Gefitinib Inhibition of Drug Resistance to Doxorubicin by Inactivating ABCG2 in Thyroid Cancer Cell Lines

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Objective: To investigate the regulation of the breast cancer resistance protein ABCG2/BRCP1 drug transporter by epidermal growth factor receptor (EGFR) kinase activity, and to determine whether gefitinib, an EGFR small molecule inhibitor, will modulate the effects of doxorubicin hydrochloride by inhibiting its extrusion from thyroid cancer cells.

Design: Extrusion assays using flow cytometry analysis were used to determine the ability of thyroid cancer cells to extrude the chemotherapy drug, doxorubicin, via the ABCG2 drug transporter in the presence or absence of gefitinib. Immunofluorescence was employed to determine the cellular expression of ABCG2. The ABCG2 expression in ARO and WRO cell lines was analyzed by Western blot analysis. Inactivation of EGFR kinase by gefitinib was analyzed by Western blot analysis and immunofluorescence. A terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay was performed to demonstrate ABCG2-mediated apoptosis in the presence of doxorubicin. Colony formation assays were performed to determine the effect of gefitinib on thyroid cancer cell survival in response to gefitinib, doxorubicin, or the combination of both drugs.

Results: Inhibition of EGFR kinase activity by gefitinib causes the translocation of the ABCG2 drug transporter away from the plasma membrane, resulting in a concomitant decrease in doxorubicin extrusion in thyroid cancer cell lines. Both ARO and WRO demonstrated differential ABCG2 expression, whereas both were sensitized to doxorubicin-induced apoptosis on ABCG2 knockdown with short interfering RNA. The addition of gefitinib increases doxorubicin-induced cell death in thyroid cancer cells as measured by colony formation assay.

Conclusions: Epidermal growth factor receptor regulates the function of the drug transporter ABCG2/BCRP1 and correlates with ABCG2 protein expression levels. Inactivation of the EGFR kinase by gefitinib potentiates the cytotoxic effect of doxorubicin in thyroid cancer, most likely by decreasing the ability of the cell to extrude doxorubicin. The expression of ABCG2 may explain in part the ineffectiveness of doxorubicin as a single modality treatment for anaplastic thyroid cancer or for treatment of metastatic follicular thyroid cancer. Use of this combination treatment of gefitinib and doxorubicin may be a promising therapy for anaplastic thyroid and metastatic follicular thyroid cancer and needs to be investigated further.

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Thyroid cancer accounts for most deaths from endocrine malignant neoplasms. Most thyroid cancers can be successfully managed by surgical resection with or without radioactive iodine ablation. However, a subset of thyroid cancers, including anaplastic thyroid cancer (ATC) and metastatic follicular thyroid cancer (FTC), can be very aggressive and resistant to therapy, often leading to death. For ATC, the traditional and mainstay treatment has been the use of doxorubicin hydrochloride, a quinone-containing anthracycline, but this treatment has proven to be ineffective primarily because of drug resistance. More recently, combination drug treatments using doxorubicin, cisplatin, and paclitaxel have been used, but none of these have shown notable promise. Even with the combination of surgery, radiotherapy, and chemotherapy, a diagnosis of ATC is linked to high rates of mortality with a mean survival of 4 to 12 months. Likewise, metastatic FTC carries a poor prognosis owing to the limited avenues for treatment that are cur-
Currently available. Although differentiated thyroid cancers usually result in a high probability of long-term survival, this is not true once distant metastases is present: the 10-year survival rate is less than 20%.\(^7\,8\) Furthermore, patients who have metastatic FTC to the bone can also experience considerable morbidity from pathologic fractures, spinal cord compression, and severe pain. With the steady increase in the incidence of thyroid cancer in the United States\(^9\) coupled with the dismal treatment options currently available for ATC and metastatic FTC, a better understanding of these malignant neoplasms as well as more effective treatment options are clearly indicated.

