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Evaluation of Novel Epothilone Analogues by means of a Common Pharmacophore and a QSAR Pseudoreceptor Model for Taxanes and Epothilones

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Taxanes (TX) and epothilones (Epo) comprise the most prominent classes of microtubule-stabilizing antimitotic agents (MSAA), which include three compounds currently in clinical use for the treatment of cancer: paclitaxel (PTX, Taxol), docetaxel (Taxotere), and the Epo B lactam ixabepilone (Ixempra). In spite of the clinical importance of these agents, the binding mode of either TX or Epo to their target protein (β-tubulin) has yet to be resolved. The interactions of taxanes (TX) and epothilones (Epo) with the tubulin/microtubule system was initially assumed to involve a common pharmacophore for both classes of compounds.[1] While this hypothesis was subsequently challenged on the basis of electron crystallography studies on a complex between tubulin polymer sheets and Epo A, the idea of a common pharmacophore for taxanes and epothilones was recently revived by results of solution NMR studies.[7]

Here, we describe the application of a 3D QSAR pseudoreceptor model, previously developed on the basis of the common pharmacophoric hypothesis, to a set of new Epo derivatives recently reported in the literature. Despite the structural differences between the molecules originally employed to build the model and the new set of analogues investigated here, predicted activities were in excellent agreement with experimental data. Moreover, the pharmacophore of Epo was found to be in agreement with the binding mode of Epo A with tubulin recently proposed on the basis of NMR studies. In fact, three out of four common pharmacophoric points between TX and Epo were satisfied by the model, thus further supporting the common pharmacophore hypothesis.

The PTX-complexed tubulin structure[2] was used to propose different binding models of Epo into the PTX binding site.[3] Amongst others, we have put forward the hypothesis of a common pharmacophoric model[4] on the basis of which a 3D QSAR pseudoreceptor model was built. Binding of Epo and TX to this model gave very promising results; predicted activity and interactions were in good agreement with both experimental activity trends and mutagenesis studies. In 2004, the structure of epothilone A (**1**, Figure 1) bound to the electron crystallography (EC) structure of tubulin, was proposed,[5] questioning the hypothesis of a common binding mode for the two classes of tubulin modulators. However, this structural model was not able to rationalize all the biological data available at that time and attempts to use the EC-derived bioactive conformation of **1** for the design of conformationally restricted derivatives of **1** was unsuccessful, thus prompting Snyder and co-workers to re-examine their Epo–tubulin binding representation.[6] Finally, Carlomagno and co-workers[7] reported the results of a solution NMR study on a complex between nonpolymerized tubulin (i.e., tubulin in an undefined, soluble, oligomeric state) and **1**. Intriguingly, the binding mode of **1** with tubulin, as derived from the NMR data, was in good agreement with our theoretical model. The different binding modes proposed by the above EC and NMR studies led us to re-assess the validity of the common pharmacophoric hypothesis for TX and Epo (and consequently the 3D QSAR model) with a series of Epo analogues recently reported, none of which had been used to build the original model. The binding mode of the new Epo analogues, predicted on the basis of their alignment to the common pharmacophoric model, led to activity predictions that were in very good agreement with the experimental data. The pharmacophoric model[4] common to Epo and PTX bound to β-tubulin suggested that the thiazole ring, the C1 carbonyl group and the C7 hydroxy substituent of Epo corresponded to the phenyl ring at C3′, the C2′ hydroxy group and the oxetane oxygen atom of PTX, respectively. As a consequence of this pharmacophoric alignment, the C12 substituent of Epo corresponded to the region between the C2 benzoyl and C4 acetyl groups of PTX (Figure 2).

While many Epo analogues had been described[8] at the time of publication of the pseudoreceptor model,[4] most of the activity data given in the literature related to their in vitro antiproliferative activity toward human cancer cells and not to binding affinity toward tubulin/microtubules. However, based on correlation between microtubule binding and antiproliferative activity,[9] we have assumed that IC_{50} values of the in vitro inhibition of human cancer cell proliferation could significantly account for and depend on the corresponding Epo–tubulin/microtubule interactions (i.e., in terms of ΔG of binding). However, to avoid possible complicating factors arising from different experimental conditions between laboratories, we have limited our analysis to a series of homogeneous biological data obtained for the human cervix carcinoma cell line KB-31.

