Effects of Patupilone (Epothilone B; EPO906), a Novel Chemotherapeutic Agent, in Hepatocellular Carcinoma: An in vitro Study

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**Key Words**
Patupilone  •  Epothilone B  •  Hepatocellular carcinoma  •  P-glycoprotein  •  Taxane  •  Doxorubicin

**Abstract**

**Purpose:** In this study, the cytotoxic effects of patupilone (epothilone B; EPO906) were assessed in a panel of hepatocellular carcinoma (HCC) cell lines, and were compared with doxorubicin and the microtubule-stabilizing taxanes. **Methods:** The following HCC cell lines were used: PLC/PRF/5, HepG2, Hep3B, SNU-387, SNU-398, SNU-423, SNU-449, and SNU-475. Cells were treated with various concentrations of patupilone, paclitaxel, docetaxel, or doxorubicin for 72 h; 50% inhibitory concentrations (IC\textsubscript{50}) were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay. P-glycoprotein expression was assessed using standard Western blotting techniques. **Results:** Patupilone was found to be the most potent drug in all 8 HCC cell lines. All cell lines except SNU-449 were 4- to 19-fold more sensitive to patupilone than to paclitaxel and docetaxel, and 59- to 208-fold more sensitive than to doxorubicin. SNU-449, the most resistant cell line and the only one overexpressing P-glycoprotein, was 3- to 39-fold more resistant to paclitaxel, docetaxel, and doxorubicin than were other cell lines. The IC\textsubscript{50} of patupilone in SNU-449 was 1.14 nmol, which was 108- to 529-fold lower than those of the other agents. **Conclusion:** Patupilone was more potent than taxanes and doxorubicin in HCC cell lines and may result in reduced clinical resistance by overcoming P-glycoprotein overexpression. A clinical study in HCC is warranted.

**Introduction**

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide \[1, 2\]. Approximately 500,000 cases of HCC are diagnosed each year, representing \(15\%\) of all cancers \[2, 3\]. Recent estimates from the United States suggested a modest increase in the incidence of HCC that could be accounted for by the growing prevalence of chronic hepatitis C infection and improved survival of patients with cirrhosis \[3–5\]. To date, only a small portion of HCC is curable by resection, and nonsurgical management of HCC remains suboptimal \[6\]. Results of systemic chemotherapy for HCC have been disappointing. Single-agent doxorubicin has been widely used to treat unresectable HCC; however, the response rate is low \((<20\%)\) and there is no compelling evidence for improved survival \[7\]. Recently, the combination of cisplatin, interferon, doxorubicin, and 5-fluorouracil was shown to produce an improved response rate (20.9\%) and an extended
median survival (8.7 months) compared with doxorubicin alone (10.5% response rate and 6.8-month survival), but at the expense of substantially greater toxicities [7]. Despite initial promise, tamoxifen has not been shown to have antitumoral or survival benefits in patients with HCC [1]. Together, these data indicate that the prognosis for patients with unresectable HCC remains poor and that effective systemic treatments are needed for patients who do not qualify for resection or transplant [7].

The efficacy of microtubule-stabilizing agents has not been studied extensively in HCC. Both paclitaxel and docetaxel have demonstrated substantial cytotoxicity in selected HCC cell lines [8, 9]. In a phase I clinical trial, a weekly 1-hour infusion of paclitaxel (70 mg/m² starting dose escalated to a maximum of 100 mg/m²) in patients with unresectable HCC resulted in a 63% disease stabilization rate [10]. However, in a phase II clinical trial, paclitaxel 175 mg/m² administered every 3 weeks failed to show an anticancer effect in patients with HCC [11].

Patupilone (epothilone B; EPO906), a macrocyclic polyketide, is a member of the epothilone class, a group of microtubule-stabilizing agents [12]. Although structurally unrelated to the taxanes, patupilone binds to a similar, although not identical, pharmacophore on the β-tubulin subunit of microtubules [13, 14]. Data also suggest that patupilone binds to this site with higher affinity than other epothilones or taxanes [15]. In vitro evidence indicates that patupilone is a more potent inducer of tubulin dimerization and is more effective in stabilizing preformed microtubules than paclitaxel [14, 16]. Patupilone has been shown to exhibit a 3- to 20-fold higher in vitro cytotoxic potency than paclitaxel, and to have cytotoxic activity in a broad range of paclitaxel-sensitive and resistant cells overexpressing the P-glycoprotein efflux pump [17]. In animal models, patupilone has produced positive results against solid tumors, including tumors that overexpress P-glycoprotein [17–20].

