ATM/ATR and SMAD3 Pathways Contribute to 3-Indole-induced G_1 Arrest in Cancer Cells and Xenograft Models

SIN-MING HUANG\textsuperscript{1,2}, KWOK-TUNG LU\textsuperscript{1} and YI-CHING WANG\textsuperscript{2}

\textsuperscript{1}Department of Life Science, National Taiwan Normal University, Taipei 11677, Taiwan, R.O.C.; \textsuperscript{2}Department of Pharmacology and Institute of Basic Medical Science, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan, R.O.C.

Abstract. Background: 3-Indole inhibits lung cancer growth by apoptosis. Here, the growth inhibition mechanism besides apoptosis was further characterized. Materials and Methods: The Comet assay was used to examine 3-indole-induced DNA damage. Cell cycle distribution and protein expression were analyzed using flow cytometry, Western blotting and immunohistochemistry in cell and animal models. Results: 3-Indole induced dose-dependent DNA damage, which was reversed by reactive oxygen species (ROS) inhibitor in lung cancer cells. Cell cycle G_1 arrest was observed in the 3-indole-treated cells. DNA damage-responsive proteins involved in the ataxia telangiectasia mutated/ataxia telangiectasia and Rad3-related (ATM/ATR) pathway and G_1 regulation proteins such as p21 and SMA- and MAD-related protein 3 (SMAD3) were induced in the cell models. The altered expression of ATM, ATR, checkpoint kinase 2 (CHK2), and cell division cycle 25 homolog A (CDC25A) were confirmed in xenograft models. Importantly, the 3-indole-induced ATM/ATR and transforming growth factor (TGF)-\(\beta\)/SMAD pathways were attenuated by ROS inhibitor. Conclusion: 3-Indole causes DNA damage and triggers ATM/ATR and SMAD3 signaling pathways to arrest lung cancer cells at the G_1-phase.

Lung cancer is the leading cause of cancer mortality in the world (1). Chemotherapy is one of the major treatments either as strategic treatment for locally advanced disease or as a palliative treatment for metastatic tumors (2, 3). However, small survival benefit and serious side-effects make some chemotherapeutic strategies still unsatisfactory for clinical use. Therefore, the development of novel and more effective anticancer drugs with fewer side-effects for cancer patients is urgently needed.

The induction of apoptosis and cell cycle arrest in cancer cells are the strategies of many anticancer drugs. Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) play important roles in maintaining genome integrity by triggering cell cycle arrest, DNA damage repair and apoptosis (4). ATM and ATR are phosphorylated at specific sites and activated when DNA damage is sensed (5). This activation of ATM and ATR results in the subsequent phosphorylation of downstream proteins such as p53, checkpoint kinase 1 (CHK1) and CHK2, p53 (Ser15) phosphorylated by ATM or ATR inhibits mouse double minute 2 homolog (Mdm2) binding and leads to the accumulation and increased transcriptional activation capacity of p53 (6). CHK1 and CHK2 are phosphorylated by ATM and ATR in response to replication stress or DNA damage (7, 8). The effects on the members of the cell division cycle 25 homolog (CDC25) phosphatase family (CDC25A, CDC25B and CDC25C) are the best understood functions of CHK1 and CHK2 (7). The activation of CHK1 and CHK2 through ATM/ATR-mediated mechanisms caused by DNA damage or replication stress, results in the inactivation of CDC25s, thereby promoting cyclin-dependent kinase (CDK) inhibition and cell cycle arrest (7).

Transforming growth factor (TGF)-\(\beta\)1 plays an important role in many biological processes, including proliferation, differentiation, extracellular matrix production and apoptosis. SMA- and MAD-related protein 3 (SMAD3) is one of the direct mediators of TGF-\(\beta\) signaling. SMAD3 has been shown to mediate transcription of the CDK inhibitors p15 and p21 and contribute to cell cycle arrest (9-11).

In our previous study, we developed an indole compound, named 3-indole, which at high dose induced apoptosis in various lung cancer cell lines via the mitochondria-mediated pathway (12). The human xenograft model in mice showed the antitumor growth ability of 3-indole. Low dose (10 μM) 3-indole induced G_1-phase arrest in treated lung cancer cells.
(12). In this study, the cancer growth inhibition mechanism of 3-indole, besides apoptosis, was further characterized.

Materials and Methods

3-Indole. The compound 3-indole was synthesized by Dr. Ching-Fa Yao at the Department of Chemistry, National Taiwan Normal University, Taipei, Taiwan. 3-Indole was obtained as a solid powder and dissolved in 100% dimethyl sulfoxide (DMSO) prior to use.

Cell culture. Human non-small cell lung cancer (NSCLC) cell lines A549 and H1437 were purchased from the American Tissue Culture Company (Rockville, MD, USA). The cells were maintained in Dulbecco’s modified Eagle medium (DMEM, pH7.4; Gibco, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). The cells were treated with solvent control (DMSO) or various concentrations of compounds for 1 h then collected and dissolved in 100% dimethyl sulfoxide (DMSO) prior to use.