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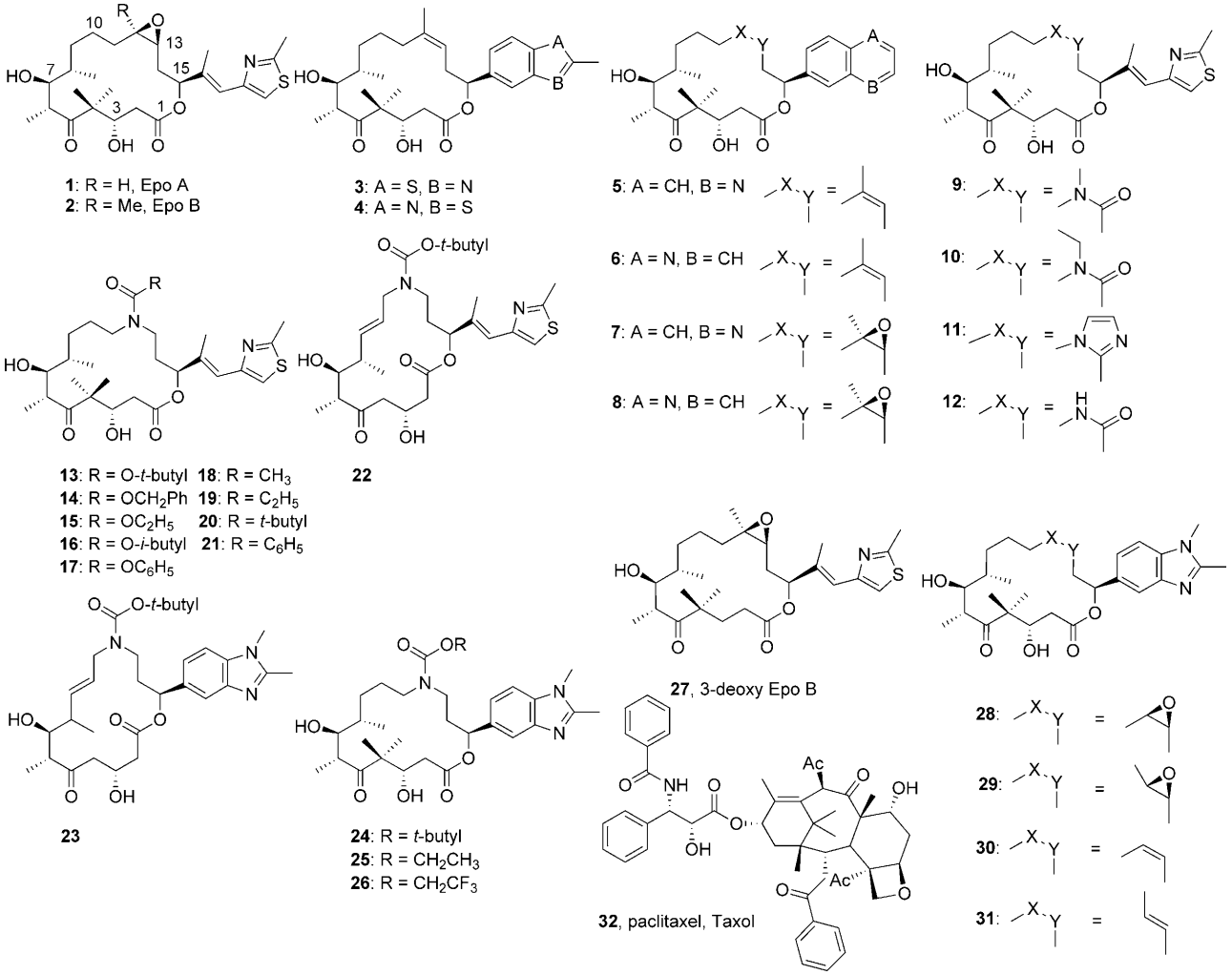

Figure 1. Paclitaxel and epothilone derivatives studied by means of the common pharmacophoric model for TX and Epo and by the pseudoreceptor model of tubulin.

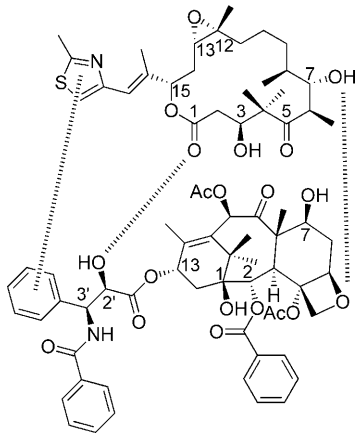

Figure 2. Schematic representation of the superposition pattern between epothilone B (top) and paclitaxel (bottom), as suggested by the common pharmacophoric model for epothilones and taxanes.

