Protective effects of schizandrins and schizandrin B towards cisplatin nephrotoxicity \textit{in vitro}

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\textbf{ABSTRACT:} Renal proximal tubular epithelial cells are the main targets of toxic drugs such as cisplatin (CisPt), an alkylating agent indicated for the treatment of solid organ tumors. Current techniques aiming at reducing nephrotoxicity in patients receiving CisPt are still not satisfactory as they can only partially prevent acute kidney injury. New nephroprotective strategies remain to be developed. In the present \textit{in vitro} study, schizandrin (Schi) and schizandrin B (Schi B), major phytochemicals from \textit{Schisandra chinensis} (Turcz.) Baill. fruits, were tested on HK-2 cells along four processes that could help alleviate CisPt toxicity. Results indicated that: (i) both Schi and Schi B enhanced cell survival via reducing apoptosis rate; (ii) only Schi showed moderate effects towards modulation of regeneration capacities of healthy cells; (iii) both Schi and Schi B limited extracellular matrix deposition; and (iv) both compounds could help preventing dedifferentiation processes via the \(\beta\)-catenin pathay. Schi and Schi B present promising activities for future development of protective agents against CisPt nephrotoxicity. Copyright © 2013 John Wiley & Sons, Ltd.

\textbf{Keywords:} Apoptosis; \(\beta\)-catenin; Cisplatin; HK-2 cells; Schizandrin; Schizandrin B; Regeneration

\section*{Introduction}

Cisplatin (cis-diaminedichloroplatinum or CisPt) is an alkylating chemotherapeutic drug commonly used for the treatment of solid organ tumors such as lung, testis or ovarian cancers. In forming DNA-adducts, it may stop tumor cell growth and trigger their death. Unfortunately, CisPt treatment is often accompanied by severe detrimental side effects, such as nephrotoxicity, which limit the use of high dosages. Clinical data indicate that a single injection of the drug at 20 mg m\(^{-2}\) induces acute kidney injury (AKI) in about a third of treated patients (Hilal \textit{et al.}, 2005; Yao \textit{et al.}, 2007) for high numbers of renal proximal tubular epithelial cells (RPTECs) die following an apoptotic process (dos Santos \textit{et al.}, 2012; Yao \textit{et al.}, 2007). The massive loss of cells triggers transient loss in kidney function and structure. The lack of efficient regeneration process of injured RPTECs can trigger the onset of severe atrophy, followed up by fibrotic repair progressively resulting in chronic kidney disease and irreversible loss of renal function (Wynn, 2010). Recurrent AKI episodes, which are partially irreversible, may become more severe and last longer with repeated injections of CisPt, leading to the progressive loss of renal function.

Numerous studies conducted worldwide aim at identifying protective molecules and/or mechanisms that could be used to prevent or alleviate CisPt insults and the onset of AKI (Barabas \textit{et al.}, 2008; dos Santos \textit{et al.}, 2012). Among these, various compounds of herbal origin have been tested (Nagwani and Tripathi, 2010; Nitha and Janardhanan, 2008; Sohn \textit{et al.}, 2009a, b). These are notably issued from traditional Chinese medicine remedies that benefit from long-term use probably justifying their safety.

The fruit of \textit{Schisandra chinensis} (Turcz.) Baill., an ancient traditional Chinese medicine drug, is nowadays acknowledged as an adaptogen, i.e., a substance having the ability to increase non-specific resistance of organisms to biological, chemical, physical or emotional stress (European Medicines Agency, 2008). Although numerous phytochemical compounds have been characterized, major biological activities seem to be attributed to schizandrin (Schi) and schizandrin B (Schi B), two lignans presented in Fig. 1 (Panossian and Wikman, 2008). These compounds have been involved in recent \textit{in vivo} and \textit{in vitro} pharmacological studies, suggesting a protective effect in various pathological models (Jeong \textit{et al.}, 2012; Lee \textit{et al.}, 2012), with some studies focusing on nephrotoxicity (Park \textit{et al.}, 2012; Sohn \textit{et al.}, 2009a, 2009b; Stacchiotti \textit{et al.}, 2011; Zhu \textit{et al.}, 2012).

