Gefitinib Reverses Chemotherapy Resistance in Gefitinib-Insensitive Multidrug Resistant Cancer Cells Expressing ATP-Binding Cassette Family Protein

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Abstract

Gefitinib inhibits the ATP-binding site of the tyrosine kinase associated with the epidermal growth factor receptor. It is conceivable that gefitinib may inhibit functions of ATP-binding cassette (ABC) transporters by binding at their ATP-binding sites. The aim of this study is to systematically explore the combined effect of gefitinib and chemotherapeutic agents in gefitinib-insensitive multidrug resistant (MDR) cells that overexpress ABC transporters. MCF7 breast carcinoma cells and CL1 lung adenocarcinoma cells were both insensitive to gefitinib. MDR cancer cells were developed by stepwise escalating concentrations of each chemotherapeutic agent in culture media. Cells that overexpress P-glycoprotein (MCF7/ADR and CL1/Pac), breast cancer–resistant protein (MCF7/TPT and CL1/Tpt), and MDR-associated protein 1 (MCF7/Vp) were used in this study. All resistant mutants were insensitive to gefitinib. Gefitinib (0.3-3 μmol/L) added to culture media had no effect on IC50 values of paclitaxel, topotecan, doxorubicin, or etoposide in wild-type MCF7 or CL1 cells. In contrast, these concentrations of gefitinib caused a dose-dependent reversal of resistance to paclitaxel in CL1/Pac cells, to doxorubicin in MCF7/ADR cells, and to topotecan in CL1/Tpt and MCF7/TPT cells. Gefitinib had no influence on sensitivity to etoposide in MDR-associated protein1 overexpressing MCF7/Vp cells. Topotecan efflux was inhibited and accumulation was partially restored in CL1/Tpt and MCF7/TPT cells when cells were incubated simultaneously with gefitinib. Our results suggest that the interaction of gefitinib and chemotherapeutic agents does occur in cells expressing one of these two proteins.

Introduction

Gefitinib is the first tyrosine kinase inhibitor approved for the treatment of advanced non–small cell lung cancer (1, 2). Gefitinib was additive or synergistic with many chemotherapeutic agents in reducing numbers of colony counts in human ovary (OVCAR-3), breast (ZR-75-1, MCF-10A ras), and colon (GEO) cancer cells grown in soft agar (3). Coadministration of gefitinib with chemotherapy agents has been shown to result in higher degree of inhibition of A549, A431, SKLC-16, TSU-PR1, PC3, and LX-1 cancer cells in vitro (4). Due to its low toxicity, combinations of gefitinib with multiple chemotherapeutic agents have been tested in clinical trials. Despite the promising preclinical results, synergistic or additive effects between gefitinib and chemotherapeutic regimens have not been observed in two large phase III clinical trials (5, 6). Furthermore, in a phase II study, gefitinib combined with vinorelbine induced unanticipated severe hematologic toxicity in non–small cell lung cancer patients (7). In an animal study, mice were intolerant to gefitinib with vinorelbine combination treatment (4). A plausible explanation for the profound myelosuppression was that unknown pharmacologic interactions occurred between gefitinib and vinorelbine in normal hematopoietic progenitor cells. We know little at present about the pharmacologic interactions that contributed to the conflicting results between many preclinical and clinical studies that combined gefitinib with chemotherapeutic agents.

The mechanism underlying gefitinib-induced chemosensitivity in cancer cells in vitro is still unclear. Gefitinib is known to inhibit the ATP-binding site of tyrosine kinase associated with the epidermal growth factor receptor. Gefitinib has been reported to interact with breast cancer–resistant protein (BCRP or ABCG2) transporter and thus may affect drug resistance patterns in cells transfected with BCRP (8). Currently, the information on gefitinib activity in laboratory-induced multidrug resistant (MDR) cancer cells is still scarce. To delineate the gefitinib-induced chemosensitivity of cancer cells that do not involve inhibition of the tyrosine kinase domain of epidermal growth factor receptor, via interaction with ATP-binding cassette (ABC) transporters, we developed several MDR cancer cells from gefitinib-insensitive lung and breast carcinoma cells. In this study, these chemosensitive cancer cells and chemoresistant cells with overexpression of ABC transporters were used to test the interactions of gefitinib with chemotherapeutic agents.

