Protective effect of pinostrobin against gentamicin-induced toxicity in human renal proximal tubular cells

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Abstract

Pinostrobin, an active ingredient found in aromatic rhizoids of the Zingiberaceae family, has shown several pharmacological activities, including anti-inflammation and anti-oxidation. This study aimed to investigate the potential of pinostrobin to prevent the toxicity of renal tubular cells caused by gentamicin. Gentamicin causes a decrease in the cell viability of human renal proximal tubule cells (RPTEC/TERT1) in a concentration-dependent manner. Our findings showed that pre-incubation with pinostrobin significantly attenuated the toxicity induced by gentamicin in RPTEC/TERT1 cells. The mechanism underlying the nephroprotective effect of pinostrobin was investigated. The protein expression of TIM-1, a kidney injury marker, was increased upon gentamicin treatment and was diminished by pinostrobin co-administration. In addition, we also showed that after 48 h of incubation, pinostrobin inhibited the uptake of OCT1 and OCT2 substrate (3H-MPP+) in CHO-K1 cells, overexpressing human OCTs, suggesting a possible mechanism of pinostrobin interfering with gentamicin accumulation in renal proximal tubule cells where OCTs were highly expressed. In summary, administering pinostrobin prior to gentamicin exposure could potentially reduce the nephrotoxicity caused by gentamicin.

Keywords

apoptosis, gentamicin, nephrotoxicity, pinostrobin, renal proximal tubule

Introduction

Gentamicin is an effective and inexpensive aminoglycoside antibiotic commonly used to treat severe gram-negative bacteria such as staphylococcal and enterococcal infections. However, the use of gentamicin is limited due to its side effects, which include nephrotoxicity and ototoxicity. Gentamicin-induced nephrotoxicity mechanisms have been shown to involve tubular, glomerular, and vascular effects. It causes apoptosis in renal proximal tubule cells, increased serum creatinine, and a decreased filtration rate (Martinez-Salgado et al. 2007). Furthermore, previous studies showed that gentamicin-induced nephrotoxicity may involve complex processes, including inflammation and oxidative stress (Lakshmi and Sudhakar 2010; Randjelovic et al. 2017). Several studies have attempted to prevent or reduce the nephrotoxicity of gentamicin, especially using natural products such as curcumin, propolis, and galangal (Aldahmash et al. 2016; Promsan et al. 2016; Laorodphun et al. 2022).

Herbal medicines have increasingly gained popularity as an alternative to the prevention and treatment of illness. They are believed to be effective and safe, with little or no side effects. However, the number of reports on interactions between prescribed drugs and herbal medicines has increased. Therefore,
it is crucial to scrutinize the mechanisms of action of these herbs as well as their interactions with medicines. Pinostrobin is found in the aromatic rhizoids of galangal (*Boesenbergia rotunda*) and black galangal (*Kaempferia parviflora*), which are widely used in East Asian cuisine. These plants are also used as traditional medicines to help ease flatulence and inflammation and as a natural aphrodisiac (Chaturapanich et al. 2012). Pharmacological activities of pinostrobin, including antiulcer, antiviral, antimicrobial, and antioxidant activities, have been reported (Fahey and Stephenson 2002; Abdelwahab et al. 2011; Wang et al. 2023). The anti-inflammatory effect of pinostrobin has been studied in vivo and in vitro (Patel and Bhutani 2014; Taechowisan et al. 2014).

Interestingly, the structurally related flavonoid, pinocembrin, showed a protective effect against gentamicin through anti-oxidant and anti-apoptotic activities in rats (Promsan et al. 2016). Thus, it is possible that pinostrobin could also attenuate the toxic effect of gentamicin. Taken together, we investigate the protective activities of pinostrobin in gentamicin-induced toxicity in renal proximal tubular cells.

