Cancer Therapeutics Insights

Alternative Cytotoxic Effects of the Postulated IGF-IR Inhibitor Picropodophyllin In Vitro

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Abstract

The insulin-like growth factor-1 (IGF-I) and its receptors play an important role in transformation and progression of several malignancies. Inhibitors of this pathway have been developed and evaluated but generally performed poorly in clinical trials, and several drug candidates have been abandoned. The cyclolignan picropodophyllin (PPP) has been described as a potent and selective IGF-IR inhibitor and is currently undergoing clinical trials. We investigated PPP’s activity in panels of human cancer cell lines (e.g., esophageal squamous carcinoma cell lines) but found no effects on the phosphorylation or expression of IGF-IR. Nor was the cytotoxic activity of PPP related to the presence or spontaneous phosphorylation of IGF-IR. However, its activity correlated with that of known tubulin inhibitors, and it destabilized microtubule assembly at cytotoxic concentrations also achievable in patients. PPP is a stereoisomer of podophyllotoxin (PPT), a potent tubulin inhibitor, and an equilibrium between the two has previously been described. PPP could thus potentially act as a reservoir for the continuous generation of low doses of PPT. Interestingly, PPP also inhibited downstream signaling from tyrosine kinase receptors, including the serine/threonine kinase Akt. This effect is associated with microtubule-related downregulation of the EGF receptor, rather than the IGF-IR. These results suggest that the cytotoxicity and pAkt inhibition observed following treatment with the cyclolignan PPP in vitro result from microtubule inhibition (directly or indirectly by spontaneous PPT formation), rather than any effect on IGF-IR. It is also suggested that PPT should be used as a reference compound in all future studies on PPP. Mol Cancer Ther; 12(8); 1–11. ©2013 AACR.

Introduction

Growth factors and their receptors play crucial roles in the establishment and development of cancer. The insulin-like growth factor receptor 1 (IGF-IR) is important in the transformation and growth of malignant cells and in preventing programmed cell death (apoptosis; ref. 1). It is often overexpressed in malignant tumors, such as breast, prostate, and lung cancers, as well as myelomas and malignant melanomas (2). The binding of IGF-I to the α-subunit of IGF-IR results in the autophosphorylation of tyrosine residues in the intracellular β-subunit of the receptor, followed by the activation of the receptor kinase. This enables the downstream activation of signaling cascades that are important for proliferation and survival, including the phosphoinositide 3-kinase (PI3K)/Akt and Ras/Erk1/2 mitogen-activated protein kinase (MAPK) pathways. Notably, these pathways are also activated downstream of most tyrosine kinase receptors, including the EGF receptor (EGFR). Like IGF-IR, EGFR is important in the control of cellular growth, proliferation, and differentiation (3). Moreover, it is often strongly expressed in esophageal cancer cells and has been associated with poor prognosis (4).

Although several inhibitors of EGFR signaling have been developed and are in current clinical use, the development of IGF-IR inhibitors has not yet produced any drugs with clinical benefits. Several recently reported phase III clinical trials of anti-IGF-IR antibodies have yielded disappointing results, clearly showing that the tested antibodies do not have substantial favorable effects in unselected patients with cancer (5). Unanticipated and dose-limiting toxicity accompanied by adverse metabolic adverse events caused significant problems in several of these trials, which prompted the abandonment of several...
antibodies and small-molecule tyrosine kinase inhibitors. It has also been suggested that the efficacy of targeted agents may be impacted by cross-talk between pathways and downregulation of negative feedback loops (6).

The cyclodimeric microtubulin (PPP) has been described as a potent and selective inhibitor of microtubule assembly using different methods and with PPP that was chemically synthesized. The results obtained are consistent with data for established tubulin inhibitors, PPT, and a low-molecular weight IGF-IR tyrosine kinase inhibitor, AEW541.

Materials and Methods

Cell culturing and counting

The human esophageal squamous cell carcinoma (ESCC) cell lines Kyse30, Kyse70, Kyse140, Kyse150, Kyse180, Kyse410, Kyse450, Kyse510, and Kyse520 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were cultivated in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg streptomycin/mL (Sigma-Aldrich Sweden AB). Cells were split twice per week and they were used for further experiments after reaching 70% to 90% confluency. Cell counting was done with a Coulter Z2 particle counter and size analyzer. Data from additional cell line panels are presented in the Supplementary Data. Because of the multitude of different cell lines used (n = 27), their diverse origins, and the lack of conclusions linked to a specific diagnosis, no authentication experiments were carried out.

