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Characterization of a Protein-Small Molecule Interaction between Microtubules and Novel Ruthenium-Polypyridyl Complexes

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Characterization of a Protein-Small Molecule Interaction between Microtubules and Novel Ruthenium-Polypyridyl Complexes

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Abbreviations

GTP	Guanosine Triphosphate
GDP	Guanosine Diphosphate
GAP	GTPase Activating Protein
RPC	Ruthenium Polypyridyl Complex
DP	($[Ru(dip)_2phen]Cl_2$)
DB	($[Ru(dip)_2bpy]Cl_2$)
PTX	Paclitaxel
MT(s)	Microtubule(s)
MSA(s)	Microtubule Stabilizing Agent(s)
MDA(s)	Microtubule Destabilizing Agent(s)
DMSO	Dimethyl Sulfoxide
FL	Fluorescence
ITC	Isothermal Titration Calorimetry
CD	Circular Dichroism

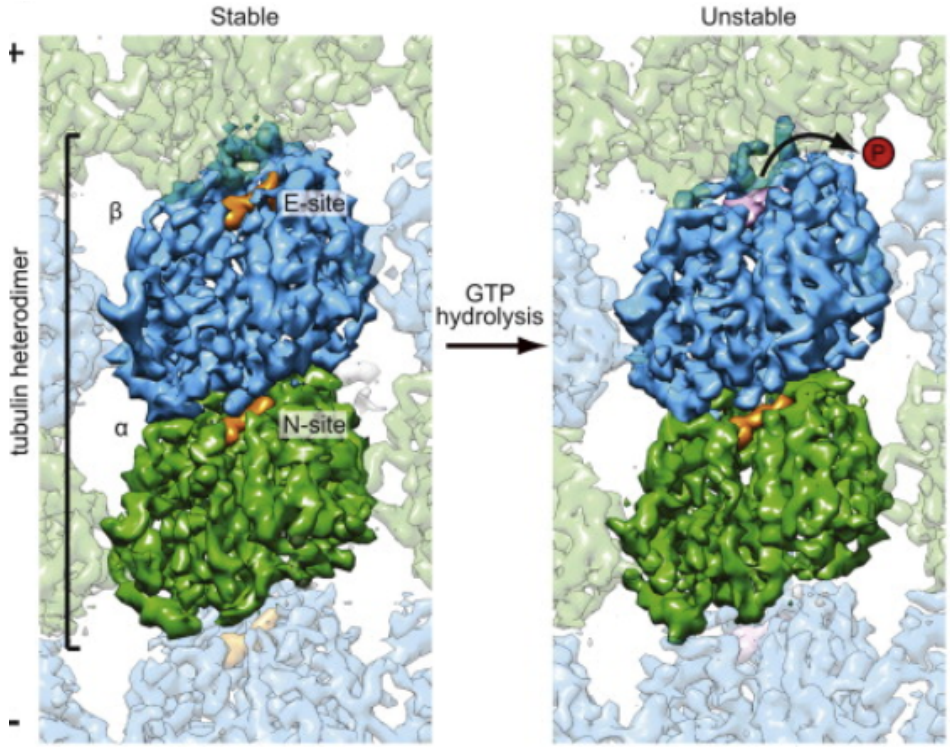
Abstract

Microtubules are cytoskeletal filaments that play a role in essential functions within the cell such as cell motility, intracellular transport, structural support and chromosome segregation. Tubulin is a heterodimeric protein that exhibits GTP dependent polymerization and self-assembles into polar microtubule filaments. Microtubules are dynamic polymers corresponding to their role in separation of duplicated chromosomes during mitosis. As the polymers cycle through rounds of polymerization and depolymerization based on their nucleotide state, the chromosomes are pulled towards the poles in the mitotic cell. Their normal function and dynamics can be disrupted in highly proliferative cells that interrupt cell cycle progression revealing microtubules as an antimitotic cancer therapy target protein. Paclitaxel, also known as Taxol, is a current chemotherapeutic agent that binds microtubules and stabilizes the polymerized state, disrupting their dynamic action, stalling the cell cycle, and leading to apoptosis. Ruthenium-Polypyridyl Complexes are metalloorganic compounds with aromatic ring structures complexed to a center Ruthenium cation, some of which have been identified as a Microtubule Stabilizing Agent (MSA). The novel RPCs under investigation in this research are ($[Ru(dip)_2bpy]Cl_2$) (DB) and ($[Ru(dip)_2phen]Cl_2$ (DP). The first goal of this research was to determine if these novel compounds bind to microtubules. Fluorescence titrations between the microtubules and each small molecule were performed with increasing concentration of the RPC to measure the binding affinity (K_d). Polymerization data reveals that each novel small molecule enhances the rate and degree of polymerization in a similar manner as Paclitaxel. Circular Dichroism reveals the binding of the novel small molecule to MTs does not result in any significant secondary structural changes. ITC data further supports the notion that the novel RPCs are tightly binding to MTs and binding to a similar degree as other known MSAs, showing the importance in futher research endeavors on these novel RPCs to determine their potential in antimitotic chemotherapy.

Introduction

Microtubules are a primary component in cytoskeletal structures and play diverse roles throughout the cell. Microtubules are formed by parallel arrangements of multiple protofilaments that are repeated units of Tubulin (1). Tubulin is a heterodimeric protein that consists of an α and β subunit, 55kDa each, exhibiting highly conserved sequence homology. The polymerization of tubulin dimers is dependent on their guanine nucleotide state with binding sites on each subunit. The α subunit contains the non-exchangeable site (N site), while the β subunit contains the exchangeable site (E site) shown in Figure 1 (1, 2).

Figure 1. Heterodimer structure within Protofilament (2)

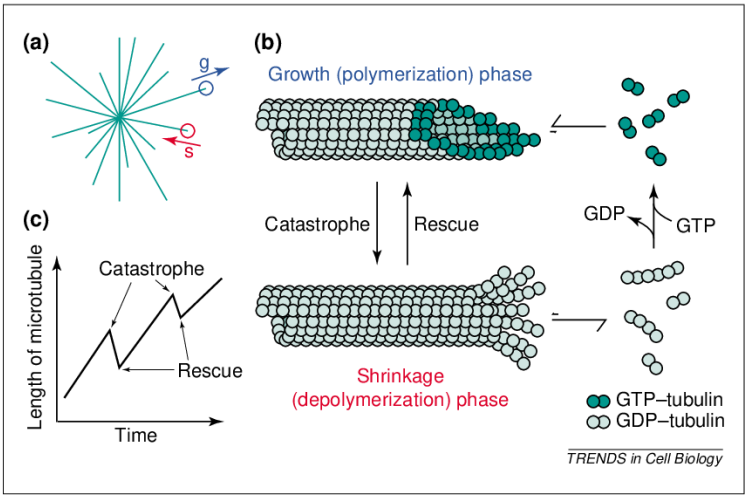


The N-site on α-tubulin is constitutively bound to GTP. Polymerization can spontaneously occur if the E site on Tubulin is also bound to GTP, and optimally occurs at 37°C. The growth phase is ensured by GTP caps at the ends of the protofilaments (1). The tubulin-GTP conformation allows a straight, parallel arrangement of the

protofilaments and creates a stable interaction (1). The GTP can be lost in the E site due to the β subunit intrinsic GTPase activity (3). This hydrolyzing activity is activated once the dimer has been added to the polymer, so the interior dimers will be GDP-tubulin while the GTP protective caps determine the ultimate fate of the polymer (4). When this protective GTP cap is lost at the β subunit end, a catastrophe event results, and the microtubules enter a state of depolymerization. The intrinsic GTPase activity is almost completely absent alone but can be potentiated by most notably $G\alpha_s$, which is a GTPase Activating Protein (GAP) for β-Tubulin (3). The α subunit of a G_S-protein is GTP activated during G-Protein Couple Receptor signaling and is involved in various signal transduction cascades (5). Therefore, the microtubule structure is coordinated with many events occurring in the cell with variability during certain periods for the cell.

