Three-Dimensional Quantitative Structure−Activity Relationship Modeling of Cocaine Binding by a Novel Human Monoclonal Antibody

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Human monoclonal antibodies (mAbs) designed for immunotherapy have a high potential for avoiding the complications that may result from human immune system responses to the introduction of nonhuman mAbs into patients. This study presents a characterization of cocaine/antibody interactions that determine the binding properties of the novel human sequence mAb 2E2 using three-dimensional quantitative structure−activity relationship (3D-QSAR) methodology. We have experimentally determined the binding affinities of mAb 2E2 for cocaine and 38 cocaine analogues. The K_d of mAb 2E2 for cocaine was 4 nM, indicating a high affinity. Also, mAb 2E2 displayed good cocaine specificity, as reflected in its 10-, 1500-, and 25000-fold lower binding affinities for the three physiologically relevant cocaine metabolites benzoylecgonine, ecgonine methyl ester, and ecgonine, respectively. 3D-QSAR models of cocaine binding were developed by comparative molecular similarity index analysis (CoMSIA). A model of high statistical quality was generated showing that cocaine binds to mAb 2E2 in a sterically restricted binding site that leaves the methyl group attached to the ring nitrogen of cocaine solvent-exposed. The methyl ester group of cocaine appears to engage in attractive van der Waals interactions with mAb 2E2, whereas the phenyl group contributes to the binding primarily via hydrophobic interactions. The model further indicated that an increase in partial positive charge near the nitrogen proton and methyl ester carbonyl group enhances binding affinity and that the ester oxygen likely forms an intermolecular hydrogen bond with mAb 2E2. Overall, the cocaine binding properties of mAb 2E2 support its clinical potential for development as a treatment of cocaine overdose and addiction.

Introduction

Despite a decline in the number of cocaine users between 1985 and 1992, cocaine abuse in the U.S. has not decreased further this past decade. In 1997, the number of cocaine users was estimated at 1.5 million, which corresponds to 0.7% of individuals older than 12 years, and cocaine abuse continues to pose a serious social and economic problem.[1] For the pharmacological therapy of cocaine addiction, a number of treatment strategies have been pursued.[2] These strategies include the development of artificial enzymes capable of rapidly breaking down cocaine[3,4] and of dopamine receptor agonists that can serve as substitute medications with a lower addiction liability.[2] Other approaches involve the design of dopamine transporter antagonists that compete with cocaine for binding to the transporter without interfering with its function. It has also been suggested that the effects of increased synaptic dopamine levels could be compensated for by dopamine receptor antagonists. Although some of the proposed drugs have performed well in animal trials, many of them had serious side effects and others lacked therapeutic effects in humans or turned out to be addictive (for review, see ref 2).

An alternative approach for the treatment of cocaine addiction may be the administration of anti-cocaine monoclonal antibodies (mAbs). Animal studies have demonstrated that the blood concentration of cocaine is an important determinant of the maintenance[5] and the reinstatement[6,7] of cocaine self-administration. Anti-cocaine mAbs reduce the concentration of free cocaine in the blood and prevent it from entering the central nervous system. Thus far, studies employing both active and passive immunization have produced encouraging results in rodents, i.e., suppression of the psychoactive effects of cocaine.[8−13] Because of the potential for eliciting adverse reactions through human immune system responses to the injection of foreign species antibodies, the use of currently available murine anti-cocaine mAbs in patients is likely to be problematic. For example, serious side effects, including anaphylactic shock, were observed in some patients that received repeated doses of the murine mAb OKT3 for immunosuppression following organ transplantation.[14] Although infrequent, allergic reactions were also reported in congestive heart failure patients who were treated with ovine anti-digoxin polyclonal Fabs (antigen-binding fragments of IgG; Digibind) for digitalis toxicity.[15] By potential elimination of adverse immune reactions, the availability of a fully human anti-cocaine mAb could be of great advantage, particularly since immunotherapy of cocaine addiction requires that the intact mAb is in circulation for an extended period of time. Fully human mAbs have been produced in transgenic mice with

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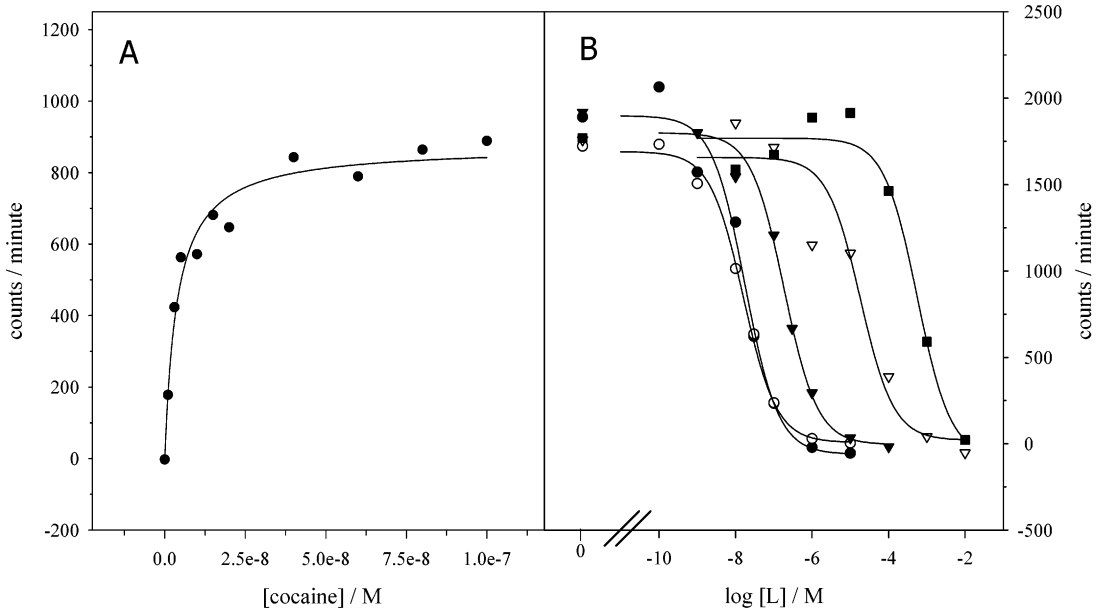


Figure 1. Radioligand binding assays with mAb 2E2 and [^{3}H]-cocaine: (A) K_d determination of cocaine binding to mAb 2E2; (B) competition assays with nonradioactive cocaine and the nonradioactive metabolites **2**, **3**, **9**, and **17** against [^{3}H]-cocaine. Symbols represent the average of two independent experimental trials (filled circles, **1**; open circles, **3**; filled triangles, **2**; open triangles, **9**; filled squares, **17**). Regression lines represent least-squares fits to the data points.