The structures of the compounds studied are depicted in Figure 1, and the corresponding IC_{50} values for inhibition of the KB-31 cell growth are summarized in table S1 in the Supporting Information. In particular, this set of compounds included Epo derivatives with a modified C15 side chain (**3**–**8**),[10] analogues with a nitrogen atom instead of the C12 (referred to as azathilones, **9**–**22**),[11] as well as compounds bearing both modifications (**23**–**26**).[12] The predicted ΔG values for tubulin binding were compared (at a qualitative level) to the binding energy of **1**. Compounds with calculated binding energies falling within a $\Delta\Delta G$ cutoff of 2.5 kcal mol^{-1} with respect to **1** were classified as active, while the remaining analogues ($\Delta\Delta G > 2.5$ kcal mol^{-1}) were classified as inactive, according to the activity classification used in our previous work.[4] Predicted activity values, calculated on the basis of the interactions between compounds and the pseudoreceptor model, are in good agreement with experimental data (table S1), thus indicating that the model provides a reliable basis for the rationalization of the experimentally observed structure–activity relationships (SAR), at least at a qualitative level. In fact, all the compounds predicted as being active showed experimental IC_{50} values lower than 97 nM; and all compounds predicted as being inactive showed IC_{50} values higher than 116 nM, with the exceptions of **13** and **19** (31 and 71 nM, respectively).

As from ligand–pseudoreceptor model,[4] interaction between Epo to β-tubulin was, in general, characterized by two hydrogen-bond interactions involving two out of three common pharmacophoric portions (Figure 2), the C7 hydroxy group and the C1 carbonyl oxygen, described by us as crucial elements for Epo activity,[4] while the remaining portions of Epo structures were characterized by lipophilic interactions with different hydrophobic pockets of the binding site.

Figure 3 depicts a comparison between the pharmacophoric points of PTX, as derived from the EC structure, and **1**, as calculated by means of the pharmacophoric-pseudoreceptor model derived from the EC structure or derived from the NMR

