SUPPLEMENTARY DATA

Xuping Wu et al: Alternative cytotoxic effects of the postulated IGF-1R inhibitor picropodophyllin (PPP) in vitro

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1. Molecular structures
The molecular structures of picropodophyllin (PPP) and podophyllotoxin (PPT) are shown in figure S1.

![Molecular structures of picropodophyllin (PPP) and podophyllotoxin (PPT).](image)

Figure 1 (Supplementary Figure S1) Molecular structures of picropodophyllin (PPP) and podophyllotoxin (PPT).

2. Determination of viability using the Fluorometric Microculture Cytotoxicity Assay
The Fluorometric Microculture Cytotoxicity Assay (FMCA) was originally developed as a method for the semi-automated determination of cytotoxicity and cellular proliferation in human tumour cell lines (1), but its clinical potential as a method for predicting treatment outcomes in leukaemia patients has been recognized (2, 3). It has also been proven to be valid for use with solid tumour specimens (4, 5). There is a significant correlation between the clinical activity profiles of anticancer drugs and the results obtained when those drugs are used in FMCA experiments with primary human tumour cultures (6, 7). This suggests that the FMCA could be used in “ex-vivo phase 2 trials”. The sensitivity and specificity of the FMCA (and similar in vitro methods) as a predictor of clinical outcomes are 0.9 and 0.7, respectively (8, 9). The FMCA is based on the measurement of fluorescence generated by the hydrolysis of fluorescein diacetate in cells with intact plasma membranes that are cultivated and exposed to drugs in 96- or 384-well microtiter plates. The level of fluorescence is linearly dependent on the number of living cells, and FMCA results have been shown to correlate with those of previously established in vitro cytotoxicity assays such as the MTT and DiSC (2, 5). A
standard protocol that is applicable to both cell lines and freshly prepared tumor cells from patients was recently published. This new method is considered to be suitable both for screening in drug development and as the basis for a predictive test that can be used to design individualized anticancer drug therapy regimes (10).

3. Additional data for the ESCC cell lines
As mentioned in the main text, the activity of PPP in the ESCC cell lines correlated well with that of PPT, but not with that of the IGF-1R tyrosine kinase inhibitor AEW-541. The activity of PPP also correlated with that of other tubulin-affecting agents, but not with that of substances having different mechanisms of action (see Figure 2).

![Figure 2](Supplementary Figure S6) Correlation analysis for the activity of PPP compared to vinorelbine (A), docetaxel (B), gemcitabine (C) and cisplatin (D). Significant correlations (p<0.05) were observed for cases A and B. The correlation coefficient was 0.62 for 5-FU (not significant; data not shown).

In addition, potential correlations between the activity of PPP and the expression and phosphorylation of IGF-1R were investigated. As mentioned in the main text and shown in Figure 3, no significant correlations (p>0.05) were identified in either case.
Figure 3 (Supplementary Figure S2) Analysis of potential correlations between PPP activity and the expression (A) or phosphorylation (B) of IGF-1R in nine ESCC cell lines. No significant correlation was identified in either case (p>0.05).

To determine whether the effects of PPP are synergistic with or opposed to those of conventional anticancer agents, FMCA experiments were conducted using fixed concentrations of PPP (0, 5, or 25µM) and variable concentrations of five different existing cancer drugs: cisplatin, docetaxel, etoposide, 5-FU, gemcitabine, and vinorelbine. IC\textsubscript{50}-values for the various combinations were determined as described above and used to create concentration-response curves. These experimental values were then compared to theoretical IC\textsubscript{50} values for the different combinations, which were calculated using an additive method. The results of this interaction analysis are presented in table S1.
Table S1. Interactions between PPP and conventional anticancer agents in the ESCC lines. Experimental survival index values were determined for various combinations of PPP (at concentrations of 0, 5, or 25 µM) and five anticancer drugs, and compared to theoretical survival index values calculated by summing the survival index values for each drug in the combination separately at their tested concentrations. The ratio of the experimental and theoretical values for each combination was used to determine whether the two drugs interacted synergistically (indicated by a ratio below 0.8), additively (ratio between 1.2 and 0.8) or antagonistically (ratio above 1.2). PPP and cisplatin interacted with antagonism in 8 of the 9 ESCC lines and additively in the ninth. The number of synergistic/additive/antagonistic interactions between PPP and the other drugs tested were 0/6/3 for 5-FU, 0/5/4 for Docetaxel, 0/8/1 for Gemcitabine, 1/7/1 for Etoposide, and 1/7/1 for Vinorelbine. The table shows the survival index ratios for the nine ESCC lines when treated with combinations of PPP at 25 µM and the other drugs at the indicated concentrations.

