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Protective effects of schizandrin and schizandrin B towards cisplatin nephrotoxicity *in vitro*

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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 *in vitro* study, schizandrin (Schi) and schizandrin B (Schi B), major phytochemicals from *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 β-catenin pathway. Schi and Schi B present promising activities for future development of protective agents against CisPt nephrotoxicity. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: Apoptosis; β-catenin; Cisplatin; HK-2 cells; Schizandrin; Schizandrin B; Regeneration

Introduction

Cisplatin (cis-diamminedichloroplatinum 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 *et al*., 2005; Yao *et al*., 2007) for high numbers of renal proximal tubular epithelial cells (RPTECs) die following an apoptotic process (dos Santos *et al*., 2012; Yao *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 *et al*., 2008; dos Santos *et al*., 2012). Among these, various compounds of herbal origin have been tested (Nagwani and Tripathi, 2010; Nitha and Janardhanan, 2008; Sohn *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 *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 *in vivo* and *in vitro* pharmacological studies, suggesting a protective effect in various pathological models (Jeong *et al*., 2012; Lee *et al*., 2012), with some studies focusing on nephrotoxicity (Park *et al*., 2012; Sohn *et al*., 2009a,2009b; Stacchiotti *et al*., 2011; Zhu *et al*., 2012).

The present *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 *et al*., 2002; Wynn, 2010); (iii) acquisition of collagen, an extracellular matrix (ECM) protein (Wynn, 2010; Yang *et al*., 2010); and (iv) dedifferentiation processes of epithelial cells via the β-catenin relocalization (Hao *et al*., 2011; Liu, 2010).

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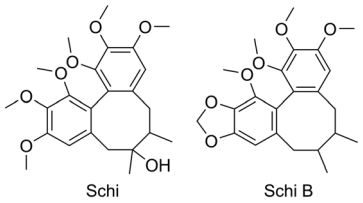

Figure 1. Structures of schizandrin (Schi) and schizandrin B (Schi B).

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 mM L-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 (4×10^5 cells), four-well chamber-slides (Lab-Tek II; Nunc, Rochester, NY, USA) (2.4×10^4 cells), 12-well plates (5×10^4 cells) or 96-well plates (1×10^4 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 IC_{25}, 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

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

Test	Why performed?	Relevance to AKI and fibrosis
Resazurin	To measure cellular viability	Cell survival is associated with improved renal function (Pabla and Dong, 2008).
Annexin V/PI	To confirm that cellular viability enhancement correlates with reduced apoptosis	CisPt induced cell death implies apoptotic and necrotic pathways *in vivo*. It is however acknowledged that only high doses of CisPt can induce necrosis (dos Santos *et al*., 2012).
Ki-67	To evaluate cellular proliferation	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).
Cell cycles	To check for eventual cell cycle arrest/confirm proliferation highlighted with Ki-67	As DNA damages can cause cell cycle arrest at G_2/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 G_2/M cells and help discriminate between proliferative or cell cycle arrest effects.
Wound healing assay	To evaluate cellular motility	Enhancing migration rate of healthy cells can help recolonizing damaged tubules (Bozic *et al*., 2011).
Collagen assessment	To assess ECM protein synthesis	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).
Membranous β-catenin	To estimate epithelial phenotype's integrity	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).
Cytoplasmic/nuclear β-catenin	To predict potential activation of genes involved in dedifferentiation	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).

AKI, acute kidney injury; ECM, extracellular matrix.

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medicine monographs prescribes the use of *S. chinensis* up to 3 qián (≈ 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:

$$\frac{(\varepsilon OX)\lambda 2.A\lambda 1 - (\varepsilon OX)\lambda 1.A\lambda 2}{(\varepsilon RED)\lambda 1.A'\lambda 2 - (\varepsilon RED)\lambda 2.A'\lambda 1}$$

Where ε_{OX} = molar extinction coefficient of resazurin (47.6 at 540 nm and 34.8 at 620 nm); ε_{RED} = molar extinction coefficient of resorufin (104.4 at 540 nm and 5.5 at 620 nm); A = absorbance of test wells; A″ = absorbance of blank well; λ1 = 540 nm; λ2 = 620 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***. Supernatant was discarded. Cells were resuspended and incubated with Annexin V-FITC detection kit (BD Pharmingen, San Diego, CA, USA) in the dark for 15 min. Suspensions were then analyzed using a BD FACSCanto II flow cytometer (BD Pharmingen). A total of 10^4 cells were recorded. The data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA); debris and cell clumps were removed and the percentages of live and apoptotic cells were calculated.

