Susan Horwitz[3]. There are, of course, important differences between these models. One is that the model of Giannakakou *et al.* makes the baccatin portion of taxanes a necessary component of their actions, whereas that of Ojima *et al.* proposes it to be non-essential. Interestingly, another pharmacophore model has since been published by the laboratories of David Kingston and Susan Horwitz *et al.*[4] The Kingston–Horwitz model was based on the microtubule-stabilizing and mitotic-blocking activities observed with 2-*m*-azidopaclitaxel and 2-*m*-azidobaccatin III, and includes hypotheses related to epothilone binding at the paclitaxel site. Interestingly, baccatin III and 2-*p*-azido-baccatin III were found to be inactive. He *et al.* suggest that the C2 benzoyl ring of paclitaxel fits into the pocket formed by βHis227 and βAsp224, which agrees with the crystallography studies. Furthermore, the authors suggest that the baccatin system is a necessary component for taxanes and that the C2 benzoyl system of taxanes and the thiazole side-chain of the epothilones do occupy the same region of the protein when bound (akin to binding mode I of Giannakakou *et al.*).

In a recent article[5], Soong-Hoon Kim and co-workers at Bristol-Myers Squibb report that four C12,13-cyclopropyl analogs of epothilones A and B are indeed active as microtubule stabilizers and as potent antiproliferative agents. Nicolaou's group previously reported that a C12,13-cyclopropyl analog of epothilone A was inactive[6] but have since revised their structural assignment of the compound they synthesized (K.C. Nicolaou, unpublished). This structure–activity revision throws something of a shadow on the pharmacophore model of Giannakakou *et al.* If, however, the C12,13 region of the epothilones is taken simply as an electron-rich region (because the C12,13-oxiranyl, -alkenyl and -cyclopropyl groups all allow activity) that happens to map to the electron-rich region of the oxetane of taxanes and is not necessarily involved in a hydrogen bond to a protein-bound water molecule, then the Giannakakou *et al.* model might still retain potential veracity. In fact, in my opinion the results of Johnson *et al.* actually strengthen the argument of Giannakakou, because a hydrophobic region of the protein (βLeu215, βLeu228, βLeu273 and βPhe270) interacts with the C12,13 region of the epothilones in the model provided by binding mode II.

Concluding remarks

Thus, even though all these laboratories are approaching the problem using different tactics, they seem to be now arriving at somewhat similar (but, granted, still divergent) conclusions. Now, it is the job of the synthetic chemists and crystallographers to test their hypotheses.

Selected references

1 Giannakakou, P. *et al.* (2000) A common pharmacophore for epothilone and taxanes: molecular basis for drug resistance conferred by tubulin mutations in human cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* 97, 2904–2909

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Reply

Rick Gussio, Tito Fojo and Paraskevi Giannakakou

We are very grateful to Billy Day for his insightful comments on our research. In his commentary, Day provides us with a brief but thorough overview of the recent accomplishments of several laboratories investigating tubulin pharmacology.

We continue to develop our pharmacophore model through utilization of any relevant empirical data as results become available. In our view, molecular models are merely three-dimensional representations of such data, and as a consequence require continuous refinement. As an example, Day has indicated that the 12,13-cyclopropyl-epothilones were recently reported to be active against tubulin by investigators at Bristol-Myers Squibb. At the time of our publication[1], these analogs were reported to be inactive and hence, conformational exploration of carbons 9–15 of the macrolide of the epothilone molecule, was limited to the formation of a hypothetical hydrogen bond between a water molecule and the tubulin backbone. This hydrogen bond would be identical to one formed by the oxetane O portion of the rigid taxanes. The new data showing that the 12,13-cyclopropyl-epothilones were active prompted us to conduct a series of molecular dynamics and minimization docking simulations on these compounds. As a result, we found that the 12,13-cyclopropyl-epithilones can maintain the pharmacologically relevant structural features of epothilone B. Yet, at the same time, carbons 9–15 can also form a conformation that displaces the water and places the 12-methyl substituent very favorably in a hydrophobic cavity formed by the side-chains of Phe270 and Leu215 of the tubulin structure (Fig. 1).

The conformational versatility of the epothilone molecular scaffold enables these types of postulations. In agreement with Day, we concur with the notion that the crystallographical coordinates of flexible molecules (such as epothilone) provide us with a configuration but not necessarily the biologically active conformation. In other laboratories, the conformation of the epothilone crystal structure served as the basis for pharmacophore formation as a result of the fact that it is one of the lowest energy conformations. We did not follow this approach because it has been shown that most (if not all) flexible ligands bind to their receptors far from