Clinical studies of patupilone in solid tumor types including lung and ovarian cancer lend further support to its anticancer activity. In a phase I dose-finding study of patupilone in 91 patients with advanced solid tumors, a disease stabilization rate (partial response plus stable disease) of 47% was observed in 60 assessable patients [21]. Further, in a phase I/II trial of 50 patients with histologically and cytologically confirmed unresectable non-small cell lung cancer who had received prior treatment with a platinum-containing regimen, a disease stabilization rate of 42% was observed [22]. Lastly, in a phase I/II trial of 45 patients with ovarian cancer who failed to respond or relapsed during taxane, platinum, or combination therapy, a disease stabilization rate of approximately 56% was observed [23].

To date, no data are available on the effects of epothilones in HCC. In the present study, the cytotoxic effects of patupilone were assessed in a panel of HCC cell lines and compared with doxorubicin, as well as the taxanes paclitaxel and docetaxel. In addition, P-glycoprotein expression of the cell lines was measured to examine its relationship to the treatment resistance pattern.

Materials and Methods

Drugs

Patupilone – provided by Novartis Pharma, Basel, Switzerland – was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, Mo., USA) as a 10-mM stock solution, divided into 5-µl aliquots, and stored at –20 °C until use. Docetaxel, paclitaxel, and doxorubicin were purchased from Aventis Pharmaceuticals, Bridgewater, N.J., USA, Bristol-Myers Squibb, Princeton, N.J., USA and EBEWE Pharma, Unterach, Austria, respectively. All drugs were diluted in culture medium immediately before addition to cell lines.

Cell Lines

The 8 HCC cell lines used in this study included PLC/PRF/5, HepG2, Hep3B, SNU-387, SNU-398, SNU-423, SNU-449, and SNU-475. All cell lines were purchased from the American Type Culture Collection, Rockville, Md., USA.

Cell Culture

Unless otherwise specified, all culture media and supplements were purchased from GIBCO®, Carlsbad, Calif., USA. PLC/PRF/5, HepG2, and Hep3B were cultured in DMEM supplement-ed with 10% fetal bovine serum, nonessential amino acids 0.1 mM, penicillin 50 units/ml, and streptomycin 50 µg/ml. The 5 SNU cell lines were cultured in RPMI 1640 with 10% fetal bovine serum, penicillin 50 units/ml, and streptomycin 50 µg/ml. All cultures were maintained in a humidified incubator at 37°C with 5% carbon dioxide.

MTT Assay

Cells at log phase of growth were seeded in 96-well culture plates at optimum density and allowed to attach for 24 h. Media were removed after 24 h and replaced with culture medium 200 µl or the same volume of medium containing various concentrations of patupilone, paclitaxel, docetaxel, or doxorubicin. After 72-hour incubation, 50 µl of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] from Sigma-Aldrich was added, incubated and removed according to standard procedure [24]. Absorbance was measured by a 96-well plate reader – Tecan Spectra (Tecan Group, Raleigh, N.C., USA) – at 570 nm with a reference wavelength of 630 nm. Each drug concentration was obtained in ≥4 replicate wells, and each experiment was repeated 3 times. Dose-response curves were generated and the 50% inhibitory concentration (IC₅₀) was determined using GraphPad Prism™ version 4.00 for Windows (GraphPad Software, San Die-
Table 1. IC\textsubscript{50} (nm) for growth inhibition of HCC cell lines by patupilone in comparison with docetaxel, paclitaxel, and doxorubicin

