Regulation of Tumor Signaling Pathways by AZD3409 *In Vitro*

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Abstract. *Background: The antineoplastic activity of AZD3409 was evaluated in relation to paclitaxel in human breast (MDA-MB-231, BT-474) and ovarian (A2780, A2780cp) cancer cell lines. Biomarkers of apoptosis, protein prenylation, survival, angiogenesis and cellular growth were determined. Materials and Methods: Cytotoxicity was evaluated by MTS assay, and apoptosis was evaluated by TUNEL. Biomarkers were measured by Western blots and ELISA. Results: The IC_{50} concentrations of AZD3409 in MDA-MB-231, BT-474, A2780 and A2780cp were 19.16, 5.69, 3.19, and 8.86 μM, respectively. The corresponding apoptogenic EC_{50} concentrations were 6.81, 4.15, 1.54 and 4.59 μM. Conclusion: Farnesylation of HDJ-2 was inhibited in all cell lines. Secretion of VEGF, bFGF and MMP-1 were inhibited in the breast lines but augmented in the ovarian lines. AZD3409 increased Akt activation in breast lines and decreased it in ovarian lines, without effect on MEK or ERK activation. AZD3409 cytotoxicity is mediated in part by inhibition of farnesylation.*

AZD3409 is a novel antiproliferative agent with a potentially broad range of anti-tumor activity. Enzymological studies have shown AZD3409 to be an inhibitor of both farnesyl transferase (FTase) and geranylgeranyl transferase-1 (GGTase-1) enzymes with modest selectivity for FTase. Studies of cultured cells, however, have shown that AZD3409 inhibits FTase almost exclusively.

AZD3409 has been shown to inhibit farnesylation of H-Ras and K-Ras in tumor cell lines. Substantial experimental evidence has linked farnesylation of Ras with malignancy. FTase-catalyzed farnesylation of Ras initiates translocation of Ras to the cell membrane and activates the Ras signal transduction cascade. AZD3409 inhibits H-Ras prenylation and causes H-Ras transformed fibroblasts in culture to revert to the contact inhibited phenotype. It also inhibits the growth of H-Ras transformed fibroblasts and several human tumor xenografts in nude mice (1). Ras mutation status does not, however, necessarily correlate with FTI sensitivity. For example, K-Ras can escape inhibition of processing by FTIs. It is now thought that Ras is probably not the most important FTI target. Downstream effectors of Ras that bind Ras in a GTP-dependent manner through the effector domain loop are likely to be affected by FTIs. These include the Raf family of proteins and the protein kinases MEK and ERK. Recently, the phosphoinositide 3-OH kinase (PI3K)/Akt2 pathway has been identified as a critical target for FTI induced apoptosis (2). FTI inhibition of tumor growth may in part be explained by induction of apoptosis through inhibition of the PI3K/Akt2 mediated cell survival and adhesion pathway (3).

It is likely that inhibition of the processing of other farnesylated proteins is responsible for the apoptotic and antineoplastic activities of FTIs (4). The effects of FTIs on the centromere-binding proteins CENP-E and CENP-F have been linked with cell cycle arrest, apoptosis, and tumor regression in preclinical studies (5). FTIs have been shown to suppress farnesylation of DJ-1 and DJ-2, homologs of the *E. coli* heat shock protein DnaJ, which are ubiquitously expressed and farnesylated. Farnesylation of human DJ-2 (HDJ-2) in peripheral blood mononuclear cells has been used as a surrogate biomarker of FTI activity in clinical trials (6).

FTIs have been shown to inhibit angiogenesis *in vitro* and *in vivo*. It has been proposed that inhibition of Ras farnesylation by FTIs can induce two events that alter host-tumor interactions; up-regulation of Fas, rendering tumors more sensitive to cytotoxic effector cells, and down-regulation of VEGF, which may inhibit tumor angiogenesis (7).

The mechanisms by which FTIs inhibit tumor growth are more complex than was originally surmised, thus, we have undertaken the determination of a range of biomarkers in an effort to cast additional light on the complexities of the cellular effects of the FTI AZD3409.

AZD3409 was evaluated in human breast cancer cells having differing levels of Her2/neu expression and in human ovarian cancer cells differing in degree of sensitivity to

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Table I. *Growth inhibitory activity of AZD3409 and paclitaxel in human tumor cell lines.*

Cell line	AZD3409 IC_{50} (μM)	Paclitaxel IC_{50} (nM)
MDA-MB-231	19.16±0.05	2.41±0.20
BT-474	5.69±0.55	6.52±1.44
A2780	3.19±0.41	3.85±1.85
A2780cp	8.86±0.12	8.35±0.63

Table II. Apoptogenic activity of AZD3409 and paclitaxel in human tumor cell lines.