Drugs and reagents

AEW541 was kindly donated by Novartis. Cisplatin, 5-fluorouracil (5-FU), gemcitabine, etoposide, docetaxel, vincristine, and vinorelbine (Apoteket AB) were used as standard agents. These standards were dissolved as specified by the manufacturer and further diluted with sterile PBS (Sigma). PPT was kindly donated by Prof. Magnus Axelson (Karolinska Institute, Solna, Sweden). PPT was purchased from Sigma-Aldrich. PPP and PPT were dissolved in dimethyl sulfoxide (DMSO) and then diluted further with sterile PBS immediately before use. PPT and PPP in cell growth medium were analyzed by high-performance liquid chromatography following purification (analysis conducted by Prof. Magnus Axelson, details of method will be published elsewhere).

Western blotting

Cell lysates were prepared according to Lennartsson and colleagues (17). Briefly, total protein concentration was determined using the BCA Protein Assay Kit (Pierce). Total cell lysates (TCL) were submitted to SDS-PAGE. Immunoprecipitation was achieved by adding antibodies against IGF-IRβ to each lysate at a concentration of 1 μg/mL. Protein A beads were then added to collect the resulting immunocomplexes. The beads were then washed and boiled in reducing sample buffer and the liberated proteins were separated by SDS-PAGE. The separated proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore), which were then blocked with 5% bovine serum albumin and incubated with the appropriate primary antibody overnight at 4°C. The primary antibodies were IGF-IRβ (C-20; Santa Cruz Biotechnology), anti-phosphotyrosine PY99 (Santa Cruz Biotechnology), and anti-actin mouse monoclonal antibodies (Sigma), and were used at the concentrations recommended by the suppliers. After washing,
the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG) antibodies (Amersham Biosciences), and the proteins were visualized using enhanced chemiluminescence (ECL) Western blotting detection systems from Roche Applied Science with a cooled charge-coupled device (CCD) camera (Fuji). The band intensities in the blots were quantified using the Aida Image Analyzer.

Cytotoxicity measurements

The fluorometric microculture cytotoxicity assay (FMCA; ref. 18) was used to investigate the cytotoxic effects of PPP alone and in combination with other cytostatic drugs in vitro. Briefly, 96- or 384-well microtiter plates (Nunclon surface; NUNC Brand Products) were prepared containing drug solutions in duplicate at 10 times the desired final drug concentration. Cells were seeded into the drug-containing microtiter plates at a cell density of 0.1 × 10^6 cells/mL and incubated for 72 hours at 37°C in a humidified 5% CO2 atmosphere. FMCA was conducted using an automated Optimized Robot for Chemical Analysis (Orca; Beckman Coulter) programmed using the SAMI software package (Beckman Coulter). The plates were washed, fluorescein diacetate (10 μg/mL; Sigma-Aldrich) was added, and the fluorescence generated was measured (485/520 nm) after 40 to 50 minutes incubation. The resulting fluorescence is proportional to the number of intact cells in each well. A successful assay required a ratio of more than 10 between the signal in the control wells and the blank wells and a coefficient of variation of less than 30% in the control wells. Cell survival between the different cell lines (PPP, 10 μmol/L; PPT, 10 μmol/L; PPP) was investigated by immunoprecipitating lysates from each cell line using an antibody against the β-subunit of the(A)OF3 non-IGF-IR Effects of PPP

Significant Non-IGF-IR Effects of PPP

Molecular modeling

Molecular modeling was conducted using the Schrödinger software package with version 9.0 (r211) of the Maestro interface (19, 20). The structure of the complex found between tubulin and PPT (PDB 1SA1; ref. 21) was used as a basis for this study. The ligand and the A and B chains of tubulin were removed from the structure, and the missing residues in the protein were added using v. 2.1 (r211) of the Prime application. The structures of tubulin, PPT, and PPP were then energy minimized in the standard way. Docking was conducted using Glide v. 5.5 (r211) with the extra precision (XP) settings and standard parameters for ligand docking. Conformation analysis was conducted using MacroModel with the OPLS 2005 force field (method: PRCG; energy limit: 21.0 kJ/mol) and was followed by Multiple Minimization, also using the OPLS 2005 force field (method: TNCG; energy limit: 21.0 kJ/mol).