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Patupilone IC\textsubscript{50}</th>
<th>Docetaxel IC\textsubscript{50}</th>
<th>relative potency vs. patupilone</th>
<th>Paclitaxel IC\textsubscript{50}</th>
<th>relative potency vs. patupilone</th>
<th>Doxorubicin IC\textsubscript{50}</th>
<th>relative potency vs. patupilone</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC/PRF/5</td>
<td>0.371 ± 0.076</td>
<td>5.293 ± 0.679</td>
<td>-14.3 ×</td>
<td>6.995 ± 1.375</td>
<td>-18.9 ×</td>
<td>56.00 ± 3.513</td>
<td>-150.9 ×</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.576 ± 0.008</td>
<td>5.945 ± 0.731</td>
<td>-10.3 ×</td>
<td>7.078 ± 0.840</td>
<td>-12.3 ×</td>
<td>33.77 ± 3.314</td>
<td>-58.6 ×</td>
</tr>
<tr>
<td>Hep3B</td>
<td>0.714 ± 0.055</td>
<td>12.52 ± 0.552</td>
<td>-17.5 ×</td>
<td>6.343 ± 0.373</td>
<td>-8.9 ×</td>
<td>46.40 ± 1.961</td>
<td>-65.0 ×</td>
</tr>
<tr>
<td>SNU-387</td>
<td>1.058 ± 0.175</td>
<td>7.377 ± 0.257</td>
<td>-7.0 ×</td>
<td>4.310 ± 1.019</td>
<td>-4.1 ×</td>
<td>220.4 ± 14.03</td>
<td>-208.3 ×</td>
</tr>
<tr>
<td>SNU-398</td>
<td>0.482 ± 0.074</td>
<td>3.844 ± 0.172</td>
<td>-8.0 ×</td>
<td>3.518 ± 0.065</td>
<td>-7.3 ×</td>
<td>75.19 ± 9.397</td>
<td>-156.0 ×</td>
</tr>
<tr>
<td>SNU-423</td>
<td>0.903 ± 0.207</td>
<td>4.904 ± 0.525</td>
<td>-5.4 ×</td>
<td>4.352 ± 0.174</td>
<td>-4.8 ×</td>
<td>108.6 ± 6.551</td>
<td>-120.3 ×</td>
</tr>
<tr>
<td>SNU-475</td>
<td>1.140 ± 0.030</td>
<td>148.1 ± 6.346</td>
<td>-129.9 ×</td>
<td>123.5 ± 10.82</td>
<td>-108.3 ×</td>
<td>602.7 ± 17.84</td>
<td>-528.7 ×</td>
</tr>
<tr>
<td>SNU-449</td>
<td>0.631 ± 0.150</td>
<td>5.829 ± 0.492</td>
<td>-9.2 ×</td>
<td>3.522 ± 0.083</td>
<td>-5.6 ×</td>
<td>61.87 ± 5.309</td>
<td>-98.1 ×</td>
</tr>
</tbody>
</table>

Results shown are the averages from 3 independent experiments ± standard deviation. IC\textsubscript{50} = 50\% inhibitory concentration.

Results

Results are summarized in table 1. All HCC cell lines demonstrated a higher IC\textsubscript{50} with doxorubicin than with the microtubule-stabilizing agents. With the exception of SNU-449, all cell lines were between 4- and 19-fold more sensitive to patupilone than to docetaxel or paclitaxel, and between 59- and 208-fold more sensitive to patupilone than to doxorubicin. The SNU-449 cell line was 3- to 39-fold more resistant to docetaxel, paclitaxel, or doxorubicin than were the other 7 cell lines. In this cell line, the IC\textsubscript{50} of patupilone was 1.14 nmol, which was 108- to 529-fold lower than the IC\textsubscript{50} of the other 3 agents. Furthermore, the IC\textsubscript{50} of patupilone was consistent across all 8 cell lines.

In addition, all HCC cell lines were found to have a higher IC\textsubscript{50} with doxorubicin than with the two taxanes, paclitaxel and docetaxel. All cell lines were between 4- and 30-fold more sensitive to docetaxel than to doxorubicin, and between 5- and 51-fold more sensitive to paclitaxel than to doxorubicin. In the SNU-449 cell line, although the IC\textsubscript{50} values of docetaxel and paclitaxel were high (148.1 and 123.5 nmol, respectively) compared with patupilone, they were 4.1- to 4.9-fold lower than the IC\textsubscript{50} of doxorubicin.

As expected, SNU-449 – the cell line demonstrating the greatest resistance to paclitaxel, docetaxel, and doxorubicin – strongly overexpressed P-glycoprotein (approximately 7-fold compared with the reference cell line PLC/PRF/5; fig. 1). P-glycoprotein was not strongly overexpressed in the remaining cell lines.

