Phe<sup>4</sup> (B1), the two allylic amino substituents (B2/3), and Leu<sup>5</sup> (B4), were evaluated separately with the use of the Leu<sup>5</sup> residue as the hydrophobic group for **17**. Regression analyses were performed iteratively as described above using combinations of the OC values obtained from the MaxD parameters yielding multiple combinations with *R*<sup>2</sup> values >0.9 and *P* values <0.05 for the regression coefficients. In all cases, the regression constant was found to be insignificant, and hence, to obtain the final model regression, analyses were performed once again restricting the intercept to zero. The resulting combinations, with higher *R*<sup>2</sup> values, obtained using different B group definitions for **18** are shown in Table 3. As seen in the table, irrespective of the B group used, most of the combinations giving good correlations are the same, in contrast to the result seen above in the model that omitted **7**. While more combinations yielding high *R*<sup>2</sup> values are seen with the Phe<sup>4</sup> group (data not shown) as the hydrophobic moiety, the best *R*<sup>2</sup> value 0.973 is obtained with the use of either the Leu<sup>5</sup> residue or the allylic substituents as the B group. The two best *R*<sup>2</sup> models were compared with the experimentally obtained efficacy values as shown in Tables 4 and 5 and Figure 4A,B. The best *R*<sup>2</sup> values in both cases (B2-3 or B4) are with the AB-ANB + AB-NBA + BN-NAB combination, and the coefficients for this model are also very similar, suggesting that either of these hydrophobic groups could provide the required hydrophobic interactions with the receptor for **18**. In the final model, all combinations involve the AB-NBA parameter, and the best combination in all three cases includes the BN-NAB parameter as well, suggesting that the structural features associated with

**Table 4.** Model Properties, Selected Pharmacophore Parameters, and a Comparison of Observed and Predicted Relative Affinities of  $\delta$  Opioid Ligands with **1** as the Reference Molecule<sup>a</sup>

model	AB-ANB	AB-NBA	BN-NAB		
<i>R</i> <sup>2</sup> = 0.973					
coefficients	-20.4463	5.0678	17.0941		
<i>P</i> values	1.35E-06	3.32E-07	9.52E-06		
biological activity		OC values			
cmpd	exp.	pred.	AB-ANB	AB-NBA	BN-NAB
<b>2</b>	0.08	0.04	0	0.007 492	0
<b>3</b>	0.36	0.29	0	0.057 046	0.000 019
<b>4</b>	0	0.01	0	0.000 996	0.000 008
<b>5</b>	0.12	0.01	0	0.001 019	0.000 008
<b>6</b>	0.18	0.22	0.001 138	0.044 116	0.001 433
<b>7</b>	1.02	1.02	0.877 801	0.906 859	0.840 738
<b>14</b>	0.59	0.63	0.008 131	0.074 764	0.024 471
<b>15</b>	0.59	0.48	0.001 921	0.070 327	0.009 454
<b>16</b>	0.8	0.80	0.064 178	0.084 515	0.098 641
<b>17</b>	0.9	0.94	0.000 554	0.180 618	0.002 153
<b>18</b>	0	0.01	0	0.002 26	0

<sup>a</sup> Model obtained using multiple regression without a constant. Compounds **7** and **18** were included in the training set with both of the allylic amino substituents (B2/B3) in **18** as the pharmacophoric B group.

