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Biochemistry

A receptor model for tumor promoters: Rational superposition of teleocidins and phorbol esters

(carcinogenesis/computer-assisted structure–activity relationships/receptor mapping/computer graphics)

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ABSTRACT **Four 12-*O*-tetradecanoyl-13-*O*-acetylphorbol-type tumor promoters—teleocidin, phorbol ester, aplysiatoxin, and ingenol ester—are superposed in an attempt to understand their common biological activity on the assumption that they may bind to the same receptor site. A method using three-dimensional computer graphics was applied for superposing molecules and receptor mapping. The main feature of the method is that molecules are superposed in terms of spatial arrangement of physical and chemical properties but not in terms of the atomic positions as in conventional methods. This led to successful extraction of common structural features required for potent tumor-promoting activity: two hydrogen donors, a hydrogen acceptor, and a large lipophilic group. Their mutual spatial arrangements are most important for biological activity.**

A two-stage model of initiation–promotion in carcinogenesis is generally accepted (1). The phorbol esters, in particular 12-*O*-tetradecanoyl-13-*O*-acetylphorbol (TPA), are well known as tumor promoters of skin and also as growth modulators of a wide variety of cells (2). Since the discovery of the tumor-promoting activity of a dihydro derivative of teleocidin B (dihydroteleocidin B) (3), a number of investigations have shown that dihydroteleocidin B and other members of the teleocidin family (4) have essentially the same activity as TPA. In addition, a marine toxin, aplysiatoxin, has also been revealed to be a potent tumor promoter (5). Thus, investigations on the relationships between chemical structures and biological activity provide an intriguing research target, because of the marked structural dissimilarities between teleocidins, aplysiatoxin, and the conventional diterpene esters (Fig. 1). Clarification of structural features essential for biological activity would offer important insights into the interaction of tumor promoters with macromolecular target(s). Recently, Jeffrey and Liskamp (6) and Wender *et al.* (7) have attempted to structurally correlate these promoters.

Here, we present our results, which lead to a different outcome. We have first determined the absolute stereochemistry of teleocidins (8) and analyzed their conformations (9, 10), and then we have attempted a computer-assisted elucidation of the structure–activity relationships. For rational superposition of different types of compounds to extract the structural features three-dimensionally, molecules are superposed in terms of physical and chemical properties (11), instead of conventional atomic positions or chemical structures. This method offers the following advantages: (*i*) Molecules with widely different structures can be superposed. (*ii*) The method takes into account the interactions of the promoters from different directions to the same receptor site. For example, the hetero atoms in different molecules interacting with the common receptor site are not necessarily superposable (Table 1). (*iii*) The method leads to the construction of a receptor cavity model by using the structural information of superposed molecules; this model provides the size, shape, surface electrostatic potentials, expected hydrogen bonding sites, etc., of the cavity.