Recently, there have been several reports\(^10,11\) on the role of epidermal growth factor receptor (EGFR) in thyroid cancer; EGFRs have been shown to be highly expressed in thyroid cancer cell lines. Schiff et al\(^10\) and En-singer et al\(^11\) have demonstrated that EGFR-targeted therapy may represent an effective method of therapy for regulating thyroid cancers. In particular, gefitinib, a small-molecule tyrosine kinase inhibitor of EGFR, has shown promise as a means of regulating the growth of highly malignant ATCs.\(^15\) We have previously demonstrated\(^13\) that EGFR kinase activity regulates the side population in head and neck squamous cell carcinoma by regulating ABCG2 function. The breast cancer resistance protein ABCG2/BCRP, a member of the adenosine triphosphate–binding cassette transporter family, has been shown to be involved in the resistance to many chemotherapeutic drugs, including doxorubicin, alvocidib,\(^14\) topotecan hydrochloride,\(^15\) and SN-38.\(^16-18\) Given this finding, and given that doxorubicin is a known substrate of ABCG2, we wished to determine whether the EGFR kinase can also regulate the extrusion of doxorubicin by the ABCG2 drug transporter, thus modulating ABCG2-mediated drug resistance. We hypothesized that inhibition of ABCG2 pump function by gefitinib could potentiate the effect of doxorubicin by preventing its extrusion, thus providing a higher effective dose to thyroid cancer cells. Drug resistance clearly is one of the main reasons for the treatment failure for ATC and metastatic FTC. Many molecular pathways are known to be implicated in cellular resistance to doxorubicin.\(^19-23\) In this study, we demonstrate that the ABCG2 drug transporter is one of the major mechanisms by which these thyroid cancers become doxorubicin resistant and that we can reverse this drug resistance by targeting EGFR. On its own, EGFR-targeted therapy can be partially effective in controlling the proliferation of ATC and FTC. We demonstrate that EGFR inhibition by gefitinib will also potentiate doxorubicin-induced cell death.

**METHODS**

**CHEMICALS**

Cell culture media were obtained from Invitrogen (Carlsbad, California). Primary antibodies against ABCG2 and IgG2B isotype control were obtained from Chemicon (Temecula, California). Gefitinib (trade name, Iressa) was obtained from AstraZeneca (London, England).

**CELL LINES**

Established human thyroid cancer cell lines were studied including the ATC line ARO and FTC line WRO, provided by Francesco Frasca, MD, PhD (Università di Catania, Catania, Italy). Cultures from these cells were grown routinely with RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin sulfate. All cell lines were maintained within incubators at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air.

**FLOW CYTOMETRY ANALYSIS OF DOXORUBICIN EFFLUX**

For doxorubicin extrusion analysis, cells were incubated with gefitinib, 2µM, for 48 hours prior to the addition of doxorubicin in Dulbecco modified Eagle medium containing 2% bovine serum albumin. Afterward, the cells that were not allowed to extrude doxorubicin were resuspended with Hanks balanced salt solution (Invitrogen) and kept in ice. A parallel set of cells was resuspended in Dulbecco modified Eagle medium with 2% bovine serum albumin and allowed to extrude the drug for 2.5 hours at 37°C. The cells were resuspended in Hanks balanced salt solution for flow cytometric analysis. Dead cells were excluded by simultaneous staining with propidium iodide. Doxorubicin efflux was analyzed using a fluorescence-activated cell sorter (FACS Vantage SE; BD Biosciences, San Jose, California) with CELLQuest (Largo, Florida) software. Statistical significance was determined by t test (P < .05 was considered statistically significant).

**IMMUNOFLOURESCENCE**

To determine the effect of gefitinib on the ABCG2/BCRP1 or phospho-EGFR protein distribution in cells, ARO cells were incubated with gefitinib, 2µM, for 48 hours. The cells were fixed with 4% paraformaldehyde and blocked in goat serum at room temperature prior to incubation with the monoclonal ABCG2/BCRP1 antibody, or anti-human phospho-EGFR (R & D Systems, Minneapolis, Minnesota). Cells tagged with the ABCG2/BCRP1 antibody were then incubated with a goat anti-mouse fluorescein isothiocyanate conjugated (FITC) secondary antibody (Chemicon). A goat anti-rabbit rhodamine conjugated secondary antibody (Molecular Probes, Eugene, Oregon) was used to detect the phospho-EGFR. The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescent images were obtained using a Leica inverted fluorescence microscope (model DMIRE2; Leica Microsystems, Deerfield, Illinois). We used the computer program Simple PCI (Compix Inc, Sewickley, Pennsylvania) for image capture.

