Inhibition of P-Glycoprotein-Dependent Multidrug Resistance by an Isoquinolinesulfonamide Compound H-87 in Rat Ascites Hepatoma AH66 Cells

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The effects of an isquinolinesulfonamide compound, H-87, on naturally acquired multidrug-resistance (MDR) in rat hepatoma AH66 cells were examined. AH66 cells were highly resistant to vinblastine, SN-38, an active camptothecin analog, adriamycin, and etoposide, compared with the sensitive variant AH66F cells. Although H-87 hardly affected the sensitivities to antitumor agents of AH66F cells, this compound completely inhibited the resistance to vinblastine, moderately inhibited the resistance to SN-38 and adriamycin and had little effect on etoposide, mitomycin C, cisplatin, and 5-fluorouracil. H-87 significantly decreased the efflux of vinblastine from the resistant cells and increased the drug accumulation. SN-38 and adriamycin also exhibited a weak but significant increase in vinblastine accumulation in AH66 cells. H-87 inhibited [3H]azidopine-photolabeling to 160 kDa P-glycoprotein in the plasma membrane of AH66 cells, as reported in acquired MDR leukemic cells. Consequently, the MDR-overcoming effect of H-87 seems to be due to its direct inhibition of the binding of antitumor agents on P-glycoprotein in the plasma membrane.

Key words: multidrug resistance; overcoming effect; H-87; P-glycoprotein; rat ascites hepatoma; AH66 cell

Many investigators have studied acquired multidrug-resistant (MDR) cell lines, which were established by prolonged exposure to some antitumor drugs, for their mechanisms and screening of overcoming drugs. The heterogeneity of tumor cells in drug sensitivity and the selection of resistant cells are major problems in cancer chemotherapy. Therefore, natural drug resistance seems to be clinically significant. There are many rat ascites hepatoma (AH) cell lines which were induced by dimethylnitrosamine and established as transplantable tumors, each with characteristic sensitivities to antitumor drugs. We have shown that the AH66 cell line is an MDR line overexpressing 160 kDa P-glycoprotein, and its variant, AH66F, is a drug-sensitive line undetectable to P-glycoprotein.

Hidaka et al. developed isquinolinesulfonamide derivatives which inhibit several protein kinase reactions to different extents. We have indicated that among them, N-[2-[N-[3-(4-chlorophenyl)-1-methyl-2-propenyl]-amino]ethyl]-5-isquinolinesulfonamide (H-87) reversed the adriamycin-induced MDR of leukemia cell lines, without interfering with its activity on protein kinases.

In this report, we investigated the effect of H-87 on the natural MDR of AH66 cells.

MATERIALS AND METHODS

Tumor Cells The AH66 and AH66F cell lines were passed weekly through female Donyu rats weighing 100 to 150 g (Nippon SLC, Hamamatsu) and harvested from the tumor-bearing rats 6 to 10 d after transplantation.

Agents The antitumor agents used were vinblastine (Shionogi & Co., Osaka), adriamycin, 5-fluorouracil, mitomycin C (Kyowa Hakko Kogyo Co., Tokyo), etoposide (Bristol-Meyers Research Institute, Tokyo), cisplatin (Nippon Kayaku Co., Tokyo), and 7-ethyl-10-hydroxy camptothecin (SN-38, given by Dr. T. Sasaki of the Cancer Research Institute, Kanazawa University, Kanazawa). [3H]Vinblastine (347 GBq/mmol) and [3H]azidopine (1.92 TBq/mmol) were purchased from American International, Buckinghamshire, UK. H-87 was synthesized by the method described before.

Cell Culture Cells were suspended in Eagle’s minimum essential medium supplemented with 10% fetal calf serum at a density of 1.5 × 106 cells/ml and cultured in the absence or presence of an antitumor agent and/or H-87 for 48 h at 37°C in a CO2 incubator. The cells were counted under a microscope, and the effects of antitumor agents were expressed by their 50% growth-inhibiting concentration (IC50).