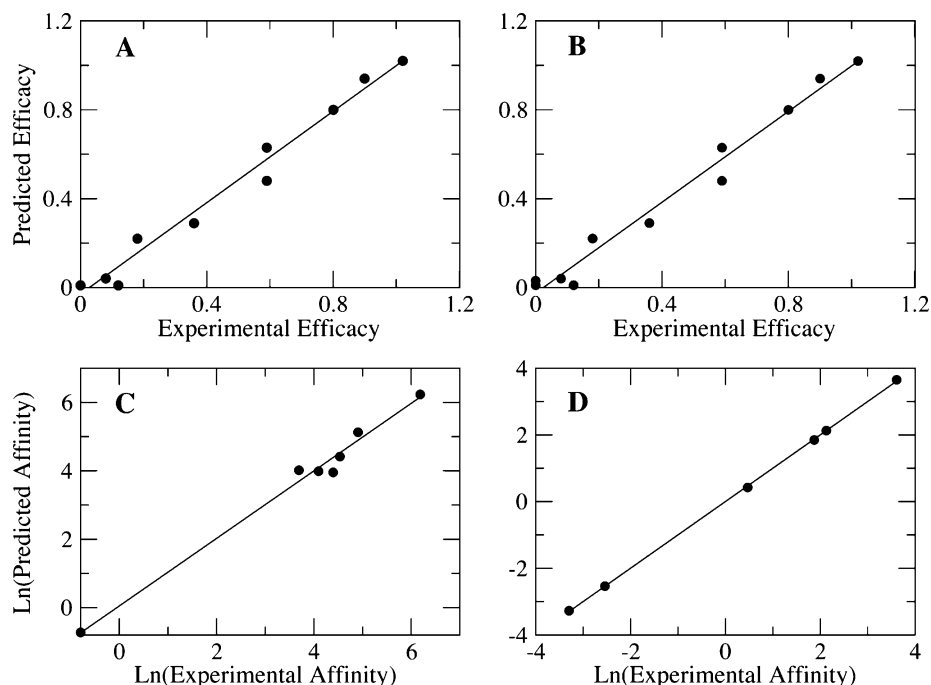
**Table 5.** Model Properties, Selected Pharmacophore Parameters, and a Comparison of Observed and Predicted Relative Efficacies of  $\delta$  Opioid Ligands with **1** as the Reference Molecule<sup>a</sup>

model	AB-ANB	AB-NBA	BN-NAB		
<i>R</i> <sup>2</sup> = 0.973					
coefficients	-20.4520	5.0703	17.0959		
<i>P</i> values	1.43E-06	3.49E-07	1E-05		
biological activity		OC values			
cmpd	exp.	pred.	AB-ANB	AB-NBA	BN-NAB
<b>2</b>	0.08	0.04	0	0.007 492	0
<b>3</b>	0.36	0.29	0	0.057 046	0.000 019
<b>4</b>	0	0.01	0	0.000 996	0.000 008
<b>5</b>	0.12	0.01	0	0.001 019	0.000 008
<b>6</b>	0.18	0.22	0.001 138	0.044 116	0.001 433
<b>7</b>	1.02	1.02	0.877 801	0.906 859	0.840 738
<b>14</b>	0.59	0.63	0.008 131	0.074 764	0.024 471
<b>15</b>	0.59	0.48	0.001 921	0.070 327	0.009 454
<b>16</b>	0.8	0.80	0.064 178	0.084 515	0.098 641
<b>17</b>	0.9	0.94	0.000 554	0.180 618	0.002 153
<b>18</b>	0	0.03	0.041 485	0.039 248	0.039 496

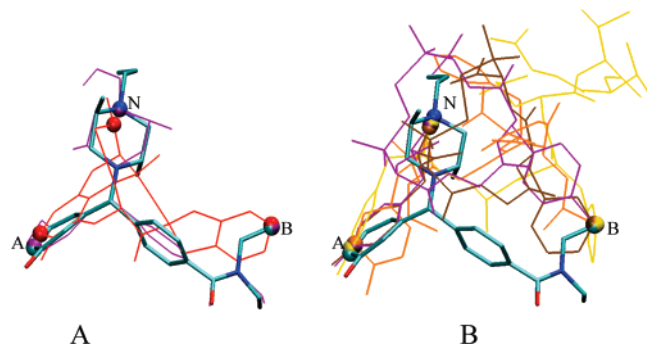
<sup>a</sup> Model obtained using multiple regression without a constant. Compounds **7** and **18** were included in the training set, with the Leu<sup>5</sup> (B4) residue in **18** as the pharmacophoric B group.

these two parameters are the most important descriptors for predicting  $\delta$  opioid efficacies.

**Determination of a Common Bioactive Conformation of the  $\delta$  Opioid Ligands.** The  $\delta$  opioid efficacy model includes the OC of the pharmacophoric parameters AB-ANB, AB-NBA, and BN-NAB with respect to the reference compound **1**. Therefore, utilizing these parameters, the regions common to all  $\delta$  opioid ligands (i.e., regions of common intersection or overlap) in these parameters were identified. As can be seen from the  $\delta$  opioid efficacy model, Table 5, the nonpeptides **6** and **7** and the peptides **14**, **15**, **16**, and **17** have finite OC values for all three parameters, and in the determination of a common bioactive conformation, regions common to these ligands as well as **1** were identified. While multiple conformations were obtained for each compound, the superimposition of one selected conformation for each ligand is shown in Figure 5 with **1** as the reference. As can be seen from the superimposition of the conformers for **6** and **7**, Figure 5A, the pharmacophore points match very well for **7**, as expected, with a slight difference in the orientation of the **6** conformer. With regard to the peptidic