Tubulin polymerization assay

The drugs’ effects on tubulin polymerization were investigated using the Tubulin Polymerization Assay Kit (porcine tubulin- and fluorescence-based), which uses fluorescent reporter enhancement (Cytoskeleton Inc.). The standard assay protocol was used (22) to determine how treatment with 10 μmol/L PPP affected tubulin polymerization, and the system’s fluorescence was measured using a FLUOstar OPTIMA instrument. The experiment was repeated twice and mean values are presented. The pharmaceutical compositions of docetaxel (Taxotere, a microtubule-stabilizing agent that was used as a positive control) and vincristine (a microtubule-destabilizing agent that was used as a second positive control) were diluted with PBS and used at final concentrations of 3 μmol/L.

Fluorescent tubulin micrographs

Cells were grown on coverslips overnight and then treated with the appropriate drug for 24 hours as indicated. Cells were fixed in 2% formaldehyde and permeabilized with 0.2% Triton X-100. Coverslips were blocked with 10 mmol/L glycine at room temperature for 1 hour, after which they were incubated with primary mouse anti-α-tubulin antibody (Sigma-Aldrich) and then with a secondary polyclonal goat anti-mouse antibody labeled with fluorescein isothiocyanate (FITC; DAKO). The nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted on object slides using a Fluoromount-G instrument (Southern Biotechnology Associates). Microtubule staining was visualized using a Zeiss immunofluorescence microscope at ×40 magnification.

Results

IGF-IR is expressed and phosphorylated in ESCC cell lines

IGF-IR is overexpressed in several malignant tumors. Its phosphorylation, induced by ligand binding, enables the downstream activation of signaling pathways that are important for proliferation and survival (1). The expression and phosphorylation of IGF-IR in 9 ESCC cell lines was investigated by immunoprecipitating lysates from each cell line using an antibody against the β-subunit of IGF-IR, followed by Western blot analysis. IGF-IR was expressed in all 9 analyzed ESCC cell lines and exhibited a
The cytostatic/cytotoxic in vitro activity of PPP in ESCC cell lines does not correlate with IGF-IR expression/phosphorylation or with the activity of AEW541

Various approaches have been used to inhibit the function of IGF-IR in malignant cells. PPP has been reported to be a potent and selective inhibitor of the tyrosine phosphorylation of the IGF-IR. Despite their expression of IGF-IR, the 9 ESCC cell lines exhibited only modest sensitivity to PPP and AEW541, as shown in Fig. 3A. Various detectable levels of pIGF-IR/IGF-IR expression were also observed in 8 different lung cancer cell lines (not shown).

The cytostatic/cytotoxic in vitro activity of PPP in ESCC cell lines does not correlate with IGF-IR expression/phosphorylation or with the activity of AEW541

Our finding that the activity of PPP did not correlate with either the level of IGF-IR expression or the activity of the IGF-IR kinase inhibitor AEW541, prompted us to analyze the effects of PPP treatment on the IGF-I–induced activation of this receptor. To this end, we treated cells with increasing concentrations of PPP (0.5, 5 and 10 μmol/L) for 24 or 48 hours, after which they were stimulated with IGF-I for 5 minutes. PPP had no effect on the phosphorylation or stability of the IGF-IR in any case (Fig. 3A). In contrast, AEW541 effectively inhibited ligand-induced IGF-IR phosphorylation in both of the tested cell lines (Fig. 3B).

These results suggest that PPP does not act as a direct IGF-IR kinase inhibitor in these 2 ESCC cell lines.

PPP activity correlates with the activity of tubulin inhibitors

Interestingly, the activity of PPP at 10 μmol/L in the 9 ESCC cell lines correlated strongly with that of PPT at 16 nmol/L ($R^2 = 0.90$; Fig. 2B), and also to that of two other standard agents that target microtubule assembly: docetaxel (20 μmol/L; $R^2 = 0.75$) and vinorelbine (10 μmol/L; $R^2 = 0.91$; for details see Supplementary Fig. S6A and S6B). The activity of PPP either did not correlate or only correlated weakly with those of three other standard cytotoxic drugs: cisplatin, gemcitabine, and 5-FU (Supplementary Fig. S6C and S6D). All the tested drug concentrations were of 4 nonresponding cell lines resulted in a weak correlation for the remaining five (data not shown). Moreover, there was no correlation of the effects of PPP and those of the unrelated IGF-IR inhibitor AEW541 ($R^2 = 0.18$; Fig. 2C).

