Journal Pre-proofs

A synthetic 2,3-diarylindole induces microtubule destabilization and G2/M cell cycle arrest in lung cancer cells

Bongkotrat Thanaussavadate, Lukana Ngiwsara, Kriengsak Lirdprapamongkol, Jisnuson Svasti, Pitak Chuawong

PII: S0960-894X(19)30742-5
DOI: https://doi.org/10.1016/j.bmcl.2019.126777
Reference: BMCL 126777

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 28 August 2019
Revised Date: 18 October 2019
Accepted Date: 23 October 2019

Please cite this article as: Thanaussavadate, B., Ngiwsara, L., Lirdprapamongkol, K., Svasti, J., Chuawong, P., A synthetic 2,3-diarylindole induces microtubule destabilization and G2/M cell cycle arrest in lung cancer cells, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.126777

A synthetic 2,3-diarylindole induces microtubule destabilization and G2/M cell cycle arrest in lung cancer cells

Bongkotrat Thanaussavadate[a], Lukana Ngiwsara[b,*], Kriengsak Lirdprapamongkol[b], Jisnuson Svasti[b], Pitak Chuawong[a,*]

[a]Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

Tel +66 2 5625555 ext. 5161; Fax +66 2 5793955; E-mail address: Pitak.C@ku.ac.th

[b]Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok 10210, Thailand

Tel +66 2 5538555 ext. 8356; Fax +66 2 2596634; E-mail address: lukana@cri.or.th

Abstract

The anticancer potential of a synthetic 2,3-diarylindole (PCNT13) has been demonstrated in A549 lung cancer cells by inducing both apoptosis and autophagic cell death. In this report, we designed to connect a fluorophore to the compound via a hydrophilic linker for monitoring intracellular localization. The best position for linker attachment was identified from cytotoxicity and effect on cell morphology of newly synthesized PCNT13 derivatives bearing hydrophilic linker. Cytotoxicity and effect on cell morphology related to the parental compound were used to identify the optimum position for linker attachment in the PCNT13 chemical structure. The fluorophore-PCNT13 conjugate was found to localize in the cytoplasm. Microtubules were found to be one of the cytosolic target proteins of PCNT13, as the compound could inhibit tubulin polymerization *in vitro*. A molecular docking study revealed that PCNT13 binds at the colchicine binding site on the α/β–tubulin heterodimer. The effect of PCNT13 on microtubule dynamics caused cell cycle arrest in the G2/M phase as analyzed by flow cytometric analysis.

Keywords

2,3-diarylindole; lung cancer; tubulin polymerization; cell cycle arrest

Lung cancer is the leading cause of cancer-associated mortality worldwide. It is divided into two types, according to histology, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 85% of lung cancers[1] and is insensitive to conventional treatments such as radiation therapy and chemotherapy. Despite advances in diagnostics and therapeutics, the outcome for patients with NSCLC remains poor. Therefore, the discoveries of effective new therapeutic drugs are still an urgent requirement for this cancer. Previously, we have reported the potential anticancer activity of the synthetic 2,3-diarylindole (PCNT13), which induces two modes of programmed cell death, autophagy, and apoptosis, toward a human NSCLC cell line, A549.[1] In the present study, we further explored the mechanism underlying the action of PCNT13 against A549 cells.

We planned to investigate the cellular localization of PCNT13. It is worth mentioning that most of the 2,3-diarylindoles possess fluorescent property, which facilitates monitoring the intracellular localization of the compounds. However, the intrinsic fluorescent intensity of PCNT13 is rather weak, rendering the compound impractical to be observed inside the cells under a fluorescence microscope. Therefore, the use of fluorophore-PCNT13 conjugate may be an alternative approach. Since structural modifications of biologically active compounds may interfere with non-covalent interactions with their biomolecular targets, the position for the hydrophilic linker attachment needs to be optimized. As illustrated in Fig.1, four different derivatives bearing a hydrophilic linker at different positions were considered. Derivatives **1** and **3** resemble PCNT13 with the linker on the indolic nitrogen and the ester moiety, respectively, while **2** and **4** resemble PCNT12, a regioisomer of PCNT13. PCNT12 was previously reported to have solubility problems with unattainable cytotoxicity.[2] Thus, introducing a hydrophilic linker might increase the solubility of PCNT12 and allows us to evaluate the anticancer activity.