**Figure 4.** Quantitative conformationally sampled  $\delta$  opioid pharmacophore (CSP) models. (A) Efficacy model based on the MaxD parameter with the Leu<sup>5</sup> residue in **17** and the allylic amino substituent in **18** as the pharmacophoric B group (see Table 4 for original data). (B) Efficacy model based on the MaxD parameter with the Leu<sup>5</sup> residue in **17** and the Leu<sup>5</sup> residue in **18** as the pharmacophoric B group (see Table 5 for original data). (C) Affinity model for high efficacy  $\delta$  opioid ligands with the Leu<sup>5</sup> residue in **17** as the pharmacophoric B group (see Table 6 for original data). (D) Affinity model for low efficacy  $\delta$  opioid ligands with the allylic amino substituent in **18** as the pharmacophoric B group (see Table 7 for original data). Affinity models were developed using the natural logarithms of experimental values.



**Figure 5.** Superimposition of conformations for (A) nonpeptidic ligands and (B) peptidic ligands identified based on the  $\delta$  opioid efficacy model. The reference compound **1** is colored based on atom type in bond format. The three atoms defining the pharmacophore points (A, B, and N using MaxD criterion) are shown as spheres. Remaining structures as wireframe are in the following colors: (A) **6** in red and **7** in purple, and (B) **14** in yellow, **15** in brown, **16** in purple, and **17** in orange.

ligands, Figure 5B, the pharmacophore points superimpose quite well, with the same conformer of **1**. Importantly, in the common conformations, these pharmacophore points are all exposed to the environment, consistent with an essential role in interacting with the receptor. However, there appears to be no significant trend in the orientation of the other residues in these peptides. Thus, the application of the CSP method permits the identification of conformations that satisfy pharmacophoric criteria, suggesting probable bioactive forms of the  $\delta$  opioid ligands. In addition, the presence of both peptide and nonpeptide conformers that satisfy the same pharmacophoric criterion may indicate similar modes of receptor activation for peptides and nonpeptides.

**Development of  $\delta$  Opioid Ligand Affinity Model.** The utility of the quantitative CSP method in the development of a model describing  $\delta$  opioid ligand affinities was evaluated by

using the experimentally obtained  $K_i$  values based on the displacement of radiolabeled **4**,<sup>65</sup> which was therefore also used as the reference for this analysis. This evaluation involved the study of the high efficacy  $\delta$  agonists (**1**, **3**, **7**, and **14–18**) as one class and the partial agonists (**2**, **5**, and **6**) and antagonists (**8**, **9**, and **18**) as a second class (Table S1 of Supporting Information). Efforts to develop a unified affinity model for both high and low efficacy ligands were unsuccessful (data not shown). OC pharmacophoric parameters were calculated with respect to **4**, as it has the highest affinity for the  $\delta$  receptor in the dataset as well as being the ligand used in the displacement assay. All possible definitions of the pharmacophoric B group for **17** and **18** were utilized for the modeling of ligand affinities. Regression analyses were performed using the OC values as the independent variables (Tables S.12 and S.13, Supporting Information) and the log of the  $K_i$  values as the dependent variable. Obtained regression models with  $R^2 > 0.9$  were then examined for the significance of the determined regression parameters with  $P$  values  $< 0.05$ .

For the high efficacy  $\delta$  agonists it was observed that the MaxD parameters based on the pharmacophore groups A and B provide models with good predictability of the ligand affinities. As mentioned above, for **17** the two definitions of the pharmacophoric B group, Phe<sup>4</sup> and Leu<sup>5</sup> were explicitly evaluated; with Phe<sup>4</sup> while satisfactory  $R^2$  values were obtained, the binding affinity for **17** was always overestimated and  $P$  values  $< 0.05$  were not obtained, indicating this group to be inappropriate for the prediction of affinity, consistent with the efficacy models presented above. Table 6 and Figure 4C present the optimal model for the prediction of affinities of the high efficacy ligands. The high  $R^2$  of 0.987 and low  $P$  values support the validity of the model, and all predicted activities are within the reported 95% confidence limits, except for **3**, for which the binding affinity is underestimated, and **14**, for which it is overestimated. The model based on the MaxD definition

