Expression of ABC Transporters in Human Hepatocyte Carcinoma Cells with Cross-resistance to Epirubicin and Mitoxantrone

NAOYA KAMIYAMA1, SAORI TAKAGI1, CHIAKI YAMAMOTO1, TAKEAKI KUDO2, TAKAHITO NAKAGAWA2, MASATO TAKAHASHI2, KAZUAKI NAKANISHI2, HIROMASA TAKAHASHI2, SATORU TODO2 and KEN ISEKI1

1Department of Clinical Pharmaceutics and Therapeutics, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812;
2Department of General Surgery, Graduate School of Medicine, Hokkaido University, Kita 15-jo, Nishi 7-chome, Kita-ku, Sapporo 060-0815, Japan

Abstract. Background: In order to understand the cross-resistance between epirubicin (EPI) and mitoxantrone (MIT), EPI- and MIT-resistant cells were established and their cross-resistance was evaluated. Materials and Methods: The degrees of growth inhibition of EPI-resistant HLE-EPI cells and MIT-resistant HLE-MIT cells by anticancer drugs were measured. The mRNA expressions of multidrug resistance protein 1 (MDR1)/ABCB1 and breast cancer resistance protein (BCRP)/ABCG2 were also measured by quantitative real-time RT-PCR. Moreover, intracellular accumulation of EPI was investigated. Results: HLE-EPI cells were resistant to EPI, MIT and docetaxel. HLE-MIT cells were resistant to EPI, MIT and SN-38. HLE-EPI cells overexpressed MDR1 and HLE-MIT cells overexpressed BCRP. The intracellular accumulation of EPI was decreased in HLE-EPI and HLE-MIT cells. Conclusion: The results suggest that both MDR1 and BCRP can up-regulate the efflux of EPI causing resistance to EPI in HLE-EPI and HLE-MIT cells.

Materials and Methods

Drugs. EPI hydrochloride was obtained from Pfizer Japan Inc. (Tokyo, Japan). MIT hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Docetaxel hydrate was obtained from Aventis Pharma Japan Ltd. (Tokyo, Japan). 7-Ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of irinotecan and cisplatin, was obtained from Yakult Honsha Co., Ltd. (Tokyo, Japan).

Cell culture. HLE, human hepatocellular carcinoma cells from JCRB Cell Bank (Osaka, Japan), were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (8). Resistant cells were developed by exposure to EPI hydrochloride or MIT hydrochloride at concentrations starting at 1 ng/mL with a gradual increase to 32 ng/mL over 4 months for 2 days before passage. The resulting HLE-EPI or HLE-MIT cells were maintained by exposing them to each drug at 32 ng/mL for 2 days before passage.

Estimation of drug resistance. Drug resistance was estimated using the collagen gel droplet embedded culture-drug sensitivity test (CD-DST) (9). The collagen solution was prepared with a collagen gel culture kit (Nitta Gelatin Inc., Osaka, Japan). A cell suspension was added to the collagen solution at a final density of 2x10^5 cells/ml. The collagen-cell mixture was dropped into 6-well culture plates at a volume of 30 µl/droplet, then placed in a 5% CO2 incubator at 37°C for 1 h to gel. Three ml of culture
medium were added to each well. After 24 h, anticancer drugs were added. After 24-h incubation for drug exposure, each well was washed twice with 4 ml of phosphate-buffered solution (PBS) by slowly shaking in the incubator for 10 min. After removal of PBS, 4 ml of culture medium were added to each well and the cells were cultured for 6 days. At the end of the incubation, neutral red was added to each well to a final concentration of 50 Ìg/ml. The cells were fixed with 10% neutral-buffered formalin for 45 min and washed in water for 10 min. The collagen gel droplets were subsequently air-dried and quantified by Primage® (Nitta Gelatin Inc.), an imaging apparatus.

Quantification of mRNA. Real-time RT-PCR was performed using a LightCycler® (Roche Diagnostics K.K., Japan) with hybridization probes. Primer sequences were as follows: MDR1 sense 5’-AAGAA GCCCT GGACA AAGCC-3’, MDR1 antisense 5’-ACAGT CAGAG TTCAC TGGCG-3’, BCRP sense 5’-ATCTT GGCTG TCATG GCT-3’, BCRP antisense 5’-TGATT CTTCC ACAAG CCC-3’, GAPDH sense 5’-GCCTC CTGCA CCACC TG-3’ and GAPDH antisense 5’-CGACG CCTGC TTCAC CACCT TCT-3’. Hybridization probes for the LightCycler® were designed and synthesized at Nihon Gene Research Laboratories Inc. (Sendai, Japan). Real-time PCR cycles started with 10 min at 95ÆC and then 45 cycles of 15 sec at 94ÆC, 15 sec at 56ÆC and 30 sec at 72ÆC for MDR1 and BCRP or 45 cycles of 10 sec at 94ÆC, 10 sec at 60ÆC and 20 sec at 72ÆC for GAPDH. The expression levels of MDR1 and BCRP were expressed as ratios (MDR1 or BCRP/GAPDH).

Uptake study. Cells were seeded on 12-well culture plates and incubated for 5 days. The cells were incubated in a neutral-pH buffered incubation medium containing a substrate (10 Ìg/ml EPI hydrochloride) at 37°C. The cells were solubilized in 1% of Tween 20. EPI was determined using a high-performance liquid chromatography (HPLC) system equipped with a fluorescence spectrophotometer (10). The settings were as follows: column, TSK-GEL CN-80Ts (TOSOH); column temperature, 40°C; mobile phase, 50 mM NaH2PO4 (pH 4.0):acetoniitrile=65:35; flow rate, 0.8 mL/min; λex 480 nm, λem 560 nm; retention time, 4.8 min.