Ki-67 immunostaining

Cells were treated on chamber slides with test substances. Slides were then 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. Cells were blocked with goat serum (PAA Laboratories) for 1 h, and then incubated with a primary rabbit anti-Ki-67 antibody (Abcam, Cambridge, UK) for 1 h. Slides were rinsed twice and incubated with a secondary Alexa Fluor 488 conjugated goat antirabbit antibody (Invitrogen, Eugene, OR, USA) for 30 min. Slides were rinsed twice and mounted with DAPI-containing mountant (Invitrogen) and examined with an Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) at magnification 400 ×. Twenty pictures per chamber were acquired; the Ki-67 index was calculated by dividing the number of Ki-67-positive cells by the total number of cells.

Cell cycle analysis

Cells were treated and harvested as for the Annexin V/propidium iodide (PI) assay, resuspended in 1 ml PBS and manually counted using a Malassez hemocytometer. Cells were then fixed by adding 2 ml of ice-cold absolute ethanol, incubated during 1 h at 4 °C, centrifuged at 500 ***g*** and rinsed with PBS. Cells were 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 10^4 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 G_0/G_1, S and G_2/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 (t_0) 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 (t_{18}).

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 t_{18} to the value obtained at t_0, 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 for 2 h with 0.1% picrosirius red staining solution. Wells were then rinsed thrice with 1% acetic acid, 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 antihuman 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 400 × 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 Inspeck™ Orange ($\lambda_{Ex} = 540$ nm/$\lambda_{Em} = 560$ 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/Cytoperm solution (BD Pharmingen) for 20 min at 4 °C. Suspension was washed twice, incubated with antihuman 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 10^4 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.

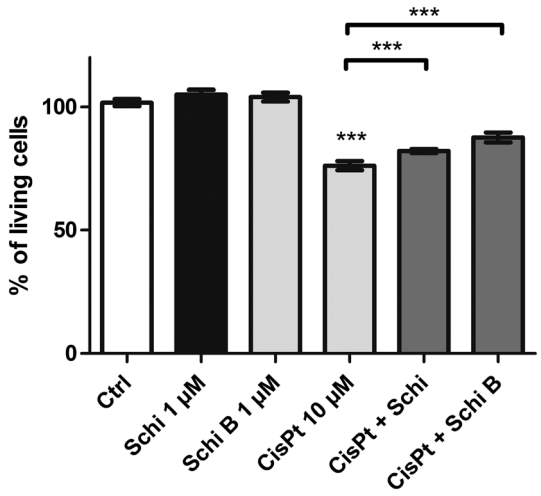

Figure 2. Protective effects of Schi (1 μM) and Schi B (1 μM) towards CisPt-treated HK-2 cells were investigated with resazurin assay after 48 h treatment. Results are displayed as means ± SD of four independent experiments (***$P < 0.001$). CisPt, cisplatin; Ctrl, control; Schi, schizandrin.

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.

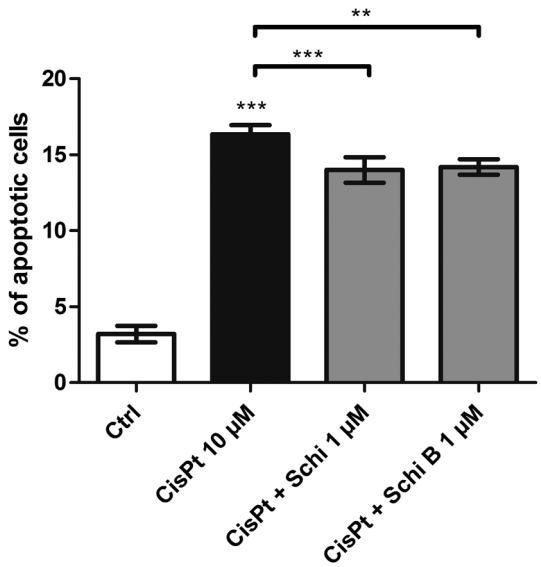

Figure 3. Proportions of apoptotic cells after treatment with 10 μM CisPt and/or Schi or Schi B (1 μM) for 48 h. Results are displayed as means ± SD of four independent experiments (**$P < 0.01$; ***$P < 0.001$). CisPt, cisplatin; Ctrl, control; Schi, schizandrin.

