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A Synthetic 2,3-Diarylindole Induces Cell Death via Apoptosis and Autophagy in A549 Lung Cancer Cells

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Abstract

A series of 2,3-diarylindoles were synthesized via the Larock heteroannulation, and evaluated for their anticancer activity against A549 lung cancer cells. The most potent compound, PCNT13 with IC_{50} = 5.17 μM, caused the induction of two modes of programmed cell death, apoptosis and autophagy.

Keywords

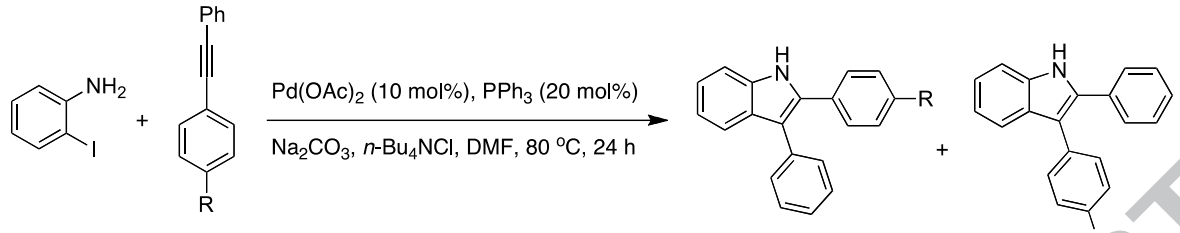
2,3-Diarylindole; Apoptosis; Autophagy; Lung Cancer

Lung cancer is one of the most common malignancies worldwide, and the long-term survival rate of lung cancer patients is one of the lowest among cancers.[1] Two major types of lung cancer have been characterized: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).[2] NSCLC is a more prevalent subtype being relatively insensitive to chemotherapy and radiation therapy compared to SCLC. The high mortality in NSCLC is due to the difficulty of early diagnosis and the high potential for local invasion and distant metastasis. Although relatively effective chemotherapeutic agents have been developed, lung cancer still has a low cure rate. Furthermore, the adverse effects of chemotherapeutic compounds often deteriorate the quality of life of lung cancer patients. Therefore, the discovery of effective novel therapeutic compounds for NSCLC treatments is urgently needed.

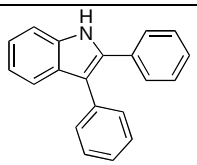
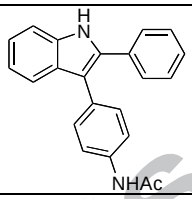
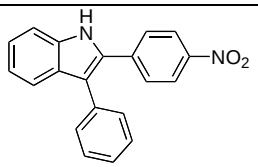
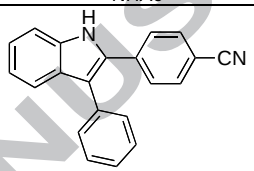
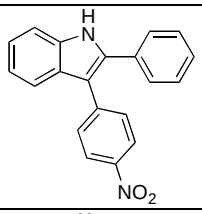
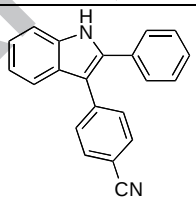
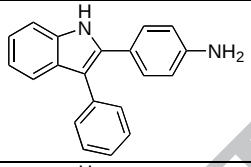
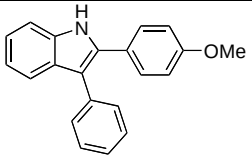
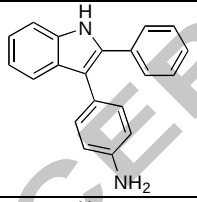
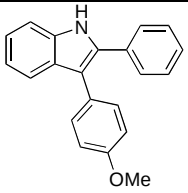
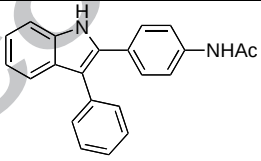
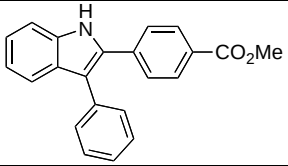
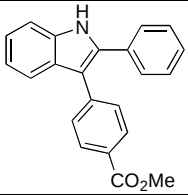
Indole alkaloids exhibit various biological activities including anti-inflammatory,[3] anticancer,[4] anti-HIV,[5] and anti-depression.[6] In particular, 2,3-disubstituted indole derivatives demonstrated inhibitory activity against cyclooxygenase I and II (COX I and II), which play crucial roles in many physiological and pathological processes including chronic inflammation, cancer, and neurodegenerative disorders.[7] In addition, 2,3-diarylindoles have been indicated to inhibit tubulin polymerization,[8] a process that has been validated for anticancer therapy.[9] In this study, we explore the potential biological activity of synthetic 2,3-diarylindoles toward a human NSCLC cell line, A549 lung adenocarcinoma.

