Discovery and Characterization of the Laulimalide-Microtubule Binding Mode by Mass Shift Perturbation Mapping

Melissa J. Bennett,[1] Khaled Barakat,[2] J. Torin Huzil,[3] Jack Tuszynski,[3] and David C. Schriemer[1,*]
[1]University of Calgary, Department of Biochemistry and Molecular Biology, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada
[2]University of Alberta, Department of Physics, 11322-89 Avenue, Edmonton, AB T6G 2G7, Canada
[3]Cross Cancer Institute, Division of Experimental Oncology, 11560 University Avenue, Edmonton, AB T6G 1Z2, Canada
*Correspondence: dschriem@ucalgary.ca

SUMMARY

Conventional approaches to site mapping have so far failed to identify the laulimalide binding site on microtubules. Using mass shift perturbation analysis and data-directed docking, we demonstrate that laulimalide binds to the exterior of the microtubule on β-tubulin, in a region previously unknown to support ligand binding and well removed from the paclitaxel site. Shift maps for docetaxel and laulimalide are otherwise identical, indicating a common state of microtubule stability induced by occupancy of the distinct sites. The preferred binding mode highlights the penetration of the laulimalide side chain into a deep, narrow cavity through a unique conformation not strongly populated in solution, akin to a "striking cobra." This mode supports the development of a pharmacophore model and reveals the importance of the C1–C15 axis in the macrocycle.

INTRODUCTION

Paclitaxel and its mimetics represent a standard of care in the treatment of solid tumors of the breast, ovary, and lung (de Bree et al., 2006; Henderson et al., 2003; Paz-Ares et al., 2008). These drugs bind to microtubules in a manner that distorts the dynamic assembly properties of this protein polymer, leading to mitotic arrest and variable cell fates, including apoptosis. The pivotal role of microtubules in cell division has rendered the assembly unit, the α/β-tubulin dimer, an enduring target for the development of chemotherapeutic drugs. Clinical inadequacies of the taxoids, including severe and persistent peripheral neuropathies, drug resistance, and vehicle-related toxicities, continue to drive new ligand development (Argyriou et al., 2008; Hunt, 2009; Singer et al., 2005). Unfortunately the diffuse nature of the taxoid binding site has presented roadblocks to pharmacophore modeling and rational ligand design (Nettles et al., 2004), prompting a search for alternative therapeutic entry points.

Studies with a new class of polyketide macrolide isolated from deep-sea sponges have proposed the existence of a nontaxoid site that, when bound, induces microtubule stabilization in a fashion similar to the taxanes (Gaitanos et al., 2004; Hamel et al., 2006; Pryor et al., 2002). Laulimalide, also known as fijianolide B (Figure 1), is representative of this new class of agents. Laulimalide has demonstrated extremely high potency against solid tumor cancer cell lines (Mooberry et al., 1999). It increases the density of interphase microtubules and causes the formation of thick, short microtubule bundles in the cytoplasm of interphase cells and abnormal mitotic spindles (Mooberry et al., 2004). This leads to G2/M arrest and eventual cell death. While the promise of a more effective alternative to paclitaxel exists, the clinical future of laulimalide as a monotherapy is uncertain. One study has demonstrated efficacy in a human colon cancer model and limited general toxicity (Johnson et al., 2007), while another has demonstrated severe toxicity and minimal tumor inhibition (Liu et al., 2007). However, in combination with other tubulin-targeting antimitotic agents it shows significant synergy at the level of cytotoxicity and antiproliferative activity (Clark et al., 2006). Although the mechanism of this synergy has yet to be established, it may derive from the combined effects on tubulin dynamics (Gapud et al., 2004; Hamel et al., 2006; Jordan and Wilson, 2004). The ligand class in general also appears less susceptible to P-glycoprotein (Pgp)-mediated drug resistance than the taxanes (Clark et al., 2006; Mooberry et al., 1999).

Collectively, these findings argue for the continued exploration of laulimalide and the minimization of adverse toxicological properties through ligand design. A thorough exploration will require knowledge of the ligand binding site and a detailed understanding of structure-activity relationships, to permit the development of analogs with altered toxicity profiles and optimized synergistic effects. In addition, the discovery of a novel site for induction of microtubule stability will provide new opportunities to study molecular mechanisms relevant to cell division, by offering a new set of chemical probes.

The identification of the paclitaxel binding site was achieved by tubulin structural analysis using electron crystallography, but this has not been successful to date for the identification of the laulimalide site (Thepchatri et al., 2005). Using displacement studies, laulimalide was unable to inhibit Flutax-2 (a fluorescent paclitaxel) or [³H] paclitaxel from binding, but this simply indicates that binding is distinct from the paclitaxel site (Pryor et al., 2002). Early efforts in site identification have involved peloruside A, a ligand of the same class that has been shown to compete with laulimalide for binding to microtubules (Gaitanos et al., 2004). A computational study has suggested that
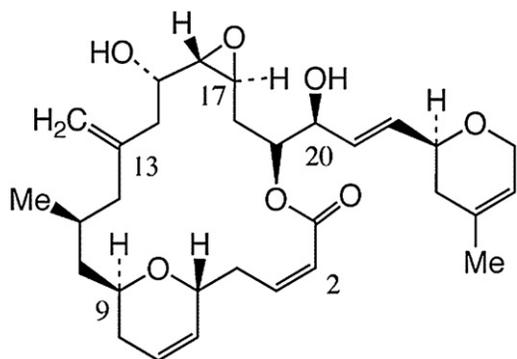
Figure 1. Chemical Structure of Laulimalide

peloruside A binds to the M-loop on α-tubulin in a region analogous to the paclitaxel site on β-tubulin (Jimenez-Barbero et al., 2006). However, a recent study in our lab proposed a site on β-tubulin on the exterior of the microtubule (Huzil et al., 2008). This was suggested upon an analysis of drug-induced microtubule stability using hydrogen/deuterium (H/D) exchange methods and mass spectrometry. An external site, if confirmed, would represent an obvious means by which endogenous protein regulators could affect microtubule stability in vivo.