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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 G_2/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 G_0/G_1 cells (71.3 ± 3.7% vs. 84.3 ± 0.9% for control condition) and a slight increase in the amount of G_2/M cells (20.2 ± 2.9% vs. 10.6 ± 1.9% for control condition).

As expected, CisPt exposure induces a cell cycle arrest at G_2/M checkpoint, resulting in a sharp increase in G_2/M cells (76.8 ± 2.4%) and decrease in G_0/G_1 phases cells (16.0 ± 2.5%).

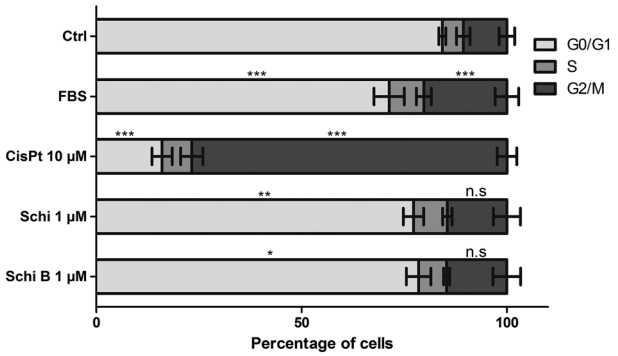

Figure 5. Cell cycle distribution after treatment of HK-2 cells for 48 h with test substances. Results are means ± SD of four independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control group). CisPt, cisplatin; Ctrl, control; FBS, fetal bovine serum; Schi, schizandrin.

Schi and Schi B treatments yielded the same patterns as observed for FBS treatment, with slight decreases of G_0/G_1 cells (77.3 ± 2.5% and 78.5 ± 3.0%, respectively); G_2/M cells show a slight increased trend, but were not statistically significant.

Schi and Schi B were not able to prevent CisPt-mediated G_2/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 G_0/G_1 cells, their ability to increase tubular

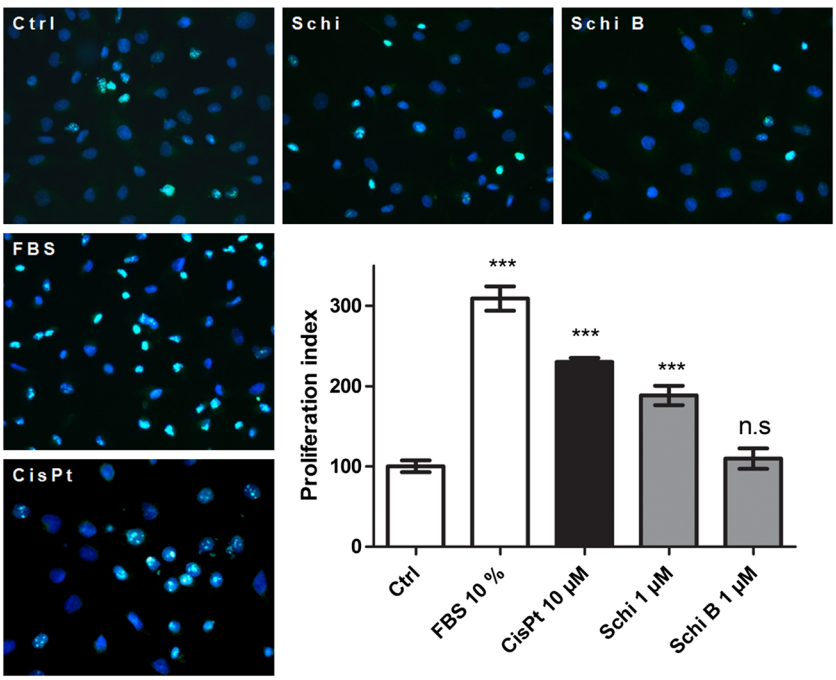

Figure 4. Fluorescence immunostaining pictures of Ki-67 antigen (green fluorescence) and nuclei (blue fluorescence) after HK-2 cells treatment with test substances for 48 h (magnification × 400). Pictures are representative of four independent experiments. The proliferation indexes assessment are displayed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

