Imatinib-Mediated Inactivation of Akt Regulates ABCG2 Function in Head and Neck Squamous Cell Carcinoma

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Objective: To investigate whether the mechanism for the reversal of ABCG2 (also known as ABCP, MXR, and BCRP)—mediated drug resistance by imatinib mesylate (Gleevec, STI571; Novartis Pharmaceuticals Corp, East Hanover, New Jersey) is caused by the downregulation of Akt kinase. The adenosine triphosphatase–binding cassette protein ABCG2 has been suggested to be involved in the resistance against various anticancer drugs. Recent studies show that imatinib reverses ABCG2-mediated drug resistance to topotecan hydrochloride and SN-38. In addition, we have previously reported that imatinib downregulates Akt kinase activity, which is elevated in head and neck squamous cell carcinoma.

Design: Flow cytometric analysis was used to determine the levels of drug or dye extrusion from the cells.

Results: We used Akt kinase inhibitors, transfection with short interfering RNA (siRNA) Akt, and the tyrosine kinase inhibitor imatinib to show that these treatments decreased the side population by 50% to 70% in Hoechst 33342 extrusion studies. Doxorubicin hydrochloride extrusion experiments also demonstrated 20% to 26% decrease in doxorubicin efflux on cells treated with imatinib, 1L6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, and transfection with siRNA Akt. With Western blot and immunofluorescence experiments, our data suggest that ABCG2 translocation is the mechanism by which imatinib and Akt regulate drug resistance. Clonogenic survival assays performed with imatinib-treated cells resulted in a dose-dependent decrease in cell survival compared with the control population.

Conclusion: Our findings demonstrate that imatinib confers greater doxorubicin retention, presumably via inhibition of Akt, which regulates ABCG2 function.

nomia (HNSCC). Akt, a downstream target in the phosphatidylinositol 3-kinase pathway, is often activated by tyrosine receptor kinases. Akt has been shown to regulate cellular growth and survival, tumorigenesis, and drug resistance such as tamoxifen resistance in the breast carcinoma cell line MCF-7.

Because the Akt kinase activity is observed to be highly activated in HNSCC, we investigated whether the reversal of ABCG2-mediated drug resistance by imatinib is caused by the downregulation of Akt. Using the Akt inhibitors LY294002 and 1L6-hydroxymethyl-chiroinositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (1L6), short interfering RNA (siRNA) Akt, and imatinib to inactivate Akt kinase activity, we have shown not only a significant decrease in side populations via Hoechst 33342 extrusion but also a decrease in doxorubicin extrusion, possibly via the translocation of the ABCG2 pumps toward the nucleus as indicated by immunofluorescence. Immunoblotting technology Inc, Beverly, Massachusetts). Statistical significance was determined by the unpaired t test.

FLOW CYTOMETRY ANALYSIS OF HOECHST STAINING AND DOXORUBICIN EFFLUX

The HNSCC cells were investigated for ABCG2 function by analyzing their Hoechst extrusion properties. Cells were stained with Hoechst 33342 (Sigma-Aldrich Corp, St Louis, Missouri) in DMEM containing 2% bovine serum albumin and 10mM HEPES buffer at 37°C. In addition, propidium iodide was added for dead cell discrimination. Verapamil hydrochloride (Sigma-Aldrich) was also added to parallel sets of samples before Hoechst staining to inhibit Hoechst efflux as a control. To determine the regulation of ABCG2 by Akt, the chemical inhibitors LY294002 (Calbiochem, San Diego), 1L6 (Calbiochem), and the tyrosine kinase inhibitor imatinib (Novartis, Basel, Switzerland) were used to inhibit Akt activity. In addition, HNSCC cells were transfected with siRNA Akt (Cell Signaling Technology Inc) or the BXP-21 clone of the ABCG2 primary antibody (Calbiochem), followed by appropriate secondary antibodies. Membranes were visualized with a chemiluminescence detection system (Pierce, Rockford, Illinois). The membranes were probed with polyclonal antibody against β-actin (Sigma-Aldrich Corp) to ensure equal protein loading.

