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# Investigation of 2-Hydroxy-4-methoxy-2',3'-benzochalcone Binding to Tubulin by Using NMR and *in silico* Docking

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**Abstract** Hydroxychalcones act as autophagy inducers and methoxy chalcones induce apoptosis. Additionally, benzoflavones inhibit the hepatitis C virus. Based on these findings, a chalcone derivative, 2-hydroxy-4-methoxy-2',3'-benzochalcone, was prepared. It showed antimitotic activity through its inhibitory effect on tubulin polymerization. Its molecular binding mode with tubulin was elucidated using *in silico* docking and nuclear magnetic resonance spectroscopy. In addition, the three-dimensional structure of 2-hydroxy-4-methoxy-2',3'-benzochalcone was determined by X-ray crystallography.

**Keywords** chalcone · *in silico* docking · nuclear magnetic resonance · tubulin · X-ray crystallography

## Introduction

Unlike flavonoids, chalcones, also known as chalconoids, have an open a,b-unsaturated carbonyl group between two benzene rings. Owing to their diverse biological activities including DNA gyrase inhibition, anti-tumorigenesis, P-glycoprotein inhibition, and cell growth inhibitory effects on the cisplatin-resistant ovarian cancer cells, their derivatization has been researched widely (Abdullah et al., 2014; Parveen et al., 2014; Shin et al., 2014; Zhou et al., 2014). Hydroxychalcones act as autophagy inducers and methoxychalcones induce apoptosis (Sun et al., 2010; Wang et al., 2014). In addition, benzoflavones inhibit the hepatitis C virus (Fukazawa et al., 2012). Therefore, attempt was made to design and synthesize a structure-fusion chalcone including hydroxyl, methoxy, and benzyl groups, namely a hydroxy-methoxy-benzochalcone, and prepared the chalcone derivative 2-hydroxy-4-methoxy-2',3'-benzochalcone (named HymnPro). HymnPro showed antimitotic activity through its inhibitory effect on tubulin polymerization (Shin et al., 2013a). From the *in vitro* tubulin polymerization assay and Western blot analysis, it was clarified that HymnPro acts as colchicine. However, its molecular binding mode is yet unknown (Shin et al., 2013a).In the present study, the molecular binding mode between HymnPro and tubulin, was determined using *in silico* docking and nuclear magnetic resonance (NMR) spectroscopy, which could help in explaining the inhibition of tubulin polymerization by 2-hydroxy-4-methoxy-2',3'-benzochalcone.

## Materials and Methods

To elucidate the molecular binding mode between HymnPro and tubulin, *in silico* docking experiments were performed on an Intel Core 2 Quad Q6600 (2.4 GHz) Linux PC with SYBYL 7.3 (Tripos, USA) (Shin et al., 2013c). Of the many 3D structures of tubulin found in the protein data bank, 3UT5 was selected because it contains a bound colchicine as its ligand. This structure includes four tubulins, A, B, C, and D, and the stathmin domain. Because colchicine resides between chains A and B and between C and D (Supplementary Data Fig. 1), chains A and B were chosen for the *in silico* experiments by using SYBYL. The NMR experiments on

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the binding of HymnPro to tubulin were performed on a Bruker Avance 400 spectrometer system (9.4 T, Karlsruhe, Germany) at 298K. HymnPro was synthesized as described previously by Hwang et al. (2011). It was dissolved in deuterated dimethyl-sulfoxide (DMSO-*d*6) and its concentration was adjusted to approximately 50 mM before transferring to a 2.5-mm NMR tube. The relaxation delay and the 90° pulse for the $^{1}$H-NMR experiments were 1 s and 10.2 μs, respectively. Experimental procedures were followed as described previously (Hwang et al., 2012). X-ray crystallographic data were collected on SMART (Bruker AXS Inc.,USA), and then solved and refined using SAINT software (Bruker) (Shin et al., 2013b). X-ray crystallography was performed in collaboration with the Korea Basic Science Institute, Korea. For X-ray crystallography, HymnPro was prepared as follows: 2 mL of 50% aqueous KOH solution was added to a 20-mL solution of 2-hydroxy-4-methoxyacetophenone (332 mg, 2 mmol) and 1-naphthaldehyde (312 mg, 2 mmol) in ethanol, and the reaction mixture was stirred at room temperature for 24 h. After completion of the reaction, this mixture was poured into 50 mL of iced water and was acidified with 3N HCl solution to yield a solid (280 mg, 46%). This synthetic procedure is summarized in Supplementary Data Scheme 1. Recrystallization of the solid in ethanol gave rise to yellow-colored crystals of HymnPro (m.p.: 391–392K). Colchicine was purchased from Sigma (USA). Tubulin was obtained from the tubulin polymerization assay kit (Cytoskeleton, USA).

