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Evidence for a distinct ligand binding site on tubulin discovered through inhibition by GDP of paclitaxel-induced tubulin assembly in the absence of exogenous GTP

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Abstract

GDP inhibits paclitaxel-induced tubulin assembly without GTP when the tubulin bears GDP in the exchangeable site (E-site). Initially, we thought inhibition was mediated through the E-site, since small amounts of GTP or Mg²⁺, which favors GTP binding to the E-site, reduced inhibition by GDP. We thought trace GTP released from the nonexchangeable site (N-site) by tubulin denaturation was required for polymer nucleation, but microtubule length was unaffected by GDP. Further, enhancing polymer nucleation reduced inhibition by GDP. Other mechanisms involving the E-site were eliminated experimentally. Upon finding that ATP weakly inhibited paclitaxel-induced assembly, we concluded that another ligand binding site was responsible for these inhibitory effects, and we found that GDP was not binding at the taxoid, colchicine, or vinca sites. There may therefore be a lower affinity site on tubulin to which GDP can bind distinct from the E- and N-sites, possibly on α -tubulin, based on molecular modeling studies.

Keywords

Tubulin; GDP; Paclitaxel; Nucleotide binding sites; ATP; Microtubule-associated proteins

The interactions of paclitaxel with tubulin and microtubules have been studied extensively. Paclitaxel, the first exemplar of a cytotoxic compound that induces rather than inhibits microtubule assembly [1], is an important chemotherapeutic agent for the treatment of human neoplasms [2]. Paclitaxel causes cells to arrest at mitosis, and, in the presence of the drug, microtubules assemble extensively, often displaying unusual intracellular distribution patterns and morphological characteristics [1,3].

In biochemical systems, paclitaxel suppresses microtubule dynamics at submicromolar concentrations [4] and hypernucleates microtubule assembly at micromolar concentrations. Hallmarks of this hypernucleation are more extensive tubulin assembly, formation of increased

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numbers of shorter polymers, and assembly at colder temperatures and in the absence of microtubule-associated proteins (MAPs)¹ and/or GTP [1,5-10]. Thus, even in the absence of exogenous GTP in the medium, tubulin with GDP bound in the exchangeable site (E-site) will assemble in the presence of paclitaxel.

We decided to explore further the role of nucleotides in paclitaxel-induced assembly to gain additional insights into tubulin-nucleotide interactions. We found that added GDP, in midmicromolar concentrations and in the absence of exogenously added GTP, inhibited paclitaxel-driven assembly of tubulin with GDP bound in the E-site. The studies presented here describe our efforts to quantitate this inhibitory effect of GDP and to understand its mechanistic basis. Our initial hypothesis, that small amounts of nonexchangeable site (N-site) GTP released by tubulin denaturation were required for paclitaxel-driven assembly, was only partially supported by experimental data. These data also argued against the effect of GDP being mediated by one of the three most extensively studied drug binding sites (taxoid, colchicine and vinca). We concluded that the inhibitory GDP must be binding elsewhere on tubulin, and we used molecular modeling to suggest a location for this binding site. Since it is hard to conceive of a physiological role for this inhibitory effect of GDP, it most likely represents binding of the nucleotide at a site meant to be occupied by another ligand. Potentially, this site could be occupied by small molecules with therapeutic potential.

Materials and methods

Materials

Electrophoretically homogeneous tubulin with one molar equivalent each of GDP in the E-site and GTP in the N-site and freed of MAPs was prepared as described previously [11], including gel filtration chromatography to remove unbound nucleotide [12], except that the last step was dialysis against 0.1 M 4-morpholineethanesulfonate (Mes) (from a 1.0 M stock solution adjusted to pH 6.9 with NaOH). The tubulin preparation has no detectable nucleoside diphosphate kinase activity [11]. Heat-treated MAPs were prepared as before [11]. The proteins were from bovine brain tissue. GTP, GDP, ATP and ITP were repurified by triethylammonium bicarbonate gradient chromatography on DEAE-cellulose. Other nucleotides and subtilisin were from Sigma. Podophyllotoxin was from Aldrich. Paclitaxel and maytansine were provided by the Drug Synthesis & Chemistry Branch, National Cancer Institute. Discodermolide was from Dr. R. Longely, Harbor Branch Oceanographic Institute. All experiments were performed in 0.1 M Mes (pH 6.9).

Methods

Tubulin assembly was followed turbidimetrically at 350 nm in Beckman model DU 7400 spectrophotometers equipped with electronic temperature controllers, modified so that they can maintain cuvette temperature at 0 °C. All components were mixed in the cuvettes at 0 °C, and baselines were established at 350 nm. The reactions were followed for 2 min at 0 °C, and the temperature was jumped over about 30 s to 30 °C. The reaction was generally followed for 30 min at 30 °C. In the figures presented here, zero time is defined as the point at which the temperature jump was initiated, since no significant turbidity change occurred at 0 °C.

For preparation of samples for electron microscopy for determination of polymer lengths, polymerization was monitored turbidimetrically. After about 30 min at 30 °C an aliquot of the reaction mixture was diluted 5- to 10-fold into a 30 °C solution of 50% (w/v) sucrose containing 0.1 M Mes (pH 6.9), 30 μM paclitaxel, and the same concentration of GDP present in the

¹Abbreviations used: MAPs, microtubule-associated proteins; E-site, exchangeable site; N-site, nonexchangeable site; Mes, 4-morpholineethanesulfonate; HPLC, high performance liquid chromatography; PDB, protein data bank; RMS, root mean square.

original reaction mixture (based on the method of Terry and Purich [13]). An aliquot of the diluted sample was applied to a 200-mesh carbon-coated, formvar-treated copper grid, followed by 5-10 drops of 1% (w/v) uranyl acetate. Excess stain was wicked from the grids, which were examined in a Zeiss model 10CA electron microscope. Microtubule lengths were determined from the micrograph negatives using the program MIPAV ("Medical Imaging, Processing, Analysis and Visualization"), developed at the Center for Information Technology, NIH, and generously provided by Dr. M. McAuliffe. For details, see <http://mipav.cit.nih.gov>

Guanosine nucleotide content of microtubules formed in the presence of paclitaxel and without added nucleotide was determined following isolation of polymer by centrifugation (30,000 rpm at 30 °C for 30 min). The pellet was dissolved in 8 M urea, and tubulin was separated from released nucleotide by gel filtration. Protein and guanine nucleotide content of the pooled excluded and included peaks were determined with the Lowry procedure and with the standard extinction coefficient for guanine nucleotides, respectively. The ratio of GDP to GTP was determined by high performance liquid chromatography (HPLC) on a Partisil SAX analytical column (0.46 × 25 cm; ammonium phosphate, pH 6.5, gradient to 0.5 M over 35 min; flow rate 2.5 mL min⁻¹). The retention times of GDP and GTP were 14 and 24 min, respectively.