humanized humoral immune systems[16−19] without the need to genetically engineer mouse−human chimeric[20] or humanized mAbs.[21]

This study presents a comprehensive characterization of the binding properties of a novel, recently generated human sequence anti-cocaine mAb (clone 2E2) through the use of three-dimensional quantitative structure−activity relationship (3D-QSAR) methods. In the absence of 3-D crystal structures of ligand/receptor complexes, insight into the ligand/receptor interactions can be provided by the 3D contour plots developed from 3D-QSAR techniques such as comparative molecular field analysis (CoMFA[22]) and comparative molecular similarity index analysis (CoMSIA[22−24]). Using radioligand binding and enzyme-linked immunosorbent assays (ELISA), we have determined the absolute and relative binding affinities of mAb 2E2 for cocaine and 38 analogues and analyzed the experimental data by CoMSIA. CoMSIA is a relatively new method[23] that adds hydrophobic and hydrogen-bond (H-bond) fields to the established steric and electrostatic CoMFA fields. We have visualized the CoMSIA results with contour plots that identify the nature and location of ligand/antibody interactions that govern cocaine binding by mAb 2E2.

Results

Determination of K_d and K_i Values of mAb 2E2 for Cocaine and Immediate Metabolites by Radioligand Binding Assays. Radioligand binding assays using the human monoclonal antibody (mAb) 2E2 and [^{3}H]-cocaine yielded an average dissociation constant (K_d) of 4.4 nM (Figure 1A). Binding inhibition constants (K_i) of the cocaine metabolites were then determined by competition assays with a constant [^{3}H]-cocaine concentration and varying concentrations of nonradioactive competitors (Figure 1B and Table 1). The results showed that mAb 2E2 binds cocaethylene (**3**) with an affinity (K_i = 3.4 nM) nearly identical to that for cocaine (**1**).

Table 1. Antibody Dissociation Constants for Cocaine (K_d) and Competition Inhibition Constants for the Metabolites (K_i) As Obtained by Radioligand Assays[a]

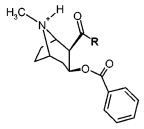
	K_i, nM	RBA by radioligand assay	RBA by ELISA
cocaine[b]	4.4 ± 0.6[b]		
cocaine	3.5 ± 0.6	1.0	1.0
cocaethylene	3.4 ± 0.5	0.97	0.74
benzoylecgonine	43 ± 8	12	9.8
ecgonine methyl ester	5200 ± 1800	1500	2500
ecgonine	95000 ± 37000	28000	28000

[a] Values represent the average ± standard deviation of three repeats. Relative binding affinities (RBAs) were calculated by dividing the K_i of a given compound by the K_i of cocaine. [b] K_d value; all other entries are K_i values.

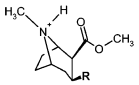
On the other hand, the affinities for the three physiologically important but inactive cocaine metabolites benzoylecgonine (**2**), ecgonine methyl ester (**9**), and ecgonine (**17**) were significantly lower (K_i values of 43 nM, 5.2 μM, and 95 μM, respectively), revealing the importance of the benzoyl moiety for high binding affinity. The K_i value for ecgonine, which lacks both functional groups, was 95 μM, suggesting that the benzoyl and methyl groups interact independently with the mAb binding site because the observed effect of their removal on binding affinity corresponds roughly to that predicted from the combination of the individual elimination effects.

Determination of the Relative IC_{50} Values for a Collection of Cocaine Analogues by Competition ELISAs. To develop a more complete understanding of how cocaine interacts with mAb 2E2, we used a competition ELISA that is faster and more convenient than the radioligand assay and enabled the large-scale screening of the binding affinities of a collection of 38 cocaine derivatives (Table 2). Figure 2 depicts a representative set of ELISA data and the corresponding inhibition binding curves. The validity of the ELISA results was established by comparing ELISA-deter-

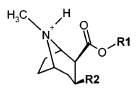
Table 2. ID Numbers, Names, Structures, and ELISA-Determined RBAs of Compounds Tested[a]



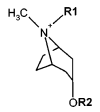
#	Name	R	RBA
1	(-) cocaine	-OCH_3	1.00
2	benzoylecgonine	-OH	*9.8 ±3.6*
3	cocaethylene	-OC_2H_5	*0.73 ±0.07*
4	cocaine propyl ester	-$O(CH_2)_2CH_3$	*0.23 ±0.05*
5	RTI-128	-NH_2	*22 ±5*
6	RTI-66	-$NH(CH_3)$	*29 ±8*
7	RTI-160	-$N(CH_3)_2$	*7.4 ±2*
8	RTI-192	-$N(CH_3)(OCH_3)$	*21 ±4*



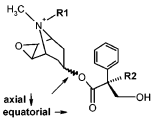
#	Name	R	RBA
9	ecgonine methyl ester	-OH	*2.5 ±0.3 x 10^3*
10	*m*-hydroxycocaine	-3-$OCOC_6H_5OH$	*8.4 ±2.2*
11	WIN 35,065-2	-C_6H_6	*29 ±6*
12	WIN 35,428	-4-C_6H_5F	*19 ±3*
13	RTI-31	-4-C_6H_5Cl	*9.3 ±1.8*
14	RTI-32	-4-$C_6H_5CH_3$	*14 ±2*
15	RTI-55	-4-C_6H_5I	*4.5 ±0.9*
16	RTI-111	-3,4-$C_6H_4Cl_2$	*8.5 ±0.5*



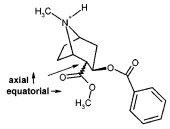
#	Name	R1	R2	RBA
17	ecgonine	-H	-OH	*2.8 ±0.9 x 10^4*
18	*m*-hydroxybenzoylecgonine	-H	-3-$OCOC_6H_5OH$	*32 ±8*
19	*p*-hydroxybenzoylecgonine	-H	-4-$OCOC_6H_5OH$	*36 ±9*
20	RTI-113	-C_6H_5	-4-C_6H_5Cl	*1.7 ±0.3*



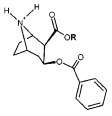
#	Name	R1	R2	RBA
21	tropine	-H	-H	*9.1 ±0.8 x 10^4*
22	benztropine	-H	-$CH(C_6H_5)_2$	*7.7 ±2.2 x 10^3*
23	4',4''-difluoro-3α-diphenylmethoxytropane	-H	-$CH(4\text{-}C_6H_4F)_2$	*8.7 ±1.8 x 10^3*
24	hyoscyamine-N-oxide	-O	-$COCHC_6H_5(CH_2OH)$	*9.0 ±1.0 x 10^4*
25	methylanisotropine	-CH_3	-$COCH(C_3H_7)_2$	*3.3 ±0.5 x 10^2*
26	tropisetronmethiodide	-CH_3	-$CO(C_8H_5N)$	*5.5 ±0.4 x 10^3*