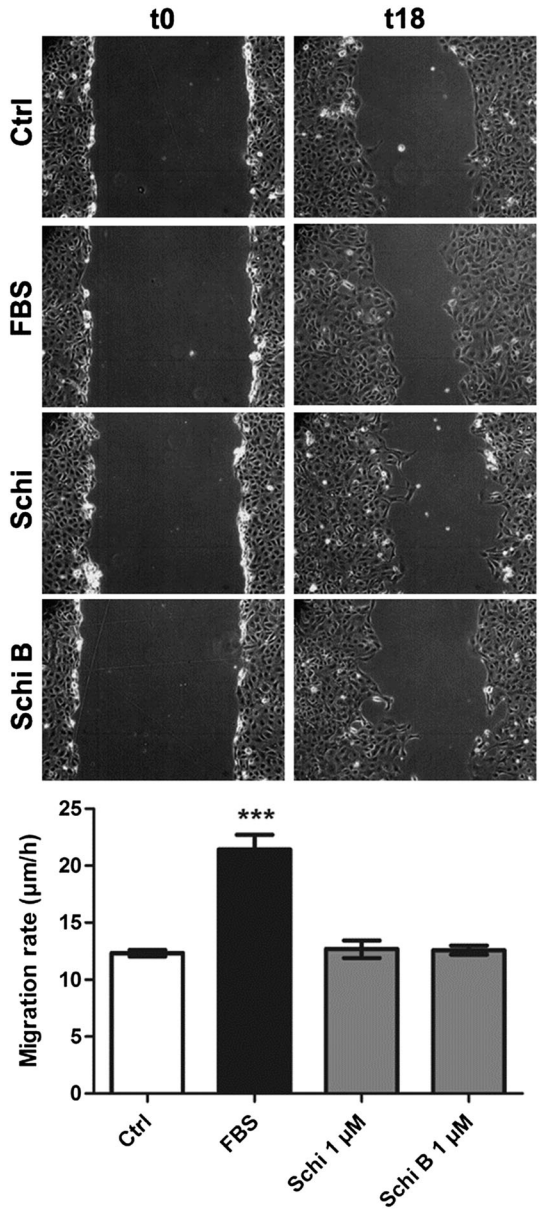

Figure 6. Wound healing assay. HK-2 cells were serum-deprived for 24 h, scratched with a pipette tip and incubated with test substances for 18 h. Phase contrast microscopy images (× 100 magnification) for control, FBS, Schi and Schi B treatments at t_0 (left pictures) and t_{18} (right pictures). Migration rates are displayed as means ± SD of four independent experiments (***$P < 0.001$ as compared to control conditions) ((graph). 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).

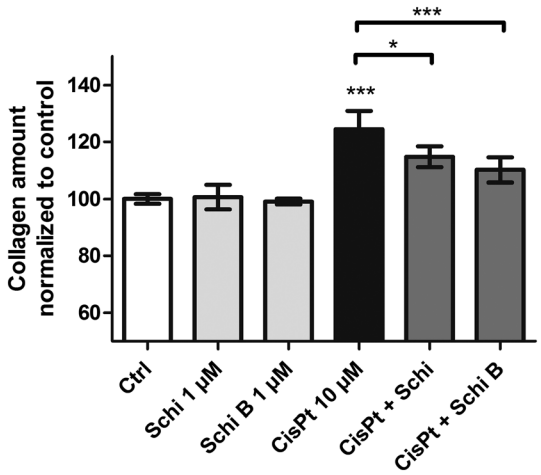

Figure 7. Collagen quantification upon treatment of HK-2 cells with test substances for 48 h and picrosirius red staining. Absorbances were normalized according to their metabolic activity (resazurin assay) and compared to control condition (means ± SD of four independent experiments; *$P < 0.05$; ***$P < 0.001$). CisPt, cisplatin; Ctrl, control; Schi, schizandrin.

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 relocalization.