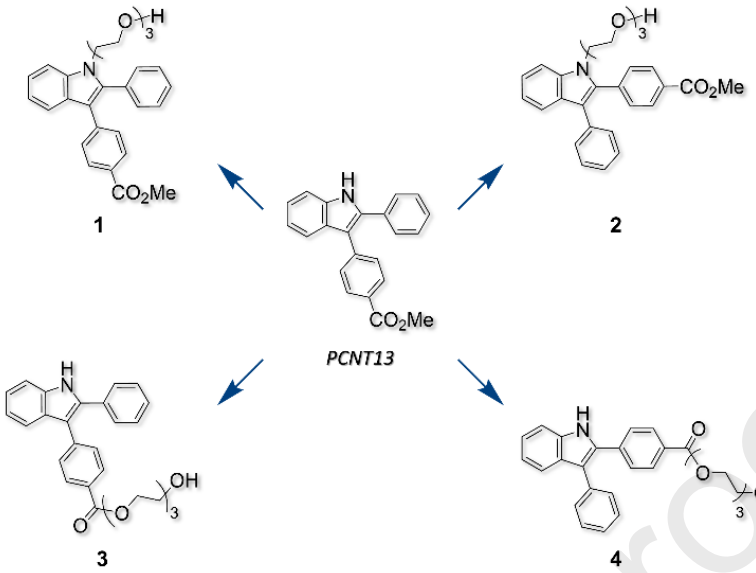
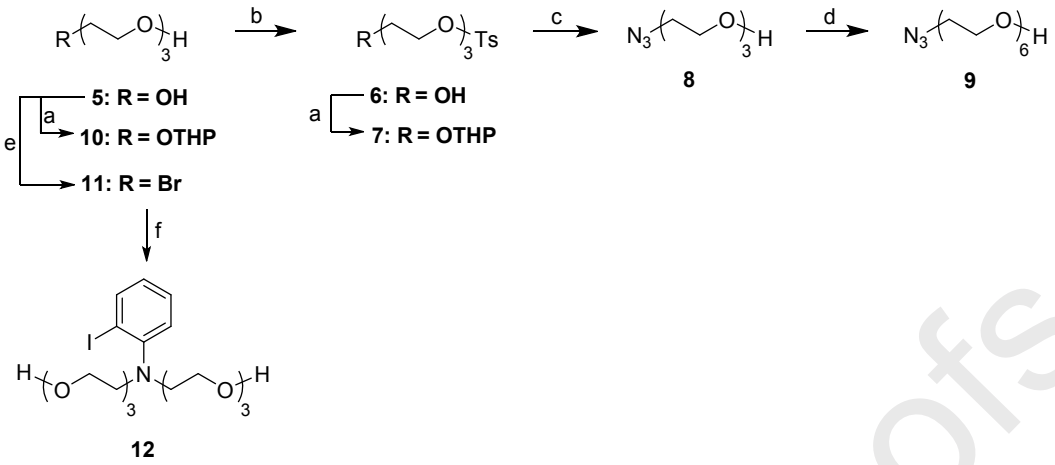


Fig. 1. Four indole derivatives utilized in the optimization of linker installation.

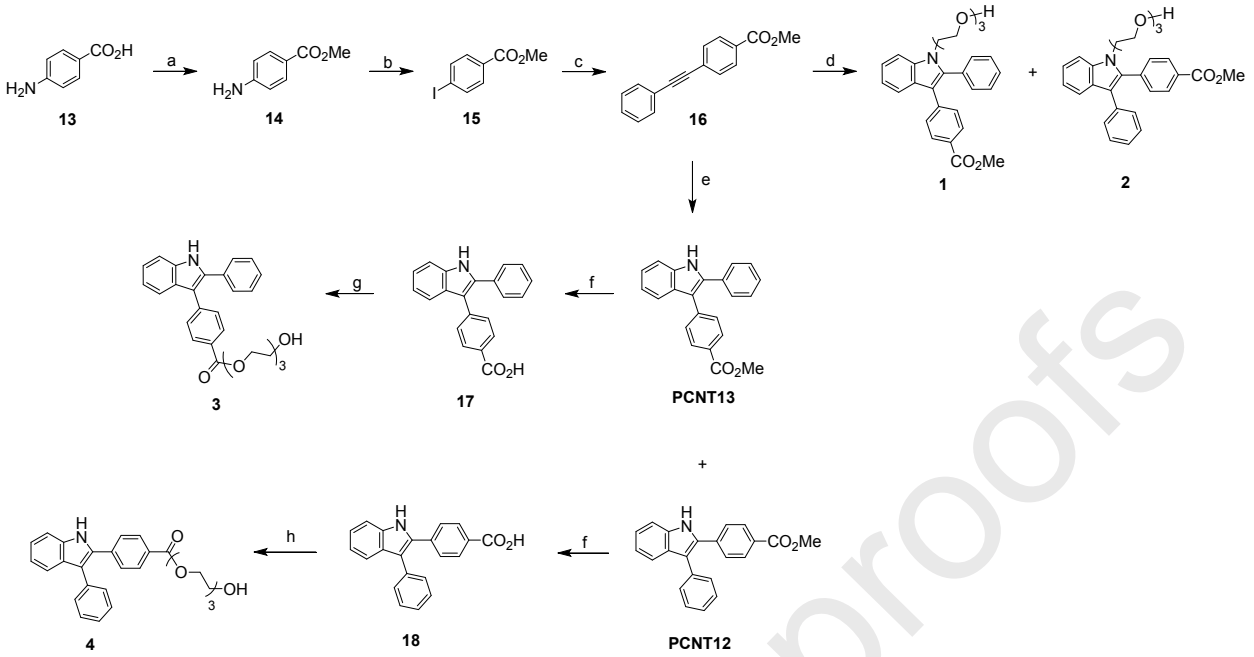
Syntheses of the hydrophilic linkers and PCNT13 derivatives were illustrated in Scheme 1 and 2, respectively. One of the two hydroxyl groups of triethylene glycol **5** was activated using tosyl chloride to afford **6** in 64% yield, followed by protection of the remaining hydroxyl group using dihydropyran under the acidic conditions to afford **7** in 85% yield. Subsequently, **6** was subjected to azide substitution using NaN_3 in refluxing acetonitrile to provide **8** in 94% yield. The reaction between **7** and **8** in the presence of NaH, followed by deprotection of the tetrahydropyranyl ether under the acidic conditions, provided the hexaethylene glycol derivative **9** in 45% yield over two steps. A hydroxyl group of **5** was also protected as the tetrahydropyranyl ether to afford **10** in 65% yield.