The current study employs a refined mass spectrometry-based method to discover and characterize the laulimalide binding mode. The approach is inspired by chemical shift perturbation (CSP) methods in NMR, which are used extensively for protein interface mapping and more recently for protein-ligand interface mapping (Stark and Powers, 2008; Zuiderweg, 2002). In place of chemical shifts, ligand-induced perturbations of backbone amide H/D exchange rates are used as sensors for the formation of new interfaces and the allosteric effects of ligation. While H/D exchange mass spectrometry is used extensively in the study of protein folding (Engen, 2009; Maier and Deinzer, 2005), recent advancements promote the automated analysis of small molecule interactions with large molecular complexes, as well as extensive protein-protein interactions (Chalmers et al., 2006, 2007; Slysz et al., 2008, 2009). Here, we describe the application of this mass shift perturbation (MSP) method for laulimalide binding site determination, and, together with data-directed computational strategies, we present a high-resolution binding mode for the ligand on natural microtubule assemblies. Using MSP data, we also show that laulimalide induces a microtubule stability profile that is virtually indistinguishable from docetaxel and suggestive of a mechanism for synergy in tubulin assembly.

RESULTS

Mass Shift Perturbation Analysis of Ligated Microtubules

Replicate measurements of the ligand-induced perturbation of H/D exchange were conducted on assembled microtubules partially stabilized by a nonhydrolyzable analog of GTP (GMPCPP), to prevent depolymerization events during our analyses (Hyman et al., 1992). Ligand binding at the paclitaxel site induces further stabilization of microtubules, manifesting as

reduced mass shifts in a large set of peptides monitored in the H/D exchange experiment (see Figures S1A and S1B available online), which is consistent with earlier findings (Huzil et al., 2008; Xiao et al., 2006). Experiments with laulimalide-stabilized microtubules revealed similarly extensive shift perturbations, rendering an objective localization of the binding site difficult (Figures S1C and S1D). We hypothesized that perturbations unique to the binding site could be detected more readily by suppressing common allosteric effects of ligand binding, using a stabilizer targeting the paclitaxel binding site. This was based on the observation that laulimalide binding is not competitive with fluorescent paclitaxel and does not appear to influence paclitaxel-tubulin stoichiometry (Pryor et al., 2002). To test this, microtubules were stabilized with a combination of laulimalide and docetaxel, the latter a hydroxylated paclitaxel binding to the taxoid site (Ringel and Horwitz, 1991). The resulting mass shift data were then compared with similar analyses of microtubules stabilized with each ligand separately and mapped to the regions in sequence space (Figure 2). We note that a direct comparison of shift data from laulimalide and docetaxel stabilized microtubules provides a similar opportunity, but referencing to a coligated, stabilized form provides reduced noise in the differential shift map and offers greater discrimination power (not shown).

The shift map arising from docetaxel applied to laulimalide-stabilized microtubules validates this strategy, as the taxoid binding site is clearly highlighted by large negative mass shifts (Figures 2A and 3B). This represents 7% of the peptides monitored, where docetaxel stabilization alone induced significant shifts in over 17% of peptides (Figure S1A). Two negative mass shifts arise from peptides defining a surface composed of the M-loop (β266–280) and the H6–H7 loop region (β213–230) (Figure 3A). The M-loop contains critical residues that are involved in the stabilization of the oxetane ring of the taxanes. In the H6-H7 loop region, Leu217 and Leu219 make hydrophobic contact with the 2-phenyl ring, assisting in stabilization (Lowe et al., 2001). As in an earlier study, the loop between βS9 and βS10 does not show significant labeling, even though this region appears in close contact with docetaxel based on established structures (Huzil et al., 2008).

Similarly, the shift perturbations arising from laulimalide applied to docetaxel-stabilized microtubules represent 5% of the peptides monitored (Figures 2C and 3B), where laulimalide stabilization alone caused shifts in at least 21% of the peptides (Figure S1C). Mapping this reduced set of perturbations to structure highlights a groove defined by the C-terminal ends of helices βH10 and βH9 on its sides and βH9' at its end (Figure 3B, boxed region). This region represents negative shifts for five overlapping peptides, defining a contiguous patch of solvent-accessible surface area that can be uniquely identified as the laulimalide binding site, as the allosteric effects of each ligand and their combination are identical under saturating conditions (see Supplemental Experimental Procedures). We note that the region encompasses the proposed site for peloruside A (Huzil et al., 2008).

Data-Directed Docking of Laulimalide

Computational routines for receptor-ligand modeling were then implemented to orient the ligand within the laulimalide binding site identified through the shift perturbation analysis. As the bound conformation of laulimalide is currently unknown, a large