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Research Paper

Deguelin, an Aurora B Kinase Inhibitor, Exhibits Potent Anti-Tumor Effect in Human Esophageal Squamous Cell Carcinoma

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ABSTRACT

Aurora B kinase has emerged as a key regulator of mitosis and deregulation of Aurora B activity is closely related to the development and progression of human cancers. In the present study, we found that Aurora B is overexpressed in human esophageal squamous cell carcinoma (ESCC), high levels of Aurora B protein were associated with a worse overall survival rate in ESCC patients. Depleting of Aurora B blunted the malignant phenotypes in ESCC cells. Importantly, we demonstrated that a natural compound, deguelin, has a profound anti-tumor effect on ESCC *via* inhibiting Aurora B activity. Deguelin potently inhibited *in vitro* Aurora B kinase activity. The *in silico* docking study further indicated that deguelin was docked into the ATP-binding pocket of Aurora B. Inhibition of Aurora B activity attenuated growth of ESCC cells, resulted in G2/M cell cycle arrest, polyploidy cells formation, and apoptosis induction. Knocking down of Aurora B decreased the sensitivity of ESCC cells to deguelin. The *in vivo* results showed that deguelin blocked the phosphorylation of histone H3 and inhibited the growth of ESCC tumor xenografts. Overall, we identified deguelin as an effective Aurora B inhibitor, which deserves further studies in other animal models and ESCC treatment.
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1. Introduction

Human esophageal carcinoma is one of the most frequently diagnosed cancers, ranked as the eighth leading causes of cancer-related mortality worldwide. Esophagus squamous cell carcinoma (ESCC) is the most common histological type of esophageal carcinomas, especially with a higher incidence in developing nations (Abnet et al., 2017; Rustgi and El-Serag, 2014). Epidemiological studies have demonstrated that alcohol consumption and tobacco use are closely linked to increased ESCC risk. ESCC is notoriously aggressive in nature, spreading by a variety of pathways including direct extension, lymphatic spread and hematogenous metastasis (Abnet et al., 2017; Lagergren and Lagergren, 2013; Liang et al., 2017). Despite advances in early detection and standard treatment, ESCC is often diagnosed at an advanced stage and has a poor prognosis. The overall 5-year survival rate for late stage ESCC is <15%, which has hardly improved during the past few decades (Liang et al., 2017). The underlying reasons for this disappointingly low survival rate remain to be greatly elucidated. Therefore, a better understanding of the molecular mechanisms of ESCC pathogenesis or identifies a novel chemical entity with activity against ESCC is expected to facilitate the development of novel therapies that can complement current traditionally therapeutic methods.

The Aurora kinases, a closely related subgroup of 3 serine/threonine kinases, including Aurora A, B, and C, are believed to play a key role in protein phosphorylation in mitosis and have been shown to contribute to the development and progression of cancer (Nguyen and Schindler, 2017; Tang et al., 2017). Aurora B is localized to the centromeres from the prophase to the metaphase-anaphase transition. Thereafter, it is localized to midzone spindle microtubules during the telophase and subsequently to midbody during cytokinesis. Small-molecule inhibitors and siRNA of Aurora B abrogate the mitotic-spindle checkpoint and cause premature mitotic exit without the completion of cytokinesis, leading to 4 N DNA-containing cells that continue to progress through the cell cycle (Carmena et al., 2009; Krenn and Musacchio, 2015; Liu et al.,

Abbreviations: ESCC, esophageal squamous cell carcinoma;; RNase A, ribonuclease A; OS, overall survival; shRNA, short nairpin RNA; DMSO, dimethyl sulphoxide.

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2009). In malignancy, alterations of Aurora kinase have been linked with genetic instability, including mitotic errors, chromosomal aneuploidy, deregulation of cell proliferation and apoptosis, which are highly associated with tumorigenesis (Mahadevan et al., 2017; Portella et al., 2011; Schecher et al., 2017). Aurora B is overexpressed in many tumor types and has been linked to poor patient prognosis in cancers (Lens et al., 2010; Otto and Sicinski, 2017). Recently, several Aurora B inhibitors have been developed, including AZD1152, BI 811283, ZM447439, and VX-680, etc., the clinical trial data demonstrated that inhibition of Aurora B kinase displayed a generally manageable safety profile and disease stabilization in some patients (Bavetsias and Linardopoulos, 2015; Falchook et al., 2015; Tang et al., 2017; Yan et al., 2016; Ziemska and Solecka, 2016).

In the present study, we identified a natural compound, deguelin, which was isolated from the Legume family, Lonchocarpus, Derris, or Tephrosia, as an effective Aurora B inhibitor for using in ESCC therapy. We investigated the therapeutic effect of deguelin in ESCC both *in vitro* and *in vivo*. Our results could provide an option for clinical targeting anti-mitotic therapies of ESCC.