The present \textit{in vitro} study compares the activity of Schi and Schi B for possible alleviation of CisPt-mediated tubulotoxicity by investigating four processes involved in AKI processes: (i) apoptosis and cellular death (Havasi and Borkan, 2011; Pabla and Dong, 2008; Wynn, 2010); (ii) tubular regeneration capacities of healthy cells (Lee and Kalluri, 2010; Megyesi \textit{et al.}, 2002; Wynn, 2010); (iii) acquisition of collagen, an extracellular matrix (ECM) protein (Wynn, 2010; Yang \textit{et al.}, 2010); and (iv) dedifferentiation processes of epithelial cells via the \(\beta\)-catenin relocalization (Hao \textit{et al.}, 2011; Liu, 2010).
Material and methods

Reagents and culture media

CisPt solutions were prepared from the marketed drug Cisplatine Hospira® (Hospira Benelux, Antwerpen, Belgium). Schi (purity 99%) and Schi B (purity 98%) were purchased from Sigma-Aldrich (St Louis, USA) and from Finetech (Wuhan, China), respectively. Cell culture medium and reagents were from (PAA Laboratories, Pasching, Austria).

Cell culture and treatment

HK-2 cells, originating from RPTECs, were obtained from American Type Culture Collection (CRL-2190), and grown in low glucose DMEM containing 10% fetal bovine serum (FBS, PAA Clone), 2 mMl-glutamine and 1% penicillin–streptomycin. Cells were subcultured or harvested for experiments when reaching about 90% confluence. For experimental purposes, cells were used between passages 6 and 25, harvested by scraping and seeded on 60-mm Petri dishes (4 × 10⁵ cells), four-well chamber-slides (Lab-Tek II; Nunc, Rochester, NY, USA) (2.4 × 10⁴ cells), 12-well plates (5 × 10⁴ cells) or 96-well plates (1 × 10⁴ cells). Cells were then incubated for 24 h in complete medium, rinsed twice with DMEM and treated with test substances for 48 h in FBS-free medium. The tests performed in this study, along with their relevance to AKI, are summarized in Table 1.

CisPt working dose corresponded to IC25, as determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and crystal violet assays (data not shown). Schi and Schi B working doses (1 μM) were calculated provided that traditional Chinese medicine (TCM) use of Schizandra is based on a ∼1000-fold lower dose than CisPt. Thus, Schi and Schi B treatments for 48 h were at concentrations that exceed what has been traditionally used.

Table 1. Explanation of the tests selected for this study and their relevance to AKI