Cell cycle distribution analysis. The assay was performed according to Kuo et al. (13). A549 cells were pretreated with nocodazole (200 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h to arrest cells at the G2/M-phase and released by changing to nocodazole (200 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA) for cell cycle distribution analysis.

Cell cycle distribution analysis. The assay was performed according to Li et al. (14). The NE-Comet assay is a modified sensitive comet assay with a NE digestion step. The DNA lesion sites are generated as strand breaks by the DNA repair proteins in the nuclear extract and are detected by the assay. A549 cells (8x10⁶ per ml) seeded in 6 cm² plates were treated with solvent control (DMSO) or various concentrations of compounds for 1 h then collected and resuspended with PBS (2.5x10⁵ cells/μl) on ice. The first layer gel was prepared by adding 70 μl of 1.2% normal melting-point agarose onto a microscope slide followed by a mixture of 60 μl of 1.2% low-melting agarose as the third layer. The slide was immersed at 37°C in a humidified incubator containing 5% CO₂ in air.

Cell cycle distribution analysis. The assay was performed according to the method described by Montecucco et al. (15). Nicked pGL4-luc plasmid was prepared by digesting with restriction enzyme HindIII, and then equilibrated at 15°C for 10 min with reaction mixture and various concentrations of the positive control doxorubicin or 3-indole. T4 ligase (2 μl) was added and the mixture was incubated at 15°C for 1 h. The 3-indole and doxorubicin were then removed by phenol/phenol-chloroform extraction and the plasmids were resuspended in TE Buffer containing 1% SDS. Electrophoresis was performed at 40 V for 6 h.

Western blot. After treating with compounds or solvent control (DMSO) for the indicated time, the cell lysates were collected and immunoblotted for various proteins with the following primary antibodies: p53, p21, CDK4 (1/1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), retinoblastoma Rb, p53 phosphoserine 15, CDC25A, CHK2 phosphothreonine 68 (1/1000; Abcam, Cambridge, UK), ATM (1/1000; Novus, Littleton, CO, USA), ATM phosphoserine 1981, ATR phosphoserine 428 (1/1000; Cell Signaling, Danvers, MA, USA), CHK1, CHK2, CHK1 phosphoserine 345 (1/1000; Cell Signaling), SMAD3 phospho (S423/425) (1/1000; Epitomics, Burlingame, CA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1/2000; Santa Cruz Biotechnology).

Immunohistochemistry (IHC). Athymic nu/nu female mice (BALB/c), 4-5 weeks of age, were obtained from the National Laboratory Animal Center (Taiwan) and raised in a pathogen-free environment. The nude mice were implanted subcutaneously with 5x10⁵ A549 cells and the tumors were allowed to reach an average size of 100 mm³ before 3-indole treatment. The animals bearing the A549 xenograft were treated with 25 mg/kg/mouse 3-indole and sacrificed 8 h later for tumor tissue resection and embedding in paraffin. Tissue slides were processed following standard procedures. Aside from the aforementioned antibodies used for Western blot, ATM phosphoserine 1981 (1/300; Epitomics) was used.

Statistical analysis. Statistical evaluation for the data was done using Student’s t-test for comparison between groups and treatments. P<0.05 was considered statistically significant.

Results

Effect of 3-indole on DNA. Since DNA damage triggers G1 arrest (17), the possibility that low dose 3-indole could induce DNA damage was investigated. The A549 lung cancer cells treated with 10 μM or 30 μM 3-indole for 1 h were assessed by NE comet assay. The data indicated that 3-indole
caused DNA damage in a dose-dependent manner. Furthermore, the DNA damage caused by 3-indole was significantly reduced by co-treatment with rotenone, suggesting that ROS play an important role in 3-indole-induced DNA damage (Figure 1A, B).

To determine whether 3-indole intercalates into and/or binds to DNA to induce DNA breaks, the DNA unwinding assay/plasmid DNA circle-ligation assay were performed. Doxorubicin, a strong DNA intercalator, was used as a positive control. In contrast to doxorubicin, which produced a concentration-dependent DNA mobility shift, there was no obvious DNA mobility shift when DNA was incubated with up to 50 μM of 3-indole (Figure 1C). Furthermore, Hoechst 33342 dye displacement showed that 3-indole was unable to quench the intensity of the fluorescence of DNA-bound Hoechst 33342, which showed that 3-indole had no competitive effect on DNA minor groove binding, suggesting that 3-indole cannot intercalate into DNA (Figure 1D).

Effect of low dose 3-indole on the cell cycle. To determine whether 10 μM 3-indole could induce cell cycle arrest, flow cytometry assay was performed. The results indicated that 10 μM 3-indole induced G1 arrest (Figure 2A). Rotenone pretreatment (30 min) partially attenuated the G1 arrest induced by 3-indole as confirmed by quantitative analysis (Figure 2B) showing a decrease of G1 phase cells and an increase of G2/M phase cells in the 3-indole and rotenone co-treatment group compared to the 3-indole treatment alone. This result suggested that ROS contributed partly to 3-indole-induced G1 arrest.