Materials and Methods

Chemotherapeutic agents and multidrug resistant cancer cells. Gefitinib was supplied by AstraZeneca (Alderley Park, Cheshire, United Kingdom). Paclitaxel was supplied by Bristol-Myers Squibb (Princeton, NJ). Topotecan was supplied by GlaxoSmithKline (Research Triangle Park, NC). Sulforhodamine B was purchased from Sigma (St. Louis, MO). MDR cancer cells were developed by stepwise escalation of chemotherapeutic agents in culture media. MCF7/WT, MCF7/ADR (9), and MCF7/Vp (10) cells are gifts from Dr. Erasmus Schneider (Wadsworth Center, NYS, Department of Health, Albany, NY) and were developed by chronic exposure of MCF7/WT cells to doxorubicin and etoposide, respectively. MCF7/TPT cells were developed in our laboratory and were described previously (11). CL1 lung adenocarcinoma cells were developed in our laboratory and were described previously (12). CL1/Pac and CL1/Tpt were developed by incubation of CL1 lung adenocarcinoma cells in escalating concentrations of paclitaxel and topotecan, respectively, for >1 year. Cells were grown as attached monolayers.

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in DMEM containing 10% fetal bovine serum at 37°C in 5% CO₂ in a humidified incubator. Drug-resistant cells were selected after cloning. Cells were grown in drug-free culture media for >2 weeks before assay.

**Cytotoxicity test.** Cytotoxicity tests were done with a sulforhodamine B assay (10). Cells were grown in 96-well microtiter plates. Various concentrations of drugs were added to the wells for 96 hours before assay. IC₅₀ values of chemotherapeutic agents were determined from survival curves. Fold of resistance of individual drugs was defined as IC₅₀ of MDR cancer cells divided by that of parental drug-sensitive cells.

**Combination of gefitinib and a chemotherapeutic agent.** To test the effect of gefitinib on the chemosensitivity of cancer cells, 0.3, 1, or 3 μmol/L of gefitinib was added to the medium with various concentrations of topotecan in CL1, CL1/Tpt, MCF7, or MCF7/TPT cells for 96 hours. These concentrations of gefitinib were added to the medium with various concentrations of paclitaxel in CL1 or CL1/Pac cell lines, doxorubicin in MCF7 or MCF7/ADR cells, and etoposide in MCF7 or MCF7/VP cells. The IC₅₀ of each test concentration of gefitinib with each chemotherapeutic agent in either chemosensitive or chemoresistant cells was determined from the survival curve. The reversing index was determined by the IC₅₀ of each chemotherapeutic agent in cells coincubated with gefitinib divided by the IC₅₀ of the same drug in the same cells without coincubation with gefitinib.

**Reverse transcription-PCR.** cDNA libraries were prepared from chemoresistant and chemosensitive CL1 and MCF7 cells. Reverse transcription was done with M-MLV reverse transcriptase (Promega Corp., Madison, WI). Oligonucleotides for PCR of mdr1, BCRP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized commercially (MDBio, Inc., Taipei, Taiwan). Sense primer for mdr1: 5'-GCTTGGCAAGCTGGGAGCAATACAAAAT-3'; antisense primer: 5'-CAGACACAGCTGACACGGGGAAGACT-3'. Sense primer for BCRP: 5'-TCTATCCTGGAATGCGAA-3'; antisense primer: 5'-TTTCACGCGGGTGAACCTTTT-3'. Sense primer for GAPDH: 5'-TCGAGTGCAACGGATGGTGGT-3'; antisense primer: 5'-GAAATTCCCATGGGTGGGAAAT-3'. Using the GeneAmp PCR system 9700 (PE Applied Biosystem, Foster City, CA), reactions were done for BCRP and GAPDH at 94°C for 1.5 minutes for initial denaturation, and then at 94°C for 40 seconds, 45°C for 40 seconds, and 72°C for 1 minute. After 30 cycles of amplification, additional extensions were done at 72°C for 10 minutes. Reactions for mdr1 was at 95°C for 5 minutes and at 95°C for 30 seconds for denaturation, 58°C for 30 seconds for annealing and 72°C for 30 seconds for elongation. After 35 cycles of amplification, additional extensions were done at 72°C for 7 minutes. Products were resolved and examined by agarose gel electrophoresis.