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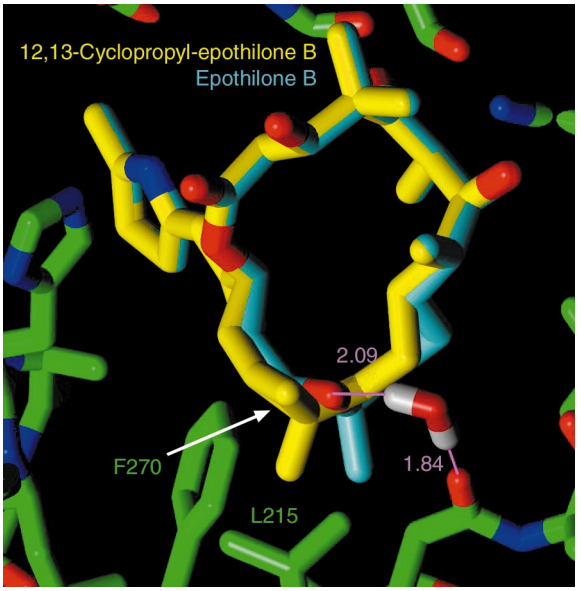

Fig. 1. Epothilone B (carbons in cyan) and 12,13-cyclopropyl-epothilone B (carbons in yellow) are docked onto tubulin in binding mode II. The tubulin backbone is shown in green. A water molecule is shown in red and white, whereas the hydrogen bonds with their respective distances in Å are shown in magenta. Notice that 12,13-cyclopropyl-epothilone B can maintain the identical pharmacophoric overlap with epothilone B. In addition, the non-polar hydrocarbon portion of the macrolide ring of 12,13-cyclopropyl-epothilone B (see arrow) adopts a new conformation that displaces the water and forms a more favorable hydrophobic contact with tubulin.

the global minimum (the most energetically favorable conformation), and not even at their local minima (higher energy conformations, still energetically feasible)[2]. This is an unattractive concept for molecular modelers because it expands the problem of identification of active conformers immensely. Furthermore, epothilones in particular have an even greater number of conformational isomers that require evaluation because they are capable of forming at least five hydrogen bonds whose favorable energy can be exchanged for intramolecular strain. In our case, the combination of the available mutation data, taxane and epothilone structure–activity relationship data, and energy-refined electron crystallographical coordinates, enabled us to narrow the conformers and form a pharmacophore concept.

As Day has emphasized, we are committed to testing and refining our pharmacophore concept. We have recently tested several epothilone analogs with a pyridine substitution, synthesized in the laboratory of K.C. Nicolaou, against our paclitaxel- and epothilone-resistant cell lines, harboring distinct β-tubulin mutations[3]. Our modeling studies had predicted that these compounds would retain activity against tubulin to a similar extent as the parent compounds. The data that we obtained confirmed our prediction and further supported the hypothesis that epothilone binding onto tubulin is most likely to occur according to binding mode II (Ref. 3).

Finally, we wish to conclude our reply with Day's final statement 'Now, it is the job of the synthetic chemists and crystallographers to test this hypothesis'.

Selected references

1 Giannakakou, P. *et al.* (2000) A common pharmacophore for epothilones and taxanes: molecular basis for drug resistance-conferred by tubulin mutations in human cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* 97, 2904–2909
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G-protein-coupled receptor dimers in the lime light

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Protein–protein interactions are crucial to all biological processes. Although it is well established that several cell-surface receptors mediate their actions as dimers[1], G-protein-coupled receptors (GPCRs) were generally considered to function as monomers. However, several recent studies have shed light on GPCR dimers.

GPCRs constitute the single largest family of cell-surface receptors and mediate physiological responses to a remarkably diverse array of stimuli. These receptors are characterized by seven transmembrane domains, with an extracellular N-terminus and a cytoplasmic C-terminus. GPCRs interact with, and signal through, heterotrimeric guanine-nucleotide-binding regulatory proteins (G proteins). On stimulation by a ligand, the receptor undergoes a conformational change that leads to activation of the G protein by GDP–GTP exchange, followed by uncoupling of the G protein from the receptor. The activated G protein can interact with a variety of effector systems and thus regulate various intracellular processes. Although classical models predict that GPCRs function as monomers, several previous studies have provided indirect evidence that the functional unit of GPCRs is a dimeric or oligomeric complex rather than a monomeric unit associated with the G protein.

Evidence in support of GPCR dimerization

Early pharmacological studies provided the initial clue that GPCRs could function as dimers and/or oligomers[2,3]. Target size analysis by radiation inactivation showed that the functional unit was a receptor with a molecular mass that was substantially greater than that predicted by denaturing techniques[4]. A variety of biochemical techniques such as crosslinking, photo-affinity labeling and size-separation chromatography provided additional support to the notion that GPCRs exist as dimers and/or oligomers[5,6]. The isolation of cDNA clones encoding these receptors enabled the crucial evaluation of GPCR dimerization. Antisera raised against epitope-tagged receptors or native receptors were used to visualize the receptor in heterologous cells and/or native tissue. Differential epitope tagging of the receptor cDNA followed by selective immunoprecipitation of the dimer was used to confirm the ability of GPCRs to dimerize[7]. However, until recently no studies had examined the formation of receptor dimers in live cells.

Bioluminescence resonance energy transfer

Using bioluminescence resonance energy transfer (BRET), a newly developed biophysical method to examine

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