The Discovery of Novel, Structurally Diverse Protein Kinase C Agonists through Computer 3D-Database Pharmacophore Search. Molecular Modeling Studies

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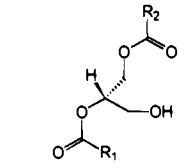
Received June 14, 1994®

A computer protein kinase C (PK-C) pharmacophore search on 206 876 nonproprietary structures in the NCI 3D-database led to the discovery of five compounds which were found to possess PK-C binding affinities in the low micromolar range and six others having detectable, but marginal, binding affinities. Molecular modeling studies showed that in addition to the presence of the defined pharmacophore, hydrophobicity and conformational energy are the two other important factors determining the PK-C binding affinity of a compound. The modeling results were confirmed by synthetic modification of two inactive compounds, producing two active derivatives. These newly discovered, structurally diverse lead compounds are being used as the basis for further synthetic modifications aimed at more potent PK-C ligands that will compete with the phorbol esters.

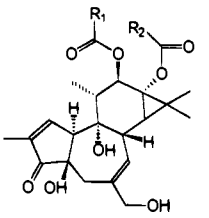
Introduction

Protein kinase C (PK-C) was first discovered by Nishizuka in 1977[1,2] and comprises a family of closely related phospholipid-dependent enzymes which vary in their activation properties and tissue distributions.[3] PK-C-mediated signal transduction, which is activated by growth factors, neurotransmitters, and hormones, has been extensively studied,[3] but our knowledge of the cell biology and cell functions of this ubiquitous family of enzymes is still limited. The involvement of PK-C isozymes in cellular signal transduction, which results in either differentiation or uncontrolled cellular proliferation, makes them attractive targets for chemotherapeutic intervention against cancer. The discovery of selective activators and inhibitors of PK-C will permit us to define more clearly the respective functional roles of each of these isozymes in intact cells.

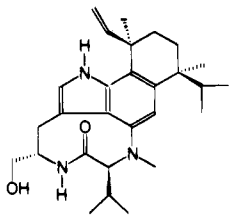
The binding of diacylglycerols (DAGs; **1**) to PK-C results in the activation of kinase activity. Phorbol esters (**2**) and other chemically diverse tumor promotors, such as teleocidin B-1 (**3**), ingenols (**4**), and debromoaplysiatoxin (**5**), also activate PK-C by acting as stable and ultrapotent DAG equivalents.[4,5] Despite the absence of 3D structural data for PK-C isozymes, efforts have been made in the past by several groups to study structure–activity relationships and to identify a common pharmacophore shared by structurally diverse PK-C activators by molecular modeling approaches.[5–11] The pharmacophore model used in our present study was first proposed by Rando, Kishi, et al.[5,6]



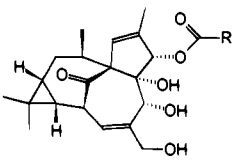
(S)-diacyl-glycerols (DAGs, 1)



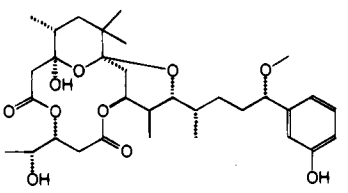
phorbol esters (2)



teleocidin B-1 (3)



3-O-acyl-ingenols (4)



debromoaplysiatoxin (5)

Three-dimensional pharmacophore searching of large databases has recently gained attention for its ability to provide new leads in drug discovery programs.[12,13] We have recently built a searchable three-dimensional database[14] of a total of ca. 407 000 structures from the 2D structures of the NCI Drug Information System (DIS) database[15] using the program Chem-X.[16] In this paper, we report the use of this 3D-database for the discovery of novel agents that compete with the phorbol esters binding to PK-C.

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® Abstract published in *Advance ACS Abstracts*, November 15, 1994.

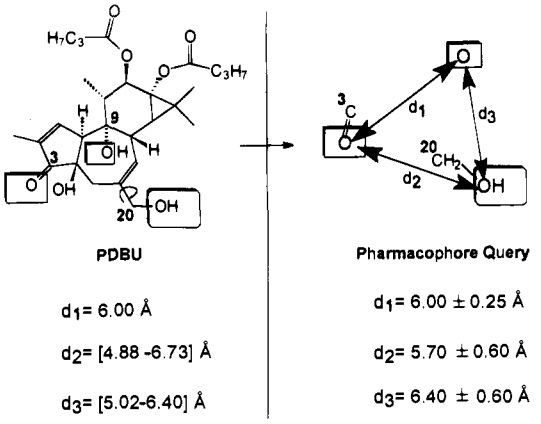


Figure 1. Constructed PK-C pharmacophore query used in the 3D-database pharmacophore search.

Methods and Materials

(A) NCI 3D-Database and Chem-X Program. The details of the NCI 3D-database and the Chem-X Chem-Model program used in both the 3D-database build and search processes have been described elsewhere.[14] Chem-X was used to build the three-dimensional structures of the compounds from the 2D information[16] (the connection tables), obtained from NCI's DIS.[15] The current version of the NCI 3D-database consists of 206 876 "open" structures and 201 036 "discreet" structures, for a total of 407 912 structures.[14] (Compounds which are provided to NCI upon a confidential basis are termed "discreet", and all data associated with "discreet" compounds are restricted. Compounds which are provided without conditions or restrictions are called "open", and data associated with "open" compounds are publicly accessible.)

(B) PK-C Pharmacophore. The 3D structure of the PK-C receptor has not yet been determined, but there is much evidence available concerning the structure of the basic pharmacophore,[5-11] defined as the three-dimensional arrangement of atoms essential for biological activity. The pharmacophore in phorbol 12,13-dibutyrate (PDBU; $R_1 = R_2 = C_3H_7$ in **2**) involves the C_3 carbonyl and the C_9 hydroxyl oxygens as hydrogen bond acceptors and the C_{20} hydroxyl group as a hydrogen bond donor[5,6] (Figure 1). It should be noted that in addition to the three hydrogen-bonding interaction sites, a hydrophobic component is essential for binding since in contrast to PDBU, which has a potent affinity, phorbol itself fails to bind to PK-C. We were unable to incorporate the hydrophobic parameter into the pharmacophore query used in the search with the current version of the Chem-X, but hydrophobicity was used successfully as a postsearch consideration in this study (vide infra).

The crystal structure of phorbol was obtained from the Cambridge Structural Database[17] and used as the basis for the construction of the pharmacophore query. Since the hydrogen atoms were not available in the X-ray structure, they were added using QUANTA.[18] The locations of the hydrogens were determined by a semiempirical quantum chemistry method, PM3,[19] in the MOPAC 6.0 package[20] running on a host mainframe Convex C240 supercomputer under Convex OS10.2 (based on BSD Unix 4.2). In the crystal structure of phorbol, the distance between the oxygen at C_3 and the oxygen at C_9 is 6.00 Å, while the distance between the C_3 oxygen and the C_{20} oxygen is 5.54 Å, and the distance between the C_9 oxygen and the C_{20} oxygen is 5.30 Å. Because the ring system of phorbol is rigid, the distance between the oxygen at C_3 and the oxygen at C_9 should remain unchanged when PDBU binds to PK-C, but the C_6-C_{20} bond is rotatable with a small energy barrier.[21] A conformational search rotating the C_6-C_{20} bond using QUANTA[18] established the range of the distance between the oxygen at C_3 and the oxygen at C_{20} to be 4.88–6.73 Å and the distance range between the oxygen at C_9 and the oxygen at C_{20} to be 5.02–6.40 Å when the C_6-C_{20} bond is rotated through 360°. These data suggest that relatively larger tolerances must be used for the two distances involving the oxygen at C_{20}, while a smaller tolerance may be used for the distance between the C_3 and C_9 oxygens in the pharmacophore query for the 3D search (see Figure 1). Previous studies[5-11] have established that a free hydroxyl group at C_{20} is required for high binding affinity in the phorbol esters. Therefore, the CH_2OH group was retained as a substructural requirement in the PK-C pharmacophore query. Many potent PK-C agonists, such as DAGs (**1**), phorbol esters (**2**), teleocidin B (**3**), ingenol (**4**), and debromoaplysiatoxin (**5**), have at least one carbonyl group in their pharmacophore, suggesting that the carbonyl group may be a necessary requirement for their high potency, and so the carbonyl group at the C_3 of phorbol esters (C_3=O) was also retained as a substructural requirement in the pharmacophore query. The oxygen corresponding to that at the C_9 in the phorbol esters can be in a hydroxyl group, as in phorbol esters (**2**), or in a carbonyl group, as in DAGs (**1**) and debromoaplysiatoxin (**5**). It seems therefore that the substructural requirement for this binding point is less restricted, except it must be able to function as a hydrogen bond acceptor. Hence, an oxygen of unspecified type was used as the substructural requirement in the 3D pharmacophore query for this binding point. The 3D pharmacophore which incorporates all these aspects including the tolerances for each of these three distances is shown in Figure 1.

