The anthelmintic agent oxfendazole inhibits cell growth in non-small cell lung cancer by suppressing c-Src activation

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Abstract. The c-Src protein family of tyrosine kinases are important in the tumorigenesis of many types of tumors, and may be a potential target for antitumor drug discovery. In the present study, immunoblotting was performed to analyze protein expression, CCK-8 assay was carried out to assess cell viability and cell cycle was analyzed using a flow cytometer. The anthelmintic agent oxfendazole was observed to be a novel c-Src inhibitor that blocked the activation of c-Src. Oxfendazole also suppressed the cell growth of non-small cell lung cancer (NSCLC) cells, and overexpression of c-Src decreased the cytotoxicity of oxfendazole against NSCLC cells. In addition, oxfendazole induced cell cycle arrest at the G_0/G_1 phase, and downregulated the protein levels of Cyclin-dependent kinase (CDK)-4, CDK6, retinoblastoma protein and E2 transcription factor 1, and upregulated the expression levels of p53 and p21 in NSCLC cells. Furthermore, oxfendazole enhanced the cytotoxicity of cisplatin against NSCLC cells. These results demonstrated that oxfendazole exerted its antitumor activity by suppressing c-Src signaling, and it was also indicated that the anthelmintic agent oxfendazole may be effective for anti-NSCLC therapy in the clinic as a single agent or in combination with other antitumor drugs.

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Introduction

Non-small cell lung cancer (NSCLC) is one of the most common malignancies, accounting for 85% of all lung cancer cases, and its treatment continues to be severe challenge in the clinic (1). Although significant progress has been made in regard to drug development, there are still few drugs that have long-term benefits in the treatment of NSCLC (2-4). Therefore, there is an urgent demand to identify novel targets and drugs to improve systemic therapy for patients with NSCLC.

The c-Src proto-oncogene belongs to the Src family of protein tyrosine kinases, which include c-Src, Fyn, Lyn, Lck, Yes, Blk and Hck (5). c-Src regulates signals from multiple cell surface molecules, including growth factors, G protein-coupled receptors and integrins (6). It has been reported previously that levels of c-Src protein were elevated or kinase activity was overactivated in many types of tumors, including NSCLC (7,8). In tumor cells, the activation of c-Src mediated cell growth, cell proliferation, cell survival and cell invasion (9). Previous studies have reported that c-Src was activated in NSCLC cells and primary tumor tissues, and its inhibition led to decreased cell growth and cell cycle arrest (10,11).

In the present study, oxfendazole potently suppressed the activation of c-Src in NSCLC cells, and inhibited NSCLC cell survival. In addition, overexpression of c-Src decreased the effects of oxfendazole on NSCLC cells. Further studies revealed that oxfendazole induced cell cycle arrest at the G_0/G_1 phase, and downregulated Cyclin-dependent kinase (CDK) signaling in NSCLC cells, including CDK4, CDK6, retinoblastoma protein (p-Rb) and E2 transcription factor 1 (E2F1) inhibition, and the upregulation of p53 and p21. In addition, oxfendazole enhanced the cytotoxicity of cisplatin against NSCLC cells by reducing c-Src activation.

Materials and methods

Cells, culture and chemicals. The NSCLC cell lines A549, H460, H1299, H1650 and H1975 were purchased from American Type Culture Collection (Manassas, VA, USA). All NSCLC cell lines were maintained in RPMI-1640 medium with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml of streptomycin and 100 μg/ml of penicillin. Oxfendazole was purchased from

Selleck Chemicals (Houston, TX, USA). Cisplatin was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Epidermal growth factor (EGF) stimulation. A549 and H1299 cells ($1x10^6$ cells/well) were seeded in 6-well plates overnight. Then, the cells were starved overnight in serum-free medium. Subsequently, starved A549 and H1299 cells were incubated with 0, 10 or 20 μM of OFD for 6 h at 37˚C, then stimulated with 10 ng/ml EGF (P5552; Beyotime Institute of Biotechnology, Haimen, China) for 20 min at 37˚C.

