Effects of polysaccharide peptide (PSP) from *Coriolus versicolor* on the pharmacokinetics of cyclophosphamide in the rat and cytotoxicity in HepG2 cells

Siu-Lung Chan *, John H.K. Yeung

Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China

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Abstract

Polysaccharide peptide (PSP), isolated from *Coriolus versicolor COV-1*, has been shown to restore the immunological effects against cyclophosphamide-induced immuno-suppression, although the mechanism(s) involved remain uncertain. This study investigated the PSP-cyclophosphamide interaction by studying the effects of PSP on the pharmacokinetic of cyclophosphamide in the rat and the effect of PSP on the cytotoxic effects of cyclophosphamide on a cancer cell line (HepG2 cells). In the pharmacokinetic studies in the rat, acute pre-treatment of PSP (4 μmol/kg/day, i.p.) decreased the clearance (CL) of cyclophosphamide by 31%, with a concomitant increase in the area under concentration–time curve (AUC) by 44%, and prolongation of the plasma half-life (*T*₁/₂) by 43%. Sub-chronic pre-treatment of PSP (2 μmol/kg/day, i.p., 3 days) decreased the CL of cyclophosphamide by 33%, with a concomitant increase in the AUC by 50%, and prolongation of the plasma *T*₁/₂ by 34%. In cytotoxicity studies using HepG2 cells, non-toxic dose of PSP (1–10 μM) enhanced the cytotoxicity of cyclophosphamide. PSP at 10 μM further decreased HepG2 cell viability by 22% compared to when cyclophosphamide was present alone. In summary, PSP enhanced the cytotoxic effect of cyclophosphamide on a cancer cell line in vitro and altered the pharmacokinetics of cyclophosphamide in vivo in the rat. Both of these effects may be beneficial in the use of PSP as an adjunct to cyclophosphamide treatment.

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Keywords: Polysaccharide peptide (PSP); *Coriolus versicolor*; Pharmacokinetics; Cytotoxicity; Cyclophosphamide

1. Introduction

Polysaccharide peptide (PSP), isolated from the Chinese fungus *Coriolus versicolor* strain COV-1, is a protein-bound polysaccharide commonly used as an adjunct to cancer chemotherapy or radiotherapy in China. Phase three clinical trials have been completed in 1997 throughout 14 hospitals in China (Liu et al., 1999). When used in combination with 5-fluorouracil, PSP improved symptoms like dryness of throat and mouth, night sweating, and vomiting in stomach cancer patients (Zhang et al., 1999). PSP also restored cyclophosphamide-induced immuno-suppression in patients, probably by increasing the level of interleukin-2 and natural killer cells functions (Qian et al., 1999). Despite the apparent beneficial effects being observed when PSP was used in combination with anti-cancer drugs, the underlying mechanism(s) of such interactions remain to be established. Improvements in anti-cancer treatment by PSP may be related to its immuno-stimulatory activities...
but it does not fully explain the improvements in the cancer chemotherapy-related symptoms as observed in the patients.

Previous pharmacokinetic studies with PSP showed that PSP exhibited protective effects on paracetamol-induced hepatotoxicity by reducing the formation of the chemically reactive metabolite of paracetamol and enhanced phase II conjugation (Yeung et al., 1994). The results indicated that PSP may affect the metabolism of other therapeutic agents when administrated concurrently, although the metabolic pathways of PSP itself remain uncertain. Cyclophosphamide is one of the most extensively used anti-cancer agents over the past 30 years (Corlett and Chrustyn, 1996) and is often used with PSP in China (Qian et al., 1999). Therefore, it would be of interest to investigate whether PSP may affect the pharmacokinetics of cyclophosphamide.