Similar results were found in a panel of 8 lung cancer cell lines and a phenotypic cell line panel designed to predict drug’s mechanism of action (Supplementary Table S2 and Supplementary Figs. S3–S5). These results strongly suggest that the cytotoxic effects of PPP identified using the FMCA method are not due to IGF-IR inhibition.

PPP does not affect the phosphorylation or total levels of IGF-IR ESCC cell lines

Figure 1. Western blot analysis result showing the expression of IGF-IR, pIGF-IR, and β-actin as a loading control in 9 different ESCC cell lines. lb, immunoblot; Ip, immunoprecipitation. The experiment was carried out twice with similar results; data from a single representative experiment are shown.

Figure 2. The cytotoxic effects of PPP measured after 72 hours in ESCC cell lines are of moderate magnitude and correlate with those of tubulin inhibitors but not an IGF-IR inhibitor. A, dose–response curves showing the sensitivity of 9 ESCC cell lines to PPP. B, correlation analysis of survival index values (%) obtained using a PPP concentration of 10 μmol/L and a PPT concentration of 16 nmol/L. C, correlation analysis of survival index values (%) obtained a PPP concentration of 10 μmol/L and an AEW541 concentration of 10 μmol/L. All experiments were carried out in triplicate, and the values presented are the mean.
selected on the basis of empirical data to give the greatest possible variation among the cell lines (23).

Results consistent with those presented earlier for ESCC lines were also obtained in a phenotypic cell line panel, which was designed to reveal drug’s mechanisms of action, and in a lung cancer cell line panel (Supplementary Data, sections 4 and 5 and Supplementary Figs. S3 and S5).

**PPP inhibits microtubule assembly**

Although PPP had no detectable effect on IGF-IR levels or phosphorylation, its activity in the ESCC panel clearly correlated with that of tubulin inhibitors. Therefore, the relevance and contribution of tubulin inhibition to its mechanism of action was explored further. ESCC cells exposed to cytotoxic levels of PPP, PPT, docetaxel, and vincristine for 24 or 48 hours were stained to visualize their microtubule networks. The resulting micrographs (Fig. 4A) revealed blurry tubulin staining after PPP exposure, which is consistent with the hypothesis that it inhibits tubulin assembly. The results obtained using PPP were similar to those seen with PPT and vincristine; docetaxel yielded a more distinct pattern of microtubules. Treatment with a PPP concentration that is too low to have cytotoxic effects in the ESCC panel (i.e., 0.5 µmol/L) did not cause dissolution of the microtubule network, which suggests that the cytotoxic effects of PPP seen in these cells are associated with tubulin inhibition. Indeed, PPP could be docked *in silico* into the colchicines-binding site of tubulin in a model constructed using the PPT–tubulin complex (PDB 1SA1) as a template (Fig. 4B–E). As described previously, PPT binds to a hydrophobic pocket of tubulin that features two hydrophobic regions. The first is surrounded by the side chains of Met259, Ala316, and Lys352 and is occupied by the benzodioxole fragment of PPT, whereas the second is surrounded by the side chains of Leu242, Ala250, and Leu255 and is occupied by the trimethoxyphenyl moiety of PPT (24). In addition, the hydroxyl hydrogen of PPT can form a hydrogen bond with the carbonyl oxygen of Thr179 in the protein backbone (2.24 Å, –OH···O=C 146°; Fig. 4D). Docking of PPP into the colchicines-binding site (Fig. 4C) revealed that it can bind in a similar way. However, because of the difference between PPP and PPT with regards to the chirality of the C2 carbon, the fused lactone ring of PPP is oriented in a different direction than in PPT. It has previously been suggested that the orientation of this lactone ring is important for the microtubule-destabilizing activity of PPT (25), which is consistent with the relatively high concentrations of PPP that must be used to induce cytotoxicity and microtubule disruption. In addition, a conformational analysis of PPT conducted using molecular mechanisms (OPLS, MacroModel) suggested that PPT is fairly rigid and has only one low-energy conformation. In contrast, PPP is more flexible and can adopt several favorable conformations, mainly due to different arrangements of the fused lactone ring and the oxazole moiety (Fig. 4E). The docking study also revealed that unlike PPT, PPP cannot form a stabilizing hydrogen bond with the tubulin backbone because the distance between its hydroxyl group and Thr179 in the protein backbone is too great.