(C) The PK-C Bioassay. PK-C binding affinity was determined by measuring the competitive displacement of [20-^{3}H]phorbol 12,13-dibutyrate from the isozyme PK-Cα by the various compounds assayed. The details of the method used have been described previously.[22]

(D) Molecular Modeling. All molecular modeling studies were carried out using QUANTA molecular modeling package (version 3.3)[18] with CHARMm 2.2 force field parameters running on a Silicon Graphics IRIS Indigo workstation unless otherwise indicated.

Results

(A) 3D Search Results. A search with the 3D pharmacophore query shown in Figure 1 was performed on all the 206 876 open compounds in the NCI 3D-database, which took approximately 1 week of CPU time. A total of 535 compounds were retrieved by the search, but samples for testing were available only for 286 compounds. These 286 structures were then visually examined for the presence of necessary hydrophobic substituents, such as a phenyl ring or a butyl group. Compounds lacking a necessary hydrophobic moiety were then excluded. The application of this restriction led to the exclusion of 161 compounds out of the 286 compounds. The remaining 125 compounds were submitted for evaluation in the PK-C binding assay.

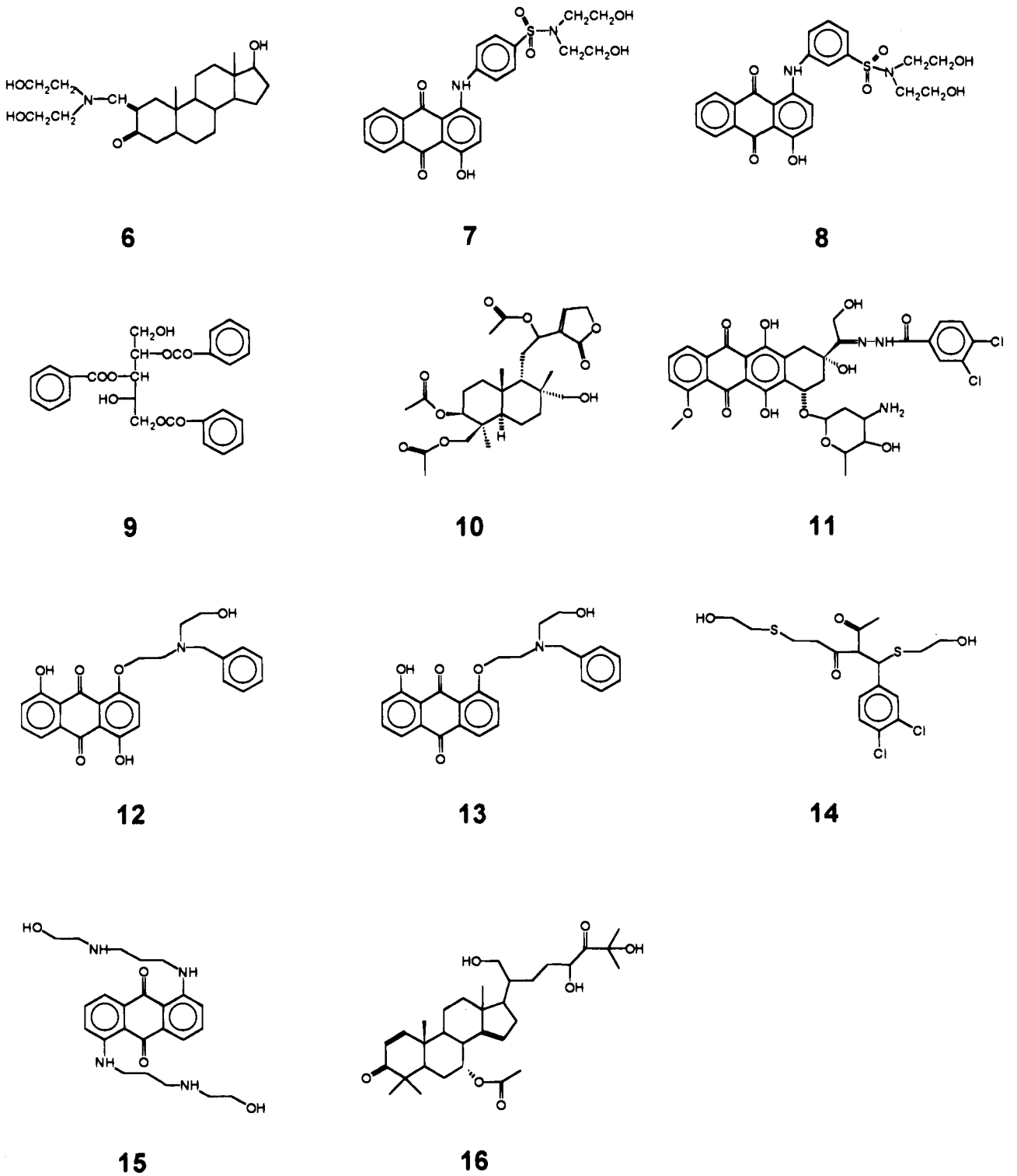


Figure 2. Chemical structures of five active and six marginally active compounds.

(B) PK-C Binding Affinity Tests. The 125 compounds were initially tested for their competitive binding affinities to PK-Cα bound to labeled [20-^{3}H]PDBU at a concentration of 30 μg/mL. If no significant amount (<10%) of bound [20-^{3}H]PDBU was displaced by the ligand, the compound was classified as inactive. Eleven of the 125 compounds (Figure 2) were classified as active (Table 1). For the five most potent compounds (**6**–**10**; Figure 2), their binding affinities, K_i values, were measured and are listed in Table 1. The dose–response curves for the two best compounds (**7** and **9**) are shown in Figure 3.

(C) Molecular Modeling Study. 1. Hydrophobicity. The vast majority of the compounds, 114 out of the 125, failed to show significant binding affinities for PK-C despite the fact that they contain the necessary pharmacophore. The exclusive presence of the PK-C pharmacophore does not therefore appear to be the sole factor controlling PK-C binding.