2. Materials and Methods

2.1. Cell Lines and Culture

Human esophageal squamous carcinoma cell line Eca109, KYSE180, and KYSE450 were purchased from Cell Bank of Chinese Academy of Sciences, Shanghai, China. Human esophageal carcinoma cell lines KYSE150 was kindly provided by Dr. Qian Tao from The Chinese University of Hong Kong, Hong Kong, China. Human normal esophageal epithelial cell Het-1A was purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin. All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Chemicals and Cell Treatments

Tris, NaCl, and SDS for molecular biology and buffer preparation were obtained from Sigma-Aldrich (St. Louis, MO). Deguelin (>98%) and Hesperadin (>95%) were purchased from Sigma-Aldrich. Cell culture media and supplements were from Invitrogen (Grand Island, NY). Subconfluent cells were treated with the compound at indicated concentrations for an indicated time. Detailed treatment procedures were described in figure legends. The final concentration of DMSO in the culture media was kept <0.1% which had no significant effect on the cell growth. Vehicle controls were prepared for all treatments.

2.3. MTS Assay

ESCC cells were seeded (2.5×10^3/well/100 mL) into 96-well plates, and proliferation was assessed by MTS assay (Promega, Madison, WI) according to the instructions provided.

2.4. Anchorage-Independent Cell Growth Assay

The anchorage-independent growth assay was conducted as described previously (Liu et al., 2016). Briefly, a total of 8000 cells were suspended between a layer of solidified basal medium Eagle/10% FBS/0.5% bottom agar with different concentrations of compound or vehicle and 1 mL basal medium Eagle/10% FBS/0.3% top agar with the same concentration of compound or vehicle in each well of six-well plates. After maintenance at 37 °C, 5% CO_2 for 2 weeks, colonies were scored under a microscope using the Image-Pro Plus software (version 6.2) program (Media Cybernetics. Rockville, MD).

2.5. Immunofluorescence Staining

The immunofluorescence staining was conducted as described previously (Li et al., 2013). Briefly, after treated with deguelin for 24 h, an asynchronous population of cancer cells were washed with PBS, and fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton X-100 for 30 min. Fixed cells were incubated with a α-tubulin rabbit antibody (#2144, Cell Signaling Technology, Danvers, MA) overnight at 4 °C followed by incubation with green fluorescent Alexa Fluor 488 dye-labeled anti-mouse IgG (Invitrogen, Grand Island, NY). Nuclei were stained with DAPI. Samples were viewed with a Nikon inverted fluorescence microscope (Melville, NY).

2.6. Flow Cytometry

The flow cytometry was performed as described previously (Yu et al., 2015). Briefly, ESCC cells were seeded into 6-well plates in RPMI 1640 containing 10% FBS. After culturing for 24 h, different concentrations of deguelin were added to each well and left on the cells for 24 h. After treatment, attached and floating cells were harvested. For apoptosis analysis, the cells were suspended in 1×10^6 cells/mL, and 5 μL Annexin V and Propidium Iodide staining solution were added to 300 μL of the cell suspension. After incubated 10–15 min at room temperature in the dark, stained cells were assayed and quantified using a FACSort Flow Cytometer (BD, San Jose, CA, USA). For cell cycle analysis, cells were harvested and washed with PBS for two times and then fixed in 70% ethanol overnight at 4 °C. Cells were counterstained in the dark with 50 μg/mL propidium iodide and 0.1% ribonuclease A (RNase A) in 400 μL of PBS at room temperature for 30 min. Stained cells were assayed and quantified using a FACSort Flow Cytometer (BD, San Jose, CA, USA).

2.7. Protein Preparation and Western Blotting

Cultured cells were harvested and whole cell lysates were prepared according to the method previously described (Yu et al., 2017a). Protein concentration was determined using the BCA Assay Reagent (Cat. no. 23228, Pierce, Rockford, IL). Western blotting was performed as previously described (Li et al., 2013). The following antibodies were used for immunodetection with appropriate dilutions: Aurora B (#3094, 1:1000), Phospho-Aurora B (Thr232) (#2914, 1:1000), Histone H3 (#4499,1:2000) and Phospho-Histone H3 (Ser10) (#3377,1:2000) antibodies were purchased from Cell Signaling Technology (Beverly, MA); β-actin (A5316, 1:10,000) was purchased from Sigma (St. Louis, MO); Ki67 (ab16667,1:200) was purchased from Abcam (Cambridge, UK).

2.8. In Vitro Aurora Kinase Assay

The *in vitro* aurora kinase assay was performed as described previously (Sheng et al., 2014). Histone H3 and active Aurora kinase B were purchased from Merck Millipore (Billerica, MA). Histone H3 (1 μg) and active Aurora kinase (100 ng) were mixed with different doses of deguelin or hesperadin in a 20 μL reaction, which was conducted in 100 μM ATP and 1× kinase buffer (Cell Signaling Technology) at 30 °C for 30 min. Reactions were stopped by boiling samples in 5 × SDS loading buffer, and proteins were analyzed by Western blot.

2.9. Lentiviral Infection

Four lentivirus plasmids targeting *Aurora B* (TRCN0000000776, TRCN0000000777, TRCN0000000778, TRCN0000000779) were purchased from Thermo Scientific. *pLKO.1-sh-GFP* (Addgene plasmid #30323), the lentiviral packaging plasmid *psPAX2* (Addgene plasmid #12260) and the envelope plasmid *pMD2.G* (Addgene plasmid #12259) were available on Addgene (Cambridge, MA). The generation of gene stable knocking down cell lines was performed as described previously (Yu et al., 2017b). Briefly, to generate Aurora B knocking down

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