The cyclical patterns of growth and shrinkage are shown in Figure 2. When tubulin contains GTP bound in both subunits, the MTs will spontaneously enter a rescue phase and increase in length by the addition of these GTP-Tubulin dimers (Figure 2). When the GTP is lost from the regulatory β subunit at the protective end, the MTs will enter catastrophe and depolymerize starting at that site (Figure 2).

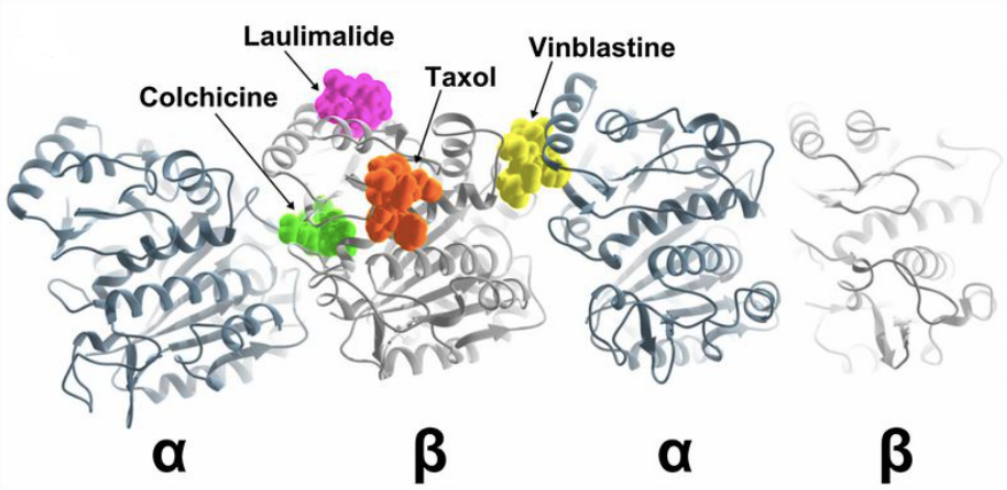
Figure 2. Guanine-Nucleotide State dependent mechanism of MT Polymerization (4)



The spontaneous dynamic activity of these polymers corresponds to their vital role in the cell, especially in mitotic events (6). During interphase, the microtubules are relatively stable with a low occurrence of catastrophe events (1). As a cell enters mitosis, the microtubules are more unstable and experiencing higher dynamic periods of shrinkage (1). Microtubules are organized in the centrosome and form the spindle apparatus, creating a cytoskeletal attachment from the duplicated chromosomes to the poles of the multiplying cell. As the microtubules cycle through these dynamic catastrophic events, the chromatids are directionally pulled to separate poles of the cell (6). Microtubules thus have an essential role in the mitotic pathway, providing a protein target for therapies treating highly proliferative cellular pathologies.

Microtubules have specific domains that small molecules can bind and interfere with normal MT function (Figure 3) (7-9). These drugs largely disrupt the equilibrium between free tubulin dimers and microtubules (8,10). Four regions on the microtubules have been well-characterized which include the taxoid, laulimalide, colchicine, and vinca site (8,11). All of these drug targeting locations are found on the regulatory β subunit or between the subunit interfaces.

Figure 3. Known Binding Domains in Microtubules (8)



Microtubule targeting molecules can be categorized into two subsets: 1) stabilizing and 2) destabilizing (12). Microtubule stabilizing agents, MSAs, bind to the microtubules and stabilize the polymer. The microtubule destabilizing agents, MDAs, bind to microtubules and increase the likelihood of depolymerizing catastrophic events (12). These protein-drug interactions disrupt the normal dynamic activity of the microtubules. A very important MSA is Paclitaxel, which is a current antineoplastic drug used in the treatment of breast cancer and many advanced carcinoma's (13). Paclitaxel interacts with MTs and disrupts their normal activity during mitosis and most commonly stalls the cell cycle at the M-phase checkpoint (13). This halt in cell cycle progression can be from abnormal MT length or tension in the spindles due to the drug interaction and interference with the normal MT dynamics (13). Cell cycle regulators precisely monitor the progress of mitosis and arresting at the M-phase checkpoint can trigger an internal cascade leading to apoptosis (10). This abnormal mitotic exit is an essential component for an MSA to trigger apoptosis (14). The induction of apoptosis is a clear mechanism for the suppression of oncogenic proliferation and tumorigenesis.

Microtubule Stabilizing Agents are complex organic molecules with many ring structures and chiral centers (7). RPCs are Ruthenium Polypyridyl Complexes which exhibit this aromatic property and are under evaluation for their activity as an MSA. Ruthenium based compounds have largely gained interest due to their potency, low toxicity, high efficacy and less overall drug resistance in comparison to more commonly used metalloorganic antitumor drugs containing Platinum (15). RPC structural subtypes, tris-chelate compounds, have been extensively investigated as they exhibit exceptional chemical stability and are kinetically inert molecules (7). They also have been identified

as metabolically stable from excretional studies in animal models (7). RPC tris-chelate complexes' strong chemical integrity is an important reason these compounds are being investigated for potential therapeutics (7). Two tris-chelate compounds complexed with Ruthenium, RPC1($[Ru(phen)_3]Cl_2$) and RPC2 ($[Ru(DIP)_3]Cl_2$), show *in vitro* enhancement of polymerization and RPC2 *in vivo* strongly disrupts MT structure and dynamics which further suggests the apoptotic pathway as the mechanism of its cytotoxicity (7). Limitations with these small molecules is their requirement for organic dissolution. Novel tris-chelate RPCs of particular interest to this research are DB ($[Ru(dip)_2bpy]Cl_2$) and DP ($[Ru(dip)_2phen]Cl_2$) (figure 4,5) which have been synthesized in efforts to make more water-soluble in comparison to previously studied RPCs (7).

