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Original Article

HSP90 inhibitor (NVP-AUY922) enhances the anti-cancer effect of BCL-2 inhibitor (ABT-737) in small cell lung cancer expressing BCL-2

Hannah Yang [a], Mi-Hee Lee [b], Intae Park [a], Hanwool Jeon [b], Junyoung Choi [b], Seyoung Seo [b], Sang-We Kim [b], Gou Young Koh [a, c], Kang-Seo Park [b, d, **], Dae Ho Lee [b, *]

[a] Biomedical Science and Engineering Interdisciplinary Program, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, 34141, Republic of Korea
[b] Department of Oncology, University of Ulsan College of Medicine, Asan Medical Center, Seoul, 05505, Republic of Korea
[c] Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, 34141, Republic of Korea
[d] Department of Biomedical Sciences, University of Ulsan College of Medicine, Seoul, 05505, Republic of Korea

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ABSTRACT

Small cell lung cancer (SCLC) cannot be efficiently controlled using existing chemotherapy and radiotherapy approaches, indicating the need for new therapeutic strategies. Although ABT-737, a B-cell lymphoma-2 (BCL-2) inhibitor, exerts anticancer effects against BCL-2-expressing SCLC, monotherapy with ABT-737 is associated with limited clinical activity because of the development of resistance and toxicity. Here, we examined whether combination therapy with ABT-737 and heat shock protein 90 (HSP90) inhibitor NVP-AUY922 exerted synergistic anticancer effects on SCLC. We found that the combination of ABT-737 and NVP-AUY922 synergistically induced the apoptosis of BCL-2-expressing SCLC cells. NVP-AUY922 downregulated the expression of AKT and ERK, which activate MCL-1 to induce resistance against ABT-737. The synergistic effect was also partly due to blocking NF-κB activation, which induces anti-apoptosis protein expressions. However, interestingly, targeting BCL-2 and MCL-1 or BCL2 and NF-κB did not induce the cytotoxicity. In conclusion, our study showed that combination of BCL2 inhibitor with HSP90 inhibitor increased activity in in vitro and in vivo study in only BCL-2 expressing SCLC compared to either single BCL2 inhibitor or HSP inhibitor. The enhanced activity might be led by blocking several apoptotic pathways simultaneously rather than a specific pathway.

Introduction

Small cell lung cancer (SCLC) accounts for 10%–15% of all lung cancers and is characterized by rapid growth, early metastasis, and rapid resistance development after an initial sensitive response. Patients with a limited disease can be treated with concurrent chemotherapy and radiotherapy and show a cure rate of 15%–20%. However, patients with an extensive disease show poor long-term survival, with a median survival of 10–12 months, after receiving existing treatment [1,2]. The lethality of SCLC might be partly associated with the development of resistance to standard cytotoxic chemotherapies, indicating the need for new therapeutic approaches or agents for treating SCLC [3,4].

Mitochondrial permeabilization is controlled by a balance of anti-apoptotic proteins and pro-apoptotic proteins. Evasion of apoptosis is a prominent factor of drug resistance in SCLC [1,4]. Multiple mechanisms might contribute to this resistance, including overexpression of the anti-apoptotic B-cell lymphoma 2 (BCL-2) protein. BCL-2 family members function through interactions with each other and the balance between the anti-apoptotic proteins, such as BCL-2, BCL-xL, and MCL-1, and the pro-apoptotic proteins, such as BH-3 only members including BAD, BID, and BIM, is crucial to prevent or initiate apoptosis [5]. Considering these complex interactions, it might be more reasonable to control the interaction or balance rather than to target one or two BCL-2 family proteins. The major function of BCL-2 is to bind and sequester BCL-2 homology

Abbreviations: CI, combination index; SCLC, small cell lung cancer.

* Corresponding author. Department of Oncology, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul, 05505, Republic of Korea.
** Corresponding author. Department of Biomedical Sciences, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul, 05505, Republic of Korea.
E-mail addresses: 77kangseo@gmail.com (K.-S. Park), leedaeho@amc.seoul.kr (D.H. Lee).