The possibility that PPP could directly affect tubulin polymerization was investigated with the commercially available Tubulin Polymerization Assay Kit (Cytoskeleton Inc.), which uses purified porcine tubulin. In this assay, PPP clearly inhibited the polymerization process at a concentration of 10 µmol/L (Fig. 4F). This suggests that while PPP can act as a tubulin inhibitor, it is not a potent one, as its effects are observed only at relatively high concentrations. This is consistent with our *in silico* docking studies, which showed that PPP can bind to tubulin but not as tightly as PPT.

The PPP used in all of the investigations discussed earlier (including the microtubule assay) was pure when the experiments were started. However, PPP may undergo nonenzymatic conversion to PPT, which could potentially have influenced the results obtained. The conversion...
of PPP into PPT was investigated by monitoring initially pure PPP solutions of various concentrations (0.5, 5, and 10 µmol/L) in cell growth medium at 37°C. After 1 hour, the level of PPT in the solutions corresponded to 0, 0.4, and 0.3% of the initially added PPP; after 24 hours, the corresponding levels were 1.8%, 1.7%, and 1.5%; and after 48 hours they were 2.1%, 2.5%, and 2.6%, respectively. The 48-hour values are very close to the expected equilibrium concentration (15). The PPP-to-PPT conversion was measured by Dr. Magnus Axelson, and used with permission.

**PPP inhibits EGFR and decreases downstream signaling from tyrosine kinase receptors**

We have previously found that the dissolution of microtubule networks using established tubulin-binding agents can result in dephosphorylation and degradation of the...

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**Figure 4.** PPP may interact with microtubule assembly in cells, in silico, and with purified tubulin in vitro. A, the effect of PPP on the microtubule system as observed by immunofluorescence microscopy. B–E, illustrations from the docking study examining the colchicine binding site of β-tubulin: PPT (B), PPP (C), and PPT (D) surrounded by various amino acids, including Thr179, with which it may form a hydrogen bond. E, the conformational flexibility of PPP, exemplified by three different conformations found in the modeling study. F, using a commercial tubulin polymerization assay, PPP inhibits microtubule assembly at a concentration of 10 µmol/L. The control curves show shifts to left (stabilizing) and right (destabilizing), respectively. The experiment was carried out twice, with similar results on both occasions.
EGFR but not IGF-IR (26). Therefore, we investigated whether PPP had similar effects on EGFR, which was expressed in all of the ESCC cell lines in the panel (not shown). PPP did indeed inhibit the phosphorylation of EGFR and reduced its stability in a dose- and time-dependent manner (Fig. 5A). It therefore seemed reasonable to investigate whether downstream signaling pathways such as Ras/Erk1/2 MAPK and PI3K/Akt were also affected by PPP. In keeping with the observed inhibition of EGFR, PPP also reduced the phosphorylation of the signaling proteins Erk1/2 and Akt in the Kyse70 cell line, whereas these effects were minimal in Kyse140 cells (Fig. 5B). This may relate to the observation that in Kyse140 cells exhibit relatively high levels of spontaneous IGF-IR phosphorylation in serum-supplemented growth medium (Fig. 1); this may be sufficient to compensate for the PPP-induced reduction in EGFR phosphorylation and to promote Akt and Erk1/2 phosphorylation even in the presence of PPP. In fact, a degree of functional cross-talk (including a physical interaction), between IGF-IR and EGFR has been described (reviewed in ref. 27). It is therefore not appropriate to use reductions in the phosphorylation of Erk1/2 and Akt (both of which are activated by most receptor tyrosine kinases) as measures of IGF-IR inhibition phosphorylation by PPP as our data show that PPP has no effect on IGF-IR despite inhibiting Erk1/2 and Akt phosphorylation in some cases. The reduced phosphorylation of Erk1/2 and Akt seen in Kyse70 cells may be partly due to the downregulation of EGFR. As the molecular target(s) of PPP have not been unambiguously identified, it remains possible that other receptors or proteins may also be affected. Moreover, we have observed that microtubule disruption in ESCC cell lines by PPT or vincristine also inhibits EGF-mediated phosphorylation of Akt and Erk1/2 (26).