**Table 6.** Model Properties, Selected Pharmacophore Parameters, and a Comparison of Observed and Predicted Affinities of High-Efficacy  $\delta$  Opioid Agonists with **4** as the Reference Molecule<sup>a</sup>

model	$R^2 = 0.987$			
Y-intercept	AB-ANB	AB-NBA	NA-NAB	
coefficients	4.0162	23.8041	18.9059	-51.6319
P values	0.0005	0.0065	0.0313	0.0016

cmpd	binding affinity		OC values		
	exp.	pred.	AB-ANB	AB-NBA	NA-NAB
<b>1</b>	0.45 (0.38-0.54)	0.48	0	0.000 996	0.092 267
<b>3</b>	40 (36-44)	56	0	0.000 001	0
<b>7</b>	60 (45-80)	54	0	0.000 165	0.000 632
<b>14</b>	81 (65-99)	52	0.093 061	0.032 653	0.056 057
<b>15</b>	487 (447-531)	510	0.080 481	0.090 259	0.027 86
<b>16</b>	135 (105-174)	168	0.170 757	0.081 941	0.087 233
<b>17</b>	93 (80-109)	83	0.003 827	0.068 911	0.019 242

<sup>a</sup> The Leu<sup>5</sup> residue (B2) in **17** was used as the pharmacophoric B group. Regression analysis was performed using the natural log of reported experimental values. Values in parenthesis are the range of experimentally determined values.

includes the OC parameters from the AB-ANB, AB-NBA, and NA-NAB terms. Notably, the AB-NBA parameter, for which all ligands have finite OC (Table S.13), and the AB-ANB parameter, which is important for the  $\delta$  opioid ligand efficacy model, both contribute to the best affinity model. Clearly, the structural features associated with these terms play essential roles in the interaction of the compounds with the  $\delta$  opioid receptor.

Regression analysis was next performed for the low efficacy  $\delta$  opioid ligands, with all possible definitions for the hydrophobic moiety **18** used for model development. Using the Phe<sup>4</sup> residue as the hydrophobic B group in **18**, models with a  $R^2$  value  $>0.9$  and  $P$  values  $<0.05$  for the coefficients were obtained with the centroid based parameters and equal weighting of the conformers (centroid=). However, these models did not give accurate affinity predictions, with the predicted values being beyond the 95% reported experimental confidence intervals. With the use of the Leu<sup>5</sup> group as the pharmacophoric group, no models with significant correlation were identified. On the other hand, with the use of the allylic substituents, B2 or B3 as the B group, models with  $R^2$  values  $>0.9$  were obtained with the MaxD based parameters. All models included the AB-NBA parameter; however, on examining the predicted affinities with the experimental values for the models with B2 as the B group, affinities for most of the compounds were outside the 95% confidence intervals for the experimental value. With the B3 group as the hydrophobic moiety and equal weighting of conformations (MaxD=), the AB-NAB + AB-NBA + BN-NAB combination yielded a model with a  $R^2$  value of 0.97 and  $P$  values  $<0.05$  for all regression parameters, but in this case, affinities for **2** and **18** were outside the experimental range. As with the efficacy prediction, the combined use of both allylic groups in determining the hydrophobic pharmacophore point was evaluated, and only one model was obtained with a  $R^2$  value  $>0.9$  and  $P$  values  $<0.05$  for the regression parameters. This model involved a combination of the AB-NAB, AB-NBA, and BN-NAB MaxD= OC values and gave a  $R^2$  value of 1, with  $P$  values well below 0.05 (Table 7). A comparison of the predicted and experimental values (Figure 4D) shows that this

**Table 7.** Model Properties, Selected Pharmacophore Parameters, and a Comparison of Observed and Predicted Affinities of Low Efficacy  $\delta$  Opioid Ligands with **4** as the Reference Molecule<sup>a</sup>

model	$R^2 = 1.000$			
Y-intercept	AB-NAB	AB-NBA	BN-NAB	
coefficients	-33.8161	-807.593	99.815 08	737.9552
P values	8.08E-05	8.58E-05	7.37E-05	8.78E-05

cmpd	binding affinity		OC values		
	exp.	pred.	AB-NAB	AB-NBA	BN-NAB
<b>2</b>	1.6 (1.5-1.8)	1.5	0	0.343 025	0
<b>5</b>	6.5 (6.0-7.1)	6.3	0.901 773	0.910 539	0.912 037
<b>6</b>	8.4 (7.3-9.6)	8.4	0.462 39	0.500 997	0.486 971
<b>8</b>	0.079 (0.069-0.086)	0.079	0.091 803	0.188 357	0.117 38
<b>9</b>	0.037 (0.034-0.042)	0.038	0.894 354	0.908 654	0.897 235
<b>18</b>	37 (29-47)	39	0	0.375 375	0

<sup>a</sup> Both allylic amino substituents (B2/B3) in **18** were used for the pharmacophoric B group. Regression analysis was performed using the natural log of reported experimental values. Values in parenthesis are the range of experimentally determined values.

