Autophagy-Dependent Survival of Mutant B-Raf Melanoma Cells Selected for Resistance to Apoptosis Induced by Inhibitors against Oncogenic B-Raf

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Abstract
Most patients with mutant B-Raf melanomas respond to inhibitors of oncogenic B-Raf but resistance eventually emerges. To better understand the mechanisms that determine the long-term responses of mutant B-Raf melanoma cells to B-Raf inhibitor UI-152, we used chronic selection to establish B-Raf (V600E) melanoma clones with acquired resistance to the new oncogenic B-Raf inhibitor UI-152. Whereas the parental A375P cells were highly sensitive to UI-152 (IC\textsubscript{50}<0.5 μM), the resistant sub-line (A375P/Mdr) displayed strong resistance to UI-152 (IC\textsubscript{50}>20 μM). Immunofluorescence analysis indicated the absence of an increase in the levels of P-glycoprotein multidrug resistance (MDR) transporter in A375P/Mdr cells, suggesting that resistance was not attributable to P-glycoprotein overexpression. In UI-152-sensitive A375P cells, the anti-proliferative activity of UI-152 appeared to be due to cell-cycle arrest at G\textsubscript{0}/G\textsubscript{1} with the induction of apoptosis. However, we found that A375P/Mdr cells were resistant to the apoptosis induced by UI-152. Interestingly, UI-152 preferentially induced autophagy in A375P/Mdr cells but not in A375P cells, as determined by GFP-LC3 puncta/cell counts. Further, autophagy inhibition with 3-methyladenine (3-MA) partially augmented growth inhibition of A375P/Mdr cells by UI-152, which implies that a high level of autophagy may protect UI-152-treated cells from undergoing growth inhibition. Together, our data implicate high rates of autophagy as a key mechanism of acquired resistance to the oncogenic B-Raf inhibitor, in support of clinical studies in which combination therapy with autophagy targeted drugs is being designed to overcome resistance.

Key Words: UI-152, B-Raf inhibitor, Melanoma, Drug resistance, Autophagy, Cell cycle arrest

INTRODUCTION
Ras/Raf/MEK/ERK signaling is frequently hyper-activated in a high percentage of tumors (McCubrey et al., 2007). Based on our understanding of the molecular mechanisms of the initiation and progression of melanoma, more than half of malignant melanomas have been known to contain B-Raf mutations, almost all of which are V600E (http://www.sanger.ac.uk/genetics/CGP/cosmic). It has been known that the activation of B-Raf promotes resistance to apoptosis (Dhomen and Marais, 2009). Thus, B-Raf inhibitors show promise for the treatment of melanomas that express mutant B-Raf (V600E). The first Raf-targeting drug to be used clinically was sorafenib, which proved ineffective in melanomas in which B-Raf is mutated in clinical trials (Eisen et al., 2006). PLX4720/4032 was developed as a selective mutant B-Raf inhibitor, which leads to growth arrest in mutant B-Raf melanoma cells (Tsai et al., 2008). We also previously demonstrated that the new oncogenic B-Raf-targeting drug, UI-152, induces G\textsubscript{1} phase cell-cycle arrest and leads to apoptosis in in vitro melanoma models (Ahn et al., 2012; Kim et al., 2012). UI-152 was developed as a potent ATP-competitive inhibitor of Raf proteins by structure-based drug design (http://www.youai.co.kr/pipeline/uai_201.html). In biochemical assays, it was found that UI-152 inhibited B-Raf (V600E) at very low nanomolar concentration (IC\textsubscript{50}=2 nM) with excellent selectivity for Raf kinases relative to other kinases and was 10-fold more potent than PLX4032/4720 in the inhibition of B-Raf (V600E) (unpublished results). Especially, in cellular assays, UI-152 was more than 1,000-fold more selective in inhibiting proliferation of tumor cell lines bearing B-Raf (V600E) than the wild-type B-Raf-bearing cells (Ahn et al., 2012).