Discussion

Growth factors and their receptors play pivotal roles in the establishment and development of cancer. The IGF-IR is an attractive target for cancer therapy because it is often overexpressed and activated in various malignant tumors (1), including esophageal cancer (28). Patients with ESCC have significantly higher serum levels of IGF-I than healthy donors, which may be an important predictor of risk for esophageal cancer (29). Strong IGF-I signaling correlates significantly with depth of invasion, pathologic stage, and poor prognosis in esophageal cancers (28, 30). Blockage of IGF signaling by dominant-negative IGF-IR...
suppresses proliferation and motility of ESCC cell lines (29). Therefore, the level of IGF can be used as a tumor marker in esophageal carcinoma, and drugs that inhibit the functioning of IGF-I could potentially be used to treat esophageal cancer (29). Presumably, candidates for such treatment would be identified on the basis of clinical immunohistochemical detection of the IGF-IR/IGF-II complex (30). In addition, laboratory studies have suggested that IGF-I stimulation plays an important role in repair processes in the esophagus (31). The IGF-I peptide antagonist JB1 is active against the esophageal carcinoma cell line CE81T/VGH in vitro and in vivo, and also seems to have favorable effects when used in combination with chemotherapy (32).

Various approaches for interfering with the IGF-IR pathway have been developed, including methods based on neutralizing antibodies, a dominant-negative receptor, IGF-IR antisense/siRNA and small-molecule tyrosine kinase inhibitors with varying degrees of selectivity. However, as IGF-IR displays 84% similarity with the insulin receptor (whose functioning is essential for most mammalian cells), the development of anticancer drug candidates targeting this receptor has been difficult. Crystallographic studies conducted to identify structural differences between IGF-IR and the insulin receptor have been used as the basis for attempts to develop selective inhibitors. Several inhibitors of the IGF-IR pathway have been evaluated in clinical trials, most of which have yielded discouraging results and resulted in the abandonment of the corresponding drug candidates. Some cyclolignans have been identified as potent and selective inhibitors of the tyrosine phosphorylation of IGF-IR. One such compound is PPP, which is almost nontoxic (oral LD$_{50}$ >500 mg/kg in rodents; ref. 8). In addition to inhibiting the tyrosine kinase activity of IGF-IR, PPP has also been claimed to inhibit IGF-IR by down-regulating the protein (33). Although most IGF-IR inhibitors have failed because of a lack of activity or adverse effects, PPP seems to be different, in that it exhibits clinical activity, with no metabolic toxicity, and only moderate general toxicity. In our view, this suggests that PPP may have a primary target (or targets) other than IGF-IR.

Our molecular modeling studies indicated that PPP can adopt several stable conformations (Fig. 4E), and it is possible that these have distinct biologic effects. Although a dual mechanism of IGF-IR antagonism has been proposed for PPP, we could not confirm either of these effects in the ESCC cell lines or in other cell lines (see the Supplementary Data for details). PPP exhibited cytotoxic/cytostatic activity at micromolar concentrations in vitro in the tested cell lines, and there was no overall correlation between PPP sensitivity and the expression or phosphorylation of IGF-IR (Supplementary Fig. S2). The exclusion of 4 nonresponding cell lines resulted in a weak correlation for the remaining five. However, the significance of these results is questionable because some of the excluded cell lines exhibited clear spontaneous IGF-IR phosphorylation. Furthermore, in experiments with recombinant IGF-I, we were unable to observe any effect of PPP on the phosphorylation or stability of IGF-IR in 2 ESCC cell lines (Fig. 3A). In contrast, AEW541 efficiently inhibited IGF-IR phosphorylation under similar conditions (Fig. 3B). A recent study using four different colon cancer cell lines, showed that all four cell lines were sensitive to PPP treatment, but PPP only inhibited the phosphorylation and stability of IGF-IR in HT-29 (34). Because all cells were sensitive to PPP, this indicates that the cytotoxic effects of PPP may be independent of its postulated IGF-IR antagonism. Another recent study showed that PPP does not inhibit the phosphorylation of IGF-IR in adrenocortical adenocarcinoma cells, but that PPP treatment nevertheless causes accumulation in $G_2$–M phase and apoptosis (35). Interestingly, the authors concluded that these effects are not mediated by antitubulin effects because PPP used was "ultra-pure," and previous investigations had shown that PPP only binds very weakly to tubulin. The PPP used in the investigations described herein was obtained from the same source as that used in the previous investigations.

