Discodermolide interferes with the binding of tau protein to microtubules

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Abstract We investigated whether discodermolide, a novel antimitotic agent, affects the binding to microtubules of tau protein repeat motifs. Like taxol, the new drug reduces the proportion of tau that pellets with microtubules. Despite their differing structures, discodermolide, taxol and tau repeats all bind to a site on β-tubulin that lies within the microtubule lumen and is crucial in controlling microtubule assembly. Low concentrations of tau still bind strongly to the outer surfaces of preformed microtubules when the acidic C-terminal regions of at least six tubulin dimers are available for interaction with each tau molecule; otherwise binding is very weak.

Key words: Tubulin; Tau; Microtubule-associated protein; Discodermolide; Taxol

1. Introduction

Discodermolide, a novel antimitotic drug isolated from a deep-sea sponge, binds to microtubules more potently than taxol, stabilises them against disassembly better than taxol and is effective even at 4°C [1,2]. Now that it can be fully synthesised [3], it has entered clinical trials as a chemotherapeutic agent for use against taxol-resistant cancers. Even though it bears no obvious structural resemblance to taxoid molecules (Fig. 1), it can displace taxol [4]. Taxol was first shown to bind to a specific site on β-tubulin in studies of zinc-induced sheets of tubulin protofilaments [5], which have since allowed the atomic structure of the complex to be resolved [6–8]. When protofilaments are assembled into microtubules, this binding pocket lies inside the lumen [9–11].

Microtubules are very labile polymers when assembled from pure tubulin but their stability can be controlled by a variety of molecules. A widespread family of structural microtubule-associated proteins (MAPs), including tau, MAP2 and MAP4, which maintain the stability of tubulin polymers, is characterised by a conserved sequence repeat motif [12,13]. By labelling one of the conserved repeat motifs in the microtubule-stabilising protein tau with nano-gold and co-assembling tubulin and labelled tau in the absence of drugs, we recently showed by three-dimensional (3D) electron cryomicroscopy that this region of the microtubule-binding domain locates to the inside surface of β-tubulin, in the region of the taxol-binding pocket [14]. If assembly occurs in the presence of taxol, there is a reduction in the proportion of tau that binds to and pellets with the microtubules. We now find that discodermolide is more consistent than taxol in reducing the proportion of tau in the pellets. The more effective properties of the new drug have also allowed us to further clarify the interactions between microtubules and tau. We conclude from our binding curves that low concentrations of tau still bind strongly to the outside surface, even when discodermolide blocks the repeat domains from binding to the inner sites, mainly because of association between each tau molecule and the acidic C-termini of six tubulin dimers. If the C-termini are removed by subtilisin digestion, tau binds only very weakly to prestabilised microtubules.

2. Materials and methods

2.1. Purification of tubulin, S-tubulin and tau

Tubulin was purified from pig brain extracts by standard methods involving cycles of warming/cooling and centrifugation, followed by chromatography on phosphocellulose resin. The buffer used for purification and assembly was BRB80 (80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)).

The C-termini of both tubulin monomers were digested with the enzyme subtilisin to produce S-tubulin using the method of Chau et al. [15]; the full extent of digestion was confirmed by Western blotting with antibodies to α- and β-tubulin [14].

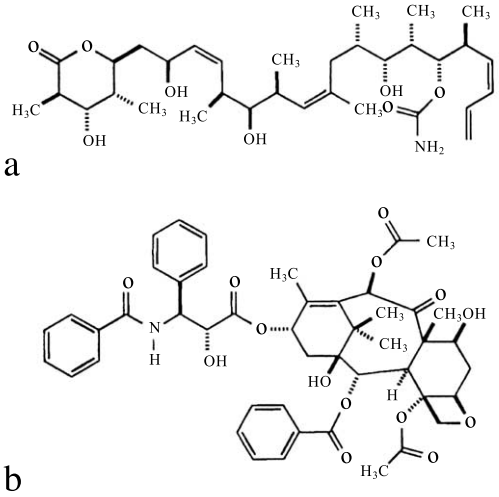

Fig. 1. The chemical structures of discodermolide (a) and taxol (b) show no obvious similarity.

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Abbreviations: MAP, microtubule-associated protein; TMAO, trimethylamine-*N*-oxide; DMSO, dimethyl sulphoxide

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Tau, containing three repeat domains (with the sequence of 37 kDa human tau), was expressed in *Escherichia coli* and purified by Method 1 in [14], in order to ensure full tubulin assembly-promoting activity.

2.2. Preparation of discodermolide

Stereocontrolled synthesis of (+)-discodermolide was carried out as described [3]. For use in these experiments it was dissolved in dimethyl sulphoxide (DMSO) to a concentration of 1 mM and subsequently diluted to 20 μM in BRB80.