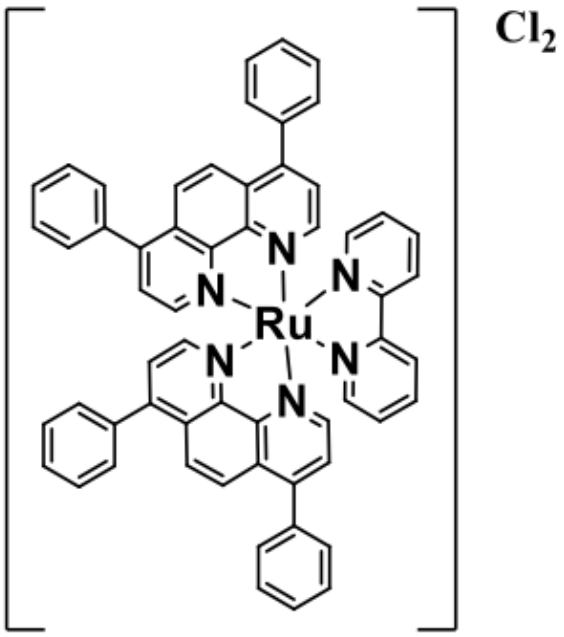


Figure 4. DB Molecular Structure (unpublished data)

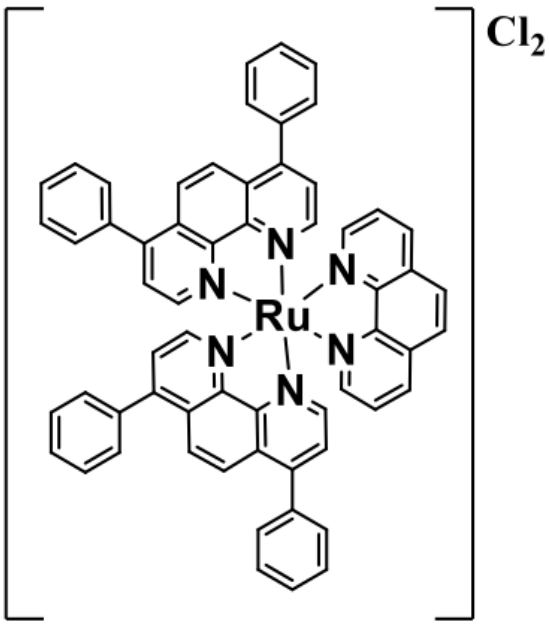


Figure 5. DP Molecular Structure (unpublished data)

Of particular interest is the characterization of the binding interaction between novel RPCs and MTs to futher understand the possible implications and effects of this

interaction on intrinsic properties and dynamics of MTs. This research will investigate whether the novel RPCs (DP and DB) bind to MTs by performing fluorescence binding studies. The thermodynamics of such binding will be characterized using ITC. The interaction will be further evaluated by a polymerization assay to determine whether DP and DB act as an MSA through measuring the kinetics and degree of polymerization in the presence and absence of the molecules. The secondary structure of the tubulin will be evaluated for effects of the small molecules on these intrinsic properties of the protein. The characterization of these parameters will allow for comparison of the novel small molecules with others Ruthenium-Polypyridyl Complexes and Paclitaxel on the mode and degree of impact on MTs which will add to our current working knowledge and guide future research trajectories for their evaluation as an antimitotic chemotherapeutic agent.

Materials and Methods

Reagents DB ($Ru(dip)_2bpy]Cl_2$) and DP ($[Ru(dip)_2phen]Cl_2$) were synthesized and provided to the laboratory in a collaborative research effort from the University of Texas. Tubulin porcine brain (>99%), GTP stock (100uM), general tubulin buffer (80mM Piperazine-N-N'-bis[2-ethanesulfonic acid] sequisodium salt; 2.0mM Magnesium chloride; 0.5mM Ethylene glycol-bis (b-amino-ethyl ether) N,N,N,N'-tetra-acetic acid, pH 6.9), 60% glycerol tubulin buffer (general tubulin buffer + 60% v/v glycerol), DMSO and Paclitaxel (10uM) were purchase from Cytoskeleton Inc. in the Tubulin Polymerization Kit.

Preparation of Aqueous RPC solutions Solubility studies completed show the novel RPCs (DP and DB) are water soluble and soluble up to concentrated stocks of 10mM. The dissolution in water was enhanced by the addition of 50^{o}C hot water bath for 2 min. DB and DP were also soluble to the same degree in 10% glycerol tubulin buffer. 10mM stocks were made in the 10% glycerol tubulin buffer to maintain solvent consistency throughout experimentation and stored at -20^{o}C.

Reconstitution of Tubulin For the first set samples, 3mg/mL (~55uM per subunit) tubulin stocks were made using 10% glycerol tubulin buffer (1x6 dilution using 60% glycerol and general tubulin buffer) with a final GTP concentration of 1mM. 10mg of lyophilized tubulin was added to 3.3 mL of 10% glycerol tubulin buffer + GTP (1mM). The stocks were aliquoted in 100uL samples and immediately snap frozen in liquid nitrogen and stored at -80^{o}C. For the second set of samples, 10mg/mL (~180uM per subunit) tubulin stocks were made using the same 10% glycerol tubulin buffer with a final GTP concentration of 1mM. 10mg of tubulin was added to 1mL of the tubulin buffer + GTP and 5 x 200uL samples were aliquoted, snap frozen and stored at -80^{o}C.

Fluorescence Titrations Intrinsic tryptophan fluorescence spectroscopy was used as tubulin contains 8 trp residues, therefore the fluorescence quenching can be observed over the 300-400nm range with excitation at 280nm to observe the protein drug interaction. 3mg/mL tubulin stocks were diluted in a 1x10 dilution using 900uL of 10% glycerol tubulin buffer + GTP (1mM) and 100uL of the 3mg/mL stock giving a final concentration of the dimer as 10mM. The small molecule concentration was optimized by running initial scans starting with 5uL injection of 1mM drug or 0.5mM drug to 400uL of 10uM MT. The fluorescence readout gave a more consistent pattern and curve using the

1mM RPC sample and was chosen for the titration studies. A JASCO J-1500 CD Spectrometer machine was used. The cell and holder temperature were set to 37^0C to maintain the protein predominantly in the polymer form. The small molecule was titrated into the 400uL of MT by 5uL injections manually (x10) and the fluorescence readout was recorded after each injection. Each small molecule was run in a triplicate series. The F_{max} is 330nm for tryptophan, thus the fluorescence absorbance was reported at this wavelength over an increasing concentration of the small molecule. The triplicates were averaged and normalized using $(F_0\text{-}F)/F_0$. The data was fit to the Hill Plot using the Origins software to report the K_d, or binding affinity.

Polymerization Assay The protocol was replicated from the manufacturers instruction for the Optical Density based polymerization assay (Cytoskeleton BK006P) and a BioTek Synergy H1 Hybrid reader was used for the study. Paclitaxel, provided in the kit, was reconstituted using 100uL of DMSO, aliquoted and frozen in -80^{o}C. The assay was temperature regulated and performed at 37^{o}C to maintain the protein in polymer form, reducing temperature effects on polymerization rates. The 96-well plate was pre-warmed in 37^{o}C for 30 minutes prior to assay. 10uL of room temperature DP, DB and PTX stocks were added in sets of three to the pre-warmed wells for a final concentration of 10uM for each molecule. The 10mg/mL stock of tubulin was thawed quickly by removal from -80^{o}C into a 1-minute water bath at room temperature and put immediately on ice. 400uL of 10mg/mL tubulin stock was diluted to 3mg/mL by the addition of 840uL of ice cold 10% glycerol + GTP buffer. With a separate 96-well plate on ice, 12 samples of 100uL (3mg/mL) of tubulin were aliquoted. A multi-channel pipettor was used to transfer the 100ul x 12 samples to the pre-warmed well plate with

well identities as follows: 3 MT, 3 MT + PTX, 3 MT + DP, 3 MT+ DB. Readings started immediately. The spectrometer was set to kinetic mode with 61 cycles and 1 reading per minute. The absorbance wavelength was set to 340nm and monochromatic light was used. Data was normalized by subtracting the initial reading to control for any absorbances due to the buffer or small molecules used. The curves were fit to the Hill Plot using the Origins software to extract kinetic parameters such as V_{max}.

