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A Novel Synthetic Indole Compound, 1,1,3-tri(3-indolyl)cyclohexane, Inhibits Microtubule Polymerization in Human Lung Cancer Cells

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ABSTRACT

BACKGROUND. Lung cancer is the most common malignancies in both men and women worldwide. Thus, the development of more effective anti-cancer drugs for lung cancer is urgently needed.

METHODS. We generated a novel indole compound, 1,1,3-tri(3-indolyl)cyclohexane (3-indole), with high purity and in large quantities. 3-indole was tested for its biological activity in A549 and H1437 lung cancer cells.

RESULTS. Our data indicated that 3-indole caused a concentration-dependent reduction in cell proliferation in human lung cancer cells but not in the normal lung cells. In addition, 3-indole induced G2-M cell cycle arrest in A549 and H1437 lung cancer cells to different extents. Using immunochemistry assay, the DMSO-treated control was shown to exhibit normal filamentous arrangement and organization of microtubule network whereas in A549 cells treated with 3-indole, almost complete loss of cellular microtubule networks throughout the cytoplasm was observed. Moreover, Western blot data showed that 3-indole dose-dependently inhibited microtubule polymerization in A549 cells.

CONCLUSIONS. Based on its potent cell growth inhibition in lung cancer cell models, our data suggest that this novel synthetic 3-indole compound of high purity and yield is a potential antimicrotubule polymerization agent for cancer treatment.

Key words: indole compound, lung cancer, tubule polymerization, antimicrotubule

Introduction

Lung cancer is the most common malignancies in both men and women worldwide (Danesi *et al.*, 2003; Jemal *et al.*, 2007). Even with the recent advent of more effective molecular targeted therapies, the clinical responses to chemotherapy in patients with lung cancer are still unsatisfactory, with a 5-year overall survival in many countries generally less than 15% (Danesi *et al.*, 2003). Thus, the development of effective anti-cancer drugs for lung cancer is urgently needed.

Microtubules are main components of the cytoskeleton and are important for a variety of cell functions including maintenance of cell shape, transportation of vesicles, mitochondria and other components throughout cells, and segregation of chromosomes during cell division (Jordan and Wilson, 2004; Pellegrini and Budman, 2005). Microtubules are extremely dynamic polymers consisting of α–tubulin and β-tubulin heterodimers arranged in the form of slender filamentous tubes that are constantly assembling (polymerization) or disassembling (depolymerization) (Jordan, 2002). Cancer cells acquire unlimited replication potential and continue to divide without progressing into immobility and senescence (Hayflick, 1997). The properties of uncontrolled proliferation and division

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make cancer cells extremely dependent upon the high dynamics of microtubule and hence sensitive to antimicrotubule compounds (Jordan and Wilson, 1998). Antimicrotubule agents (with various tubulin-binding sites), which have been found to interfere with tubulin/microtubules dynamic equilibrium, induce G2-M cell cycle arrest and trigger apoptosis (Woods *et al.*, 1995; Jordan *et al.*, 1996). These findings indicate that microtubule is an important target for the development of novel anticancer drugs (Giannakakou *et al.*, 2000).

The clinically used antimicrotubule drugs generally fall into two main groups. One group includes *vinca* alkaloids, known as the microtubule-destabilizing agents such as vinorelbine, vincristine, and vinblastine. This type of agent inhibits microtubule polymerization and lead to the depolymerization of existing microtubules. The other group is known as the microtubule-stabilizing agents including taxanes, such as taxol (paclitaxel) and docetaxel, they stabilize microtubules and induce a net polymerization (Li and Sham, 2002). Despite the efficiency of antimicrotubule drugs in inhibiting the progression of some tumors, the important unsolved questions about the antitumor activities of antimicrotubule drugs concern the basis of their tissue specificities in many cancer types and the basis for the development of drug resistance to these agents usually occur during therapy (Gottesman, 2002).

Several microtubule polymerization inhibitors, such as *Vinca* alkaloids, characterized by the presence of an indole core nucleus have been obtained from natural products or have been prepared by semi-synthesis (Brancale and Silvestri, 2007). We recently synthesized a novel indole compound, 1,1,3-tri(3-indolyl)cyclohexane (3-indole), with high purity and in large quantities (Ko *et al.*, 2006). In the present study, we analyzed the biological activities especially the mechanisms involved in the anti-cancer growth activities of 3-indole in cell models. 3-indole induced G2-M cell cycle arrest in A549 and H1437 lung cancer cells to different extents. Furthermore, we found that cell cycle arrest was induced via inhibition of microtubule polymerization in A549 cells. Together, these results indicated that 3-indole is a potential lead compound based on its antimicrotubule properties.