Akt KINASE ACTIVITY ASSAY

To assay for Akt kinase activity, we used a nonradioactive Akt kinase kit (Cell Signaling Technology Inc). Imatinib was added to the HNSCC cell lines at concentrations of 0, 5, and 10 µmol/L. After 24 hours of incubation with imatinib, cells were harvested and lysed with 1mM phenylmethylsulfonyl fluoride and 0.5 mL of cell lysis buffer as described previously. The amount of total protein was standardized using a bicinchoninic acid protein assay reagent kit (Pierce). Samples were subjected to Western blot analysis as mentioned previously. Glycogen synthase kinase 3 (GSK-3) phosphorylation was detected using phospho-GSK-3α/β (Ser21/9) antibody, an appropriate secondary antibody, and chemiluminescent detection.

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Cells were washed with ice-cold phosphate-buffered saline solution and incubated with 10mM NHS-SS-biotin (Pierce) at 4°C for 20 minutes. After washing with ice-cold phosphate-buffered saline solution containing 10mM glycine, cells were incubated with the same buffer for 20 minutes at 4°C. After removing the buffer, cells were lysed with 100 µL of lysis buffer, mentioned previously for Western blot analysis, at 4°C for 20 minutes. Then, 100 µL of streptavidin-agarose beads (Amer sham Biosciences, Pittsburgh, Pennsylvania) were added to the lysate and incubated overnight at 4°C with rotation. After centrifugation, the beads were washed 3 times with lysis buffer. The biotinylated proteins were released with 1% sodium dodecyl sulfate loading buffer. Samples of total cell lysates and immunoprecipitated proteins were subjected to Western blot analysis.

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The HNSCC cells were grown on coverslips. The cells were fixed with 4% paraformaldehyde and blocked in goat serum at room temperature before incubation with the BXP-34 clone of Chemicon) of the antihuman ABCG2 antibody. The cells were then incubated with a goat antimouse fluorescein isothiocyanate-conjugated secondary antibody (Chemicon) and were counterstained with 4’,6-diamidino-2-phenylindole. Fluorescent images were obtained using an inverted fluorescence microscope (Leica DMIRE2; Leica Microsystems Inc, Wetzlar, Germany) and a commercially available computer program (Simple PCI; Compix Inc, Sewickley, Pennsylvania) for image capture.
CLONOGENIC SURVIVAL ASSAY

Clonogenic survival assays were performed to assess the efficacy of the combination of imatinib and doxorubicin compared with doxorubicin alone. The HNSCC cells were plated in triplicate at $10^3$ cells per 60×15-mm culture plate and incubated in DMEM supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. After 24 hours, the cells were incubated with imatinib (2µM or 6µM) and varying concentrations of doxorubicin (0.01µM, 0.02µM, 0.03µM, 0.05µM, 0.08µM, and 0.10µM). Colonies were fixed and stained with a crystal violet/methanol solution and counted after 7 to 10 days.

RESULTS

ALTERATION OF ABCG2 ACTIVITY BY INHIBITION OF Akt KINASE ACTIVITY

We performed flow cytometry analysis of Hoechst 33342 extrusion, a method used to distinguish side populations based on the presence of ABCG2, to determine the effects of the Akt kinase activity in regulating ABCG2 activity in HNSCC cell lines. Various Akt kinase inhibitors were used to demonstrate that the downregulation of Akt kinase activity results in decreased ABCG2 activity. Because the Hoechst 33342 extrusion analysis is gated for the side population, which consists of cells with high ABCG2 activity, a decrease in the gated population serves as an indication of decreased ABCG2 activity. The tyrosine kinase inhibitor imatinib, the phosphatidylinositol 3-kinase chemical inhibitor LY294002, and the direct inhibitor of Akt 1L6 reduced the relative side population in HNSCC by 57.7%, 70.2%, and 52.2%, respectively. We further observed that transfection with siRNA Akt also reduced the side population by 69.6% (Figure 1).

These experiments were performed in duplicate, and the results reported are the averages of the 2 trials. Representative data were selected and displayed in Figure 1.