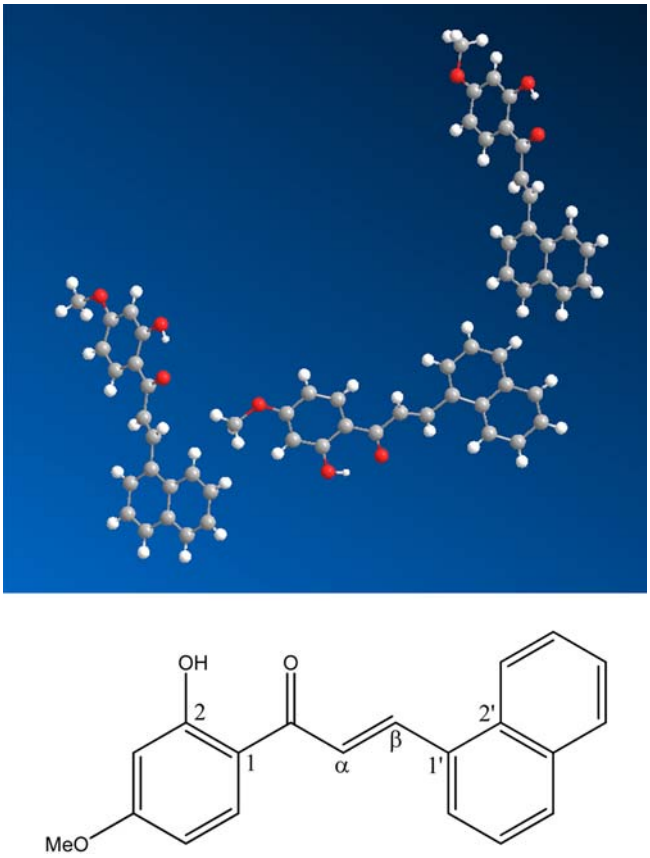


**Fig. 1** The crystal structure (top) and nomenclature (bottom) of HymnPro.

## Results and Discussion

*In vitro* tubulin polymerization assay demonstrated that HymnPro binds to tubulin (Shin et al., 2013a), and this molecular binding mode was elucidated using *in silico* docking. The three-dimensional (3D) structure of HymnPro (Fig. 1) was determined using X-ray crystallography in the present study. Its crystal data and structure refinement details are listed in Supplementary Data Table 1. Atomic coordinates and equivalent isotropic displacement parameters are listed in Supplementary Data Table 2. The dihedral angles of C-1/C=O/Ca/Cb and C=O/Ca/Cb/C-1' are 173.39° and 179.80°, respectively. As a result, the three rings contained in HymnPro are present in the same plane. The hydrogen bond (H-bond) distance between the proton of the 2-hydroxyl group and the oxygen of the ketone group is 1.77 Å. However, the three molecules contained in the unit cell (Fig. 1) show that HymnPro does not form a stacked structure inspite of its flat shape.