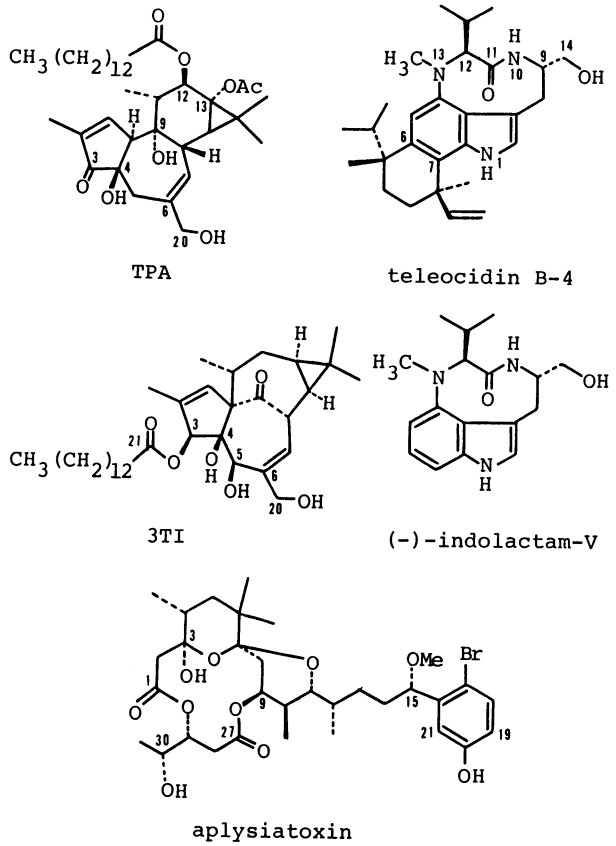

FIG. 1. Chemical structures of tumor promoters: TPA, teleocidin B-4, 3-*O*-tetradecanoylingenol (3TI), (−)-indolactam-V, and aplysiatoxin.

MATERIALS AND METHODS

Stereochemistry of Teleocidins. The structure of teleocidin B (teleocidin B-4) was determined by Hirata and coworkers by x-ray crystallography (12). The absolute stereochemistry was determined by comparison of the CD spectrum of

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Abbreviations: TPA, 12-*O*-tetradecanoyl-13-*O*-acetylphorbol; 3TI, 3-*O*-tetradecanoylingenol.

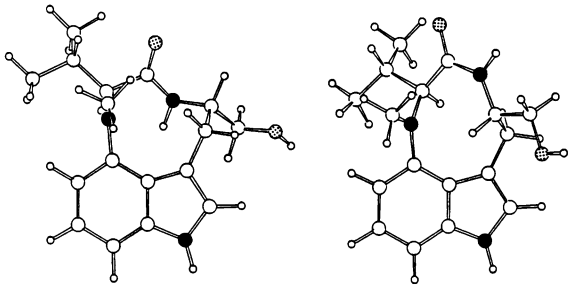

FIG. 2. Sofa and twist forms of (−)-indolactam-V.

teleocidin B-4 with that of (−)-indolactam-V (Fig. 1), whose absolute stereochemistry was synthetically determined (8, 9). This conclusion is further supported by the isolation of (−)-indolactam-V from the culture medium together with teleocidins (13) and of *N*-methyl-L-valyl-L-tryptophanol together with olivoretins (*O*-methyl derivatives of teleocidins) (14, 15). The NMR spectra of (−)-indolactam-V revealed that the molecule exists in two stable conformational states, "sofa" and "twist" forms (Fig. 2) (9). Furthermore, all teleocidin and olivoretin derivatives and lyngbyatoxin A (16) were shown to equilibrate rapidly between sofa and twist forms in solution, although a single conformation of each of them was observed in x-ray crystallographic analyses (14, 15). The low energy barriers between the two conformers [the observed free energy of activation, $\Delta G^{\ddagger} = 18$ kcal/mol (1 cal = 4.18 J)], and the short half-life of interconversion (estimated as 1.2 sec at 37°C) raised the question as to which is the important conformer for biological activity.

Structure–Activity Relationships. Structure–activity relationships of phorbol esters have been well documented (2, 17). Briefly, (*i*) the OH group at C-4 must be β-oriented, and its methylation leads to disappearance of activity, (*ii*) a free 6-CH_2OH group is preferred, (*iii*) reduction of the 3-C=O to 3β-OH decreases the activity, and (*iv*) a large lipophilic group at C-12 is necessary.

The activity of (−)-indolactam-V is 0.1–0.01 of that of teleocidin B in several kinds of bioassays, including skin tumor promotion (18, 19); the reduced but significant activity clearly shows that the structure satisfies the minimum requirement as a tumor promoter. Therefore, the terpenoid side chain in teleocidin B-4, which can be substituted by simpler alkyl groups without loss of activity (18, 20), appears to be intimately involved in increasing the biological potency. The isopropyl substituent at C-12 is preferably replaced by a larger lipophilic group (21), and this substituent presumably constitutes the lipophilic moiety of these molecules. Methylation at N-1 diminished the biological activity, but N-prenylation or N-geranylation increased the activity (19). These facts indicate that the presence of the hydrogen at N-1 is not essential for the activity. The methylation of the 9-CH_2OH group completely abolishes the activity. We have never found any kind of activity of olivoretins in a [^{3}H]TPA binding assay, induction of ornithine decarboxylase, and HL-60 cell adhesion (22), though an activity in the induction of Epstein–Barr virus antigen has been reported (23). These facts strongly suggest that the 9-CH_2OH group is indispensable as a hydrogen donor for interaction with the receptor. The study of indolactam-V isomers revealed that a single stereochemical inversion at C-9 or C-12 [(+)- and (−)-epi-indolactam-V] results in loss of activity (18).