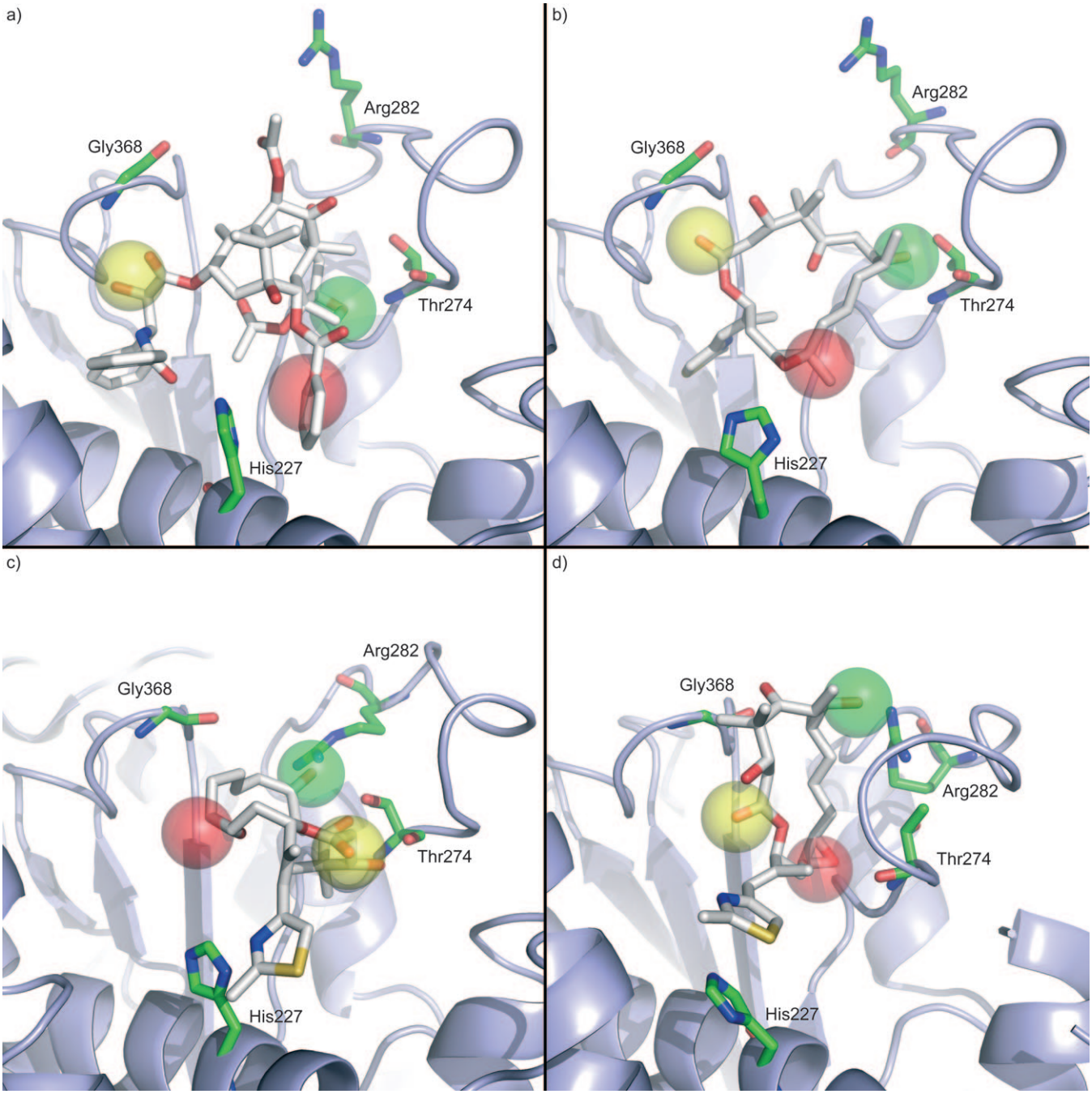

Figure 3. Comparison of the binding mode of paclitaxel and epothilones. Ligands (white) and binding-site key residues (green) are shown in stick representation. The remaining part of the β-tubulin secondary structure is rendered with pale blue ribbons. Pharmacophoric features are shown as semitransparent spheres. Hydrogens are omitted for sake of clarity. a) PTX (**32**) as found in the electron crystallography model (PDB: 1JFF).[2] Oxetane oxygen (green), C2′ hydroxy oxygen (yellow), C2 benzoyl group (red); b) Epo B (**2**) resulting from the 3D-QSAR pseudoreceptor model.[4] C7 hydroxy oxygen (green), C1 carbonyl oxygen (yellow), C12 substituent region (red); c) Epo A (**1**) as found in the electron crystallography model (1TVK).[5] C7 hydroxyl oxygen (green), C1 carbonyl oxygen (yellow), C12 substituent region (red); d) Epo A (**1**) as found in the tubulin-bound NMR model.[7] C7 hydroxy oxygen (green), C1 carbonyl oxygen (yellow), C12 substituent region (red).

study. In detail, the 7-OH group of Epo seemed to play a pivotal role in driving the overall binding, mainly by interacting with Thr 274. A conformational analysis of **2** in the binding site[4] suggested that the OH group could constitute an acceptor/donor motif with the backbone NH and the side chain of Thr 274, mimicking the role of the oxetane oxygen atom of PTX (see Figure 3 a and 3 b). The correspondence of the Epo 7-OH and the oxetane ring is in agreement with the results of mutagenesis studies,[3b] which showed that a βT274I mutation had a detrimental effect on activity, and this effect is more pronounced on Epo than on PTX activity. This observation suggests that it may be possible for PTX to use functional groups other than the oxetane ring to make additional interactions with the protein, thus overcoming the loss of the Thr-dependent hydrogen bond with the oxetane ring. In contrast, Epo did not show any possibility to replace the hydrogen bond donor–acceptor motif involving its 7-OH group upon βT274I mutation.[3b] Moreover, the inversion of configuration at C7 in Epo was expected to be associated with a decrease in activity, as hydrogen-bond interactions with Thr 274 would no longer be possible. While this hypothesis was supported experimentally with analogues where both the configurations at C6 and C7 were inverted, no Epo analogues have been described so far with the inversion of configuration at C7 as the sole modification.

