A common pharmacophore for epothilone and taxanes: Molecular basis for drug resistance conferred by tubulin mutations in human cancer cells

Paraskevi Giannakakou†, Rick Gussio‡, Eva Nogales§, Kenneth H. Downing§, Daniel Zaharevitz‡, Birgit Bollbuck¶, George Poy, Dan Sackett**, K. C. Nicolaou††, and Tito Fojó*†

*Medicine Branch, National Cancer Institute, and †Genetics and Biochemistry Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892; ‡Target Structure-Based Drug Discovery Group, Information Technology Branch, and §Laboratory of Drug Discovery Research and Development, Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, Frederick, MD 21702; ¶Life Science Division, Lawrence Berkeley National Laboratory, and Molecular and Cell Biology Department, University of California, Berkeley, CA 94720; and †Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037

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The epothilones are naturally occurring antimitotic drugs that share with the taxanes a similar mechanism of action without apparent structural similarity. Although photoaffinity labeling and electron crystallographic studies have identified the taxane-binding site on β-tubulin, similar data are not available for epothilones. To identify tubulin residues important for epothilone binding, we have isolated two epothilone-resistant human ovarian carcinoma sublines derived in a single-step selection with epothilone A or B. These epothilone-resistant sublines exhibit impaired epothilone- and taxane-driven tubulin polymerization caused by acquired β-tubulin mutations (β274Thr→Ile and β282Arg→Gln) located in the atomic model of αβ-tubulin near the taxane-binding site. Using molecular modeling, we investigated the conformational behavior of epothilone, which led to the identification of a common pharmacophore shared by taxanes and epothilones. Although two binding modes for the epothilones were predicted, one mode was identified as the preferred epothilone conformation as indicated by the activity of a potent pyridine-epothilone analogue. In addition, the structure–activity relationships of multiple taxanes and epothilones in the tubulin mutant cells can be fully explained by the model presented here, verifying its predictive value. Finally, these pharmacophore and activity data from mutant cells were used to model the tubulin binding of sarcodictyins, a distinct class of microtubule stabilizers, which in contrast to taxanes and epothilones interact preferentially with the mutant tubulins. The unification of taxane, epothilone, and sarcodictyin chemistries in a single pharmacophore provides a framework to study drug–tubulin interactions that should assist in the rational design of agents targeting tubulin.

The clinical success of paclitaxel (PTX) and docetaxel has stimulated a search for compounds with a similar mode of action and has resulted in the identification of three nontaxane chemical classes of natural products: the soil bacteria-derived epothilones A and B (Epo A and Epo B) (1), the marine sponge-derived discodermolide (2, 3), and the coral-derived eleutherobins/sarcodictyins (4, 5). All three classes stabilize microtubules (MTs) and competitively inhibit the binding of PTX to tubulin polymers, indicating overlap of binding sites (1–3, 6, 7). The epothilones, however, display some superior qualities: they are water soluble, can be produced in large quantities through bacterial fermentation, and retain activity against multidrug-resistant (MDR) cell lines and tumors (5, 8, 9).

The PTX-binding site on β-tubulin has been identified both by electron crystallography, showing PTX bound on the αβ-tubulin dimer (10), and by photoaffinity labeling, which has identified amino acids β1–31 and β217–233 as important areas for PTX binding (11, 12). Similar studies, however, for epothilones are not available. In this study, we present a report of epothilone-resistant (EpoR) human cancer cell lines with acquired β-tubulin mutations. The residues involved, β274 and β282, both map near the taxane-binding site in the atomic model of tubulin (10). These mutations affect the ability of epothilones to induce tubulin polymerization as well as inhibit cell growth. Using molecular modeling and guided by the mutation data and the activity profile of several MT-stabilizing agents against the cell lines with mutant tubulins, we were able to identify a common pharmacophore shared by taxanes and epothilones and in turn model Epo binding onto tubulin. In addition, we have identified an MT-active agent of the sarcodictyin family which, in contrast to taxanes and epothilones, is preferentially active against the tubulin mutants, and we have modeled the way it binds tubulin.