Preparation of Structural Data. The atomic coordinates for the twist form of teleocidin B-4 used in this study were taken from the x-ray crystal analysis (14). The sofa structure of teleocidin B-4 was obtained by replacing the indolactam part of teleocidin B-4 with that of olivoretin B (*O*-methyl derivative of teleocidin B-1), whose structure was crystallographically determined (15), and then replacing the methoxyl group at C-9 by a hydroxyl group. The structure of TPA was constructed by connecting a tetradecanoyl group to the 12-OH of phorbol (24), whose atomic coordinates were taken from the Cambridge Crystallographic Database. The structure of aplysiatoxin was built from Maruzen HGS molecular models, based on the crystal structure of 19,21-dibromoaplysiatoxin (25), and was refined by using Allinger's MM2 program (26). The structure of 3TI was also constructed in the same manner as with aplysiatoxin, based on the crystal structure of ingenol triacetate (27), followed by replacing the acetyl group at C-3 by a tetradecanoyl group. All hydrogen atoms were relocated at the geometrically expected positions in all molecules. Atomic charges in all molecules were calculated in advance by molecular orbital calculations using the MNDO program (28).

Computational Methods. In this study, we made use of the RECEPS program¶ (11), where molecules are superposed in terms of spatial arrangements of physical and chemical properties memorized on three-dimensional grid points.

A molecule, whose structure is rigid or conformationally defined, is chosen as the template molecule among the molecules that should be superposed. For each grid point generated three-dimensionally with an adequate interval around the template molecule, the following physical and chemical properties are calculated and stored: electrostatic potential, charge distribution, hydrogen bonding character, flag on occupancy by each molecule, and flag for molecular surface. New molecules (named trial molecules) are super-

¶The program system RECEPS is written in FORTRAN 77 and runs on a HITAC M-680H computer and a Daikin DS361B color raster-type three-dimensional computer graphic display. Details of this program system will be presented elsewhere and will be distributed to other users on request.

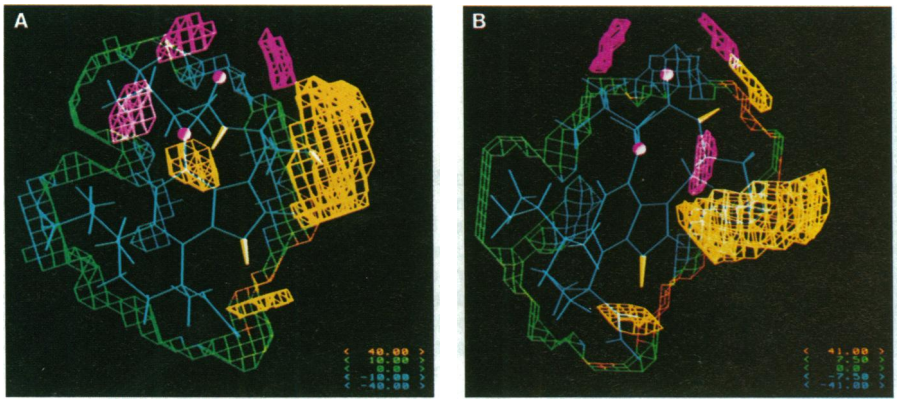

FIG. 3. Expected possible regions for receptor hydrogen bonding atoms and the van der Waals molecular surface (color coded by the electrostatic potentials) for teleocidin B-4. (*A*) Sofa form. (*B*) Twist form. Colors for potentials: blue, positive; green, approximately zero; orange, negative. Colors for hydrogen bonding regions: red, hydrogen acceptor; yellow, hydrogen donor. A painted ball indicates an acceptor hetero atom, and a yellow cone represents an X-H bond that donates a hydrogen.