All known PK-C agonists contain a hydrophobic moiety.[6] We have used this information in a qualitative way for the selection of the final 125 compounds out of the 286 compounds for the PK-C test. However, further insight on the relationship between hydrophobicity and

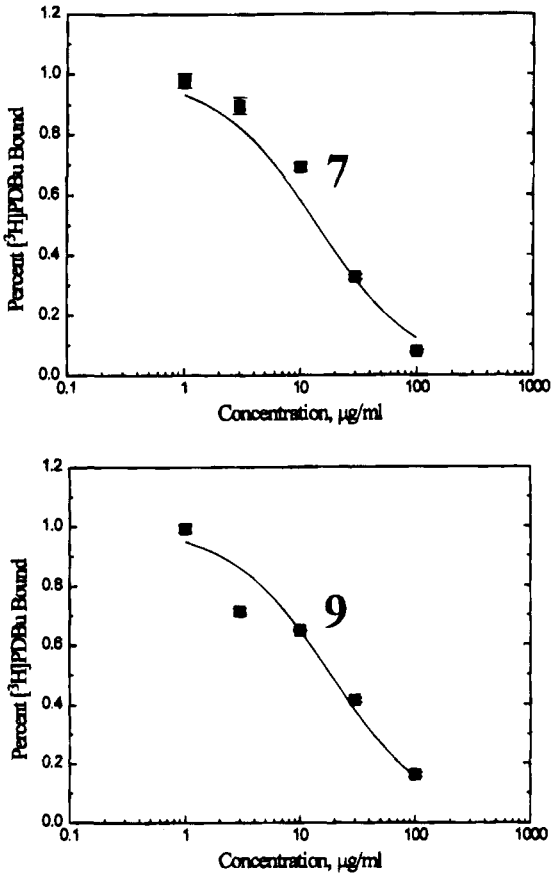


Figure 3. Dose–response curves for compounds **7** and **9**.

Table 1. K_i Values for the Five Active Compounds and Binding Affinities for Six Other Marginally Active Compounds

active		marginally active	
compd	binding affinity, K_i (μM)	compd	binding affinity (% inhib)[a]
6	16.1 ± 3.3	**11**	30
7	7.8 ± 0.9	**12**	34
8	26.6 ± 3.4	**13**	25
9	12.9 ± 1.7	**14**	30
10	37.7 ± 4.8	**15**	30
		16	27

[a] Percentage of inhibition at a concentration of 30 μg/mL in media.

PK-C binding affinity could be gained if hydrophobicity is expressed in a quantitative manner. We thus evaluated the hydrophilic–hydrophobic balance for the 125 tested compounds in terms of their water solubility, or its logarithm [$\log_{10}$(WS)]. A recently developed numerical method[24] for the estimation of $\log_{10}$(WS), which has been successfully applied in this laboratory in connection with the study of structure–activity relationships of a series of ribonolactone PK-C ligands,[21,23] was utilized to estimate the $\log_{10}$(WS) values for these 125 compounds.

The lack of quantitative binding affinity data for the 114 inactive compounds prevented us from obtaining a quantitative correlation between the binding affinities and the $\log_{10}$(WS) values. In Figure 4, the binding affinities (active/marginally or inactive) of these 125 compounds are plotted as a function of their $\log_{10}$(WS) values. All the 11 active compounds have a $\log_{10}$(WS) value less than 0.0, and all the 89 compounds with a $\log_{10}$(WS) value greater than 0.0 are inactive. This qualitative correlation demonstrates the importance of the hydrophobicity in PK-C binding affinity. In our

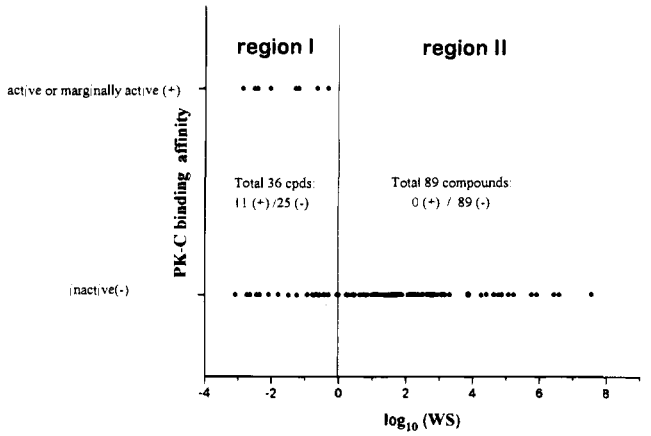


Figure 4. Correlation between PK-C binding affinities and $\log_{10}$(WS) values of all 125 compounds tesed in the PK-C bioassay.

previous study of ribonolactone ligands,[23] the optimal $\log_{10}$(WS) value of a ligand for PK-C binding was found to be around −3.0. Although for such a diverse group of 125 compounds it is difficult to determine what the optimal $\log_{10}$(WS) value would be, because the $\log_{10}$(WS) value in this case is not the sole factor determining the difference of their binding affinities, our results suggest that for a compound to show measurable PK-C binding affinity it must be hydrophobic, with an estimated $\log_{10}$(WS) value less than 0.0.

The $\log_{10}$(WS) values for the 161 compounds excluded from the PK-C bioassay were also calculated. It was found that all these 161 compounds have a $\log_{10}$(WS) value larger than 0.0, indicating that they are indeed quite hydrophilic and unlikely to have any significant PK-C binding affinity. This provided a strong support for their exclusion from the PK-C bioassay.

2. Five Active Compounds. The structures of these five active compounds (Figure 2; **6**–**10**) allow multiple possible arrangements that fit the pharmacophore defined in Figure 1. Identification of the most probable pharmacophore in these compounds represents a significant challenge because many conformations for each compound were generated and searched and multiple conformations for each of these compounds were found to meet the pharmacophore query requirements defined in Figure 1. Detailed conformational analysis and pharmacophore detection were carried out for this purpose on these five active compounds using QUANTA/CHARMm.[18] Table 2 summarizes the results of this conformational analysis, together with the results of fitting the three pharmacophore oxygens in the low-energy conformations of all possible pharmacophore patterns of each of the five active compounds on the three corresponding oxygens in a restricted phorbol pharmacophore. In this restricted phorbol pharmacophore, the three critical distances used correspond to the median values, being 6.00, 5.70, and 6.40 Å, respectively, as defined in the pharmacophore query of Figure 1. The term "geometric fit to the phorbol pharmacophore" is used to refer to this operation.

Only one of the four pharmacophore patterns that were examined for compound **6** was found to have a low-energy conformation [<1.0 kcal/mol above the global minimum ("The global minimum" refers to the lowest energy conformation found in the conformational search.)] with an excellent geometric fit [root mean square (rms) = 0.28 Å] to the phorbol pharmacophore (Figure 5a).

Table 2. Results of the Comparisons between the Conformations of the Five Active Compounds and the Phorbol Pharmacophore

compd	no. of conformations sampled	no. of pharmacophore patterns examined	rms (global)	rms (+4 kcal/mol)	energy diff between global minima and conformation with best fit (kcal/mol)	$\log_{10}$(WS)[a]
6	1000	4	0.28	0.27	0.06	−1.30
7	1000	12	0.24	0.21	1.47	−0.32
8	1000	12	0.19	0.19	0.00	−0.32
9	5000	12	0.74	0.25	5.29	−2.03
10	3000	12	0.46	0.45	0.02	−1.19

[a] Estimated logarithm of water solubility. The unit of the water solubility is mol/M[3].