We started our investigation with the synthesis of 2,3-diarylindoles. Several synthetic methods have been reported for indole derivatives[10] including palladium-catalyzed reactions,[11] which have become very attractive due to their high efficiency. Among them, the Larock heteroannulation is a palladium-catalyzed reaction utilizing 2-iodoaniline and disubstituted alkynes as substrates.[12] The reaction results in the formation of carbon-carbon and carbon-nitrogen bond in a single operation. When unsymmetrical disubstituted alkynes are used, two regioisomeric products can be obtained. The 2,3-diarylindoles bearing different substituents at the *para* position of one of the phenyl moieties (PCNT01-13) were synthesized via the Larock heteroannulation according to our previous report (Table 1).[13]

Table 1. Synthesis of 2,3-diarylindoles and their IC_{50} values against A549 lung cancer adenocarcinoma cell line.



R = H, NO_2, NH_2, NHAc, CN, OMe, CO_2Me

Compound	Structure	IC_{50} (μM)	Compound	Structure	IC_{50} (μM)
PCNT01		58.70	PCNT07		N/A[a]
PCNT02		24.81	PCNT08		15.0
PCNT03		38.37	PCNT09		18.3
PCNT04		54.70	PCNT10		288.31
PCNT05		83.38	PCNT11		30.0
PCNT06		N/A[a]	PCNT12		N/A[a]
			PCNT13		5.17

[a]Compounds become insoluble during the assay.

These compounds were first screened for their cytotoxic activity at 72 h using the MTT assay.[14] Three compounds (PCNT06, PCNT07, and PCNT12) had solubility problems hence their cytotoxic activity could not be evaluated. Among all compounds tested, PCNT13 showed the highest cytotoxic activity with IC_{50} value of 5.17 μM (Table 1). Therefore, this compound was chosen for further study. Cell viability was reduced in a time- and dose-dependent manner when treated with compound PCNT13 for 24, 48, and 72 h at a concentration range of 6.3-100 μM (Fig. 1).

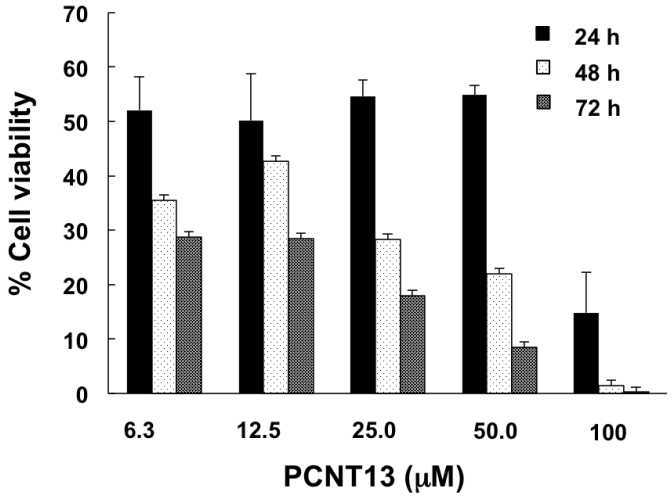


Figure 1. Cytotoxic effect of PCNT13 against A549 cells. The viability of cells after exposed to PCNT13 at various concentrations (6.3-100 μM) for 24 h was determined by MTT assay. Data are reported as average values from three independent experiments with standard deviations (SD).