Currently, the limited clinical benefit of anticancer drugs therapies arises from the fact that responsive tumors even-
ultimately acquire drug resistance. Also, the duration of clinical response with selective B-Raf inhibitors is short in many patients due to acquired resistance to oncogenic B-Raf inhibitor (Flaherty et al., 2010). 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 overexpression of MAP kinase kinase 8 (MAP2K8; COT) (Johannessen et al., 2010), mutations in N-Ras, and PDGFRα overexpression (Nazarian et al., 2010). A recent study has suggested that autophagy is required for the induction of necrotic cell death in cells that are unable to activate apoptosis (Ullman et al., 2008). The Ras/ Raf-1/MEK/ERK cascade is the signaling pathway recently associated with autophagy regulation. Ng and Huang (2005) reported that the inactivation of Raf-1 resulted in the significant decrease in autophagy induction. One study has also reported that amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 (Pattingre et al., 2003).

Thus, understanding the resistance mechanism can provide clues for the potential of UI-152 as an effective anticancer agent to treat malignancies that resistant, either used alone or in association with autophagy-blocking treatments. Here, we used chronic UI-152 treatment in vitro to generate resistant derivatives of B-Raf (V600E) melanoma cell lines. This model cell line was used in vitro to understand acquired resistance mechanisms after the initial response to UI-152. The present study implicates high rates of autophagy as a key mechanism of acquired resistance to the oncogenic B-Raf inhibitor. Moreover, our data suggest that inhibition of autophagy in combination with a selective Raf inhibitor offers a more effective therapeutic strategy for melanoma.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Polyclonal anti-p21(Cip1), anti-p27(Kip1) and anti-MDR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Apoptosis kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS) and penicillin-streptomycin were purchased from GIBCO-Invitrogen (Carlsbad, CA, USA). Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Hercules, CA, USA). Wortmannin and 3-methyladenine (3-MC) were obtained from Sigma (St. Louis, MO, USA). B-Raf targeting drug UI-152 was obtained from YOUI Co., Ltd. (Suwon-Si, Gyeonggi-Do, Korea). UI-152 was dissolved in DMSO and freshly diluted for each experiment. DMSO concentrations were less than 0.1% in all experiment.

**Generation of melanoma cells resistant to Raf inhibitors-induced apoptosis from B-RAFV600E melanoma cell lines**

Human A375P melanoma cells harboring B-Raf (V600E) were cultured in DMEM supplemented with 10% FCS, penicillin-streptomycin, and glutamine. Cell lines with acquired resistance to UI-152 were generated by propagating parental A375P cells in medium containing 1 μM UI-152.

**Cell growth assay**

The cells were plated in quadruplicates into 96-well microplates (Costar, Cambridge, MA, USA) at 5x10^3 cells/well and then treated with either UI-152 or PLX470 at 37°C in a humidified 5% CO,95% air incubator. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, MTT dissolved in 0.8% NaCl solution at 5 mg/ml was added to each well (0.2 ml) on day 3, and the cells were incubated at 37°C for 3 h. The supernatants in the wells were carefully aspirated and replaced with 100 μl of isopropanol supplemented with 0.05 N HCl to solubilize the reacted dye. The absorbance of the samples against a background control (medium alone) as a blank was measured at 450 nm using a microculture 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 cycle distribution was examined by measuring the DNA content using a Gallios flow cytometer and Kaluza analysis software (Beckman Coulter, Inc., Brea, CA, USA). A minimum of 10^4 cells per data point were examined.

**Immunofluorescence staining**

For immunofluorescence experiments, cells were grown on chamber slides (Nunc), and fixed in 10% formalin solution for 10 min as described (Ahn et al., 2010). Samples were blocked with blocking solution (1% BSA, 0.6% Triton X-100 in PBS) for 1 h and incubated with primary antibodies diluted in blocking solution for 16 h at 4°C. Texas Red-coupled anti-rabbit IgG was used to detect MDR proteins while DAPI was used to stain nuclei. Photographs were taken at 1000X magnification through a Zeiss Axio Scope. A1 epifluorescence microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA).