Tubulin polymerization assay of HymnPro showed a behavior similar to that of colchicine (Shin et al., 2013a). The residues surrounding the binding site, from both chains A and B were determined based on LigPlot analysis (Kramer et al,. 1999; Shin et al., 2013c). Colchicine resides between chains A and B, which participate in the binding site. Thr179(A), Ala180(A), Leu242(B), Ala250(B), Asp251(B), Lys254(B), Leu255(B), Asn258(B), Met259(B), Thr314(B), Val315(B), Ala316(B), Ile318(B), Asn350(B), Lys352(B), and Ile378(B) are involved in the hydrophobic interactions with colchicine, and the nitrogen of Val181(A) as well as hydrogen of thiol group of Cys241 formed H-bonds with an oxygen of the ketone group and two oxygens of the methoxy groups of colchicine, respectively. Their distances are 2.91, 2.77, and 3.03 Å, respectively (Supplementary Data Fig. 2). The 3D image representation of the binding region of the colchicine-tubulin complex was generated using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC, USA) (Fig. 2A). The docking radius for the flexible docking was set to 6.5 Å, and the docking process was performed using SYBYL 7.3 software. After 30 iterations, the corresponding numbers of HymnPro-tubulin complexes were generated with docking scores ranging from 20.39 to 16.90 kcal/mol. Of these, complex 19 with a docking score of 18.00 kcal/mol showed the best docking pose. The residues surrounding the binding site between HymnPro and tubulin were analyzed by LigPlot and classified based on their mode of interaction. The residues displaying hydrophobic interactions were Thr179(A), Ala180(A), Gln247(B), Leu248(B), Asn249(B), Ala250(B), Lys254(B), Leu255(B), Asn258(B), and Lys352(B), whereas Ser178(A), Tyr224(A), and Lys254(B) were involved in H-bonds. The H-bonds were found between the nitrogen of Lys254(B) and the oxygen of the ketone group of HymnPro, the oxygen of the hydroxyl group of

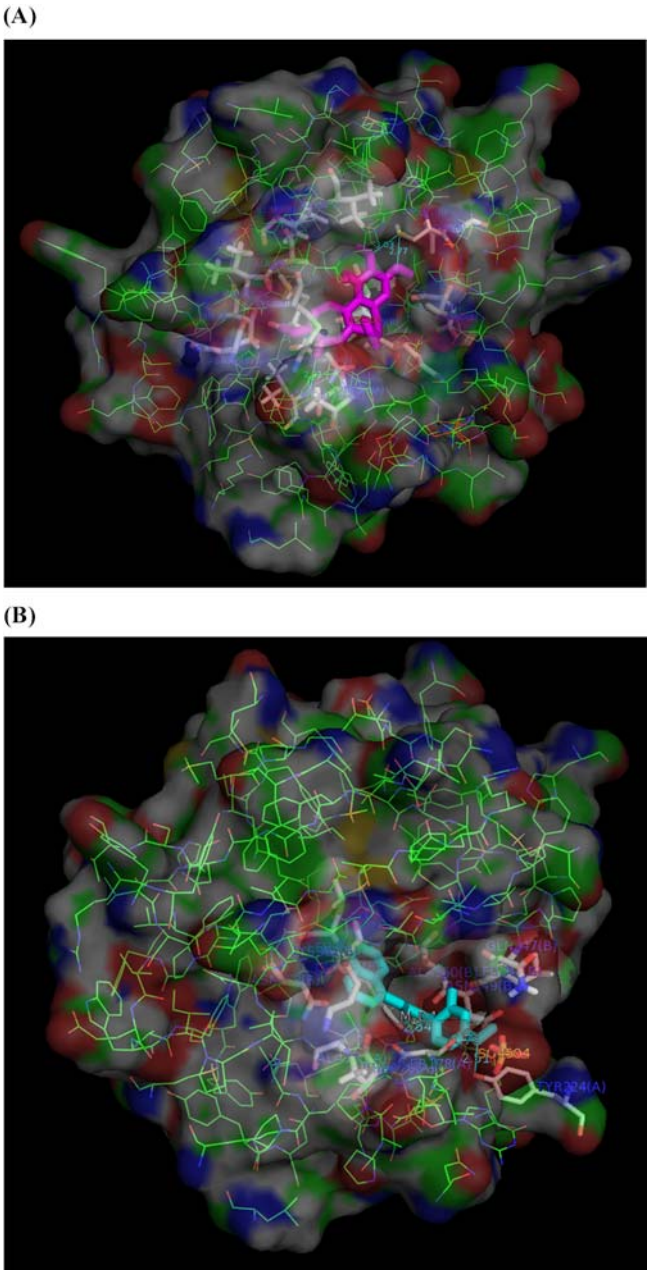


**Fig. 2** (A) 3D image of colchicine (magenta) docked into the binding site of tubulin and (B) that of HymnPro (cyan), as viewed in PyMOL.