Apart from the clinical studies carried out in patients, the cytotoxic effects of PSP on cancer cells has been studied in vitro and in tumor-bearing animal models. PSP suppressed Erlich ascitic carcinoma, monocytic leukemia, P-388 leukemia, lymphosarcoma, human gastric cancer, and lung cancer cell lines in vitro (Yang, 1993). PSP also inhibited the growth of sarcoma 180 (Yang, 1993), lung adenocarcinoma (Zeng et al., 1993), and Lewis lung cancer (Wang et al., 1993) bearing mice. However, the combined effects of PSP with other anti-cancer agents on cytotoxicity of cancer cells have not been carried out in this study. In the direct study, the direct cytotoxic effects of PSP alone, or in combination with cyclophosphamide, were investigated in HepG2 cells, selected because it is not known if PSP may exert a direct cytotoxic effect on hepatoma cell lines, alone or in combination with other cytotoxic anti-cancer agents.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (250–300 g) were supplied by the Laboratory Animal Service Centre, The Chinese University of Hong Kong (CUHK). The animals were kept in an animal holding room, under temperature control (23 ± 2 °C) with 12-h light-dark cycle, with free access to water and food (Glen Forrest Stockfeeders, Australia). All animal procedures were carried out with approval by the Animal Experimentation Ethics Committee (CUHK) in accordance to the Department of Health (Hong Kong) guidelines in Care and Use of Animals.

2.2. Materials

Polysaccharide peptide (PSP), isolated from deep-layer cultivated mycelia of Coriolus versicolor, was a gift from Winsor Health Products Ltd. (Hong Kong). The PSP sample was composed of 90% polysaccharides (74.6% glucose, 4.8% xylose, 2.7% galactose, 1.5% mannose, and 2.4% fucose) and 10% peptides (18 different amino acids, mostly aspartic acid and glutamic acid) and was authenticated by Professor Q.Y. Yang (Shanghai Teachers’ University, China). The PSP sample used in this study has a molecular weight of 10 kDa, analyzed by gel electrophoresis, according to the method by Zhou and Yang (1999). Cyclophosphamide, cimetidine, and urethane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade solvents were from Fluka Chemicals (Buchs, Switzerland). Cell culture medium and reagents were purchased from Gibco Laboratories (Grand Island, NY, USA). HepG2 (HB 8065) cell line was purchased from American Type Culture Collection.

2.3. Effects of PSP on the clearance of cyclophosphamide in vivo

For the acute study, six animals were treated with PSP (2 or 4 μmol/kg, i.p.), four animals with cimetidine (258 μmol/kg, i.p.) and six animals with saline as control. For sub-chronic study, six animals in each group were pre-treated with PSP (2 or 4 μmol/kg, i.p.) for 3 days. At this dose range, PSP has been shown to protect paracetamol-induced hepatotoxicity in the rat, with underlying pharmacokinetic mechanisms (Yeung et al., 1994, 1995). During the pre-treatment period, the rats were kept in a 12-h light/dark cycle animal room with controlled temperature and humidity. Free access to laboratory rodent diet and tap water was allowed. After the pre-treatment (1 h for acute study and day 4 for sub-chronic study), the rats were anesthetized with urethane (20% w/v, 6 ml/kg, i.p.). The left carotid artery, right jugular vein and trachea were cannulated. The pharmacokinetics of a single dose of cyclophosphamide (358 mmol/kg, i.v.) was measured. At this dose, the initial plasma concentration was around 100 μg/ml and gradually decreased to around 20 μg/ml after 360 min, which is close to the detection limit of 10 μg/ml of the system. Blood samples (0.3 ml) were collected via the carotid artery just before drug administration as the control blank sample, and at various intervals from 10 to 360 min. Heparinized saline (0.2 ml, 100 units/ml) was given before the start of the experiment to prevent blood coagulation. Saline was replaced via jugular vein after each blood sample collection. Blood samples were centrifuged to obtain plasma for analysis. In another set of animals, the body weight was recorded for 28 days with treatment of PSP (4 μmol/kg/day, i.p.) or saline.

2.4. Determination of plasma cyclophosphamide by high performance liquid chromatography (HPLC)

Purification procedures for the samples were based on the methods of Busse et al. (1997), with modifications. Briefly, plasma sample (200 μl) was mixed with 5 μl phenacinet (internal standard; 2.5 μg/ml final concentration) and 1 ml of ethyl acetate. The mixture was shaken for 30 min followed by centrifugation at 2500 rpm for 15 min. The solvent was dried under a gentle stream of nitrogen at 40 °C. The pellet was resuspended in 200 μl of the mobile phase (25:75 acetonitrile-phosphate buffer, pH 4.0). The HPLC system included a Hewlett Packard 1050 series pumping system and a multiple wavelength detector at 195 nm. Eluates (50 μl) were analyzed by reversed-phase C8 (Agilent Zorbax Eclipse XDB-C8, 150 × 4.6 mm) column at room temperature. The mobile phase was 25:75 acetonitrile-phosphate buffer, pH 4.0 (Corlett and Chrustyn, 1996). The retention time of cyclophosphamide and internal standard were 6.9 min and 7.4 min, respectively. The assay sensitivity was around 10 μg/ml.