**Western blot.** Cellular total protein was prepared from cell lysates of CL1, MCF7, and chemoresistant cell lines. Proteins were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes in buffer containing 48 mmol/L Tris base, 39 mmol/L glycine, 0.05% SDS, and 20% methanol. Membranes were blocked with a 5% solution of powdered skim milk dissolved in Tris-buffer saline-Tween [50 mmol/L Tris, 150 mmol/L NaCl (pH 7.5), and 0.2% Tween 20], incubated with primary antibody of BCRP, P-glycoprotein or actin (Chemicon, Inc., Temecula, CA) at 4°C overnight followed with a goat anti-mouse antibody conjugated to horseradish peroxidase at 4°C for 3 hours (Chemicon) and detected with chemiluminescence (Santa Cruz, Inc., Santa Cruz, CA).

**Topotecan accumulation.** Topotecan accumulation and efflux were determined by flow cytometry as described previously (11). Wild-type and topotecan-resistant MCF7 or CL1 cells growing in culture medium were trypsinized and washed with PBS. Cells resuspended in HBSS were incubated with or without 0.3, 1, or 3 μmol/L of topotecan and gefitinib for 30 minutes as described above, the topotecan-accumulated cells were centrifuged and resuspended in HBSS. After resuspension for different periods of time, each sample was subjected to flow cytometric measurement. The efflux process was carried out in a 37°C water bath. Intracellular topotecan of topotecan-treated cancer cells was estimated by fold of fluorescence intensity after normalization to topotecan accumulation in the presence of 10 μmol/L of topotecan without gefitinib in each chemosensitive parental cells. Fold of fluorescence intensity equals to (mean FL2-H unit of topotecan incubated with or without gefitinib in each chemosensitive parental cells) / (mean FL2-H unit of untreated parental cells).

**Topotecan efflux.** Topotecan efflux of cancer cells with or without the presence of gefitinib was determined by flow cytometry (11). After loading of topotecan and gefitinib for 30 minutes as described above, the topotecan-accumulated cells were centrifuged and resuspended in HBSS. After resuspension for different periods of time, each sample was subjected to flow cytometric measurement. The efflux process was carried out in a 37°C water bath. Intracellular topotecan of topotecan-treated cancer cells was estimated by mean FL2-H unit of 5,000 cells at each time point after efflux. Background level (mean FL2-H unit of topotecan-untreated control cells) was subtracted from the mean FL2-H unit of topotecan-treated cells. The first time point recorded was 10 seconds after efflux.
Breast cancer–resistant protein amino acid position 482 determinations. The expression of amino acid position 482 of BCRP in CL1/Tpt and MCF7/TPT cells was determined by sequencing cDNA from these cells. Reverse transcription of BCRP mRNA was described as above. Products were resolved by 1.2% agarose gel electrophoresis. A 407 bp product was purified and sequenced using ABI 3100 Autosequencer (PE Applied Biosystems).

Results

Cross-resistant pattern of multidrug resistant lung adenocarcinoma CL1 cells. IC_{50} of each chemotherapeutic agent in each cell type was determined at least thrice. Table 1 showed the fold of resistance of CL1/Pac and CL1/Tpt in various chemotherapeutic agents. CL1/Tpt was resistant only to topotecan and was only slightly cross-resistant to doxorubicin and VP16. CL1/Pac was cross-resistant to classic P-glycoprotein substrates, such as doxorubicin and VP16.