The E-site GDP in our tubulin preparation is derived from GTP hydrolysis during an assembly cycle [11]. One hypothesis examined to explain inhibition by exogenous GDP was that exchange of GDP into the E-site resulted in a functional difference in the tubulin as compared with GDP derived from *in situ* hydrolysis. Tubulin at 30 μM was incubated with or without 1.0 mM GDP in 0.1 M Mes (pH 6.9) for 45 min on ice. The tubulin was precipitated with 2.5 M monosodium glutamate [12] and recovered by centrifugation at 0 °C for 30 min at 30,000 rpm. Each tubulin pellet was resuspended as a slurry with 0.5 mL of cold water. Each slurry was dialyzed against 0.1 M Mes (pH 6.9) for 2 h. The tubulin concentrations of the resulting solutions were determined, and the effect of 20 μM paclitaxel on assembly of each tubulin preparation at 20 μM was evaluated.

Magnesium content of reaction components was determined by atomic absorption spectroscopy with a Perkin-Elmer model 1100B instrument. The standard magnesium solution was also obtained from Perkin-Elmer. Exogenous Mg²⁺ was not used in the preparation of the tubulin [11], nor was it included in reaction mixtures, unless indicated (see Results section).

Treatment of tubulin with subtilisin was performed essentially as described by Sackett et al. [14], with tubulin at 10 mg/mL plus subtilisin at a weight ratio of 1:100 in 0.1 M Mes (pH 6.9). After 2 h at 30 °C, the reaction mixture was placed on ice, and the enzyme was inactivated with a 4-fold molar excess, relative to subtilisin, of phenylmethanesulfonyl fluoride. The reaction mixture was centrifuged at 15,000 rpm for 10 min. The supernatant, protein concentration determined with the Lowry assay, was used as subtilisin-treated tubulin.

Molecular modeling

Tubulin has no amino acid sequences that correspond to guanine nucleotide binding sites in other proteins [15-19], a conclusion confirmed by the localization of the E- and N-sites in the electron crystallographic model of tubulin [20]. Our modeling approach began with a search of the Protein Data Bank (PDB) for conformational overlaps between tubulin [20,21; PDBcodes, 1TUB and 1JFF] and over 460 entries with bound GDP, focusing on the α-carbon architecture of the polypeptide backbones. We used the 1TUB and 1JFF electron crystallographic structures because they both have the most complete α- and β-subunit sequences. We did not use the X-ray structures of tubulin co-crystallized with stathmin-4 [22,23] because, relative to the 1TUB and 1JFF structures, significant changes were observed in the tertiary features at the colchicine site and elsewhere in the tubulin α,β-dimer. Moreover, the resolution provided in the X-ray crystal structures added no significant advantage to the

resolutions reported for 1TUB and 1JFF. From the GDP binding site entries, we separated out the GDP binding sites by amino acids that had first neighbor contacts with the GDP itself. The binding complexes served as structures to be compared with the α -carbon trace of both tubulin subunits by three-dimensional structural sequence alignments with the 1TUB and 1JFF structures.

These proteins were further analyzed using the Homology Program from Accelrys [24], performing three-dimensional structural sequence alignment of backbone geometries of the GDP binding sites with both tubulin structures. The structural alignment employed a minimum block length of four amino acids and a probe size for the backbone geometry search that was set to six amino acids. The zone lengths and scan limits were systematically varied to search the α - and β -tubulin sequences in their entirety, using the various GDP binding sequences as probes. During probing, the root mean square (RMS) threshold parameters were initially set to a very high value and lowered for each compared pair to a point just above elimination of the match. Segments of the known GDP binding sites that satisfied threshold values for backbone alignment were identified and tabulated for each GDP binding search performed. Alignment sets for which the identified segments did not span the entire known GDP binding site of the protein were considered incomplete matches and discarded.

Structure matches were scattered over large portions of both tubulin subunits, but a single, dense “hot spot” with 30 matches occurred at the α -tubulin sequence spanning residues 15-35 (see Results section). We observed very little difference in our searches in this hot spot whether 1TUB or 1JFF was used as the scaffold for comparisons. The best four matches were selected for further evaluation, with GDP being modeled into this region of α -tubulin, using an energy-refined model derived from 1TUB [20]. Our criteria for the final model were that the proposed binding site be solvent accessible, to permit ligand entry, with the diphosphate moiety of GDP closer to the solvent than the purine and ribose moieties; and that a spatial rearrangement of the amino acid side chains be possible to permit accommodation of the volume of the GDP molecule. A final criterion was that chemical complementarity must exist between GDP and the amino acid side chains of the candidate binding site without imposing significant changes in the secondary structure of tubulin to build the new binding site. With these restrictions, the best result was obtained with the sequence 1RQ7, residues 101-107 [25], corresponding to α -tubulin residues 27-33. The initial model of the α -tubulin GDP binding site was created by first manually altering dihedrals of amino acid side chains near residues 27-33 to create an adequate space to accommodate GDP. Next, a molecular model of GDP was oriented in the space to obtain the best steric and chemical complementarity between the nucleotide and α -tubulin, so

that violations of van der Waals contacts greater than $0.25 \text{ \AA}$ did not occur. In an iterative manner, all possible orientations and conformations were examined within the steric cavity formed from the best 1RQ7/ α -tubulin overlap. The GDP-tubulin model was optimized by applying a force of $2000 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$. This force was stepped off the structure in $100 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ decrements by minimizing with conjugate gradients until the norm of the gradient was $0.1 \text{ kcal mol}^{-1}$. This process was repeated until all applied external force was removed. The cff91 potentials were used for the molecular mechanics optimizations [26].