70

71 **Scheme 1.** Synthesis of hydrophilic linkers. Reagents and conditions: (a) DHP, conc. HCl, rt, 16 h; (b)
72 TsCl, Et_3N, CH_2Cl_2, rt, 5 h; (c) NaN_3, CH_3CN, reflux, 16 h; (d) i) **7**, NaH, THF, reflux, 26 h; ii) 1 M HCl,
73 MeOH, rt, 6 h; (e) PBr_3, reflux, 4 h; (f) 2-iodoaniline, Na_2CO_3, CH_2Cl_2, rt, 3 h. (Note: DHP = 3,4-dihydro-
74 2*H*-pyran; THP = tetrahydro-2*H*-pyran)

75 An *N*,*N*-disubstituted derivative of 2-iodoaniline **(12)** was prepared in 2 steps. First, a hydroxyl
76 group of **5** was converted to a bromide using PBr_3 to provide **11** in 49% yield, followed by the *N*-alkylation
77 of 2-iodoaniline using **11** in the presence of Na_2CO_3 to provide **12** in 40%. Compound **12**, along with the
78 synthesized hydrophilic linkers, were utilized in the synthesis of 2,3-diarylindole derivatives **1**, **2**, **3**, and **4,**
79 as described in Scheme 2.



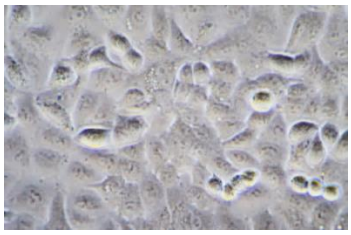
Scheme 2. Synthesis of PCNT13 derivatives bearing hydrophilic linker. Reagents and conditions: (a) MeOH, H_2SO_4, reflux, 5 h; (b) H_2SO_4, $NaNO_2$, 0 °C, 5 h, then KI/H_2O, rt, 16 h; (c) Phenyl acetylene, $Pd(OAc)_2$, PPh_3, CuI, Et_3N, THF, rt, 4 h; (d) **12**, $Pd(OAc)_2$, PPh_3, *n*-Bu_4NCl, Na_2CO_3, DMF, 80 °C, 16 h; (e) 2-iodoaniline, $Pd(OAc)_2$, PPh_3, *n*-Bu_4NCl, Na_2CO_3, DMF, 80 °C, 16 h; (f) EtOH, sat. KOH, reflux, 3 h; (g) **5**, PPh_3, DIAD, THF, rt, 3 h; (h) i) **10**, PPh_3, DIAD, THF, rt, 3 h; ii) 5 M HCl, MeOH, rt, 30 min.

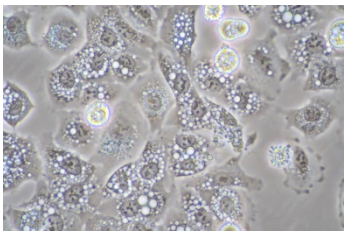
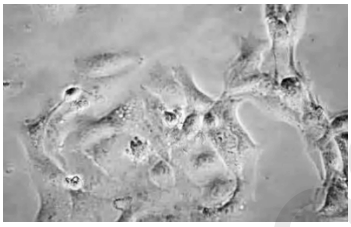
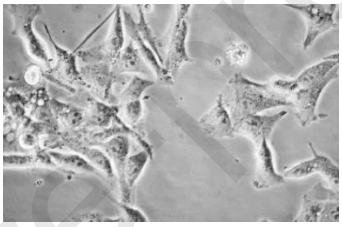
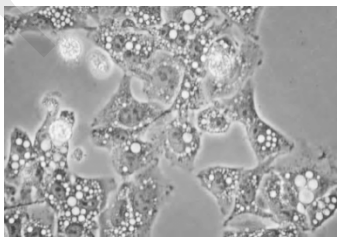
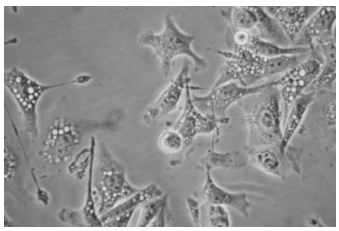
The synthesis of **1**, **2**, **3**, and **4** started with esterification of **13** to afford methyl ester **14** in 98% yield (Scheme 2). Subsequently, the Sandmeyer reaction was utilized to convert arylamine **14** to iodobenzene derivative **15** in 66% yield.[3] Sonogashira coupling reaction between **15** and phenylacetylene provided diarylacetylene derivative **16** in 85% yield.[4] Then, the Larock heteroannulation between **16** and **12** afforded the regioisomeric products **1** and **2** in 15% and 47% yields, respectively.[5, 6] It is worth mentioning that one of the two alkyl groups on the nitrogen atom of **12** was removed during the catalytic cycle as previously reported.[7] Compound **16** was also subjected to the Larock heteroannulation using 2-iodoaniline to afford **PCNT13** and **PCNT12** in 37% and 47% yields, respectively. The methyl ester in **PCNT13** and **PCNT12** was hydrolyzed under basic conditions using saturated KOH in ethanol to afford carboxylic acids **17** and **18** in 99% yields. Esterification of **17** and **18** using carbodiimide reagents was