Accumulation and Eflux of [3H]Vinblastine Cells (1 × 106/ml) were incubated in the culture medium in the presence of 10 nM [3H]vinblastine with or without a test agent for 30 min at 37°C. The cells were washed, dissolved in 0.5 N NaOH and neutralized. The radioactivity was counted in a toluene/Triton X-100 (2/1, v/v) scintillation cocktail by a liquid scintillation counter.

In the study on influx, to obtain a high intracellular concentration of [3H]vinblastine, cells (2 × 107/ml) were preloaded with 20 nM [3H]vinblastine in a glucose-deprived Hank’s solution containing 10 mM sodium azide for 30 min at 30°C. The cells were washed and resuspended in the fresh culture medium with or without a test agent at 30°C, and the radioactivity retained in the cells was counted.

Photoaffinity Labeling Plasma membranes prepared by a Percoll sedimentation method as previously reported were incubated with 200 nM [3H]azidopine for 30 min at room temperature in the presence or absence of H-87 and irradiated at 366 nm for 20 min. The sample was solu-
bibilized in sodium dodecylsulfate (SDS) and 8 M urea and fractionated by electrophoresis on a 7.5% polyacrylamide/4.5 M urea gel, with a stacking gel. After being fixed and dried, the gel was autoradiographed on a Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY, U.S.A.) with an intensifying screen at −70 °C.

Statistics Experiments were done at least three times in triplicate. Statistical analysis was done using Student’s t-test and Welch’s t-test.

RESULTS AND DISCUSSION

Table 1 shows that AH66 cells were highly resistant to vinblastine, SN-38, adriamycin, and etoposide, compared with AH66F cells. The resistant indices of AH66 for mitomycin C, cisplatin, and 5-fluorouracil cells were below 3. Since the cytotoxicity of H-87 on these cells was not different, with IC_{50} values ranging from 6 to 8 μM, the concentration of H-87 used in the combination study was fixed at 2 μM, a level at which it was non-toxic to both cells. Although H-87 only slightly increased the sensitivity to vinblastine of AH66F cells, this compound completely reversed the vinblastine resistance of AH66 cells (Table 1 and Fig. 1). And H-87 partially but significantly inhibited the resistance to SN-38 and adriamycin of AH66 cells, without influencing the sensitivity of AH66F cells. The growth-inhibitory activities of other antitumor agents on both cell types were not affected by H-87 (Table 1).

Previously, we reported that H-87 inhibited vinblastine binding to P-glycoprotein and suppressed the drug extrusion from the acquired MDR leukemia cells. This compound increased the intracellular drug concentration and reversed the drug resistance. This study confirmed the effects of H-87 in natural MDR AH66 cells. Namely, H-87 inhibited the efflux of vinblastine from the cells and significantly increased the drug accumulation in AH66 cells (Fig. 2). AH66 cells overexpress a 160 kDa P-glycoprotein in the membrane, and this protein was clear-

Table 1. Effects of H-87 on the in Vitro Sensitivities to Antitumor Agents of AH66F and AH66 Cells

<table>
<thead>
<tr>
<th>Antitumor agent</th>
<th>AH66F</th>
<th>AH66</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>H-87 (2 μM)</td>
<td>H-87 (2 μM)</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>3.4±0.3 (1.0)</td>
<td>1.6±1.0 (1.1)</td>
</tr>
<tr>
<td>SN-38</td>
<td>13±9 (1.0)</td>
<td>14±3 (1.1)</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>24±5 (1.0)</td>
<td>35±5 (1.1)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>45±6 (1.0)</td>
<td>62±4 (1.4)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>24±3 (1.0)</td>
<td>19±4 (1.4)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>222±36 (1.0)</td>
<td>200±15 (1.0)</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>490±84 (1.0)</td>
<td>427±78 (0.9)</td>
</tr>
</tbody>
</table>

Each value is the mean IC_{50}±S.E. (μM) of at least three experiments. Number in parenthesis is the relative resistance index. * Significantly different from the IC_{50} value for each antitumor agent alone in AH66 cells at p<0.001.