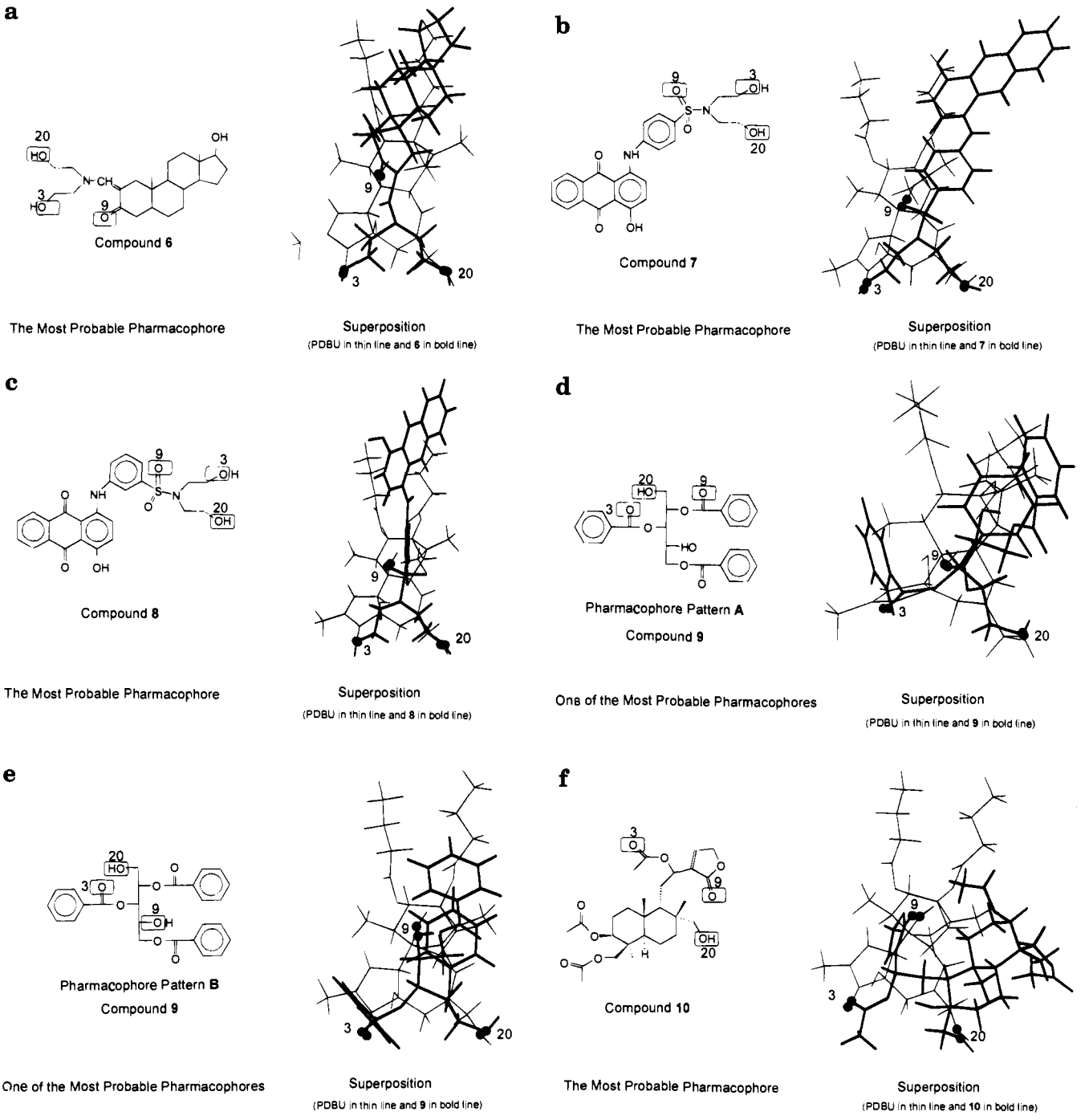


Figure 5. (a–f) Superpositioning of the most probable pharmacophores in compounds **6–9** in their low-energy conformations on the PDBU pharmacophore.

None of the eight possible pharmacophore patterns for compounds **7** and **8**, that would satisfy the phorbol pharmacophore with a conformational energy penalty of <10 kcal/mol above the global minimum, gave a reasonably good geometric fit to the phorbol pharmacophore (rms value = 1.9 Å in the best case). Such poor fits would predict poor binding affinities for these two compounds; however, their low K_i values, 7.8 and 26.6 μM, respectively, indicated otherwise. The alternative use of other functional groups in **7** and **8** to match the pharmacophore revealed that if the SO_2 group replaced the C=O group in the pharmacophore, excellent three-

point geometric fits could be obtained with rms values of 0.24 and 0.19 Å for **7** and **8**, respectively. The three-point (oxygen) superimpositions of a low-energy conformer (<2 kcal/mol above the global minimum) of **7** and **8** to the phorbol pharmacophore is shown in Figure 5b,c, respectively. The results suggest the most probable pharmacophore in these two compounds was made of one of the S=O oxygens in the sulfonyl group, one of the two hydroxymethyl (CH_2OH) groups, and the oxygen in the other hydroxymethyl.

Twelve pharmacophore patterns were examined for compound **9**. The two pharmacophore patterns that provided the best fits were labeled A and B. Superimpositions of a low-energy conformation (<4 kcal/mol above the global minimum) of compound **9** to the phorbol pharmacophore according to patterns A and B gave rms values of 0.31 and 0.25 Å, respectively, as shown in Figure 5d,e.

For compound **10**, 12 pharmacophore patterns were examined. In this case, the best fit to the phorbol pharmacophore with its low-energy conformations (<1 kcal/mol above the global minimum) was found to have an rms value of 0.46 Å, as shown in Figure 5f. A slightly improved fit (rms = 0.39 Å) was obtained when using a conformer with a conformational energy 9.7 kcal/mol above the global minimum. Among the five active compounds, the worst geometric fit to the phorbol pharmacophore is given by compound **10**, which is consistent with its weakest binding affinity among these five active compounds.

Although all the five active compounds studied have a good three-point geometric fit to the phorbol pharmacophore with their low-energy conformations, a good geometric fit to the three specific oxygen atoms in the phorbol pharmacophore is not the sole parameter for accurately predicting the PK-C binding affinity of a compound. We have shown recently[21] that the specific spatial orientation of these atoms, which is crucial to the strength of the requisite hydrogen bonds with the receptor, and other factors such as entropic changes during the binding and hydrophobicity (vide supra) are also important. The key element of the present approach, however, is that it provides a rapid access to the prediction of qualitative binding affinities of lead compounds without the need to perform lengthy binding energy calculations. It is of interest to note from Figure 5 that the hydrophobic portions in all the five active compounds are oriented into the same region as the hydrophobic portion in PDBU, which may suggest the importance of the orientation of the hydrophobic portion in determining the binding affinity for a PK-C agonist.

3. Thirty-one Hydrophobic Inactive Compounds. The results discussed above clearly indicate that a combination of proper hydrophobicity and a good geometric fit to the phorbol pharmacophore is essential to endow a ligand with PK-C binding affinity. It was therefore postulated that for the six marginally active (**11**–**16**; Figure 2) and 25 inactive compounds (**17**–**41**; structures not shown), whose estimated $\log_{10}$(WS) values are well below zero, the most important if not the sole factor for their weak binding affinities must be the conformational energy, i.e., no low-energy conformation for any of these compounds had a good geometric fit to the phorbol pharmacophore. In order to test this hypothesis, modeling studies were also performed on these 31 compounds.

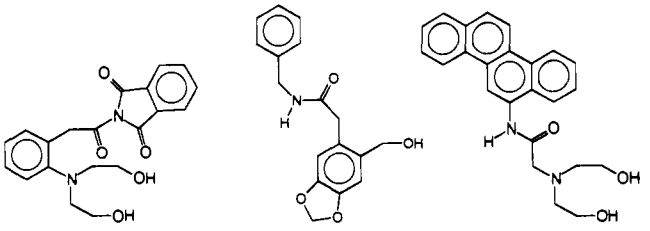
Table 3. Conformational Analyses of the 31 Inactive/Marginally Active Compounds

compds	no. of conformations sampled	no. of pharmacophore patterns examined	rms[a]	rms[b]	$\log_{10}$(WS)[c]
11	1000	12	0.67	0.65	−2.88
12	5000	12	2.62	2.09	−2.07
13	1000	10	1.76	1.64	−2.07
14	5000	8	3.08	2.62	−2.52
15	1000	8	0.77	0.70	−0.64
16	1000	12	1.15	0.71	−2.43
17	1000	6	0.49	0.49	−2.08
18	1000	6	0.49	0.49	−2.08
19	3000	4	0.93	0.55	−0.30
20	2000	4	0.45	0.39	−0.43
21	3000	6	0.85	0.32	−0.31
22	3000	8	0.38	0.22	−0.56
23	1000	8	0.91	0.87	−0.92
24	2000	12	1.46	1.46	−0.39
25	2000	10	5.78	5.72	−2.36
26	1000	4	0.15	0.15	−0.93
27	5000	2	0.19	0.17	−2.73
28	5000	8	0.91	0.90	−1.47[d]
29	5000	8	0.93	0.93	−0.04
30	5000	10	1.46	1.00	−2.70
31	5000	2	0.49	0.30	−0.01
32	1000	8	0.90	0.90	−1.49
33	1000	4	2.24	2.24	−1.24
34	5000	2	0.88	0.88	−0.77
35	5000	4	0.42	0.24	−0.64
36	5000	4	1.75	1.65	−0.06
37	2000	10	0.69	0.60	−2.45
48	2000	12	0.43	0.14	−2.63
39	2000	10	10.14	9.84	−3.09
40	3000	2	0.85	0.70	−0.70
41	3000	6	0.60	0.47	−0.66