**WESTERN BLOT ANALYSIS**

Cell lysates were separated on a 10% sodium dodecyl sulfate polyacrylamide protein gel (Invitrogen) and transferred onto a polyvinylidene fluoride microporous membrane. The membrane was blocked for 1 hour at room temperature with 5% nonfat dry milk in phosphate-buffered saline with 0.1% Tween-20 (PBS-T) and incubated with either affinity-purified rabbit phospho-EGFR (R & D Systems), ABCG2/BCRP (Calbiochem, San Diego, California), or β-actin (Cell Signaling, Danvers, Massachusetts) at 1:1000 dilution overnight. After a cycle of three 10-minute washes with PBS-T, the membrane was incubated with anti-rabbit IgG with a
1:10 000 dilution at room temperature. After 3 additional washes, the protein-antibody complexes were visualized by enzyme chemofluorescence (Pierce, Rockford, Illinois).

TUNEL ASSAY

Apoptosis was measured by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. For this assay, cultures of cells were grown on dishes 10 cm in diameter, treated with agents as indicated, and harvested by trypsinization. Cells were fixed with 1% paraformaldehyde, and cytoplasmic DNA fragments with 3'-hydroxyl ends were detected with an APO-Direct TUNEL kit (Phoenix Flow Systems, San Diego) by following the manufacturer’s protocol.

CLONOGENIC SURVIVAL ASSAY

To test the hypothesis that gefitinib can sensitize thyroid cancer cells to doxorubicin, ARO and WRO cells were incubated with gefitinib, 0.01µM, 0.05µM, or 0.1µM, 24 hours prior to exposure to varying doses of doxorubicin. Colonies were counted and adjusted to account for growth inhibition due to gefitinib alone. Cells were plated in triplicate at 500 cells per 60 × 15-mm culture plate and grown in RPMI medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin streptomycin. Colonies were fixed and stained with a crystal violet and formalin solution and counted after 7 to 10 days.

RESULTS

ALTERATION OF PHOSPHO-EGFR MORPHOLOGIC CHARACTERISTICS AND EXPRESSION IN THE PRESENCE OF GEFITINIB

To verify that the target of gefitinib, EGFR, was present in the thyroid cancer cells, immunofluorescence was performed in the presence or absence of gefitinib (Figure 1). After incubation with gefitinib for 24 hours, both ARO and WRO cells showed an increase in the stippling pattern of staining for phospho-EGFR compared with the control. In addition, there was a decrease in the intensity of the staining for phospho-EGFR seen in the WRO cells on addition of gefitinib. Western blot analysis was performed to further verify the down-regulation of phospho-EGFR protein levels by gefitinib. Both ARO and WRO cell lines displayed a notable decrease in phospho-EGFR levels on incubation with gefitinib, 2µM, relative to the control (Figure 1).

REDUCTION OF THE EFFLUX OF DOXORUBICIN

To determine whether EGFR can mediate extrusion of doxorubicin, a direct substrate of ABCG2, cells were incubated with gefitinib, 2µM, 48 hours prior to doxorubicin...
bicin extrusion analysis. Our results demonstrate that gefitinib decreased doxorubicin efflux in ARO and WRO cells by 51.5% and 20.0%, respectively (Table 1).

**DIFFERENTIAL ABCG2 EXPRESSION IN ARO AND WRO CELLS AND ALTERATION OF ABCG2 LOCALIZATION**

Relative expression of ABCG2/BCRP1 in ARO and WRO cell lines was measured via Western blot, with ARO demonstrating considerably higher expression levels relative to WRO (Figure 2). The ABCG2/BCRP1 protein levels and distribution were analyzed using immunofluorescence in the presence and absence of gefitinib. The fluorescein isothiocyanate–tagged ABCG2/BCRP1 transporter in ARO cells is present on the cytoplasmic membrane, forming a ring around the cell (Figure 2). On the addition of gefitinib, the ABCG2/BCRP1 transporter localizes toward the nucleus as shown in the merged images, consistent with the observed decreased function of the drug transporter.