The unification of taxane, epothilone, and sarcodictyin chemistries in a single pharmacophore, and the fact that their structure–activity relationship profile against both the parental and mutant tubulins can be successfully explained by our modeling and docking studies, provide a framework to study drug–tubulin interactions that should assist in the rational design of agents targeting tubulin.

Materials and Methods

Materials. PTX and docetaxel were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, Bethesda, MD. Epo A and B (13), Epo B pyridine analogue,†† and sarcodictyin A analogue (14) were prepared by chemical synthesis.

Abbreviations: PTX, paclitaxel; MT, microtubule; Epo A, epothilone A; Epo B, epothilone B; EpoR, epothilone-resistant.
†To whom reprint requests should be addressed at: Medicine Branch, NCI, NIH, Bldg. 10, Room 12N226, 9000 Rockville Pike, Bethesda, MD 20892. E-mail: evigi@box-e.nih.gov.
††The synthesis of this Epo B analogue will be reported elsewhere.

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Baccatin and mouse monoclonal anti-β-tubulin antibody (Tub2.1) were from Sigma, and horseradish peroxidase-conjugated sheep anti-mouse IgG antibody was from Amersham.

**Cell Culture and Cytotoxicity Assay.** The EpoR cell lines, 1A9/A8 and 1A9/B10, were isolated in a single step after exposure of the human ovarian carcinoma cell line A2780 (1A9) to lethal concentrations (IC50) of either Epo A or Epo B (IC50 concentrations: 6 nM for Epo A and 0.5 nM for Epo B). After an initial expansion, the concentration of epothilones in the culture medium was gradually increased to 30 nM for Epo A and 5 nM for Epo B. Cells were maintained in drug, in a 5% CO2 humidified atmosphere at 37°C in RPMI medium 1640 (GIBCO/BRL) containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) (GIBCO/BRL). Before an experiment, the 1A9/A8 and 1A9/B10 cells were cultured for at least 7–10 days in drug-free medium. Cytotoxicity assays using the protein-staining sulfonfodamine B method were performed in 96-well plates as described previously (15), by seeding 500 cells per well and incubating with cytotoxic agents for 4 days.

**Tubulin Polymerization Assay.** Quantitation of the degree of in vivo tubulin polymerization in response to MT-stabilizing agents was performed as previously described (15). Briefly, cells were plated in 24-well plates and the following day were exposed to increasing concentrations of PTX or epothilones for 5 h. Then, cells were lysed in a hypotonic buffer (1 mM MgCl2/2 mM EGTA/0.5% Nonidet P-40/2 mM phenylmethylsulfonyl fluoride/200 units/ml aprotinin/100 μg/ml soybean trypsin inhibitor/5.0 mM e-aminocaproic acid/1 mM benzamide/20 mM Tris-HCl, pH 6.8), the cytoskeletal and cytosolic fractions containing polymerized (p) and soluble (s) tubulin, respectively, were separated by centrifugation, resolved by electrophoresis through SDS/10% polyacrylamide gels, and immunoblotted with an antibody against β-tubulin.

**PCR and Sequencing of β-Tubulin.** PCR amplification and sequencing of the predominant β-tubulin isotype (protein class I/gene M40) from the EpoR clones (1A9/A8 and 1A9/B10) were performed with overlapping sets of primers as previously described (15).

**Molecular Modeling Methods.** For molecular modeling methods and docking of epothilones and sarcodictyins on the αβ tubulin crystal structure, please refer to the supplemental data on the PNAS web site, www.pnas.org.

In brief, the initial model of Epo B was the crystallographic coordinates (16) and was optimized (PM3 Hamiltonian) (rms deviation = 0.087 Å). Molecular mechanics potentials (CFF91 force field) (17) were selected to optimize as close as possible to the crystal structure. High-temperature (1000 K) molecular dynamics (MD) was used to generate Epo conformations. Conformers of Epo were analyzed by tracking distances between the epoxide O and other atoms to identify a common pharmacophore with taxanes. The Epo/docetaxel overlap model was formed by using template forcing. The deposited tubulin structure (10) (Refcode 1TUB) was energy refined by using tethered minimizations. Epo was docked into the binding site formerly occupied by docetaxel and optimized. Docetaxel was completely ligated by water to the tubulin structure and optimized.