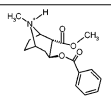
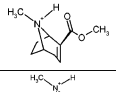
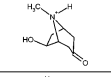
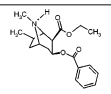
#	name	R1	R2	C3-position	RBA
27	anisodine	-H	-OH	equatorial	*5.9 ±0.8 x 10^4*
28	scopolamine	-H	-H	axial	*4.9 ±0.3 x 10^4*
29	scopolamine-N-oxide	-O	-H	axial	*1.3 ±0.3 x 10^5*
30	methylscopolamine	-CH_3	-H	axial	*5.9 ±0.2 x 10^3*
31	N-butylscopolamine	-C_4H_9	-H	axial	*3.7 ±0.3 x 10^3*



#	name	C4-position	RBA
32	(-) pseudococaine	Equatorial	*1.8 ±1.2 x 10^3*
33	(+) cocaine	Axial	*3.4 ±1.3 x 10^2*



#	Name	R	RBA
34	norcocaine	-CH_3	*4.3 ±1.1*
35	benzoylnorecgonine	-H	*1.2 ±0.3 x 10^2*

#	Name	Structure	RBA
36	(+) pseudococaine		*1.1 ±0.2 x 10^3*
37	ecgonidine		*2.1 ±0.2 x 10^4*
38	*exo*-6-hydroxytropinone		*2.2 ±0.4 x 10^5*
39	methylcocaethylene		*0.21 ± 0.08*

[a] Values represent the average ± standard deviation of at least three independent assays. RTI is Research Triangle Institute.

mined relative binding affinities (RBAs) for the cocaine metabolites to the RBAs obtained from the competition radioligand assays. The comparison showed excellent agreement between results obtained from the two methods (Table 1).

The observed RBAs covered a broad range of approximately 6 orders of magnitude (Table 2), illustrating the dramatic effects of changes in ligand structure upon binding affinity. It is noteworthy that the RBAs for the set of tested compounds were evenly distributed over this range without significant gaps or clusters. A large variation and even distribution of values within a set of data is advantageous for the establishment of robust quantitative structure−activity relationship models.

A simple inspection of the ELISA data for **2** shows that the removal of the methyl group of the methyl ester reduces the cocaine affinity for mAb 2E2 about 10-fold. The requirement for steric bulk in this position for high binding affinity is further illustrated by the RBAs of compounds **3** (RBA = 0.73), **39** (RBA = 0.21), and **4** (RBA = 0.23), all of which bind to mAb 2E2 with a higher affinity than cocaine. The importance of the stereochemistry at C2 is indicated by (+)-pseudococaine (**36**) whose drastically lowered affinity (RBA = 1100) results from shifting the methyl ester group from the axial into the equatorial position. It is also evident that the phenyl group plays a pivotal role in binding, with **9** binding 2500-fold more poorly than cocaine. The importance of the carboxyl group linking the phenyl residue to the tropane ring is low as exemplified by the affinity of **11**, which is only about 30-fold lower than that of cocaine.

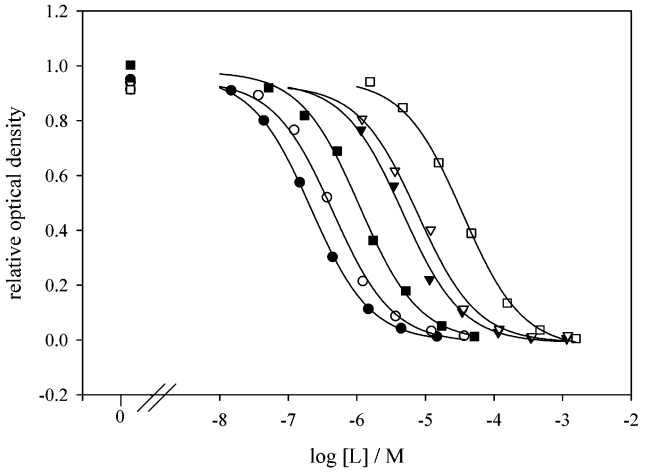


Figure 2. Representative competition ELISA for the determination of the relative binding affinities of mAb 2E2 for compounds **1** (filled circles), **20** (open circles), **12** (filled triangles), **11** (open triangles), **34** (filled squares), and **35** (open squares). Symbols represent the average of two independent experiments, and regression lines were obtained from least-squares fitting.

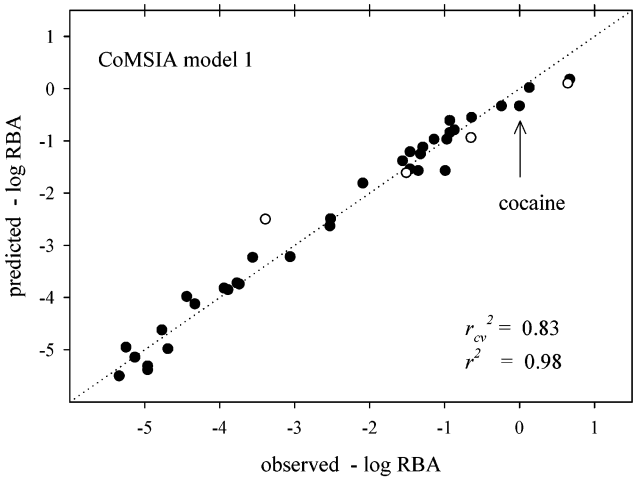


Figure 3. Correlation between CoMSIA-predicted (model 1; see Supporting Information) and observed values of −log RBA. Solid circles represent training set compounds. Open circles indicate test set compounds that were excluded from the training set.

Perhaps the most surprising finding is the fact that the amide analogues of both cocaine and benzoylecgonine, compounds **6** and **5**, respectively, bind with a 29- and 2-fold lower affinity to mAb 2E2 than their respective parent compounds. Most antibodies display their highest affinity for the immunizing hapten, but exceptions have been reported in the literature.[19,25] We initially hypothesized that the amide nitrogen of the benzoylecgonine−KLH conjugate would be an acceptable mimic of the methyl ester oxygen, predicting that the hapten-raised mAbs would cross-react with cocaine reasonably well but that the affinity of the mAbs for cocaine would be somewhat poorer than that for the amide compounds. Instead, the reverse was found, with cocaine binding better than the immunogen.