**REGULATION BY ABCG2 OF DOXORUBICIN-INDUCED APOPTOSIS IN ARO AND WRO CELLS**

We performed TUNEL assays to determine whether the observed ABCG2-mediated efflux of doxorubicin ultimately regulates cell survival in thyroid cancer cell lines.

<table>
<thead>
<tr>
<th>Type of Treatment</th>
<th>Pre-extrusion</th>
<th>Postextrusion</th>
<th>Change (Relative to Control)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARO control</td>
<td>88.0</td>
<td>67.5</td>
<td>−20.5</td>
<td>.045</td>
</tr>
<tr>
<td>ARO + 2µM gefitinib</td>
<td>83.9</td>
<td>74.0</td>
<td>−51.5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>WRO control</td>
<td>51.6</td>
<td>39.6</td>
<td>−20.0</td>
<td>.045</td>
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<tr>
<td>WRO + 2µM gefitinib</td>
<td>53.3</td>
<td>43.8</td>
<td>−20.0</td>
<td>.045</td>
</tr>
</tbody>
</table>

Abbreviation: EGFR, epidermal growth factor receptor.

*Data are presented as percentages except where indicated.

**Table 1. Inactivation of EGFR Kinase With Gefitinib Resulting in Decreased Extrusion of Doxorubicin Hydrochloride in Thyroid Cancer Cells**

Figure 2. Gefitinib promotes translocation of the ABCG2/BCRP1 transporter from the cellular membrane to the intracellular compartment. A, The ARO and WRO cell lines demonstrate differential expression of ABCG2/BCRP1. Expression of ABCG2/BCRP1 was determined by Western blot analysis. B, The ARO cells were immunolabeled for the ABCG2/BCRP1 transporter as shown by green fluorescence. The ARO cells were incubated with 2µM of gefitinib for 24 hours. Cells were also labeled with 4,6-diamidino-2-phenylindole (DAPI) to identify nuclei. Immunofluorescent microscopy; original magnification ×40.
Both ARO and WRO cells were transfected with either an empty vector or short interfering RNA ABCG2 for 72 hours and thereafter exposed to 0.5µM of doxorubicin hydrochloride for 24 hours. Both cell lines demonstrate a considerably higher percentage of TUNEL-positive cells for short interfering RNA ABCG2-treated cells relative to an empty vector, suggesting that knockdown of ABCG2 confers greater sensitivity to doxorubicin-induced DNA damage. As expected, neither cell line demonstrated notable TUNEL-positive populations in the absence of doxorubicin incubation.

**GEFITINIB AND REVERSAL OF ABCG2-MEDIATED DRUG RESISTANCE**

Clonogenic colony formation assays were performed to determine whether gefitinib can decrease doxorubicin resistance by inhibiting ABCG2 activity. Cells were pre-treated with gefitinib, 0.01µM, 0.05µM, or 0.1µM, and analyzed with varying doses of doxorubicin. Once colonies were counted, survival was adjusted to exclude the antiproliferative effects of gefitinib, which were minimal at the doses used. Figure 3 shows that gefitinib, in combination with doxorubicin, notably decreased colony numbers in ARO and WRO compared with doxorubicin treatment alone. Cells treated with gefitinib showed the largest decline in survival occurring at 0.03µM of doxorubicin hydrochloride for WRO and 0.03µM of doxorubicin hydrochloride for ARO. Our findings demonstrate that gefitinib confers greater sensitivity to doxorubicin, in part via reduced ABCG2 function as a result of EGFR inhibition.