Cell line	AZD3409 EC_{50} (μM)	Paclitaxel EC_{50} (nM)
MDA-MB-231	6.184	5.886
BT-474	4.145	4.517
A2780	1.542	3.914
A2780cp	4.593	11.77

cytotoxic drugs and radiation. MDA-MB-231 (not Her2/neu overexpressing), BT-474 (Her2/neu overexpressing) breast cancer lines and A2780 (radiation and cisplatin sensitive) and A2780cp (radiation and cisplatin resistant) ovarian cancer cell lines were treated with AZD3409 and paclitaxel.

Materials and Methods

Cell culture. BT-474 and MDA-MB-231 human breast cancer cells (American Type Culture Collection, Rockville, MD, USA) were maintained in a 1:1 mixture of DMEM : F12 (Mediatech Inc., Herndon, VA, USA), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone (Invitrogen Corp., Carlsbad, CA, USA) and 10% fetal bovine serum (Invitrogen), and incubated in a humidified incubator at 37˚C in 5% CO_2/95% HEPA filtered air.

Drug treatment. AZD3409 (AstraZeneca, Cheshire, UK) and paclitaxel (Sigma Chemical Company, St. Louis, MO, USA) were prepared as stock solutions in dimethylsulfoxide (DMSO; Sigma Chemical Co.). The drug solutions were diluted into media to a final DMSO concentration of 0.1%, and added on day zero to the cells.

Apoptosis assay. The apoptogenic effects of the drug treatments were evaluated by TUNEL analysis (8). Cells were exposed to the drugs for 48 hours. Drugs were administered at eight serial dilutions with six replicates at each concentration. The EC_{50} values were calculated using PRISM™ GraphPad Software.

Growth inhibition assay. The MTS assay was used to evaluate the growth inhibitory effects of the drugs using an established protocol (8). Isoeffect plots were generated as previously described (9). The results are the average of three determinations.

Antibodies. Polyclonal antibodies were obtained from the following sources: anti-MAP kinase and anti-ERK1/2 (Sigma Chemical Co.); anti-MEK1/2 and anti-phospho-MEK1/2 (Ser217/221), anti-Raf-1 (C-12) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-phospho-Raf (Ser259), anti-Akt and anti-phospho-Akt (Ser473) (Cell Signaling Technology, Beverly, MA, USA); and anti-PI 3-kinase p85 (Upstate Biotechnology, Lake Placid, NY, USA). Monoclonal antibodies used were: anti-RhoB (C-5) (Santa Cruz Biotechnology), anti-HDJ-2/DNAJ (Neomarkers, Fremont, CA, USA) and anti-β-actin (Ab-1) (Oncogene Research Products, Boston, MA, USA). The antibodies were used for immunoblotting as described by Izbicka *et al.* (9).

Immunodetection of biomarkers. Cells were seeded in six0well plates (Falcon, Fisher Scientific, Pittsburgh, PA, USA) and treated with 1 nM paclitaxel, 2 μM AZD3409, or the vehicle on day zero. Four sets of plates for each cell type were prepared with one set of plates taken for analysis each day. Media were collected for quantitative analysis of secreted factors by ELISA. Cell processing, protein assays, gel electrophoresis and immunodetection of biomarkers were performed as described by Izbicka *et al.* (9). The densitometric intensity of analyte bands was normalized to the intensity of the actin band in each sample lane. The ratios of phosphorylated or prenylated species to the total protein were calculated for selected proteins. The results are the averages of three determinations.

ELISA. Cell culture media were collected once every 24 hours for 4 days. The drugs were added on day zero. The collected culture media were centrifuged to remove cellular debris and analyzed by ELISA using the following human-specific kits: MMP-2 (Amersham Biosciences, Piscataway, NJ, USA), MMP-1, MMP-9, VEGF and basic FGF/FGF-2 (R&D Systems, Minneapolis, MN, USA) according to suppliers' instructions. All assays were performed in duplicate.

Results

Cytotoxicity. MTS assays were conducted after 72 hours of continuous exposure of the cells to AZD3409 or paclitaxel. As presented in Table I, BT-474 cells were about three-fold more sensitive to AZD3409 (5.69 μM) than MDA-MB-231 cells (19.16 μM). On the other hand, paclitaxel was approximately three times more toxic to MDA-MB-231 cells (2.41 nM) than it was to BT-474 cells (6.52 nM).