<table>
<thead>
<tr>
<th>Drug combination (25 µMPPP plus partner shown below)</th>
<th>KYSE30</th>
<th>KYSE70</th>
<th>KYSE140</th>
<th>KYSE150</th>
<th>KYSE180</th>
<th>KYSE410</th>
<th>KYSE450</th>
<th>KYSE510</th>
<th>KYSE520</th>
</tr>
</thead>
<tbody>
<tr>
<td>CisP 20 µM</td>
<td>1.36</td>
<td>2.90</td>
<td>1.78</td>
<td>1.74</td>
<td>10.3</td>
<td>1.34</td>
<td>3.34</td>
<td>3.50</td>
<td>1.18</td>
</tr>
<tr>
<td>Doce 20 µM</td>
<td>1.23</td>
<td>1.00</td>
<td>0.98</td>
<td>1.22</td>
<td>0.86</td>
<td>1.31</td>
<td>0.87</td>
<td>1.27</td>
<td>0.97</td>
</tr>
<tr>
<td>Etop 50 µM</td>
<td>0.86</td>
<td>0.94</td>
<td>0.81</td>
<td>1.06</td>
<td>1.06</td>
<td>0.73</td>
<td>1.44</td>
<td>0.88</td>
<td>1.12</td>
</tr>
<tr>
<td>5-FU 5 mM</td>
<td>0.91</td>
<td>0.94</td>
<td>1.38</td>
<td>1.28</td>
<td>1.24</td>
<td>1.16</td>
<td>0.87</td>
<td>1.07</td>
<td>1.01</td>
</tr>
<tr>
<td>Gem 12.5 mM</td>
<td>0.96</td>
<td>0.88</td>
<td>1.25</td>
<td>0.95</td>
<td>1.06</td>
<td>1.04</td>
<td>0.85</td>
<td>0.78</td>
<td>1.00</td>
</tr>
<tr>
<td>Vino 10 µM</td>
<td>0.86</td>
<td>0.94</td>
<td>0.81</td>
<td>1.06</td>
<td>0.73</td>
<td>1.44</td>
<td>0.88</td>
<td>1.12</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Abbreviations: CisP: Cisplatin; 5-FU: 5-Fluorouracil; Doce: Docetaxel; Gem: Gemcitabine; Etop: Etoposide; Vino: Vinorelbine

4. Data from a phenotypic cell line panel
In 1989, a rationally designed “disease-oriented” panel consisting of more than 60 different human tumor cell lines selected to represent the most common and important types of human tumors was established at the US National Cancer Institute (NCI). Compounds were tested for their toxicity and ability to inhibit growth in this panel using semi-automated non-clonogenic in vitro assays. Similar patterns of activity were observed for compounds with similar modes of action (11, 12), which suggested that the panel might be useful in preliminary investigations into the mode of action of new anticancer substances. In addition, a smaller panel consisting of ten cell lines that exhibit different types of resistance to cytotoxic drugs has been established. Experiments using this smaller panel can provide mechanistic information comparable to that obtained using the larger NCI cell line panel (13).

Table S2. The phenotypic ten cell-line panel (13).