Immunoblotting. Immunoblotting was conducted as previously described (12,13). Briefly, cells ($1x10^6$ cells/well) were lysed by RIPA lysis (P0013B; Beyotime Institute of Biotechnology), and whole cell lysates were extracted. Then, proteins were determined by BCA method (BCA kit, P0011; Beyotime Institute of Biotechnology) and 30 μg of total proteins were subjected to SDS-PAGE separation by using 10% or 12% acrylamide gel, and the proteins were transferred onto 0.2 μm polyvinylidene difluoride (PVDF) membrane (1620177; Bio-Rad Laboratories, Inc., Hercules, CA, USA), followed by immunoblotting with specific antibodies. The primary antibodies phosphorylated (p)-c-Src (Tyr416) (6943; 1:1,000), c-Src (2109; 1:1,000), CDK4 (12790; 1:1,000), CDK6 (13331; 1:1,000), p-Rb (Ser807/811) (8516; 1:1,000), E2F1 (3742; 1:1,000), p53 (2527; 1:1,000) and p21 (2947; 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-GAPDH antibody (AM1020A; 1:5.000) was purchased from Abgent Biotech Co., Ltd. (Suzhou, China). Anti-mouse (A0216; 1:1,000) and anti-rabbit (A0208; 1:1,000) immunoglobulin G (IgG) horseradish peroxidase-conjugated antibodies were purchased from Beyotime Institute of Biotechnology (Haimen, Nantong, China). All immunoblotting signals were further assessed by using ECL (BeyoECL Plus kit, P0018M; Beyotime Institute of Biotechnology) and analyzed with Quality One software (version number: 4.0.1; Bio-Rad Laboratories, Inc.).

Cell growth and viability. Viable cells (8,000 cells/well) were analyzed by Cell Counting Kit-8 (CCK-8; BioTools, Inc., Jupiter, FL, USA) assay according to the manufacturer's instruction, as described previously (14). To assess the effect of OFD on the survival rates of different NSCLC cell lines, 5 NSCLC cell lines were treated with 0, 5, 10 or 20 μM of OFD for 24 h at 37˚C, or cells were treated by 5 μM OFD for 0, 24, 48 or 72 h at 37˚C, followed by a CCK-8 assay. For the transfected cells, A549 or H1299 cells were treated with 0, 2.5, 5 or 10 μM of OFD for 24 h at 37˚C. In addition, A549 and H1299 cells were treated with 5 μM cisplatin for 24 h at 37˚C in the presence or absence of 10 μM OFD, and then the cells were assessed by a CCK-8 assay.

Plasmids construction and gene transfection. The human c-Src gene was generated and cloned into the pcDNA3.1 vector as previously described (15,16). The primers for c-Src amplification were as follows: Forward, 5'-ATGGGTAGC AACAAGAGCAAGC-3' and reverse, 5'-CTAGAGGTTCTC CCCGGGCTGGTA-3'. A549 or H1299 cells were transfected with 1 μg/μl empty vector (pcDNA3.1 vector) or 1 μg/μl c-Src plasmids for 24 h by Lipofectamine® 2000™ (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Cell cycle analysis. Cell cycle analysis was performed as previously described (17). A549 and H1299 cells ($1x10^6$ cells/well) were treated with 0, 5 or 10 μM of oxfendazole for 24 h at 37˚C prior to cell cycle analysis. Then, cells were fixed with 70% cold ethanol overnight at -20˚C and washed with cold PBS, followed by being resuspended in 100 μl PBS containing 100 μg/ml RnaseA (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China) for 30 min at 37˚C. Cells were then washed with cold PBS and incubated with propidium iodide (PI) for 5 min at room temperature by using the Cell Cycle Detection kit (C1052; Beyotime Institute of Biotechnology). Cell cycle distribution was analyzed on a flow cytometer (Attune® NxT; Thermo Fisher Scientific, Inc.) and the data was analyzed by FlowJo 7.6.1 (FlowJo LLC, Ashland, OR, USA).

