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ABT-737 Synergizes with Cisplatin Bypassing Aberration of Apoptotic Pathway in Non-small Cell Lung Cancer

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Abstract

A subset of non-small cell lung cancer (NSCLC), which does not have a druggable driver mutation, is treated with platinum-based cytotoxic chemotherapy, but it develops resistance triggered by DNA damage responses. Here, we investigated the effect of activation of STAT3 by cisplatin on anti-apoptotic proteins and the effectiveness of a co-treatment with cisplatin and a BH3 mimetic, ABT-737. We analyzed the relationship between cisplatin and STAT3 pathway and effect of ABT-737, when combined with cisplatin in NSCLC cells and K-ras mutant mouse models. The synergism of this combination was evaluated by the Chou-Talalay Combination Index method. In *vivo* activity was evaluated by micro-CT. In NSCLC cells, there was a time and dose-dependent phosphorylation of SRC-JAK2-STAT3 by cisplatin, followed by increased expression of anti-apoptotic molecules. When the expression of the BCL-2 protein family members was evaluated in clinical samples, BCL-xL was most frequently overexpressed. Dominant negative STAT3 suppressed their expression, suggesting that STAT3 mediates cisplatin mediated overexpression of the anti-apoptotic molecules. ABT-737 displaced BCL-xL from mitochondria and induced oligomerization of BAK. ABT-737 itself showed cytotoxic effects and a combination of ABT-737 with cisplatin showed strong synergistic cytotoxicity. In a murine lung cancer model, co-treatment with ABT-737 and cisplatin induced significant tumor regression. These findings reveal a synergistic cytotoxic and anti-tumor activity of ABT-737 and cisplatin co-treatment in preclinical models, and suggest that clinical trials using this strategy may be beneficial in advanced NSCLC.

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Introduction

Lung cancer is a common and leading cause of cancer death worldwide. In the year 2012, 1,824,701 new lung cancer were diagnosed, and 1,590,000 patients died of this devastating disease worldwide [1]. Advanced stage of lung cancer, which does not have a druggable driver mutation, is treated with platinum based-cytotoxic chemotherapy, but clinical outcome is suboptimal.

Cisplatin is a prototypic platinum chemotherapeutic agent and one of the most commonly prescribed drugs for the treatment of solid malignancies; however, its therapeutic benefits are often limited because of multiple resistance mechanisms. These resistance mechanisms can be classified by the alterations of steps (1) delivering cisplatin to DNA (pre-target resistance), (2) forming DNA-cisplatin adducts (on-target resistance) (3) related to cell death pathways elicited by DNA damage responses (post-target resistance) and (4) affecting signaling pathways that do not have obvious links with cisplatin treatment (off-target resistance) [2].

Overexpression of antiapoptotic members of the BCL-2 protein family is a cause for poor responses to chemotherapeutic agents and is

Abbreviations: CI, combination index; DFS, disease-free survival; FFPE, formalin-fixed paraffin embedded; IHC, immunohistochemistry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSCLC, non-small cell lung cancer; OS, overall survival; RT-PCR, real-time PCR.

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related to cisplatin resistance and diseases recurrence in cancer [3,4]. Alterations in cell death pathway induced by DNA damage responses is one of the critical mechanisms related to chemoresistance, and therefore, modulation or bypass of these responses might be an effective way in improving treatment outcomes [5,6]. Influence of MAPK pathway activation on cisplatin mediated apoptotic pathway is well established, but the relationship between JAK2-STAT3 pathway and response to cisplatin mediated cytotoxicity is limited (7). Considering the role of STAT3 as an oncogene and that activation of its signaling confers resistance to apoptosis, it is important to identify the relationship and to develop effective control modality.

Inactivation of BCL-2 family proteins might overcome the resistance against cisplatin-mediated apoptosis and may improve clinical outcomes. BH3-only proteins of BCL-2 family can directly or indirectly activate the effector protein of the mitochondrial membrane permeabilization. In the indirect (displacement) model, the BH3-only proteins (NOXA, BAD and BIM) insert their a-helical BH3 domain into the hydrophobic groove of prosurvival proteins. In the direct activation model, BH3-only proteins are classified into "sensitizer" and "activator". In this model, the activator BH3-only proteins (BIM, tBID) are sequestered by prosurvival proteins. The subsequent interaction between the prosurvival proteins and "sensitizer" BH3-only proteins (BAD, NOXA) releases the "activator" proteins [7]. In both model, the binding of BH3-only proteins to prosurvival molecules results in BAX/BAK conformation change, oligomerization and mitochondrial membrane permeabilization [8,9]. BH3 mimetics show a biochemical affinity for specific anti-apoptotic BCL-2 proteins and this is linked to their ability to kill specific cells. Several clinical trials have been performed using BH3 mimetics, such as ABT-737, ABT-263, AT-101, GX15-070, and TW-37, with limited success [10]. We hypothesize that a combination of cisplatin with prototypic BH3 mimetics, ABT-737, would overcome the cisplatin resistance caused by STAT3 activation.