Tyr224(A) and the oxygen of the methoxy group of HymnPro, as well as the oxygen of the hydroxyl group of Ser178(A) and the oxygen of the methoxy group of HymnPro (Supplementary Data Fig. 3). The 3D image representing the binding site of the HymnPro-tubulin complex was generated using PyMOL (Fig. 2B). Comparing the binding sites of the colchicine-tubulin and the HymnPro-tubulin complexes revealed that whereas 18 residues surrounded colchicine, only 12 residues surrounded HymnPro owing to the larger volume of colchicine. HymnPro formed H-bonds with three different residues, and colchicine formed H-bonds with two residues. However, the number of H-bonds in both complexes is the same, indicating that the binding interactions of both ligands can be expected to be same. In addition, the 3D images of the binding sites of colchicine and HymnPro show similar docking poses. As a result, the inhibitory effect of HymnPro on tubulin polymerization, determined using *in vitro* tubulin polymerization assay kit, could be explained based on the *in silico* docking results.

Furthermore, *in silico* docking and *in vitro* tubulin polymerization assays demonstrated that HymnPro binds to tubulin, and the results were further confirmed by NMR experiments. Because HymnPro and colchicine are not soluble in $D_2O$, they were dissolved in DMSO, whereas tubulin was dissolved in $D_2O$. First, the $^1$H NMR spectrum of 20 μM tubulin in $D_2O$ was compared with that of 20 μM tubulin in DMSO-$d_6$. Upon observation of upfield shifts of several peaks, tubulin in DMSO-$d_6$ still showed proton signals, and all samples for NMR experiments were prepared in DMSO-$d_6$ (Supplementary Data Fig. 4). Subsequently, the $^1$H NMR spectra of colchicine dissolved in DMSO-$d_6$ and that of HymnPro in DMSO-$d_6$ were collected. A comparison of the former with the latter provided a few distinguishable signals. Peaks between 6.5–7.5 ppm were observed in colchicine, whereas peaks between 7.5–8.5 ppm were observed only in HymnPro (Supplementary Data Fig. 5). Because it is known that colchicine binds to tubulin, we tested whether our NMR experimental condition can detect the binding between tubulin and its ligand. NMR spectra of a mixture of tubulin and colchicine in DMSO-$d_6$ were collected. Initially, the $^1$H NMR spectrum of 100 μM colchicine alone was collected (Fig. 3A). Various amounts of tubulin were added into colchicine solution, and $^1$H NMR spectra were collected after every 1.5-μL addition of 100 μM tubulin to colchicine. There were no observable chemical shifts upon addition of 1.5 μL of 100 μM tubulin to 148.5 μL of 100 μM colchicine (Fig. 3B). However, several changes in the spectrum were observed when 15 μL of tubulin was added to 135 μL of colchicine (Fig. 3C). The peak at 7.06 ppm shifted downfield to 7.12 ppm, and two peaks at 6.98 and 7.00 ppm shifted downfield by 0.02 and 0.03 ppm, respectively. The peak at 6.74 ppm shifted upfield by 0.02 ppm. However, the addition of DMSO-$d_6$ into colchicine solution did not change any peaks in the spectrum, implying that the chemical shift changes were caused by the binding interaction between colchicine and tubulin. Therefore, our NMR experimental condition can detect the binding between tubulin and its ligand.

Similar experiments were performed with HymnPro instead of colchicine. $^1$H NMR spectra were collected after every 1.5-μL addition of 100 μM tubulin to HymnPro. The $^1$H peaks 7.5–8.5 ppm were dispersed, and several peaks could be observed separately (Fig. 4A–F). NMR can be used to detect the binding of small compounds such as colchicine and HymnPro to a protein such as tubulin, because the signals caused by a small compound alone are distinct from those caused by a complex of a small