**Results and Discussion**

Two EpoR clones were isolated by selecting 1A9 human ovarian carcinoma cells with Epo A or Epo B. The two clones, designated 1A9/A8 and 1A9/B10, are 25- to 57-fold resistant to Epo A and B and 5- to 10-fold cross-resistant to PTX, docetaxel, and their precursor, baccatin (Table 1). Neither of the EpoR clones express the resistant phenotype most likely resulted from impaired Epo gene (MDR1) in both Epo R clones and the 1A9 cells from which they were derived. Class I β-tubulin is one of six different β-tubulin isotypes expressed in human cells, and it accounts for 90% of total β-tubulin mRNA in 1A9 cells (15) and the EpoR clones (data not shown). A different point mutation was iden-

![Epothilone B-Pyridine Analogue](image)

![Sarcodictylin A Analogue](image)

### Table 1. Cytotoxicity profile of 1A9/EpoR cells to drugs acting on MTs

<table>
<thead>
<tr>
<th>Drug</th>
<th>1A9 IC50† nM</th>
<th>1A9/A8 (μ274) IC50†</th>
<th>1A9/B10 (μ282) IC50†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX</td>
<td>2 ± 0.23</td>
<td>19.5 ± 1.14 (10)</td>
<td>13 ± 0.58 (6.5)</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>1 ± 0.15</td>
<td>7 ± 0.45 (7)</td>
<td>5 ± 0.25 (5)</td>
</tr>
<tr>
<td>Baccatin</td>
<td>550 ± 70</td>
<td>3950 ± 1060 (7)</td>
<td>3000 ± 870 (6.4)</td>
</tr>
<tr>
<td>Epo A</td>
<td>3 ± 0.42</td>
<td>127 ± 14.7 (40)</td>
<td>182 ± 6.25 (57)</td>
</tr>
<tr>
<td>Epo B</td>
<td>0.22 ± 0.03</td>
<td>5.4 ± 0.64 (25)</td>
<td>5.2 ± 0.35 (24)</td>
</tr>
<tr>
<td>B-pyridine</td>
<td>0.1 ± 0.015</td>
<td>3 ± 0.25 (30)</td>
<td>6 ± 0.60 (60)</td>
</tr>
<tr>
<td>Sarc. A analogue</td>
<td>3.75 ± 0.02</td>
<td>1.18 ± 0.12 (0.3)</td>
<td>0.45 ± 0.018 (0.12)</td>
</tr>
</tbody>
</table>

*IC50 values (±SEM) represent the mean of 3–13 independent 96-h growth inhibition assays. Values in parentheses are fold resistance factors, defined as the IC50 value obtained for the β-tubulin mutant line divided by that obtained for the parental line.

†Sarcodictyin A analogue is methylated at the C-3 oxygen atom of the parent compound. This analogue is shown as an active member representative of the sarcodictyin family.

‡The fold resistance factors in parentheses are less than 1 because the IC50 values for the β-tubulin mutant cells are lower than the IC50 for the 1A9 parental cells.

**PHARMACOLOGY**
A template to fit the Epo molecule onto the baccatin ring system. Next, because the C12,C13 epoxide is the most rigid feature. This is in agreement with our findings that in the absence of drug the Epo B-selected clone, I9/10, harboring the 282Arg→Gln mutation, has a significantly slower growth rate than the parental I9/10 cells. The doubling times for I9, I9/A8, and I9/B10 cells are 24 h, 30 h, and 50 h, respectively. A combined effect on both drug binding and lateral interactions is also possible.

The data presented thus far do not allow one to determine a preference between the two modes of Epo binding shown in Fig. 2. However, drug sensitivity data in I9 cells and the previously described PTX-selected cell line (PTX10), containing a 270Phe→Val mutation (15), provided us with a tool to test this conformational hypothesis. Specifically, drug sensitivity data using an Epo B analogue†† with a pyridine moiety in place of the thiazole ring of the parent compound (Table 1) allowed us to identify binding mode II (Fig. 2) as the preferred conformation of epothilones. This preference is because the pyridine ring is bulkier than the thiazole side chain, which in binding mode II is in close proximity to Phe-270. If binding mode II is preferred, one would predict that the 270Phe→Val mutation would have a greater impact on the sensitivity of the pyridine-containing Epo B derivative compared with the thiazole-containing molecule.