<table>
<thead>
<tr>
<th>Test</th>
<th>Why performed?</th>
<th>Relevance to AKI and fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resazurin</td>
<td>To measure cellular viability</td>
<td>Cell survival is associated with improved renal function (Pabla and Dong, 2008).</td>
</tr>
<tr>
<td>Annexin V/PI</td>
<td>To confirm that cellular viability enhancement correlates with reduced apoptosis</td>
<td>CisPt induced cell death implies apoptotic and necrotic pathways <em>in vivo</em>. It is however acknowledged that only high doses of CisPt can induce necrosis (dos Santos et al., 2012).</td>
</tr>
<tr>
<td>Ki-67</td>
<td>To evaluate cellular proliferation</td>
<td>Enhancing proliferation rate of healthy cells can improve kidney repair following AKI-induced cell loss (Price et al., 2009; Wynn, 2007). Ki-67 antigen is present at the nuclear surface of proliferative cells commonly used to assess cellular proliferation capacity (Urruticoechea et al., 2005).</td>
</tr>
<tr>
<td>Cell cycles</td>
<td>To check for eventual cell cycle arrest/confirm proliferation highlighted with Ki-67</td>
<td>As DNA damages can cause cell cycle arrest at G2/M phases – as it is the case for CisPt (Jamieson and Lippard, 1999) – they lead to an increased Ki-67 index not correlated to enhanced proliferation. Cell cycle analysis can evaluate the proportion of G2/M cells and help discriminate between proliferative or cell cycle arrest effects.</td>
</tr>
<tr>
<td>Wound healing assay</td>
<td>To evaluate cellular motility</td>
<td>Enhancing migration rate of healthy cells can help recolonizing damaged tubules (Bozic et al., 2011).</td>
</tr>
<tr>
<td>Collagen assessment</td>
<td>To assess ECM protein synthesis</td>
<td>Collagen is a major constituent of ECM, replacing functional tissues by permanent fibrotic scar during abnormal wound healing. Limiting fibrogenesis and collagen accumulation can counter the irreversible loss of kidney function and structure (Wojcikowski et al., 2004; Wynn, 2007).</td>
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<tr>
<td>Membranous β-catenin</td>
<td>To estimate epithelial phenotype's integrity</td>
<td>β-catenin links E-cadherin to the cytoskeleton, thus ensuring intercellular adhesion. Preserving epithelial phenotype can prevent cell loss along the tubules (Bozic et al., 2011; Hao et al., 2011).</td>
</tr>
<tr>
<td>Cytoplasmic/nuclear β-catenin</td>
<td>To predict potential activation of genes involved in dedifferentiation</td>
<td>Inhibiting β-catenin relocalization is associated with hampered expression of genes involved in cellular de-differentiation, a phenomena involved in fibrogenesis (Bozic et al., 2011; Hao et al., 2011; He et al., 2011).</td>
</tr>
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</table>

AKI, acute kidney injury; ECM, extracellular matrix.
medicine monographs prescribes the use of *S. chinensis* up to 3 qian (= 10 g) per intake (Bensky et al., 1986) and that the minimal content of Schi in crude drug is 0.4%, as stated by the European Pharmacopoeia (Council of Europe, 2007) (leading to the oral ingestion of at least 40 mg of Schi per intake). A pharmacokinetic study reports plasma levels of 1.17 μM after oral administration of Schi to rats at a dose of 5 mg kg⁻¹ body weight (Xu et al., 2008), corresponding to a human equivalent dose of about 50 mg per intake (Reagan-Shaw et al., 2008). Assuming that plasma and the glomerular filtrate have similar compositions, we decided to treat the cells with a concentration close to that reported above (i.e., 1 μM).

**Cell viability assay**

Cells were treated with CisPt and/or Schi or Schi B in 96-well plates, washed twice with phosphate-buffered saline (PBS), and assessed for their viability by incubation for 1.5 h with 0.44 mM resazurin solution (Sigma-Aldrich, St Louis, MO, USA). Absorbances were measured at 540 and 620 nm using iEMS Reader MF spectrophotometer (Thermo Labsystems, Breda, The Netherlands). Percentage of reduced dye was calculated with the following formula:

\[
\frac{(\varepsilon_{OX}) \cdot 2 \cdot A_{540} - (\varepsilon_{OX}) \cdot 1 \cdot A_{540}}{(\varepsilon_{RED}) \cdot 1 \cdot A_{540} - (\varepsilon_{RED}) \cdot 2 \cdot A_{540}}
\]

Where \( \varepsilon_{OX} \) = molar extinction coefficient of resazurin (47.6 at 540 nm and 34.8 at 620 nm); \( \varepsilon_{RED} \) = molar extinction coefficient of resorufin (104.4 at 540 nm and 5.5 at 620 nm); \( A = \) absorbance of test wells; \( A^{\prime} = \) absorbance of blank well; \( \lambda_1 = 540 \text{ nm}; \lambda_2 = 620 \text{ nm}. \) Metabolic activity was normalized against control conditions.