Materials and Methods

Cell Culture

Human non-small cell lung carcinoma cells (A549 and H1437) were maintained in DMEM. Media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 g/ml of streptomycin (Invitrogen, Eugene, OR). The cells were maintained at 37°C in a humidified incubator containing 5% CO_2 in air.

Cell Viability Assay

Cells were treated with DMSO or various concentrations of 3-indole (1, 5, or 10 μM) for 72 h. After treatment, the cells were washed with 1× PBS and then treated with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in appropriate medium for 30 min at 37°C; cells generate a blue color when dissolved in DMSO. The intensity of the absorbance was measured using a reader for an enzyme-linked immunosorbent assay at a wavelength of 540 nm.

Analysis of Cell Cycle Distribution

Cells were incubated in DMSO, 1 μM taxol (paclitaxel), 1 μM vinorelbine or various concentrations of 3-indole (10, 20, or 30 μM) for 24 h. Cells were collected by trypsinization, washed with 1× PBS, and fixed with ice-cold 80% ethanol at least overnight at -20°C until analysis. Fixed cells were collected by centrifugation, washed with 1× PBS, resuspended in 1 ml of 1× PBS containing 20 g/ml propidium iodide, 200 g/ml RNase A, and 0.1% triton X-100, and then incubated in the dark for 20 min. Determination of cell cycle distribution was performed by FACScan flow cytometer (BD, MountainView, CA) and calculated using ModFit LT software, version 2.0 (BD).

Immunocytochemistry

Cells were incubated in DMSO, 1 μM taxol, 1 μM vinorelbine or various concentrations of 3-indole (10, 20, or 30 μM) for 24 h. Cells were washed with 1× PBS, fixed with 1% formaldehyde for 20 min at room temperature, and washed twice with 1×PBST (1×PBS + 0.1% tween20) for 5 min. Cells were then incubated with 1× PBS containing primary antibodies α-tubulin (Cell Signaling Technologies, Beverly, MA) for 1 h at 37°C. After washing with 1×PBS, cells were reincubated with FITC-conjugated secondary antibody (Upstate

Biotechnology Inc., Lake Placid, NY) and DAPI (4'-6'-Diamidino-2-phenylindole, Sigma) in the dark room for 1 h at 37°C. Cells then were washed with 1×PBS three times. Cellular microtubules were observed with an Olympus BX50 fluorescence microscope (Optical Elements Corporation, Dulles, VA).

Western Blot Analysis

Cells were washed with 1×PBS before adding lysis buffer containing 20 mM Tris-HCl (pH 6.8), 1 mM $MgCl_2$, 2 mM EGTA, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 0.5% Nonidet. Supernatants were collected after centrifugation at 13,000 rpm for 10 min at 4°C. The pellets were dissolved in an SDS-PAGE sampling loading buffer and heated at 95°C for 10 min. Cell lysates were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skim milk/1×PBST for 1 h at room temperature and probed with appropriate dilutions of primary antibody overnight at 4°C, as recommended by the manufacturers. The primary antibodies used were α-tubulin (Cell Signaling Technologies, Beverly, MA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Novus Biologicals, Littleton, CO). Membranes were then washed three times with 1×PBST and subsequently incubated with appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After a further three washes with 1×PBST, immunoreactive proteins were visualized using Western blot chemilluminescent reagents.

Results

3-indole Inhibited the Growth of A549 lung cancer cells but not of normal lung cells

3-indole is a novel, 2-step synthetic indole-like compound with high purity and yield. To test the cytotoxicity effect and future clinical use of 3-indole for anti-cancer treatment, IMR-90 normal human lung fibroblast cells and A549 human lung cancer cells were treated with 1, 5 or 10μM of 3-indole for 72 h and cell viability was assessed by the MTT assay. Fig. 1 shows that 3-indole caused a dose-dependent reduction in cell viability in A549 lung cancer cells. 3-indole achieved an IC50 value at 5 μM in A549 human lung cancer cells, whereas

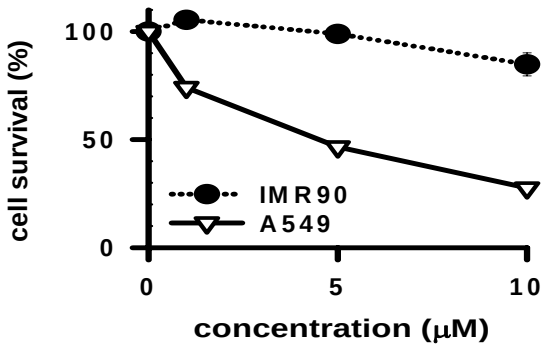

Figure 1. Viability assays of 3-indole in IMR-90 normal human lung cells and A549 human lung cancer cells. Cells were treated with 1, 5 or 10μM of 3-indole for 72 h and cell viability was assessed by the MTT assay.