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

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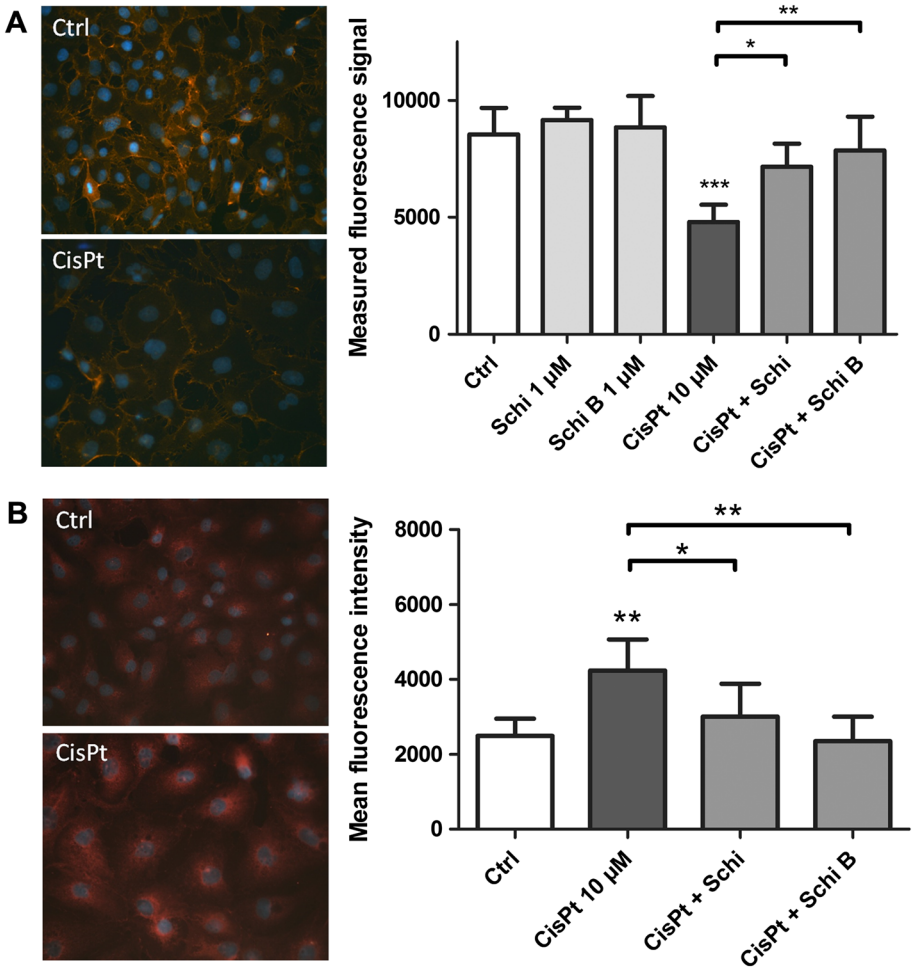

Figure 8. Immunostained membranous β-catenin after treatment for 48 h for control and CisPt conditions (A). Ten images were acquired per condition and fluorescence intensities were measured; DAPI counterstaining was subtracted to remove eventual β-catenin translocated to the nucleus. Luminance values 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.

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 G_1, S, G_2 and M phases of the cell cycle) and remains absent in quiescent cells (G_0) (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 G_2/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 G_2/M cells.

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 G_2/M cells, whereas neither Schi nor Schi B induces a significant accumulation of cells in this phase. However, G_0/G_1 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 (G_1), 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 tubule. 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 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.

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Conflicts of interest

The Authors did not report any conflict of interest.

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References

Barabas K, Milner R, Lurie D, Adin C. 2008. Cisplatin: a review of toxicities and therapeutic applications. *Vet. Comp. Oncol.* **6**: 1–18.

Bensky D, Gamble A, Kaptchuk T, Bensky L. 1986. *Chinese Herbal Medicine: Materia Medica*. Eastland Press: Seattle.

Bozic M, de Rooij J, Parisi E, Ortega MR, Fernandez E, Valdivielso JM. 2011. Glutamatergic signaling maintains the epithelial phenotype of proximal tubular cells. *J. Am. Soc. Nephrol.* **22**(6):1099–1111.

Council of Europe. 2007. European Pharmacopoeia 6.0 Strasbourg, Council of Europe.