Results

GDP inhibits paclitaxel-induced assembly of tubulin: evidence in favor of an interaction at the E-site

In the absence of both GTP and MAPs, our tubulin preparations yield a relatively high critical concentration for paclitaxel-induced tubulin assembly. With tubulin and paclitaxel both at 10

μM , there was negligible assembly. With the tubulin preparation used here, the critical concentration with $10 \mu\text{M}$ paclitaxel at 30°C was about $15 \mu\text{M}$, somewhat lower than the values of $22\text{--}28 \mu\text{M}$ obtained previously at 37°C [10,27]. (Adding both MAPs and GTP to the reaction reduced the critical concentration to less than $1 \mu\text{M}$ tubulin.)

We therefore used $30 \mu\text{M}$ concentrations of tubulin and paclitaxel to examine the effect of GDP on this assembly reaction (Fig. 1). As little as $5 \mu\text{M}$ GDP had a significant inhibitory effect, and assembly was almost totally eliminated with $100 \mu\text{M}$ GDP. A series of experiments (Fig. 2, squares) yielded an IC_{50} of 15 ± 6 (SD) μM GDP.

This inhibitory effect suggested a GTP requirement for paclitaxel-driven tubulin assembly, even when no nucleotide was added to a tubulin preparation containing no unbound GDP and GTP. We confirmed that the tubulin used here was comparable to previous preparations of tubulin [11]. As before [11], there were two molar equivalents of guanine nucleotide relative to the 100 kDa $\alpha\beta$ -heterodimer, with half as GDP (presumably extracted from the E-site) and half as GTP (presumably from the N-site). (This molecular weight of tubulin is based on the primary sequences of the subunits [28,29].) Tubulin, however, is a labile protein, so we postulated that small amounts of GTP had been released from the N-site during tubulin denaturation and had re-equilibrated into the E-site of active dimers.

If a small amount of GTP of N-site origin were essential for this assembly reaction, then the inhibitory effect of GDP should be countered by adding small amounts of GTP. This was the case (Fig. 2). With increasing GTP, GDP became less inhibitory. The IC_{50} values obtained for GDP increased from $15 \mu\text{M}$ with no exogenous GTP, to 58 ± 20 (SD) μM with $5 \mu\text{M}$ GTP, to $260 \pm 50 \mu\text{M}$ with $20 \mu\text{M}$ GTP, to $1.8 \pm 0.4 \text{ mM}$ with $150 \mu\text{M}$ GTP.

If the inhibitory effect of GDP were being exerted through the E-site, adding Mg^{2+} should reduce the inhibitory effect of GDP, since Mg^{2+} enhances the affinity of GTP, but not GDP, for the E-site [30,31]. This, too, was observed. Fig. 3 shows that the GDP IC_{50} was increased almost 4-fold, from 15 to $57 \pm 4 \mu\text{M}$ when 2 mM MgCl_2 was added to the reaction.

Finally, we examined other nucleotides for inhibitory effects on paclitaxel-induced tubulin assembly. No monophosphate or cyclic monophosphate examined had any inhibitory effect, nor did ADP, CDP, UDP, or IDP (data not presented). A weak inhibitory effect, however, was observed with ATP (see below for further description and discussion).

The inhibitory effect of GDP is unlikely to be caused by chelation of Mg^{2+} cations required for paclitaxel-induced tubulin assembly

In the studies described above, most reaction mixtures contained no exogenously added Mg^{2+} , nor were Mg^{2+} salts used in the preparation of the tubulin [11]. Consequently, the total Mg^{2+} concentration of the reaction mixtures, as determined by atomic absorption spectroscopy, was not even stoichiometric with the $\alpha\beta$ -heterodimer concentration, with most of the Mg^{2+} derived from the tubulin added to the reaction mixtures, probably bound to N-site GTP [32].

The inhibitory effect of GDP might be caused by its chelation of small amounts of Mg^{2+} required for assembly. This seemed unlikely, considering that other ribonucleoside diphosphates had no inhibitory effect. Nevertheless, we also evaluated the inhibitory effect of EDTA, which binds divalent cations much more strongly than nucleotides [33], on the paclitaxel-induced assembly reaction. While EDTA also had a progressive inhibitory effect on assembly, it was much weaker than GDP. We obtained an average IC_{50} value (extent of assembly after 30 min) for EDTA of $0.44 \pm 0.1 \text{ mM}$, almost 30-fold higher than the IC_{50} for GDP, excluding Mg^{2+} chelation as causing the inhibitory effect of GDP.

There is no evidence for a specific requirement for GTP for nucleation in paclitaxel-induced tubulin polymerization

The above findings presented a dilemma. GDP inhibited the polymerization of tubulin induced by paclitaxel when the E-site was occupied by GDP and when there was no free GTP in the reaction mixture. If a trace amount of E-site GTP was required for assembly with paclitaxel, it was most reasonable that the GTP requirement was for polymer nucleation rather than propagation, since far fewer $\alpha\beta$ -heterodimers would be involved in the nucleation phase of assembly. While Fig. 1 demonstrates inhibition of both nucleation and elongation by GDP, inhibition of elongation could reflect a decreased number of active nuclei in the presence of GDP.

A specific effect on nucleation would require that, as the added GDP concentration increased, the microtubules formed would increase in length. This did not occur (Table 1). There was essentially no change in average microtubule length whatever the GDP concentration. This implies that a significant inhibitory effect must occur during the elongation phase of assembly. There was also no significant change in polymer morphology when GDP was included in the reaction mixture.