Far-UV Circular Dichroism CD was performed on the JASCO J-1500 CD Spectrometer at 37°C to investigate the secondary structural changes in the presence of the small molecule. 50uL of the 10mg/mL tubulin stock was diluted to 0.5mg/mL by the addition 950uL of 10% glycerol + GTP buffer. The 10% glycerol + GTP buffer used was initially diluted 10x by the addition of water to minimize the concentration of salt in solution. CD readings were taken over the range of 250nm to 190nm with a scan speed of 50nm/min. 5 readings were taken of 300uL of 0.5mg/mL MT at 37°C, averaged and reported using Origins. 5 readings were taken of 300uL MT (0.5mg/mL) + 3uL small molecule (10uM), averaged and reported using Origins. MT and small molecule were incubated together on ice for 5 minutes before CD was started.

Isothermal Titration Calorimetry ITC was performed using a GE MicroCal iTC_{200}. The temperature was set to 37°C for preformed MT. 50uL of 10mg/mL tubulin stock was diluted with 1.75mL of ice cold 10% glycerol buffer + GTP for a final concentration of 10uM. The small molecule was prepped in a 1x100 dilution of the 10mM stock to 100uM in preparation for a 1x10 concentration difference between protein and small molecule. 50uL of the molecule was titrated by 30 injections (1.66uL each) into 300uL of MT (10uM) over 30 minutes. Blanks were completed before

experimental ITC to equilibrate and normalize. The data was analyzed in the Origins software and provided thermodynamic parameters such as the binding constant (K), enthalpy (ΔH), entropy (ΔS) and stoichiometric coefficient of binding (N).

Results

Tubulin Reconstitution

Tubulin was reconstituted with DI water, aliquoted and snap frozen in liquid nitrogen and stored at -80^{0}C. Before experimentation, a 15% SDS-PAGE was performed under reducing conditions. A strong band is visualized at 55kDa, the molecular weight of each subunit. The concentration of tubulin run on the gel was 3mg/mL (~55uM per subunit), with 35uL in each lane. Successful reconstitution is seen, without denaturation occurring during storage time frame.

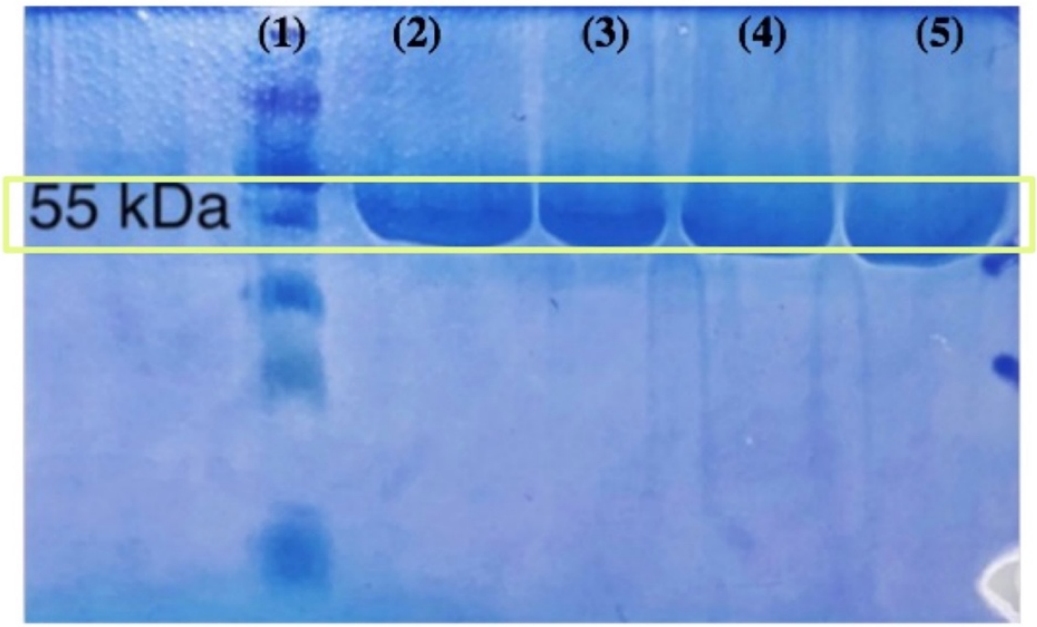


Figure 6. SDS-Page. Molecular weight marker Lane 1. Pure Tubulin monomers visualized in lanes 2-5 at 55kDa.

Novel-RPCs are water soluble

DP and DB were synthesized in efforts to make more water-soluble RPCs, which proved successful. Each compound was initially dissolved in a hot water bath at 50^0C for 2 minutes with stirring, in 1mM concentrations. Specific to this research, these compounds were soluble in the 10% glycerol tubulin buffer and soluble up to concentrations of 10mM without the addition of heat, further proving the solubility of the novel RPCs in hydrophillic solvents.

DP and DB addition to preformed MT exhibit a fluorescence redshift

The fluorescence readout was measured during the titration studies after each addition of 5uL (x 10) of the small molecule to the preformed MT at 37ºC. Fluorescence quenching was observed which is a decrease in the intensity of FL emission (Figure 7,8) that can be the result of energy transfer, molecular remodeling, or complex formation. The results also show a fluorescence redshift which is a shift to longer wavelength, lower intensity F_{max}. This can be the result of the tryptophan amino acids in the dimer being more exposed to the polar solvent, indicating some protein structural changes with the addition of the small molecules.

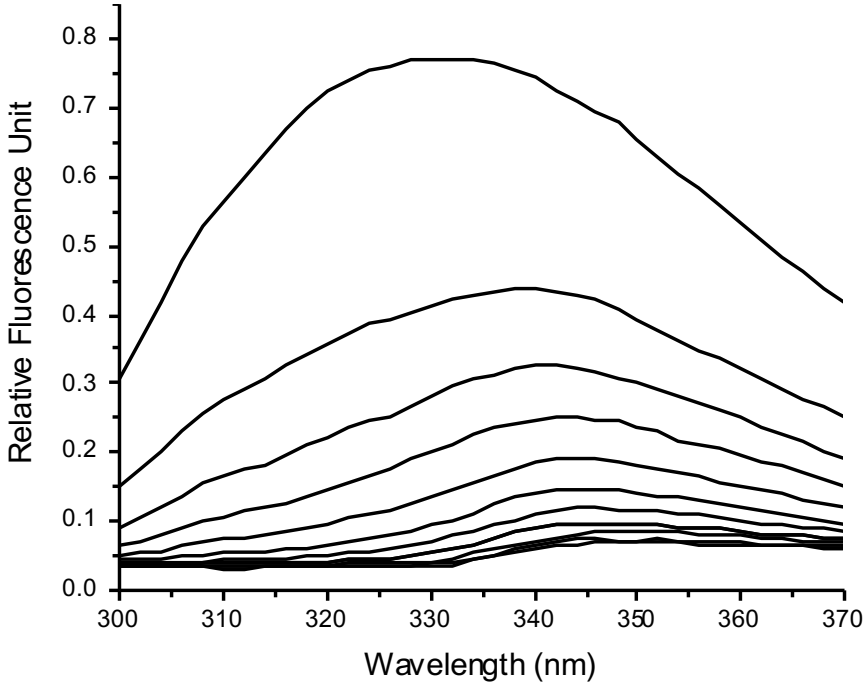


Figure 7. Fluorescence quenching observed for the interaction between MT and DP. As the concentration of the small molecule increases, the FL signal decreases. The Fmax of the first reading is approximately 330nm. The final reading with highest concentration of the small molecule is around 345-350nm, indicating a redshift.