After 24 h treatment, the morphology of A549 cells became more rounded in appearance, with more vacuoles appearing in the cells when concentrations of PCNT13 were increased (10, 50, and 100 μM) (Fig. 2A). The cell morphology began to change upon treatment of PCNT13 after 3 h (data not shown). We further examined whether PCNT13 induced apoptosis in A549. Changes in apoptosis markers were analyzed by flow cytometric technique with Muse Cell Analyzer using Annexin-V and Dead Cell assay kit.[15] By increasing the concentrations of PCNT13 for 24 h, treated cells showed increased Annexin-V and 7AAD-positive cell population, indicating the activation of apoptosis (Fig. 2B). The highest proportion of late apoptosis cells showing both Annexin-V and 7AAD positive was about 40% when cells were treated with PCNT13 at a concentration of 100 μM (Fig. 2C).

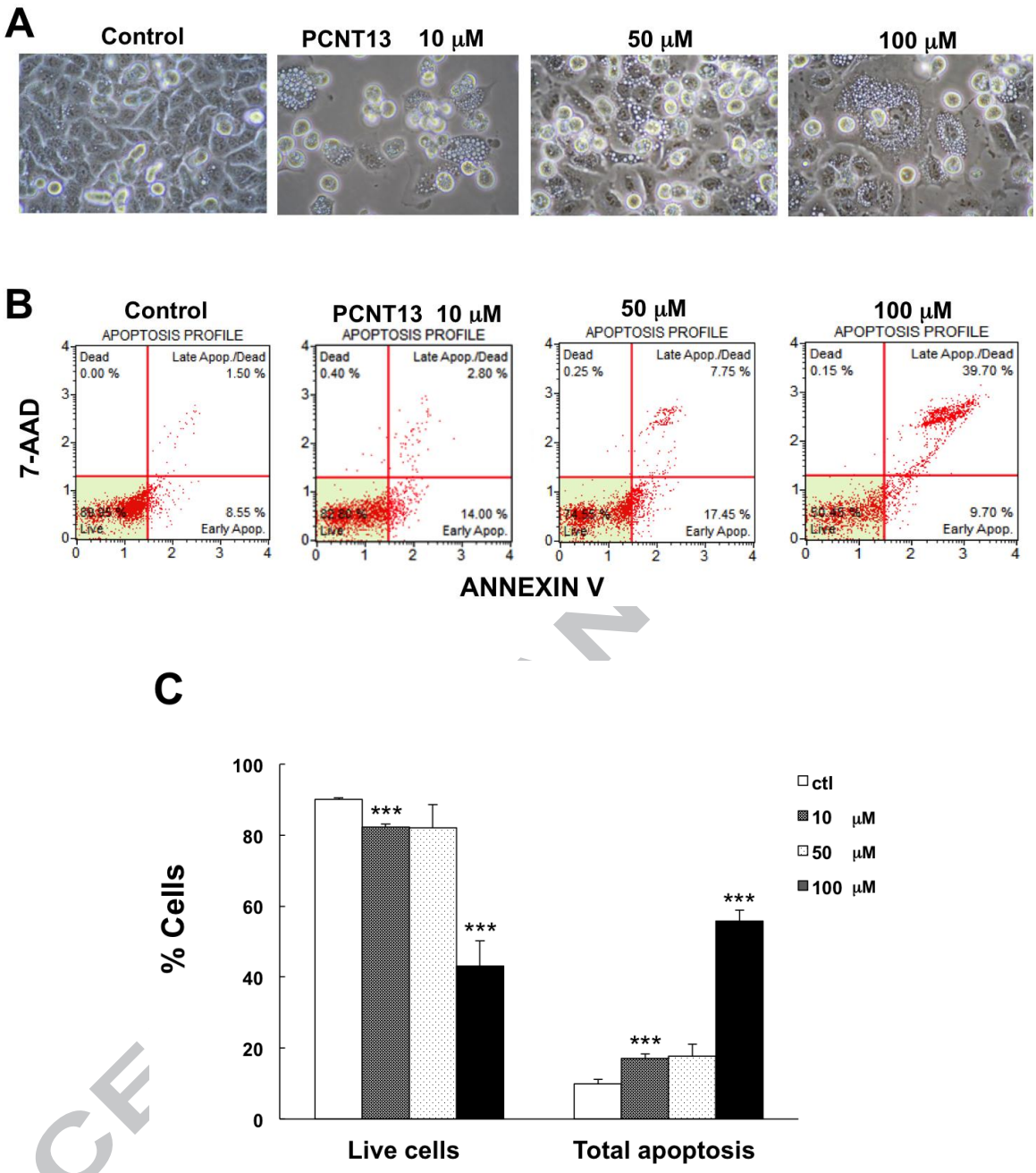


Figure 2. Effect of PCNT13 on cell morphology and apoptosis induction in A549 cells. Cells were treated with PCNT13 at 10, 50, and 100 μM for 24 h and photographs were taken, after which cells were analyzed for apoptosis using Muse Cell Analyzer with Annexin-V and Dead Cell assay kit. (A) Cell morphology changes, original magnification of ×400. (B) Dot plots recording Annexin-V and 7-AAD positive cells in a representative experiment. (C) Quantitative analysis of percent cell populations. Data are reported as average values from three independent experiments with standard deviations (SD), ***$p < 0.001$ vs. control cells.