[a] The lowest rms value of 3-point (the three pharmacophore oxygens) fit of all the conformations, which have a conformational energy within 4 kcal/mol of the lowest conformational energy, on the phorbol pharmacophore. [b] The lowest rms value of 3-point (the three pharmacophore oxygens) fit of all the conformations, which have a conformational energy within 10 kcal/mol of the lowest conformational energy, on the phorbol pharmacophore. [c] Estimated logarithm of water solubility. The unit of the water solubility is mol/M³. [d] In the $\log_{10}$(WS) calculation, compound **28** has a missing parameter (N^+); therefore, the calculated $\log_{10}$(WS) value for this compound is not reliable.

Since very high energy conformations are not relevant to PK-C binding, two conformational energy cutoff values, 4 and 10 kcal/mol, were used to select conformations, respectively. If the conformational energy cutoff value of 4 kcal/mol was used, all the conformations with a conformational energy within 4 kcal/mol of the global minimum were selected. All the qualified conformations in this cluster essentially represent the low-energy conformations. Similarly, if the conformational energy cutoff value of 10 kcal/mol was used, all the conformations with a conformational energy within 10 kcal/mol of the global minimum were selected. We considered that 10 kcal/mol is probably the upper limit for the conformational energy penalty when a ligand binds to PK-C. If a compound was found to have a poor fit to the phorbol pharmacophore even with the conformations in this cluster, it would be predicted to be a weak PK-C ligand. These selected conformations using two different conformational energy cutoff values for each of these 31 inactive compounds with all the possible pharmacophore patterns in their structures were then geometrically fitted to the phorbol pharmacophore to obtain the rms values. The results are summarized in Table 3.

When the conformational energy cutoff value of 4 kcal/mol was used, three compounds, **22**, **26**, and **27**, show



22 26 27

Figure 6. Three inactive compounds with excellent fits on the phorbol pharmacophore and proper hydrophobicity.

good geometric fits on the phorbol pharmacophore, with rms values of 0.38, 0.15, and 0.19 Å, respectively. These three compounds (**22**, **26**, and **27**; Figure 6) represent the false positives in the group of 31 compounds. Nine other compounds have intermediate fits on the phorbol pharmacophore, with an rms value between 0.40 and 0.80 Å, and all other 19 compounds have a relatively bad fit, with an rms value >0.8 Å. Therefore, for 28 of the 31 compounds (91%), their lack of significant binding affinities is at least in part because the low-energy conformations of these compounds have a poor fit (rms > 0.4 Å) to the phorbol pharmacophore. It is not entirely clear to us at this stage why the three compounds (**22**, **26**, and **27**) do not show significant binding affinity despite having a proper hydrophobicity and a good three-point geometric fit to the phorbol pharmacophore.

When the conformational energy cutoff value of 10 kcal/mol was used, seven compounds, **20**–**22**, **26**, **27**, **31**, and **35** (Table 3), were found to have a good geometric fit to the phorbol pharmacophore (rms < 0.40 Å). The other 24 compounds have a relative poor geometric fit (rms > 0.4Å) to the phorbol pharmacophore even with this fairly large conformational energy of 10 kcal/mol as the cutoff value to select conformations to fit to the phorbol pharmacophore. This clearly demonstrates that the conformational energy is indeed one of the most important factors responsible for the weak binding affinities of these 31 compounds.

4. Conformational Energy. All the compounds retrieved by the 3D-database search contain a "potential" pharmacophore. The differences that exist between active and inactive compounds with proper hydrophobicity are due to the energy cost needed to achieve a good fit to the pharmacophore. We have observed cases where at least some of the low-energy conformers for each of the active compounds meet the geometrical requirements demanded by the ideal pharmacophore while none of the low-energy conformations for the majority of the inactive compounds satisfied these demands. The fundamental question is, then, what is the relationship between the rms (the goodness of the fit to the pharmacophore) and the conformational energy of a ligand? Our recent studies[25,26] on the conformational changes of ligands upon binding on protein receptors have shown that ligands do not have to be in their global or local energy minima to bind and that the deformation of a ligand upon binding to a receptor is a common phenomenon. Obviously, the binding of a ligand to its receptor will be more effective if this interaction occurs through a low-energy conformer of the ligand since other modes of interactions will be energetically more costly. Our goal here is to shed some light on the relationship between the rms value, the conformational energy, the binding energy, and the binding affinity, by detailed studies of one active compound (**7**; Figure 2) and one inactive compound (**32**; structure not shown).

The best rms values of the three-point fit of the low-energy conformations to the phorbol pharmacophore for **7** and **32** are 0.24 and 0.96 Å, respectively. Since the most critical parameters of the phorbol pharmacophore are the three distances defined in Figure 1, these distance constraints were thus imposed on these molecules using the CHARMm/CONSTRAIN option in QUANTA during the minimization process in order to optimize their structures to the ideal pharmacophore arrangements. By varying the strength of these constraints, different conformations were generated which represent different conformational states with varied conformational energies. These conformations were then fit to the phorbol pharmacophore, and the rms values were obtained. The energy differences between these constrained conformations and the global minimum were also recorded.

We propose that the interaction energy between a ligand and the binding site of the PK-C receptor can be probably estimated by the following equation:

$$E_{\text{inter}} = E_o * \exp(\text{-rms}) \quad (1)$$

where E_{inter} is the interaction energy between the ligand and the PK-C receptor, E_o is the maximum interaction energy between a ligand and the receptor, and rms is the root mean square value of fitting a conformation on the pharmacophore. From eq 1, when rms = 0, $E_{\text{inter}} = E_o$ (the maximum interaction) and when rms $\rightarrow \infty$, $E_{\text{inter}} \rightarrow 0$ (no interaction at all).

Therefore, upon binding, the net gain energy of the whole system will be

$$E_{\text{net gain}} = E_{\text{inter}} - E_{\text{conf}} \quad (2)$$

where $E_{\text{net gain}}$ is the net gain energy (enthalpy) of the whole ligand–receptor complex, E_{inter} is the interaction energy, and E_{conf} is the conformational energy for the ligand.

Our previous modeling study[21] on the binding site of the PK-C suggested that up to four hydrogen bonds can be formed between the ligand and the binding site of the PK-C for maximum interaction. If each of these four hydrogen bonds can provide 4 kcal/mol interaction energy (enthalpy) for the maximum interaction, then E_0 is equal to 16 kcal/mol.

