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BH3-mimetic gossypol-induced autophagic cell death in mutant BRAF melanoma cells with high expression of $p21^{Cip1}$

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ABSTRACT

Aims: The aim of the present study was to identify the potential therapeutic effects of BH3-mimetic gossypol on melanoma cells with acquired resistance to BRAF inhibitors.
Main methods: The IC_{50} values of gossypol were determined using MTT assays in three melanoma cell lines with different resistances to BRAF inhibitor. The effects of gossypol on three melanoma cell lines were further examined by immunoblotting analysis, cell cycle analysis, flow cytometric apoptotic assay and autophagy assay. The functional role of autophagy in gossypol-induced growth inhibition was investigated using siRNA-mediated knockdown of Beclin-1.
Key findings: Gossypol retained its efficacy in BRAF-V600E melanoma clones with acquired resistance to BRAF inhibitors through a mechanism independent of MEK–ERK inhibition. Gossypol caused G_2/M arrest in both BRAF mutant A375P and A375P/Mdr cells with high expression of $p21^{Cip1}$, regardless of their drug resistance. Interestingly, we determined that the lack of gossypol-induced mitotic arrest in BRAF-WT-harboring SK-MEL-2 cells was associated with a low level of $p21^{Cip1}$ expression. In addition, gossypol preferentially induced autophagy and apoptosis in the gossypol-sensitive cells and not in the gossypol-resistant SK-MEL-2 cells. In particular, alleviation of autophagy by knockdown of Beclin-1 partially caused a resistance to gossypol-induced cell cycle arrest at G_2/M in BRAF-V600E cells with a concomitant decreased induction of apoptosis.
Significance: Taken together, these results suggest that gossypol may exhibit potential for the treatment of BRAF inhibitor-resistant tumors, but a functional $p21^{Cip1}$ is a prerequisite for a positive response to its clinical application.

Introduction

The limited clinical benefit of chemotherapies generally arises from the fact that responsive tumors acquire drug resistance. The activating mutations of BRAF have been identified in a large proportion of human cancers (http://www.sanger.ac.uk/genetics/CGP/cosmic). Notably, BRAF somatic missense mutations are observed in 66% of malignant melanomas (Davies et al., 2002). The duration of the clinical response in many melanoma patients treated with selective BRAF inhibitors has also been shown to be short due to acquired resistance (Flaherty et al., 2010). Thus, the largest concern regarding BRAF inhibitors arises from the fact that responsive tumors eventually acquire resistance to BRAF inhibitors. The precise causes that underlie the therapeutic resistance of melanoma are not well-understood and are likely to be mediated by diverse mechanisms. These mechanisms include the overexpression of MAP kinase kinase kinase 8 (MAP3K8; COT (Johannessen et al., 2010)), mutations in N-Ras, and PDGFRβ overexpression (Nazarian et al., 2010). In addition, several aberrant events including mutant *BRAF* amplification or alternative splicing (Romano et al., 2013; Shi et al., 2012), and *MEK* mutation (Emery et al., 2009) have been reported as acquired BRAF inhibitor resistance mechanisms. As new mechanisms of resistance are identified, novel drugs may increase the sensitivity of melanoma to chemotherapeutic treatments. However, the prevalence of these mechanisms remains uncertain because insufficient numbers of patient samples have been analyzed. In fact, Jiang et al. (2011) suggested that rebound melanoma growth after initial BRAF-targeted chemotherapy may not be responsive to MEK inhibitors, although the addition of MEK inhibition to supplement the ongoing inhibition of mutated B-Raf has been suggested to re-suppress the pathway optimally and, consequently, overcome resistance (Su et al., 2012). Moreover, our previous study suggested that the acquisition of resistance may be due to the activation of other pathways that reduce the dependence of the cell on BRAF signaling (Ahn and Lee, 2013).

Gossypol, which is a natural product from cottonseeds, has been identified as a BH3-mimetic small-molecule pan-inhibitor of Bcl-2/Bcl-xL/Mcl-1 (Etxebarria et al., 2008; Priyadarshi et al., 2010) that can induce apoptosis in various cancer cell lines (Liu et al., 2002; Oliver

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et al., 2004). In particular, Lei et al. (2006) demonstrated that gossypol potently induces the Bax/Bak-independent activation of apoptosis by converting Bcl-2 from an inhibitor to an activator of apoptosis. In fact, the potent anticancer activity of gossypol has been demonstrated in many types of malignancies (Balakrishnan et al., 2008; Balci et al., 1999; Patel et al., 2009; Zhang et al., 2007). We recently found that Ras-NIH 3T3/Mdr cells, which exhibit a strong cross-resistance to many chemotherapeutic agents, exhibited essentially no resistance to gossypol (Ahn et al., 2013).

We previously generated resistant derivatives of BRAF-V600E A375P melanoma cell lines through chronic treatment with UAI-201 (described as UI-152 in previous reports), which we recently reported as a potent ATP-competitive inhibitor of RAF proteins (Ahn et al., 2012; Kim et al., 2012). In this study, we demonstrate that gossypol retained its efficacy in A375P/Mdr cells with acquired resistance to BRAF inhibitors and that the growth inhibition attained is similar to that observed in the parental A375P cells. Moreover, we provide the first demonstration that the gossypol resistance of wild-type (WT) BRAF-harboring SK-MEL-2 cells is due to the low expression of $p21^{Cip1}$ proteins. Collectively, our data suggest that gossypol offers a more effective therapeutic strategy for BRAF-V600E melanoma with acquired resistance to BRAF inhibitors.