**Annexin V/propidium iodide staining assay**

Cells were incubated in 12-well plates with 1 μM Schi or Schi B and/or 10 μM CisPt. At the end of treatment, cells were rinsed with PBS, harvested with trypsin/EDTA (0.5/0.2%) and centrifuged at 500 g, resuspended in 400 μl PBS, harvested with trypsin/EDTA (0.5/0.2%) and centrifuged at 500 g, resuspended in PI/RNase Staining Buffer (BD Pharmingen) following the manufacturer’s instructions, incubated 30 min at room temperature, centrifuged at 500 g, resuspended in 400 μl BD FACSFLOW (BD Pharmingen) and analyzed using a BD FACSCanto II flow cytometer (BD Pharmingen). A minimum of 10⁶ events corresponding to HK-2 cells were recorded using the BP 585/42 filter at a maximum rate of 200 events s⁻¹.

Cell cycle analysis was achieved with FlowJo software (Tree Star): debris and cell clumps were removed and proportions of G0/G1, S and G2/M were determined according to the Dean–Jett–Fox model.

**Wound healing assay**

Cells were seeded on 60 mm Petri dishes, incubated for 48 h, yielding approximately 90-100% confluence and were growth arrested for 24 h in serum-free medium. Monolayers were scratched in a linear fashion with a sterile 200 μl pipette tip and gently rinsed with serum-free medium to remove cell debris. Ten pictures of the initial scratch were taken (t₀) using Motic AE21 microscope equipped with a Moticam 2300 camera (Motic, Wetzlar, Germany) at magnification 100 ×. Cells were then treated with test substances for 18 h, and 10 pictures of the scratch were taken (t₁₈).

The area of the scratch was measured using TScratch (Geback et al., 2009) and expressed as a mean width per image (μm). For each condition, the migration rate (μm h⁻¹) was calculated by subtracting the value obtained at t₁₈ to the value obtained at t₀ and reported to the duration of incubation.

**Assessment of collagen synthesis**

Cells were treated in 96-well plates for 48 h, rinsed with PBS and assessed for their metabolic activity with the resazurin assay as described above. Next, cells were washed with PBS, fixed with ice-cold methanol for 1 h at 4 °C, rinsed twice with 1% acetic acid, and stained with 2% w/v picrosirius red staining solution. Wells were then rinsed thrice with 1% acetic acid, and dyes were solubilized in 0.1 M NaOH, and absorbances of the wells measured at 540 nm. Absorbances attributed to picrosirius red staining were normalized according to the metabolic activity of each well (the correlation between the number of cells, metabolic activity and protein amount determined by the bicinchoninic acid method was verified in preliminary experiments).

**β-catenin determinations**

**Membranous β-catenin determination**

Cells were treated on chamber slides for 48 h in medium containing Schi or Schi B and/or CisPt. Slides were rinsed twice...
in PBS, fixed in 4% paraformaldehyde solution for 20 min, rinsed and permeabilized with 0.01% Triton X for 5 min. Slides were rinsed again and cells blocked with donkey serum (sc-2044; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. They were then incubated with mouse antimouse β-catenin primary antibody (sc-7963; Santa Cruz Biotechnology) for 1 h, washed twice, incubated with cyanine-3 conjugated donkey antimouse secondary antibody (715-166-151, Immuno Research Lab Jackson, West Grove, USA) for 30 min, rinsed twice, mounted with DAPI-containing Prolong Gold antifade reagent (Invitrogen) and examined at magnification 400x with an Axioskop fluorescence microscope (Zeiss) equipped with a DP200 camera (Deltapix, Maalov, Denmark).

Before evaluation of the fluorescence intensity, the linearity and reproducibility of the method were controlled using InspeckTM Orange (λex = 540 nm/λem = 560 nm) fluorescently labeled microbeads (6.0 μm diameter) (Molecular Probes, Eugene, OR, USA).

For each condition, 10 pictures were taken, and analyzed using Fiji (Fiji Is Just ImageJ) software. The fluorescence intensity was evaluated by the measurement of luminance. DAPI counterstaining allowed removing the signal corresponding to eventual β-catenin translocated in the nucleus.