Apoptosis assay. The apoptogenic effects of AZD3409 and paclitaxel as measured by EC_{50} are presented in Table II. The EC_{50} values for AZD3409 are similar in the breast cancer cell lines. For MDA-MB-231 cells this comparability of EC_{50} values stands in contrast to the very different IC_{50} values discussed above, which lends support the hypothesis that AZD3409 exerts pharmacological effects on several different targets and/or that there are alternative prenylation or cellular survival escape pathways (10). In the ovarian cancer cell lines similar two- to three-fold differential effects for both drugs were observed between the A2780 line and

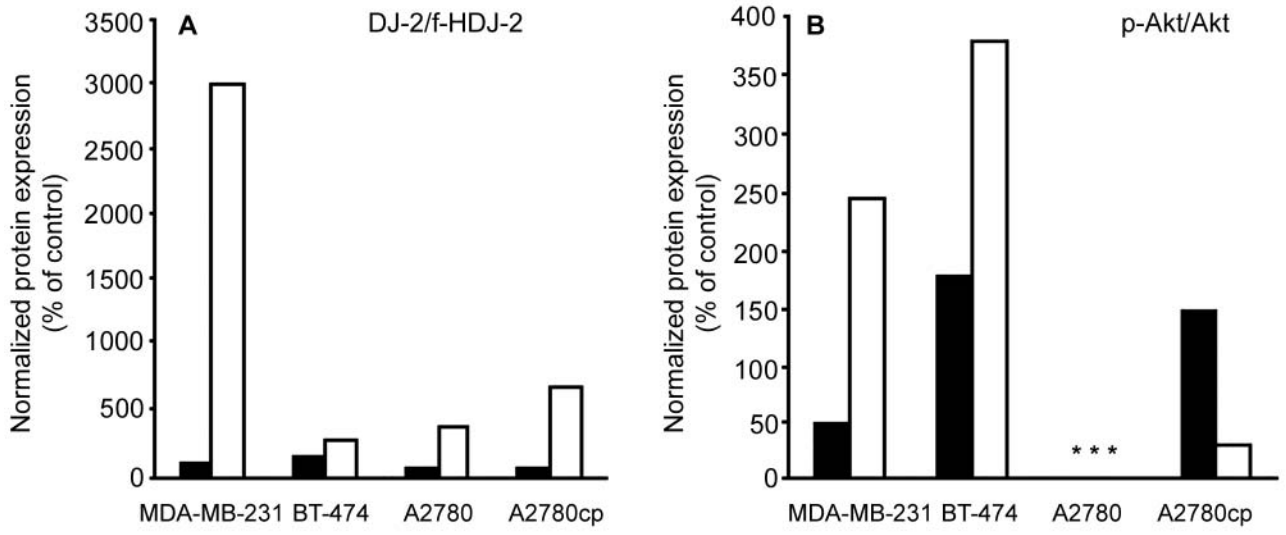

Figure 1. ■ *Paclitaxel,* □ *AZD 3409. A) HDJ-2 farnesylation relative to control following 4 days' treatment with AZD3409 or paclitaxel derived from densitometric analysis of western blot data normalized to β-actin. All lines demonstrated significant suppression of HDJ-2 farnesylation. B) Activation of Akt derived from densitometric analysis of western blot data normalized to β-actin relative to control. Cells were exposed to the drugs for four days. AZD3409 significantly suppressed Akt signaling in both ovarian cancer cell lines and augmented activation in both breast cancer cell lines.*

the A2780cp line, *i.e.,* 1.54 μM *vs.* 4.59 μM for AZD3409 and 3.91 nM *vs.* 11.77 nM for paclitaxel. Thus, the A2780cp cells were approximately three times more resistant to the apoptogenic effects of both drugs.

Prenylation biomarkers. To determine effect of the drugs on prenylation of HDJ-2, cells were treated with AZD3409 or paclitaxel and harvested daily for four consecutive days. Quantitative analysis of the data is summarized in Figure 1. As shown by gel-shift farnesylation assays of the cell extracts, paclitaxel had little effect on HDJ-2 post-translational modification by FTase in all of the cell lines studied. In contrast, after treatment with AZD3409 the ratios of unfarnesylated to the farnesylated HDJ-2 were over 30-fold higher than the controls in the BT-474 line. AZD3409 increased the ratio of HDJ-2/fHDJ-2 significantly less than that observed for MDA-MB-231 cells. AZD3409 increased the HDJ-2/fHDJ-2 ratio 5- and 7-fold in the A2780 and A2780cp lines, respectively.