Fig. 1. Growth-Inhibitory Effects of Vinblastine and the Combined Effects of H-87 on AH66F and AH66 Cells

AH66F cells (□, ■) or AH66 cells (○, ●) were incubated in the absence (open symbols) or presence (closed symbols) of 2 μM H-87 for 48 h. Data are the means±S.E. (bar) of three independent experiments done in triplicate.

Fig. 2. Effects of H-87 on the Accumulation and Efflux of Vinblastine in AH66F and AH66 Cells

(A) Accumulation of vinblastine in the absence (open symbols) or presence (closed symbols) of varying concentrations of H-87 for 30 min. (B) Percentage of retained vinblastine in the absence (open symbols) or presence (closed symbols) of 10 μM H-87 for the indicated time after the forced accumulation. AH66F, □, ■; AH66, ○, ●. Data are the means±S.E. (bar) of three experiments done in triplicate. * Significantly different from the untreated control at p<0.01.
ly photolabeled with $[^3$H]azidopine, which is a ligand for P-glycoprotein\(^{19}\); also, H-87 inhibited the labeling in a concentration-dependent manner (Fig. 3). On the other hand, H-87 increased vinblastine accumulation and potentiated the drug sensitivity of AH66F cells, to only a small extent. P-glycoprotein was not detectable in the plasma membrane of AH66F cells by immunostaining and photolabeling methods (our previous reports\(^{7-9}\) and Fig. 3), but it is possible that AH66F cells have a low level of P-glycoprotein in the membrane.

In this study, the growth-inhibitory activities of SN-38, an active form of camptothecin analog with inhibitory activity of topoisomerase I (Topo I)\(^{20}\) and adriamycin, a Topo II inhibitor,\(^{21}\) were also potentiated by H-87. It is well known that adriamycin is a substrate of P-glycoprotein with low affinity, and that it causes P-glycoprotein-dependent resistance.\(^{31}\) Whether the resistance to SN-38 is related to P-glycoprotein is not reported. We then examined the effects of these agents on the accumulation of vinblastine in the AH cells. As shown in Fig. 4, both agents significantly and concentration-dependently increased the vinblastine content in AH66 cells but not in AH66F cells. These results suggest that not only adriamycin but also SN-38 may be extruded through P-glycoprotein. This resistance in AH66 cells, however, seems to be less dependent upon P-glycoprotein than that of vinblastine. Indeed, the resistance to SN-38 and adriamycin was partially inhibited by H-87 (Table 1).

Another Topo II inhibitor, etoposide, has been reported to be concerned with P-glycoprotein-dependent and non-P-glycoprotein-dependent resistance.\(^{3,4}\) In this study, its resistance was not influenced by H-87 (Table 1), and it never affected the vinblastine transport in the AH cells (Fig. 4). Consequently, the fraction of resistance to SN-38, adriamycin, and etoposide in AH66 cells, which could not be reversed by H-87, may be based on mechanisms other than P-glycoprotein, such as changes in the structure and activity of Topo I and II.

This study indicates that AH66 cells were resistant not only to vinblastine and adriamycin, but also to SN-38 and etoposide. In addition, an isoquinolinesulfonamide compound, H-87, inhibited the natural MDR, as well as acquired MDR, except for the etoposide resistance. There have been many reports describing the relation of protein phosphorylation to the mechanisms of MDR.\(^{22,23}\) Isoquinolinesulfonamide compounds, including H-87, show inhibitory activities on several protein kinases.\(^{14}\) While these compounds possess a high affinity for P-glycoprotein\(^{24}\) and overcome MDR without involving their activities on protein kinases,\(^{11-13}\) new MDR-overcoming drugs, which act on both P-glycoprotein and signal transduction in MDR cells, may be developed from isoquinolinesulfonamide derivatives.

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