It is now well known that the reduction in tumor size caused by chemotherapy is a function of apoptotic cell death (type I programmed cell death). However, apoptosis is suppressed in certain tumors that progress to stages with high-grade malignancy and resistance to therapy.[16] Recent evidence has pointed to the existence of autophagy, or type II programmed cell death, which is normally activated in response to nutrient deprivation, but is also activated by exposure to anticancer agents.[17] Autophagy is characterized by the formation of autophagosomes in the cytoplasm, which fuse with lysosomes, organelles, so that cytoplasmic macromolecules are sequestered and ultimately degraded within the vesicle by lysosomal enzymes.[18] Autophagy in response to cellular stress serves as a potent death signal, as in the case of chemotherapy. Since the morphology of PCNT13 treated cells exhibited vacuole containing cells as a hallmark of autophagy (Fig. 2A), we assessed whether PCNT13 also induces autophagy in A549 cells. After treatment for 24 h, PCNT13 treated cells displayed positive results for staining with monodansylcadaverin (MDC), an autophagic vacuole-specific fluorescent probe (Fig. 3A). Moreover, PCNT13 treatment induced MDC accumulation in A549 cells in a dose-dependent manner (Fig. 3B). The results suggest that PCNT13 induces both autophagy and apoptosis in A549 cells.