**Quantitation of autophagy**

The cells were grown on chamber slides (Nunc), washed with PBS, and fixed in 10% formalin solution for 10 min. Cells were transfected with pEGFP-LC3 (Addgene, Cambridge, MA, USA) for 48 h and then treated with UI-152 for 24 h. Fixed cells were classified as cells with predominantly diffuse GFP-LC3 fluorescence or 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 among 200 GFP-positive cells in two independent fields.

**Caspase-3 activity for apoptosis assay**

Activation of caspase-3 was determined by detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA (ApoAlert Caspase-3 Colorimetric Assay; Clontech Laboratories, Mountain View, CA, USA). Briefly, an aliquot of cell suspension (2×10^5 cells/ml) after treatment with UI-152 for 24 h was washed with PBS and centrifuged (400×g, 10 min, 4°C). Cells were then lysed in 50 μl lysis buffer. After centrifugation at 1,000×g, 3 min, 4°C, the supernatants were incubated with DEVD-pNA for 1 h at 37°C, and then optical density was measured at 405 nm. Caspase activity was defined as nmol
pNA/h/mg of protein. The protein concentration was determined with a BCA protein assay reagent kit as described by the manufacturer (Pierce; Rockford, IL, USA).

**Preparation of cell lysates and immunoblot analysis**

For the preparation of cell lysates, cells were harvested by scraping the cells into RIPA lysis buffer. The cell lysates were clarified by centrifugation at 15,000 × g for 10 min, and the protein concentrations were determined using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). The whole-cell lysates were subjected to immunoblot analysis using the appropriate primary antibodies. The immune complexes were detected with the ECL-Plus chemiluminescent system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Fluorescent images were captured using the KODAK Image Station 4000R (Carestream Health, Inc., Rochester, NY, USA). The protein bands were quantified with Kodak Molecular Imaging software, version 4.5.0 (Carestream Health, Inc.).

**RESULTS**

**Chronic B-Raf inhibition leads to acquired drug resistance**

To investigate whether chronic B-Raf inhibition could lead to acquired drug resistance, human A375P melanoma cell lines harboring the V600E mutation in the B-raf gene were chronically treated with increasing concentrations of the specific B-Raf inhibitor UI-152. The MTT assay showed that, whereas parental A375P cells were highly sensitive to B-Raf inhibition by UI-152 (IC	extsubscript{50} < 0.5 μM), melanoma cells that had been chronically treated with UI-152 required higher doses of the drug for partial growth inhibition (IC	extsubscript{50} > 20 μM) (Fig. 1A). UI-152-resistant A375P/Mdr cells exhibited growth rates similar to those of UI-152-sensitive A375P cells, even when grown in the presence of UI-152. Additionally, they became resistant to another Raf inhibitor PLX4720 (Fig. 1B); therefore, a typical MDR phenotype was induced. This model cell line was then used to investigate the molecular mechanisms of acquired resistance after the initial response to oncogenic Raf inhibitor.

**The drug resistance observed in A375P/Mdr cells is not mediated through overexpression of the P-glycoprotein**

The loss of efficacy of anticancer therapy is predominantly correlated with the overexpression of P-glycoproteins that actively efflux chemotherapeutic drugs (Gottesman, 2002). Thus, we investigated whether the increased expression of P-glycoprotein plays a role in conferring acquired resistance. We first examined whether verapamil affects cell proliferation in A375P/Mdr cells. A calcium channel blocker verapamil has been reported to inhibit the transport function of P-glycoprotein (Nobili et al., 2006). The addition of verapamil had no effect on the UI-152 resistance of A375P/Mdr cells (Fig. 2A), suggesting that upregulation of MDR activity does not contribute to resistance. Additionally, immunofluorescence staining showed no increase in the levels of the P-glycoprotein MDR transporter in A375P/Mdr cells (Fig. 2B).