**Materials and methods**

**Materials**

Pinostrobin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and gentamicin sulfate were purchased from Sigma-Aldrich (USA). Rabbit Tim-1 antibody was purchased from Abcam (UK). Goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was purchased from KPL International Limited (India). Enhanced chemiluminescence (ECL) substrate was purchased from Thermo Fisher Scientific (USA). All the other chemicals were purchased from standard sources.

**Cell cultures**

Human renal proximal tubule cells (RPTEC/TERT1) and Chinese hamster ovary (CHO)-K1 cells were obtained from ATCC. RPTEC/TERT1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F-12 medium supplemented with 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL sodium selenite, 100 units/mL penicillin, 100 µg/mL streptomycin, 10 ng/mL epithelial growth factor, and 36 ng/mL hydrocortisone. CHO-K1 cells were cultured in Kaighn’s Modification of Ham’s F-12 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained in a humidified atmosphere of 5% CO₂ and 95% O₂ at 37 °C.

**Overexpression of hOCT1 and hOCT2 in CHO-K1 cells**

The CHO-K1 cells overexpressing human organic cation transporter 1 (hOCT1) and hOCT2 were generated by transfection of parent CHO-K1 cells with hOCT1 or hOCT2 plasmids (OriGene Technologies, Inc., MD, USA). After transfection, the hOCT1-CHO-K1 cells and hOCT2-CHO-K1 cells were selected by complete media containing 3 mg/mL of G418. The CHO-K1 and hOCT2-CHO-K1 cells were maintained in complete media with 1 mg/mL G418 for 8 weeks before usage.

**MTT cell viability assay**

RPTEC/TERT1 cells were seeded at 5×10⁴ cells/well in a 96-well plate. After 24 h, the culture medium was replaced with fresh media containing test compounds or DMSO as a vehicle control. The final concentration of DMSO used with the cells was less than 0.1%. The cells were incubated for a period of treatment prior to an addition of 10 µl MTT solution (Sigma Aldrich; 5 mg/mL in PBS) and further incubated for 2–4 h at 37 °C. Subsequently, the media with MTT were removed, and 100 µl of DMSO was added to dissolve the formazan crystal produced by live cell mitochondria. The absorbance was measured at 550 nm using a spectrophotometer (Perkin-Elmer VICTOR Nivo) and used to calculate % cell viability compared with the control.

**Western immunoblotting**

RPTEC/TERT1 cells were grown in 6-well plates until they reached 80% confluence. Fresh growth-factor-free medium containing 5 and 10 µg/mL of pinostrobin was then added to the cells. After 24 h, 2 mg/mL of gentamicin was added and further incubated for another 24 h. Subsequently, cells were harvested and lysed with lysis buffer [in mM: 50 Tris HCl, pH 7.4, 150 NaCl, 0.5% Na deoxycholate, 0.1% SDS, and 1% Triton X-100] containing protease inhibitors (Thermo Fisher Scientific). The lysates were then centrifuged at 12,000 xg for 10 min at 4 °C (Haraes Biofuge 15R). The supernatant was collected, and protein concentrations were measured using a BCA assay (Thermo Scientific). Thereafter, total protein lysates (25 µg) were separated on SDS-PAGE using a 12% acrylamide gel. The proteins were then transferred to PVDF membranes and, consequently, blocked for non-specific binding with 5% skimmed milk in Tris buffer saline, containing 0.1% tween 20 (TBS-T) at room temperature for 1 h. Next, the blot was probed with Tim-1 antibody (1:1,000) overnight with gentle shaking at 4 °C. The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (1:10,000) in 5% skimmed milk/TBS-T, which was probed for 1 h at room temperature. The protein bands were detected using an enhanced chemiluminescence (ECL) substrate.