ATP-binding cassette transporters expression in multidrug resistant cancer cells. Reverse transcription-PCR (RT-PCR) of the mdr1 and BCRP genes was done in chemosensitive and chemoresistant CL1 and MCF7 cells. As shown in Fig. 1A, mdr1 was highly expressed in MCF7/ADR and CL1/Pac cells. BCRP was highly expressed in CL1/Tpt and MCF7/TPT cells. Western blots of BCRP and P-glycoprotein were done in chemosensitive and chemoresistant MCF7 and CL1 cells. BCRP was overexpressed in CL1/Tpt and MCF7/TPT cells and P-glycoprotein was overexpressed in MCF7/ADR and CL1/Pac cells (Fig. 1B). The expression of P-glycoprotein, MDR-associated protein 1 (MRP1) or BCRP in MDR CL1 and MCF7 cells are listed in Table 1.

Cytotoxic effect of combining gefitinib and chemotherapy in CL1 and MCF7 cells. In human pharmacokinetic studies, the highest peak plasma gefitinib level was roughly 3 μmol/L (13). Higher concentrations were considered inactive against cancer cells. Gefitinib was ineffective against CL1/WT, CL1/Pac, CL1/Tpt, MCF7/WT, and MCF7/ADR cells (IC_{50} values were all >5 μmol/L; data not shown). The cytotoxic effect of chemotherapeutic agents in the presence of 0, 0.3, 1, or 3 μmol/L gefitinib was measured by sulforhodamine B assay. Representing cytotoxicity curves are shown in Fig. 2. The average IC_{50} values of chemotherapeutic agents in chemosensitive and chemoresistant CL1 or MCF7 cells in different concentrations of gefitinib are shown in Table 2. The reversing capability of gefitinib was estimated by reversing index (see Materials and Methods). Gefitinib at all concentrations tested had no effect on the cytotoxicity curves (Fig. 2) or IC_{50} values in all chemosensitive wild-type CL1 or MCF7 cells (Table 2). In MDR BCRP-overexpressing cells (MCF7/TPT and CL1/Tpt), gefitinib dose-dependently reversed topotecan resistance (Fig. 2A and B). The lowest concentration of gefitinib tested (0.3 μmol/L) was able to reverse resistance to topotecan in CL1/Tpt cells (reversing index = 0.25 and P = 0.002) and in MCF7/TPT cells (reversing index = 0.36 and P = 0.006). Similarly, gefitinib dose-dependently reversed paclitaxel and doxorubicin resistance in P-glycoprotein overexpressing CL1/Pac and MCF7/ADR cells (Fig. 2C and D). However, gefitinib was able to reverse resistance to doxorubicin in MCF7/ADR cells or paclitaxel to CL1/Pac cells only at concentrations >1 μmol/L. Low-dose gefitinib (0.3 μmol/L) had no reversing effect on either CL1/Pac (reversing index = 0.79, P = 0.33) or MCF7/ADR cells (reversing index = 1.0, P = 0.975). On the other hand, gefitinib...
at highest tested concentration of 3 μmol/L had only a small reversing effect on MRP1-overexpressing MCF7/VP cells for etoposide resistance (Fig. 2E; Table 2). In summary, reversing capability of gefitinib was highest in BCRP-overexpressing cells, modest in P-glycoprotein–overexpressing cells, and minimal in MRP1-overexpressing cells. In all MDR cells tested, gefitinib at the highest tested concentration of 3 μmol/L did not completely reverse chemoresistance (they were still 2.6- to 27-fold resistant compared with parental cells).