unsuccessful. We then turned to Mitsunobu esterification utilizing PPh_3, diisopropyl azodicarboxylate (DIAD) in THF.[8] Gratifyingly, **3** was obtained in 50% yield using triethylene glycol **5** as a nucleophile. Interestingly, Mitsunobu esterification of **18** using **5** as alcohol provided an inseparable mixture. To overcome this problem, **10** was used instead of **5**, and the reaction proceeded to give the ester intermediate, followed by deprotection of the tetrahydropyranyl ether to afford **4** in 23% yield over two steps. (See synthetic details and spectroscopic data of synthesized compounds in the Supporting Information.)

The derivative with an optimum position for linker attachment should display cytotoxicity similar or relatively close to the parent compound, to ensure a similar cytotoxic mechanism. With four indole derivatives in hand, we proceeded to evaluate cytotoxicity against A549 lung cancer cells. As illustrated in Table 1, all four compounds exhibited cytotoxic activity, with **3** showing the lowest IC_{50} value of 10.2±1.04 μM, closet to the 5.17 μM of the parent compound PCNT13.[2] In addition, the changes in cell morphology observed after 48 h treatment with **3** were similar to those treated with PCNT13.[2] Therefore, an ester functional group at the 4-position of the aromatic ring of PCNT13 was the best site for hydrophilic linker attachment.

Table 1. IC_{50} values of compound **1**-**4** against A549 cells and their effect on cell morphology

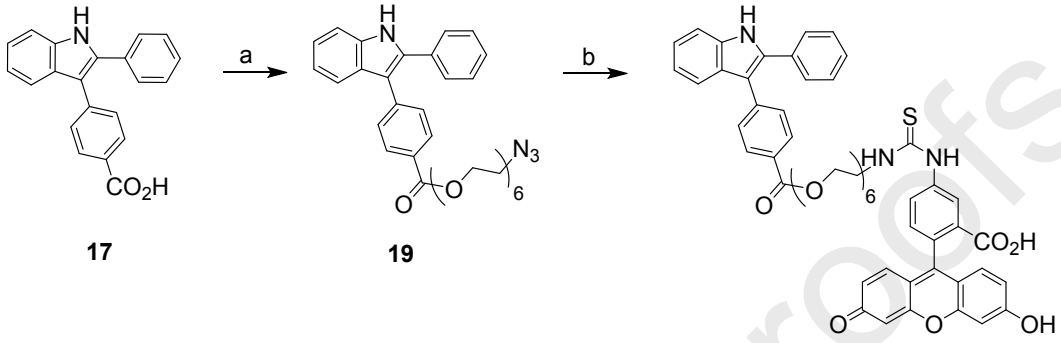
Compound	**IC_{50} (μM)**	**Cell morphology (48 h)**
No compound	-	

PCNT13	5.17±1.61	
1	30.8±2.56	
2	41.3±2.08	
3	10.2±1.04	
4	29.5±1.50	

111

112 Fluorescein isothiocyanate (FITC), a commonly used fluorophore, was chosen to connect to
113 PCNT13 through a hydrophilic linker. Synthesis of the fluorophore-PCNT13 conjugate (FL-PCNT13) was
114 illustrated in Scheme 3. The synthesis started from Mitsunobu esterification of **17** using a hydrophilic linker
115 **9** to afford **19** in 75%.[8] A longer linker (**9**, 12 carbon atoms) was used to provide ample space between the

PCNT13 indole moiety and the fluorescein to minimize any steric hindrance upon target binding. Subsequently, the azido group of **19** was reduced via catalytic hydrogenation, followed by a coupling reaction with FITC to afford FL-PCNT13 in 19% over two steps.



Scheme 3. Synthesis of FL-PCNT13. Reagents and conditions: (a) **9**, PPh_3, DIAD, THF, rt, 3 h; (b) i) Pd/C, MeOH, H_2, rt, 3 h, ii) FITC, Et_3N, MeOH, rt, 8 h in the dark.

The FITC moiety allows observing the intracellular localization of FL-PCNT13 by confocal laser microscopy. The A549 cells were incubated with 250 μM FL-PCNT13 for 3 h, then stained with a nuclear probe Hoechst 33342 (0.1 μg/mL) for 5 min before subjecting to live-cell imaging under a confocal laser microscope. As shown in Fig. 2, the observed fluorescent signal of FL-PCNT13 was distributed throughout the cytoplasm while the fluorescent signal in the nucleus was faintly detected, suggesting that PCNT13 and its primary target proteins are localized in the cytoplasm of the cells.