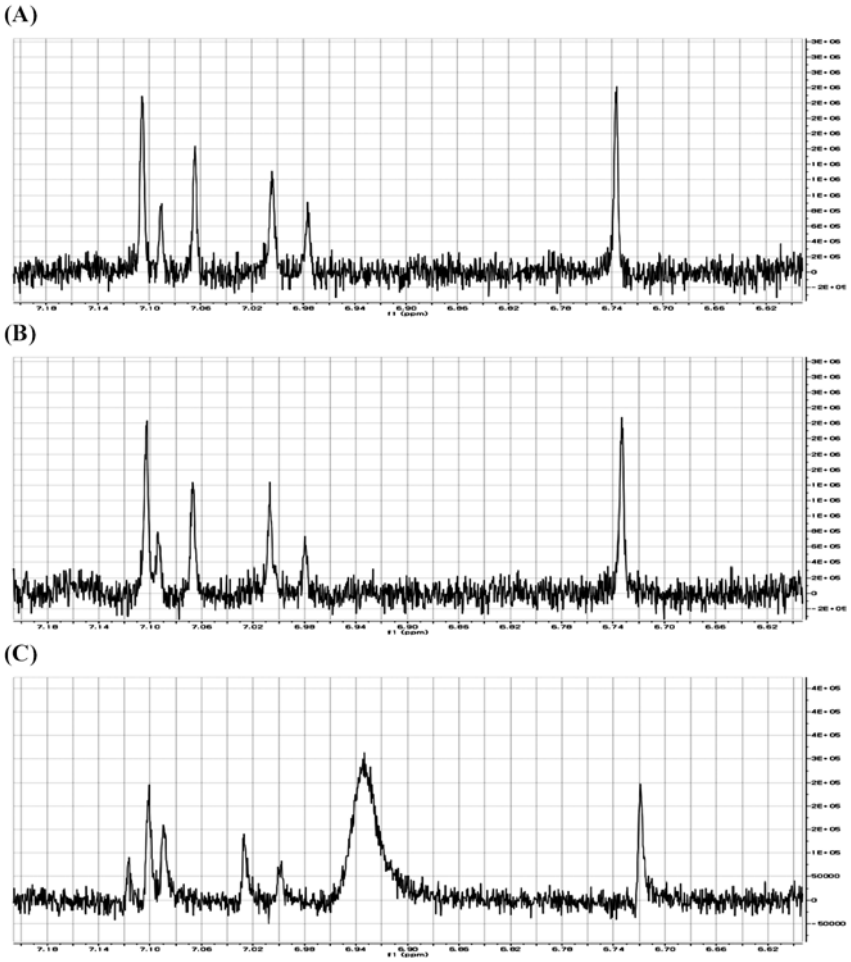


**Fig. 3** (A) The $^{1}$H NMR spectrum of 100 μM colchicine in DMSO-$d_6$, (B) the $^{1}$H NMR spectrum of colchicine collected after addition of 1.5 μL of 100 μM tubulin to colchicine, and (C) the $^{1}$H NMR spectrum of colchicine collected after addition of 15 μL of 100 μM tubulin.

molecule and a protein (Kim et al., 2008). Therefore, it can be concluded that HymnPro binds to tubulin based on the interpretation of the $^{1}$H NMR data collected in the present study. To determine whether HymnPro binds more effectively to tubulin than to colchicine, these experiments were repeated with a mixture of HymnPro and colchicine. Supplementary Data Fig. 6A shows the $^{1}$H NMR spectrum of a mixture of HymnPro and colchicine without tubulin, where peaks between 7.5–8.5 ppm belong to HymnPro and peaks between 6.6–7.2 ppm belong to colchicine. As shown in Supplementary Data Fig. 6B, while the peaks of HymnPro were dispersed, the peaks of colchicine were not changed.

Our previous results demonstrated that a chalcone derivative, HymnPro, binds to tubulin *in vitro*, as determined by tubulin polymerization assay (Shin et al., 2003a). To confirm this result, *in silico* docking and NMR experiments were carried out. For the docking study, the 3D structure of HymnPro was initially determined by X-ray crystallography. *In silico* docking results showed that HymnPro binds well to the binding pocket of colchicine in the crystallographic structure of a tubulin-colchicine complex. NMR data confirmed that HymnPro binds to tubulin as well. Therefore, the inhibitory effect of HymnPro (2-hydroxy-4-methoxy-2',3'-benzochalcone) on tubulin polymerization was demonstrated using *in silico* docking experiments and NMR spectroscopy.