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domain 3 mimetic (BH3)-only pro-apoptotic activator proteins, leading to weakening of the pro-apoptotic response. A therapeutic strategy to target this interaction comprises the development of BH-3 mimetic agents that can bind the hydrophobic groove on BCL-2, displace the BH3-only protein, and finally induce apoptosis [6–8].

BCL-2 inhibitors, including ABT-737 and its orally bioavailable derivative ABT-263, exert anticancer effects in BCL-2-expressing SCLC and chronic lymphocytic leukemia [9–11].

However, the limitation of BCLcl-2 inhibitors was observed in SCLC because of dose-limiting toxicity [10–13]. In addition, ABT-263 shows limited clinical activity against SCLC, with a response rate of 2.6% (1 of 39) and median progression-free survival of 1.5 months [14]. To overcome these limitations, BCL-2 inhibitors ABT-737 and ABT-263 have been examined in combination with other chemotherapeutic agents [15–17] to effectively enhance their cytotoxicity against cancer cells. However, limited activity of these combination therapies has highlighted the need for improved rational combination strategies.

NVP-AUY922 (AUY922), a potent non-geldanamycin heat shock protein 90 (HSP90) inhibitor, exerts significant inhibitory effects in various tumor cells [9,18,19]. HSP90 inhibitors promote proteasomal degradation of HSP90 target proteins, including EGFR, IGF1R, AKT, RAF-1, IKK, c-KIT, v-SRC, NPM-ALK, and P53 [20], and indirectly downregulate MCL-1 expression by inhibiting AKT and MAPK signaling [19].

Although HSP90 inhibitors in clinical trials also faced a limit of single agent activity, AUY922 is still being developed as a component for combination therapies in various clinical trials [18,20,21]. Considering NF-κB activation is known as a inducer of anti-apoptosis [22], the blockage of NF-κB might promote the synergistic anti-cancer effect with BCL-2 inhibitors.

Therefore, we examined whether HSP90 inhibitors improved the anticancer effects of BCL-2 inhibitors and exerted synergistic apoptotic effect in BCL-2-expressing SCLC cells. We also attempted to determine mechanism(s) underlying the anticancer effects of this combination therapy.

Materials and methods

Cell culture, drugs, and reagents

Three SCLC cell lines, namely, NCI-H146, NCI-H187, and NCI-H69, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) [9] and were cultured in RPMI-1640 medium (Welgene, Seoul, Korea) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 37 °C in 5% CO_2. ABT-737 and AUY922 were purchased from Selleckchem and were dissolved in dimethyl sulfoxide to produce 100 mmol/L stock solution that was aliquoted and stored at −80 °C.

Western blotting analysis

Whole cells were harvested and lysed in a lysis buffer (Thermo Fisher Scientific) containing phosphatase and proteinase inhibitors (Thermo Fisher Scientific). Protein concentration in the cell lysates was determined using bicinchoninic acid method (Thermo Fisher Scientific). Proteins present in the cell lysates (10 μg) were resolved by performing SDS-PAGE on 8% gels. Bromophenol blue and mercaptoethanol were added to the samples before loading. Western blotting analysis was performed using standard procedures and primary antibodies against the following proteins: ERK, phosphorylated ERK (pERK), MEK, phosphorylated MEK (pMEK), AKT, phosphorylated AKT (pAKT), IκB-α, phosphorylated NF-κB (pNF-κB), MCL-1, BCL-2, phosphorylated BCL-2 (pBCL-2), BCL-xL, BAX, BIM, cleaved BID, cleaved caspase-3, cleaved caspase-7, cleaved PARP, and β-actin (all from Cell Signaling Technology, Danvers, MA, USA). The primary antibodies were detected using horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies. Proteins were visualized using ECL Plus enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA).