**COMMENT**

For patients who have the subset of thyroid cancer that is resistant to conventional therapy, the prognosis is quite dismal. Currently, although combination chemotherapy drugs are being used in an effort to control ATC, the new regimens have not proved to be effective. Doxorubicin is still the drug of choice even though most ATCs are resistant to this compound. In this study, we have identified a major mechanism for the resistance to doxorubicin through the activity of the drug transporter ABCG2. We have also demonstrated how the receptor tyrosine kinase EGFR modulates the activity of this drug transporter. Incubation with gefitinib, which by itself has an antiproliferative effect on thyroid cancer cells, caused inhibition of EGFR kinase activity. The EGFR inhibition via gefitinib also considerably decreased the extrusion of doxorubicin in ARO, the ATC cell line, and modestly in WRO, the FTC cell line, by regulating ABCG2/BCRP1 activity via translocation of ABCG2 from the plasma membrane to the nucleus. This variation in extrusion efficiency may be partially explained by the differential expression of ABCG2 in ARO and WRO cell lines, considering that ARO has considerably higher levels of ABCG2, which may confer greater efflux capacity. With a longer extrusion period, we anticipate a greater difference in extrusion levels between treated cells and the control cells. Furthermore, we have demonstrated that ABCG2

### Table 2. TUNEL-Positive FITC-Stained Cells in ARO and WRO Treated With Doxorubicin Hydrochloride or siRNA ABCG2 in Combination With Doxorubicin

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>Treatment With Doxorubicin</th>
<th>Treatment With siRNA ABCG2 + Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARO</td>
<td>1.5</td>
<td>26.6</td>
<td>47.7</td>
</tr>
<tr>
<td>WRO</td>
<td>0.8</td>
<td>72.4</td>
<td>88.0</td>
</tr>
</tbody>
</table>

Abbreviations: FITC, fluorescein isothiocyanate conjugated; siRNA, short interfering RNA; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

aData are presented as percentages.
is directly implicated in the regulation of doxorubicin-induced cell death, because incubation with short interfering RNA ABCG2 sensitized both ARO and WRO cells to apoptosis in the presence of doxorubicin. Increased concentration of doxorubicin in these thyroid cells owing to inhibition of the ABCG2 drug transporter resulted in increased death in both cell lines.

The presence of the drug transporter ABCG2 may explain why current drug therapies could be ineffective in the treatment of thyroid cancer. This would also suggest that any future curative therapies should be designed to selectively target the regulators of the drug transporters to avoid efflux by ABCG2 proteins, in this case, EGFR, which has also been implicated in drug resistance.

One of the difficulties in developing effective curative therapies stems from the fact that the methods commonly used to combat normal thyroid cancers are non-responsive in ATC. For example, radioactive iodine therapy, currently a prominent treatment for thyroid cancer, is rendered ineffective in ATC because they lack the necessary human sodium iodide I131 symporter proteins required for uptake of radioactive iodine. Therefore, a better understanding of the molecular biologic characteristics of thyroid cancer is needed to be able to tailor treatments to this disease. The advent of targeted therapy using small molecule inhibitors to EGFR would allow regulation not only of these drug resistance pumps but also of cancer cell survival itself, with hopefully minimal adverse effects. Ultimately, a combined treatment of surgical resection, combination chemotherapy, and external radiotherapy is likely to have the greatest impact on this disease.

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Author Contributions: Mr Lopez and Dr Wang-Rodriguez contributed equally to this study. Messrs Lopez and Aguilera; Drs Wang-Rodriguez, Pardo, and Ongkeko; and Mss Chang and Chen had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Lopez, Wang-Rodriguez, Pardo, and Ongkeko. Acquisition of data: Lopez, Chang, Chen, Aguilera, and Ongkeko. Analysis and interpretation of data: Lopez, Chang, and Ongkeko. Drafting of the manuscript: Lopez, Wang-Rodriguez, Pardo, and Ongkeko. Critical revision of the manuscript for important intellectual content: Lopez, Wang-Rodriguez, Chang, and Ongkeko. Obtained funding: Wang-Rodriguez, Pardo, and Ongkeko. Administrative, technical, and material support: Lopez, Chen, Aguilera, and Ongkeko. Study supervision: Lopez, Wang-Rodriguez, and Ongkeko.

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