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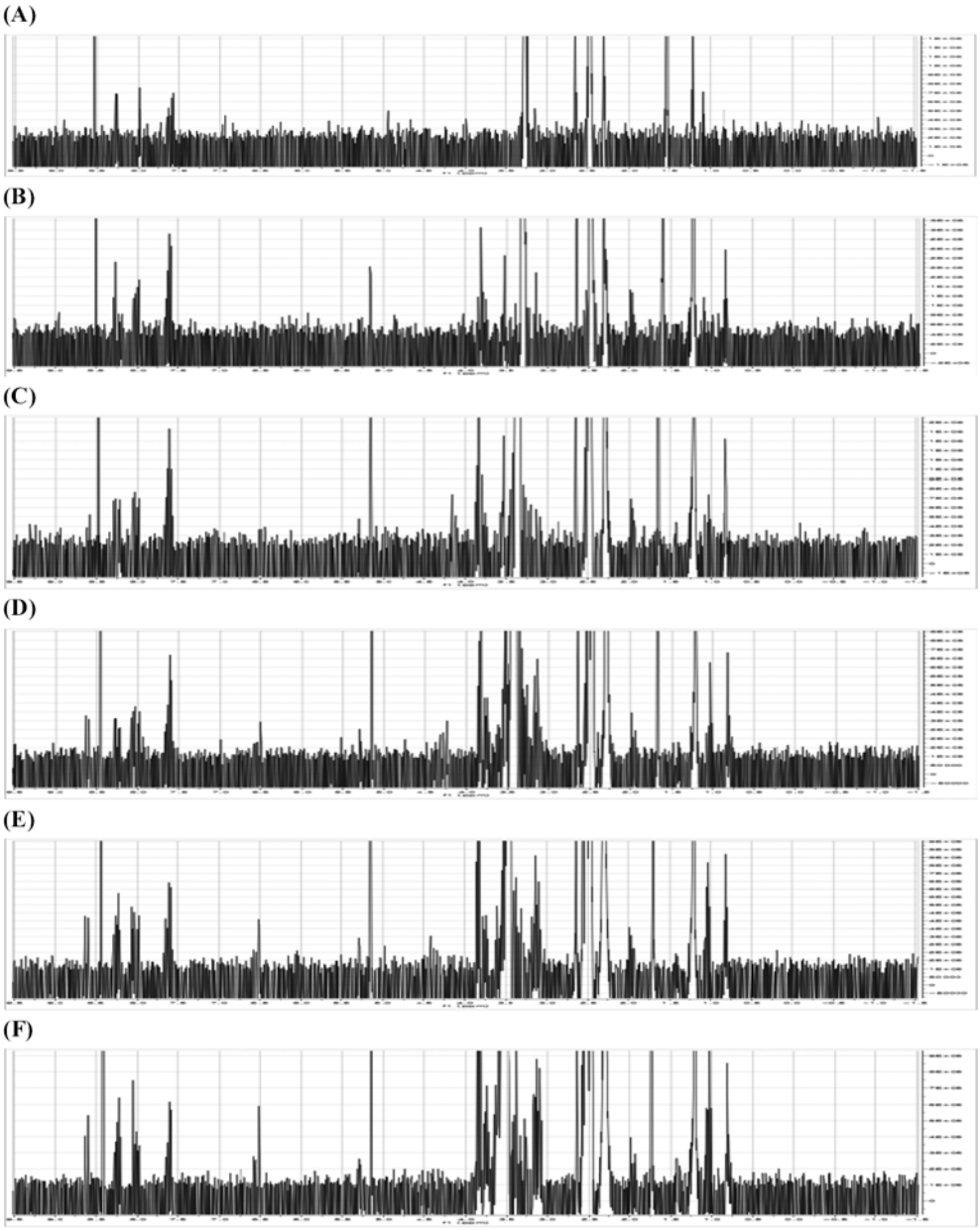


**Fig. 4** (A) The $^{1}$H NMR spectrum of 100 μM HymnPro in DMSO-$d_6$, and the $^{1}$H NMR spectra of HymnPro collected after the addition of (B) 1.5 μL, (C) 3.0 μL, (D) 4.5 μL, (E) 6.0 μL, and (F) 7.5 μL of 100 μM tubulin to HymnPro.

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