Biomarkers of tumor growth. The Ras/Raf/MEK (mitogen-activated protein kinase/ERK kinase)/ERK (extracellular-signal-regulated kinase) pathway is central to signaling networks that control proliferation, differentiation and tumor cell survival. It has been proposed that inhibition of FTase activity would translate to decreased phosphorylation of Raf, MEK and ERK. No significant effect was observed on the p-Raf to Raf ratio relative to controls at any time-point in any of the cell lines. Activation of MEK, expressed as the p-MEK to MEK ratio, was not affected by the drug treatments in any of the cell lines studied. Activation of ERK, expressed as the ratio of p-ERK to ERK showed a significant difference between AZD3409 treatment and control groups in the BT-474 and A2780 cells. Paclitaxel produced no significant alteration of Raf, MEK or ERK activation in any of the cell lines studied (data not shown).

Biomarkers of tumor survival. Akt signaling, as measured by the ratio of p-Akt to Akt, was dramatically increased by AZD3409 in both breast cancer lines and significantly suppressed in both ovarian cancer lines (Figure 1). Paclitaxel suppressed Akt activation in MDA-MB-231 but augmented it in BT-474. Paclitaxel also significantly augmented Akt activation in A2780cp cells. This observation is reflected in the higher relative EC_{50} value for paclitaxel determined in the apoptosis assay. Both paclitaxel and AZD3409 suppressed Akt activation to levels below the method detection limit in A2780 cells.

Biomarkers of angiogenesis. Since FTIs modulate tumor signaling pathways responsible for angiogenesis, we examined the effects of the drugs on secretion of pro-angiogenic growth factors. The levels of secreted bFGF and MMP-9 were below the detection limits of the assay in all treatment groups in all cell lines. MMP-2 levels were all either below the method detection limit (MDL), or were not significantly different from background. As shown in Figure 2, VEGF secretion in MDA-MB-231 and BT-474 cells was not significantly affected by either drug treatment. In both breast cancer lines MMP-1 was the only marker measured

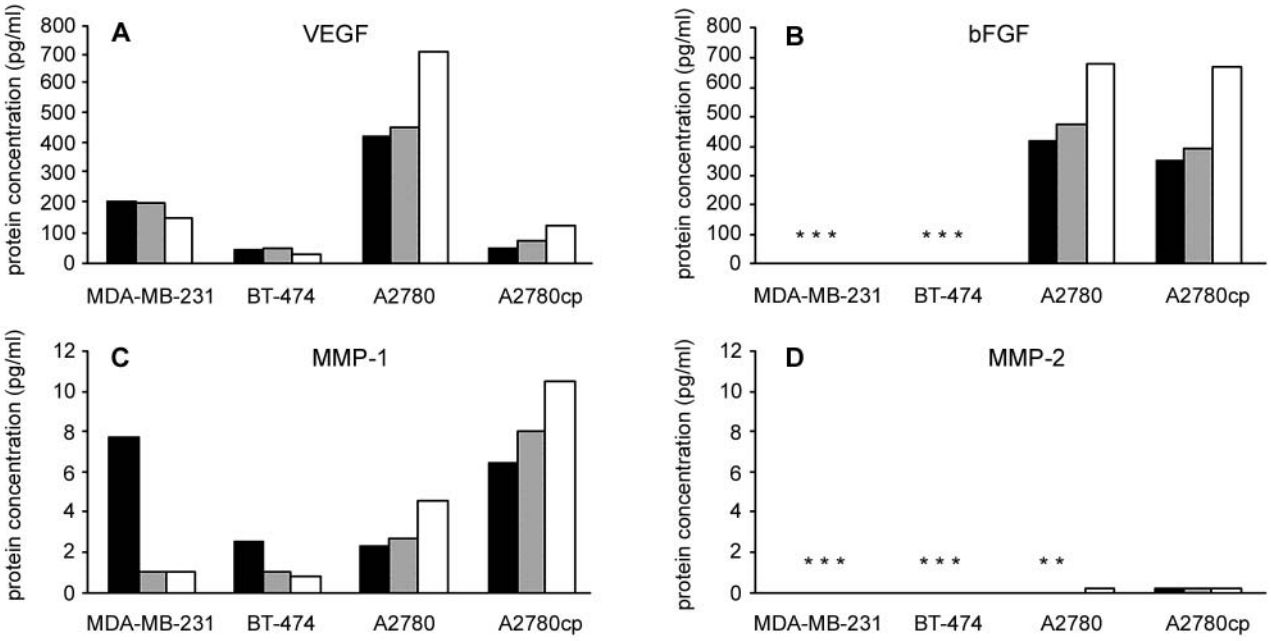

Figure 2. ■ *Control,* ▒ *Paclitaxel,* □ *AZD 3409. Effect of 4 days' treatment with AZD3409 or paclitaxel on the expression of VEGF (A), bFGF (B), MMP-1 (C) and MMP-2 (D) in examined cell lines as determined by ELISA. Data are the mean of two determinations. Markers of neovascularization are significantly augmented by both drugs in the ovarian cancer lines. MMP-1, indicative of pro-metastatic activity, was suppressed by both drugs in the breast cancer lines and augmented in both ovarian cancer lines.*

that was significantly suppressed relative to control by the drugs. The VEGF, bFGF and MMP-1 levels measured in the ovarian cancer models A2780 and A2780cp all increased in response to both drug treatments.