**Cell cycle and apoptosis**

Since ERK activity in B-Raf-mutated melanoma cells is capable of driving cellular proliferation through dysregulation of the cell cycle, cell cycle and apoptosis assays were performed before and after exposure to UI-152. Flow cytometric analysis of UI-152-treated populations revealed significantly higher numbers of cells in the G	extsubscript{0}/G	extsubscript{1} phases as compared to fractions of untreated cells. UI-152 treatment induced G	extsubscript{0}/G	extsubscript{1} cell-cycle arrest and decreased the percentage of cells in the S and G	extsubscript{2}/M phases in the A375P cell line (Fig. 3A). UI-152 treatment of A375P/Mdr cells also similarly increased the proportion of G	extsubscript{0}/G	extsubscript{1} cells, but to a lesser extent. To examine the molecular mechanisms underlying the changes in cell cycle progression, we investigated the expression of the cyclin-dependent kinase inhibitors (CKIs) p21	extsuperscript{Cip1} and p27	extsuperscript{Kip1} (Fig. 3B). We detected a marked induction of p27	extsuperscript{Kip1} in A375P cells. However, UI-152 only moderately increased the levels of p27	extsuperscript{Kip1} in A375P/Mdr cells, which caused a lower extent of cell-cycle arrest than that seen in A375P cells. UI-152 only moderately increased the levels of p21	extsuperscript{Cip1} in both cell lines, implying that the senescence signals mediated by UI-152 selectively target p27	extsuperscript{Kip1}. Evaluation of apoptosis showed that UI-152 induced a predominantly apoptotic response in A375P cells but not in A375P/Mdr cells, as measured by caspase-3 activity, one of the hallmark events in apoptotic processes (Fig. 3C). Together, these data show that prolonged exposure to UI-152 renders the A375P/Mdr cell line resistant to apoptosis.
Autophagy induction leads to drug resistance in A375P/Mdr cells

Recent studies reported that melanoma cells with oncogenic B-Raf and hyper-activation of ERK show higher levels of autophagy (Maddodi et al., 2010). In addition, our previous studies have shown that autophagy might serve as a protective mechanism in cancer cells (Ahn and Lee, 2011; Ahn et al., 2012). Thus, to define the cellular response associated with UI-152 resistance, we investigated the effects of UI-152 on autophagy in A375P/Mdr cells. Quantitation of autophagy was performed based on the percentage of GFP-LC3-positive autophagic vacuoles or cells with GFP-LC3 punctate dots.

LC3 is now believed to be a reliable marker for autophagy, the lysosome-mediated form of cell death (Kabeya et al., 2000). As shown in Fig. 4A, UI-152 caused a dose-dependent increase in the number of cells with GFP-LC3 punctate dots in both of A375P and A375P/Mdr cells. However, most sur-

Fig. 2. P-glycoprotein was not involved in the resistance to UI-152 in A375P/Mdr cells. (A) To determine the effect of verapamil on the resistance to UI-152, increasing concentrations of UI-152 with or without verapamil were added to A375P/Mdr cells. The cell growth rate was measured by the MTT assay. The relative proliferation rate of cells treated with vehicle alone was regarded as 100%. Values represent the mean ± SD of quadruplicate determinants from 1 of 3 representative experiments. (B) Immunofluorescence analysis was performed to detect the expression of MDR proteins (P-glycoprotein) in A375P/Mdr cells. MDR proteins were immunostained with anti-MDR antibody, and subsequently reacted with anti-rabbit IgG-Texas Red, respectively. Nuclei were counterstained with DAPI. Fluorescence was captured by fluorescence microscopy. Red depicts MDR expression; blue depicts DAPI nuclear staining. The results presented are representative of at least 3 independent experiments performed under the conditions described.