3-indole did not show apparent cytotoxicity to the IMR-90 normal lung cells at this dose.

3-indole induced G2-M cell cycle arrest in A549 and H1437 cells

Indole-like compounds are known to arrest cells in G1 or G2/M, and substantially induce apoptosis (Brandi *et al.*, 2003; Kuo *et al.*, 2004). To determine whether the anti-cancer effect of 3-indole was associated with cell cycle deregulation, the cell cycle distribution was analyzed by flow cytometry. We studied whether G2-M cell cycle arrest could be induced in A549 cells treated with 10, 20 and 30 μM of 3-indole for 24 h. Flow cytometry results indicated that 10 μM of 3-indole caused A549 cancer cells to accumulate in G1 and partially G2-M cell cycles, and that a substantial increase in the sub-G1 region (an apoptosis indicator) resulted from treatment with 30 μM of 3-indole at 24 h (Figs. 2A-F). The G2-M arrest of 30 μM of 3-indole was also noted in H1437 lung cancer cells (Figs. 2G-H). These results indicate that 3-indole may induce cell death partly via G1 and G2-M arrests.

Effect of 3-indole on the cellular microtubule distribution in A549 cells

Microtubules are highly dynamic polymers composed of α–tubulin and β-tubulin heterodimers that are constantly assembling (polymerization) or disassembling (depolymerization). Microtubules are crucial in G2-M phase and cell division. Antimicrotubule agents are known to arrest cells in G2-M, and substantially induce apoptosis (Giannakakou *et al.*, 2000). To confirm that the partially G2-M arrest was caused by interference

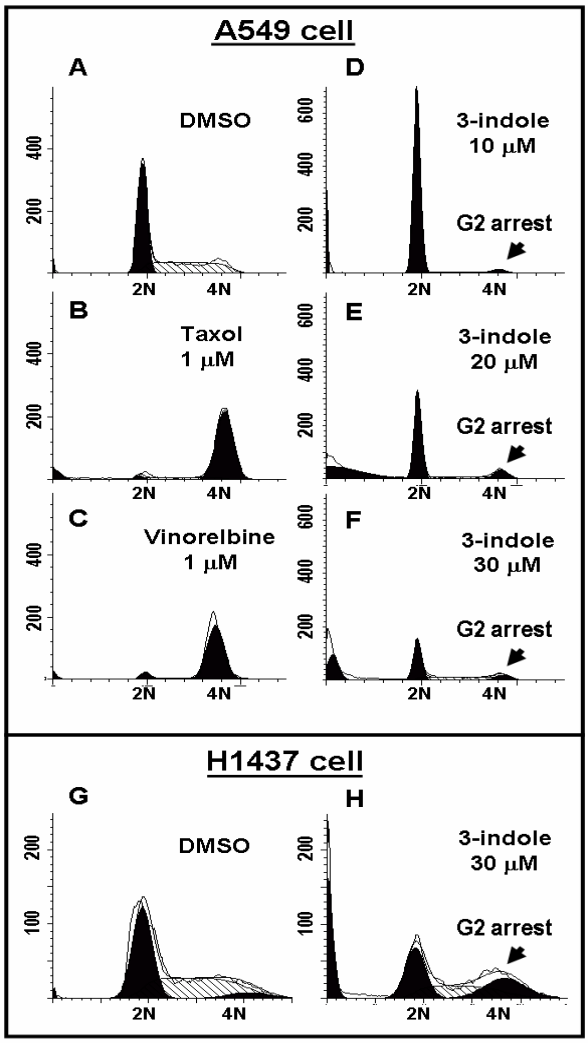

Figure 2. 3-indole induced G2-M cell cycle arrest in A549 and H1437 lung cancer cells. A549 cells were treated with DMSO **(A),** 1 μM Taxol **(B)**, 1 μM Vinorelbine **(C)**, or 3-indole (10-30 μM) for 24 h **(D-F)**, whereas H1437 cells were treated with DMSO or 3-indole (30 μM) for 24 h **(G-H)**. 3-indole blocks cell cycle at G2-M phase (indicated by arrow heads) similar to that of known anti-microtubule agents (Taxol and Vinorelbine). Determination of cell cycle distribution was performed by FACScan flow cytometer.