Docherty NG, O'Sullivan OE, Healy DA, Murphy M, O'Neill AJ, Fitzpatrick JM, Watson RW. 2006. TGF-beta1-induced EMT can occur independently of its proapoptotic effects and is aided by EGF receptor activation. *Am. J. Physiol. Renal Physiol.* **290**(5):F1202–F1212.

EMEA (European Medicines Agency) 2008. *Reflection Paper on the Adaptogenic Concept*. EMEA. HMPC: London. **EMEA/HMPC/102655/2007**.

Geback T, Schulz MM, Koumoutsakos P, Detmar M. 2009. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. *Biotechniques* **46**(4):265–274.
Hao S, He W, Li Y, Ding H, Hou Y, Nie J, Hou FF, Kahn M, Liu Y. 2011. Targeted inhibition of beta-catenin/CBP signaling ameliorates renal interstitial fibrosis. *J. Am. Soc. Nephrol.* **22** (9):1642–1653.
Havasi A, Borkan SC. 2011. Apoptosis and acute kidney injury. *Kidney Int.* **80**:29–40.
He W, Kang YS, Dai C, Liu Y. 2011. Blockade of Wnt/beta-catenin signaling by paricalcitol ameliorates proteinuria and kidney injury. *J. Am. Soc. Nephrol.* **22**:90–103.
Hilal G, Albert C, Vallée M. 2005. Mécanismes impliqués dans la néphrotoxicité. *Ann. Biol. Clin. Qué.* **42**(3):29–35.
Jamieson ER, Lippard SJ. 1999. Structure, recognition, and processing of cisplatin-DNA adducts. *Chem. Rev.* **99**(9):2467–2498.
Jeong SI, Kim SJ, Kwon TH, Yu KY, Kim SY. 2012. Schizandrin prevents damage of murine mesangial cells via blocking NADPH oxidase-induced ROS signaling in high glucose. *Food Chem. Toxicol.* **50**(3–4): 1045–1053.
Lee SB, Kalluri R. 2010. Mechanistic connection between inflammation and fibrosis. *Kidney Int.* **78** (Suppl. 119): S22–S26.
Lee TH, Jung CH, Lee DH. 2012. Neuroprotective effects of Schisandrin B against transient focal cerebral ischemia in Sprague–Dawley rats. *Food Chem. Toxicol.* **50**(12):4239–4245.
Liu Y. 2010. New insights into epithelial-mesenchymal transition in kidney fibrosis. *J. Am. Soc. Nephrol.* **21**(2):212–222.
Megyesi J, Andrade L, Vieira JM, Jr, Safirstein RL, Price PM. 2002. Coordination of the cell cycle is an important determinant of the syndrome of acute renal failure. *Am. J. Physiol. Renal Physiol.* **283**(4):F810–816.
Nagwani S, Tripathi YB. 2010. Amelioration of cisplatin induced nephrotoxicity by PTY: a herbal preparation. *Food Chem. Toxicol.* **48**(8–9): 2253–2258.
Nitha B, Janardhanan KK. 2008. Aqueous-ethanolic extract of morel mushroom mycelium Morchella esculenta, protects cisplatin and gentamicin induced nephrotoxicity in mice. *Food Chem. Toxicol.* **46**(9): 3193–3199.
Pabla N, Dong Z. 2008. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney Int.* **73**(9):994–1007.
Panossian A, Wikman G. 2008. Pharmacology of Schisandra chinensis Bail.: an overview of Russian research and uses in medicine. *J. Ethnopharmacol.*. **118**(2):183–212.
Park EJ, Chun JN, Kim SH, Kim CY, Lee HJ, Kim HK, Park JK, Lee SW, So I, Jeon JH. 2012. Schisandrin B suppresses TGFbeta1 signaling by inhibiting Smad2/3 and MAPK pathways. *Biochem. Pharmacol.* **83**(3): 378–384.
Pozdzik AA, Salmon IJ, Debelle FD, Decaestecker C, Van den Branden C, Verbeelen D, Deschodt-Lanckman MM, Vanherweghem JL, Nortier JL. 2008. Aristolochic acid induces proximal tubule apoptosis and epithelial to mesenchymal transformation. *Kidney Int.* **73**(5):595–607.