The C1 carbonyl group was suggested as a hydrogen-bond acceptor for the backbone NH of Gly 368, thus stabilizing the overall Epo–tubulin complex. The important role of this carbonyl group as indicated by the model was in agreement with the experimental SAR of Epo showing that modifications of this region were not tolerated.[3c, 13, 14]

In addition, the C3 hydroxy group seemed to be exposed to the solvent and not involved in polar interactions with the protein. As a consequence, removal of this group was predicted to have only a limited effect on the binding energy and on cellular potency, in agreement with the available experimental data on 3-deoxy-Epo, such as **27**.[15–17] In contrast, in the EC model[5] this group was proposed to stabilize the binding of Epo A by establishing direct hydrogen-bond interactions with Thr 274 (see Figure 3 c).

Moreover, the C9–C10 region appeared to be highly important for the overall conformation of the macrolactone ring, and the presence of conformational constraints in this part of the macrocycle seemed to affect the activity. While the incorporation of a *trans* double bond (C9=C10) in natural Epo has been reported to enhance antiproliferative activity in the majority of cell lines investigated,[18] somewhat surprisingly, the opposite trend was found for 12-aza-Epo **22** and **23**. In fact, the strain introduced by the double bond (C9=C10) influenced molecular alignment and the formation of the hydrogen bond with the C7 hydroxy group, ultimately resulting in a loss of activity compared with the saturated parent compounds. However, further studies on unsaturated C9–C10 analogues are required to recalibrate the model and to account for the appropriate weight of this feature in activity estimation and prediction.

The effects on binding energy resulting from modifications to the C12–13 region seemed to be related to steric properties of substituents and to their lipophilicity rather than conformational variations of the macrocycle. In particular, binding interactions were not affected by changes from a *cis* to *trans* epoxide moiety (**28** versus **29**) or double bond (**30** versus **31**). On the other hand, polar groups appeared to be less tolerated (**9**–**12**).[12b] In fact, the lack of activity found in molecules incorporating (substituted) amide moieties (**9**, **10**, **12**) or an imidazole ring (**11**) can be related to unfavorable interactions of the oxygen or nitrogen at C13 and the hydrophobic pocket that is usually occupied by the C12 methyl group of **2** or remains empty, in the case of **1**.

According to our common pharmacophoric model, substituents at the C12–C13 region of Epo correspond to the portion of the PTX structure between the C2 benzoyl and the C4 acetyl groups.[4] In particular, Epo derivatives bearing a C12 methyl group are generally more active than the corresponding desmethyl analogues,[13] while groups bulkier than methyl are known to be tolerated only to a limited degree.[13, 19–20] The hydrophobic cavity engaged by these groups is delimited by His 229 that is known to prevent hydrophobic collapse of PTX C3′ and C2 phenyl rings.[2b] As a consequence of its role, that residue has been considered as not protonated in the pseudoreceptor model. Due to the hydrophobic character of the cavity into which the C12 substituents protrude, nonpolar groups are favored at this position. Short and flexible lipophilic groups can engage the pocket without compromising the overall binding mode because potential clashes with these substituents can be avoided by conformational adaptations of the macrocycle as a whole.

Although this region of the binding site was able to tolerate a substituted nitrogen atom at position 12 of Epo, activity of each analogue depended on the specific substituent on the nitrogen. In detail, a decreased activity was observed for 12-aza-Epo bearing substituents of increasing size (from a 12-ethoxycarbonyl, **15**, to a 12-*i*-butoxycarbonyl, **16**, and to a 12-phenoxycarbonyl group, **17**), mainly due to the difficulty to arrange bulkier groups within the pocket. Extended and/or bulky substituents (e.g., in **13**, **14**, **16** and **20**) generally led to a destabilization of the modeled complexes, while **17** and **21** were completely unable to form stable complexes with the pseudoreceptor model. Moreover, the model predicted that compound **13** would be inactive, in disagreement with its experimental antiproliferative activity, while the activity of **19** was underestimated, and the antiproliferative effect of **15** was overestimated. These discrepancies in predicted activity may indicate that the bioactive conformation of 12-aza-Epo deviates from that of the polyketide-based natural products and closely related analogues, and therefore may not be appropriately described by our pharmacophoric model. Alternatively, parameters such as cellular uptake, intracellular distribution, or cellular stability may be significantly different between analogues based on a (modified) polyketide scaffold or on an aza macrolide core, but no data on these issues are available in the literature.