Further evidence against a specific effect on nucleation is that, as nucleation was made more facile, the inhibitory effect of GDP was reduced. A well-known role of MAPs is to enhance microtubule nucleation, substantially reducing the critical concentration required for assembly. This also occurs when paclitaxel induces assembly. With 10 μM paclitaxel at 30 °C, adding MAPs alone reduced the critical concentration from about 15 μM to about 3.5 μM (previously, at 37 °C, with different protein preparations, the reduction was even greater, from 22-28 to 2-5 μM [10,27]). Fig. 4 presents a typical experiment in which the inhibitory effect of GDP on paclitaxel-induced assembly with MAPs was examined. Because of the lower tubulin critical concentration, the tubulin and paclitaxel concentrations were reduced to 10 μM . Despite this reduction, much more GDP was required to inhibit assembly. The IC_{50} was 1.6 ± 0.3 mM, over 100-fold higher than the IC_{50} without MAPs and with a 3-fold higher concentration of tubulin.

Another approach to evaluating inhibition of nucleation vs elongation is to examine the effects of paclitaxel and GDP on assembly of subtilisin-treated tubulin. Partial digestion of tubulin with the protease resulted in a major reduction in the critical concentration for assembly dependent on GTP [14]. With our bovine brain tubulin, treatment with subtilisin led to almost a 10-fold reduction in the critical concentration with 10 μM paclitaxel, from 15 μM (see above) to 1.6 μM (about half the critical concentration of undigested tubulin with MAPs). We therefore examined the effect of GDP with 10 μM subtilisin-treated tubulin and 10 μM paclitaxel. The inhibitory effect of GDP was substantially reduced relative to undigested tubulin (IC_{50} , 1.0 ± 0.2 mM). This is about 40% lower than the value obtained with MAPs (1.6 mM) at the same tubulin concentration and about 70-fold higher than the 15 μM value obtained with 3-fold higher undigested tubulin.

We also performed an evaluation of the effects of GDP on polymerization induced by epothilone B [27] and discodermolide [34], both of which cause formation of shorter microtubules and lower critical concentrations for tubulin than occurs with paclitaxel. Thus, both compounds enhance microtubule nucleation more effectively than does paclitaxel. Our experiments clearly demonstrated that polymerization reactions induced with either epothilone B or discodermolide were substantially more resistant to GDP inhibition than the paclitaxel-induced reaction. Limited amounts of epothilone B prevented quantitation of its effects, but we were able to study 30 μM discodermolide + 30 μM tubulin. The IC_{50} for GDP was 50 ± 3 mM, over 3000-fold higher than the 15 μM IC_{50} obtained with paclitaxel.

The inhibitory effect of GDP is not caused by the preferential assembly of tubulin with an empty E-site in the presence of paclitaxel

Might the inhibitory effect of GDP be due to paclitaxel's preferentially favoring assembly of tubulin with an empty E-site? Then paclitaxel-induced polymer should have reduced nucleotide content. This seemed unlikely in view of the full nucleotide content of zinc-induced, paclitaxel-stabilized sheet polymer reported by Nogales et al. [20]. Still, we were using quite different reaction conditions. Paclitaxel-induced polymer was isolated by ultracentrifugation, and the tubulin to nucleotide and GDP to GTP ratios were determined. Nucleotide content did not differ from the starting tubulin: two molar equivalents of guanine nucleotide per $\alpha\beta$ -heterodimer, with equal amounts of GDP and GTP.

GDP that enters the E-site directly is equivalent to GDP derived from GTP hydrolysis during assembly

We postulated that tubulin with GDP bound in the E-site might exist in different states, depending on whether it entered the site as GDP by exchange or entered as GTP with conversion to GDP during an assembly cycle. This model implies different binding mechanisms for GTP and GDP to tubulin, presumably with both nucleotides binding to tubulin with a transiently vacant E-site. At high exogenous GDP concentrations, the amount of bound GDP derived from GTP should be negligible. This model would require that paclitaxel-induced assembly would be optimal with tubulin bearing GDP derived from GTP hydrolysis.

To test this hypothesis, 1.0 mM GDP was added to 30 μ M tubulin (33-fold excess of GDP), and the tubulin was recovered by precipitation. Control tubulin without exogenously added GDP was processed similarly. When these two tubulin preparations were evaluated for paclitaxel-induced assembly, there was little difference between them (data not shown). Therefore, the origin of E-site GDP does not seem to affect the ability of paclitaxel to induce tubulin assembly.

Does the inhibitory effect of GDP result from its binding to a site distinct from the E- and N-sites?

There have been several reports of unusual nucleotide effects on microtubule assembly reactions, leading investigators to postulate the existence of a third nucleotide binding site on tubulin (e.g., refs. [35-38]), and this is a difficult model to exclude for the inhibitory effects of GDP that we observed. We were reluctant to invoke it but decided to investigate additional nucleotide effects on paclitaxel-induced assembly.

As noted above, no ribonucleoside monophosphate (highest concentration examined, 2 mM) affected paclitaxel-induced assembly. Unexpectedly, extensive inhibition was observed with ATP (Fig. 5A), but ATP was about 140-fold less effective than GDP, with an IC_{50} of 2.2 ± 0.3 mM. Still weaker effects were observed with CTP (not shown). In contrast, ITP, UTP, and XTP were stimulatory (Fig. 5B) ($ITP > XTP \approx UTP$), but this presumably reflects weaker interactions at the E-site [39,40]. None of these nucleotides was as effective as micromolar GTP in enhancing assembly (Fig. 5B).

Molecular modeling identifies a potential GDP binding site on α -tubulin

Even the E- and N-sites of tubulin do not have the primary sequences common to typical GDP/GTP binding proteins [15-21]. We therefore searched proteins with bound GDP in the PDB for polypeptide chain backbone conformational similarity in their GDP binding domains with the entire polypeptide backbones of α - and β -tubulin. The only recurring structural homology was with the α -tubulin sequence spanning residues 15-35. Fig. 6 shows the best four architectural matches resulting from the alignment analysis with this segment of α -tubulin.