Statistical analysis. Statistical analysis was performed using Statistical Package for Social Sciences 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). The results were presented as the mean ± standard deviation. One-way analysis of variance with Bonferroni post hoc tests were used to determine significance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Oxfendazole inhibits c-Src activation in NSCLC cells. To evaluate the effects of oxfendazole on c-Src activation in NSCLC cells, a panel of NSCLC cell lines were treated with oxfendazole for 24 h, followed by immunoblotting against p-c-Src (Tyr416) and c-Src. As shown in Fig. 1A and B, c-Src was activated in all five NSCLC cell lines, and oxfendazole markedly inhibited the phosphorylation of c-Src. In addition, oxfendazole downregulated the phosphorylation of c-Src in a concentration-dependent manner in A549 and H1299 cells (Fig. 1C). Next, A549 and H1299 cells were starved overnight in serum-free medium followed by oxfendazole treatment and epidermal growth factor (EGF) stimulation. As shown in Fig. 1D, EGF, a key trigger of c-Src signaling, markedly activated c-Src, but this action was suppressed by oxfendazole in a concentration-dependent manner. These results suggested that oxfendazole inhibited the activation of c-Src in NSCLC cells, and that it may be a novel c-Src inhibitor.

Oxfendazole inhibits cell survival in NSCLC cells. It was previously reported that the activation of c-Src promoted the cell survival of tumor cells (9). In the present study, to evaluate the effects of oxfendazole on NSCLC cell survival, a panel of NSCLC cell lines were treated with increased concentrations of oxfendazole for 24 h, followed by a CCK-8 assay. As shown in Fig. 2A, oxfendazole decreased NSCLC cell viability in a concentration-dependent manner. In addition, oxfendazole also inhibited the cell survival of A549 and H1299 cells in a time-dependent manner (Fig. 2B). Furthermore, when A549 and H1299 cells were transfected with c-Src plasmids, oxfendazole-induced cell death was significantly attenuated (Fig. 2C and D). For example, in A549 cells treated with 10 μM of oxfendazole, the fraction of surviving cells was

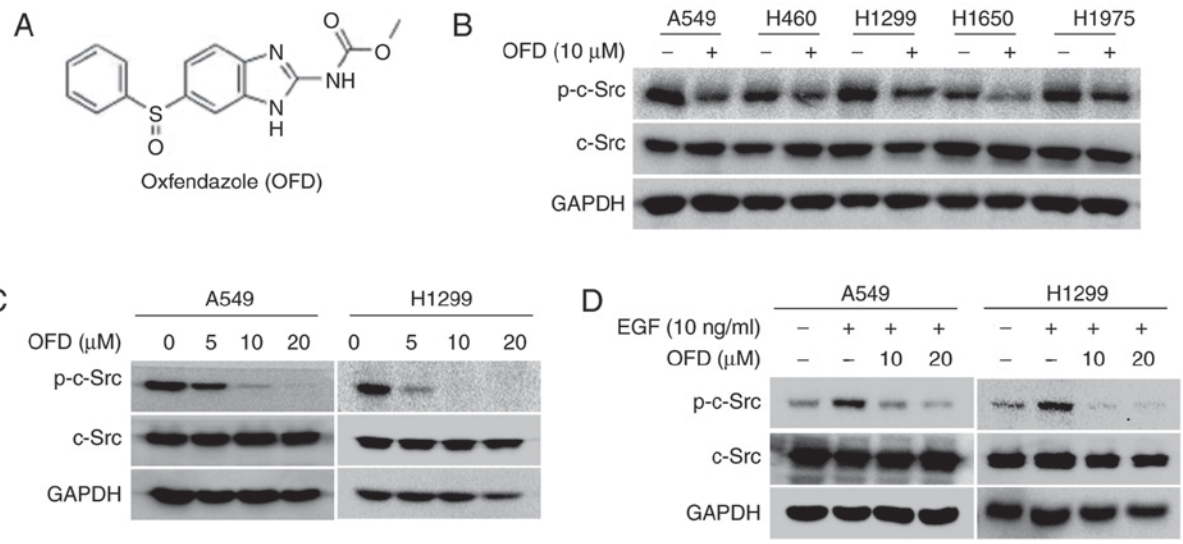

Figure 1. Oxfendazole inhibits c-Src activation in NSCLC cells. (A) The chemical structure of OFD. (B) Five NSCLC cell lines were treated with 10 μM OFD for 24 h at 37˚C, and then cells were prepared for immunoblotting against p-c-Src and c-Src. GAPDH was used as an internal control. (C) A549 and H1299 cells were treated with increased concentrations of OFD for 24 h at 37˚C, followed by immunoblotting against p-c-Src, c-Src and GAPDH. (D) Following starvation overnight in serum-free medium, A549 and H1299 cells were incubated with increased concentrations of OFD for 6 h, then stimulated with EGF (10 ng/ml) for 20 min at 37˚C. The cells were then prepared for immunoblotting against p-c-Src, c-Src and GAPDH. NSCLC, non-small cell lung cancer; OFD, oxfendazole; p-, phosphorylated; EGF, epidermal growth factor.