Compounds bearing fused heterocycles at C15 showed an alignment very similar to that found for **1**, characterized by the thiazole ring embedded in a hydrophobic region close to the Phe 270 side chain, which also accommodates the C3′ phenyl

ring of PTX. On this basis, different rings attached to C15, even if bulkier than the original thiazole moiety, were tolerated and led to activity retention or enhancement because they satisfied the requirement for hydrophobic interactions with the binding site. The shape of the benzimidazole (**23**–**26**, **28**–**31**), benzothiazole (**3** and **4**), and quinoline (**5**–**8**) moieties are comparable with each other and can all be overlapped to the side chain of Epo in our binding mode. As such, the model is able to account for the high activity of compounds bearing fused aromatic heterocycles instead of the thiazolylvinyl moiety. In contrast, the model is unable to explain differences in activity that are caused by changing the nitrogen position in the heterocycle (see compounds **3**–**8**).

On the basis of EC studies on tubulin polymer sheets complexed with **1**,[5] it was proposed that TX and Epo, although sharing the same binding site, did not have a common pharmacophore for their interactions with β-tubulin. In contrast, more recent results from an NMR study in solution on Epo bound to tubulin[7] suggested that, although the tubulin-binding site adopted a different conformation with **1** bound, compound **1** and PTX actually share the same essential interactions, thus supporting the hypothesis of a common pharmacophore (see Figure 3a and 3d). Surprisingly, despite substantial differences between the Epo bioactive conformation obtained from the QSAR model and that proposed on the basis of NMR studies, the key protein–ligand interactions identified by NMR are in good agreement with the common pharmacophore proposed by us (Figure 3b and 3d). All the pharmacophoric points, with the exception of the aromatic portions (see below), are clustered in the same regions of the protein structure with their counterparts of the NMR-derived model.

In particular, the 7-OH group of Epo was found to form essential interactions in both the model and the NMR-derived structure; it forms crucial hydrogen bonds with the electrostatic network composed of Thr 274 and Arg 282. As alluded to above, this finding was also supported by the results of mutagenesis studies.[3a] Moreover, both the model and the NMR structure showed that the 3-OH group was not involved in any direct interaction with the protein, thus suggesting it is not essential for activity, in agreement with additional experimental data.[15–17] The theoretical model also suggested the C1 carbonyl oxygen forms part of a hydrogen bond with Gly 368 and mimics the C2′ hydroxy group of the NMR PTX structure. In the NMR structure, this group was too far from any protein hydrogen-bond donor to form a direct hydrogen bond, but NMR study suggested[7] that interactions between the C1 carbonyl group and the protein were likely to be mediated by a water bridge. The most pronounced differences between our model and the NMR-derived structure were found for the position of the thiazole ring at the C15 side chain. In the experimental structure, the thiazole ring occupied the region corresponding to the C3′ benzamido moiety of PTX and interacted with the imidazole ring of His 229. In contrast, in the pharmacophoric model, this group corresponded to the C3′ phenyl ring of PTX, engaging the same hydrophobic cavity.

Finally, both the NMR and the theoretical models suggested that the C12 substituents of Epo should be located in the middle of a hydrophobic cavity close to Phe 270, suggesting that small apolar substituents could give profitable interactions in this region, in agreement with SAR analysis.

While the original hypothesis of a common pharmacophore for PTX and Epo was questioned due to results of an EC study on tubulin polymer sheets complexed with Epo A, the same hypothesis was recently revived through findings of NMR experiments with soluble tubulin. Our original common pharmacophoric model and the resulting pseudoreceptor model are in good agreement with the model proposed on the basis of the solution NMR studies. In particular, three out of the four pharmacophoric points are comparable between the two models. The predictive performance of the pseudoreceptor model has been confirmed by the good agreement with the experimental biological data of a series of recently synthesized Epo analogues. The model is able to account for activity changes associated with major modifications in the Epo structure (i.e., C15 side chain and C12 position)[4] further validating the hypothesis of a common pharmacophore between PTX and Epo. Due to the complex character of this interaction, additional experimental data will be necessary to clarify the details. Despite the intrinsic limitations and the level of detail of the pseudoreceptor model, the ability to identify most of the essential interactions for both TX and Epo, and to discriminate between active and inactive molecules, confirm its validity as a useful tool for designing derivatives targeting the paclitaxel-binding site. Further biological data and new Epo derivatives will be necessary to clarify the details involved in the interaction with the tubulin.

Computational Methods

The ligands were prepared and processed following the previously reported protocol.[4] For details on ligand alignment, equilibration and evaluation procedures, see the Supporting Information.

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Keywords: antitumor agents · epothilones · molecular modeling · pharmacophores · taxanes

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