**Topotecan accumulation and efflux in CL1 and MCF7 cells.** Topotecan accumulation was measured by flow cytometry in CL1, CL1/Tpt, MCF7, and MCF7/TPT cells. MCF7/TPT cells accumulate less topotecan than MCF7/WT cells due the presence of the BCRP efflux pump in the cell membrane (11). Gefitinib at 0.3, 1, or 3 μmol/L had no effect on the topotecan accumulation in chemosensitive MCF7 or CL1 cells. On the contrary, gefitinib increased topotecan accumulation in chemoresistant MCF7/TPT and CL1/Tpt cells in a dose-dependent manner (Fig. 3). In BCRP-overexpressing cells, topotecan efflux was rapid, resulting in a sharp decline of the fluorescence in flow cytometric measurement. When 3 μmol/L gefitinib was added to the media, topotecan efflux was decreased in CL1/Tpt cells (Fig. 4A) and MCF7/TPT cells (Fig. 4B). However, gefitinib had no effect on the topotecan efflux curves of CL1 or MCF7 cells that did not overexpress BCRP.

**Breast cancer–resistant protein amino acid position 482 determinations.** Sequence of cDNA encoding for amino acid position 482 in both MCF7/TPT and CL1/Tpt cells were determined as AGG, translating to arginine. Therefore, both BCRP-overexpressing cells express wild-type BCRP.

### Table 2. Combined cytotoxic effects of chemotherapeutic agents with gefitinib

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC_{50} (nmol/L)</th>
<th>RI*</th>
<th>P †</th>
<th>Cells</th>
<th>IC_{50} (nmol/L)</th>
<th>Fold ‡</th>
<th>RI</th>
<th>P †</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPT</td>
<td>13.6 ± 1.4</td>
<td></td>
<td></td>
<td>CL1/Tpt</td>
<td>277 ± 22</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPT + 0.3 μmol/L Gef</td>
<td>14.5 ± 1.3</td>
<td>1.1</td>
<td>0.74</td>
<td>CL1/Tpt</td>
<td>68.0 ± 8.1</td>
<td>5.0</td>
<td>0.25</td>
<td>0.002</td>
</tr>
<tr>
<td>TPT + 1 μmol/L Gef</td>
<td>13.8 ± 1.3</td>
<td>1.0</td>
<td>0.94</td>
<td>CL1/Tpt</td>
<td>40.1 ± 3.4</td>
<td>2.9</td>
<td>0.14</td>
<td>0.001</td>
</tr>
<tr>
<td>TPT + 3 μmol/L Gef</td>
<td>13.8 ± 1.4</td>
<td>1.0</td>
<td>0.97</td>
<td>CL1/Tpt</td>
<td>34.7 ± 2.6</td>
<td>2.6</td>
<td>0.13</td>
<td>0.001</td>
</tr>
<tr>
<td>TPT</td>
<td>13.8 ± 5.3</td>
<td></td>
<td></td>
<td>MCF7/TPT</td>
<td>432 ± 37</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPT + 0.3 μmol/L Gef</td>
<td>13.5 ± 7.4</td>
<td>1.0</td>
<td>0.97</td>
<td>MCF7/TPT</td>
<td>157 ± 18</td>
<td>11</td>
<td>0.36</td>
<td>0.006</td>
</tr>
<tr>
<td>TPT + 1 μmol/L Gef</td>
<td>12.8 ± 9.8</td>