![Figure 1](http://archotol.jamanetwork.com/pdfaccess.ashx?url=/data/journals/otol/9822/ on 06/25/2017)
REDUCTION OF DOXORUBICIN EFFLUX
WITH GLEEVEC AND siRNA Akt

As a direct substrate of ABCG2, we used doxorubicin extrusion analysis by means of flow cytometry to measure the drug extrusion level by ABCG2. Our results demonstrate that imatinib decreased doxorubicin efflux by 22.2% in doxorubicin compared with untreated UMSCC10B cells. To further investigate whether the downregulation of Akt results in decreased doxorubicin extrusion, 1L6, a direct Akt inhibitor, and siRNA Akt were used to inhibit Akt kinase activity. The treatment with 1L6 resulted in a decrease of 26.3% in doxorubicin extrusion levels. In addition, a 21.0% decrease in doxorubicin extrusion levels was observed when cells were transfected with siRNA Akt (Figure 2).

REGULATION OF LOCALIZATION OF ABCG2
BUT NOT PROTEIN LEVELS BY Akt

Western blot analysis of cells treated with the various Akt kinase inhibitors confirmed the downregulation of the Akt kinase activity (Figure 3A). Cells treated with 10µM LY294002 and 10µM imatinib, and transfected with siRNA Akt all resulted in decreased levels of phospho-Akt, which is the active form of Akt and serves as a measure of the Akt kinase activity. In addition, an Akt kinase assay was performed on cells treated with 0µM, 5µM, and 10µM imatinib, further confirming the inhibitory effects imatinib exerts on the Akt kinase activity (Figure 3B).

Treatment of UMSCC10B cells with 10µM imatinib, LY294002, or 1L6 for 24 hours demonstrated no significant change in protein levels of ABCG2 on Western blot analysis, regardless of treatment. However, on analyzing biotinylated ABCG2 proteins isolated by immunoprecipitation, cells treated with imatinib or LY294002 displayed a reduction in cell surface ABCG2 levels, with imatinib eliminating most of the detectable levels of surface ABCG2 (Figure 3C).

Immunofluorescence was performed to analyze ABCG2 localization in the cell. In the untreated cells, ABCG2
forms a ringlike structure around the cell membrane. However, on the addition of imatinib, the ringlike structure becomes less defined, with ABCG2 protein concentrated toward the nucleus. Cells incubated with LY294002 or 1L6 exhibited an even less-defined ABCG2 ring around the cell membrane, with more ABCG2 localized in the nuclear region (Figure 3D).

ENHANCED SENSITIZATION OF HNSCC CELLS TO DOXORUBICIN AFTER TREATMENT WITH SI1571 IN AN IN VITRO CLONOGENIC SURVIVAL ASSAY

To test the hypothesis that the addition of imatinib can sensitize HNSCC cells to doxorubicin, HN30 and UMSCC10B cells were pretreated with 2µM and 6µM imatinib, respectively, and varying doses of doxorubicin and plated for clonogenic cell survival. Once the colonies were counted, survival was adjusted to exclude the primary apoptotic effects of imatinib. Figure 4 shows that imatinib greatly suppressed the clonogenic survival of both cell lines. Incubation with imatinib resulted in a dose-dependent decrease in cell proliferation and survival compared with cells in the control population. The largest decline in survival occurred at doxorubicin concentrations ranging from 0.03µM to 0.08µM; however, cells treated with imatinib showed decreased survival for all concentrations of doxorubicin.

Recent studies show that the adenosine triphosphatase–binding cassette protein ABCG2 can confer resistance against several chemotherapeutic agents commonly used in cancer treatment.1-3 Previous studies have shown that imatinib, a small-molecule protein tyrosine kinase inhibitor, can reverse ABCG2-mediated drug resistance and sensitize cancer cells to the cytotoxic effects of chemotherapeutic agents.7 Although imatinib is known to inhibit a number of protein tyrosine kinases in various cancers,8,9 the molecular mechanism through which imatinib ultimately regulates ABCG2-mediated drug resistance has not yet been demonstrated.

A possible mechanism through which imatinib regulates drug resistance is the serine/threonine kinase Akt, known to be highly expressed in numerous drug-resistant cancer cell lines, including tamoxifen-resistant MCF-7 breast carcinomas and HNSCC. We have demonstrated that the addition of imatinib causes a significant decrease of phosphorylated Akt levels in HNSCC cell lines via an Akt kinase assay and Western blot analysis of phospho-Akt. Hence, we continued to demonstrate in subsequent experiments that downregulation of Akt kinase activity leads to decreased ABCG2 function and thus to increased drug resistance of the HNSCC cells.