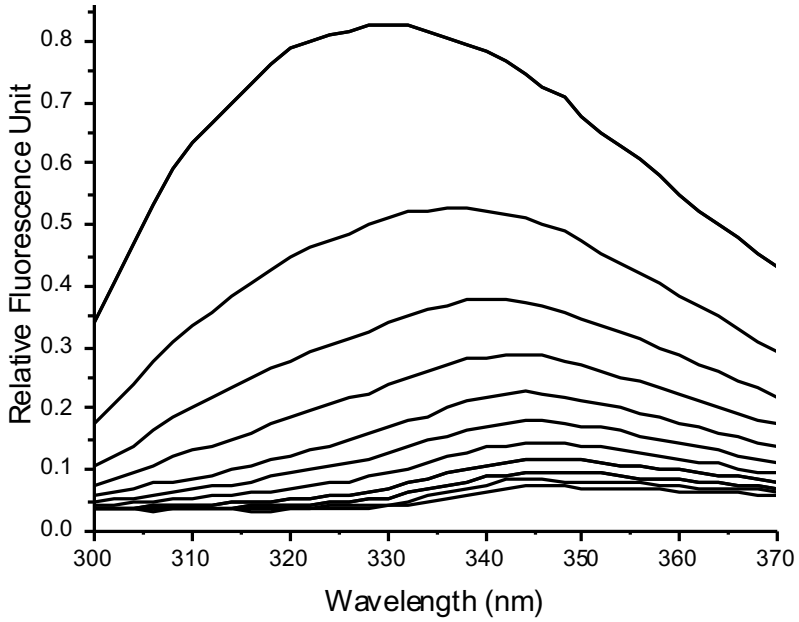


Figure 8. Fluorescence quenching observed for the interaction between MT and DB. As the concentration of the small molecule increases, the FL signal decreases. The Fmax of the first reading is approximately 330nm. The final reading with highest concentration of the small molecule is around 345-350nm, indicating a redshift.

DP and DB bind to preformed MTs with moderately strong binding affinities

DP and DB were evaluated for a possible binding interaction with preformed MT using intrinsic tryptophan fluorescence titrations. The fluorescence was measured for a constant concentration of the MT (10uM) and an increasing concentration of the small molecule (0-110uM), by titrating a small amount and reading the FL readout after each addition. The FL at the F_{max}=330nm was reported for each triplicate, averaged and fit to the Hill Plot (Figure 9,10). DP binds to MT with a K_d = 15uM. DB binds to MT with a K_d=19uM. The hyperbolic curve and K_d in the micromolar range show the novel RPC molecules are binding to the MT with a moderately strong binding affinity, which is in agreement to previously studied non-soluble RPCs (7).

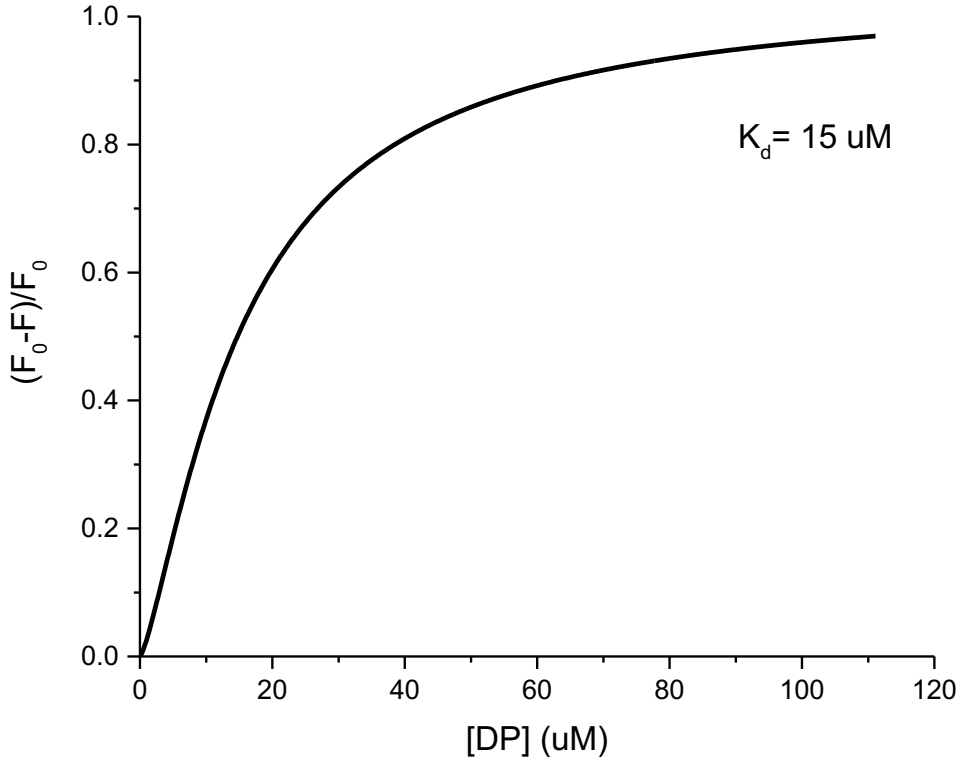


Figure 9. Hill Plot binding Curve for DP + MT (37° C). Data was normalized using $(F_o-F)/F_o$. The FL unit is reported at 330nm for an increasing concentration of the small molecule. A hyperbolic curve and binding site saturation is visualized. The Kd=15uM which suggests a moderate to strong binding affinity between DP and MT.