**Uptake assay**

hOCT1 and hOCT2 overexpressed CHO-K1 cells were cultured in a 24-well plate at a density of 1×10⁴ cells/mL until confluent. Then, the cells were washed by replacing the media with Dulbecco’s modified phosphate buffer saline or DPBS (in mM: 137 NaCl, 3 KCl, 0.5 Na₂HPO₄, 7H₂O, 1 KH₂PO₄, 0.5 MgCl₂, 6H₂O, 5.6 D-glucose, pH 7.4) and incubated for 15 min. After the second wash, the cells were
incubated in DPBS buffer at 37 °C for 30 min, followed by buffer containing OCT substrates, 3H-MPP+, alone or with treatments for 1 min. Subsequently, the reaction was stopped by removing the buffer, and the cells were washed three times with ice-cold DPBS. The cells were then lysed overnight with a lysis solution containing 0.4 N NaOH and 10% SDS, followed by 100 µl of 1 N HCl to neutralize the lysates. The radioactivity was measured using a liquid scintillation beta counter. The OCT-mediated uptake of 3H-MPP+ was calculated and expressed as fmol/min/cm² of the confluent monolayer surface.

Data analysis

All data are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism software, Version 7.0. Significant differences among groups were compared using a one-way ANOVA, followed by Tukey’s comparison of pairs. A value of \( P < 0.05 \) was considered statistically significant.

Results

Concentration-dependent effect of gentamicin and pinostrobin on RPTEC/TERT1 cell viability

RPTEC/TERT1 cells were treated with gentamicin at various concentrations for 24 h, followed by measurement of cell viability using the MTT assay. Gentamicin-induced cytotoxicity was concentration-dependent. The data showed that gentamicin at 0.5 mg/mL did not significantly alter cell survival compared to vehicle control, whereas gentamicin at concentrations of 1 mg/mL significantly decreased the viability of the cells. An increase in the concentration of gentamicin produced a greater effect on cell viability (Fig. 1A). In addition, when the incubation time was extended to 48 h, the results were comparable with the 24 h incubation but had a slightly higher toxicity. However, exposing the cells to pinostrobin at 1.25–20 µg/mL for 24 and 48 h did not cause toxicity to the cells (Fig. 1B).

Effect of pinostrobin on expression of renal cell injury markers in gentamicin treatment

Next, we tested the effect of pinostrobin on the toxicity of renal tubular cells induced by gentamicin. RPTEC/TERT1 cells were treated with vehicle, pinostrobin (5 and 10 µg/mL) alone, gentamicin (2 mg/mL), or a combination of gentamicin and pinostrobin for 24 h. The results showed that gentamicin significantly reduced cell viability compared to the vehicle control. Co-treatment with pinostrobin did not show protection against cell death caused by gentamicin (Fig. 2A). Pretreatment of the cells with pinostrobin at 5 µg/mL for 4 h, followed by a 24-h treatment of gentamicin, did not prevent the toxicity of gentamicin. Interestingly, the increased duration of pinostrobin treatment for 24 h led to a decreased toxicity induced by gentamicin (Fig. 2B). Having observed the potential of the cytoprotective effect of pinostrobin, we tested whether growth factors in the culture medium were involved. The cells were treated with growth factor-free medium containing pinostrobin for 24 h, followed by 2 mg/mL of gentamicin for another 24 h. The result showed that pinostrobin could prevent gentamicin-induced cytotoxicity, and this effect did not contribute to growth factors in the culture medium (Fig. 2C).

Effect of pinostrobin on expression of renal cell injury markers in gentamicin treatment

To investigate the mechanism underlying the protective effect of pinostrobin, we determined its effect on the Tim-1 protein expression level, a marker of renal cell injury. The RPTEC/TERT1 cells were treated with vehicle, gentamicin (2 and 4 mg/mL), and gentamicin plus pinostrobin (50 µM) for 24 h. As shown in Fig. 3, the incubation of cells with gentamicin significantly increased the Tim-1 expression level compared with the vehicle control. Moreover, pre-incubation of pinostrobin for 24 h could attenuate the up-regulation of Tim-1 induced by gentamicin. Interestingly, gentamicin did not affect the expression level of Bcl-2 (data not shown).