Cytoplasmic/nuclear β-catenin determination

Cells were treated in 12-well plates, rinsed with PBS, harvested with trypsin/EDTA (0.5/0.2%), centrifuged at 500 g and fixed in Cytofix/Cytoprot solution (BD Pharmingen) for 20 min at 4°C. Suspension was washed twice, incubated with antimouse β-catenin–phycoerythrin monoclonal antibody (R&D Systems, Minneapolis, MN, USA) in the dark for 30 min. Cells were washed twice and analyzed using a BD FACSCanto II flow cytometer (BD Pharmingen). A minimum of 10⁴ cells were recorded. The data were analyzed with FlowJo software (Tree Star); debris and cell clumps were removed and mean fluorescence intensities (geometric mean) were calculated using BP 585/42 filter for phycoerythrin.

Statistical analysis

Unless stated otherwise, experiments were performed four times using independent samples. Results needing to be normalized versus controls were treated as described by (Valcu and Valcu, 2011). Data were compared through a one-way ANOVA with post-hoc Student’s t test (Bonferroni correction) using Prism 5 software (GraphPad, San Diego, USA). P < 0.05 was considered significant.

Results

Effects of schizandrin and schizandrin B on cisplatin-induced cell death

Figure 2 shows the effects of CisPt incubation with or without Schi or Schi B (1 μM) on the viability of HK-2 cells determined with resazurin assay. After 48 h incubation with CisPt, the proportion of live cells dropped to 76.2 ± 1.9%, whereas Schi and Schi B did not induce any statistically significant difference at tested concentrations. Upon cotreatment with CisPt and Schi or Schi B, cells exhibited significantly higher survival rates (82.1 ± 0.8% and 87.7 ± 2.0%, respectively; P < 0.001), as compared to CisPt treatment alone.

Schizandrin and schizandrin B moderately reduce cisplatin-induced HK-2 cells apoptosis

To confirm further the protective effects observed with Schi and Schi B towards CisPt-induced cell death, apoptosis rates were investigated. Annexin V/PI staining was used to identify cells in both early and late stages of apoptosis; necrotic cells were not taken into account as necrosis is normally induced with higher concentrations in CisPt (dos Santos et al., 2012).

Treatment of HK-2 cells with 10 μM CisPt induced an increase in number of apoptotic cells from 3.2 ± 0.5% for control conditions to 16.4 ± 0.6% (Fig. 3). Upon cotreatment with Schi and Schi B (1 μM), the ratios of apoptotic cells were significantly reduced to 14.0 ± 0.8% and 14.2 ± 0.5%, respectively.

Figure 2. Protective effects of Schi (1 μM) and Schi B (1 μM) towards CisPt-treated HK-2 cells were investigated with resazurin assay after 48 h treatment. Results are displayed as means ± SD of four independent experiments (**P < 0.001). CisPt, cisplatin; Ctrl, control; Schi, schizandrin.

Figure 3. Proportions of apoptotic cells after treatment with 10 μM CisPt and/or Schi or Schi B (1 μM) for 48 h. Results are displayed as means ± SD of four independent experiments (***P < 0.001). CisPt, cisplatin; Ctrl, control; Schi, schizandrin.
Altogether, these results indicate that both Schi and Schi B, when given concomitantly with CisPt, are able to reduce cellular death, probably via lowering the apoptosis rate.

**Schizandrin but not schizandrin B enhances cellular proliferation**

Immunofluorescence staining of the Ki-67 marker after treatment for 48 h with test substances is displayed in Fig. 4. FBS was used as a positive control; indeed, HK-2 cell growth has been shown to be epidermal growth factor-dependent (Ryan et al., 1994). As compared to control grown in the absence of serum, FBS induced a 3.1-fold increase in the number of cells staining positive for Ki-67. CisPt (10 μM) also increased the Ki-67 index (2.3-fold over control). However, this effect probably does not correspond to enhanced cellular proliferation and could be a consequence of a G2/M cell cycle arrest induced by CisPt-triggered DNA damages (Jamieson and Lippard, 1999).

Schi treatment also raised the Ki-67 index to about 1.9-fold over control. To determine whether this effect is a consequence of a proliferation enhancement, or a toxic effect as presumed for CisPt, an analysis of cell cycle distribution was carried out.