Fig. 3. Detection of G0/G1 arrest and apoptosis in cells treated with UI-152. (A) Cell cycle progression was assessed by staining fixed cells with propidium iodide to estimate the percentage of cells in the G0/G1 (2N DNA content), G2/M (4N DNA content), and S phases (2 to 4N DNA content). The percentage of cells in each phase of the cell cycle was quantitated using Kaluza analysis software. (B) The expression of p21Cip1 and p27Kip1 were assessed by immunoblotting using whole cell lysates prepared from cells treated with UI-152 (1 μM) for the indicated times. The pro-apoptotic activity of caspase-3 was determined by detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The caspase-3 activity of cells treated with vehicle alone was regarded as 100%. Values represent the mean ± SD of duplicate determinants from 1 of 3 representative experiments. **p<0.01 as determined by the Dunnett’s T-test.
prisingly, autophagy was more highly induced in A375P/Mdr cells. Autophagic cells were observed in 30.5% of A375P/Mdr cells, compared with 12.3% of A375P cells at 72 h after UI-152 treatment. These results suggest that highly induced autophagy in response to UI-152 may lead to cell survival in A375P/Mdr cells. To further investigate whether autophagy contributes to UI-152 resistance in A375P/Mdr cells, 3-methyladenine (3-MA) and wortmannin were introduced as specific autophagy inhibitors (Seglen and Gordon, 1982). A group of phosphoinositide 3-kinase (PI3K) inhibitors, such as 3-methyladenine (3-MA) and wortmannin, have been widely used as autophagy inhibitors based on their inhibitory effect on class III PI3K activity, which is known to be essential for the induction of autophagy (Petiot et al., 2000; Wu et al., 2010). Autophagy inhibition with 3-MA augmented the growth inhibition of A375P/Mdr cells exposed to UI-152 at the lower dose range (Fig. 4B), suggesting that autophagy is an important survival mechanism. However, wortmannin failed to sensitize A375P/Mdr cells towards UI-152 (Fig. 4C). Overall, these data do not offer strong support for the PI3K pathway contributing to the growth and survival of UI-152-resistant A375P/Mdr cell line.

**DISCUSSION**

Drug resistance is a common problem associated with chronic treatment with anticancer drugs (Pao et al., 2005; Engelman and Jänne, 2008). Regardless of the encouraging results in the treatment of B-Raf (V600E) melanoma cells, the emergence of acquired resistance to inhibitors of oncogenic B-Raf also limits their effectiveness in the treatment of melanoma (Flaherty et al., 2010). The mechanisms by which cancer cells become multidrug-resistant are widely known to be correlated predominantly with the overexpression of the P-glycoprotein efflux pump (Gottesman, 2002). Thus, the development of compounds that block P-glycoprotein-mediated efflux has been the conventional approach used to overcome MDR (Wu et al., 2008). However, we demonstrated that the drug resistance observed in A375P/Mdr cells was not mediated through the overexpression of MDR proteins. Recent reports have suggested several mechanisms that counteract PLX4032 effectiveness, all of which bypass B-Raf (V600E). These mechanisms include the overexpression of COT proteins (Johannessen et al., 2010), mutations in N-Ras that activate Raf-1, or the expression of PDGFRβ (Nazarian et al., 2010). However, the prevalence of these mechanisms remains uncertain, as too few patient samples have been analyzed. In any case, the fact that melanoma develops acquired resistance to the oncogenic B-Raf inhibitor emphasizes the importance of simultaneously targeting several pathways. Reactivation of the ERK signaling pathway as a resistance mechanism may warrant the addition of MEK inhibition to supplement the ongoing inhibition of mutated B-Raf to optimally re-suppress the pathway and consequently overcome resistance. Actually, Su et al. (2012) reported that combination treatment with PLX4032 and MEK inhibitor synergistically inhibited the proliferation of resistant cells. However, Jiang et al. (2011) suggested that rebound melanoma growth after initial treatment with a B-Raf targeted drug may not be responsive to MEK inhibitors. In our resistant cell lines, ERK signaling is at least partially sensitive to Raf inhibitors (data not shown), suggesting that the acquisition of resistance might be due to the activation of other pathways that reduce the dependence of the cell on B-Raf signaling.