We have previously shown that the disruption of the microtubule system by PPT or vincristine causes the dephosphorylation and degradation of the EGFR (26). We obtained similar results in this work using PPP (Fig. 5A). This inhibition correlated with reductions in the activity of the downstream signaling proteins Erk1/2 and Akt in Kyse70 cells but not in Kyse140 cells (Fig. 5B), which exhibit relatively high levels of spontaneous IGF-IR phosphorylation (Fig. 1); this spontaneous phosphorylation was unaffected by exposure to PPP. The existence of this effect on EGFR means that care should be taken when using secondary reporters such as reductions in the activity of Erk1/2 or Akt to monitor IGF-IR inhibition in vitro. The cross-talk between IGF-1 and EGFR signaling is another factor that could confound such evaluations, and it has been shown that the activation of Erk1/2 by IGF-I requires the EGFR (36, 37). These 2 factors mean that loss of EGFR function would have results that could be misinterpreted as evidence for the inhibition of IGF-I–induced signaling to Erk1/2. This significantly complicates the interpretation of studies in which the inhibition of Erk1/2 was used as a measure of PPP-mediated IGF-IR inhibition.

The cytotoxic activity of PPP in various cell line panels measured by the FMCA method correlated strongly with that of various tubulin inhibitors but not with that of the IGF-IR inhibitor NVP-AEW541 (Fig. 2B and C). Using computer modeling, we showed that PPP is theoretically able to fit into the colchicine-binding pocket of tubulin (Fig. 4F). This is partly due to structural flexibility of PPP allowing it to adopt conformations that are compatible with tubulin binding (Fig. 4E). In keeping with observation, PPP inhibited microtubule assembly in a cell-free assay (Fig. 4F) and disrupted the tubulin staining patterns at relevant (i.e., cytotoxic and clinically achievable in patients) concentrations in cultured cells (Fig. 4A). However, PPP did not fit as tightly as PPT into the binding pocket of tubulin; together with the relatively
high PPP concentration needed to interfere with tubulin polymerization, this suggests that PPP is a weak tubulin inhibitor.

An equilibrium between PPP and PPT was established rather rapidly when PPP solutions were left to stand in cell culture media, and concentrations of PPT that would be expected to have biologic effects were detected after only 1 hour of incubation. It is therefore possible that the results observed in our experiments were partly due to the presence of PPT in small quantities. Importantly, this would also be the case for all previous experimental investigations using PPP (as an IGF-IR inhibitor), and our results strongly suggest that the equilibrium between PPP and PPT should be considered in future studies on these compounds. PPT is a known and potent inhibitor of microtubule assembly, and even small amounts of PPT in experimental samples could potentially have dramatic effects on the results obtained, especially in assays based on whole cells. The impact of the PPP-to-PPT conversion on the results will depend on the concentration of PPP that is needed to observe cytotoxic effects. It has previously been suggested that PPP may have no effect whatsoever on tubulin (38), and data were presented suggesting that highly purified PPP does not bind to tubulin or binds very weakly to at concentrations up to 50 μmol/L. This is in sharp contrast with our results, which confirmed that PPP has direct effects on the microtubule assembly in a 45-minute cell-free assay (Fig. 4F). The PPP used in this assay was pure at the beginning of the experiment (coming from freshly thawed DMSO stock), but it is certainly possible that minute amounts of PPT could be formed under the experimental conditions, as this seems to happen spontaneously under standard laboratory conditions. Furthermore, it cannot be excluded that PPT is actively being removed from solution due to its strong binding to tubulin [the binding constant for the formation of tubulin–PPT complex is 1.8 × 10^6 (μmol/L)^{-3} (13)], and that this removal drive the further conversion of PPP into PPT to maintain the equilibrium between the 2 isomers in solution. It is possible that this conversion also happens in vivo, but may not be detected as the levels of free PPT would be negligible: tubulin is very abundant in all cells and would rapidly sequester the PPT as it is formed, thus removing it from serum. Moreover, the interaction between tubulin and PPP may enhance the rate of PPP to PPT conversion due to the tighter binding of PPT to tubulin. In such a scenario, one would expect to find negligible or very low levels of PPT in serum as it is formed in the binding pocket of tubulin. Indeed, no circulating PPT was detected in a recent investigation (with a limit of quantification of 0.01 μmol/L) that examined plasma from patients treated with oral PPP in a phase I study (39). Plasma levels PPP in excess of 1 μmol/L were detected following an oral dosing with 520 mg PPP, which justifies the selection of the concentrations used in our experiments. However, the cellular concentrations and PPP or PPT (i.e., their concentrations in the tubulin-rich compartments of the body) were not analyzed.