**Cell cycle analysis**

Figure 5 shows the cell cycle phases distribution of cells treated in the same conditions as for the Ki-67 index assessment. The positive control (FBS 10%) induced a slight decrease in the amount of G0/G1 cells (71.3 ± 3.7% vs. 84.3 ± 0.9% for control condition) and a slight increase in the amount of G2/M cells (20.2 ± 2.9% vs. 10.6 ± 1.9% for control condition).

As expected, CisPt exposure induces a cell cycle arrest at G2/M checkpoint, resulting in a sharp increase in G2/M cells (76.8 ± 2.4%) and decrease in G0/G1 phases cells (16.0 ± 2.5%).

Schi and Schi B treatments yielded the same patterns as observed for FBS treatment, with slight decreases of G0/G1 cells (77.3 ± 2.5% and 78.5 ± 3.0%, respectively); G2/M cells show a slight increased trend, but were not statistically significant.

Schi and Schi B were not able to prevent CisPt-mediated G2/M arrest (data not shown), which suggests that these compounds probably do not protect HK-2 cells against DNA damages.

**Schizandrin and schizandrin B have no effect on wound healing**

As Schi shows a Ki-67-enhancing effect, and as both Schi and Schi B promote the progression in cell cycle by reducing the proportion of G0/G1 cells, their ability to increase tubular

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**Figure 4.** Fluorescence immunostaining pictures of Ki-67 antigen (green fluorescence) and nuclei (blue fluorescence) after HK-2 cells treatment with test substances for 48 h (magnification × 400). Pictures are representative of four independent experiments. The proliferation indexes assessment are displayed as means ± SD of four independent experiments (**P < 0.001 compared to control group). CisPt, cisplatin; Ctrl, control; FBS, fetal bovine serum; Schi, schizandrin.

**Figure 5.** Cell cycle distribution after treatment of HK-2 cells for 48 h with test substances. Results are means ± SD of four independent experiments (*P < 0.05; **P < 0.01; ***P < 0.001 compared to control group). CisPt, cisplatin; Ctrl, control; FBS, fetal bovine serum; Schi, schizandrin.
regeneration was investigated through the study of cellular migration enhancement.

FBS induced a 1.7-fold increase over control, but no effect could be observed upon treatment with 1 μM Schi or 1 μM Schi B (Fig. 6).

Both schizandrin and schizandrin B reduce cisplatin-induced collagen synthesis

Total collagen amounts, assessed with picrosirius red staining, were not modified upon treatment of HK-2 cells with both Schi and Schi B as compared to control condition (Fig. 7). However, if CisPt induced an increased collagen synthesis (125 ± 6%), both Schi and Schi B cotreatments were able to restrain this effect (115 ± 4% and 110 ± 4%, respectively).

Assessments of β-catenin

Membranous β-catenin

When HK-2 cells were treated with 10 μM CisPt, the membranous β-catenin expression was reduced by 1.8-fold as compared to control conditions (Fig. 8A). Upon cotreatment with Schi and Schi B, the β-catenin losses were limited to 1.2 and 1.1 respectively; this indicates that both compounds could prevent the loss of epithelial phenotype.

Intracytoplasmic/nuclear β-catenin

HK-2 cells grown in the same conditions were stained for their intracytoplasmic/nuclear β-catenin and analyzed by flow cytometry. The observed loss of membranous β-catenin with CisPt treatment (Fig. 8A) correlates with an increased intracytoplasmic/nuclear isoform of 1.7-fold over control (Fig. 8B). Both Schi and Schi B cotreatments were effective in limiting this increase to 1.2- and 1.0-fold over control respectively. This indicates that both compounds are effective in preventing β-catenin relocation.

Discussion

CisPt is an efficient chemotherapeutic agent, for which adverse effects, notably renal toxicity, remain highly detrimental to patients and often lead to a dose reduction of the drug, compromising the therapy. Until now, ensuring adequate hydration and diuresis represents the only strategy implemented for limiting the nephrotoxicity of the treatment.