3D-QSAR Modeling of Cocaine Binding to mAb 2E2 by CoMSIA. For a more detailed and rigorous analysis of the drug/mAb interactions, we developed 3D-QSAR models for cocaine binding. A robust CoMSIA model with good predictive capabilities for cocaine binding by mAb 2E2 was successfully established by the PLS algorithm implemented in SYBYL. Five principal components (PCs) were sufficient to develop CoMSIA models, which was a sufficiently small number to avoid overfitting the dataset of 39 observations. The quality of the final CoMSIA model was assessed by inspection of the logarithmic plots of the predicted versus observed values of RBAs (Figure 3). Although the data covered an affinity range of more than 6 orders of magnitude, none of the predicted RBAs deviated from the experimentally determined ones by more than 1 logarithmic unit. Other parameters indicative of the quality of the models are the cross-validated correlation coefficient, r_{cv}, which serves as an indicator of the internal robustness and consistency of a model, and the conventional correlation coefficient, r, which is a measure of the extent to which the variation in the data is accounted for by the models. The obtained squares of both r_{cv} (0.83) and r (0.98) were well above the generally accepted threshold values of 0.5 for r_{cv}^2 and 0.9 for r^2.

The models were also able to closely predict RBAs of each compound when four test sets consisting of four molecules each were selected for exclusion from the PLS procedure in order to determine the models' external predictive abilities (see Figure 3 and Supporting Information, Table 3). It is noteworthy that the obtained values for r_{cv}^2 and for r^2 were not dependent on the selected molecules assigned to the external test sets, which emphasizes robustness and stability of the model. The final CoMSIA model was derived by including all of the experimentally tested compounds in the training set. The CoMSIA-calculated contributions of the molecular fields were 21% steric, 16% electrostatic, 24% hydrophobic, 28% H-bond donor, and 9% H-bond acceptor.

CoMSIA Contour Maps for Cocaine Binding. The composite structure diagram presented in Figure 4 shows the structures of all tested drugs superimposed and illustrates how the structurally diverse set of compounds allowed for a comprehensive probing of the effects of structural modifications in all regions about the central tropane core. Structural effects on binding affinity are then visualized by color contour maps (Figures 5 and 6) that highlight the areas about the bound ligand where changes in ligand molecular fields are associated with variations in the binding affinity. For optimum visual clarity, the cutoff threshold in the contour plots was set to a ratio of 90:10.

The CoMSIA contour map in the upper portion of Figure 5 shows how changes in the steric bulk of the ligand in specified areas affect its binding affinity. The small yellow region near the equatorial position of C2 of cocaine and the extensive yellow area lining almost the entire side of the cocaine molecule about the equatorial positions at C4, C5, C6, and the phenyl ester carbonyl group indicate regions where an increase in ligand bulk reduces binding affinity. These areas were probed extensively by the cocaine isomers (**32**, **33**, **36**), tropine-related compounds (**21**−**26**), and scopolamine-related compounds (**28**−**31**) that all displayed poor affinities. The green area near the methyl ester methyl group reflects the observed increase in ligand binding

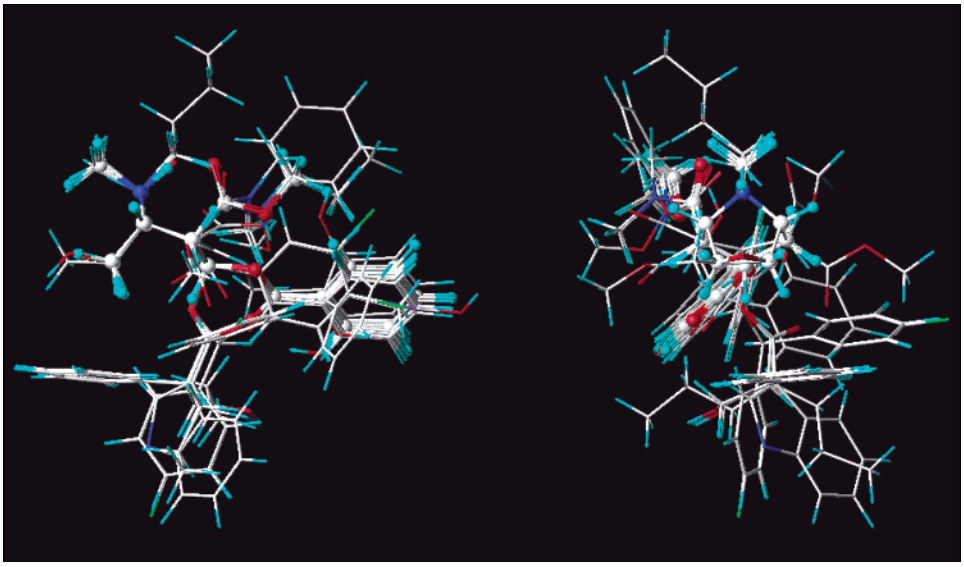


Figure 4. Orthographic views of the alignment of all compounds on the cocaine tropane ring system. As a reference, cocaine is shown as a ball-and-stick model.