When comparing the complete binding site architectures, 1R5N [41] and 1JNY [42] possessed the most common type of GDP binding site structure found in the PDB, encompassing over 90% of the entries, while 1RQ7 [25] and 1JFF [21] (when compared with 1TUB) possessed structurally unique GDP binding sites. 1R5N and 1JNY are PDB entries for the GDP bound forms of proteins involved in protein synthesis. 1JFF is a PDB entry for tubulin, and the peptide backbone overlap involved part of the β -tubulin E-site with α -tubulin. 1RQ7 is the PDB entry for the GDP bound form of the *Mycobacterium tuberculosis* FtsZ protein. The match described in Fig. 6 is between the GDP site of the A polypeptide chain in 1RQ7 and a segment of the α -tubulin backbone. (Although the original FtsZ structure [43] was not included in our original data set, it closely overlaps 1RQ7 and has homology with the same region of α -tubulin.)

The best model accommodating GDP was constructed based on the secondary structure alignment of α -tubulin residues 27-33 with residues 101-107 of the A polypeptide chain in 1RQ7. This model is shown in Fig. 7. Key features of the model include (1) a bifurcated hydrogen bond from the guanine amino group and the N-1 atom of guanine to the backbone carbonyl of His-61, (2) a water-mediated hydrogen bond from the backbone carbonyls of Lys-60 and His-28 and the carbonyl oxygen of the guanine moiety, (3) a water-mediated hydrogen bond from the backbone carbonyl of Thr-41 to the N-3 atom of the guanine moiety, (4) a water-mediated hydrogen bond from the backbone carbonyl of Tyr-24 to the N-7 atom of the guanine moiety, and (5) a conformation of the ribosyldiphosphate portion of GDP oriented to permit ion-ion and ion-dipole interactions between a solvated diphosphate and Lys-40 and Arg-84 of α -tubulin.

Discussion

We observed that paclitaxel-induced assembly of tubulin with GDP bound in the E-site was inhibited by adding GDP to the reaction mixture in the absence of GTP other than that bound in the N-site. The IC_{50} for GDP was 15 μ M with 30 μ M tubulin. We initially explained the inhibitory effect of GDP by assuming the GDP was in competition with a small amount of contaminating GTP of N-site origin. This was supported by observations that adding either Mg^{2+} , which enhances GTP but not GDP binding to the E-site, or low concentrations of GTP reduced the inhibitory effect of GDP. Experiments to define the mechanism in greater detail, however, failed to support the concept that the GDP inhibition occurred through the E-site.

In particular, we reasoned that if such small amounts of GTP were required for paclitaxel-induced assembly, it could only be the nucleation step for which this GTP was required. Most important in excluding this idea was that microtubule length was little affected by the inhibitory GDP, rather than increasing GDP leading to progressively longer microtubules if nucleation were inhibited by GDP preventing GTP from binding in the E-site. Moreover, modification of reaction conditions to enhance nucleation resulted in reduced inhibitory effects for GDP. Adding MAPs resulted in a 107-fold increase in the IC_{50} for GDP. Treating the tubulin with subtilisin resulted in a 67-fold increase in the IC_{50} for GDP. Most dramatically, replacing paclitaxel with discodermolide, which enhances nucleation more extensively than does paclitaxel [34], resulted in a 3300-fold increase in the IC_{50} for GDP.

An alternate interpretation for the common effect of MAPs and subtilisin treatment is that GDP has some interaction with the carboxy terminal regions of α - and β -tubulin, since these termini interact with MAPs and are removed from tubulin by subtilisin digestion. However, discodermolide binds in the same β -tubulin site as paclitaxel, far from the carboxy terminal regions of both subunits [20]. It also seems unlikely that the highly acidic carboxy terminal regions of tubulin [28,29] could interact with the negatively charged GDP.

Our thinking changed when we found that ATP (but not ADP or IDP) inhibited the paclitaxel-induced assembly reaction. It thus seems possible that the specific reaction condition used here unmasks a distinct nucleotide binding site on tubulin with relatively high affinity for GDP and low affinity for ATP. However, the affinity of GDP for this postulated site is low compared with the very high affinity for GDP at the E-site (15 μM IC_{50} vs the 20-60 nM K_d for GDP binding to the E-site [44,45]).

Our conclusion about an additional nucleotide site is somewhat reminiscent of the third nucleotide site proposed by Zabrecky and Cole [35-37], who reported that purified tubulin in a high glycerol/high Mg^{2+} assembly condition formed an aggregated ring polymer, with ATP and GTP acting synergistically. Moreover, 8-azidoadenosine 5'-triphosphate formed a covalent bond with α -tubulin [37]. However, in the glycerol/ Mg^{2+} system GDP inhibited while ATP as well as GTP stimulated assembly [35,36], while in our paclitaxel system both GDP and ATP were inhibitory.

Our findings correlate better with the findings of Jameson and Caplow [38], who examined the effects of millimolar concentrations of nucleotides on the assembly of porcine microtubule protein. These workers also concluded that there was an additional, low affinity nucleotide binding site on tubulin. They found that inhibition of assembly by 3 mM GDP could not be overcome by GTP, arguing against an E-site effect, and that even high concentrations of GTP were inhibitory. They, like us, also found weaker inhibitory effects on assembly of ATP and CTP, but not ADP. The chief difference in their findings versus ours is that we observed inhibitory effects with paclitaxel and without exogenous Mg^{2+} at micromolar GDP and millimolar ATP concentrations, while the effects they observed with microtubule protein and 0.5 mM Mg^{2+} occurred with millimolar GDP and ATP concentrations. Adding MAPs to our system reduced the effectiveness of GDP at least 100-fold, consistent with the findings of Jameson and Caplow [38] with microtubule protein, but we did not examine MAPs with ATP. A confounding factor in the work of Jameson and Caplow [38] is the presence of high amounts of nucleoside diphosphate kinase in microtubule protein, but this enzyme is not present in either our tubulin preparation or in heat-treated MAPs [11].