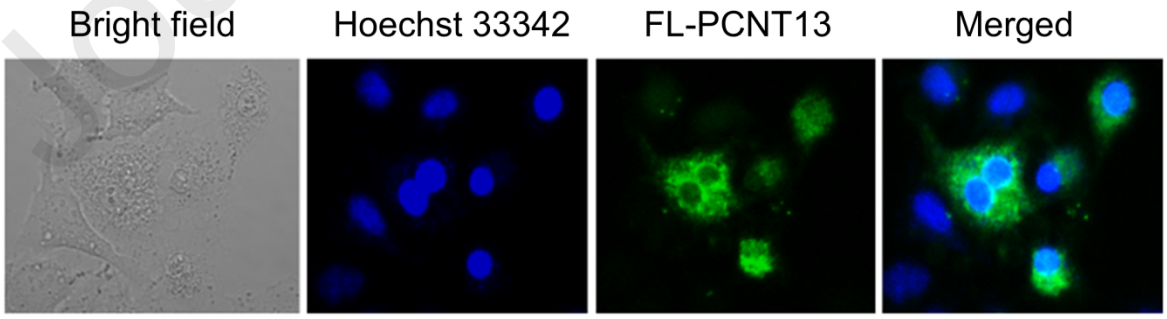


Fig. 2. Intracellular localization of FL-PCNT13 in living A549 cells. Confocal fluorescent images were taken after the cells were incubated with 250 μM FL-PCNT13 (Green) for 3 h, followed by 5-min staining with 0.1 μg/mL Hoechst 33342 nuclear dye (Blue).

Since the chemical structure of PCNT13 was similar to arylindoles, which have been reported as tubulin polymerization inhibitors,[9-20] we examined the effect of PCNT13 on tubulin polymerization by using the Tubulin Polymerization Assay Kit (Cytoskeleton, Inc.). Tubulins purified from the porcine brain were allowed to polymerize in a buffer containing 80 mM PIPES pH 6.9, 0.6 mM EGTA, 2 mM $MgCl_2$, and 1 mM GTP at 37 °C. Polymerization was monitored by recording the absorbance at 340 nm as a result of microtubule formation. As shown in Fig. 3, an anticancer drug Taxol (10 μM), which is known to be a microtubule-stabilizing agent, enhanced tubulin polymerization. In contrast, the polymerization was drastically inhibited in the presence of 10 μM PCNT13, indicating that PCNT13 might act as a microtubule-destabilizing agent. Since PCNT13 was found to localize in the cytoplasm of cells where the microtubules are formed, we suggested that the cytotoxicity of PCNT13 might result from inhibiting microtubule formation, and tubulin is one of the target proteins of this compound.

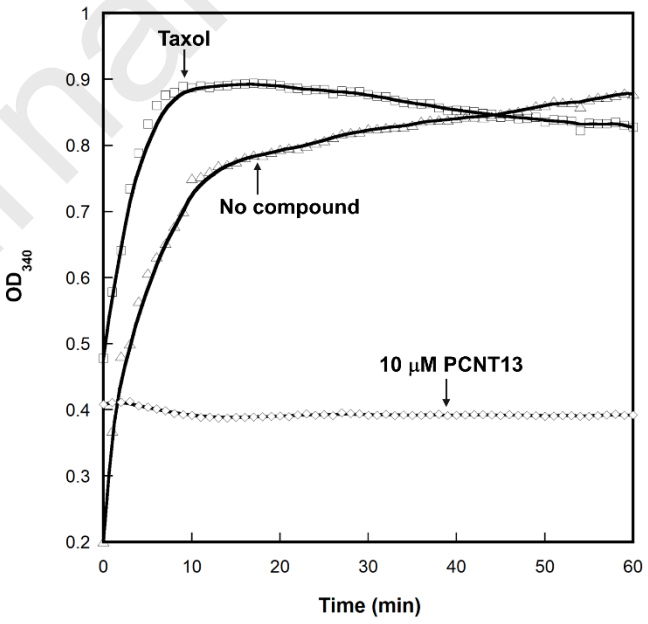


Fig. 3. Effect of PCNT13 on microtubule formation *in vitro*. Tubulins were allowed to polymerize in the presence of 10 μM PCNT13 or 10 μM Taxol. The microtubule formation was monitored by measuring absorbance at 340 nm.

Molecular docking simulation was employed to reveal binding mode between PCNT13 and tubulins. The three-dimensional structure of PCNT13 was obtained from DFT calculations using the B3LYP method with 6-31G(d,p) basis set implemented in Gaussian03 package.[21] Crystal structures of tubulin α and β were obtained from the Protein Data Bank (PDB ID: 1SA0).[22] Molecular docking was performed using AutoDock 4.0.[23] Ligands and protein structures were prepared using AutoDockTools 1.5.6.[24] Grid box was assigned to adequately cover the tubulin α and β subunits and the interface between them. Docking analysis reveals that PCNT13 binds to the colchicine binding site located at the interface between α and β subunits of tubulin as illustrated in Fig. 4. (See detailed analysis of the binding site in the Supporting Information.) Colchicine is known to destabilize microtubules. The docking results are consistent with the microtubule-destabilizing activity of PCNT13 observed *in vitro*.