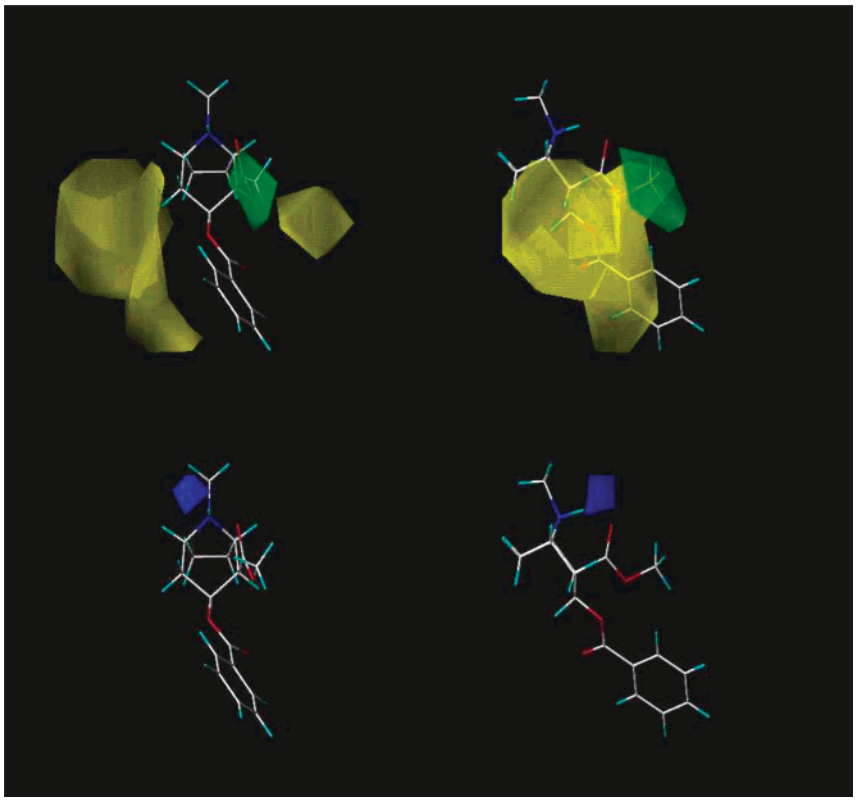


Figure 5. Orthographic views of the steric and electrostatic CoMSIA contour maps for ligand binding to mAb 2E2. The structure of cocaine is displayed as a reference. The upper portion shows the steric component, and the lower portion shows the electrostatic component. Addition of steric bulk in green regions and increase in positive charge in blue areas enhance binding affinity. Addition of steric bulk in yellow regions and increase in positive charge in (potential) red areas reduce binding affinity. Contour cutoff levels are set at 90:10.

affinity upon the addition of larger aliphatic residues in this position such as the ethyl and propyl groups of compounds **3** and **4**. This is also consistent with the higher affinity of **20** in comparison to **13**, which is the methyl ester analogue of the bulkier phenyl ester **20**.

The electrostatic CoMSIA contour plot (Figure 5, lower portion) delineates a single blue region in which increases in partial positive ligand charges increase the binding affinity. The map properly describes the higher binding affinity of scopolamine (**28**) compared to scopolamine *N*-oxide (**29**), which places an unfavorable partial negative charge (oxygen atom) inside the blue area. Interestingly, there is no indication of partial negative charges such as those of the ester groups contributing to cocaine binding interactions (absence of red areas).

The hydrophilic contour map (Figure 6) emphasizes the importance of the hydrophobic phenyl group for tight binding and also reflects the relatively high binding affinities of some compounds whose large hydrophobic methyl ester substituents protrude into the yellow

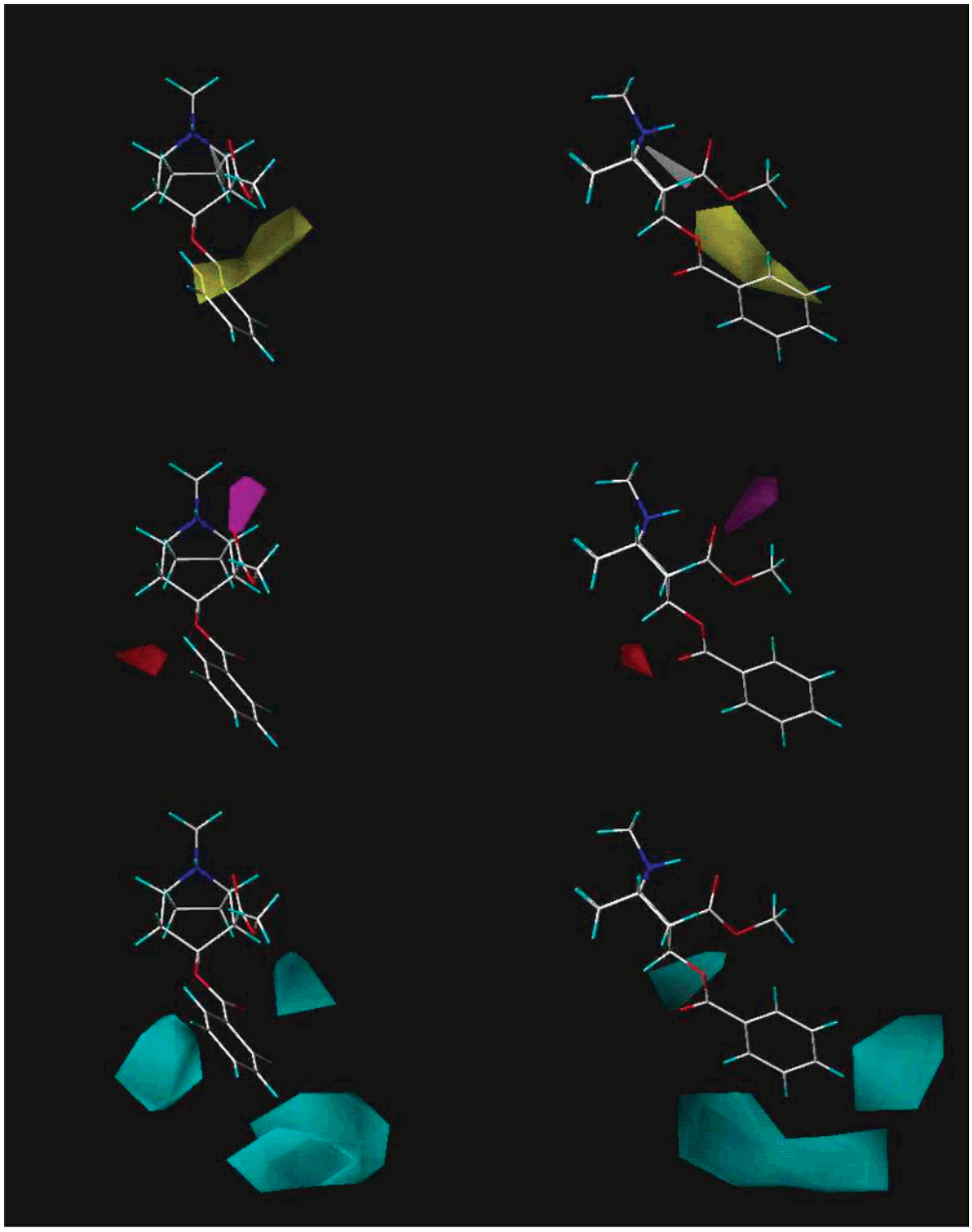


Figure 6. Orthographic views of the hydrophobic, H-bond donor, and H-bond acceptor CoMSIA contour maps for ligand binding to mAb 2E2. The structure of cocaine is displayed as a reference. The upper portion shows the hydrophobic component. Binding affinity increases upon an increase in hydrophobicity in the yellow areas and upon a decrease of hydrophobicity in the gray areas. The middle portion shows the H-bond donor component. The presence of H-bond donor groups on mAb 2E2 in the magenta area increases binding affinity, whereas their presence in the red area decreases binding affinity. The lower portion shows the H-bond acceptor component. The presence of H-bond acceptor groups on mAb 2E2 in the cyan areas increases binding affinity. Contour cutoff levels are set at 90:10 except for the lower portion where the ratio was 92:8.

region (for example, **20** versus **13** or **7** versus **5**). In addition, the small gray hydrophilic contour positioned between the tropane nitrogen and the methyl ester carbonyl group suggests the presence of polar antibody residues near this region.