Assessment of P-Glycoprotein Expression

P-glycoprotein expression was assessed using standard Western blotting techniques. A subconfluence monolayer of HCC cells was trypsinized and washed with PBS 3 times. Cell pellets were lysed in a standard manner and lysates were further incubated on ice for 40 min with occasional low-speed vortexing. After centrifugation at 14,000 rpm for 15 min at 4°C, the supernatant was collected and protein concentration was determined by Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, Calif., USA). Protein extracts (10 μg) from each cell line were separated by 10% SDS-PAGE (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in TBST and probed with anti-P-glycoprotein antibodies (Calbiochem, Darmstadt, Germany) in 1% skimmed milk dissolved in TBST. The membrane was then stripped and reprobed with anti-GAPDH antibodies (Ambion, Austin, Tex., USA) to ensure equal loading. Expression levels of P-glycoprotein for individual cell lines were measured by Quantity One® 1-D Analysis software (Bio-Rad Laboratories) and normalized by their respective expression levels of GAPDH. The PLC/PRF/5 cell line, which does not overexpress P-glycoprotein, was used as a reference [25].

Discussion

Doxorubicin is a standard systemic therapy for patients with unresectable HCC. The drug is frequently used despite its low response rates and lack of evidence for survival prolongation [26]. Our findings have demonstrated that all 8 HCC cell lines had the highest sensitivity to patupilone, moderate sensitivity to taxanes and least sensitivity to doxorubicin. However, two phase II trials on taxane had demonstrated minimal activities...
against HCC while the response rate to doxorubicin was persistently above 10% [6, 7, 27, 28]. Difference in in vitro drug sensitivity may not reflect clinical efficacy but it can provide insight into the mechanism of drug resistance such as multidrug resistance.

Intrinsic multidrug resistance caused export of chemotherapeutic drugs across the plasma membrane by transporters such as P-glycoprotein [29]. In vitro studies have found that up to 85% of cell lines derived from HCC demonstrate significant expression of P-glycoprotein, even in the absence of pretreatment with doxorubicin or taxane [30, 31]. Moreover, these same studies suggest a direct correlation between P-glycoprotein status and clinical outcomes. In another study, patients with P-glycoprotein-positive tumors were found to have a significantly shorter disease-free interval (38.5 months) than patients with P-glycoprotein-negative tumors (86.2 months; p < 0.05), as well as a shorter (although nonsignificant) survival time (47.0 vs. 72.3 months) [32]. It should be noted that there was no difference in survival between P-glycoprotein-positive patients who had chemotherapy and those who did not, suggesting that chemotherapy had little benefit in these patients.

Data suggest that the mechanisms of intrinsic drug resistance in HCC cell lines may also extend beyond P-glycoprotein expression. Certain members of the multidrug resistance protein (MRP) family – a group of integral membrane glycoproteins that confer resistance to chemotherapeutic drugs – have been shown to be overexpressed in up to 87% of HCC cell lines [33]. Clinically, the mean survival of patients with MRP-negative tumors was found to be significantly longer than that of patients with MRP-positive disease (p < 0.05) [34].

In view of the intrinsic resistance to doxorubicin and taxanes in HCC cell lines [35], it is reasonable to suggest that microtubule-stabilizing agents such as patupilone, which is a weak substrate for the P-glycoprotein efflux pump, may have greater efficacy. The results of this study indicate that patupilone was substantially more potent than either paclitaxel or docetaxel in a series of HCC cell lines with intrinsic multidrug resistance. In this study, the SNU-449 cell line demonstrated significant overexpression of P-glycoprotein compared with the other cell lines examined. Consistent with these data, SNU-449 was significantly more resistant to paclitaxel, docetaxel, and doxorubicin in comparison with the other cell lines. In addition, the IC₅₀ of patupilone for SNU-449 was significantly lower than that of the other three agents. It should also be noted that the IC₅₀ of patupilone in this cell line was consistent with that of the other cell lines. These results are in accordance with earlier studies showing that patupilone had 3- to 20-fold higher cytotoxic potency than paclitaxel across a broad range of paclitaxel-sensitive and resistant cells that overexpress the P-glycoprotein efflux pump [19]. However, observation from in vitro experiments was not sufficient to conclude on efficacy of patupilone on HCC and our encouraging findings should be further investigated in a human tumor xenograft model.

In conclusion, patupilone demonstrated substantially greater potency than paclitaxel, docetaxel, and doxorubicin in this panel of 8 HCC cell lines that had intrinsic re-
sistance to a variety of chemotherapeutic agents. In view of the limited options for systemic chemotherapy and the poor outcomes in patients with unresectable HCC, a clinical study of this novel chemotherapeutic agent in such patients could prove successful.

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References