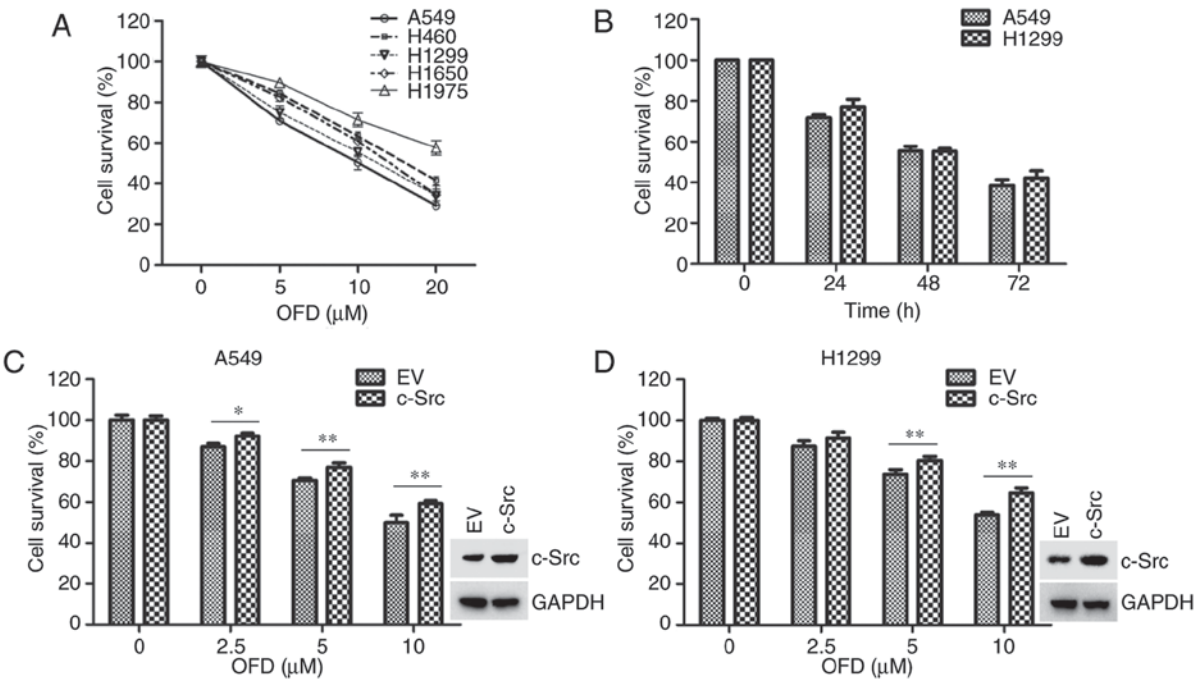

Figure 2. OFD inhibits cell survival in NSCLC cells. (A) The NSCLC cell lines A549, H460, H1299, H1650 and H1975 were treated with the indicated concentrations of OFD for 24 h at 37˚C, followed by a CCK-8 assay. (B) A549 and H1299 cells were treated by 5 μM OFD for the indicated times at 37˚C, and then cells were prepared for the CCK-8 assay. (C) A549 and (D) H1299 cells were transfected with 1 μg/μl EV (pcDNA3.1 vector) or 1 μg/μl c-Src plasmids for 24 h, and then cells were treated with increasing concentrations of OFD for 24 h at 37˚C, followed by a CCK-8 assay and immunoblotting. *P<0.05 and **P<0.01, as indicated. EV, empty vector; NSCLC, non-small cell lung cancer; OFD, oxfendazole; CCK, Cell Counting Kit.

increased from 50% in vector-transfected cells to 60% in c-Src-transfected cells (Fig. 2C). In H1299 cells treated with 10 μM of oxfendazole, the fraction of surviving cells was increased from 55% in vector-transfected cells to 65% in c-Src-transfected cells (Fig. 2D). These results indicated that c-Src signaling may be involved in the underlying mechanism of the action of oxfendazole.