Using eqs 1 and 2, with the rms values and the conformational energies obtained from the modeling studies, E_{inter} and $E_{\text{net gain}}$ can be readily computed. Figures 7 and 8 plot the conformational energy, the interaction energy, and the net gain energy against the rms values for the active compound **7** and the inactive compound **32**, respectively. Figures 7 and 8 show that for both the active and the inactive compounds, as the conformational energy increases, the rms value decreases and the interaction energy increases, although the actual curve for each compound is different. The net gain energy ($E_{\text{net gain}}$), however, first increases and reaches a peak value in both cases and then decreases as the conformational energy increases and the rms value decreases. Both cases clearly show that the conformational deformation of a ligand does help the whole system (ligand–receptor complex) gain additional interaction energy. Therefore, it is a thermodynami-

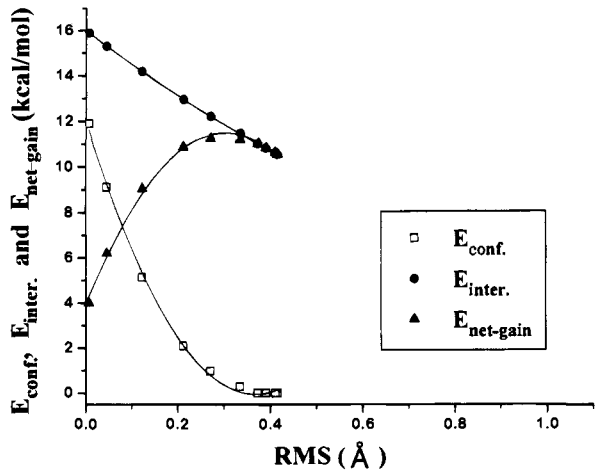


Figure 7. Plot of conformational energy, interaction energy, and net gain energy against rms for active compound **7**.

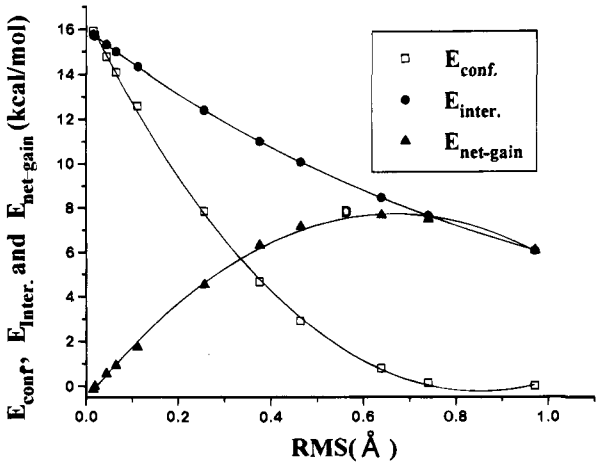


Figure 8. Plot of conformational energy, interaction energy, and net gain energy against rms for inactive compound **32**.

cally favorable process for a ligand to deform to some extent in order to have more enthalpy gain for the ligand–protein complex.

The peak value of the $E_{\text{net gain}}$ (the maximum interaction) for **7** is ca. 11.5 kcal/mol from Figure 7, while the peak value of the $E_{\text{net gain}}$ for **32** is ca. 7.5 kcal/mol from Figure 8. As a first approximation, without considering other factors, such as binding entropy, solvation energy, and hydrophobicity, for two compounds, the binding affinity difference (ΔK_i) can be expressed by:

$$-2.303RT \log_{10}(\Delta K_i) = \Delta E_{\text{net gain}} \quad (3)$$

where $\Delta E_{\text{net gain}}$ is the net gain energy difference for two compounds.

Using eq 3, at room temperature (25 °C), the 4 kcal/mol net gain energy gap between compounds **7** and **32** can be translated into a ca. 850-fold difference in their binding affinities. Given the K_i value of 7.8 μM for **7**, the estimated K_i value for **32** would be ca. 6600 μM, indicating a virtually inactive compound, a conclusion consistent with the experimental results.

(D) Synthetic Modifications. The 89 compounds which were inactive due to their inadequate hydrophobicity [estimated $\log_{10}$(WS) > 0.0] can also be grouped according to their goodness of fit (rms value) to the phorbol pharmacophore. The structures with rms values of ≤0.4 Å when fitting their low-energy conformations to the phorbol pharmacophore should be potentially active ligands if proper hydrophobicity is provided. In contrast, the structures with large rms values will not be active even if proper hydrophobicity is provided. To test this hypothesis, two compounds, **42** and **43** (Figure 9), were selected for synthetic modifications to promote their hydrophobicity to the desired range while leaving the pharmacophores intact. Molecular modeling studies showed that **42** has a low-energy conformation with a conformational energy only 0.1 kcal/mol above the global minimum, which fits well to the phorbol pharmacophore (rms = 0.40 Å); the best fit among the low-energy conformations of **43** (within 10 kcal/mol of the conformational energy of the global minimum) to the phorbol pharmacophore has an rms value of 0.78 Å. The calculated $\log_{10}$(WS) values for **42** (2.95) and **43** (3.03) clearly indicate they both are too hydrophilic to bind effectively to PK-C. Both of these compounds are acids and can be readily converted to an ester or amide with a long aliphatic chain to achieve the proper hydrophobicity. Such modifications led to two new compounds, **44** and **45** (Figure 9), with estimated $\log_{10}$(WS) values of −2.80 and −3.00, respectively.

The modified compound (**44**) was able to displace 36 ± 6% of PK-C-bound PDBU at 100 μg/mL, as compared to only 3% for the original compound (**43**) with the identical pharmacophore. This data was consistent with the expectation that although hydrophobicity is an essential component for binding to PK-C, the less than ideal pharmacophore arrangement in **44**, as suggested by the rms value of 0.78 Å, leads to only marginal activity. On the other hand, compound **42**, which itself was able to displace 9 ± 2% (estimated K_i > 420 μM) of bound PDBU at a concentration of 30 μg/mL, was converted into a much improved ligand (**45**), with a K_i value of 42 μM. This more than 10-fold increase in binding affinity of **45** over **42** is due entirely to the increase in hydrophobicity.

Discussion

(A) Modifications of the Pharmacophore Query. In the development of the PK-C pharmacophore query for the 3D search, besides the chemical substructural requirements, i.e., the carbonyl (C=O), the hydroxymethyl (CH_2OH), and the type unspecified oxygen (O), the other factor which has a impact on the results of the 3D search is the distance tolerance used in the pharmacophore. In the PK-C pharmacophore query used in the present search, as shown in Figure 1, the three distance tolerances were set to be 0.60, 0.60, and 0.25 Å. The reasons for assigning different distance tolerances in the pharmacophore query were already discussed in the Methods and Materials section. However, another important and different issue which also needs to be addressed is how many possible conformations should be considered for each structure during the 3D-database pharmacophore search.

Chem-X employs a rule-based algorithm in both database build and search stages. Each single bond is rotated with a step size of 120°, and each conjugated bond is rotated with a step size of 180° as the default.[16] Chem-X thus covers a much wider conformational space than some 3D-database build programs, such as Concord,[28] in which only a single conformer is stored. But with such large step sizes, 120° for single bonds and 180° for conjugated bonds, Chem-X can only have a