Interestingly, genomic arrays of cells selected for PPP resistance show commonly altered expression of tubulin or microtubule-associated genes including overexpression of MARK1 and TUBA4A and underexpression of EML5 and MICAL2 (40), although other changes in gene expression was also found.

Conclusion

The viability of the ESCC cells used in this investigation was affected by PPP at micromolar concentrations, which are high but may be achieved in vivo after oral dosing of PPP (39). On the basis of the results presented herein, we propose that under such conditions, PPP’s primary mechanism of action does not involve IGF-IR inhibition and that it instead causes microtubule destabilization. This is associated with EGFR downregulation and decreased activity of downstream signaling pathways. Similar results were obtained in a lung cancer cell line panel (n = 8), and a phenotypic cell line panel (n = 10; Supplementary Table S2), which have different resistance mechanisms to those of the ESCC lines. PPP may act directly on microtubules, as it inhibited tubulin polymerization in a cell-free assay (Fig. 4F) and molecular modeling investigations did not exclude the possibility that PPP might bind directly to tubulin (Fig. 4C and E). However, it is likely that at least some of PPP’s apparent effects on microtubules are indirect and stem from its conversion into PPT, which was observed to occur within an hour under standard laboratory conditions. Interestingly, although several publications have reported that PPP affects the phosphorylation and/or stability of IGF-IR, we could not reproduce this in our ESCC cells despite using relatively high PPP concentrations (Fig. 3A). However, we did observe an analogous effect on EGFR (Fig. 5A). The mechanism responsible for this effect is not readily apparent, and it is possible that the microtubule-destabilizing agents may affect different receptors in different cell types. In addition, the effect on EGFR was only observed after prolonged incubation with PPP (24 hours or more), suggesting that the tubulin-mediated effect targets receptor turnover. Consequently, treatment with PPP also downregulates tyrosine kinase receptor signaling via proteins such as pAkt (Fig. 5B). This could potentially mislead investigators into believing that PPP has anti-IGF-IR effects in various cell lines.

In summary, we suggest that the postulated IGF-IR inhibitor PPP primarily acts by disrupting the microtubule cytoskeleton, either by directly binding to tubulin or via conversion into its isomer, PPT, and that this indirectly results in EGFR downregulation. We strongly suggest that the alternative effect on microtubules should be accounted for in all further studies with that use PPP. Furthermore, our results suggest that it is not appropriate to use levels of phosphorylated Akt or Erk1/2 as a readout for anti-IGF-IR activity in vitro as we clearly show that EGFR is inhibited by PPP. Our results are in sharp contrast to previous reports, and we have put forward a model that explains the inconsistent results presented in some
previous publications. Although the results presented in this article strongly suggest that PPP does not have an IGF-IR-mediated mechanism of action, we would like to emphasize that this in no way means that it is necessarily without therapeutic effects, potentially beneficial to patients. On the contrary, the continuous moderate inhibition of tubulin assembly (either by PPP itself or via its gradual conversion to PPT, which would simulate a slow-release preparation) is of high interest and could be a promising strategy for treating solid malignancies. For example, in a study using metronomic oral vinorelbine dosing in 62 patients with advanced refractory cancer, 13% of the patients exhibited objective antitumor responses, and 32% of patients experienced disease stability for at least 6 months. Three responders received nonstop treatment for more than 3 years without overt toxicity (including absence of neurotoxicity). The authors suggest an antiangiogenetic mechanism (41), which would be consistent with the disease stabilization and the central necrotic areas observed in patients with non–small cell lung carcinoma (NSCLC) treated with PPP (10).

Because PPP has shown activity in clinical trials, investigations aimed at identifying its cellular target(s) are highly warranted.

Disclosure of Potential Conflicts of Interest

J. Gullbo is co-founder with ownership interest (including patents) in Oncopetides AB, which is a small research and development company that for a long period of time (until July 2012) shared the same principal investor/owner (Karolinska Development AB) as Axellar AB (now developing PPP for clinical use). J. Gullbo has no ownership interest in Karolinska Development AB or Axellar AB. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: X. Wu, M. Wickström, J. Lennartsson, J. Gullbo
Development of methodology: J. Gullbo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Sooman, M. Wickström, J. Lennartsson, J. Gullbo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wu, L. Sooman, M. Wickström, M. Frykman, C. Dyrager, J. Gullbo
Writing, review, and/or revision of the manuscript: X. Wu, L. Sooman, M. Wickström, M. Frykman, C. Dyrager, J. Lennartsson, J. Gullbo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Frykman
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