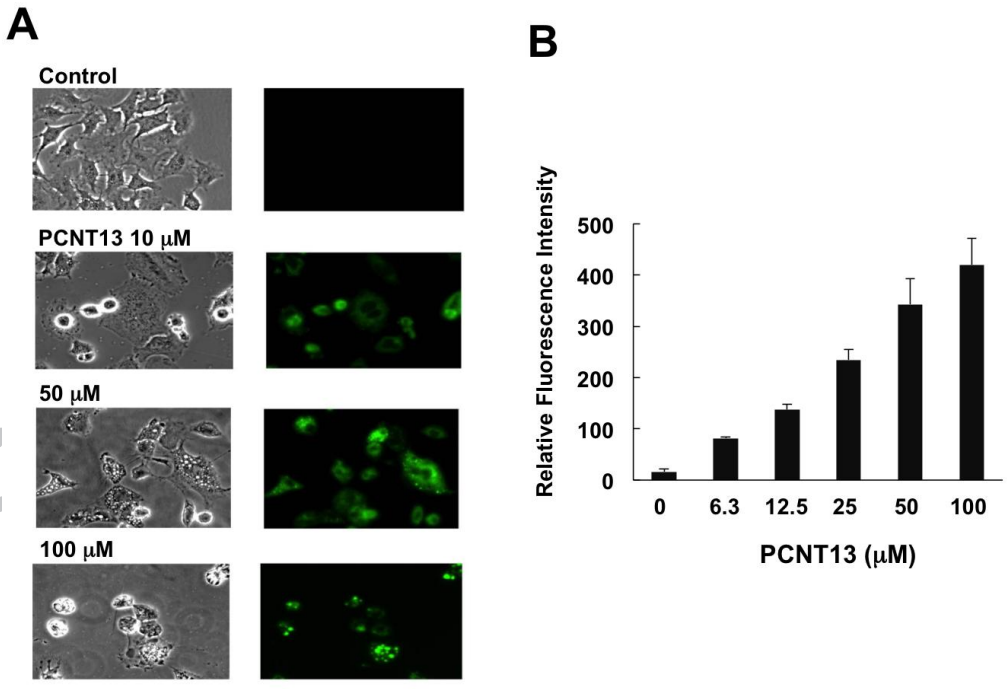


Figure 3. Induction of autophagy by PCNT13 in A549 cells. Cells were treated with PCNT13 at different concentrations (10, 50, and 100 μM) for 24 h. (A) Fluorescent imaging of the treated cells after staining with an

autophagic vacuole-specific fluorescent probe, monodansylcadaverin (MDC). (B) The relative fluorescent intensity of MDC staining, Ex/Em at 335/512 nm, in treated cells after exposure to PCNT13 at various concentrations (6.2-100 μM) for 6 h. Data are reported as average values from three independent experiments with standard deviations (SD).

We further confirmed the occurrence of both apoptosis and autophagy processes by monitoring the alteration of key proteins required for those two specific events. During the execution phase of apoptosis, PARP is specifically cleaved by caspase-3. Autophagy is initiated by conjugation of Agt12, Atg5, and Atg8/microtubule-associated protein light chain 3 (LC3) to the nascent autophagosome membrane. The increase of LC3-II levels has been used to represent the autophagy extent.[19] After 24 h treatment with PCNT13, A549 cells were harvested and subjected to Western blot analysis. As shown in Figure 4, PCNT13 effectively enhanced PARP cleavage in a dose-dependent manner, and this corresponds to the elevated conversion of LC3-I to LC3-II.

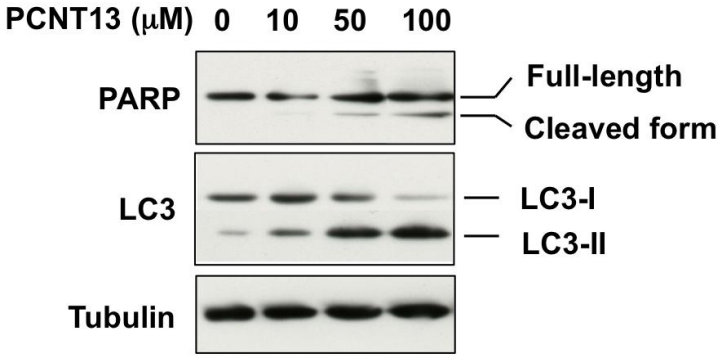


Figure 4. Western blot analysis of apoptosis and autophagy progression in PCNT13-treated A549 cells. Cells were exposed to PCNT13 at different concentrations (10, 50, and 100 μM) for 24 h, and then harvested and subjected to Western blot analysis. Results shown are representative of at least three independent experiments. PARP cleavage and LC3 conversion are the hallmark of apoptosis and autophagy, respectively. Tubulin is used as a loading control.