If such a third nucleotide binding site exists, its potential physiological role is unclear. It is only unmasked under highly atypical reaction conditions and, moreover, has a much lower affinity for GDP as compared with the E-site. Most likely, such a site would have a more specific ligand with higher affinity. If better defined, it might be exploited as a therapeutic target.

Another possible explanation for the inhibitory effects of GDP and ATP is that the nucleotides could bind in one of the well defined drug binding sites on tubulin, including the paclitaxel site itself. We examined inhibitory effects of podophyllotoxin and maytansine, as representatives of colchicine and vinca site ligands, with and without MAPs under the reaction conditions used to study GDP inhibition of paclitaxel-dependent assembly. As noted above, the ratio of IC_{50} values with and without MAPs for GDP was over 100 (1.6 mM vs 15 μM). Preliminary IC_{50} values (<5 μM in all experiments) obtained for both podophyllotoxin and maytansine were nearly identical under the two reaction conditions. We conclude that the effect of GDP on paclitaxel-induced assembly is not exerted through either the colchicine or vinca site.

We examined potential binding of GDP in the taxoid site by using fluorescence anisotropy to determine whether GDP, like numerous taxoid site ligands, could displace a fluorescent taxoid analog from microtubules [46]. These studies showed that GDP was unable to displace the fluorescent taxoid (personal communication, Dr. Fernando Díaz, Centro de Investigaciones

Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain). Thus, GDP is unlikely to interact at the taxoid site.

Molecular modeling provides an approach to locate an unknown binding site on tubulin. Since the E- and N-sites of tubulin are unique in their amino acid sequences [15,19,20], the primary structures of α - and β -tubulin provided no clue where GDP might bind to produce the inhibitory effect we observed on GTP-independent, paclitaxel-dependent assembly. Our approach was to compare the conformations of the polypeptide backbones of GDP binding sites of over 460 proteins in the PDB with the conformations of short segments of the backbones of α - and of β -tubulin. Matching conformations were scattered over both tubulin subunits, but only the sequence in α -tubulin from residues 15 through 35 recurred frequently, with 30 matches. The best four backbone conformation matches in this region were examined further for the feasibility of entry of GDP into a reasonable binding pocket, and the best model is shown in Fig. 7. Criteria for this model included (1) minimal disruption of backbone conformation or overall secondary structure, (2) placement of the diphosphate moiety so that it is closer to the solvent accessible protein surface compared with the guanine and ribose moieties, (3) amino acid side chain charge neutralization of the diphosphate moiety, and (4) biochemically feasible complementarity between the binding site amino acid contacts and the atoms of the ribose and guanine moieties.

Even though ATP was 140-fold less active than GDP as an inhibitor of paclitaxel-driven assembly, we also performed a preliminary evaluation of how adenine nucleotides would fit in the new GDP pocket. Somewhat surprisingly, ATP fit relatively well, and ADP fit poorly. The amino group of the adenine moiety formed hydrogen bonds directly with the polypeptide chain backbone of α -tubulin, as opposed to the water-mediated hydrogen bond between the guanine carbonyl group and the polypeptide backbone described above. This direct hydrogen

bonding resulted in a 1.5–2.0 Å shift in the position of the adenine ring, which in turn caused changes in the relative positions of the ribose and polyphosphate moieties. As a consequence, the β -phosphate group of ADP, unlike that of GDP, did not have an effective interaction with Lys-40 and approached the carboxylate group of Glu-22, causing electrostatic repulsion between two negative charges. In contrast, the γ -phosphate group of ATP preserved the electrostatically favorable interaction with Lys-40.

In summary, the inhibitory effect of GDP on paclitaxel-driven tubulin assembly in the absence of exogenous GTP is unlikely to be mediated either through the E-site or through one of the standard drug binding sites on tubulin. While the affinity of GDP for this site appears to be in the mid-micromolar range ($>15 \mu\text{M}$, based on the IC_{50} for inhibiting assembly), the inhibitory effect of GDP is most noticeable when tubulin assembly is feeble. Enhancing nucleation reduced inhibition by GDP. The inhibitory effect of GDP appears to be mediated by a distinct site on tubulin that may represent a site for the regulation of microtubule assembly or function. The relatively low affinity of GDP for this site, as compared with GDP/GTP affinity for the E- and N-sites, suggests that this site usually accommodates another ligand. If the site can be better defined, it may be possible to design or discover pharmacologically active agents with high affinity for the site. Such agents presumably would inhibit tubulin assembly and therefore should have therapeutic potential.