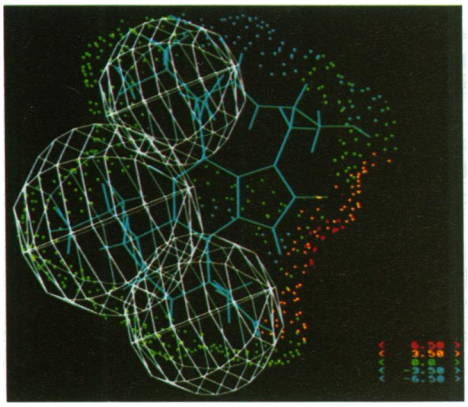

FIG. 4. Hydrophobic spheres generated on the solvent-accessible molecular surface of teleocidin B-4 (sofa form). Colors of dots indicate the electrostatic potentials divided into five levels. They are coded from blue (< −6.5 kcal/mol) to red (>6.5 kcal/mol).

posed on the graphic expression of these three-dimensionally tabulated data.

The system is used to (*i*) represent the spatial arrangement of various properties of molecules that facilitates the initial location of the trial molecules and (*ii*) calculate the goodness of fit values (11) on the basis of spatial similarity of the physical and chemical properties between molecules. They are displayed on a three-dimensional computer graphic display and are updated during interactive manipulation of the trial molecule, such as rotation, translation, and bond rotation.

With regard to hydrogen bonds, matching of the expected locations and character of the hydrogen bonding partner are judged between two molecules during the superposing process. Allowable locations were assumed to be from 2.5 to 3.1 Å in distance, whereas the allowable deviation from the orientation vector of X-H or Y-lone pair electrons (X, Y = N and O) was taken to be 30°. For all groups capable of hydrogen bonding the program provides the functions for generating the positions of lone-pair electrons automatically and for predicting the possible locations of hydrogen bonding partners, taking into account the bond-rotational allowance of the C-X bond in C-X-H and of the C-Y bond in C-Y-lone-pair electrons. The new atomic coordinates of trial molecules superposed successively are stored in a file from which the grid point data are calculated. The surface environment of the receptor cavity is described by the united grid point data resulting from those of superposed molecules.

RESULTS

Superposition of Teleocidin and Phorbol Ester. The two representative conformers of teleocidin B-4, sofa and twist forms, were used as the template molecules. The procedures of superposing the TPA molecule on the teleocidin B-4 molecule were performed independently for both forms. First, a three-dimensional grid with a regular interval of 0.5 Å was generated around the template molecule. For each grid point, various physical and chemical properties were calculated and stored (Fig. 3). Then, the TPA molecule was superposed interactively on the graphic expression of these properties characterized as above and was manipulated so as to attain better superposition as judged from the goodness of fit indices. The rotational degree of freedom in the bond between C-6 and C-20 was adjusted interactively. As a result of superposing the hydrophilic moieties, it became clear that the TPA molecule can interact with the receptor presumed for the sofa form through three hydrogen bonds, whereas through only two hydrogen bonds for the twist form. In the sofa form, the CH_2OH groups at C-9 in teleocidin and at C-6 in TPA (as hydrogen donors), the C=O groups at C-11 in teleocidin and at C-3 in TPA (as hydrogen acceptors), and amide NH in teleocidin and OH at C-4 in TPA (as hydrogen donors) can interact well with the same hydrogen bonding sites in the receptor. On the other hand, the twist form lacks the three-dimensional correspondence between amide NH in teleocidin and 4-OH in TPA, which are pointed in opposite directions.