Effect of pinostrobin on the drug transporter responsible for gentamicin uptake

Gentamicin is transported into the renal proximal tubular cells via OCTs (Gai et al. 2016). We tested whether the protective effect of pinostrobin on the drug transporter involved the uptake of gentamicin. Incubation of CHO-K1 cells overexpressing human OCT1 and OCT2 with buffer containing pinostrobin (10, 50, and 100 µM) and 3H-MPP+ for 5 min did not affect OCT1- and OCT2-mediated transport of 3H-MPP+. These results showed that pinostrobin did not interact with the transporters (Fig. 4A). However, when the cells were pre-incubated with pinostrobin for 48 h, the uptake of 3H-MPP+ was inhibited in a concentration-dependent manner (Fig. 4B).

Discussion

Gentamicin is an effective aminoglycoside that is active against a wide range of gram-negative bacteria. However, its side effects, including nephrotoxicity, have limited the use of this antibiotic. Co-administration of a nephroprotective agent with gentamicin is intended to lower the risk of drug-induced toxicity and to achieve the full potential of the medicine. Therefore, our study aimed to investigate the potential and mechanism of pinostrobin as a supportive agent with gentamicin is intended to lower the risk of its side effects, including nephrotoxicity, have limited the full potential of the medicine. Therefore, our study aimed to investigate the potential and mechanism of pinostrobin as a supportive agent with gentamicin.
Figure 1. Viability of RPTEC/TERT1 cells upon treatment with gentamicin (A) and pinostrobin (B) for 24 and 48 h. Each value represents the mean ± SD; n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with the control (Ctrl).

Figure 2. Effect of pinostrobin on gentamicin-induced toxicity of RPTEC/TERT1 cells. A. Cells were co-treated with gentamicin at 2 mg/mL and pinostrobin (5 or 10 µg/mL) for 24 h; B. Cells were treated with gentamicin at 2 mg/mL for 24 h following pre-incubation of pinostrobin at 5 µg/mL for 4 and 24 h; C. Cells were pre-incubated with growth factor-free media containing pinostrobin (5 µg/mL) for 24 h, followed by treatment with gentamicin (2 mg/mL) for 24 h. Each value represents the mean ± SD; n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, compared with control, #P < 0.05.
was reported that gentamicin caused inflammation and renal tubular injury in animal models, which were mediated by superoxide anion, hydrogen peroxide, NF-κB, iNOS, TNF-alpha, and eNOS (Quiros et al. 2011; Abd-Elhamid et al. 2018). A previous study showed the anti-inflammatory activity of pinostrobin as it inhibited TNF-α and IL-1 production in LPS-stimulated cell lines and rats (Patel and Bhutani 2014). In our study, the co-treatment of pinostrobin and gentamicin did not protect cells from gentamicin-induced toxicity. However, pretreatment with pinostrobin 24 h prior to gentamicin could lower gentamicin toxicity. This effect was not observed when the time of pre-incubation was reduced to 4 h. The different results with treatment duration (4 vs. 24 h) suggest that the mechanism is less likely mediated by pre-synthesized proteins but probably involves a change in protein expres-

**Figure 3.** Representative immunoblotting (A) and densitometry data (B) showing protein expression of TIM-1 in RPTEC/TERT1 cells. GAPDH was used as a loading control. Data are expressed as the mean of percent control ± SD; n =3. *P<0.05, **P< 0.01.

**Figure 4.** Effect of pinostrobin on the transport function of OCT1 and OCT2 in CHO-K1 cells overexpressing hOCT1 and hOCT2. A. The cells were incubated for 5 min with buffer containing 'H-MPP' and pinostrobin (10–50 µM); B. The cells were incubated with buffer containing 'H-MPP' for 5 min, following pre-incubation with media containing pinostrobin (10–50 µM) for 48 h. Each value represents the mean ± SD; n = 3. **P<0.01, ****P< 0.0001 compared with control.
tion. One of our speculations was that the effect may result from growth factors supplemented in the culture medium. Nevertheless, it was ruled out because the results were not changed when growth factors were eliminated.