2.5. Effects of PSP on the cytotoxicity of cyclophosphamide on HepG2 cells using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay

HepG2 cells (180 μl, 1 × 10^5 cells/ml) were pre-incubated with PSP (0.1–10 μM) in 96-well culture plates for 1 h or 24 h. This dose range of PSP has been shown to be cytotoxic to a number of cancer cell lines in vitro (Yang, 1993). Since the cytotoxicity of PSP on HepG2 cells has never been studied, the incubation time was selected, based on other toxicity studies using extract of plant origin (Kao et al., 2001; Koo et al., 2000; Slameno et al., 2004). At the end of the incubation period, PSP was washed away and the HepG2 cells were exposed to cyclophosphamide (10 μM) for a further 48 h (Wang et al., 2002). Control experiments were set up with the HepG2 cells being exposed to phosphate buffered saline (control), PSP alone or cyclophosphamide alone. At the end of the incubation period, MTT (20 μl; 5 mg/ml) was added and incubated for a
further 4 h. The medium was then discarded and 200 μl of DMSO was added and shaken for 1 h. The absorbance at 570 nm was measured by spectrophotometry (Carmichael et al., 1987). Results were the mean of 4 individual experiments and calculated as percentage of the control.

2.6. Data analysis

The area under concentration–time curve (AUC) ratio of cyclophosphamide and internal standard was used to obtain the corresponding concentration of cyclophosphamide in the standard curve. The Microsoft Excel program Pharmacokinetic Solutions 2.0 (Summit Research Services, Ashland, USA) was used to calculate the pharmacokinetic parameters of AUC, initial concentration (C_{initial}), volume of distribution (V_d), total clearance (CL), and half-life (T_{1/2}). The significance of difference between groups were analyzed by Students’ t-test for 2 groups of data or one-way analysis of variance (ANOVA) followed by Fisher’s Protected Least Significant Difference (PLSD) test for more groups in Statview version 9.0 (Abacus Concepts, USA). Differences were considered statistically significant when p < 0.05.

3. Results

3.1. Effects of PSP on the clearance of cyclophosphamide in vivo

The relative standard deviation of the HPLC system for cyclophosphamide was 5.2% at 100 μg/ml and 2% at 200 μg/ml. The accuracy of the standard curve is 98%, 96% and 99% at 25, 100 and 200 μg/ml cyclophosphamide, respectively. Cimetidine, the enzyme inhibitor, decreased the clearance of cyclophosphamide (Fig. 1). Acute administration of PSP at 2 μmol/kg only minimally affected (P > 0.05) the plasma concentration–time profile of cyclophosphamide. At a higher dose of 4 μmol/kg, the pharmacokinetics of cyclophosphamide was altered. There was an increase in the area under the curve, AUC (44%), a decrease in clearance, CL (31%), and an increase in the plasma half-life of cyclophosphamide, T_{1/2}, by 43% (Table 1a). The effective dose range was similar to those previous reported in which PSP (3 μmol/kg) protected paracetamol-induced hepatotoxicity (Yeung et al., 1994). The C_{initial} and V_d remained unchanged indicating that PSP decreased cyclophosphamide clearance without affecting its distribution and protein binding. After sub-chronic treatment of PSP for three days, a decrease in cyclophosphamide clearance was observed at 2 μmol/kg and 4 μmol/kg (Fig. 2). As shown in Table 1b, PSP (2 μmol/kg) increased the AUC by 50%, decreased the CL by 33%, and prolonged the T_{1/2} by 34%. The effect of the higher dose PSP (4 μmol/kg) produced similar changes.