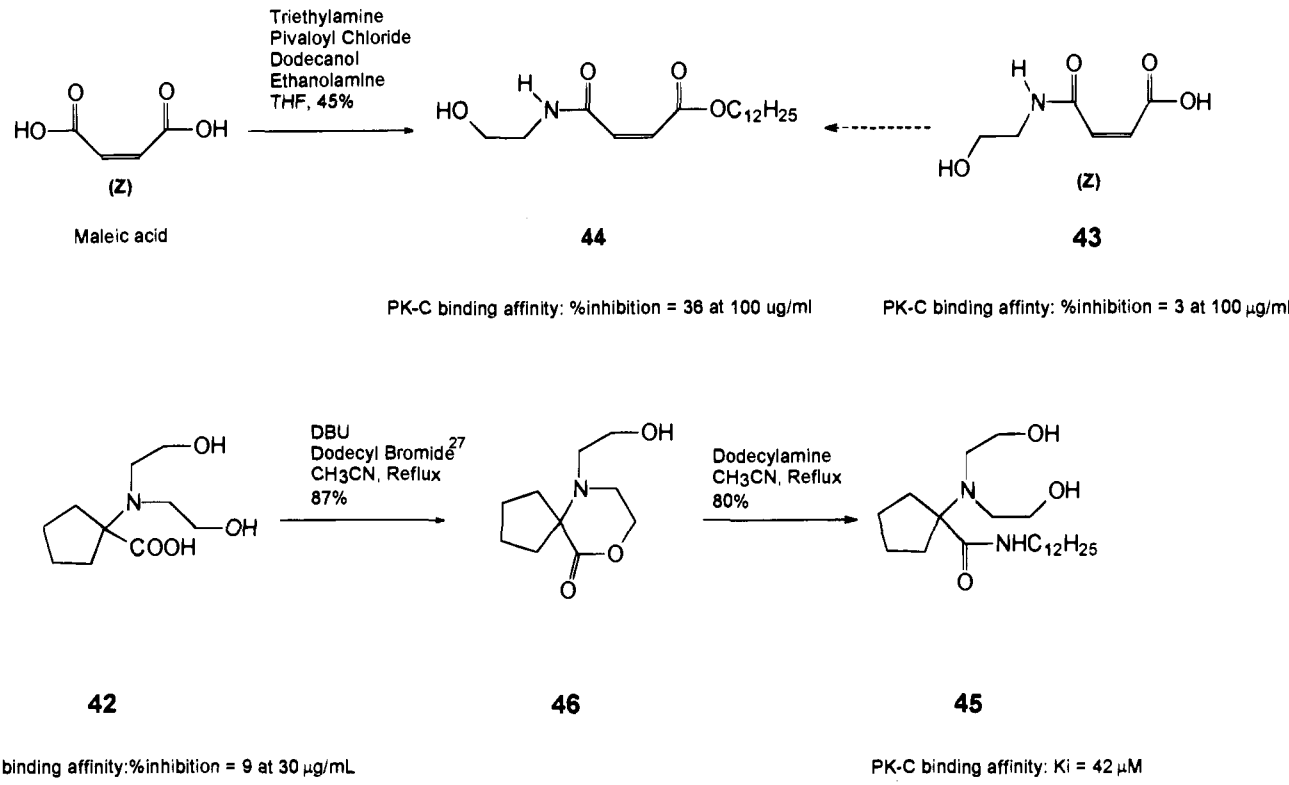


Figure 9. Synthetic schemes for the conversion of two inactive compounds into two active derivatives.

limited coverage of the entire conformational space for each compound. If smaller angle increments, such as 30° for a single bond, are employed in the generation of conformations, Chem-X would cover a much wider conformational space, but for a database of 450 000 compounds, this would take too much CPU time in both build and search stages and is not feasible. An alternative is to allow larger distance tolerances in the pharmacophore query. By doing so, the conformations, which have close but not exact geometric parameters (distances), would be accepted as valid hits. This alternative will result in larger number of false positives, but it will be useful if the purpose of the search is to discover lead compounds and the biological evaluation is not very time consuming nor expensive.

Molecular modeling results suggested that in compounds **7** and **8** the pharmacophore is made of a sulfonyl group (SO_2), a hydroxymethyl group (CH_2OH), and another hydroxyl oxygen as shown in Figure 5b,c. This differs constitutionally from the pharmacophore defined in Figure 1. The sulfonyl group apparently can effectively replace the carbonyl group in a PK-C agonist, and this modified pharmacophore could be confirmed by the design, synthesis, and biological evaluation of new compounds, in which the only possible pharmacophore is that proposed in compounds **7** and **8**. This modified pharmacophore can now be used as the new query to search the NCI 3D-database and could lead to the discovery of more novel ligands that compete with the phorbol esters to bind to PK-C.

(B) Improving Search Efficiency. Improvement of 3D-database search efficiency can make the 3D-database search more cost effective and speed up the process of drug discovery. However, it should be kept in mind that although it is desirable to cut down the number of false positives, it is extremely important to make sure that a 3D-database search does not miss the active compounds.

The molecular modeling results show that the majority of the 31 inactives/marginally actives were structures that had to adopt a high energy conformation in order to satisfy the pharmacophore query. The presence of high-energy conformations as hits is not surprising because the criterion in Chem-X for judging the acceptability of a conformation was a simple set of rules, which use the three consecutive torsion angles defined by six consecutive atoms.[16] Close van der Waals contacts between atoms separated by more than six atoms will not be detected. It is possible to perform molecular mechanics energy calculation for every conformation generated, but the time to search through hundreds of thousands of structures would be prohibitive. The Chem-X program thus provides a compromise in the form of a simple "bump check", which simply calculates the number of atom pairs that have overlapping van der Waals radii.[16] It will be of interest to investigate in the future if by implementing this type of energy calculation the number of high-energy hits could be cut down while still keeping the pharmacophore search of this large database fast enough for practical use.

The modeling results clearly showed that the hydrophobicity of a compound is an important factor in determining its PK-C binding affinity. Since the $\log_{10}$(WS) calculations are fast, water solubility can be used as a criterion to screen out those hydrophilic compounds, which are all inactive. The $\log_{10}$(WS) estimation is quite accurate,[24] normally with an error less than 0.4 log unit. Hence, it is safe to screen out those compounds with $\log_{10}$(WS) values >0.5. This exercise has been routinely used in our subsequent work whenever new modified PK-C pharmacophore queries were used and new searches were performed.

Conformational analysis would definitely screen out false positives, as was shown in the molecular modeling studies, but unfortunately, it is very time consuming. On average, the CPU time on the Silicon Graphics

Indigo workstation spent on conformational analysis for one single compound was 1–4 days, depending upon the size of the compound. Therefore, it is not practical to perform conformational analyses on a large number of compounds routinely. The PK-C assay used in our test is fortunately not very time consuming and permits us to test a fairly large number of compounds (~100).

(C) Problems. Some problems exist with the current database build and search. The first problem is related to the NCI DIS 2D-database.[14,15] In the entire NCI DIS 2D-database, the chirality in approximately 122 000 chiral compounds is unknown and Chem-X has no choice but to arbitrarily assign chirality to each chiral center in these compounds. Compounds such as phorbol esters, which are known PK-C agonists and are present in the database, were missed in the present pharmacophore search because the stereochemistry was assigned incorrectly. When these compounds were rebuilt with correct stereochemistry, they were indeed identified as valid hits by Chem-X using the same pharmacophore query as shown in Figure 1. It might be possible to build every possible configuration for chiral compounds, but the total number of 3D structures stored in the 3D-database for each chiral compound with unknown chirality would be very large. The size of the 3D-database and the search time would probably be increased by a factor of 10–100 (the current 3D-database occupies nearly 1000 Mbytes of disk space).

The second problem is related to Chem-X. The largest ring fragment in the fragment library of the model builder module in Chem-X is a seven-membered ring.[16] Thus, for some known PK-C agonists such as debromoaplysiatoxin (**5**) analogs, in which a 12-membered ring is present, and the teleocidin (**3**) analogs, in which a nine-membered ring is present, Chem-X failed to build 3D structures due to the lack of the necessary fragments. The current version of the NCI 3D-database does not contain the structures for these compounds.[14]

The third problem is also somewhat related to Chem-X. We limited the maximum number of rotatable bonds in a structure to 15 in the keying step in the database build. Therefore, if a compound has more than 15 rotatable bonds in its structure, it is excluded from the keying step in the 3D-database build. This kind of compound will not be searched in the pharmacophore search. Should the maximal number of rotatable bonds be increased in the database build stage in the future, this problem would be minimized.