VEGF expression in both breast cancer cell lines was relatively unaffected by treatment with either drug. MMP-1 expression was suppressed by both AZD3409 and paclitaxel to nearly the same degree. MMP-2, MMP-9 and bFGF levels were all below the MDL in both lines.

Expression of VEGF, bFGF and MMP-1 were significantly increased by both drugs in the ovarian cancer lines. AZD3409 increased the expression of these markers to a greater degree than did paclitaxel in both lines. MMP-2 and MMP-9 were both below or insignificantly above the limit of detection.

Discussion

AZD3409 has been shown to inhibit prenylation of the biomarker HDJ-2 in the human cancer cell lines that were tested. The inhibition of prenylation was more pronounced in the breast cancer model with a low expression level of HER2/*neu* than in the overexpressing cells. The extent of inhibition most likely reflects the stoichiometric ratio of the enzyme (FTase) to the inhibitor (AZD3409) in the cells. In both breast cancer cell lines, no clear effect was observed with paclitaxel, consistent with the expected mechanism of action of AZD3409. The ovarian cancer lines both showed increases in the HDJ/f-HDJ ratio, with a 4 fold increase observed for A2780 cells and a 7-fold increase for the A2780cp cells.

Several studies have investigated HDJ-2 as an end-point of FTI activity (6, 11-13). The use of HDJ-2 farnesylation in PBMC as a surrogate pharmacodynamic assay has been shown to be feasible in the context of Phase I evaluations of AZD3409, but the correlation of the farnesylation of HDJ-2 to antitumor effects is unclear.

FTIs block the post-translational modification of Ras and interfere with subsequent activation of downstream effectors. Although initially developed to target Ras, the effects of FTIs extend beyond Ras, involving RhoB, centromere binding proteins and other farnesylated proteins. The hypothesis that therapy with AZD3409 could be of benefit in the treatment of patients with HER2/*neu* expressing breast cancers has been examined. Amplification of the HER2 gene and overexpression of the HER2 protein induces cell transformation and has been demonstrated in 10% to 40% of human breast cancers. HER2 overexpression is associated with high tumor aggressiveness, poor clinical prognosis and poor clinical response to hormonal and cytotoxic agents. The mechanisms that underlie these observations are unclear, although increased tumor cell proliferation, increased blood vessel formation, and invasiveness or metastatic potential have all been suggested.

The cell viability studies demonstrated that AZD3409 is more toxic to human breast cancer cell lines overexpressing HER2/*neu* than it is to cells with low HER2/*neu* levels. HER2 overexpression seems to be a significant predictor of response to taxanes. Given the resistance of HER2/*neu*-overexpressing cancers to many therapies, this finding may be of significance for the application of AZD3409 in the management of these types of tumors. The EC_{50} values for all cell lines in response to AZD3409 treatment were similar with the exception of A2780 cells which were roughly three times more sensitive to the apoptogenic effects of the drug. A2780cp cells were approximately half as sensitive to AZD3409 relative to the A2780 cells. In the case of MDA-MB-231 cells the observed EC_{50} value is in contrast to the relatively high IC_{50} value measured. The most profound effect of AZD3409 on prenylation was also observed in the MDA-MB-231 cells. These observations imply the existence of an alternative prenylation pathway or some other compensatory escape or survival mechanism in this line.

Overexpression of HER2 in human tumor cells is closely associated with increased angiogenesis and expression of VEGF. Up-regulation of VEGF in epithelial cancer cells is likely to support angiogenesis, sustaining and promoting survival and metastasis. Inhibition of the VEGF pathway can suppress tumor growth (14). Measurements of angiogenesis and lymphangiogenesis may have utility for breast cancer prognosis, particularly in the estimation of metastatic risk (15). Recent evidence confirms the prognostic value of VEGF, UPA and PAI-1 expression in women with early breast cancer and suggests that genotype should be considered when selecting systemic therapy (16).

In conclusion, the activity of AZD3409 *in vitro* on protein farnesylation is consistent with the expected drug mechanism of action.

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