In this study, we investigated the relationship between cisplatin and STAT3 pathway and effect of ABT-737, prototype of BH3 mimetics, when combined with cisplatin in non-small cell lung cancer (NSCLC) cells and a *K-ras* mutant mouse models. The synergism of this combination was evaluated by the Chou-Talalay Combination Index (CI) method. *In vivo* activity was evaluated by microCT and showed that this combination can be effectively applied for the treatment of lung cancer.

Materials and Methods

Cell Lines, Plasmids, Clinical Specimens, Chemicals, and Antibodies

A549 and H1703 cells were purchased from ATCC (Manassas, VA, USA) in 2012. H460, H1299, H358, H2009, and H596 cells were obtained from the Korean Cell Line Bank in 2012 (https://cellbank.snu.ac.kr/main/, Seoul, Korea), which provides cell test and authentication by DNA fingerprinting analysis by short tandem repeat markers and mycoplasma contamination test. Except for the experiment for revision, cells were used within six months after purchase. EF.GFP (#17616), EF.STAT3DN.Ubc.GFP (#24984), pCDNA3 Flag MKK7B2Jnk1a1 (#19726), and, pCDNA3 Flag MKK7B2Jnk1a1(APF) (#19730), were obtained from Addgene (Cambridge, MA, USA) and pcDNA3 were obtained from Invitrogen (Carlsbad, CA, USA). Anisomycin (ab120495) was purchased from Abcam (Cambridge, UK) and dasatinib (# S1021) was purchased from Selleckchem (Houston, TX, USA). To evaluate expression of anti-apoptotic proteins in human NSCLC, 12-paired lysates from adjacent normal appearing lung tissue and cancer-enriched tissue were analyzed by immunoblotting. Another set of 117 formalin-fixed paraffin embedded (FFPE) NSCLC tissue were used for immunohistochemistry (IHC). This study was approved by the IRB of Gangnam Severance Hospital (IRB #3–2014-0299) and was carried out in accordance with the Declaration of Helsinki and Korean GCP guidelines. ABT-737 was purchased from the AdooQ™ Bioscience (Irvine, CA, USA) and its chemical and crystal structure was described in elsewhere [11,12]. Antibodies, unless otherwise stated, were obtained from Cell Signaling Technology (Danvers, MA, USA).

Immunoblotting

Cells were harvested on ice using 2×Laemmli sample buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). After sonication, 30–50 mg of lysate was separated by gel electrophoresis on 7.5 to 12% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The expression level of each protein was measured using ImageJ (http://rsbweb.nih.gov/ij/) and quantified relative to that of β-actin [5].

RT-PCR

The RT-PCR was performed as described elsewhere [13]. Total RNA was extracted using TRI reagent (Ambion, Austin, TX, USA). Quantitative RT-PCR analysis was performed using TaqMan Gene Expression assay reagents and the StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) using an inventoried primer-probe set (http://bioinfo.appliedbiosystems.com/genome-database/gene-expression.html).

Mitochondrial Cytochrome c Release Assay

Cells were harvested and suspended in cell permeability buffer and incubated on ice for 10 minutes [14]. Cell disruption was performed by pipetting and vortexing. The homogenates were spun at 700×*g* for 10 min at 4°C. The supernatants were transferred to a fresh tube and spun at 13,000×*g* for 10 min at 4°C. The supernatants (cytosolic fraction) were transferred to a new tube and the mitochondrial pellets were resuspended in cell permeability buffer. After sonication, lysates were resolved on a 15% polyacrylamide gel, and analyzed by immunoblotting using a mouse anti-cytochrome c antibody.

Cell Death, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay, and Drug Combination Study

To measure cell death, cells were treated with the indicated dose of ABT-737 and cisplatin for 48 h, then stained with annexin-V and propidium iodide (PI) and analyzed using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The effect of treatment on cell proliferation was assessed by the MTT assay. Briefly, 5×10^5 cells per well were treated with either ABT-737 (0–80 μM), cisplatin (0–160 μM), or a combination of both drugs at fixed concentration ratios of 1:0.5, 1:1, and 1:2 (cisplatin:ABT-737). After 48 h, MTT was added at a final concentration of 0.5 mg/mL, and cells were incubated for an additional 2 h at 37°C. Formazan complexes were dissolved in DMSO, and absorbance was measured at 550 nm with a spectrophotometer (Thermo Scientific, Rockford, IL, USA). The effect of combining the therapies was evaluated with a CI previously proposed by Chou-Talalay using CompuSyn software (CompuSyn) [15].

Immunocytochemistry

Cells (5×10^5) were plated in 6-well plates containing a sterilized coverslip. On the following day, cells were fixed with 4% formaldehyde in PBS, incubated in blocking solution containing 5% BSA in PBS, and then incubated with anti-cytochome c, anti-BAK and anti-Bcl-xL antibodies for 16 hours. On the following day, the cells were washed, and