CisPt concentrates in tumor cells, as well as in RPTECs and binds covalently to DNA, resulting in apoptosis and cell cycle arrest (dos Santos et al., 2012), both of which are starting points for AKI and fibrotic repair (Wynn, 2010). It is yet recognized that CisPt is highly nephrotoxic and that there is a need for adjuvants to reduce the damage associated with this treatment.
that preventing AKI may contribute to restrain fibrosis onset and progression to end-stage renal disease.

The present in vitro study focused on four of the key elements involved in AKI.

**Prevention of cell death via apoptosis**

This is a mechanism leading to the loss of functional units of the tubules. Both Schi and Schi B enhanced the survival of cells treated with CisPt. This was confirmed for both compounds by a reduction in the number of cells undergoing apoptosis at end-point (48 h incubation) of experiment. As both Schi and Schi B showed similar efficacy in reducing the apoptosis rate, this factor alone does not explain the higher enhancement in cell survival for Schi B; other mechanisms may be involved. Nevertheless, these promising results are to be related to a potential reduction in the loss of tubular cells in vivo, what could reduce tubular atrophy and AKI. This has notably been demonstrated for Schi B in a cyclosporine A-mediated nephrotoxicity model (Zhu et al., 2012).

**Promotion of tubular regeneration**

Enhancing cellular proliferation and migration capacities could help tubules recover after CisPt insults and could thus reduce the duration and severity of AKI in avoiding functional and structural losses (Price et al., 2009; Yamamoto et al., 2010).

The proliferation enhancement was assessed by the Ki-67 index, commonly used to determine whether tumors have a high proliferative potential or not. Ki-67 is present at the nuclear surface of proliferating cells (i.e., in G1, S, G2 and M phases of the cell cycle) and remains absent in quiescent cells (G0) (Urruticoechea et al., 2005). Ki-67 has previously been used in vitro and in vivo for the assessment of RPTEC’s proliferation/ regeneration (Docherty et al., 2006; Pozdzik et al., 2008).

CisPt increased the Ki-67 index by 2.3-fold over control conditions, but this increase is a consequence of its toxicity rather than an effect on proliferation; CisPt’s binding to DNA strands is known to trigger cell cycle arrest at G2/M checkpoint (Jamieson and Lippard, 1999), resulting in Ki-67-positive cells. This was confirmed by flow cytometry analysis of cell cycle phases that indicated a 7.2-fold increase in G2/M cells.

**Figure 8.** Immunostained membranous β-catenin after treatment for 48 h for control and CisPt conditions (A). Ten images were acquired per condition and fluorescence intensities were measured; DAPI counterstaining was subtracted to remove eventual β-catenin translocated to the nucleus. Luminance values were then recorded (graph A). Results are displayed as means ± SD of four independent experiments (*P < 0.05; **P < 0.01; ***P < 0.001 compared to control group). Fluorescence images confirming the nuclear/cytoplasmic β-catenin immunostaining (B). Cells were analyzed with a flow cytometry technique and geometrical mean fluorescence intensity was evaluated (graph B). Means ± SD of four independent experiments (*P < 0.05; **P < 0.01; ***P < 0.001). CisPt, cisplatin; Ctrl, control; Schi, schizandrin.
The addition of FBS (10%) or Schi (1 μM) induce 3.1- and 1.9-fold increases respectively in the Ki-67 index, whereas Schi B treatment does not produce any modification. Cell cycle analysis indicates that FBS treatment yields a 1.9-fold increase in G2/M cells, whereas neither Schi nor Schi B induces a significant accumulation of cells in this phase. However, G0/G1 phases were significantly reduced upon Schi and Schi B treatment, suggesting that both compounds may promote cellular proliferation. The results obtained for the Ki-67 index assessment suggest that Schi actively instigates cell cycle entrance (G1), thus lowering the amount of quiescent cells. Schi B could influence the duration of definite phases of the cell cycle, without influencing the proportion of quiescent/proliferating cells. This should be confirmed by further investigations.