In this study, B-Raf (V600E) melanoma clones with acquired resistance were derived by chronic selection with increasing doses of the new oncogenic B-Raf inhibitor UI-152. In UI-152-sensitive A375P cells, flow cytometric analysis of UI-152-treated populations revealed significantly higher numbers...
of cells in the G1/G0 fractions with a concomitant decrease in S-phase cells compared with the cell cycle profile of the vehicle control group. It has previously been shown that an aberrant mitotic arrest triggers the intrinsic apoptotic pathway, independent of the cellular target of the drug (Marcus et al., 2005; Blagosklonny, 2007). Actually, UI-152 resulted in the progressive emergence of apoptotic cells in A375P cells. Unexpectedly, UI-152 treatment of A375P/Mdr cells also similarly increased the proportion of G1/G0 cells, but to a lesser extent. However, A375P/Mdr cells were found to be resistant to the apoptosis mediated by UI-152. More interestingly, UI-152 preferentially induced autophagy in A375P/Mdr cells but not in A375P cells, as determined by GFP-LC3 puncta/cell counts, suggesting that highly induced autophagy in response to UI-152 may lead to cell survival in A375P/Mdr cells. In addition, these results indicate that the growth inhibitory activity of UI-152 is predominantly due to a cytotoxic, rather than a cytostatic, effect.

Understanding autophagic changes in B-Raf mutant cells has important therapeutic implications due to the ability to both inhibit and stimulate autophagy in malignant melanoma using clinically available drugs. However, whether autophagy induction in cancer cells is cytoprotective or cytotoxic is an ongoing debate in the literature. Our previous results indicated that the inhibition of autophagy might offer a more effective therapeutic strategy for v-Ha-ras-transformed cells (Ahn and Lee, 2011; Ahn et al., 2012). Recent studies also suggest that a high “autophagic index” in melanoma patient tumor biopsies can be linked to poor response to therapy and shorter survival (Ma et al., 2011). Consistent with these previous reports, our results suggest that autophagy induction might play an important role in drug resistance to B-Raf inhibitors.

On the other hand, the role of B-Raf in the regulation of autophagy is controversial; ERK signaling is associated with autophagosome-lysosome fusion (Corcelle et al., 2006), and autophagic cell death (Cagnol and Chambard, 2010). Furthermore, overexpression of mutant B-Raf in melanoma cells results in autophagy induction (Maddodi et al., 2010), suggesting that activated B-Raf promotes autophagy. Especially, the crosstalk between the Ras/Raf and the LKB1/AMPK/mTOR signaling pathways appears to regulate the cellular response to autophagy-inducing signals (Karbowniczek et al., 2004). Activation of the PI3K-Akt-mTOR signaling pathway is known to promote necrotic cell death via suppression of autophagy (Wu et al., 2010). However, PI3 kinase inhibitor wortmannin failed to sensitize A375P/Mdr cells towards UI-152. Conversely, autophagy inhibition with 3-MA partially augmented the growth inhibition of A375P/Mdr cells exposed to UI-152. These results imply that regulation of autophagy by UI-152 is PI3K/mTOR-independent. In fact, several autophagy inducers are known to employ an mTOR-independent pathway (Shintani et al., 2010). In particular, calpain-regulated autophagy, where cAMP regulates inositol 1,4,5-trisphosphate (IP3) levels, appeared to be independent of the known pathway that is negatively regulated by mTOR (Williams et al., 2008). However, a more detailed mechanism by which UI-152 induces autophagy remains unclear.

Our data support autophagy as an important mechanism of resistance to oncogenic B-Raf inhibitors in mutant B-Raf melanoma cells, implying the therapeutic potential of autophagy inhibitors in resistance to B-Raf inhibition. However, acquired resistance mechanisms to these agents still need to be investigated, as well as combination therapies with simultaneous inhibition of different pathways. Understanding the network of signal transduction pathways and the heterogeneity within tumor specimens will direct drug development for efficient B-Raf targeting in the future.

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REFERENCES