Chronic treatment of PSP for 28 days significantly decreased the rate of body weight gain compared to control rats (Fig. 3). After 28 days of treatment, the body weight of the control group (448 ± 5.7 g) was 25% higher than the PSP-treated group (336 ± 7.1 g). The average daily increase in body weight was dropped by 76% from 5.8 ± 0.8 g to 1.6 ± 0.8 g.

3.2. Effects of PSP on the cytotoxicity of cyclophosphamide on HepG2 cells

Cyclophosphamide (10 μM) alone was minimally cytotoxic to HepG2 cells, with a small percentage decrease in cell viability (7%) in all groups, results similar to those reported for rat primary hepatocytes (Wang et al., 2002). PSP at low doses (0.1–1 μM) did not show prominent cytotoxicity after 1 h but the cytotoxic effect became significant

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Table 1a

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Cimetidine</th>
<th>PSP 2 μmol/kg</th>
<th>PSP 4 μmol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{initial} (μg/ml)</td>
<td>94.2 ± 2.78</td>
<td>102.6 ± 7.01</td>
<td>92.4 ± 2.66</td>
<td>94.3 ± 1.01</td>
</tr>
<tr>
<td>AUC (mg min/ml)</td>
<td>14.7 ± 1.07</td>
<td>28.3 ± 2.88 ***</td>
<td>17.1 ± 1.24</td>
<td>21.1 ± 0.31 ***</td>
</tr>
<tr>
<td>V_d (ml/kg)</td>
<td>1056.7 ± 31.8</td>
<td>979.9 ± 70.1</td>
<td>1066.0 ± 30.9</td>
<td>1045.9 ± 12.6 ***</td>
</tr>
<tr>
<td>CL (ml/min/kg)</td>
<td>6.94 ± 0.52</td>
<td>3.66 ± 0.40 ***</td>
<td>6.10 ± 0.70</td>
<td>4.75 ± 0.07 ***</td>
</tr>
<tr>
<td>T_{1/2} (min)</td>
<td>107.0 ± 5.16</td>
<td>189.1 ± 14.56 ***</td>
<td>123.1 ± 9.96</td>
<td>152.9 ± 2.55 ***</td>
</tr>
</tbody>
</table>

*: initial concentration of cyclophosphamide, AUC: area under concentration–time curves, V_d: volume of distribution, CL: clearance, T_{1/2}: half-life. **p < 0.001 and ***p < 0.01 when compared to control.
at a higher dose of 10 μM (cell viability decreased by 18%, Fig. 4a). PSP (1 μM, 1 h) had no significant cytotoxic effects but enhanced the cytotoxic effects of cyclophosphamide. The combination of cyclophosphamide and PSP (10 μM) decreased cell viability by 22% and 10% when compared with cyclophosphamide or PSP alone, respectively. PSP incubated alone for 24 h showed no toxic effects at all concentrations (0.1–10 μM). The results suggested that PSP enhanced the cytotoxic effect of cyclophosphamide (Fig. 4b).

4. Discussion

The present study represented the first report on the pharmacokinetic interaction between PSP and the anticancer agent, cyclophosphamide. We found that PSP decreased cyclophosphamide clearance in the rat after both acute and chronic administration of PSP. The effective doses of PSP were similar to previous pharmacokinetic
study in which PSP decreased paracetamol-induced hepatotoxicity in the rat (Yeung et al., 1994). Cyclophosphamide is metabolised to a number of metabolites, particularly 4-hydroxy-cyclophosphamide (Dolley, 1991), through P450-mediated pathways involving CYP2B6, CYP2C9, and CYP3A4 (Chang et al., 1993). It is believed that the pharmacokinetic interaction between PSP and cyclophosphamide may be related to multi-factors. Firstly, the inhibitory effect of acute dose PSP (prolonged T1/2 by 43%) was less effective than cimetidine (T1/2 prolonged by 76%), a well established P450 enzyme inhibitor which formed an inactive complex with P450 (Gibson and Skett, 1994). Secondly, sub-chronic PSP pre-treatment also decreased cyclophosphamide clearance, by similar extents when compared to the acute treatment. The subchronic effects of PSP were not dose-dependent, and one of the possible explanations is that PSP exerted immuno-stimulating effects by inducing the release of cytokines, which are known to modulate the regulation of cytochrome P450.