<td>0.93</td>
<td>0.91</td>
<td>MCF7/TPT</td>
<td>53 ± 7.4</td>
<td>3.9</td>
<td>0.12</td>
<td>0.001</td>
</tr>
<tr>
<td>TPT + 3 μmol/L Gef</td>
<td>14.2 ± 5.2</td>
<td>1.0</td>
<td>0.96</td>
<td>MCF7/TPT</td>
<td>40 ± 10</td>
<td>2.9</td>
<td>0.09</td>
<td>0.001</td>
</tr>
<tr>
<td>Pac + CL1/WT</td>
<td>0.76 ± 0.11</td>
<td></td>
<td></td>
<td>CL1/Pac</td>
<td>272 ± 27</td>
<td>358</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pac + 0.3 μmol/L Gef</td>
<td>0.74 ± 0.07</td>
<td>0.97</td>
<td>0.88</td>
<td>CL1/Pac</td>
<td>215 ± 31</td>
<td>282</td>
<td>0.79</td>
<td>0.33</td>
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<tr>
<td>Pac + 1 μmol/L Gef</td>
<td>0.69 ± 0.09</td>
<td>0.91</td>
<td>0.71</td>
<td>CL1/Pac</td>
<td>77.4 ± 17.6</td>
<td>102</td>
<td>0.28</td>
<td>0.01</td>
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<tr>
<td>Pac + 3 μmol/L Gef</td>
<td>0.61 ± 0.08</td>
<td>0.80</td>
<td>0.43</td>
<td>CL1/Pac</td>
<td>9.17 ± 1.60</td>
<td>12</td>
<td>0.033</td>
<td>0.001</td>
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<tr>
<td>ADR</td>
<td>10.0 ± 1.3</td>
<td></td>
<td></td>
<td>MCF7/ADR</td>
<td>1,380 ± 85</td>
<td>139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADR + 0.3 μmol/L Gef</td>
<td>9.1 ± 0.8</td>
<td>0.91</td>
<td>0.66</td>
<td>MCF7/ADR</td>
<td>1,376 ± 55</td>
<td>138</td>
<td>1.0</td>
<td>0.975</td>
</tr>
<tr>
<td>ADR + 1 μmol/L Gef</td>
<td>9.2 ± 1.2</td>
<td>0.92</td>
<td>0.74</td>
<td>MCF7/ADR</td>
<td>819 ± 42</td>
<td>82</td>
<td>0.59</td>
<td>0.008</td>
</tr>
<tr>
<td>ADR + 3 μmol/L Gef</td>
<td>8.1 ± 0.7</td>
<td>0.81</td>
<td>0.37</td>
<td>MCF7/ADR</td>
<td>272 ± 22</td>
<td>27</td>
<td>0.20</td>
<td>0.001</td>
</tr>
<tr>
<td>VP</td>
<td>158.8 ± 22.6</td>
<td></td>
<td></td>
<td>MCF7/VP</td>
<td>3,867 ± 335</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP + 0.3 μmol/L Gef</td>
<td>135.5 ± 20.3</td>
<td>0.85</td>
<td>0.566</td>
<td>MCF7/VP</td>
<td>2,761 ± 159</td>
<td>17</td>
<td>0.90</td>
<td>0.538</td>
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<tr>
<td>VP + 1 μmol/L Gef</td>
<td>134.9 ± 16.0</td>
<td>0.85</td>
<td>0.520</td>
<td>MCF7/VP</td>
<td>2,509 ± 173</td>
<td>16</td>
<td>0.82</td>
<td>0.294</td>
</tr>
<tr>
<td>VP + 3 μmol/L Gef</td>
<td>112.4 ± 19.1</td>
<td>0.71</td>
<td>0.271</td>
<td>MCF7/VP</td>
<td>1,768 ± 156</td>
<td>11</td>
<td>0.58</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Abbreviations: RI, reversing index; Gef, gefitinib.