This prediction is confirmed by the cytotoxicity data, which show that the 270Phe→Val mutation has only a 3-fold effect on the sensitivity of the thiazole-containing Epo B (15) compared with a 10-fold change for the pyridine Epo B (IC50 values: 0.1 nM for I9 and 1 nM for the 270Phe→Val mutant cells). While these data, together with the fact that the Epo B-pyridine analogue is equipotent to Epo B, provide strong evidence in support of binding mode II, further empirical crystallographic evidence will be required to determine which binding mode is more likely.

The model presented here is in agreement with studies of both taxane and Epo structure–activity relationships (SAR). For example, SAR studies have shown that the C9–C5 oxetane ring of taxanes is absolutely required for activity (20), whereas in epothilones replacement of the C12,C13 epoxide with a cyclopropane ring results in total loss of activity both in vitro with purified tubulin and in whole cells (21). In the proposed Epo/tubulin pharmacophore model, the oxygen atoms contained in the oxetane of taxanes and the epoxide of epothilones overlap (Fig. 2). Both the epoxide and the oxetane are restricted ethers to reiterate, these two conformations were the only ones that met the intramolecular pharmacophore requirements and at the same time fit the docetaxel–tubulin binding site model in a manner consistent with the tubulin mutation data. We emphasize that in the absence of additional biological or crystallographic data, the two different conformations of epothilones presented here appear equally likely.

Using this pharmacophore and guided by the mutations in the EpoR cells, we succeeded in docking Epo on an energy-refined model of the 3.7-Å density map of the docetaxel-binding site on β-tubulin (Fig. 3). In agreement with the cross-resistance data and the in vivo polymerization studies, the model predicts that the change in residue 274 from Thr to Ile should have a greater impact on the binding of epothilones as compared with that of taxanes. The model predicts that the reason for this is that epothilones use their C7-OH group to hydrogen bond in the vicinity of the Thr-274; when Thr-274 is mutated to Ile, this hydrogen bond is disrupted. In contrast, taxanes, which have hydrogen bond donors or acceptors at the C10, C9, and C7 positions, can form alternate hydrogen bonds. The 282Arg→Gln mutation sits on the M loop (Fig. 3), identified as the major region in lateral contacts between protofilaments (19), and is located near the common binding site of taxanes and epothilones. As the N terminus of the M loop is part of the taxane ring-binding region, the mutation of Arg-282 could directly affect the binding of taxanes and epothilones. In addition, the mutation could cause a disruption of lateral contacts. This is in agreement with our findings that in the absence of drug the Epo B-selected clone, I9/B10, harboring the 282Arg→Gln mutation, has a significantly slower growth rate than the parental I9/10 cells and the Epo A-selected clone, I9/A8. The doubling times for I9, I9/A8, and I9/B10 cells are 24 h, 30 h, and 50 h, respectively. A combined effect on both drug binding and lateral interactions is also possible.