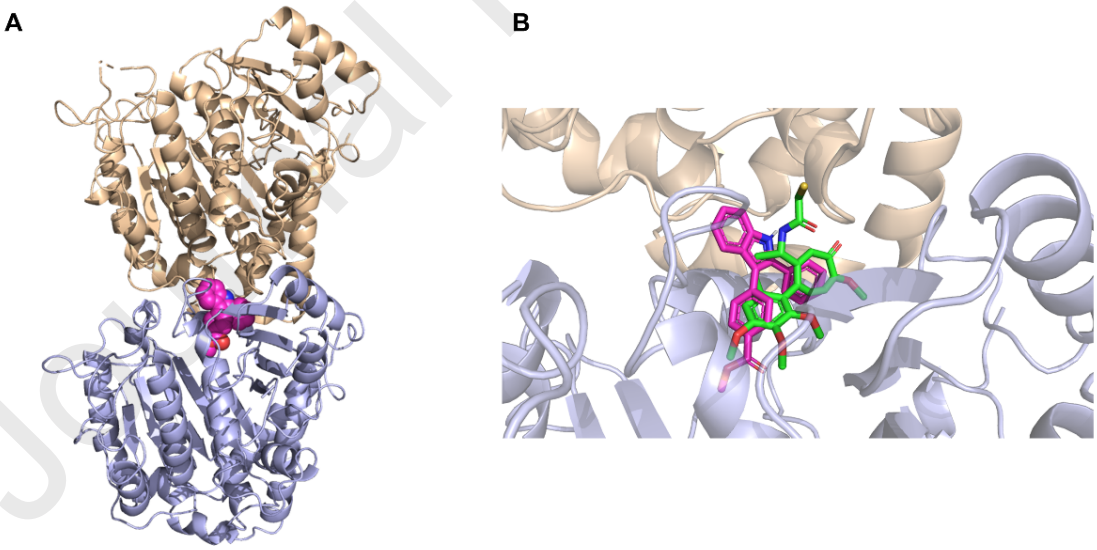


Fig. 4. The best-docked conformation of PCNT13 on the α/β–heterodimer of tubulins. (A) PCNT13 (Magenta) binds to the interface of tubulin α (Light brown) and β (Light blue). (B) Comparison of PCNT13

(Magenta) binding mode with that of colchicine (Green), a ligand co-crystallized with tubulin α and β subunits in 1SA0 crystal structure.

Since microtubule formation plays a critical role in the mitotic (M) phase of cell division, therefore, we further determined the effect of PCNT13 on A549 cell cycle distribution by flow cytometric technique using Muse Cell Analyzer (Merck Millipore) and Muse Cell Cycle Assay kit. After 24 h treatment, the A549 cells treated with PCNT13 (2.5 and 5 μM) exhibited a dose-dependent increase in the proportion of cells in the G2/M phase as 42.8±1.77% and 70.0±1.0%, respectively, compared with 24.0±3.44% of the untreated cells (Fig. 5). The results indicated that the mitosis phase of A549 cell division was disrupted by the microtubule-destabilizing activity of PCNT13, resulting in the cell cycle arrest in G2/M phase.

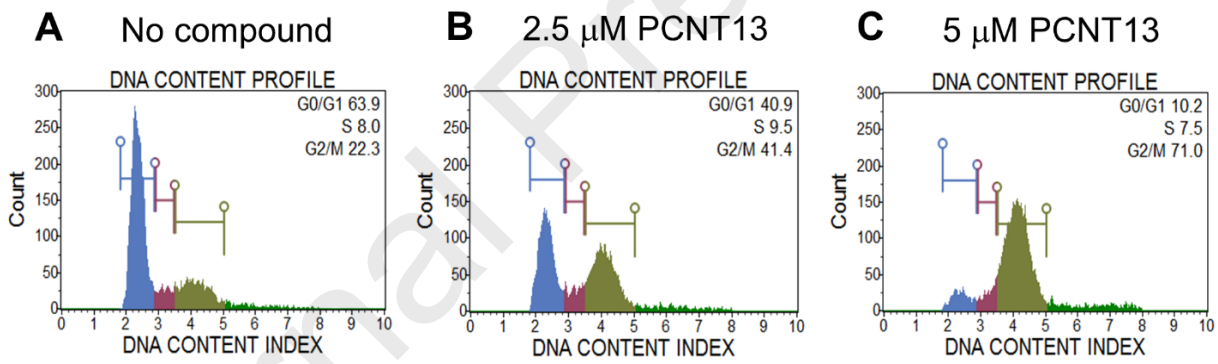


Fig. 5. Effect of PCNT13 on cell cycle distribution of A549 cells. The cells were treated with vehicle (A) or 2.5 and 5 μM PCNT13 (B and C) for 24 h. The percentages of cell populations in G0/G1, S, and G2/M phases were determined by using Muse Cell Analyzer and Muse Cell Cycle kit. DNA content histograms from a representative experiment were shown. Similar results were obtained in three independent experiments.

The effect of FL-PCNT13 on microtubule dynamics was similar to that of PCNT13 treatment as G2/M cell cycle arrest was observed when the cells were treated with 200 μM FL-PCNT13 for 24 h, by

53.3% increase in G2/M phase compared with 16.2% of untreated cells. The fluorophore FITC has been shown by other groups to localize in cytosol, and when tagged with compounds of interest, the FITC exhibited minimal interference to the binding of the compounds to the molecular targets.[25, 26] These results are consistent with our finding that the FL-PCNT13 conjugate was found to localize mainly in the cytoplasm, where microtubules, one of the cytosolic target proteins, were located. Nonetheless, FL-PCNT13 exhibited less cytotoxic potency (IC_{50} = 238±7.63 μM) compared with the parent compound PCNT13 (IC_{50} = 5.17±1.61 μM), presumably due to steric hindrance of the FITC moiety.