Oxfendazole inhibits cell cycle progression and suppresses CDK signaling in NSCLC cells. It has been previously reported that c-Src activation regulated the cell growth and cell cycle of tumor cells (18,19). Therefore, the present study evaluated whether oxfendazole regulated the cell cycle of NSCLC cells. As shown in Fig. 3, flow cytometry revealed that oxfendazole induced cell cycle arrest at the G_0/G_1 phase in

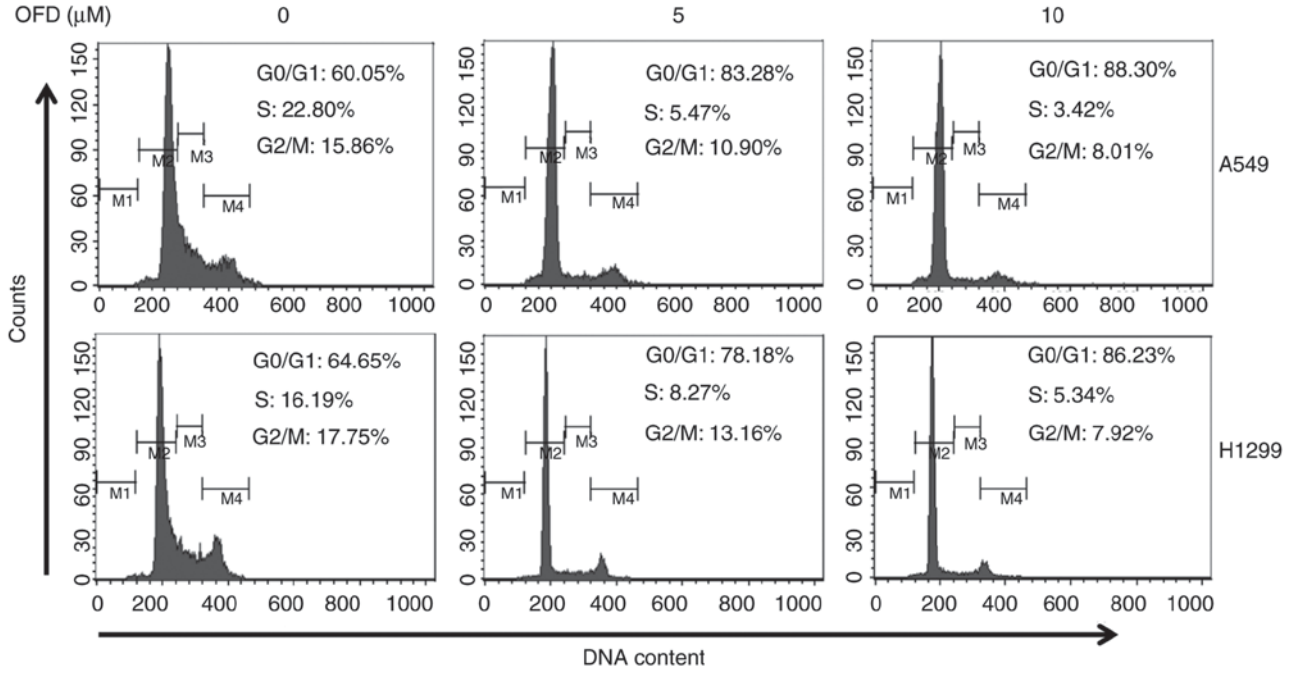

Figure 3. OFD inhibits cell cycle progression in non-small cell lung cancer cells. A549 and H1299 cells were treated with the indicated concentrations of OFD for 24 h at 37˚C, and then cells were collected for DNase-free RNase treatment, followed by propidium iodide staining and analyzed on a flow cytometer. OFD, oxfendazole.

A549 and H1299 cells. In A549 cells, the fraction of the G_0/G_1 cells was increased from 60.05% in the control to 88.30% in cells treated with 10 μM oxfendazole, coupled with a decrease in the fraction of S phase cells from 22.80 to 3.42% (Fig. 3; upper panel). In H1299 cells, the percentage of G_0/G_1 cells was increased from 64.65 to 86.23% following treatment with 10 μM oxfendazole, coupled with a decrease in the fraction of S phase cells from 16.91 to 5.34% (Fig. 3; lower panel).