with tubulin/microtubules dynamic equilibrium, we employed immunocytochemistry to further examine the effect of 3-indole on cellular microtubule networks in A549 cells treated with 1 μM taxol, 1 μM vinorelbine or various concentrations of 3-indole (10, 20, or 30 μM) treatment for 24 h. As shown in Fig. 3, the microtubule network exhibits normal filamentous arrangement and organization in A549 cells in the DMSO-treated control. However, 1 μM of vinorelbine caused cellular

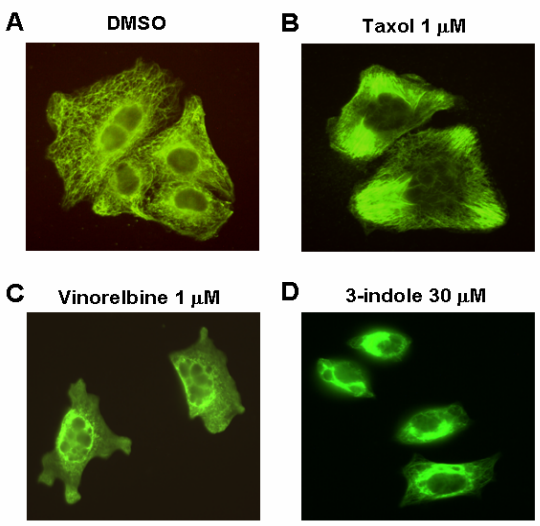

Figure 3. Effect of 3-indole on the cellular microtubule distribution in A549 cells. Cells were treated with DMSO **(A)**, 1 μM Taxol **(B)**, 1 μM Vinorelbine **(C)**, or 30 μM 3-indole **(D)** for 24 h. The short depolymerized microtubules are presented in Vinorelbine- and 3-indole-treated cells, and the long polymerized microtubules are found in Taxol-treated cells.

microtubule depolymerization as most cells had short microtubule fragments scattered throughout the cytoplasm. In contrast, 1 μM of taxol promoted microtubule polymerization with an increase in the density of cellular microtubules and formation of long thick microtubule bundles. Furthermore, 30 μM of 3-indole treatment resulted in findings similar to those of vinorelbine. We observed an almost complete loss of microtubules throughout the cytoplasm after 30 μM of 3-indole treatment. These results indicated that 3-indole may be an antimicrotubule polymerization agent.

Validation of 3-indole-induced microtubule depolymerization by Western blot

We thus used Western blot analysis to confirm the inhibition of microtubule polymerization by 3-indole. The effect of 3-indole on microtubule assembly was compared with those of taxol and vinorelbine. Inhibition of microtubule assembly was observed in A549 cells treated with 1 μM of vinorelbine. In contrast, 1 μM of taxol promoted tubulin polymerization. Similar to the effect of vinorelbine, 3-indole inhibited tubulin polymerization in a concentration-dependent manner (Fig. 4). Together, these results confirmed the antimicrotubule effect of 3-indole through inhibition of microtubule polymerization.

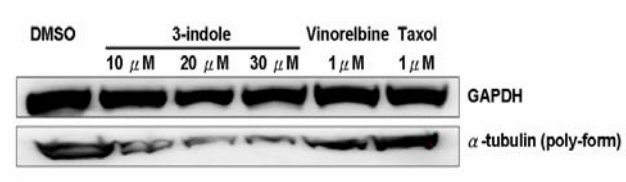

Figure 4. 3-indole dose-dependently inhibits microtubule polymerization. A549 cells were treated with DMSO**,** 3-indole (10-30 μM), Vinorelbine, or Taxol for 24 h. Cell lysates were centrifuged to separate polymerized microtubules as described in "Materials and Methods."

Discussion

In the present study, we show for the first time that a novel synthetic indole structure compound, 3-indole, exhibits anti-cancer growth activities and inhibits tubulin polymerization in cell model. 3-indole causes an accumulation in the G1 phase and partially increases in the G2-M phase in A549 human lung cancer cells. The G2-M arrest of 3-indole was also apparent in H1437 human lung cancer cells. Microtubules are crucial in G2-M phase and cell division (Jordan and Wilson, 2004; Pellegrini and Budman, 2005). The mechanism of action of many antimicrotubule drugs is interference with the normal formation of the mitotic spindle by either increasing microtubule depolymerization or tubulin polymerization leading to cell cycle arrest (Sorger *et al.*, 1997). Our results show that treatment of A549 cells with 3-indole results in disruption of intracellular microtubule network as demonstrated in the immunocytochemistry studies. Furthermore, dose-dependent inhibition of tubulin polymerization by 3-indole in A549 cells is validated by Western blot assays. Together, these results suggest that 3-indole induces G2-M cell cycle arrest may be through the inhibition of microtubule polymerization.