Apoptosis and autophagy are considered to be two different, but closely related programmed cell death mechanisms. Apoptotic signaling can regulate autophagy, and conversely autophagy can regulate apoptosis. However, the molecular connections between autophagy and cell death are complicated and, in different contexts,

autophagy may either promote or inhibit cell death.[20] We used an autophagy inhibitor, Bafilomycin A1 (Baf), to explore the role of autophagy in the growth inhibition and apoptosis induced by PCNT13. Baf prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes.[21] Our results showed that Baf co-treatment was able to block PCNT13-promoted autophagy in A549 cells, as shown by a lower MDC fluorescent intensity (Fig. 5A). Interestingly, Baf co-treatment partially reversed the apoptosis-inductive effect of PCNT13 (Fig. 5B), implying that autophagy contributes to PCNT13-induced apoptosis in A549 cells.

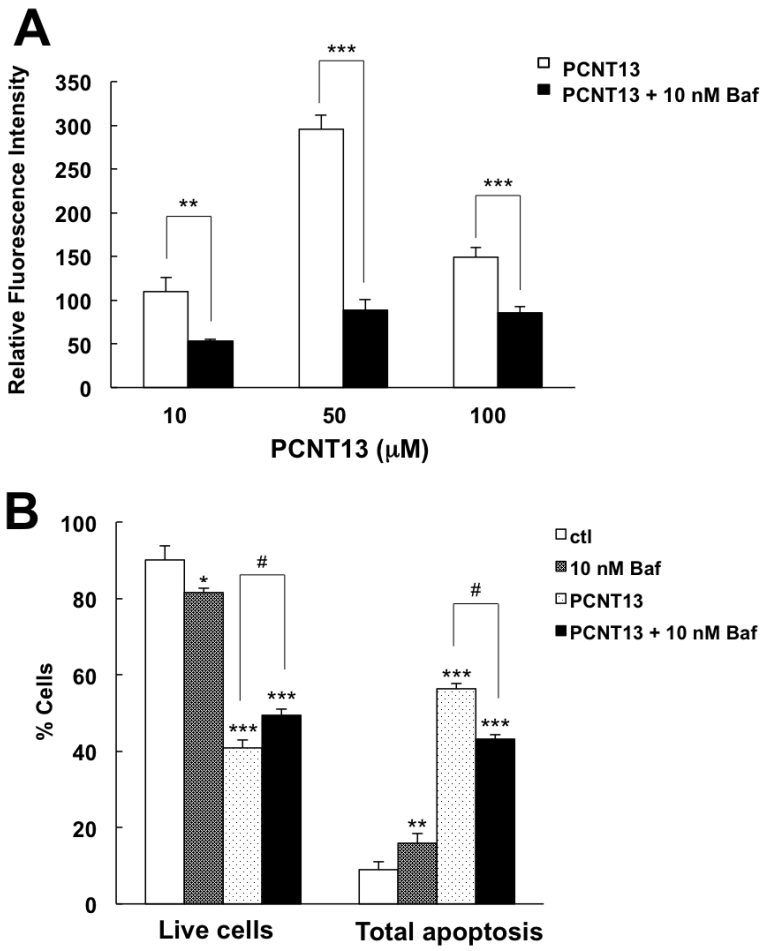


Figure 5. Effect of autophagy inhibition on apoptosis rate in PCNT13 treated A549 cells. The cells were exposed to PCNT13 for 24 h, at various concentrations (10, 50, and 100 μM) with or without 10 nM Bafilomycin A1 (Baf). (A) Quantitative analysis of autophagy in PCNT13 treated A549 cells using MDC staining. (B) The rate of apoptosis was analyzed using Muse Cell Analyzer with Annexin-V and Dead Cell assay kit. Data are reported as

average values from three independent experiments with standard deviations (SD). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control cells, and significance between PCNT13 and PCNT13 + Baf are indicated by $^{\#}$, $p < 0.05$.

In conclusion, this study sheds light on the anticancer potential of 2,3-diarylindole derivatives against human NSCLC cells *via* promoting autophagy and apoptosis induction. Further studies are in progress on the exact cytotoxic mechanism(s) exerted by this class of compounds, and the results should expand the scope for further investigation on target-based anticancer drug design derived from two modes of programmed cell death.

Acknowledgments

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Graphical Abstract