With regard to the role of hydrophobic or lipophilic moieties in TPA-type promoters, although nonstereospecific interactions with membranes are supposed, it is also possible that they are required for strong interactions with the receptor. In this case, macroscopic coincidences of the shapes and

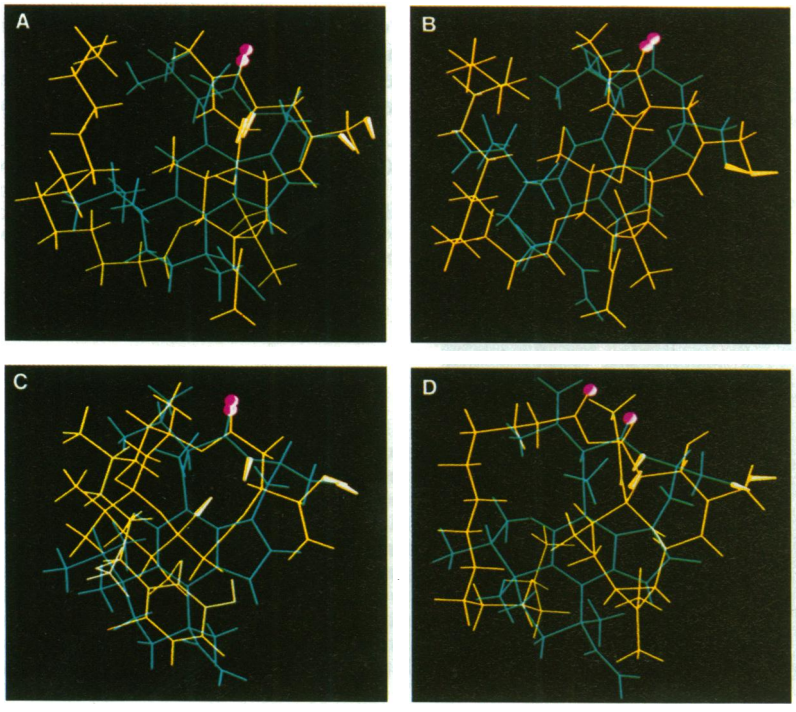

FIG. 5. The best superposition of TPA, aplysiatoxin, and 3TI on teleocidin B-4. For the definitions of painted balls and cones, see the legend to Fig. 3; those participating in hydrogen bonds to the common receptor are displayed. (*A*) TPA molecule (yellow) superposed on teleocidin sofa form (blue). (*B*) TPA molecule (yellow) superposed on teleocidin twist form (blue). (*C*) Aplysiatoxin molecule (yellow) superposed on teleocidin sofa form (blue). (*D*) 3TI molecule (yellow) superposed on teleocidin sofa form (blue).

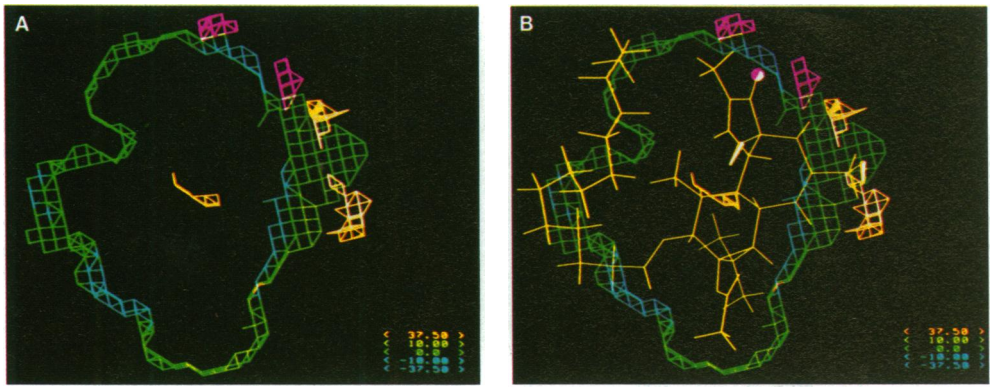

FIG. 6. (*A*) Constructed receptor cavity model based on the superposed structures of teleocidin B-4 (sofa form) and TPA molecules. The size and shape of the receptor cavity are shown by bird cage expression using color coding of electrostatic potentials with reversed signs from those of the promoter molecules (blue, negative; green, approximately zero; orange, positive). The converged sites and characters of hydrogen bonding hetero atoms in the receptor cavity are represented by cages (red, hydrogen acceptor; orange, hydrogen donor). Clipping was done only to the cage expression for the cavity. (*B*) TPA molecule incorporated in the receptor cavity.