*Reversing index = IC_{50} of gefitinib plus chemotherapy in cancer cells divided by IC_{50} of chemotherapy-treated cells.

† Unpaired t test comparing IC_{50} of cells in gefitinib with IC_{50} of cells without gefitinib.

‡ Fold, fold of resistance, IC_{50} of drug-resistant cells divided by IC_{50} of drug-sensitive untreated cells.

### Discussion

Gefitinib is the first tyrosine kinase inhibitor marketed for the treatment of advanced stage non–small cell lung cancer patients who failed previous chemotherapy (2). Unlike other chemotherapeutic agents that develop cross-resistance readily both in vitro and in the clinic toward other structurally related or unrelated compounds, gefitinib has not been reported to develop cross-resistance to prior chemotherapeutic agents either in vivo or in vitro (14). Gefitinib sensitivity in chemosensitive and chemoresistant breast carcinoma and lung adenocarcinoma cells was tested in this study. There were no changes in the IC_{50} values to gefitinib after cells acquired MDR phenotypes in MCF7/ADR, MCF7/VP, MCF7/TPT, CL1/Pac, and CL1/Tpt cells.

Gefitinib may enhance chemosensitivity of cancer cells in vitro. Gefitinib was synergistic with SN38 in HT-29 and LoVo colorectal cancer cells (15). A strong synergism was noted between gefitinib and 5′-deoxy-5-fluorouridine in CAL33 head and neck cancer cells (16). Gefitinib had sequence-dependent synergy to oxaliplatin (17). In animal studies, gefitinib was synergistic to several chemotherapeutic agents in various cancer cell lines (4). Cooperative antitumor activity was noted in animals treated with p.o. taxane and gefitinib (18). Despite all these promising observations, no clear mechanisms of synergism between gefitinib and chemotherapeutic agents were proposed in those studies. This study showed that sensitivity to chemotherapeutic agents in MDR cancer cells were increased at the presence of clinically relevant concentrations of gefitinib. Gefitinib, however, did not modulate chemosensitivity in parental chemosensitive cells. Because all the resistant cells tested in this study contain at least one overexpressing ABC transporter that...
conferred resistance to the parental cancer cells, it is likely that gefitinib affects chemosensitivity of MDR cancer cells through interaction with ABC transporters.

Interaction of gefitinib with chemotherapeutic agents in P-glycoprotein, BCRP, and MRP1-overexpressing cancer cells were tested previously. A low concentration of gefitinib (0.2 μmol/L) sensitized BCRP-overexpressing HL60 cells to mitoxantrone toxicity. However, gefitinib may sensitize P-glycoprotein-overexpressing HL60 cells only at concentrations higher than 10 μmol/L. In addition, gefitinib had essentially no effect on the mitoxantrone sensitivity of MRP1-overexpressing HL60 cells (8). In another study, gefitinib at submolar concentrations was shown to enhance sensitivity to taxanes in P-glycoprotein-overexpressing bcl2-transfected MCF7/ADR cells in a 10 to 14 days exposure cytotoxicity assay (19), suggesting that gefitinib did interact with P-glycoprotein. No comparisons to wild-type MCF7 cells were made in that study. Furthermore, gefitinib at 1 to 5 μmol/L was shown to reverse SN38 resistance in Saos2 cells overexpressing functional BCRP. Gefitinib enhanced antitumor activity of irinotecan to mice bearing BCRP-overexpressing tumor (20). Our study showed that gefitinib interact with BCRP and P-glycoprotein in MDR cancer cells. Gefitinib was able to inhibit P-glycoprotein at clinically relevant concentration (1 μmol/L) in chemoresistant MCF7 and CL1 cells. Gefitinib may even inhibit MRPI in MCF7/VP cells at very high concentrations.

Mutation on the amino acid position 482 of BCRP may change substrate specificity and alter MDR phenotypes (21, 22). Gefitinib-sensitive A431 cells transfected with wild-type BCRP acquired resistance to gefitinib and protected cells from gefitinib-induced cell death. The protective effect was not present when the same cells were transfected with BCRP nonfunctional mutants (23). These findings suggest that gefitinib is itself a wild-type BCRP substrate. However, efflux and accumulation of radioactive [14C]gefitinib were the same in Saos2 cells overexpressing functional BCRP or nonfunctional BCRP, indicating that gefitinib is not a BCRP substrate (20). In our study, both CL1/Tpt and MCF7/TPT cells express wild-type BCRP. Although CL1/Tpt and MCF7/TPT cells are not more resistant to gefitinib compared with their parental counterpart, CL1 or MCF7 cells are not sensitive to gefitinib. We cannot find out from our study whether gefitinib is or is not a wild-type BCRP substrate.