Interestingly, the calculated contribution of H-bond acceptor field to the model (28%) is larger than those of the other fields. The contour map shown in Figure 6 (middle portion) defines a single, magenta area where H-bond donor groups from mAb 2E2 interact with the cocaine methyl ester oxygens and favor ligand binding. In contrast to the H-bond donor field, the H-bond acceptor field contributes only moderately (9%) to ligand binding. Its visualization resulted in a rather diffuse contour plot that could only be focused by increasing the contour cutoff level (92:8) above the standard value of 90:10 used in all other representations (Figure 6, lower portion). The resultant contour map only contains cyan areas near the benzoyl moiety, indicating where H-bond acceptor groups of mAb 2E2 enhance ligand binding.

Another noteworthy feature of the plots is the absence of any contours in the region near C6, C7, and C9 of cocaine. Because a number of the ligands tested had structural modifications in this area (compounds **27**–**31**, **33**, **38**, and **39**), this observation is not the consequence of a lack of screening in this area. Instead, it

suggests an antibody binding pocket that leaves cocaine solvent-accessible in this position.

Discussion

Evaluation of Cocaine Affinity and Specificity of mAb 2E2. To be an efficient immunotherapeutic agent, a cocaine-binding mAb must have an affinity for cocaine sufficiently high to reduce free drug concentrations in the serum to nontoxic levels and restrict its access to the central nervous system. It is desirable to reduce the cocaine concentration to levels below 150 nM, which has been shown to be the threshold above which effects such as increased pulse and blood pressure are observed.[26] A human antibody with a K_d of 4 nM, such as mAb 2E2, would be saturated at those cocaine levels with a high binding efficiency and would minimize the mAb required.

Another important factor determining the therapeutic suitability of an anti-cocaine mAb is its cocaine specificity. The half-lives of the initial two major cocaine metabolites, ecgonine methyl ester and benzoylecgonine, are longer than that of cocaine (several hours versus less than 30 min[27]), and as a result, the blood levels of the metabolites can easily exceed that of cocaine in the case of repeated drug intake. Because ecgonine methyl ester and benzoylecgonine are physiologically inactive, their binding to the mAb should be kept at a minimum. The affinity of mAb 2E2 for ecgonine methyl ester is clearly low enough (ELISA-determined RBA of 2500) to avoid such complications. The affinity of mAb 2E2 for benzoylecgonine, however, is about 10 times lower than that for cocaine, which may lead to a reduced mAb efficiency in extreme cases of chronic cocaine abuse. In contrast, a high mAb affinity for the metabolite cocaethylene would be useful because this psychoactive metabolite is produced when cocaine users ingest alcohol,[27] which occurs frequently. Since its binding affinity for cocaethylene is even higher than for cocaine, mAb 2E2 fully meets this treatment requirement.

A 3D-QSAR-Based Description of the mAb 2E2 Cocaine Binding Site. CoMSIA is a relatively new 3D-QSAR method that has been developed from the widely used CoMFA approach.[23] Compared to CoMFA, CoMSIA adds hydrophobic and H-bond fields to the established steric and electrostatic CoMFA fields and thereby facilitates a more detailed breakdown of the types of ligand/receptor interactions. Moreover, its use of Gaussian functions to account for the distance dependence of the molecular similarity index has been reported to provide smoother contour maps that are less fragmented than typical CoMFA maps and therefore more readily interpreted. The ability of 3D-QSAR methodology to correlate molecular structure expressed in terms of molecular fields with variations in biological activity has made them an extremely useful tool for the study of ligand/receptor interactions.[22] As a comparative technique, CoMSIA does not provide absolute values for binding constants or energies but rather accounts for the effects of changes in ligand structure upon relative biological activities. Although their primary applications are in the field of prediction-based drug design and discovery, 3D-QSAR can provide valuable information on the nature of the receptor binding site.[28] Moreover, 3D-QSAR models allow initial predictions of the potential for cross-reactivity with thus far untested therapeutic drugs and drugs of abuse that a patient could encounter during immunotherapy.

The steric contour map of our CoMSIA model (Figures 5, upper portion) suggests a highly restricted binding site of mAb 2E2 whose borders are most likely defined by bulky amino acid residues. Possible sources of the unfavorable interactions between the mAb and some cocaine derivatives are bulky amino acids such as the side chains of the six tyrosine residues that have been located in the heavy chain complementarity determining regions (CDRs) of mAb 2E2 (unpublished results). The origin of the favorable steric interactions that account for the well-defined green area in the contour maps is presumably the amino acid residues that line the border of these areas and engage in van der Waals dispersion interactions with the ligand. The presence of attractive interactions in this particular location is expected because the immunogenic cocaine analogue was tethered to the carrier protein at this site, and consequently, mAb 2E2 was raised against a compound with significant steric bulk in this region.

The preference of the binding site for ligands with high partial positive charges may be a reflection of charge complementarity between a binding site with an overall negative surface charge and cocaine, which carries a net positive charge at its tropane nitrogen at neutral pH ($pK_a = 8.6$ [29]). Amino acid side chains with an overall negative charge such as aspartate and glutamate or with polar residues such as the hydroxyl groups of serine, threonine, and tyrosine may be the origin of this negative surface charge. All these residues are represented in the CDRs of mAb 2E2. A negatively charged binding site surface may partially account for the cocaine specificity of mAb 2E2 because the metabolites benzoylecgonine and ecgonine methyl ester have a net charge of zero while cocaine carries a net charge of +1.

As suggested by the hydrophobic CoMSIA contour map (Figure 6, upper portion), the cocaine phenyl group likely stacks against hydrophobic amino acid residues. This would result in an energetically favorable overall reduction of solvent-exposed hydrophobic areas on ligand and antibody upon binding.