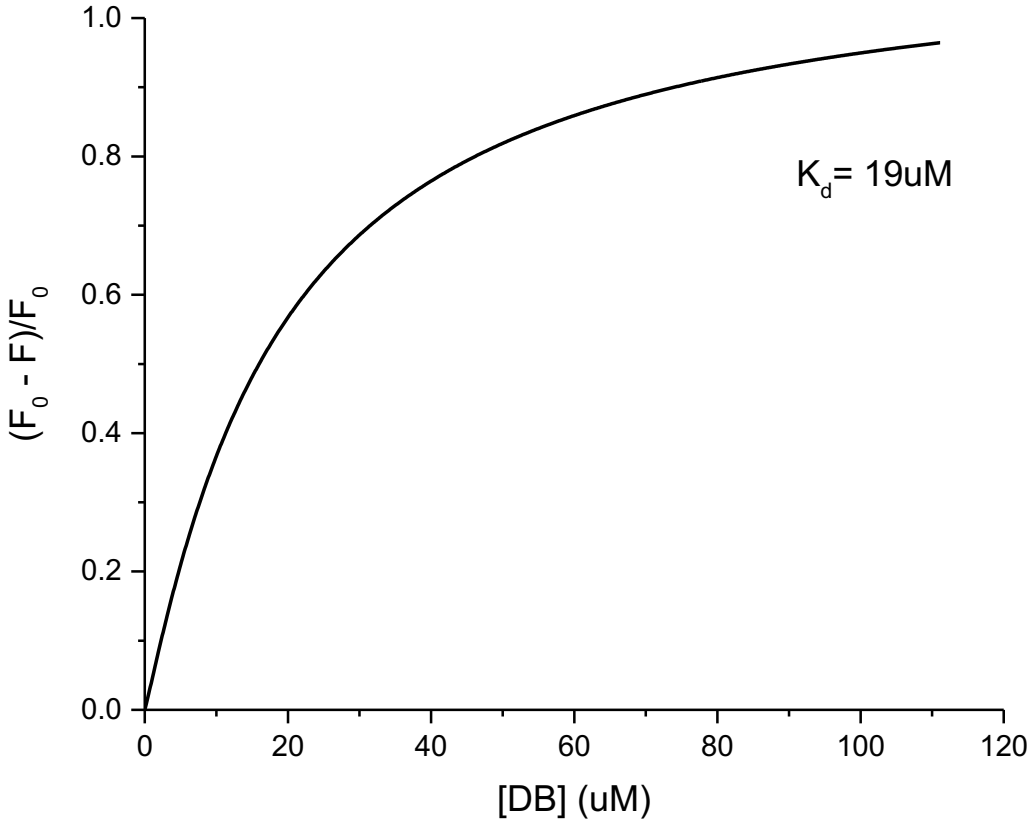


Figure 10. Hill Plot binding Curve for DB + MT (37° C). Data was normalized using (F_o-F)/F_o. The FL unit is reported at 330nm for an increasing concentration of the small molecule. A hyperbolic curve and binding site saturation is visualized. The Kd=19uM which suggests a moderate to strong binding affinity between DB and MT.

DP and DB acts as an MSA *in vitro*

The polymerization assay *in vitro* show the novel water-soluble RPCs enhance the rate and degree of polymerization with comparison to the positive (MT + PTX) and negative controls (MT) (Figure 11). The assay was performed at 37°C for preformed MT. The V_{max} for this experiment represents the maximal rate at which polymerization is occurring. The MT + Paclitaxel with a V_{max}=79mOD/min acts as the positive control as Paclitaxel is a current MSA used in the treatment of cancer. The MT alone acts as the negative control with a V_{max}=22mOD/min. There is approximately a 3-fold enhancement of the rate of polymerization in the presence of Paclitaxel supporting its identity as an

MSA. DP was found to have a V_{max}=43mOD/min, which is about a 2-fold increase in the rate of polymerization, suggesting DP *in vitro* has microtubule-stabilizing properties. DB was found to have a V_{max}=56mOD/min which is 2.5 faster than the MT alone, suggesting DB also has microtubule-stabilizing properties. The total OD corresponds to the length of the polymer, which shows at saturation, these novel RPCs (DP and DB) also enhance the degree of polymerization with respect to the MT alone, further suggesting their identify as an MSA.

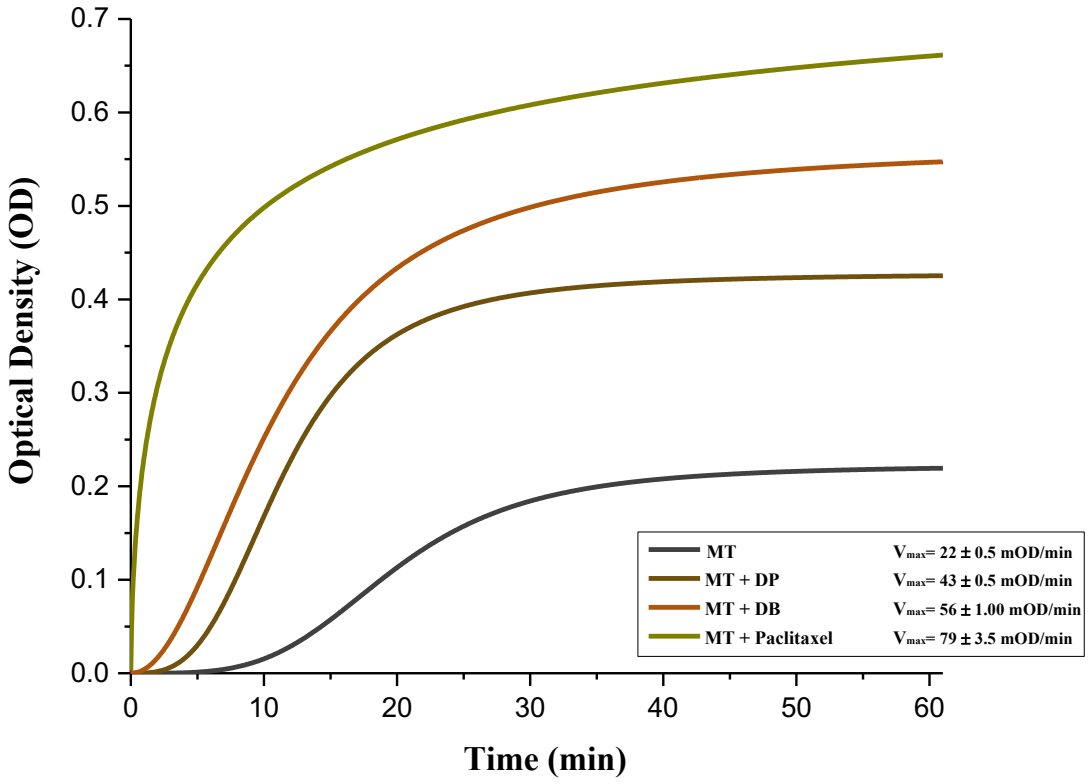
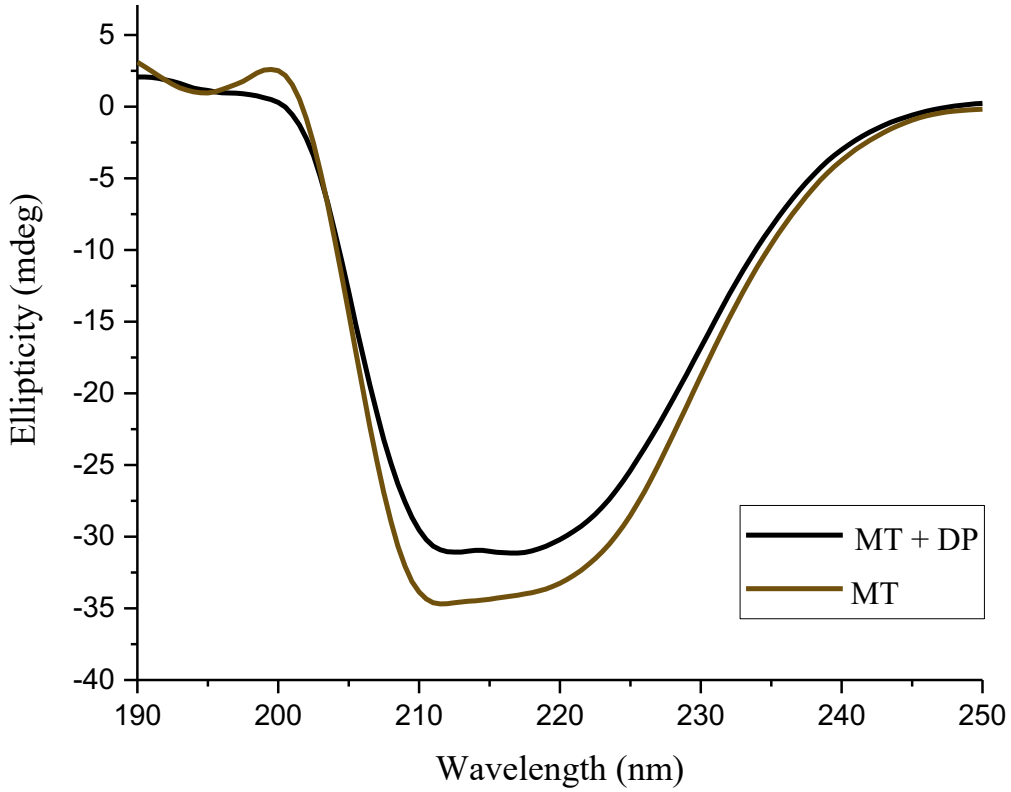