**Table 8.** Prediction of Efficacy of Test Compounds Using the CSP Based  $\delta$  Opioid Efficacy Model<sup>a</sup>

cmpd	biological activity	
	experimental	predicted
<b>10</b> <sup>b</sup>	1.1	2.07
<b>11</b> <sup>b</sup>	0.96	1.68
<b>12</b> <sup>c</sup>	0.89	0.49
<b>13</b> <sup>d</sup>	0.15	0.03

<sup>a</sup> The reported experimental efficacy values were normalized with respect to the common reference compound **7**. <sup>b</sup> From Wei et al. [6a and 6b]. <sup>c</sup> From Thomas et al. [(-)-23a]. <sup>d</sup> From Burkey et al. [TAN-67].

model gives very accurate predictions for all the low efficacy  $\delta$  opioid ligand affinities.

The AB-NBA parameter is also seen to be important for the affinity of the low efficacy  $\delta$  opioid ligands. In addition, the BN-NAB parameter that was important in efficacy prediction is important for the affinity of the low efficacy ligands. From the previously published CSP models used for qualitative analysis of  $\delta$  opioid agonists and antagonists<sup>27,28</sup> it was observed that 2D combinations of the BN distance with the angle parameters showed the best discrimination, and the inclusion of the BN-NAB parameter in both the efficacy model and the model for the affinities of low efficacy ligand suggest that this parameter may differentiate between the high and the low efficacy  $\delta$  ligands. The importance of the hydrophobic B group in  $\delta$  opioid ligands is thus observed for both efficacy and affinity models, including its contribution to the AB and BN distances, with the latter consistent with previous conclusions based on the qualitative  $\delta$  opioid CSP models.<sup>27,28</sup>

**Prediction of Efficacy for Test Compounds.** To more rigorously test the final efficacy model, it was used to predict the activity of four test compounds (Figure 1) obtained from literature with reported experimental results.<sup>68-70</sup> Compounds **10**, **11**, and **12** were selected as the effective agonists that are structurally similar to the reference compound, **1**, while **13** was selected as it has a structure that differs from those included in the training set as well as being a low efficacy ligand. Applying the same simulation protocol as used for the other compounds and defining the pharmacophore groups as shown in Figure 1, the efficacy for each ligand was obtained using the developed



$\delta$  opioid efficacy model (Table 4), with the resulting predictions along with the experimental data shown in Table 8. As **7** was common to the three studies from which the test compounds were selected as well as part of the present study, experimental values were normalized with respect to **7**. Analysis of Table 8 shows the predictions to be consistent with the experimental values. The overall ordering of the activities is in agreement with experiment, though differences in the absolute values exist. The predicted efficacies for compounds **10** and **11** are much higher than the experimental values reported, while those for **12** and **13** are underestimated. Compounds **10** and **11**, as mentioned, have similar structural features as compound **7**. However, an important difference is the presence of an alkene bond in these compounds that restricts the conformational flexibility of these molecules, particularly with respect to the relative orientation of the pharmacophoric point N. This conformational restraint gives higher overlap values in the BN–NAB parameter, resulting in an over estimation of the efficacy. While these results indicate that additional refinement of the model may be achieved via the inclusion of conformationally restricted agonists in the training set, differences in experimental methods may have an impact. For example, the use of membranes in some studies,<sup>68,69</sup> as opposed to the transfected C6 glioma cell data used in the present work, could contribute to some of the discrepancy in predicted versus experimental values. Compounds **12** and **13** are predicted to be less efficacious in comparison to **7**, which is consistent with the experimental data. The predicted efficacy of 0.03 for **13** agrees well with the reported efficacy of 0.15<sup>70</sup> with respect to **7**. The extent of agreement is similar to **5**, which had a predicted efficacy of 0.01 versus an experimental value of 0.12 (Table 4). Thus, the developed CSP efficacy model effectively predicts **13** to be a relatively poor agonist, although it appears that the model is limited in accurately separating low efficacy agonists from full antagonists, as discussed above. Overall, the quality of the predictions, while not ideal, indicates the utility of the developed model as well as the path toward improvements in the model.