PSP has been shown to stimulate the release of interleukin-1β, interleukin-6 and tumor necrosis factor-α in human blood mononuclear cells (Wang et al., 1999), cytokines which have been shown to down-regulate the transcription of P450 subtypes (Renton, 2001), subsequently leading to a reduction of P450. Another possible explanation is that PSP may directly interact with hepatic P450 enzyme.

MTT assay was used to evaluate the cytotoxic effect of PSP and cyclophosphamide in this study. Cyclophosphamide at 10 μM was found to have some cytotoxic effects on HepG2 cells, result similar to those reported previously (Wang et al., 2002). The reported IC50 of cyclophosphamide on HepG2 was 22 mM (Wang et al., 2002), which was considered very high compared to the concentrations used in this study. The concentrations of cyclophosphamide selected in this in vitro study was based on the fact that the therapeutical doses, at the milligram level, would be expected to produce significant cytotoxic effects on cancer cells in vivo. In this study, we found that pre-incubation of PSP, at non-cytotoxic dosages, enhanced the cytotoxicity of cyclophosphamide. The combined effect of PSP with cyclophosphamide on HepG2 cytotoxicity was significantly greater than the cytotoxic effect of cyclophosphamide alone or the cytotoxic effect of high concentration of PSP alone. This observation represented a positive implication on the use of PSP as adjunct with cancer chemotherapy. When PSP alone was exposed for 1 h, cytotoxic effect was observed only at the highest dose of 10 μM. The result was in line with those observed in previous studies that PSP suppressed the growth of cancer cell lines such as Enrlich ascitic carcinoma, monocytic leukemia, P-388 leukemia, lymphosarcoma, human gastric cancer, and lung cancer (Yang, 1993). A more recent study showed that PSP inhibited the growth of breast cancer cells by increasing p21 gene expression and decreasing cyclin D1 expression (Chow et al., 2003), suggesting that apoptosis may be one of the mechanisms responsible for the cytotoxic effect of PSP.

PSP administration slowed down the weight gain over the duration of treatment (28 days), particularly with a weight loss in the first 4 days of treatment. Weight loss was generally considered as one of the determinants of in vivo toxicity, but we have to be cautious in the interpretation of the current finding. A previous report showed that oral PSP treatment at 6 g/day for 62 days had no toxic effects on rats. Rats in both sexes were studied and the body weight, food consumption, blood parameter, serum biochemical indexes, histopathological changes in major organs have not been altered (Jiang et al., 1999). Since there is a lack of data in terms of the bioavailability of PSP, it may not be appropriate to directly compare the effects of PSP through different routes of administration. In this study, each animal was administrated with 1 μmol of PSP, which was 500-fold higher than that reported to produce toxic effect in vitro (at 2 nmol, equivalent to 10 μM in 200 μl system). As much higher doses of PSP would be used in vivo, it would not be possible to exclude the possibility that PSP may exhibit cytotoxic effects and, on the other hand, produced pharmacokinetic changes that would alter the disposition of other anticancer agents. An alternative explanation to the PSP-induced weight loss could be related to the suppressive effect of PSP on appetite, leading to a reduction of food consumption. This assumption would again be related to the reported effects of PSP in enhancing the release of cytokines such as interleukins and tumor necrosis factors (Chan and Yeung, in press), which were reported to modulate appetite and energy homeostasis (Wong and Pinkney, 2004).

In conclusion, PSP has been shown to enhance the cytotoxic effects of cyclophosphamide on a cancer cell line in vitro. The synergistic effect may be an advantage for the use of PSP as an adjunct in cancer chemotherapy. Pharmacokinetic studies have shown that PSP decreased the cyclophosphamide clearance and increased the plasma half-life of the anticancer drug. This pharmacokinetic interaction may also be beneficial to the concomitant use of PSP and cyclophosphamide as a combined therapy in patients, such that the doses of cyclophosphamide may be adjusted to prevent anticancer drug-induced side effects. Whether the pharmacodynamic and pharmacokinetic effects observed in this study are related is uncertain, but would merit further investigations.

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