Figure 11. Ligand binding effects on the polymerization of tubulin. Increase in turbidity (OD) is a measure of polymer elongation. The rate of polymerization was measured in the assay over 60 minutes, in steady state 37°C. The positive control is (MT + PTX) which is a known MSA. The negative control is (MT) which will show polymerization under experimental conditions but would lack enhancement not in the presence of a small molecule. Novel-RPCs (DP and DB) show an increase in the rate of polymerization (V_{max}) and the degree of total polymerization at saturating conditions (OD_{final}). The novel small molecules show MSA properties *in vitro*.

Secondary Structural effects on MT in the presence of DB and DP

Far-UV Circular Dichroism was used to qualitatively analyze the secondary structure of the tubulin with and without the small molecules present. α-helices show a large negative band at 220nm and β-sheets show a positive peak around 205nm. It has been established through computation analyses using CD signals that Tubulin consists of 33% α-helices, 21% β-Sheets, and 45% random coils (16). The structural features are seen in Figure 12, with a large negative peak around 220nm (α-helix) and a small positive peak around 200-205nm (β-sheet). Small changes in the CD signal are seen with and without the small molecule but suggests overall, there are no large structural changes occurring when the MT is bound to DP (Figure 12).

Figure 12. Far-UV Circular Dichroism in the presence of the novel small molecule show no large changes within the protein secondary structure



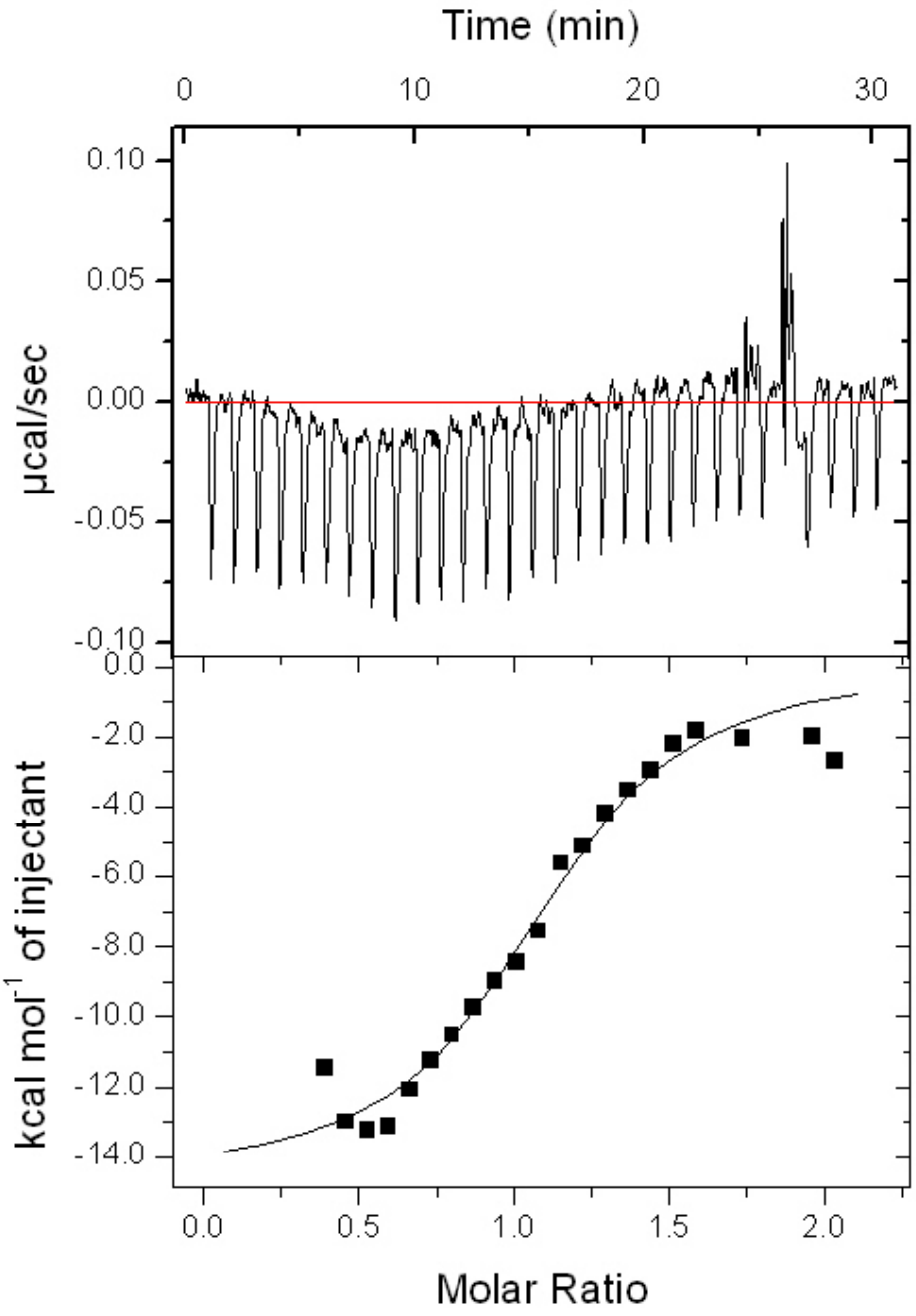
Thermodynamics of DP binding to MT show similarities to other known RPCs

Isothermal titration calorimetry was completed to characterize the thermodynamics of small molecule binding to preformed MTs (37^0C) by 50uL injection of the water-soluble (100uM) small molecule into 300uL of (10uM) of MT. For comparison, RPC2 (non-soluble MSA RPC) data has been reported in Table 1 (7). Paclitaxel is known to bind to preformed MTs with a binding affinity (K_d) of 9.35E-8 M (7). The binding affinity extracted from ITC isotherms for the novel small molecule is in great correspondence with RPC2 and PTX and thermodynamic similarities between RPC2 and DP would suggest these compounds exhibit similar modes of binding. A stoichiometric coefficient (N) of 1.11 ± 0.003 was measured for the interaction between DP and MTs, suggesting a 1:1 mole ratio for binding (Figure 13). This is in agreement with other MSA/MDAs (docetaxel & RPC2 / colchicine) that also exhibit a N=1. Isotherm data can be seen in Figure 13 for the DP + MT study.