<table>
<thead>
<tr>
<th>Parental cell line</th>
<th>Subline(s)</th>
<th>Origin</th>
<th>Selecting agent</th>
<th>Proposed resistance mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>CEM/VM-1</td>
<td>Leukaemia</td>
<td>Teniposide</td>
<td>TopoII-associated</td>
</tr>
<tr>
<td>NCI-H69</td>
<td>H69/AR</td>
<td>SCLC</td>
<td>Doxorubicin</td>
<td>MRP</td>
</tr>
<tr>
<td>RPMI8226/S</td>
<td>8226/Dox40</td>
<td>Myeloma</td>
<td>Doxorubicin</td>
<td>Pgp</td>
</tr>
<tr>
<td></td>
<td>8226/LR5</td>
<td></td>
<td>Melphalan</td>
<td>GSH</td>
</tr>
<tr>
<td>U-937 GTB</td>
<td>U-937 Ver</td>
<td>Lymphoma</td>
<td>Vincristin</td>
<td>Tubulin-associated</td>
</tr>
<tr>
<td>ACHN</td>
<td>-</td>
<td>Renal</td>
<td></td>
<td>Primary resistant</td>
</tr>
</tbody>
</table>


The FMCA results obtained in this work using the ten-line panel are presented as IC50 values (which reflect the drug concentration required to yield a survival rate equal to 50% of that
seen in control experiments). The IC\textsubscript{50} values for the cell line panel can be used to calculate resistance factors (RF) for the resistance mechanisms interrogated by the panel, and to create “delta” patterns of logIC\textsubscript{50}-values for the panel as a whole, which facilitates mechanistic analysis (12, 13). RF values are determined by dividing the IC\textsubscript{50} value for a given subline by that for the parental line (13). The activity of PPP correlated strongly with that of PPT in this panel (Figure 4).

![Figure 4 (Supplementary Figure S3)](A: Survival-concentration curves for PPP in the phenotypic cell line panel. B: Correlation analysis for the effects of PPT (16 nM) and PPP (5 µM) in the phenotypic cell line panel (p < 0.001).)

5. Data from eight lung cancer cell lines

The activity of PPP was also investigated in five non-small cell lung cancer cell lines: U-1810 (primary large-cell lung carcinoma (14)), U-1752 (squamous cell lung carcinoma (15)), NCI-H23 (adenocarcinoma(16)), NCI-H125 (adenosquamous carcinoma, purchased from American Type Culture Collection), NCI-H157 (squamous cell carcinoma, purchased from American Type Culture Collection), and three small cell lung cancer cell lines: U-1285 (17), U-1906L (18), and U-1690 (14). These cell lines have not been authenticated.

Cell lysates were prepared according to Lennartsson et al (19). Briefly, total protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Total cell lysates (TCL) were then subjected to SDS polyacrylamide gel electrophoresis. For immunoprecipitation, antibodies against IGF-1R\(\beta\) were added to each lysate at a concentration of 1 \(\mu\)g/ml, after which Protein A beads were added to collect the resulting immunocomplexes. The beads were then washed and boiled in reducing sample buffer to liberate the proteins, which were separated by SDS polyacrylamide gel electrophoresis. The separated proteins were electroblotted to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were then blocked using 5% BSA and incubated with a primary antibody overnight at 4 °C. The primary antibodies were IGF-1R\(\beta\) (C-20, Santa Cruz), anti-phosphotyrosine PY99 (Santa Cruz) and anti-actin mouse monoclonal antibodies (Sigma), and were used with the concentrations recommended by the suppliers. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Amersham Biosciences), and proteins were visualized using an ECL Western blotting detection systems from Roche Applied Science with a cooled charge-coupled device (CCD) camera (Fuji, Minami-Ashigata, Japan). The band intensities in the blots were quantified using the Aida Image Analyzer.

The activities of PPP, PPT and a panel of standard drugs were investigated using the FMCA method, and correlated with the expression and phosphorylation of IGF-1R (Figure 5). In
addition, correlations between the drugs’ effects were analyzed (Figure 6). As was found previously, the activity of PPP correlated strongly with that of tubulin stabilizing agents but not with the phosphorylation or expression of IGF-1R.

Figure 5 (Supplementary Figure S4) The activity of PPP at 10 µM did not correlate with the expression (A) or phosphorylation (B) of IGF-1R (p>0.05) in eight lung cancer cell lines.
The activity of PPP correlated significantly (p<0.05) with that of tubulin destabilization agents PPT, colchicine, and vinorelbine, but not with that of the other tested drugs in eight lung cancer cell lines.

REFERENCES