Figure 3. Flow cytometric assay of topotecan accumulation in chemosensitive and chemoresistant cells exposed to various concentrations of topotecan and gefitinib. Intracellular topotecan is estimated by fold of fluorescence intensity. Fluorescence intensity in each parental chemosensitive cells exposed to 10 μmol/L of topotecan without gefitinib is normalized to 1. Columns, average values of at least three measurements for CL1 and CL1/Tpt (A), and MCF7 and MCF7/TPT cells (B); bars, SD. There are no changes in the fluorescence intensity after gefitinib treatment in CL1 and MCF7 cells. Topotecan accumulation increases in CL1/Tpt and MCF7/TPT cells in the presence of gefitinib.
It seems that no added efficacy or toxicity was noted when gefitinib was added to chemotherapy in two large phase III studies (5, 6). In theory, a synergistic effect of gefitinib and chemotherapeutic agents may be harmful to hematopoietic progenitor cells that highly express BCRP or P-glycoprotein (24). In an animal study, profound myelosuppression was noted with a combination of gefitinib and vinorelbine (4). Profound and unexpected myelosuppression was shown in a single phase II study that combined gefitinib with vinorelbine (7). It is possible that inhibition of gefitinib of the P-glycoprotein or other ABC transporters of hematopoietic progenitor cells increased accumulation of vinorelbine in these cells and resulted in profound myelosuppression.

Drug pharmacokinetics that is affected by BCRP or P-glycoprotein may be altered in the presence of gefitinib. For example, it was shown that topotecan bioavailability might be increased when taken p.o. with a BCRP and P-glycoprotein inhibitor GF120918 (25). Imatinib mesylate, another clinically available tyrosine kinase inhibitor, is also a potent BCRP transporter inhibitor (26). In an animal study, bioavailability of p.o. administered irinotecan was dramatically increased when gefitinib was administered simultaneously (20). Gefitinib or imatinib may modulate pharmacokinetics of BCRP or P-glycoprotein substrates and result in increased gastrointestinal absorption and central nervous system penetration of the ABC transporter substrates. Increased intracellular concentration of BCRP or P-glycoprotein substrates in BCRP or P-glycoprotein–overexpressing cells, such as hematopoietic progenitor cells, can be expected. As more tyrosine kinase inhibitors become available, pharmacologic interactions between these drugs and ABC transporter substrates in the above-mentioned tissues as well as in cancer cells cannot be overlooked.

Despite the failure of combining small molecule tyrosine kinase inhibitors with chemotherapy in several large phase III clinical

![Graph](image-url)

**Figure 4.** Representative topotecan accumulation and efflux curves measured by flow cytometry. Intracellular topotecan is estimated by mean FL2-H unit as described in Materials and Methods. A, topotecan efflux out of CL1 cells at the same rate without (●) or with (○) gefitinib. Topotecan was expelled out of the CL1/Tpt cells rapidly without gefitinib (▼) as opposed to slower efflux in cells exposed to 3 μmol/L gefitinib (▲). Similarly, in (B), topotecan efflux out of MCF7 cells at the same rate without (●) or with (○) gefitinib. Topotecan was expelled out of the MCF7/TPT cells rapidly without gefitinib (▼) as opposed to slower efflux in cells exposed to 3 μmol/L gefitinib (▲).
trials, the chemotherapy and targeted therapy combination approach is still a viable clinical research paradigm (27). It is possible that a synergistic or additive effect of gefitinib and chemotherapy only exists in small targeted populations. Patients who are extremely sensitive to gefitinib, such as those who had mutations or deletions on the tyrosine kinase domain of the epidermal growth factor receptor in their tumor (28, 29), may have benefited from the combined approach. On the other hand, the result of this study has suggested that patients bearing tumors overexpressing BCRP or P-glycoprotein may benefit from a combination approach if an ABC transporter substrate, such as irinotecan or paclitaxel, is included in the chemotherapy regimen. BCRP is a predictive factor for survival in non–small cell lung cancer patients treated with chemotherapy (30). A study combining gefitinib with irinotecan may be beneficial for patients whose tumors express high level of BCRP.

In conclusion, gefitinib may inhibit cellular BCRP or P-glycoprotein functions at clinically relevant concentrations. Reversal of chemo-resistance was shown in cells overexpressing P-glycoprotein or BCRP. Gefitinib reverses topotecan resistance in BCRP-overexpressing cells by increasing topotecan accumulation in MDR cancer cells. These observations may have multiple clinical impacts.

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