TIM-1, also called KIM-1 or kidney injury molecule-1, is a membrane receptor that is upregulated in the proximal tubule epithelium of injured kidneys. It is commonly used as a kidney cell injury marker after exposure to nephrotoxic agents. In our study, TIM-1 protein expression was increased in the proximal tubule epithelial cells upon gentamicin treatment and decreased when pinostrobin was present. This finding is consistent with the study in Sprague-Dawley rats that received a 30-day treatment of cadmium (Ijaz et al. 2023). In their experiments, cadmium caused an increase in several inflammatory mediators as well as TIM-1, which were reduced when pinostrobin was co-administered, suggesting nephroprotective effects. Furthermore, the activity of COX-2, an important inflammatory player, was inhibited by pinostrobin in these rats. In our study, however, the expression of COX-2 protein did not alter in cells co-treated with gentamicin and pinostrobin (data not shown). A previous study showed that the combined use of gentamicin and a selective COX-2 inhibitor, rofecoxib, did not improve rat renal function, whereas a nonselective COX inhibitor, indomethacin, caused an increased kidney injury in gentamicin-treated animals (Hosaka et al. 2004). Hence, COX-2 might not be a major contributor to gentamicin-induced kidney injury.

Next, we explored the possibility of apoptosis, a biological process triggered by cellular damage. We found that the protein expression of Bcl-2, an anti-apoptotic marker, in cells pre-treated with pinostrobin did not change compared to those treated with gentamicin or control (data not shown). Moreover, Worakajit et al. (2021) showed that human renal proximal tubule cells were protected when pinocembrin was co-administered with colistin through anti-apoptotic and anti-oxidative mechanisms (Worakajit et al. 2021). Colistin decreased anti-apoptotic protein Bcl-2 expression in RPTEC/TERT1 cells, which was up-regulated when supplemented with pinocembrin.

In addition, Promsan et al. showed that the related flavonoid pinocembrin protected against gentamicin-mediated reduction of the anti-apoptotic protein Bcl-XL in the renal cortical tissues of rats. The protective mechanisms of pinocembrin were suggested to be through anti-oxidative and anti-inflammatory activities (Promsan et al. 2016). It is noteworthy that the structure of pinostrobin is different from that of pinocembrin only at carbon position 7, where the methoxy group of pinostrobin is replaced by a hydroxy group of pinocembrin. Therefore, the structural difference could contribute to the different activities of these two compounds.

Organic cation transporters (OCT) 1 and 2 play a major role in transporting drugs or xenobiotics in several organs, including the kidneys. Specifically, they affect the efficacy and toxicity of many pharmacological agents. Our study showed that pinostrobin inhibited OCT1 and OCT2 uptake activity at 48 h. However, in the short-term exposure, the uptake of OCT substrate did not change, suggesting that pinostrobin did not directly interact with the transporters. Thus, this evidence implies that, when given together, pinostrobin reduced gentamicin uptake and lessened the toxicity of gentamicin in renal cells. In addition, gentamicin accumulation in the kidney is related to the increased expression of OCT2 (Gai et al. 2016). Therefore, another possible mechanism is that pinostrobin may decrease OCT expression in these cells and reduce the gentamicin nephrotoxicity effect.

**Conclusion**

Taken together, this study shows that pinostrobin protects renal tubule cells from gentamicin-induced toxicity. The mechanism underlying the protective effect of pinostrobin was also explored. We showed that it may partly involve OCT functions. Although the precise mechanisms were not completely elucidated, our findings support the potential benefit of galangal and black galangal ingredients in preventing the toxicity caused by gentamicin.

**References**


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