relative sites of lipophilic moieties to other properties are important for the binding nature to the receptor. Thus, we attempted to define the conformation of the superflexible lipophilic chains so as to superpose them on the lipophilic moiety of the template molecule rationally by the following computational procedures. We have made use of spheres with various radii on a three-dimensional computer graphic display (named hydrophobic spheres) in order to specify the position and range of lipophilic regions. The surface regions where the absolute values of the electrostatic potentials, displayed by color-coded dots with the potentials (29), are continuously small were judged to be lipophilic. In the case of teleocidin B-4, three spheres were set up so as to cover not only the terpenoid moiety but also the isopropyl group at C-12 for each form (Fig. 4).

After superposing the hydrophilic parts of the trial molecule (TPA) on the corresponding parts of the template molecule (teleocidin), the conformations of the lipophilic chains were determined through a systematic conformational search. All possible conformers were generated by rotating all rotatable bonds with an appropriate angular interval and tested for geometrical constraints and/or conformational stability; the program CONFOM, which was developed for rapid conformational search with the supercomputer HITAC S-810, was employed. The conformers, whose lipophilic chains can be included within the spheres and also be energetically stable, were chosen for each trial molecule. Thus, the conformations of the lipophilic long chain of the TPA molecule were chosen for both forms of teleocidin, so that as much as possible of the chain could be enclosed within the spheres and be energetically stable.

One of the best superposing results of TPA on both conformers of teleocidin B-4 is shown in Fig. 5 *A* and *B*. Comparison of the surface areas and occupied volumes between the two superposed structures did not yield any significant difference between the sofa and the twist form. Consequently, the better agreement between the hydrophilic character of the sofa form and that of TPA leads to the conclusion that this form is the crucial conformer for the tumor-promoting activity.

A receptor cavity model was then constructed on the basis of the superposed structures of TPA and the sofa form of teleocidin B-4 as shown in Fig. 6*A*; the same receptor model incorporating the TPA molecule is shown in Fig. 6*B*. The receptor provides two hydrogen acceptor sites and one hydrogen donor site in one side of the cavity and a large lipophilic region in the other side of the cavity. Fig. 7 *A* and *B* illustrate stereoscopic views of the receptor cavity together with the trapped teleocidin and TPA molecules, respectively.

The superposition of the aplysiatoxin and 3TI molecules on teleocidin sofa form are shown in Fig. 5 *C* and *D*. The lipophilic chains of both molecules were also arranged similarly to the case of TPA, using the hydrophobic spheres on the teleocidin sofa form. The lipophilic chains of the two molecules can be stably included in the hydrophobic spheres to a similar extent to that of the TPA molecule. Both