Price PM, Safirstein RL, Megyesi J. 2009. The cell cycle and acute kidney injury. *Kidney Int.* **76**(6):604–613.
Reagan-Shaw S, Nihal M, Ahmad N. 2008. Dose translation from animal to human studies revisited. *FASEB J.* **22**(3):659–661.
Ryan MJ, Johnson G, Kirk J, Fuerstenberg SM, Zager RA, Torok-Storb B. 1994. HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int.* **45**:48–57.
dos Santos NA, Carvalho Rodrigues MA, Martins NM, dos Santos AC. 2012. Cisplatin-induced nephrotoxicity and targets of nephroprotection: an update. *Arch. Toxicol.* **86**(8):1233–1250.
Sohn SH, Lee EY, Lee JH, Kim Y, Shin M, Hong M, Bae H. 2009a. Screening of herbal medicines for recovery of acetaminophen-induced nephrotoxicity. *Environ. Toxicol. Pharmacol.* **27**(2):225–230.
Sohn SH, Lee H, Nam JY, Kim SH, Jung HJ, Kim Y, Shin M, Hong M, Bae H. 2009b. Screening of herbal medicines for the recovery of cisplatin-induced nephrotoxicity. *Environ. Toxicol. Pharmacol.* **28**(2):206–212.
Stacchiotti A, Li Volti G, Lavazza A, Schena I, Aleo MF, Rodella LF, Rezzani R. 2011. Different role of Schisandrin B on mercury-induced renal damage in vivo and in vitro. *Toxicology* **286**(1–3):48–57.
Urruticoechea A, Smith IE, Dowsett M. 2005. Proliferation marker Ki-67 in early breast cancer. *J. Clin. Oncol.* **23**(28):7212–7220.
Valcu M, Valcu CM. 2011. Data transformation practices in biomedical sciences. *Nat. Methods* **8**(2):104–105.
Wojcikowski K, Johnson DW, Gobe G. 2004. Medicinal herbal extracts – renal friend or foe? Part two: herbal extracts with potential renal benefits. *Nephrology* **9**(6):400–405.
Wynn TA. 2007. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J. Clin. Invest.* **117**(3):524–529.
Wynn TA. 2010. Fibrosis under arrest. *Nat. Med.* **16**(5):523–525.
Xu M, Wang G, Xie H, Huang Q, Wang W, Jia Y. 2008. Pharmacokinetic comparisons of schizandrin after oral administration of schizandrin monomer, *Fructus Schisandrae aqueous extract and Sheng-Mai-San to rats. J. Ethnopharmacol.* **115**(3):483–488.
Yamamoto E, Izawa T, Juniantito V, Kuwamura M, Sugiura K, Takeuchi T, Yamate J. 2010. Involvement of endogenous prostaglandin E2 in tubular epithelial regeneration through inhibition of apoptosis and epithelial-mesenchymal transition in cisplatin-induced rat renal lesions. *Histol. Histopathol.* **25**(8):995–1007.
Yang L, Besschetnova TY, Brooks CR, Shah JV, Bonventre JV. 2010. Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury. *Nat. Med.* **16**(5):535–543, 531p following 143.
Yao X, Panichpisal K, Kurtzman N, Nugent K. 2007. Cisplatin nephrotoxicity: a review. *Am. J. Med. Sci.* **334**(2):115–124.
Yoo JH, Lee HJ, Kang K, Jho EH, Kim CY, Baturen D, Tunsag J, Nho CW. 2010. Lignans inhibit cell growth via regulation of Wnt/beta-catenin signaling. *Food Chem. Toxicol.* **48** (8–9):2247–2252.
Zeisberg M, Neilson EG. 2009. Biomarkers for epithelial-mesenchymal transitions. *J. Clin. Invest.* **119**(6):1429–1437.
Zeisberg M, Neilson EG. 2010. Mechanisms of tubulointerstitial fibrosis. *J. Am. Soc. Nephrol.* **21**(11):1819–1834.
Zhu S, Wang Y, Chen M, Jin J, Qiu Y, Huang M, Huang Z. 2012. Protective effect of schisandrin B against cyclosporine A-induced nephrotoxicity in vitro and in vivo. *Am. J. Chin. Med.* **40**(3):551–566.