2.3. Pelleting assays

Tubulin was thawed from liquid nitrogen and spun at 77 300×*g* for 20 min. Its concentration was determined spectroscopically. Since tau has a very low absorbance, its concentration was estimated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), against a bovine serum albumin (BSA) standard and tau protein of known concentration. Varying concentrations of tau were incubated with 10 μM tubulin, 0.1 M trimethylamine-*N*-oxide (TMAO), 1 mM guanosine triphosphate (GTP), and 5% DMSO and incubated for 20 min at 37°C. After centrifugation at 96 600×*g* for 40 min the supernatants were carefully removed and the pellets were resuspended in 40 μl of BRB80. Pellets and supernatants were run on SDS–PAGE, and the gels were stained with Page Blue83. For pelleting in the presence of discodermolide, the drug was added to tubulin prior to polymerisation and tau was added either at the same time or after microtubule assembly.

3. Results and discussion

3.1. Tau in microtubule pellets, with and without discodermolide

We carried out microtubule pelleting assays with a constant amount of tubulin and varying amounts of a tau isotype con-

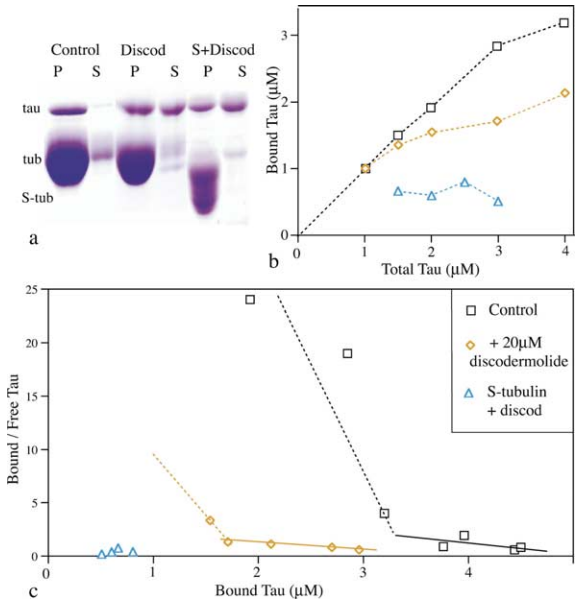

Fig. 2. a: SDS gel of proteins in pellets (P) and supernatants (S) when 3 μM of three-repeat tau was incubated at 37°C with 10 μM tubulin in the absence (control) or presence of 20 μM discodermolide and then centrifuged [14]. Tubulin was either native or digested with subtilisin (S-tub). b: Binding curves for varying amounts of tau added to 10 μM microtubules under the conditions shown in a. c: Scatchard plots better display the transition from strong binding (dissociation constant $\sim 10^{-7}$ M, from slope of line), at low ratios of tau to tubulin, to weak binding ($\sim 10^{-6}$ M) at higher ratios. Without any drug, one repeat motif of tau binds strongly to each tubulin dimer (3.3 μM tau to 10 μM tubulin). With discodermolide present, one complete tau molecule binds fairly strongly to ~6 tubulin dimers (1.7 μM tau to 10 μM tubulin).

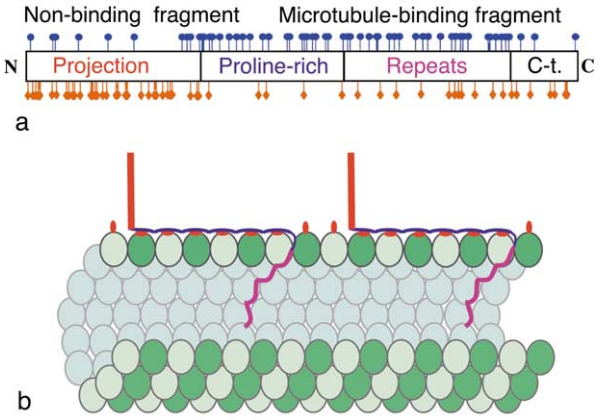

Fig. 3. a: Distribution of positively charged (blue circles) and negatively charged (red diamonds) protein residues within the various domains of htau40, the longest form of human tau (diagram modified from [26]). Note that the concentration of negative charges is lower in the proline-rich domain. The htau37 used for the experiments in Fig. 2 is identical except it has only three repeat motifs instead of four. b: Tentative scheme for the preferred interactions of different regions of tau with a microtubule. Tubulin heterodimers are shown green. The N-terminal region (red line) of tau projects from the outer surface. The proline-rich region (blue) of tau interacts with each tubulin monomer via the negatively charged tubulin C-termini (red ovals, shown for only one protofilament). The repeat region (purple) of tau interacts with the inner surface of the microtubule [14]. Current data do not provide information about the preferred location of the C-terminal region of tau.

taining three conserved repeats in its microtubule-binding domain, as described previously [14]. Before centrifugation, the proteins were incubated together at 37°C, in the presence or absence of 20 μM discodermolide. The pellets and supernatants were run on 10% SDS gels (Fig. 2a). At low ratios of tau:tubulin dimer, tau bound tightly to the pelleted microtubules. Without drugs, tau appeared in the supernatant when the ratio was about 1:3 [14] but in the presence of discodermolide, unbound tau was detected at a 1:4 ratio and larger amounts were seen in each supernatant at higher concentrations (Fig. 2b). The results were similar whether the drug was added to soluble tubulin at the same time as tau, or was used to stabilise preformed microtubules before the addition of tau. The reduction in bound tau is more distinct at all concentrations than we found in the presence of taxol [14], consistent with discodermolide's stronger stabilisation of microtubules. TMAO, an agent that stabilises the folding of numerous proteins [16] including tau [17], was included in our buffers, though it is not essential if tau is purified in a way that ensures full activity [14].