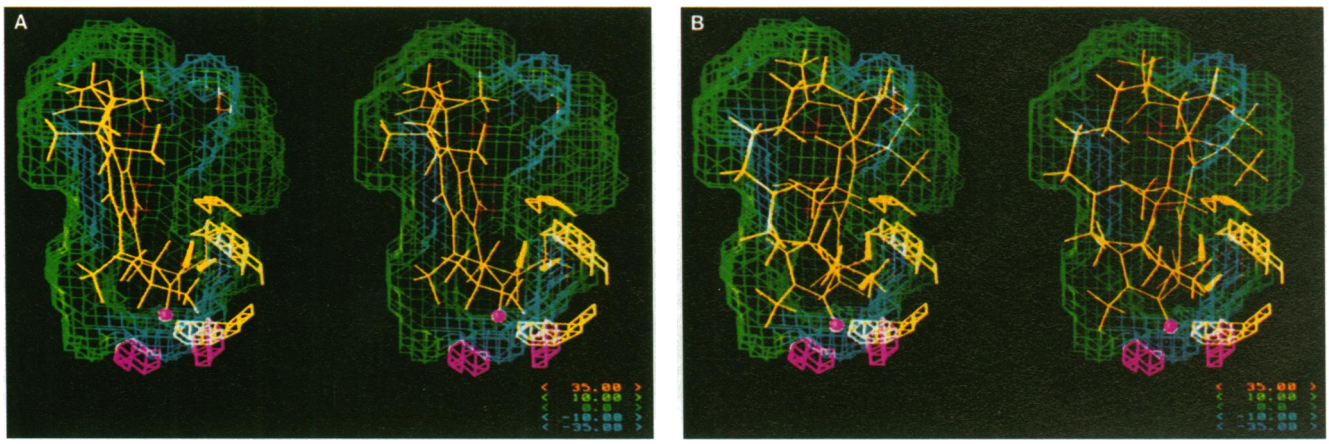

FIG. 7. Stereoscopic views of the receptor cavity incorporating teleocidin (*A*) and TPA (*B*) molecules. The cages are defined as in the legend to Fig. 6. The front side of the receptor cavity was clipped.

Table 1. Interatomic distances (Å) between the hetero atoms in teleocidin and the corresponding ones in TPA, aplysiatoxin, and 3TI molecules, which are superposed in this study

Compound	Interatomic distances (Å) relative to specific atoms in teleocidin		
	Amide NH	9-CH_2OH	11-C=O
TPA	4-OH (1.38)	6-CH_2OH (0.51)	3-C=O (0.48)
Aplysiatoxin	3-OH (3.53)	30-OH (0.60)	27-C=O (0.96)
3TI	4-OH (1.63)	6-CH_2OH (1.64)	21-C=O (3.16)

molecules can form three hydrogen bondings with the expected sites in the receptor. In aplysiatoxin, the OH groups at C-30 and C-3 and the C=O at C-27 function as the hydrogen bonding sites, and, in 3TI, the two OH groups at C-20 and C-4 and the ester C=O at C-21 participate. The interatomic distances between the hetero atoms in teleocidin and the corresponding ones in TPA, aplysiatoxin, and 3TI are listed in Table 1. This shows that the atoms involved in the hydrogen bonding to the same receptor sites are not necessarily superposed on one another positionally, as expected.

DISCUSSION

From the combination of chemical results and computer-assisted analyses of structure–activity relationships, the following conclusion was drawn. Of the two stable conformers of teleocidins, the sofa form can better bind to the same receptor as TPA, because hydrogen bondings seem to be most important for the specific binding. A model of the receptor pocket was constructed based on the superposition of teleocidins and TPA. The model shows the size and shape of the cavity, the expected sites and character of hydrogen bonding, and the surface electrostatic potentials. As the aplysiatoxin and 3TI molecules can be well adapted to this receptor cavity, it is clear that these four compounds possess common structural features required for tumor-promoting activity.

Two models have been proposed by superposing teleocidins and phorbols (6, 7). Both were based on the superposition of atomic positions of hetero atoms: such a superposition fundamentally does not make sense. The principal defect in Jeffrey's model is adoption of the wrong absolute stereochemistry. Another point is the assumption of correspondence between the phorbol ester 4-OH and teleocidins N-13. However, structure–activity studies of phorbol esters show that the 4-OH must be a hydrogen donor, whereas the teleocidins N-13 cannot be a hydrogen donor. A similar shortcoming is also seen in Wender's model. Although it is claimed that the indole NH of teleocidins corresponds to the phorbol 9-OH, our structure–activity studies indicate that the hydrogen at N-1 is not essential; moreover, this type of a nitrogen atom cannot be a hydrogen acceptor.

The proposed receptor model represents a binding site or a possible active region of the enzymes or other macromolecules that associate with these specific tumor promoters. However, we need further reliable information regarding structure–activity relationships, in particular, the role of hydroxyl groups in TPA (at C-4 and C-9). One approach will be to synthesize compounds in which the nine-membered ring is restricted in the sofa or twist form and to check the importance of the sofa conformation of teleocidins.

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