The cellular migration, assessed by a scratch assay, did not highlight any effect as compared to control conditions.

The regeneration potential of the two tested compounds appears somewhat limited. Only Schi seems capable of promoting the proliferation of tubular cells.

**Cisplatin-induced collagen production**

This ECM protein is secreted soon after injury to produce acellular non-functional scar tissue, filling eventual gaps left by dead cells along the tube. However, collagen acts as a pro-fibrosis factor (Zeisberg and Neilson, 2010). The amounts of deposited collagen depend on the balance between its production by RPTEC and its degradation by matrix metalloproteinases. Preventing its deposition is likely to help recovery following CisPt-induced AKI and to avoid fibrosis onset.

In our model, we showed that both Schi and Schi B were effective at reducing CisPt-induced collagen synthesis. However, Schi B showed a higher effect than Schi. Evidence suggested that lowering the ECM protein synthesis correlates to attenuated fibrotic lesions in an obstructive nephropathy in vivo model (Hao et al., 2011).

**Localization of β-catenin**

This is a protein playing a dual role in cell adhesion as well as in the transcription of genes. In adherens junctions, β-catenin links E-cadherin to the cytoskeleton, ensuring the epithelial phenotype of RPTECs. In case of cellular injury, these links disrupt and β-catenin may relocalize into the nucleus where it serves as a co-transcriptional activator with T-cell factor (Zeisberg and Neilson, 2009), a complex governing the transcription of genes involved in fibrosis (Bozic et al., 2011). Evidence suggested that blockade of the β-catenin pathway could reduce the onset and limit the progression of renal fibrosis (Hao et al., 2011; He et al., 2011). Moreover, Schi and Schi B are lignans, a class of compounds that have already proven their ability to inhibit the β-catenin transcription pathway in colorectal adenocarcinoma cells (Yoo et al., 2010).

**Membranous β-catenin**

Quantitative fluorescence image analysis demonstrated that HK-2 cells treatment with CisPt induced a 1.8-fold decrease in the amount of membranous β-catenin, as compared to control condition. Cotreatment with Schi and Schi B restrained this loss to 1.2- and 1.1-fold, respectively. Both compounds have rather similar efficacy towards prevention of this epithelial phenotype loss. However, none had the ability to increase membranous β-catenin after 48 h treatment, indicating that they were not able to promote the protein's expression.

It is conceivable that, in preserving the adherens junction's tightness, Schi and Schi B can prevent cell detachment and thus influence the overall cellular viability.

**Internal β-catenin**

The amounts of nuclear/cytoplasmic isoforms of β-catenin were assessed by flow cytometry. Both Schi and Schi B could restrain the relocalization of the protein in the presence of CisPt; this indicates that they may be effective in reducing the transcription of genes involved in fibrosis development.

Altogether, these results point out a promising nephroprotective potential of Schi and Schi B towards CisPt toxicity. The prevention of cell death, along with the slight enhancement in tubular regeneration, restrained ECM deposition and prevention of β-catenin relocalization could help alleviate the severity and duration of CisPt-induced AKI, and, in the longer term, avoid the onset of kidney fibrosis.

**Conclusion**

Altogether, these results point out a promising nephroprotective potential of Schi and Schi B towards CisPt toxicity. The prevention of cell death, along with the slight enhancement in tubular regeneration, restrained ECM deposition and prevention of β-catenin relocalization could alleviate the severity and duration of CisPt-induced AKI, and, in the longer term, avoid the onset of kidney fibrosis.

**Acknowledgments**

Thanks are expressed to E. De Prez, M. Faes and O. Vaillant for technical assistance, T. Baudoux and C. Husson for their valuable advices on flow cytometry.

**Conflicts of interest**

The Authors did not report any conflict of interest.

**Funding**

Valérain Bunel is a fellow of the Fonds de la Recherche Scientifique – FNRS (FRIA grant).

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Nephroprotection of Schisandra compounds against cisplatin toxicity