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References

- [1]. Schiff PB, Fant J, Horwitz SB. *Nature (London)* 1979;277:665–667. [PubMed: 423966]
- [2]. Eisenhauer EA, Vermorken B. *Drugs* 1998;55:5–30. [PubMed: 9463787]
- [3]. Toyama Y, Forry-Schaudies S, Hoffman B, Holtzer H. *Proc. Natl. Acad. Sci. USA* 1982;79:6556–6560. [PubMed: 6128733]
- [4]. Derry WB, Wilson L, Jordan MA. *Biochemistry* 1995;34:2203–2211. [PubMed: 7857932]
- [5]. Schiff PB, Horwitz SB. *Biochemistry* 1981;20:3247–3252. [PubMed: 6113842]
- [6]. Kumar N. *J. Biol. Chem* 1981;256:10435–10441. [PubMed: 6116707]
- [7]. Hamel E, del Campo AA, Lowe MC, Lin CM. *J. Biol. Chem* 1981;256:11887–11894. [PubMed: 6117556]
- [8]. Thompson WC, Wilson L, Purich DL. *Cell Motil* 1981;1:445–454.
- [9]. Díaz JF, Andreu JM. *Biochemistry* 1993;32:2747–2755. [PubMed: 8096151]
- [10]. Grover S, Rimoldi JR, Molinero AA, Chaudhary AG, Kingston DGI, Hamel E. *Biochemistry* 1995;34:3927–3934. [PubMed: 7696257]
- [11]. Hamel E, Lin CM. *Biochemistry* 1984;23:4173–4184. [PubMed: 6487596]
- [12]. Grover S, Hamel E. *Eur. J. Biochem* 1994;222:163–172. [PubMed: 8200341]
- [13]. Terry BJ, Purich DL. *J. Biol. Chem* 1980;255:10532–10536. [PubMed: 6253478]
- [14]. Sackett DL, Bhattacharyya B, Wolff J. *J. Biol. Chem* 1985;260:43–45. [PubMed: 3965457]
- [15]. Sternlicht H, Yaffe MB, Farr GW. *FEBS Lett* 1987;214:226–235. [PubMed: 3106086]
- [16]. Hesse J, Theiraug M, Ponstingl H. *J. Biol. Chem* 1987;262:15472–15475. [PubMed: 3680207]
- [17]. Linse K, Mandelkow E-M. *J. Biol. Chem* 1988;263:15205–15210. [PubMed: 3170578]
- [18]. Shivanna BD, Mejillano MR, Williams TD, Himes RH. *J. Biol. Chem* 1993;268:127–132. [PubMed: 8416920]
- [19]. Burns RG, Farrell KW. *Trends Cell. Biol* 1996;6:297–303. [PubMed: 15157437]
- [20]. Nogales E, Wolf SG, Downing KH. *Nature London* 1998;391:199–203. [PubMed: 9428769]
- [21]. Löwe J, Li H, Downing KH, Nogales E. *J. Mol. Biol* 2001;313:1045–1057. [PubMed: 11700061]
- [22]. Ravelli RBG, Gigant B, Curmi PA, Jourdain I, Lachkar S, Sobel A, Knossow M. *Nature (London)* 2004;428:198–202. [PubMed: 15014504]
- [23]. Gigant B, Wang C, Ravelli RBG, Roussi F, Steinmetz MO, Curmi PA, Sobel A, Knossow M. *Nature (London)* 2005;435:519–522. [PubMed: 15917812]
- [24]. Accelrys, Insight 2005 Molecular Modeling Workbench.
- [25]. Leung AKW, White EL, Ross LJ, Reynolds RC, DeVito JA, Borhani DW. *J. Mol. Biol* 2004;342:953–970. [PubMed: 15342249]
- [26]. Maple JR, Hwang M-J, Jalkanen KJ, Stockfisch TP, Hagler A. *J. Comput. Chem* 1998;19:430–458.
- [27]. Kowalski RJ, Giannakakou P, Hamel E. *J. Biol. Chem* 1997;272:2534–2541. [PubMed: 8999970]
- [28]. Ponstingl H, Krauhs E, Little M, Kempf T. *Proc. Natl. Acad. Sci. USA* 1981;78:2757–2761. [PubMed: 7019911]
- [29]. Krauhs E, Little M, Kempf T, Hofer-Warbinek R, Ade W, Ponstingl H. *Proc. Natl. Acad. Sci. USA* 1981;78:4156–4160. [PubMed: 6945576]
- [30]. Huang AB, Lin CM, Hamel E. *Biochim. Biophys. Acta* 1985;832:22–32. [PubMed: 3931683]
- [31]. Mejillano MR, Himes RH. *Arch. Biochem. Biophys* 1991;291:356–362. [PubMed: 1952949]
- [32]. Correia JJ, Baty LT, Williams RC Jr. *J. Biol. Chem* 1987;262:17278–17284. [PubMed: 2826416]
- [33]. Sillén, LG.; Martell, AE. *Stability Constants of Metal-Ion Complexes*. The Chemical Society; London, UK: 1971.
- [34]. Kowalski RJ, Giannakakou P, Gunasekera SP, Longley RE, Day BW, Hamel E. *Mol. Pharmacol* 1997;52:613–622. [PubMed: 9380024]
- [35]. Zabrecky JR, Cole RD. *J. Biol. Chem* 1980;255:11981–11985. [PubMed: 7440578]
- [36]. Zabrecky JR, Cole RD. *J. Biol. Chem* 1982;257:4633–4638. [PubMed: 7068656]
- [37]. Zabrecky JR, Cole RD. *Arch. Biochem. Biophys* 1983;225:475–481. [PubMed: 6688710]
- [38]. Jameson L, Caplow M. *J. Biol. Chem* 1980;255:2284–2292. [PubMed: 6102089]

- [39]. Farr GW, Yaffe MB, Sternlicht H. Proc. Natl. Acad. Sci. USA 1990;87:5041–5045. [PubMed: 2367522]
- [40]. Chakrabarti G, Mejillano MR, Park Y-H, Vander Vel de DG, Himes R. Biochem 2000;39:10269–10274. [PubMed: 10956016]
- [41]. Kong C, Ito K, Walsh MA, Wada M, Liu Y, Kumar S, Barford D, Nakamura Y, Song H. Mol. Cell 2004;14:233–245. [PubMed: 15099522]
- [42]. Vitagliano L, Masullo M, Sica F, Zagari A, Bocchini V. EMBO J 2001;20:5305–5311. [PubMed: 11574461]
- [43]. Löwe J, Amos LA. Nature (London) 2001;391:203–206. [PubMed: 9428770]
- [44]. Zeeberg B, Caplow M. Biochemistry 1979;18:3880–3886. [PubMed: 486401]
- [45]. Hamel E, Batra JK, Huang AB, Lin CM. Arch. Biochem. Biophys 1986;245:316–330. [PubMed: 3954356]
- [46]. Buey RM, Barasoain I, Jackson E, Meyer A, Giannakakou P, Paterson I, Mooberry S, Andreu JM, Díaz JF. Chem. Biol 2005;12:1269–1279. [PubMed: 16356844]

Fig. 1.

Progressive inhibition of tubulin assembly induced by paclitaxel by increasing concentrations of GDP. Each 200 μL reaction mixture contained tubulin at 3.0 mg mL^{-1} (30 μM), 30 μM paclitaxel, 0.1 M Mes (pH 6.9), and 3% (v/v) dimethyl sulfoxide (the drug solvent). GDP concentrations were as follows: curve 1, none; curve 2, 5.0 μM ; curve 3, 10 μM ; curve 4, 25 μM ; curve 5, 50 μM ; curve 6, 100 μM .