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containing conformationally rigid oxygens that are likely to function as hydrogen bond acceptors. In the tubulin structure, the C4–C5 oxetane of docetaxel is located near a cluster of polar backbone atoms from tubulin residues 273, 275, and 276, with the hydroxyl side chain of Thr-β274 also located in this hydrophilic area. In the Epo tubulin model the epoxide is similarly disposed. In addition, studies have shown that with the C12,C13 epoxide present, some substitutions at C12 lead to analogues with increased biological activity (13). Included among these is the naturally occurring Epo B, which differs from Epo A by a single methyl group substitution at C12, a modification that increases its potency by 14-fold (see Table 1). According to the model presented here, the C12 methyl group stabilizes a favorable hydrophobic interaction in the vicinity of the side chains of Leu-β273, Leu-β215, Leu-β228, and Phe-β270; this may account for the increased potency of Epo B compared with Epo A. Furthermore, Epo analogues in which the C12,C13 epoxide is removed and replaced by a double bond (deoxy-12,13-olefinic derivative), display intact enhancement of in vitro tubulin-polymerization-enhancing activity, likely resulting from its weak binding to tubulin. Enhanced baccatin binding has been achieved in the taxanes with the introduction of the C13 and the C2 side chains, the importance of which is underscored by the electron crystallographic data showing the docetaxel side chains in contact with β-tubulin (10). Although epothilones lack the overall bulk of taxanes, their macrolide ring system provides a larger hydrophobic core compared with the diterpene ring system of baccatin. This, coupled with the occupancy of epothilones' side chain in the vicinity of the C13 side chain of taxanes and the critical hydrogen bonds formed by the C1-OH, C7-OH, and epoxide oxygens, enables epothilones to interact with high affinity with tubulin even though they have significantly less molecular volume than taxanes. A recent report of a common pharmacophore between nonataxel (a non-aromatic PTX analogue) and epothilones proposes that baccatin is a nonessential component of these drugs (23). This conclusion was based on a relatively inactive analogue that was designed before data on the taxane-binding site, available from the crystal structure of α/β tubulin dimer (10), could be considered. Thus, our modeling in combination with the mutational data reinforces our belief that baccatin is an essential moiety for tubulin binding and should not be viewed as a passive scaffold holding functional groups. We would

One way to conceptualize these observations is to compare the epothilones with baccatin, the “inactive” taxane precursor. Baccatin is a structurally rigid molecule, with poor in vitro tubulin-polymerization-enhancing activity, likely resulting from its weak binding to tubulin. Enhanced baccatin binding has been achieved in the taxanes with the introduction of the C13 and the C2 side chains, the importance of which is underscored by the electron crystallographic data showing the docetaxel side chains in contact with β-tubulin (10). Although epothilones lack the overall bulk of taxanes, their macrolide ring system provides a larger hydrophobic core compared with the diterpene ring system of baccatin. This, coupled with the occupancy of epothilones' side chain in the vicinity of the C13 side chain of taxanes and the critical hydrogen bonds formed by the C1-OH, C7-OH, and epoxide oxygens, enables epothilones to interact with high affinity with tubulin even though they have significantly less molecular volume than taxanes. A recent report of a common pharmacophore between nonataxel (a non-aromatic PTX analogue) and epothilones proposes that baccatin is a nonessential component of these drugs (23). This conclusion was based on a relatively inactive analogue that was designed before data on the taxane-binding site, available from the crystal structure of α/β tubulin dimer (10), could be considered. Thus, our modeling in combination with the mutational data reinforces our belief that baccatin is an essential moiety for tubulin binding and should not be viewed as a passive scaffold holding functional groups. We would

Fig. 2. Proposed common overlaps of PTX and Epo B. (A and B) Atoms shown in red represent the pharmacophore shared by the two drugs. X is a pseudoatom at the centroid of the 4-gem-dimethyl, 3-OH, 7-OH system of Epo B, as well as the corresponding groups in PTX. The average distance of X from the epoxide O of Epo B is 6.93 Å (SD = 1.18 Å) as measured in the 100 lowest-energy conformations extracted from 10 separate 100-ps molecular dynamics simulations under 10 different dielectric conditions. Displayed in blue are atoms overlapping as a result of a template-forced minimization of Epo B onto PTX. (C and D) Three-dimensional overlap of the common substituents of Epo B (carbon atoms shown in cyan) with those of docetaxel (carbon atoms shown in yellow). For both molecules, oxygen atoms are displayed in red and nitrogen atoms are displayed in blue. Functional groups have been identified in white for docetaxel and black for Epo B. The two Epo conformations presented here reflect the two potential binding modes for Epo interaction with tubulin. Binding mode I in A and C and binding mode II in B and D.
emphasize that our proposed conformation of the binding of Epo to the docetaxel-binding site of tubulin is distinct from both the crystal structure (16) and the reported solution conformations (24). This difference is not surprising, given the finding that ligands frequently undergo conformational changes upon binding to proteins (25).

