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Combination effects of arsenic trioxide and fibroblast growth factor receptor inhibitor in squamous cell lung carcinoma

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ABSTRACT

Objectives: Lung cancer remains the top cancer killer worldwide, with squamous cell carcinoma (SCC) as the second commonest histologic subtype. Arsenic trioxide (ATO) was previously shown to suppress growth of lung cancer. Fibroblast growth factor receptor (FGFR) amplification was recently demonstrated in lung SCC, with specific FGFR inhibitor (e.g. PD173074) developed as a potential targeted therapy. Therefore the combination effects of ATO and PD173074 in SCC was studied.

Materials and methods: The combination of ATO/PD173074 was studied in a proof-of-principle model using a lung SCC cell line with FGFR1 overexpression: SK-MES-1. The effects of ATO and/or PD173074 on cell viability and protein expression were studied by MTT assay and Western blot respectively. Cell cycle analysis, phosphatidylserine externalization and mitochondrial membrane depolarization were monitored by flow cytometry. FGFR1 knockdown was performed with siRNAs. Proteasome inhibitor (MG-132) was used to study the degradation mechanism. *In vivo* effect of ATO and/or PD173074 was investigated using a nude mice xenograft model.

Results: Combined ATO/PD173074 reduced cell viability along with increased sub-G1 population, phosphatidylserine externalization and mitochondrial membrane depolarization more significantly than single treatments. Downregulation of FGFR1, p-Akt, Akt, p-Src, Src, p-c-Raf, c-Raf, Erk and survivin as well as upregulation of p-Erk and cleaved PARP were observed upon ATO and/or PD treatment. MG-132 partially reversed the degradation of Akt, Src, c-Raf and Erk induced by ATO/PD, suggestive of ubiquitin-independent proteasome-dependent degradation. However, the mechanism of FGFR1 downregulation remained unknown. Downregulation of FGFR1, Akt, Src, c-Raf and Erk as well as cleaved PARP elevation induced by ATO and/or PD were confirmed *in vivo*.

Conclusion: Massive protein degradation (FGFR1, Akt, Src, c-Raf and Erk) was induced by ATO and/or PD173074 treatment mainly mediated by activation of proteasomal degradation in SCC cell line SK-MES-1 *in vitro* and *in vivo*.

1. Introduction

Based on the GLOBOCAN project of the World Health Organization in 2012, lung cancer was the third most common cancer with incidence and mortality rates 16.7% and 23.2% of all cancers worldwide respectively (http://globocan.iarc.fr/). Lung cancer can be divided into non-small cell carcinoma (NSCLC) and small cell carcinoma (SCLC). Squamous cell lung carcinoma (SCC) represents the second most common histologic subtype of NSCLC after adenocarcinoma [1]. Doublet chemotherapy regimens remain the cornerstone first-line systemic treatment in SCC [2]. Unfortunately, majority of patients with SCC do not benefit from established targeted therapeutics. Immune checkpoint inhibitors have recently emerged as a promising treatment, but the benefit is confined to a minority of patients with lung SCC [3]. Fibroblast growth factor receptor (FGFR) amplification was found in 13% to 21% of SCC [4–6]. In particular, the incidence of FGFR1 amplification is higher in SCC than in other subtypes, associated with poor 5-year overall survival [4]. There have been recent interests to explore FGFR1 as a potential therapeutic target in SCC.

PD173074 (PD) is a relatively specific inhibitor of FGFR1 which has demonstrated pro-apoptotic effect in FGFR1-amplified SCC [7]. Arsenic trioxide (ATO) has been adopted as a therapy for

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acute promyelocytic leukemia with an intravenous formulation approved by the US Food and Drug Administration more than a decade ago [8]. Although ATO has yet to be used clinically for treatment of lung cancer, it has demonstrated *in vitro* and *in vivo* anti-cancer effects in lung adenocarcinoma [9,10], SCLC [11] and mesothelioma [12]. Nonetheless, the inhibitory activity of ATO in SCC has not been studied. We proposed that ATO would have anti-cancer activity in SCC. Furthermore, FGFR1 amplification has been shown biologically important in SCC, supporting a postulated benefit when combining ATO with FGFR1 inhibitor (PD173074) in FGFR1-overexpressed lung SCC model. The findings in this study would provide a scientific basis for the future clinical development of such combination in the treatment for FGFR1-overexpressed SCC.

2. Materials and methods

2.1. Cell lines and reagents

SK-MES-1, SW900 and H2170 cells were purchased from American Type Culture Collection (Manassas, VA, USA). SK-MES-1, SW900 and H2170 cells were cultured in MEM, L-15 and RPMI-1640 medium respectively (Gibco®, Life Technologies, Carlsbad, California, USA) and supplied with 10% fetal bovine serum (FBS) (Gibco®) in a humidified atmosphere of 5% CO_2 at 37 °C. ATO (Sigma-Aldrich, St. Louis, Missouri, USA), PD173074 (PD), MG-132, lactacystin (Cayman, Teaduspargi, Tallinn, Estonia) and FGF1 (Peprotech, Rocky Hill, USA) were obtained.