Western blot analysis of ATM/ATR pathway. To further characterize the expression statuses and activities of DNA damage response proteins and the regulators of the G1-phase arrest pathway induced by 3-indole, proteins in the ATM/ATR pathways were analyzed by Western blot. The results showed that ATM and ATR were activated (by phosphorylation) within 0.5-1 h and their downstream proteins CHK1, CHK2 and p53 were also activated in 1-4 h in both A549 and H1437 cancer cell lines upon 10 μM 3-indole treatment (Figure 3, left panels). CDC25A, a substrate of CHK1 and CHK2, decreased from 8 h post-treatment with 3-indole (Figure 4A). The effect of 3-indole (10 μM) on the status of the G1-phase-related CDK inhibitor p21 was to increase the expression level from

Figure 1. Effect of 3-indole on DNA. A, B: NE Comet assay. ROSI, ROS inhibitor rotenone co-treatment. C: DNA unwinding assay/plasmid DNA circle-ligation assay. T4 ligase, negative control; doxorubicin, positive control. D: Hoechst 33342 dye displacement assay; doxorubicin, positive control.
8 h post-treatment along with a decreased expression of the G1-phase-related CDK4 (Figure 4A). The expression of Rb was also increased (Figure 4A). To verify whether ROS inhibitor could repress the signaling involved in the 3-indole-induced G1 arrest, the cancer cells were pretreated with rotenone for 30 min followed by 3-indole treatment. The results indicated that the phosphorylation levels of ATM and ATR were reduced along with a decrease or delay of phosphorylation of p53, CHK1 and CHK2 proteins (Figure 3, right panels).

**Effect of 3-indole on the SMAD pathway.** The data showed that SMAD3 protein was activated as manifested by phosphorylation from 1 h post-treatment with 10 μM 3-indole. In addition, expression of its downstream protein p15 increased after 3-indole treatment (Figure 4B).

**Effect of 3-indole on signaling pathways in animal tumor tissue.** To investigate whether 3-indole-induced ATM/ATR signaling pathway also occurred in vivo, IHC was performed to determine protein expression in the mouse tumor tissues. The phosphorylated ATM, ATR and CHK2 proteins were induced along with a decrease of CDC25A protein level in the xenograft tumors from animals treated with 3-indole (Figure 5). This result indicates that 3-indole indeed induced the ATM/ATR signaling pathway in vivo.

**Discussion**

3-Indole induced ROS production and caused DNA damage leading to G1-phase arrest. The 3-indole-induced DNA damage activated the ATM/ATR pathway and subsequently altered the expression of downstream proteins including the up-regulation of p21 and Rb and the down-regulation of CDC25A and CDK4. SMAD3 and downstream protein p15 were also activated in the 3-indole treated cells. Moreover, the changes in protein level of ATM, ATR, CHK2 and CDC25A were confirmed in the tumor xenografts, indicating that the ATM/ATR and SMAD3 pathways contributed to the 3-indole-induced G1 arrest in both models.

The induction of intracellular ROS to achieve cancer cell apoptosis is a new anticancer strategy and it has been found to be the mechanism of several anticancer compounds such as amitriptyline, edelfosine and motexafin gadolinium (18-21). Many types of cancer cells have higher ROS levels (20, 22) and less antioxidative enzyme activity (23-25) compared to their normal counterparts. Therefore, the reduced ability to protect against ROS stress in cancer cells provides a way to kill cancer cells specifically. The potential anticancer compound 3-indole induced ROS, thus it could induce cancer-specific death.

Drug resistance due to the genome instability of cancer cells leading to escape from a single type of inhibition (26) is a major cause of failure of many anticancer chemotherapeutic drugs. Combination therapy or multi-target drugs may resolve the drug resistance problem. In the present study, 3-indole activated multiple pathways such as ATM/ATR and SMAD3 signaling to induce cell cycle arrest. This activation of ATM and ATR resulted in the subsequent activation of downstream proteins such as p53, CHK1 and CHK2 and degradation of CDC25A. The activation of SMAD3 leads to an increase of CDK inhibitors p15 and p21 along with a decrease of CDC25A, which contribute to cell cycle G1 arrest (9-11, 27). The Rb protein, a negative regulator of the cell cycle, acts by
repressing the transcription activity of E2 promoter binding factor and results in G1-phase arrest (28). The total Rb level was increased presumably due to a decrease of phosphorylation as a result of an inhibition of CDK4 (29), thus confirming that 3-indole induced G1 arrest. In addition, we previously showed that 3-indole induces apoptosis via the intrinsic mitochondria pathway (12). Our data suggest that the multi-target drug, 3-indole, is a promising candidate to be tested as a lead pharmaceutical compound for cancer treatment.

References


Received October 23, 2010
Revised December 13, 2010
Accepted December 14, 2010