With flow cytometry analysis of Hoechst 33342 extrusion, we demonstrate that inactivation of Akt causes significant reduction in the side population, gated for the cells that have elevated levels of ABCG2 function.

To determine whether Akt can also mediate direct extrusion of chemotherapeutic agents via ABCG2, we investigated the drug extrusion properties of HNSCC cells by incubating them with doxorubicin, a direct substrate of ABCG2. This assay allowed us to compare the intracellular doxorubicin levels of cells by flow cytometry. Our results demonstrate that chemical inhibition of Akt with imatinib and 1L6 showed an approximately 20% decrease in doxorubicin efflux. Transfection with siRNA Akt also decreased doxorubicin extrusion in a comparable manner, which further suggests that Akt is a key modulator of ABCG2 function. With a longer extrusion period, we anticipate a greater difference in doxorubicin efflux between treated and untreated cells. Examination of Hoechst 33342 and doxorubicin extrusion results lead to the observation that the downregulation of ABCG2 pump activity is mostly likely due to the inhibition of the Akt kinase.

On investigating a possible mechanism by which Akt regulated ABCG2, we found that regulation of ABCG2 function by Akt was not associated with changes in protein levels. Treatment of UMSCC10B cells with 10µM imatinib, LY294002, or 1L6 for 24 hours demonstrated no significant change in protein levels of ABCG2 on Western blot analysis. However, when we analyzed biotinylated cell surface ABCG2 proteins isolated by immunoprecipitation, we discovered that cells treated with
imatinib or LY294002 displayed a reduction in cell surface ABCG2 levels, with imatinib eliminating most of the detectable levels of surface ABCG2. Thus, immunofluorescence was performed to visualize specific ABCG2 localization in the cell. In the untreated HNSCC cells, ABCG2 forms a ringlike structure around the cell membrane because most of the pumps collect near the cell surface. However, with the addition of the chemical Akt inhibitors, fewer aggregates of the ABCG2 pump localize on the cellular membrane. These results suggest that inhibition of Akt decreases ABCG2 function by inducing localization of active ABCG2 transporters away from the cell membrane.

Ultimately, we performed a clonogenic assay to test the synergistic effects of imatinib with doxorubicin on HNSCC. Cells were pretreated with imatinib and subjected to varying concentrations of doxorubicin. Colony survival was adjusted to exclude the primary apoptotic effects of imatinib, which were minimal at the concentrations used. Imatinib, in combination with doxorubicin, significantly decreased colony numbers in UMSCC10B and HN30 compared with doxorubicin treatment alone.

Our findings demonstrate that imatinib confers greater sensitivity to doxorubicin, presumably in part via reduced ABCG2 function as a result of Akt inhibition. In addition, our results suggest that downregulation of Akt leads to internal localization of ABCG2, thus decreasing doxorubicin resistance as shown in the extrapolation and clonogenic assays. These experiments produced similar results in other HNSCC cell lines.

Imatinib continues to be studied in preliminary clinical trials for possible synergistic effects with common chemotherapeutic agents. Ultimately, further investigation of the downstream targets of Akt in the imatinib-mediated reversal of drug resistance will need to be explored. Future studies will also be needed to investigate other multidrug resistance genes involved in cancer drug resistance and the implications Akt may have on other drug resistance pathways.

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Author Contributions: Dr Ongkeko had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Ms Chu and Chen and Mr Lopez contributed equally to this work. Study concept and design: Chu, Lopez, Pardo, and Ongkeko. Acquisition of data: Chen, Lopez, Aguilera, and Ongkeko. Analysis and interpretation of data: Chen and Ongkeko. Drafting of the manuscript: Chu, Chen, Lopez, and Ongkeko. Critical revision of the manuscript for important intellectual content: Pardo, Aguilera, and Ongkeko. Statistical analysis: Chu. Obtained funding: Pardo and Ongkeko. Administrative, technical, and material support: Chen, Lopez, Aguilera, and Ongkeko. Study supervision: Chu and Ongkeko.

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REFERENCES