Fig. 2.

Effect of GTP on the inhibitory effect of GDP on tubulin polymerization induced by paclitaxel. Each 200 μ L reaction mixture contained tubulin, paclitaxel, Mes, and dimethyl sulfoxide as described in the legend for Fig. 1 and the indicated concentrations of GDP and GTP. Symbols as follows: squares, no GTP; circles, 5.0 μ M GTP; upright triangles, 20 μ M GTP; diamonds, 150 μ M GTP; inverted triangles, 500 μ M GTP. Standard errors indicated by the error bars, unless the standard error value was smaller than the symbol.

Fig. 3. Effect of Mg^{2+} on the inhibitory effect of GDP on tubulin polymerization induced by paclitaxel. Each 200 μL reaction mixture contained tubulin, paclitaxel, Mes, and dimethyl sulfoxide as described in the legend for Fig. 1 and the indicated concentrations of GDP and MgCl_2 . Symbols as follows: squares, no MgCl_2 (same curve shown in Fig. 2); circles, 2.0 mM MgCl_2 . Standard errors indicated by the error bars, unless the standard error value was smaller than the symbol.

Fig. 4.

Progressive inhibition of tubulin assembly with MAPs and paclitaxel by increasing concentrations of GDP. Each 200 μL reaction mixture contained tubulin at 1.0 mg mL^{-1} ($10 \mu\text{M}$), 0.75 mg mL^{-1} heat-treated MAPs, $10 \mu\text{M}$ paclitaxel, 0.1 M Mes (pH 6.9), and 1% (v/v) dimethyl sulfoxide (the drug solvent). GDP concentrations were as follows: curve 1, none; curve 2, 0.25 mM ; curve 3, 0.5 mM ; curve 4, 1.0 mM ; curve 5, 2.0 mM ; curve 6, 5.0 mM .

Fig. 5. Effects of nucleoside 5'-triphosphates on paclitaxel-induced assembly of tubulin. (A) Weak inhibition by ATP. Each 200 μ L reaction mixture contained tubulin, paclitaxel, Mes, and dimethyl sulfoxide as described in the legend for Fig. 1. Additional components as follows: curve 1, no further addition; curve 2, 1.0 mM ATP; curve 3, 1.5 mM ATP; curve 4, 2.0 mM ATP; curve 5, 2.5 mM ATP. (B) Stimulatory effects of UTP, XTP, and ITP compared with that of GTP. Each 200 μ L reaction mixture contained tubulin, paclitaxel, Mes, and dimethyl sulfoxide as described in the legend for Fig. 1. Additional components as follows: curve 1, no further addition; curve 2, 50 μ M GTP; curve 3, 50 μ M ITP; curve 4, 50 μ M UTP; curve 5, 50

μM XTP; curve 6, 2.5 mM GTP; curve 7, 2.5 mM ITP; curve 8, 2.5 mM XTP; curve 9, 2.5 mM UTP.

Fig. 6. GDP binding domain protein backbone alignments with α -tubulin. The α -tubulin backbone secondary structure from residues 17 through 34 (taken from 1TUB, or tub, of the PDB) is shown in white, and the amino acid sequence is shown the diagram, a17 indicating the sequence begins at residue 17. Superimposed on the α -tubulin backbone are the four best matches of the 30 sequences that met computational criteria for this sequence of α -tubulin. Highly homologous sequences from 1R5N (or r5n) and 1JNY (or jny) are shown in green, along with the corresponding backbone conformation, with the initial residue position and sequences shown below the α -tubulin sequence. Similarly, the segments of 1JFF (or jff) and 1RQ7 (or rq7) are shown in dark blue and cyan, respectively, with the backbone conformations superimposed on the corresponding segments of the α -tubulin backbone and the initial amino acid residue positions and overlapping sequences shown below the α -tubulin sequence. Similar results were obtained with 1JFF.

Fig. 7.

A molecular model of a hypothetical additional nucleotide binding site in α -tubulin, with GDP bound in the site. In green, a ribbon backbone rendering of a portion of α -tubulin, taken from the electron crystallographic-derived tubulin structure (PDB refcode = 1TUB). The cyan ribbon superimposed on the green ribbon demonstrates the minimal conformational change in the polypeptide backbone required to accommodate the GDP molecule (for further details, see Materials and methods), based on the 1RQ7 conformational homology that led us to this area on α -tubulin. GDP and selected side chains are shown as space-filling models, with the side chain carbons shown in cyan and the GDP carbon and phosphorus atoms shown in magenta. Oxygen and nitrogen atoms are shown in red and blue, respectively. For orientation, the N-site GTP is faintly shown (space-filling model, with the carbon and phosphorous atoms in gray-yellow, the nitrogens in dark blue, and the oxygens in maroon), along with the side chains of

Pro-63 and Phe-87. The centroid of the N-site guanine moiety is approximately 15 Å from the β -phosphate of the GDP bound in the proposed new site. The charge neutralization of the GDP phosphate groups by Lys-40 and Arg-84 is also shown. Almost identical results were obtained when the 1JFF tubulin structure was used to model the α -tubulin GDP binding site.

Table 1

Lengths of paclitaxel-induced microtubules formed with and without GDP.

GDP(μ M)	Mean microtubule length (μ m) $\pm$ SD ^a			Total sample size
	Experiment I	Experiment II	Average	
0	3.2 $\pm$ 1.9	2.1 $\pm$ 1.4	2.5 $\pm$ 1.7	1872
5	3.7 $\pm$ 2.2	2.1 $\pm$ 1.5	2.8 $\pm$ 2.1	1065
10	3.5 $\pm$ 2.3	2.2 $\pm$ 1.6	2.8 $\pm$ 2.1	1225
50	4.5 $\pm$ 3.2	2.0 $\pm$ 1.5	2.7 $\pm$ 2.4	1271

^a Microtubule lengths were determined as described in the text.