The different sensitivity of tumor and normal cells to antimicrotubule agents could possibly be due to (a) deficient function of G1 checkpoint (Trielli *et al.*, 1996) and (b) deficiency of p53 tumor suppressor genes (Di Leonardo *et al.*, 1997) in tumor cells. *p53* is one of the most commonly mutated genes found in human tumors (Friend, 1994). The function of p53 as a tumor suppressor has been demonstrated by experiments showing that the loss of p53 correlates with the loss of G1-S cell cycle transition regulation after DNA damage (Kastan *et al.*, 1991; Park *et al.*, 2001; Liu *et al.*, 2003). In contrast to synthetic small-molecule compounds with an indole structure, such as vinorelbine, which induce almost complete G2-M arrest, 3-indole causes different extents of G2-M arrest in various human lung cancer cells with different p53 statuses, including A549 (*p53*-wild) and H1437 (*p53*-mut). The multi-effect of an anti-cancer drug on G1 or G2/M cell cycle arrest has also been shown for other compounds (Blajeski *et al.*, 2002). Characterization of 3-indole-induced G2-M arrest in more cells with null or mutant p53 backgrounds with various treatment time of 3-indole is under investigation. In addition, microtubulin binding site of 3-indole will be further verified.

The tumor vasculature is a new target for cancer therapy. Tumor cells die rapidly unless they are supplied with oxygen and nutrients through the blood. Antimicrotubule compounds that bind to the colchicine or *Vinca* domain on microtubules, have undergone extensive development as antivascular agents, such as CA-4-P. CA-4-P induces cell death through rapid depolymerization of microtubules and formation of actin stress fibres with no evidence of apoptosis (Kanthou and Tozer, 2002). The difference between classical antimicrotubule agents and the novel vascular-targeting agents might be that the effects of potential vascular-targeting agents can (a) enter cells rapidly, (b) rapidly reverse its binding to tubulin or microtubules, (c) rapidly depolymerize microtubules, and (d) rapidly be metabolized or excreted (Tozer *et al.*, 2002). Our preliminary data indicated that DNA damage induced by 3-indole can be rapidly reversed in cell model (Lee *et al.*, 2008). Whether 3-indole might act as an antivascular agent is under investigation.

In conclusion, our data indicated that 3-indole, a novel synthetic indole compound, with high purity and yield, increases G2-M cells in A549 and H1437. In addition, 3-indole inhibits tubulin polymerization in A549 cells. Such effects of an anti-cancer drug have also been shown for other indole compounds, such as vinorelbine. Vinorelbine has been shown to affect different targets including tumor vasculature during cancer therapy. Characterization of 3-indole on various targets including vasculature of cancer cell is under investigation.

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新穎的吲哚結構合成化合物 1,1,3-tri(3-indolyl)cyclohexane 抑制肺癌細胞株 microtubule 微管聚合作用探討

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摘　要

目的：肺癌在世界各地無論男性或女性都是發病率、死亡率名列前茅的惡性腫瘤。因此，發現與合成新穎的肺癌治療抗癌藥物是刻不容緩的工作。

材料與方法：本研究團隊發展了一種新穎的吲哚結構合成化合物 1,1,3-tri(3-indolyl)cyclohexane (3-indole)，並藉由 A549 及 H1437 人類肺癌細胞株來探討新穎抗癌藥物對於肺癌細胞的毒殺作用及其機制。

結果：新穎的抗癌藥物 3-indole 可以抑制 A549 和 H1437 肺癌細胞株細胞生長並誘導細胞週期停滯在 G2-M 期。在 IMR90 正常肺細胞內，3-indole 無法抑制其細胞生長，且細胞骨架 microtubule 微管為呈現網狀之完整分佈於整個細胞體內；而經由免疫細胞染色法發現 3-indole 處理 A549 細胞後，其 microtubule 微管網絡可見到幾乎受到破壞且聚集於細胞核周圍，無法順利延伸散佈於整個細胞體內。此外，西方墨點法分析顯示 3-indole 處理可抑制 A549 細胞 microtubule 微管聚合作用並呈現劑量相關性。

結論：3-indole 具有抑制 A549 和 H1437 肺癌細胞株細胞生長及抑制 A549 肺癌細胞株細胞骨架 microtubule 微管聚合作用，顯示具有發展作為新穎的抗微管作用癌症用藥的價值。

關鍵詞：吲哚結構化合物、肺癌、微管聚合作用、抗微管作用

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