D-cyclins combine with CDK4/6 to phosphorylate p-Rb, allowing for the release of E2F transcription factors, which activate G1/S-phase gene expression (20). Thus, the present study next evaluated the effects of oxfendazole on the expression of these proteins. As shown in Fig. 4A and B, oxfendazole downregulated the expression levels of CDK4, CDK6, p-Rb and E2F1 in a concentration-dependent manner.

The p53 tumor suppressor protein serves a major role in arresting cell cycle progression (21). To determine whether p53 was associated with oxfendazole cytotoxicity in cell survival, the present study then examined the expression of p53. As shown in Fig. 4C, oxfendazole upregulated p53 expression. The expression of p21 expression was also detected, which is a potent CDK inhibitor and a target of p53 (22). As shown in Fig. 4C, oxfendazole markedly upregulated p21 expression.

These results indicated that oxfendazole inhibited cell cycle progression by suppressing CDK signaling in NSCLC cells, and its activity in survival inhibition in NSCLC cells was observed.

Oxfendazole enhances the cytotoxicity of cisplatin against NSCLC cells. Cisplatin is a common chemotherapeutic drug for NSCLC therapy, but resistance is frequently observed during the process of NSCLC treatment in the clinic (23-25). It has been reported that overactivation of c-Src could result in drug resistance to NSCLC treatment (10,26); thus, the present study then evaluated whether oxfendazole could enhance the effect of cisplatin in the treatment of NSCLC cells. As shown in Fig. 5A, the CCK-8 assay demonstrated that oxfendazole enhanced the cytotoxicity of cisplatin against A549 and H1299 cells. In addition, the immunoblotting analysis revealed that the combination of oxfendazole and cisplatin could enhance the inhibition of c-Src activation, as well as the upregulation of p53 (Fig. 5B). Therefore, oxfendazole enhanced the cytotoxic activity of cisplatin by suppressing c-Src activation, and oxfendazole could utilized for anti-NSCLC therapy in the clinic in combination with other antitumor drugs, such as cisplatin.

Discussion

The current strategies being investigated for NSCLC treatment have focused on new targeted therapies against epidermal growth factor receptor, angiogenesis and immune checkpoints. However, these therapies have exhibited limited benefits for patients with NSCLC (27-29). Therefore, novel and effective drugs are urgently required to treat NSCLC, and one possible strategy is to utilize previously discovered drugs that are currently used to treat different diseases (14). In the present study, the anthelmintic agent oxfendazole significantly displayed antitumor activity in NSCLC cells; thus, the effect of oxfendazole *in vivo* will be tested in future work.

In epithelial cancers, including NSCLC, c-Src and other Src-associated kinases (including Fyn, Yes and Lyn) are overexpressed and activated, and their levels are closely associated with tumor progression (30). As expected, suppressing Src family kinases in these tumors led to the inhibition of cell

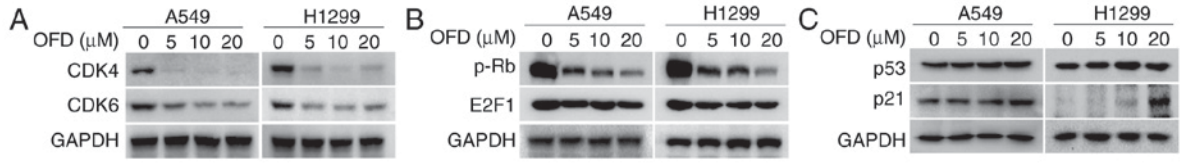

Figure 4. OFD inhibits CDK signaling in non-small cell lung cancer cells. (A) A549 and H1299 cells were treated with the indicated concentrations of OFD for 24 h at 37˚C, and then cells were prepared for immunoblotting against CDK4, CDK6 and GAPDH. (B) A549 and H1299 cells were treated with the indicated concentrations of OFD for 24 h at 37˚C, followed by immunoblotting against p-Rb, E2F1 and GAPDH. (C) Following treatment with the indicated concentrations of OFD for 24 h at 37˚C, A549 and H1299 cells were analyzed by immunoblotting against p53, p21 and GAPDH. OFD, oxfendazole; CDK, Cyclin-dependent kinase; p-Rb, protein retinoblastoma; E2F1, E2 transcription factor 1.