## Conclusions

The CSP approach includes all sampled conformers of a ligand in the development of a pharmacophore, thus maximizing the probability of including the receptor bound conformations in the model. The application of this method to the study of  $\delta$  opioid ligands, including both nonpeptides and peptides, resulted in qualitative pharmacophore models capable of distinguishing  $\delta$  opioid agonists from antagonists.<sup>27,28</sup> The present study extends the application of the CSP method to allow quantitative predictions of ligand efficacies and affinities using regression analyses of overlap coefficients of the 2D pharmacophoric parameters. An important aspect of the CSP method is that it does not require conformational alignment of the molecules, but rather includes all conformers in the analysis, and the essential pharmacophoric parameters are verified during refinement. This allows for the development of models that span a diverse range of structures, including both peptidic and non-peptidic ligands. In addition, the approach appears to be able to distinguish substituent effects as both the efficacy and the affinity models are able to distinguish **15** from **16**, which differ only by the single chlorine atom. Similarly, with the test compounds **10** and **11**, which differ in just a fluorine substituent, the relative efficacies<sup>68</sup> of these ligands are predicted correctly. The application of the model for the prediction of the efficacy of other external compounds **12** and **13**, indicated them to be weaker agonists compared to **7**, consistent with experimental data.<sup>69,70</sup>

Application of the CSP approach to **17** and **18** led to the re-evaluation of the identity of the hydrophobic B group essential for  $\delta$  opioid activity. Traditionally, the Phe<sup>4</sup> side chain is considered to be the hydrophobic moiety.<sup>24</sup> However, with **17**, the side chain of the Leu<sup>5</sup> residue yielded better models of both efficacy and affinity. Similarly, with **18**, it was seen that groups other than the Phe<sup>4</sup> side chain could serve as the hydrophobic B group, with the present results indicating that the allylic substituents on the basic nitrogen in this linear peptide fulfill that role. It is hoped that the present observation will motivate the design of novel ligands to test the hypothesis that alternate hydrophobic groups are substituting for Phe<sup>4</sup>.

Use of either **1** or **7**, both high efficacy ligands, as the reference for calculation of the overlap coefficients indicated the AB–NBA parameter as the primary predictor of  $\delta$  opioid activity. However, the lower efficacy **6** as the reference compound yielded a model where the NA–NBA parameter had better correlation with  $\delta$  opioid ligand efficacies. Use of a lower activity compound as the reference required a third parameter to yield a predictive model, apparently due to the lower population of active conformations sampled by the reference compound. This is also the probable cause that the OC values based on equal weighting to all conformational points is required for efficacy prediction. These observations show that the success of the quantitative CSP approach is not dependent on the selected reference compound, but the use of high efficacy or high affinity compounds is preferable.

The obtained quantitative models (Figure 4), indicating the importance of the hydrophobic B group, are consistent with the qualitative observations from previous studies.<sup>27,28,35,66,67</sup> This is based on the contribution of terms, including the AB distance in the best efficacy and affinity models and the importance of the BN distance in the affinity model of the low efficacy ligands, suggesting a role of this pharmacophoric parameter in discriminating low and high efficacy ligands. In addition, it is seen that the overall dimensions of the molecules are important because the best predictions of activity and affinity are obtained using the MaxD based calculation of OC. The development of the quantitative efficacy model further enabled the determination of the probable bioactive conformations of the  $\delta$  opioid ligands, where both peptide and nonpeptide agonists were found to satisfy identical pharmacophoric requirements, suggesting similar binding modes for the two classes of compounds.

While the limited availability of suitable experimental data does not permit further refinement of the models at present, this study indicates the applicability of the CSP approach for pharmacophore development. The present study involves a set of structurally diverse molecules, including both peptides and nonpeptides, and does not rely on molecular alignment techniques for determination of pharmacophoric groups. Thus, the method does not require the use of a rigid reference compound, and even the use of a structurally flexible ligand as the reference (i.e., **1**) yields models with high predictability of  $\delta$  opioid ligand efficacies and affinities. The quantitative CSP approach is therefore suggested to be appropriate for general application in ligand-based drug development methods for structurally diverse ligands.

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**Supporting Information Available:** Biological data used for efficacy and affinity modeling; overlap coefficients for all pharmacophoric parameters for each compound for efficacy and affinity models; statistical analysis of initial efficacy models; efficacy models with **7** or **6** as reference compounds; and efficacy models for various B group definitions for **18**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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