Results

The HLE-MIT cells showed strong resistance to MIT and SN-38 (69- and 76-fold, respectively) and moderate resistance to EPI (6.9-fold). On the other hand, the HLE-EPI cells showed 18-fold resistance to docetaxel and moderate resistance to EPI and MIT (5.9- and 3.4-fold, respectively). Neither cell line showed cross-resistance to fluorouracil or cisplatin (Table I). These spectra of multidrug resistance indicated that HLE-EPI and HLE-MIT overexpressed multidrug resistance transporters. To clarify the expression levels of multidrug resistance transporters, MDR1 and BCRP mRNA levels were measured by real-time RT-PCR. MDR1 mRNA was overexpressed in HLE-EPI cells (21-fold) but not in HLE-MIT cells. On the other hand, BCRP mRNA was overexpressed in HLE-MIT cells (18-fold) but not in HLE-EPI cells (Figure 1). To determine whether the intracellular accumulation of EPI was altered in HLE-EPI and HLE-MIT cells, the intracellular accumulation of EPI was evaluated by an HPLC system with a fluorescence spectrophotometer. Accumulation of EPI in HLE-EPI and HLE-MIT cells was significantly less than that in the HLE cells. The accumulation of EPI in the HLE-EPI cells was the same as that in the HLE-MIT cells from 15 min to 60 min (Figure 2).

Discussion

EPI is an anthracycline known to be a substrate of MDR1 and BCRP (5, 6). To understand cross-resistance and the expression status of ABC transporters in EPI-selected cell lines, EPI-selected sublines were established from human hepatocellular carcinoma HLE cells (8). The HLE-EPI cells showed cross-resistance to MIT and docetaxel, while the HLE-MIT cells showed strong resistance to MIT and SN-38. MIT is a substrate of MDR1 and BCRP. Docetaxel is the substrate of MDR1 (11) but not of BCRP. SN-38 is a substrate of BCRP (12) but not of MDR1. These results strongly suggest that the multidrug resistance of the HLE-EPI cells mainly depended on the overexpression of MDR1 and that of the HLE-MIT cells depended mainly on the overexpression of BCRP.

EPI was selected at 10 Ìg/ml in the absence of serum. The 18-fold resistance to docetaxel in the HLE-EPI cells was lower than the 21-fold resistance to docetaxel in the HLE-EPI cells selected at 10 Ìg/ml with FBS (10% v/v). To evaluate the overexpression of transporters, mRNA levels of MDR1 and BCRP were measured with real-time RT-PCR and intracellular accumulation of EPI. The

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<table>
<thead>
<tr>
<th>IC50 (ÌM) and fold resistance.</th>
<th>HLE</th>
<th>HLE-EPI</th>
<th>HLE-MIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epirubicin</td>
<td>0.034±0.005</td>
<td>0.17±0.033</td>
<td>(5.9) 0.20±0.051</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.006±0.002</td>
<td>0.019±0.003</td>
<td>(3.4) 0.39±0.35</td>
</tr>
<tr>
<td>Fluourouracil</td>
<td>40±5.7</td>
<td>40±2.5</td>
<td>(1.0) 40±10</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.63±0.20</td>
<td>0.54±0.083</td>
<td>(0.86) 0.47±0.097</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.002±0.0002</td>
<td>0.036±0.011</td>
<td>(18) 0.001±0.0003</td>
</tr>
<tr>
<td>SN-38</td>
<td>0.008±0.003</td>
<td>0.010±0.002</td>
<td>(1.2) 0.62±0.22</td>
</tr>
</tbody>
</table>

Fifty percent inhibition concentration (IC50) was calculated with sigmoid fitting. The fold resistance is shown in parenthesis.
HLE-EPI cells overexpressed MDR1 with no change in the BCRP mRNA expression level. Furthermore, accumulation of EPI in HLE-EPI cells was significantly less than that in HLE cells. These findings suggest that HLE-EPI cells acquire multidrug resistance by the overexpression of MDR1. On the other hand, the HLE-MIT cells overexpressed BCRP with no change in the MDR1 mRNA expression level and with reduced accumulation of EPI. Although different transporters are overexpressed in these resistant cells, the degrees of accumulation of EPI were almost the same, providing an explanation as to why the degrees of resistance of HLE-EPI and HLE-MIT to EPI are almost the same. Robey et al. showed that the relative resistance value of EPI was less than that of MIT or SN-38 in codon 482 wild-type (482R) and mutants (482G and 482T) (13). The sequence of the BCRP codon 482 in HLE-MIT cells has not been revealed, but the cross-resistant status we examined is consistent with the results of their experiment. It is possible that cross-resistance beyond the analogs of an anticancer agent mainly depends on multidrug efflux transporters, such as MDR1 or BCRP.

In conclusion, the EPI-selected human hepatocellular carcinoma cell line HLE overexpressed MDR1 with no change in BCRP mRNA. On the other hand, the MIT-selected HLE cells overexpressed BCRP with no change in MDR1 mRNA. However, the degree of resistance and the intracellular accumulation of EPI declined in both HLE-EPI and HLE-MIT cells. These findings suggest that both MDR1 and BCRP can up-regulate the efflux of EPI, causing resistance to EPI in HLE-EPI and HLE-MIT cells.
References


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