The identification of a common pharmacophore between taxanes and epothilones prompted us to examine whether sarcodictyins, a distinct class of coral-derived MT-stabilizing agents, would fit in the same model. However, in contrast to taxanes and epothilones, which have reduced activity in the mutant cells compared with parental cells, the activities of sarcodictyins A and B (not shown) and a methyl ketal analogue of sarcodictyin A (7) were enhanced (Table 1). This favorable interaction of sarcodictyins with the EpoR cells harboring mutant tubulins can be explained according to our pharmacophore model. The sarcodictyin A analogue was modeled into the taxane-binding site in a manner similar to that described for the epothilones (Molecular Modeling Methods) (Fig. 4). According to the pharmacophore model, the isopropyl group at C14 of sarcodictyin corresponds to the gem-dimethyl groups of the epothilones and the taxanes. Furthermore, the restricted ether oxygen attached to C4 and C7 of sarcodictyins is within 2 Å from the epoxy and oxetane oxygens of the epothilones and the taxanes, respectively, and is also favorably placed to accept the same hydrogen bond from the single water molecule. The methylimidazole side chain of sarcodictyins lies in the vicinity of Phe-β270 (Fig. 4). This location is similar to those of the aromatic side chain in binding mode II of the epothilones and the C13 side chain of taxanes. The sarcodictyins contain a methyl group at C7, which makes an unfavorable yet tolerable hydrophobic–polar interaction with the alcohol side chain of Thr-β274 (Fig. 4). However, in the β274Thr→Ile mutant, this same interaction becomes a highly favorable hydrophobic interaction between the C7 methyl group of sarcodictyin and the Ile-β274 aliphatic side chain. This explains why the potency of sarcodictyins is substantially increased in the 1A9/A8 cells with the β274Thr→Ile mutation compared with parental 1A9 cells with a Thr at position 274.

To further understand the molecular mechanism of drug binding, we utilized an energy-refined model of the 3.7-Å crystal structure of the docetaxel-binding site on β-tubulin that includes water interactions with the protein (Fig. 5). In this energy-refined model, water molecules were added to portions of tubulin that were sterically and electrostatically feasible as evidenced by their energy-minimized coordinates. The model must be interpreted with caution and may provide a possible explanation for the mutational analysis. We postulate that, in the apo-structure of tubulin (unbound tubulin), solvent may occupy a large portion of the binding pocket, forming a hydrogen-bonded network that is coordinated by the spatial disposition of
neighboring polar amino acid side chains and backbone residues. For example, in such a model, Arg-β282 is linked to this solvent network by a salt bridge with Glu-β288 (Fig. 5). At the apex of this water network, the OH group of Thr-β274 controls the location of water molecules that form a critical hydrogen bond with the C7-OH of Epo. According to this model, the β274Thr→Ile mutation is disruptive because it shifts the location of these waters, resulting in the loss of the C7-OH hydrogen bond with tubulin. The β282Arg→Gln mutation may also be disruptive because loss of the salt bridge with Glu-β288 enhances the anionic charge of the latter. In an attempt to screen this anionic activity of sarcodictyin in the vicinity of the water network, the OH group of Thr-274 side chain. At the same time it also explains the reason for enhanced activity of sarcodictyin caused by the favorable hydrophobic–hydrophobic interaction between the C7-methyl group of sarcodictyins and the methyl group of the Thr-274 side chain. At the same time it also explains the increased activity of sarcodictyin caused by the favorable hydrophobic–hydrophobic interaction between the C7-methyl group of sarcodictyins and the methyl group of the Thr-274 side chain.

The clinical success of taxanes has made tubulin a very attractive target for cancer chemotherapy (26) and has prompted a worldwide search for compounds with similar mechanism of action. Here we present acquired β-tubulin mutations in human carcinoma cells that confer resistance to taxanes and taxanes by impairing their binding to tubulin. The biological importance of this finding is underscored in a recent study reporting that patients with non-small-cell lung cancer not responding to PTX harbored β-tubulin mutations near the taxane-binding site (27). The identification of a common pharmacophore for taxanes and epothilones presented here is a valuable example of molecular modeling guided by mutational/biological data that helps us to better understand the interaction of these compounds with their common intracellular receptor, tubulin. We believe our data should assist in the engineering of MT-stabilizing molecules with improved characteristics, such as the Epo B-pyridine analogue and the sarcodictyin A analogue, setting the stage for advances in cancer chemotherapy.