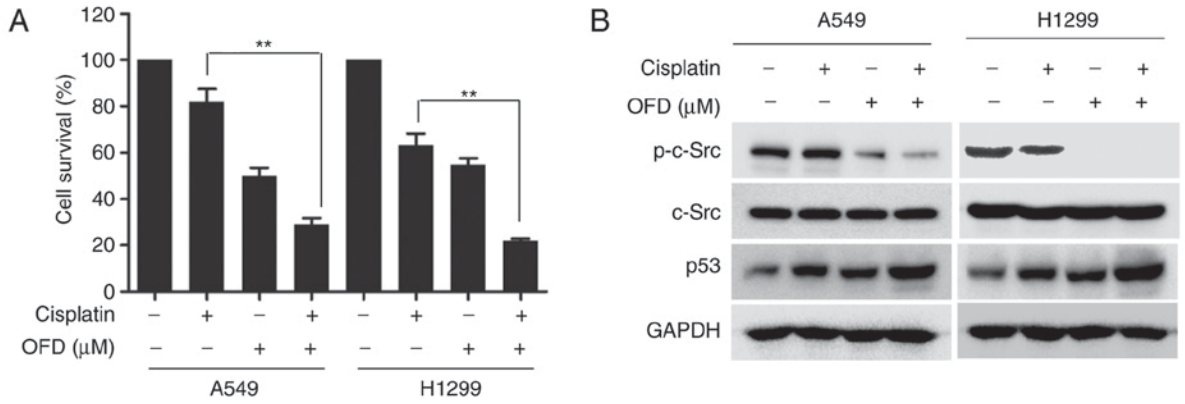

Figure 5. OFD enhances the cytotoxicity of cisplatin against non-small cell lung cancer cells. (A) A549 and H1299 cells were treated with 5 μM cisplatin for 24 h at 37˚C in the presence or absence of 10 μM OFD, and then the cells were assessed by CCK-8 assay. (B) Cells were also prepared for immunoblotting against p53, p-c-Src, c-Src and GAPDH. **P<0.01, as indicated. OFD, oxfendazole; CCK, Cell Counting Kit; p-, phosphorylated.

growth, and c-Src has been considered to be an ideal drug target for various cancer treatments (31). For example, Dasatinib, a small molecule tyrosine kinase inhibitor, inhibited cell migration and invasion, and induced cell cycle arrest (blocking the G1-S transition) by suppressing c-Src activation in head and neck squamous cell carcinoma and NSCLC cells (9). In the present study, oxfendazole inhibited the activation of c-Src in different NSCLC cell lines, and overexpression of c-Src decreased the cytotoxicity of oxfendazole in A549 and H1299 cells, which suggested that oxfendazole could be used as a novel c-Src inhibitor.

Signaling through c-Src has been reported to be involved in tumor progression, including cell cycle regulation, angiogenesis, cell survival, cell invasion and metastasis (32). Therefore, the present study analyzed the cell cycle of NSCLC cells following treatment with oxfendazole via flow cytometry. The results revealed that oxfendazole induced cell cycle arrest at the G_0/G_1 phase in A549 and H1299 cells, and further immunoblotting demonstrated that oxfendazole inhibited CDK signaling, which is the downstream signaling pathway of c-Src.

Cisplatin is a common chemotherapeutic drug in the treatment of lung cancer, but patients frequently develop resistance to it partly due to the overactivation or overexpression of c-Src (10). As described above, oxfendazole inhibited the activation of c-Src, so the present study next investigated whether oxfendazole could enhance the cytotoxicity of cisplatin in NSCLC cells. The results demonstrated that oxfendazole enhanced the cytotoxic activity of cisplatin against NSCLC cells, which suggested that oxfendazole may be effective in NSCLC therapy in the clinic in combination with other drugs, such as cisplatin.

In conclusion, the present study demonstrated that the anthelmintic agent oxfendazole exerted its antitumor action by suppressing c-Src activation in NSCLC cells. The results suggests that oxfendazole may be effective as a novel type of NSCLC treatment in the clinic in the future.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

DX, WT and CJ performed the experiments. DX and SZ wrote and edited the manuscript. ZH and SZ designed the research project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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