Observations that taxol reduces the amounts of tau that bind to microtubules and 3D electron microscopy of microtubules co-assembled with tau molecules that had been labelled with nano-gold on one of the repeat motifs originally suggested that binding of the repeat motifs overlaps the drug-binding sites on β-tubulin [14]. Because discodermolide binds more tightly than taxol does to tubulin, the binding curves shown here support this model more clearly. Discodermolide and taxol compete for binding to microtubules [1,2] and electron crystallographic studies also indicate they have overlapping binding sites (K.H. Downing, personal communication). We conclude that the binding sites of both drugs overlap with that of tau repeat motifs. The corresponding site on the α-tubulin subunit is filled by an extended loop with the pri-

mary sequence TVVPGGDL. The most similar sequence within the tau repeat domain is THVPGGGN. However, there is no obvious relationship between the structures of these amino acids and either of the drug molecules.

3.2. How does tau interact with the microtubule outer surface?

In our model for the incorporation of tau into microtubules during co-assembly, the C-terminal one-third of a tau molecule binds to the inner surfaces of tubulin subunits in such a way that, ideally, each tau repeat motif interacts with one tubulin dimer. The N-terminal projection domain extends from the outer surface, while some part of the central region must thread its way through the microtubule wall via the gaps between tubulin protofilaments, in order to connect the N-terminal and C-terminal portions. In the presence of discodermolide or taxol, low concentrations of tau still bind fairly well (Fig. 2c), even if the tau is added to preassembled closed tubes, when it seems likely that it can only bind to the outside. Binding to the outer surface primarily involves regions of tau other than the repeats, since a three-repeat domain by itself (K19) bound poorly to taxol-stabilised microtubules, while a construct that lacked any repeats (K23) bound almost as well as complete three-repeat tau [18].

When we incubated prestabilised microtubules overnight with the enzyme subtilisin to completely remove the acidic carboxyl terminals of all α- and β-tubulin monomers, tau bound only weakly (Figs. 2b, c), in agreement with earlier findings [15,19–22]. The distribution of tau's charged amino acids (Fig. 3a) suggests that the proline-rich region is most likely to partner the acidic regions on the outer surface of a microtubule (Fig. 3b). The position at which the line in the Scatchard plot (Fig. 2c) abruptly changes its slope when sites on the inside surface are blocked by drugs indicates that binding remains strong only if each tau molecule can interact on the outside surface with six or more tubulin dimers (i.e. at least 48 nm along a protofilament). This contrasts with the need for interactions with only three tubulin dimers for tau to be very strongly bound in the absence of drugs. The proline-rich region alone is not long enough to contact six separate tubulin dimers, either along a protofilament or even across adjacent protofilaments. But when bound drugs restrict the entire tau molecule to interacting with the outer surface of the microtubule, the repeat motifs and the C-terminus of tau can also stick to tubulin's highly charged C-termini and thus contribute to the overall binding strength [23]. Indeed, when taxol-stabilised microtubules decorated with tau or MAP2c under such conditions were studied by 3D electron cryomicroscopy [22], density that could be attributed to the MAPs or to a gold label attached to the repeat domain was detected only on the outermost ridges of the protofilaments, in contact with the C-termini of the tubulin subunits.

3.3. A versatile binding site on β-tubulin?

Discodermolide reduces the proportion of total tau that binds to microtubules even more effectively than taxol, suggesting that these two dissimilar drugs and the repeat motifs of MAPs all have a common binding site on the inside surface of β-tubulin. Being highly conserved across all eukaryotic species, it cannot have adapted to bind a range of different ligands that are found only in specific organisms (like marine sponges or yew trees) and thereby induce cell death. More probably, it is so versatile because it has evolved to accommodate a variety of proteins that control microtubule stability under different circumstances. Since this site on β-tubulin is ideally situated to control the conformation of the tubulin protofilament and its lateral interactions with neighbouring protofilaments [24], it may be used to bind a range of alternative protein sequence motifs in other MAPs. Interestingly, taxol has been shown to reversibly abolish the binding of EB1, a protein found concentrated on the plus ends of microtubules [25]. But this protein family has no conserved sequence motif that is obviously similar to tau or MAP2 repeats and it remains to be shown whether any other types of MAPs bind to microtubules in an equivalent way to the repeat motifs of tau.

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