Materials and methods

Antibodies and reagents

Rabbit polyclonal anti-Beclin-1, anti-Bcl-2, anti-MEK, anti-$p21^{Cip1}$, and anti-$p27^{Kip1}$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-LC3 was obtained from Sigma (St. Louis, MO, USA). The anti-phospho-MEK was obtained from Cell Signaling Technology (Danvers, MA, USA). For the apoptosis assay, the FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and penicillin–streptomycin were purchased from GIBCO-Invitrogen (Carlsbad, CA, USA). The reagents for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Hercules, CA, USA). The BRAF-targeting drug UAI-201 (described as UI-152 in previous reports) was obtained from YOUAI Co., Ltd. (Suwon-Si, Gyeonggi-Do, Korea). Gossypol and 3-methyladenine (3-MA) were obtained from Sigma (St. Louis, MO, USA).

Cell lines and cell culture

Melanoma cell lines (A375P and SK-MEL-2) were obtained from either the Korean Cell Line Bank (KCLB; Seoul, Korea) or YOUAI Co., Ltd. (Suwon-Si, Gyeonggi-Do, Korea). The development of BRAF inhibitor-resistant A375P melanoma cells (A375P/Mdr) was previously described (Ahn and Lee, 2013). The A375P/Mdr cells and their parental counterparts were maintained at 37 °C in DMEM supplemented with 10% FCS, penicillin–streptomycin, and glutamine. The A375P/Mdr cells were further propagated in growth medium containing 1 μM UAI-201. Before their use, the A375P/Mdr cells were maintained in a UAI-201-free culture medium and subcultured at least three times.

Plasmid DNA and siRNA transfection

The vectors encoding pEGFP-LC3 were obtained from Addgene (Cambridge, MA, USA). For Beclin-1 knockdown, a pool of three target-specific Beclin-1 siRNAs was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The targeted sequences were the following: CAGCUCAACGUCACUGAAAtt, GAGAUCUUAGAGCAAAUGAtt, and GGAUGACAGUGAACAGUUAtt. The targeted sequence of the siRNA used for the knockdown of $p21^{Cip1}$ was UGUCAGAACCGGCUGGGGA. A non-targeting siRNA (Santa Cruz Biotechnology, CA, USA) was used as a control. Where indicated, the cells were transiently transfected with either the siRNAs or pEGFP-LC3 using Lipofectamine 2000 in Opti-minimal essential medium I (Invitrogen).

Cell growth assay

The cells were plated in quadruplicates in 96-well microliter plates (Costar, Cambridge, MA, USA) at a density of 5×10^3 cells/well and then treated with gossypol at 37 °C in a humidified 5% CO_2/95% air incubator. On day 3, the cells were incubated with MTT at 37 °C for 3 h. The absorbance of the samples against a background control (medium alone), which was used as a blank, was measured at 450 nm using a microliter plate (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA).

Cell cycle assay

The cells were washed once with PBS, trypsinized, and collected by centrifugation at 400 ×*g* for 5 min. The cells (10^6 cells per sample) were fixed with 70% ethanol and stained with 50 μg/ml propidium iodide (PI) for 5 min. The cell cycle distribution was examined by measuring the DNA content using a Gallios flow cytometer and the Kaluza analysis software (Beckman Coulter, Inc., Brea, CA, USA).

Quantitation of autophagy

The cells were grown on chamber slides (Nunc), washed with PBS, and fixed in 10% formalin solution for 10 min. The cells were then transfected with pEGFP-LC3 for 48 h and treated with gossypol for 24 h. The fixed cells were classified as cells with predominantly diffuse GFP-LC3 fluorescence or a punctate GFP-LC3 pattern using a Zeiss Axio Scope.A1 epifluorescence microscope. The percentage of cells exhibiting autophagy was quantified by counting the number of cells expressing the punctate pattern of GFP-LC3 out of 200 GFP-positive cells in two independent fields.

Apoptosis assay using Annexin V staining and flow cytometry

After treatment with gossypol, 2×10^6 cells were harvested, washed with ice-cold PBS, resuspended in 200 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$), and incubated with 5 μl of Annexin V conjugated with FITC for 10 min at room temperature in the dark. The samples were then washed with binding buffer, resuspended in PBS, counterstained with PI, and analyzed with a Gallios flow cytometer and the Kaluza analysis software. The Annexin V^-/PI^+ cells were considered to be necrotic, whereas the Annexin V^+/PI^+ cells were considered to be late apoptotic, and the Annexin V^+/PI^- cells were identified as apoptotic cells.

Preparation of cell lysates and immunoblot analysis

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested by scraping the cells into RIPA lysis buffer. The lysate protein concentrations were determined with a BCA protein assay reagent kit. For immunoblotting, the whole cell lysates were denatured in Laemmli sample buffer and resolved by SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose, and the immune complexes were detected using the ECL-Plus chemiluminescent system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The fluorescent images were captured using KODAK Image Station 4000R (Carestream Health, Inc., Rochester, NY, USA). The bands were quantified using the Kodak Molecular Imaging software (version 4.5.0).