Conclusion

Despite a number of deficiencies in the current 3D-database pharmacophore search, our success in discovering a number of novel, structurally diverse PK-C agonists has demonstrated that computer 3D-database pharmacophore searching is an effective and promising tool to discover novel lead compounds in drug development. The well-defined PK-C pharmacophore query, the large size of the NCI 3D-database, and the ability of the search software, Chem-X, to generate multiple conformations for each structure all seem to be important to our success.

Molecular modeling is useful to gain further insight about the structure–activity relationships and to help adjust the 3D-database search strategies in order to make the search more effective. Through molecular modeling studies, it was found that in addition to the presence of the basic pharmacophore components in the chemical structure the hydrophobicity and the conformational energy also play very important roles in determining the PK-C binding affinity of a compound. The conclusion of molecular modeling studies has been confirmed by the binding affinities of two new synthetic compounds. Our study suggests that the combined approach of computer 3D-database pharmacophore search and molecular modeling may be more effective in the discovery of new leads, as compared to using computer 3D-database pharmacophore search alone.

It is our opinion that the main purpose of a 3D-database pharmacophore search is not to find compounds with ultrapotent activity. A realistic goal of a 3D-database pharmacophore search should be the discovery of new leads which can be subsequently used as the basis for further synthetic modifications.

Experimental Section

Molecular Modeling. All the molecular mechanics calculations were carried out with QUANTA molecular modeling package (version 3.1) with CHARMm 2.2 parameter set running on a Silicon Graphics IRIS Indigo workstation. Since the molecular mechanics calculation depended upon the atom types, the atom type for every atom was carefully examined to ensure it was correct before any molecular calculations were conducted. All the conformational analyses were performed with the conformational search module within QUANTA. Monte Carlo random sampling algorithm was used to generate conformations, usually 2000~5000 conformations for each structure. For each generated conformation, 1000 steps of adopted-basis Newton Raphson (ABNR) minimization was carried out or until convergence (with a convergence criterion equal to 0.01 kcal $Å^{-1}$). Rotatable bonds involved in the Monte Carlo conformation sampling are only those sp^3–sp^3 or sp^3–sp^2 bonds relevant to the possible pharmacophores, and an angle increment of 60° was used for each defined rotatable bond. A dielectric constant of 1 was used (CDIE = 1) in all the calculations. All the possible pharmacophore patterns in a compound were evaluated in order to identify correctly the most probable pharmacophore in a structure. For each conformer, the relevant distance information, as well as the conformational energy, for each of the possible pharmacophore patterns was downloaded into an ASCII file. A program was then developed to compare automatically these conformations to the phorbol pharmacophore template and to identify the best ones according to considerations of both the rms value and the conformational energy.

Synthesis. General Methods. All chemicals and reagents were obtained commercially and used without further purification. Flash column chromatography was carried out using silica gel 60 (230–400 mesh). Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA. NMR data were obtained on a Bruker AC250 (250 MHz) and are referenced to the solvent in which they were run.

Dodecyl 4-[(2-Hydroxyethyl)amino]4-oxo-2-butenoate (44). A solution of maleic acid (0.3 g, 2.58 mmol) in anhydrous THF (25 mL) at 0 °C was treated with triethylamine (1.8 mL, 12.9 mmol) followed by pivaloyl chloride (1.59 mL, 12.9 mmol). After the mixture was stirred for 45 min at 0 °C, dodecanol (0.49 mL, 2.6 mmol) was added and the reaction mixture was stirred at 0 °C for 2 h and then at room temperature for 6 h. At this time, the reaction mixture was cooled to 0 °C, ethanolamine (0.23 mL, 3.9 mmol) was added, and the mixture was brought to room temperature in 2 h. The reaction mixture was further stirred at room temperature for 16 h and diluted with water (5 mL), concentrated under vacuum, and extracted with EtOAc (3 × 15 mL). The combined organic extract was washed with water (2 × 10 mL) and dried (Na_2SO_4). The oil obtained on concentration was purified by flash chromatography on silica gel using EtOAc as eluant. Pure **44** was obtained as a white solid (0.422 g, 45%): mp 52–53 °C (EtOAc/hexane); ^1H NMR ($CDCl_3$) δ 0.86 (distorted triplet, 3 H, -CH_3), 1.20–1.40 (m, 20 H, -CH_2-), 1.60–1.70 (m, 2 H, -$COOCH_2CH_2$-), 2.75 (br, 1 H, -O*H*), 3.45 (m, 2 H, -$NHCH_2CH_2OH$), 3.73

(distorted triplet, 2 H, -CH_2OH), 4.12 (t, 2 H, J = 6.7 Hz, -COOCH_2-), 6.10 (d, 1 H, J = 12.8 Hz, -CH=CH-), 6.35 (d, 1 H, J = 12.8 Hz, -CH=CH-), 8.05 (br, 1 H, -NH); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.64, 25.8, 28.3, 29.1, 29.3, 29.4, 29.5, 29.6, 31.9, 42.7, 61.8, 65.9, 125.2, 138.4, 165.4, 166.2. Anal. ($C_{18}H_{33}NO_4$) C, H, N.

N-(2-Hydroxyethyl)-6-oxo-7-oxa-10-azaspiro[4.5]decane (46). A solution of [N,N-bis(2-hydroxyethyl)amino]-cyclopentane-1-carboxylic acid (**42**) (122 mg, 0.56 mmol), DBU (86 mg, 0.56 mmol), and 1-bromododecane (168 mg, 0.67 mmol) in acetonitrile (15 mL) was refluxed for 2.3 h. The reaction mixture was cooled to room temperature and concentrated under vacuum. The resulting oil was flash chromatographed on silica gel using EtOAc/MeOH (98/2) as eluant to give **46** (98 mg, 87%) as a white solid: mp 91.5–92.5 °C (EtOAc/hexane); ^{1}H NMR ($CDCl_3$) δ1.60–1.90 (m, 6 H, -CH_2-), 2.10–2.30 (m, 2 H, -CH_2-), 2.40 (br, 1 H, -OH), 2.60 (t, 2 H, J = 5.2 Hz, -NCH_2CH$_2$OCO-), 2.90 (t, 2 H, J = 5.1 Hz, -NCH_2CH$_2$-OH), 3.60 (m, 2 H, -CH_2OH), 4.40 (t, 2 H, J = 5.2 Hz, -CH_2-OCO-); ^{13}C NMR ($CDCl_3$) δ 26.7, 35.3, 44.5, 50.7, 58.5, 68.0, 72.9, 175.0. Anal. ($C_{10}H_{17}NO_3$) C, H, N.

N-Dodecyl-1-[N,N-bis(2-hydroxyethyl)amino]cyclopentane-1-carboxamide (45). A solution of **46** (36 mg, 0.18 mmol) and dodecylamine (67 mg, 0.36 mmol) in acetonitrile (5 mL) was refluxed for 24 h. After cooling to room temperature, the reaction mixture was concentrated, and the residue obtained was purified by flash chromatography on silica gel using EtOAc/hexane (95/5) as eluant to obtain **45** as a white solid (56 mg, 80%): mp 50–51 °C (EtOAc/ hexane); ^{1}H NMR ($CDCl_3$) δ 0.85 (distorted triplet, 3 H, -CH_3), 1.20–1.40 (m, 18 H, -CH_2-), 1.50 (m, 2 H, -NHCH$_2$CH_2-), 1.60–1.80 (m, 6 H, cyclic -CH_2-), 2.00 (m, 2 H, cyclic -CH_2-), 2.60 (m, 4 H, -NCH_2-CH$_2$OH), 3.10–3.30 (m, 4 H, -CONHCH_2- and -OH), 3.60–3.70 (m, 4 H, -CH_2OH), 7.40 (br, 1 H, -CONH-); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.6, 25.5, 27.1, 29.3, 29.5, 29.6, 29.62, 31.9, 33.4, 39.5, 52.9, 62.1, 70.5, 177.6. Anal. ($C_{22}H_{44}N_2O_3$) C, H, N.

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