The H-bond contour maps (Figure 6, middle and lower portions) provide convincing evidence for a favorable interaction between the cocaine methyl ester oxygens and a H-bond donor on mAb 2E2, such as the side chains of tyrosine, serine, threonine, lysine, and arginine or a backbone amide group. In fact, the presence of such an H-bond donor group could account for the higher affinity of mAb 2E2 for esters compared with that for their amide analogues because a comparison of binding energies reveals that H-bonds to an oxygen acceptor atom tend to be energetically more stable than H-bonds that have a nitrogen as the acceptor but are otherwise identical.[30] The presence of H-bonding polar residues in this location of the binding pocket is also implied by the hydrophobic CoMSIA map whose gray area (Figure 6, upper portion) is near the magenta area of the H-bond donor map. The chemical basis of the less important H-bond acceptor contours are possibly H-bond-donating hydroxyl groups attached to the benzene ring of certain

cocaine and benzoylecgonine analogues (compounds **10**, **18**, and **19**) interacting with appropriate residues of mAb 2E2.

A Qualitative Comparison of Cocaine Binding Models by mAbs 2E2, 3P1A6, MM0240PA, and GNC92H2. Our previously developed CoMFA models for cocaine binding by the murine mAbs 3P1A6 and MM0240PA[31] with a similar, yet somewhat smaller set of tested compounds permit a comparison with the 3D-QSAR model derived for mAb 2E2. Overall, the contour maps obtained for mAb 2E2 and mAb MM0240PA resemble each other to a larger extent than that for mAb 3P1A6. For example, both CoMFA contour plots for mAb 2E2 and MM0240PA reveal attractive steric interactions that are restricted to a single small region close to the methyl ester methyl group. This is likely the result of the antibodies being raised to an amide benzoylecgonine derivative that has a linker in this position. Surprisingly, the third mAb, 3P1A6, shows no sign of attractive steric interactions in this region. All three mAbs possess sterically restricted areas about the lower half of the cocaine molecule (axial position of C3 and equatorial position of C4), but the exact shapes of these areas clearly differ. mAb 3P1A6 has the least sterically confined binding site, whereas steric restrictions are more distinct for mAb MM0240PA, and mAb 2E2 appears to bury the ligands deep inside a binding pocket likely lined with bulky amino acid residues. Compared to the murine mAbs, the sterically restricted (yellow) areas in the contour map of mAb 2E2 are significantly larger and display an additional restricted area about the region that is occupied by substituents in the equatorial positions at C2 of the tropane ring. The latter result is not observed in either of the murine mAbs.

The electrostatic contour plots of all three mAbs are dominated by several regions indicative of enhanced binding to the antibody site upon an increase in partial positive charge on the ligand. In all three cases, corresponding contours are observed between the carbonyl oxygen of the methyl ester group and the hydrogen atom of the tropane nitrogen. As stated above, such charge complementarity of the binding sites with a positively charged ligand will certainly enhance cocaine specificity. Finally, the suggested solvent accessibility of the binding site of mAb 2E2 about C9 differs somewhat from that of mAbs 3P1A6 and MM0240PA, which are solvent-exposed at C6 and C7, respectively.[31] With respect to their therapeutic potentials, the specificity and affinity of human mAb 2E2 for cocaine over benzoylecgonine are clearly superior (RBA = 9.8) to those of murine mAb MM0240PA (RBA = 1.0) but not as good as those of mAb 3P1A6 (RBA = 41), indicating a potential for future improvements of the binding properties of mAb 2E2.

The recently obtained crystal structure of the moderate affinity human/mouse chimeric anti-cocaine mAb GNC92H2 (K_d = 400 nM) complexed to cocaine[20] allows for a further comparison of cocaine binding patterns used by different mAbs. In contrast to the three mAbs discussed above, mAb GNC92H2 has a binding pocket that is solvent-exposed at the cocaine benzoyl residue. Further, the crystal structure reveals no intermolecular H-bonds between ligand and antibody but instead shows an intramolecular hydrogen bond that is formed by the methyl ester carbonyl and protonated ring nitrogen. The shape and charge complementarity that convey high cocaine specificity, however, seems to be a general feature that is shared by all four mAbs.

Summary and Future Directions

In this study, we have systematically explored the binding site of the novel human anti-cocaine mAb 2E2 using a set of 39 cocaine analogues as probes and 3D-QSAR techniques. The antibody has a high cocaine affinity and specificity and is therefore a good prototype for a therapeutic agent against cocaine addiction and relapse. Future research efforts will be directed toward homology modeling of the three-dimensional structure of the cocaine binding site, followed by computational ligand docking studies providing models for the prediction of modifications of specific amino acid residues of mAb 2E2 that result in a further improvement of cocaine affinity and specificity. The 3D-QSAR results will assist the evaluation of different cocaine binding modes during docking by providing geometrical constraints for possible ligand orientations, such as the location of solvent-exposed parts of the ligand, the location of hydrophobic amino acid residues interacting with cocaine phenyl group, and the presence of H-bond donor groups near the methyl ester oxygen.

Materials and Methods

Cocaine Analogues. Cocaine (both optical isomers), cocaethylene, pseudococaine (both optical isomers), benzoylecgonine, ecgonine methyl ester, ecgonine, norcocaine, and the compounds **5**−**8**, **11**−**16**, and **20**, synthesized by Dr. Ivy Carroll of the Research Triangle Institute,[32] were graciously provided through the drug supply program of the National Institute on Drug Abuse (NIDA). Tritiated cocaine (levo-[benzoyl-3,4-^{3}H(N)]-cocaine, specific activity of 50 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA). With the exception of **39**, which was purchased from SPECS and BioSPECS (Rijswijk, The Netherlands), all other compounds were obtained from Sigma-Aldrich (St. Louis, MO) and were of at least 98% purity.

Generation of Human Sequence Anticocaine Monoclonal Antibody 2E2. A human sequence anti-cocaine γ-immunoglobulin (IgG) response was generated in transgenic mice [(C57Bl/6J × CBA/J)F_2] that have been described previously.[16−18] The hybridoma cell line (2E2/3.5) that secreted the human anti-cocaine mAb 2E2 was obtained from one of several mice (strain HCo7/Ko5) that had been immunized with the hapten benzoylecgonine coupled via an amide linkage[10,33] to 1,4-butanediamine-derivatized keyhole limpet hemocyanin (KLH). Mouse splenic lymphocytes were fused with P3X63-Ag8.653 nonsecreting mouse myeloma cells (American Type Culture Collection, Manassas, VA, CRL 1480), and hybridomas were selected essentially as previously described for human anti-digoxin mAbs[19] except that 10 μg/mL of a benzoylecgonine−ovalbumin conjugate in 10 mM PBS was used to coat microtiter plates as the screening antigen.