2.2. Cell viability assay

Briefly, cells (2500/well) were incubated with different concentrations of ATO and/or PD for 72 h as previously described [10]. FGF1 (50 ng/ml) was added if indicated. The combination effect of ATO and PD was analyzed with CalcuSyn software (Version 12 2.0, Biosoft, Cambridge, UK) [13].

The combination index (CI) equation was established from the multiple drug-effect equation of Chou and Talalay derived from enzyme kinetic models [14]. CI values are automatically generated by the software and defined as indicating strong synergism (CI = 0.1–0.3), synergism (CI = 0.3–0.7), moderate synergism (CI = 0.7–0.85), additive effect (CI = 0.9–1.1) or antagonism (CI > 1.1).

2.3. Cell cycle analysis

Cells were fixed at 4 °C for overnight with 75% ethanol, washed with PBS and stained at 37 °C for 20 min with propidium iodide (PI, 25 μg/ml) and RNase A (50 μg/ml). Flow analysis was carried out and signals were detected by FL-3 (570 nm) channel (Beckman FC500). Data was analyzed by WinMDI (The Scripps Research Institute, CA, USA).

2.4. Phycoerythrin (PE)-conjugated annexin-V and 7-(aminoactinomycin D) AAD staining

Phosphatidylserine externalization (PS) (loss of membrane asymmetry) was studied using the PE-conjugated annexin-V and 7-AAD staining [9].

2.5. Measurement of mitochondrial membrane potential by JC-1 staining

The fluorescent dye 5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolycarbocyanine iodide (JC-1, Sigma-Aldrich) was used for the determination of mitochondrial transmembrane potential [9].

2.6. Western blot of whole-cell lysate

Western blot was performed [15]. Specific primary antibodies [anti-β-actin (Sigma-Aldrich), anti-p-Akt, anti-Akt, anti-p-c-Raf^{338}, anti-c-Raf, anti-p-Erk, anti-Erk, anti-p-Src, anti-Src, anti-FGFR1, anti-PARP and anti-survivin, anti-ubiquitin (Cell Signaling Technology, Danvers, Massachusetts, USA) antibodies) and corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) were purchased. An enhanced chemiluminescence (ECL) kit (GE Healthcare) was used to detect protein expression. Beta-actin was selected as a reference protein.

2.7. FGFR1 siRNA knockdown

FGFR1 siRNA (sc-29316, Santa Cruz Biotechnology, Santa Cruz, California, USA) or FGFR1 siRNA (SR301583, OriGene Technologies, Rockville, USA) or control siRNA (sc-37007, Santa Cruz Biotechnology) was allowed to transfect cells for 6 h using a transfection reagent (Santa Cruz Biotechnology) in MEM medium, followed by replenishment with new MEM medium enriched with 1% FBS for 3 days. Cell viability and FGFR1 protein expression were measured [9].

2.8. Combination effect of FGFR1 siRNA and ATO

There were 4 groups for each FGFR1 siRNA: control siRNA, ATO/control siRNA, FGFR1 siRNA and ATO/FGFR1 siRNA. Cells (2500/well) were transfected with different concentrations of control siRNA or FGFR1 siRNAs (Santa Cruz or OriGene) for 6 h, followed by incubated with different concentrations of ATO for 72 h. FGF1 (50 ng/ml) was added if indicated. Cell viability assay was carried out. The CI values of ATO and FGFR1 siRNAs were then calculated.

2.9. Effect on protein expression after ATO and/or PD treatment followed by proteasome inhibitor MG-132

Cells were treated with ATO and/or PD for 72 h. MG-132 (10 μM) was added 8 h before harvest.

2.10. Tumor growth inhibition in vivo

The SK-MES-1 xenograft model was developed by subcutaneous injection of 10^7 cells with Matrigel (BD, Bio-science, San Jose, CA, USA) into the upper back of 40 nude mice (female, 4–6-week-old, 10-14 g, BALB/cAnN-nu, Charles River Laboratories, Wilmington, USA). Mice were randomized into 4 groups after tumor growth was established. PBS (served as control), ATO (5 mg/kg), PD (7 mg/kg) or combination of ATO/PD was administrated intraperitoneally and daily. Tumor dimension (using standard calipers) and body weight of mice were measured on alternate days and tumor volume calculated [volume = (length × width × width)/2]. For humane reasons, mice were killed when diameter of tumor size achieved 17 mm. Tumor xenografts were removed from mice and lysed for Western blot. The study protocol was approved by the institutional Animal Ethics Committee (approval reference number: CULATR 2860-12), and standard humane endpoints for animal research were applied.

2.11. Statistical analysis

Experiments were repeated for at least 3 times and data presented in mean ± standard deviation. The difference between groups was analyzed using Student's two-tailed *t*-test by Prism (GraphPad Software, La Jolla, Southern California, USA). A p-value < 0.05 defined statistical significance (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).