Table 1. Thermodynamic properties of binding between MT and RPCs measured using ITC.

Complex	Ligand	K_d (uM)	ΔG (kcal/mol)	ΔH(kcal/mol)	-TΔS (kcal/mol)
MT	DP	0.6	-8.86	-14.6 ± 0.75	5.6
MT	RPC2 (7)	0.2	–9.1 ± 0.9	-16.6 ± 2.3	7.5

Figure 13. ITC with preformed MT and DP. Isothermal inflection point shown at approximately 1 (N).



Discussion

Small molecules that target proteins with essential physiological functions have been under investigation as a potential route for antitumor therapies, including metalloorganic compounds. Platinum based compounds became the leading drug for treating cancer with high efficacy, however there are reports of severe side effects of bone marrow suppression and peripheral neuropathy thus there has been an initiative to find novel metal-based compounds for cancer therapy (15). Research has revealed that Ruthenium-based complexes show promising advantages over previously used Platinum containing drugs regarding reduced drug resistance and lower toxicity (15). This research has been focused on identifying Novel-RPCs that are more water-soluble and display characteristic microtubule-stabilizing activity.

Novel RPCs, DP and DB, are water soluble compounds and soluble in concentrations of 10mM. Ruthenium-based compounds have large aromatic ring structures and have previously been known to have low solubility in aqueous systems and with modification of the ligand structures (RPC1 and RPC2)(7), DB and DP show remarkedly enhanced water-solubility. This development is significant progress in the field of anticancer drugs as many are hydrophobic with strong limitations on the efficacy and therapeutic safety (17). To enhance the solubility of these drugs, conjugation with hydrophillic carriers has been a method to avoid this issue but this application is not without consequences (17). The identification of DP and DB as water-soluble is a unique feat for a possible anticancer drugs and would allow for increased bioavailability, reducing the dose required for effective treatment and overall, reducing side effects and increasing quality of life for a patient (17,18).

Titrations with DP and DB to preformed MTs exhibits a fluorescence redshift, a higher emission wavelength with increasing concentration, which supports the notion that the novel molecules are interacting with the polymer. Exhibition of a redshift is indicative of the tryptophan's being more exposed to the hydrophillic solvent, indicating there are molecular and structural changes in the MT occurring upon binding of the RPCs. These molecular changes associated with fluorescence quenching can indicate complex formation or energy transfer, which would be expected to occur if the novel molecules bind the MTs. Research published in 2019 (7) was the first to characterize Ruthenium (II) compounds interacting with MTs as their previous actions targeted DNA, mitochondria, ER and different signaling pathways (15). Therefore, DP and DB are included in a small subset of RPCs that have been recently identified as interacting with microtubules which is a newly realized mode of action. Furthermore, no significant secondary structural changes were seen in the dimer on CD spectra supporting the notion that overall structural integrity is maintained even with DP binding.

DP and DB are binding to preformed MTs with a similar binding affinity as other known MT interacting small molecules. Fluorescence data fit to a Hill Plot gave a K_d=15uM for DP and K_d=19uM for DB which shows moderately strong binding to preformed MTs. Preliminary ITC data for DP + MT gave a K_d=0.6uM, which is in strong agreement with ITC data of MT+ RPC2 (7) with a K_d=0.2uM. This suggests that these RPC compounds are binding with almost identical affinities. Reported K_d for Paclitaxel is 90nM (7) which is in exceptional agreement with the K_d's for the novel water-soluble RPCs (DP and DB), proving their importance in follow up work.

The thermodynamics of binding reported from ITC suggest the non-soluble RPC2 and the novel water-soluble DP have similar modes of binding MTs due to very similar magnitudes of Gibb's Free Energy, enthalpy and entropy of binding. DP binds to microtubules spontaneously through an exothermic process that results in more ordered molecular products. This is in comparison to Paclitaxel with a reported ΔG=-41 kcal/mol, ΔH=-57kcal/mol and $-T\Delta S$= 8.99 kcal/mol (19). Through competitive binding ITC, it has been concluded that RPC2 binds to another region other than the taxane-binding domain which is supported through the difference in magnitudes of the thermodynamics of binding between RPC2 and Paclitaxel (7). This could possibly extend to our novel DP since the thermodynamics are more similar to the RPC2, however this must be confirmed with its own set of competitive binding experiments. The stoichiometric coefficient of binding for DP to MT is N=1 and is consistent with all other known MSA/MDAs.

DP and DB act as a microtubule-stabilizing agent *in vitro.* The polymerization assay shows an elevation in the V_{max} and also the degree of polymerization/length of polymer (total OD) for both small molecules. Paclitaxel is a current antineoplastic drug that enhances the stability of microtubules and can induce apoptosis via mitotic arrest. In my research, PTX acts as the positive control and shows 3-fold enhancement of the V_{max} of polymerization and degree of polymer formation. DP and DB novel molecules show respectively 2 and 2.5-fold enhancement of the rate and degree of polymerization which suggests their identity as an MSA. RPCs only recently were identified as interacting with microtubules, such that DP and DB may be the first known water-soluble Ruthenium-Polypyridyl Complexes that act as an MSA.

The preliminary characterization of novel water-soluble RPCs that bind, stabilize and enhance polymerization with respect to microtubules is a promising feat in the development of new cancer drugs. One of the biggest barriers to cancer therapy success is the progression of drug resistance. Proposed methods of overcoming such resistance includes developing novel drugs, combinations of drugs with different modes of actions and improved pharmalogical targeting (deeper response, bioavailability) (20). The novel RPCs (DP and DB) touch on aspects of all those notions. RPCs targeting microtubules has been a new development in the last few years and our data shows a strong interaction and stabilization effects. The MSA activity of DB and DP can be expected to follow such a mechanism *in vivo* via the apoptotic pathway such as Paclitaxel (MSA). Furthermore, the hydrophillic solubility is a positive feature for clinical applications and improved penetrance in tissues. Future work can include competitive binding titrations with other known MSA/MDAs to exclude/include known binding sites as a possible location for the interaction between DP and DB with MTs. This can also be evaluated using computerized binding simulations. Tumor microenvironments have a lower pH and therefore a pH denaturation experiment would also be another avenue to consider. Overall, there are many positive properties these novel RPCs exhibit when thinking of tumorigenic applications and should be further investigated with their role in future clinical treatment.

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