Each cloned hybridoma cell line was tested for its production of a human anti-benzoylecgonine γ1 heavy and κ light chain immunoglobulin and the absence of any mouse chain IgGs. In the case of mAb 2E2, the human γ1 heavy chain was easily identified by both species-specific and isotype-specific immunoreagents using ELISA and Western blot analysis. Amino terminal amino acid sequencing indicated that both heavy and light chains were human sequence chains. This was also

confirmed by DNA sequencing of the hybridoma genomic DNA encoding the variable regions of the immunoglobulin chains and identifying them respectively as human VH3 family gene DP-50 and VK3 family gene L6 derived proteins (a detailed presentation will be given in a separate publication). The human mAb 2E2 used for these studies was obtained from hybridoma-generated ascites fluid from SCID (severe combined immunodeficiency disease) mice and partially purified by sodium sulfate precipitation and protein A-Sepharose column chromatography.[19]

Radioligand Binding Assays. The dissociation constant for the mAb 2E2/cocaine complex was determined by saturation radioligand binding assays according to a previously described double antibody precipitation method[34] in combination with liquid scintillation counting. The only modification was the substitution of the goat anti-mouse antibody by a goat anti-human Fc-region antibody in the first step of the protocol. Data points were the average of two independent measurements, and each experiment was repeated three times. Dissociation constants (K_d) were determined by fitting the measured counts per minute as a function of total cocaine concentration to a binding isotherm.

The cocaine specificity of mAb 2E2 was determined by a radioligand competition binding assay for the four cocaine metabolites **2**, **3**, **9**, and **17**. The experimental conditions were identical to those described above except that the specific activity of all samples (1 nM [^{3}H]-cocaine) was diluted with 14 nM nonradioactive cocaine, with varying concentrations of the nonradioactive metabolite competitors, and 0.4 nM mAb 2E2. The points of a graph showing radioactive counts versus varying competitor concentration from experiments performed in duplicate were fit to a three-parameter logistic equation. IC_{50} was the concentration of competitor required to displace half of the cocaine initially bound to the mAb 2E2 binding site. The IC_{50} values were then converted to inhibition constants (K_i) according to Cheng and Prusoff.[35]

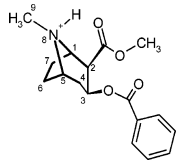
Competition Enzyme-Linked Immunosorbent Assays (ELISAs). For the rapid screening of relative binding affinities, competition ELISAs were employed according to a procedure described previously.[31] The only modification was the use of biotinylated goat anti-human IgG (γ-chain-specific, 500-fold dilution of 1 mg/mL stock in PBS) instead of biotinylated goat anti-mouse IgG. IC_{50} values were determined by fitting the data to a three-parameter logistic equation. The relative binding affinity (RBAs) of the competitors were defined as their IC_{50} divided by that of cocaine. Without exception, compounds presented in this study were able to inhibit cocaine binding to levels of 50% or greater within the concentration range used in the assays. Results are reported as the average of three or more independent experiments done in duplicate.

Molecular Modeling and Structural Alignment. Molecular modeling of the compounds under study and 3D-QSAR analysis were performed with the SYBYL software package from Tripos (versions 6.7 and 6.8) essentially as described previously.[31] Because the binding studies were performed at essentially neutral pH and the pK_a of the cocaine ring nitrogen is 8.6,[29] all ring nitrogens were modeled in their protonated form. In agreement with the crystal structure of cocaine hydrochloride[36] and cocaine complexed to an antibody,[20] the proton was added on the side of C2 and C3 of cocaine. Although there is some degree of conformational flexibility in the two ester groups, we have assumed the same conformation for each compound. The excellent fit of the 3D-QSAR model to the data (see Results) indicated that this assumption was reasonable.

The ligands were aligned by superimposing their structures onto a template by means of a least-squares procedure. Because it is common to all compounds tested, the part of the tropane ring containing atoms 1, 2, 4, 5, 6, 7, and 8 (as labeled in Chart 1) served as the structural template. This alignment has served as the basis for a number of previous successful CoMFA studies.[31,37,38]

CoMSIA Modeling. For CoMSIA modeling, the five molecular fields (steric, electrostatic, lipophilic, H-bond donor, and H-bond acceptor) were calculated by computing molecular similarity indices between a probe atom and the ligands according to[39]

Chart 1. Cocaine and Its Structural Alignment



$$A^q_{F,k}(j) = -\sum_i w_{\text{probe},k} w_{ik}\, e^{-\alpha r_{iq}^2}$$

At grid point q (2 Å spacing interval), A represents the molecular similarity index calculated for all atoms (summation index i) of the ligand j and a probe atom using the molecular field F. r_{iq} is the distance between the probe atom at grid point q and atom i of the ligand. w_k is a parameter describing the appropriate physicochemical property of the ligands and the probe atom (radius, 1 Å; charge, +1; hydrophobicity index, +1; H-bond donating parameter, +1; H-bond accepting parameter, +1) at each grid point. For the steric field, w_k was the third power of the atomic radius, for the electrostatic field, w_k represented the Gasteiger−Hückel charges, for the hydrophobic field, w_k was equal to the atom-based hydrophobicity parameter according to Viswanadhan,[40] and for H-donor and H-acceptor fields, w_k reflected a value assigned to suitably placed pseudoatoms.[39] The attenuation factor α in the exponent was set to its default value of 0.3.[23,39]

For further analysis, correlations between the logarithms of the RBA values and the molecular fields of the compounds were established by the partial-least squares (PLS) method implemented in SYBYL.[22] PLS reduced the large number of molecular field descriptors to a few principal components (PCs) that are linear combinations of the original descriptors. The optimum number of PCs was defined as the value that resulted in the lowest standard error of prediction, s_{cv}, and was determined in cross-validated PLS runs based on the "leave-one-out" procedure. This number of PCs also corresponded to a cross-validated correlation coefficient (r_{cv}^2) that did not increase significantly upon the addition of further PCs. Non-cross-validated PLS analyses were carried out with the optimum number of PCs. All PLS runs were executed with SYBYL's CoMFA standard scaling feature activated and with a column filter set to 2 kcal/mol, causing columns with an energy variation below that threshold value to be excluded from analysis for the purpose of noise reduction.

A further test for the CoMSIA model was the exclusion from PLS analysis of four different sets of four randomly selected test compounds representative of the structurally diverse types of molecules in the training set. For example, compounds **4**, **9**, **15**, and **18** were assigned to the test compound set for CoMSIA model 1 (see Supporting Information, Table 4). The ability of the models to properly predict the relative binding affinities of molecules "unknown" to them served as a measure of the external predictive ability of the models.

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Supporting Information Available: Table of ELISA-determined and CoMSIA-predicted RBA values and residuals for mAb 2E2. This material is available free of charge via the Internet at http://pubs.acs.org.

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