Cell growth and viability assay

SCLC cells were plated at a density of 5×10^3 cells/well in white 96-well plates (Corning), with three replicate wells for each condition; treated with each drug; and assayed at 72 h after the drug treatment. Metabolic activity of the cells was determined by performing a luminescent ATP-based assay (CellTiter-Glo; Promega), according to the manufacturer's instructions. Results were obtained using a fluorescent plate reader (VICTOR), with a read time of 1 s per well.

Calculation of combination index

Activity of monotherapy or combination therapy with the indicated drugs was estimated using CalcuSyn software program (Biosoft, Ferguson, MO, USA). This program calculates ED50 and combination index, a quantitative measure of the degree of drug interaction. For each given endpoint, combination index (CI) of 1 indicated an additive effect, CI of <1 indicated a synergistic effect, and CI of >1 indicated an antagonistic effect.

Apoptosis assay

The extent of apoptosis was measured using Muse™ Annexin V & Dead Cell Assay Kit (Millipore), according to the manufacturer's protocols. The kit allows quantitative analysis of live, early apoptotic, and late apoptotic cells. At 24 h after the drug treatment, the cells were collected in RPMI-1640 medium supplemented with 10% FBS (final concentration, 1×10^5 cells). Next, 100 μL cell suspension was mixed with 100 μL Muse Annexin V & Dead Cell reagent, incubated in the dark for 20 min at room temperature, and analyzed using Muse Cell Analyzer (Millipore).

Xenograft experiment

Five-week-old BALB/C nude mice were purchased from Central Lab. Animal Inc. (Seoul, Korea). The mice were subcutaneously injected with NCI-H146 cells (density, 2×10^7 cells/mouse) and were maintained until the tumor reached a volume of approximately 600 mm^3. Next, the mice were intraperitoneally injected with 50 mg/kg AUY922, 50 mg/kg ABT-737, 50 mg/kg AUY922 and 50 mg/kg ABT-737, or control twice per week. Tumor sizes were measured twice per week. All animal procedures were approved by the institutional review board of Asan Medical Center (Seoul, Korea).

Results

ABT-737 and AUY922 shows anti-cancer effect in SCLC cells expressing BCL-2

First of all, out of 5 cell lines tested we first selected three SCLC cell lines, H146, H187 and H69, which express BCL-2 and checked various signaling in the three SCLC cell lines (Fig. 1A). To evaluate the anti-cancer effect of ABT-737 and AUY922 as a single agent, we evaluated the efficacy of ABT-737 or AUY922, separately. As expected, all three cell lines showed sensitivity to BCL-2 inhibitor, ABT-737 (Fig. 1B) while, interestingly the HSP90 inhibitor, AUY922, did not completely kill these cell lines (Fig. 1C).

Dual targeting of BCL-2 and HSP90 shows synergistic anti-cancer effects in SCLC cells expressing BCL-2

To determine whether AUY922 can enhance the anti-cancer effect of ABT-737, we exposed the cell lines to different concentrations of AUY922 with ABT-737. This combination showed synergistic effect in those cell lines expressing BCL-2 (H146, H187 and H69) (Fig. 2A) while it was not observed in BCL-2 absent SCLC cell lines (H82 and H128) (Supplementary Fig. 1). In addition, another HSP inhibitor, STA-9090, also enhanced the anti-cancer effect of BCL-2 inhibitor (ABT-737) (Supplementary Fig. 2). In a tumor xenografts model using NCI-H146 treated by intraperitoneal injection with either single drugs or combination of both drugs, at 50 mg/kg twice per week, the combination treatment was superior to either single drug treatment or the control, without body weight change or significant toxicity. However each single drug did not show superiority to another single treatment nor the control (Fig. 2B and C). In addition, combination group showed increased cleaved PARP cleavage rather than control or single drug treatments (Fig. 2D and E).

Co-targeting of BCL-2 and HSP90 shows synergistic apoptotic effect in SCLC cells expressing BCL-2

Mechanism underlying the synergistic anticancer effects of the combination treatment with BCL-2 and HSP90 inhibitors was