In conclusion, we have elucidated a mechanism underlying the apoptosis-inducing activity of 2,3-diarylindole PCNT13 using the FITC fluorophore connected to the optimum position of the compound through a hydrophilic linker. This study demonstrated a correlation between cytoplasmic localization, inhibition of tubulin polymerization, and G2/M arrest effect of PCNT13 in A549 cells. Our results are consistent with the reported biological effects of other microtubule inhibitors that induce G2/M arrest and subsequently lead to apoptosis in cancer cells.[27] Hence, we conclude that the microtubule destabilization is the mechanism underlying G2/M cell cycle arrest and apoptosis induction by PCNT13 in A549 lung cancer cells.

Acknowledgments

This project was supported by the Thailand Research Fund (MRG5180073), by the Kasetsart University Research and Development Institute (KURDI), by the Faculty of Science, Kasetsart University, and a research grant from the Chulabhorn Research Institute. BT is supported by the Development and Promotion of Science and Technology Talents Project (DPST). Financial support from the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation is gratefully acknowledged.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at XXX.

References

1. Inamura K. Lung Cancer: Understanding its molecular pathology and the 2015 WHO classification. *Front. Oncol.* 2017;7:193.

2. Rukkijakan T, Ngiwsara L, Lirdprapamongkol K, Svasti J, Phetrak N, Chuawong P. A synthetic 2,3-diarylindole induces cell death via apoptosis and autophagy in A549 lung cancer cells. *Bioorg. Med. Chem. Lett.* 2016;26(9):2119-2123.

3. Shao M, Zhao Y. TTFAQ-cored D/A ensembles: synthesis, electronic properties, and redox responses to transition metal ions. *Tetrahedron Lett.* 2010;51(21):2892-2895.

4. Sonogashira K. Development of Pd–Cu catalyzed cross-coupling of terminal acetylenes with sp^2-carbon halides. *J. Organomet. Chem.* 2002;653(1-2):46-49.

5. Phetrak N, Rukkijakan T, Sirijaraensre J, et al. Regioselectivity of larock heteroannulation: a contribution from electronic properties of diarylacetylenes. *J. Org. Chem.* 2013;78(24):12703-12709.

6. Larock RC, Yum EK. Synthesis of indoles via palladium-catalyzed heteroannulation of internal alkynes. *J. Am. Chem. Soc.* 1991;113(17):6689-6690.

7. Hao W, Geng W, Zhang WX, Xi Z. Palladium-catalyzed one-pot three- or four-component coupling of aryl iodides, alkynes, and amines through C-N bond cleavage: efficient synthesis of indole derivatives. *Chem. Eur. J.* 2014;20(9):2605-2612.

8. Lepore SD, He Y. Use of sonication for the coupling of sterically hindered substrates in the phenolic Mitsunobu reaction. *J. Org. Chem.* 2003;68(21):8261-8263.

9. Kazan F, Yagci ZB, Bai R, Ozkirimli E, Hamel E, Ozkirimli S. Synthesis and biological evaluation of indole-2-carbohydrazides and thiazolidinyl-indole-2-carboxamides as potent tubulin polymerization inhibitors. *Comput. Biol. Chem.* 2019;80:512-523.

10. Chen P, Zhuang Y-X, Diao P-C, et al. Synthesis, biological evaluation, and molecular docking investigation of 3-amidoindoles as potent tubulin polymerization inhibitors. *Eur. J. Med. Chem.* 2019;162:525-533.

11. La Regina G, Bai R, Coluccia A, et al. New 6- and 7-heterocyclyl-1*H*-indole derivatives as potent tubulin assembly and cancer cell growth inhibitors. *Eur. J. Med. Chem.* 2018;152:283-297.

12. La Regina G, Bai R, Rensen W, et al. Design and synthesis of 2-heterocyclyl-3-arylthio-1*H*-indoles as potent tubulin polymerization and cell growth inhibitors with improved metabolic stability. *J. Med. Chem.* 2011;54(24): 8394-8406.

13. Ahn S, Hwang DJ, Barrett CM, et al. A novel bis-indole destabilizes microtubules and displays potent in vitro and in vivo antitumor activity in prostate cancer. *Cancer chemother. pharmacol.* 2011;67(2):293-304.

14. Romagnoli R, Baraldi PG, Sarkar T, et al. Synthesis and biological evaluation of 1-methyl-2-(3′,4′,5′-trimethoxybenzoyl)-3-aminoindoles as a new class of antimitotic agents and tubulin inhibitors. *J. Med. Chem.* 2008;51(5):1464-1468.

15. Kaufmann D, Pojarová M, Vogel S, et al. Antimitotic activities of 2-phenylindole-3-carbaldehydes in human breast cancer cells. *Bioorg. Med. Chem.* 2007;15(15):5122-5136.

16. De Martino G, La Regina G, Coluccia A, et al. Arylthioindoles, potent inhibitors of tubulin polymerization. *J. Med. Chem.* 2004;47(25):6120-6123.

17. Li Q, Sham HL. Discovery and development of antimitotic agents that inhibit tubulin polymerisation for the treatment of cancer. *Expert Opin. Ther. Pat.* 2002;12(11):1663-1702.

18. Flynn BL, Hamel E, Jung MK. One-pot synthesis of benzo[*b*]furan and indole inhibitors of tubulin polymerization. *J. Med. Chem.* 2002;45(12):2670-2673.

19. Bacher G, Beckers T, Emig P, Klenner T, Kutscher B, Nickel B. . New small-molecule tubulin inhibitors. *Pure Appl. Chem.* 2001;73(9):1459–1464.

20. Gastpar R, Goldbrunner M, Marko D, von Angerer E. Methoxy-substituted 3-formyl-2-phenylindoles inhibit tubulin polymerization. *J. Med. Chem.* 1998;41(25):4965-4972.

21. Frisch MJ, Trucks GW, Schlegel HB, et al. Gaussian 03, Revision C.02, Gaussian Inc., Wallingford, CT, USA, 2004.

22. Ravelli RB, Gigant B, Curmi PA, et al. Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain. *Nature.* 2004;428(6979):198-202.

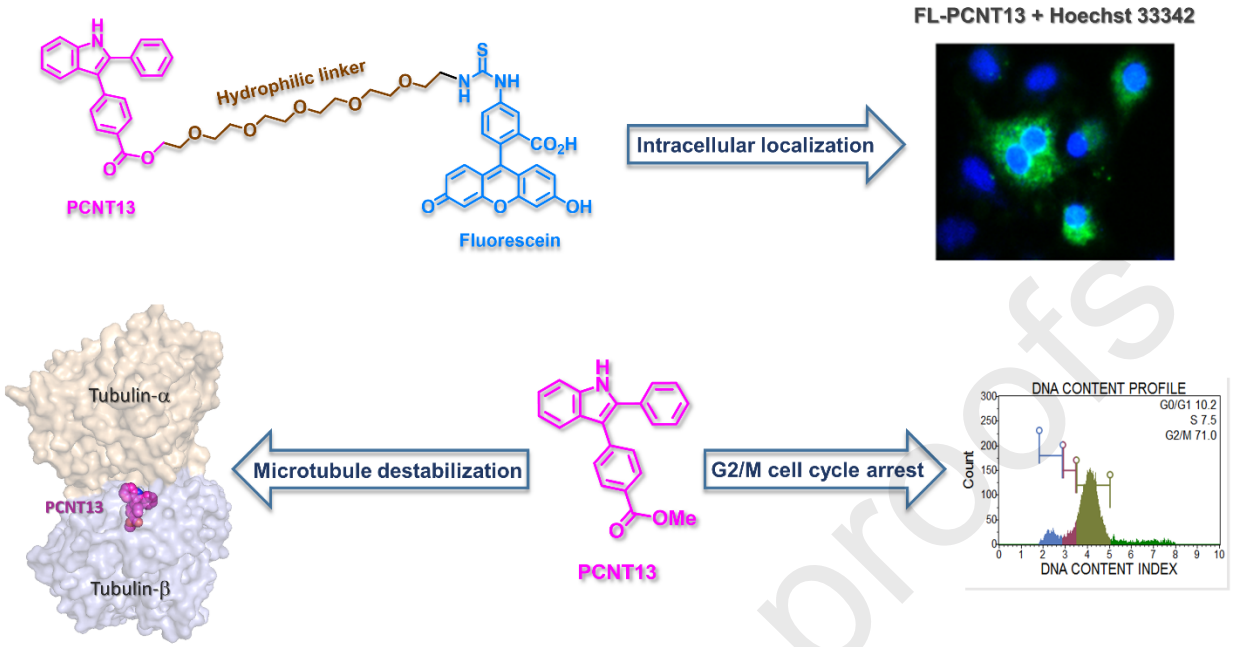
23. Morris GM, Goodsell DS, Halliday RS, et al. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* 1998;19(14):1639-1662.

24. Sanner MF. Python: a programming language for software integration and development. *J. Mol. Graph. Model.* 1999;17(1):57-61.

25. Kirkham S, Hamley IW, Smith AM, et al. A self-assembling fluorescent dipeptide conjugate for cell labelling. *Colloids Surf. B Biointerfaces.* 2016;137:104-108.

26. Best TP, Edelson BS, Nickols NG, Dervan PB. Nuclear localization of pyrrole-imidazole polyamide-fluorescein conjugates in cell culture. *Proc. Natl. Acad. Sci. U.S.A.* 2003;100(21):12063-12068.

27. Bates D, Eastman A. Microtubule destabilising agents: far more than just antimitotic anticancer drugs. *Br. J. Clin. Pharmacol.* 2017;83(2):255-268.



269

270 **Declaration of interests**

271

272 ☒ The authors declare that they have no known competing financial interests or personal relationships
273 that could have appeared to influence the work reported in this paper.

274

275 ☐The authors declare the following financial interests/personal relationships which may be considered
276 as potential competing interests:
277

278
279
280

281

282