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Design, synthesis and evaluation of PD176252 analogues for ameliorating cisplatin-induced nephrotoxicity†

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Cisplatin is a clinical chemotherapy drug for cancers; however, its remarkably high kidney toxicity and other toxicities pose a danger to patients. As the small molecule inhibitor of GRPR, PD176252 can inhibit the growth and proliferation of various cancer cells, but the characteristics of high toxicity and poor water solubility has limited its use as a drug. When we studied PD176252 for the reduction of toxicity of cisplatin, we modified its structure to synthesize 16 analogues. Surprisingly, the analogues showed reduced cisplatin-induced renal toxicity, and unlike PD176252, the analogues **5d** and **5m** were almost non-toxic to the normal HK2 cells. Furthermore, the analogue **5d** and PD176252 were subjected to cisplatin-induced inflammatory response *in vitro*. The results showed that **5d** was able to better prevent this condition by effectively inhibiting its inflammatory response. Thus, this study will help in clinically reducing the side effects of cisplatin.

Introduction

Cisplatin, one of the main pillars of clinical chemotherapy drugs, is widely used to treat many kinds of human cancers, including lung, head and neck, breast, bladder, ovarian, and prostate cancers.[1,2] The complications of cisplatin have been gradually discovered. In particular, kidney toxicity has been a main side effect in cancer patients undergoing cisplatin therapy.[3,4] Furthermore, a high dose or prolonged use of cisplatin is prone to cause kidney failure[5–7] and even death.[8,9]

At present, it has been found that cisplatin-induced kidney inflammation is one of the important factors of renal toxicity.[10–12] In addition, the inhibition of this inflammatory response could significantly reduce kidney toxicity.[13–15] Researchers have demonstrated that monocyte chemotactic protein (MCP-1), inflammatory cytokine (IL-6), and proinflammatory cytokine TNF-α[16] play an important role in renal toxicity induced by cisplatin. Thus, they can be used as important markers for the diagnosis of renal toxicity *in vitro* and *in vivo*.[17–19]

Bombesin/gastrin-releasing peptide receptor (GRPR),[20–23] which is a G protein-coupled receptor,[24] is involved in the regulation of the release of inflammatory factors and plays a role in inflammatory diseases.[22,25] In recent studies, GRPR inhibitors have been known to ameliorate some diseases by inhibiting the inflammatory response.[26,27]

PD176252 is a nonpeptide GRPR antagonist[28,29] that can inhibit the growth and proliferation of various cancer cells, including head and neck cancer[30] and lung cancer,[21,31] but it was not studied in the field of inflammatory diseases. In this study, PD176252 has been used as a subject to ameliorate cisplatin-induced nephrotoxicity. The result was pleasing with PD176252 effectively inhibiting the cisplatin-induced apoptosis of human renal proximal tubular cells (HK-2 cells). However, the characteristics of high cytotoxicity and poor water solubility of PD176252 have limited its application and development as a medicine.

Subsequently, a series of PD176252 analogues were designed and synthesized. 16 analogues were efficiently synthesized and evaluated for their activity of inhibition of cisplatin-induced apoptosis of human renal proximal tubular cells. To our delight, many of the analogues could reduce cisplatin-induced renal toxicity, and the more active analogues did not cause toxicity to the normal cells in HK2 cells. In further studies, analogues **5d**, **5m**, and PD176252 were

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studied as compounds for the inhibition of cisplatin-induced inflammatory responses in HK2 cells *in vitro*.

Results and discussion

Design and chemistry

As shown in Fig. 1, the PD176252 structure consisted of three fragments. In order to reduce the cytotoxicity and improve water solubility, the left part was replaced by phenylacetic acid derivatives, which have commonly been used as fragments in various lead compounds[32] and drugs with anti-inflammatory activity such as benzylpenicillin and diclofenac sodium. Furthermore, natural amino acids containing L-phenylalanine, L-tyrosine or L-tryptophan were inserted as an intermediate part. Last but not the least, the unique structure of the morpholine ring and imidazole ring with the desired electron-rich feature is conducive to derivatives that readily bind to receptors and various enzymes in biological systems *via* a variety of weak interactions and exhibit a wide range of biological activities.[33,34] The structure of the compounds used in this study is shown in Table 1.

The procedure for the synthesis of the target compounds **5a–p** is illustrated in Scheme 1. Phenylacetic acid derivatives and the amino acid methyl ester hydrochloride were reacted to form chemical intermediates, and the intermediates were subsequently hydrolyzed. Then, the compounds **4a–h** and 2-morpholinoethan-1-amine (or 2-(1*H*-imidazol-1-yl)ethan-1-amine) were converted to the target compounds **5a–p** (Table 1). The results obtained confirm that the synthesis route was fast and efficient. The synthesis route of the compounds used in this study is shown in Scheme 1.

Table 1 The structure of the compounds

Compound	R_1	R_2	R_3
5a	NO_2	Phenyl	Imidazole
5b	NO_2	3-Indolyl	Imidazole
5c	CH_3	*p*-Hydroxyphenyl	Imidazole
5d	CH_3	Phenyl	Imidazole
5e	F	*p*-Hydroxyphenyl	Imidazole
5f	F	Phenyl	Imidazole
5g	F	3-Indolyl	Imidazole
5h	CF_3	3-Indolyl	Imidazole
5i	NO_2	Phenyl	Morpholine
5j	NO_2	3-Indolyl	Morpholine
5k	CH_3	*p*-Hydroxyphenyl	Morpholine
5l	CH_3	Phenyl	Morpholine
5m	F	*p*-Hydroxyphenyl	Morpholine
5n	F	Phenyl	Morpholine
5o	F	3-Indolyl	Morpholine
5p	CF_3	3-Indolyl	Morpholine

Activity of ameliorating cisplatin-induced nephrotoxicity

The effect of the synthesized compounds on the cell viability of HK2 cells was evaluated by the MTT assay. The HK2 cells were treated with cisplatin alone or be treated with a combination of the compound and cisplatin. The results are shown in Fig. 2. The lethality of cisplatin was detected in the normal HK2 cells; nevertheless, a majority of analogues synthesized in this work showed an inhibitory effect on cisplatin-induced cell death. In particular, **5d** and **5m** obviously enhanced the survival rate compared with PD176252 and increased it by more than 40% and 30%, respectively, compared with the control group of cisplatin. A dose–response study of the analogues **5d** and **5m**, which was performed on HK2 cells, further demonstrated that the analogues exhibited more

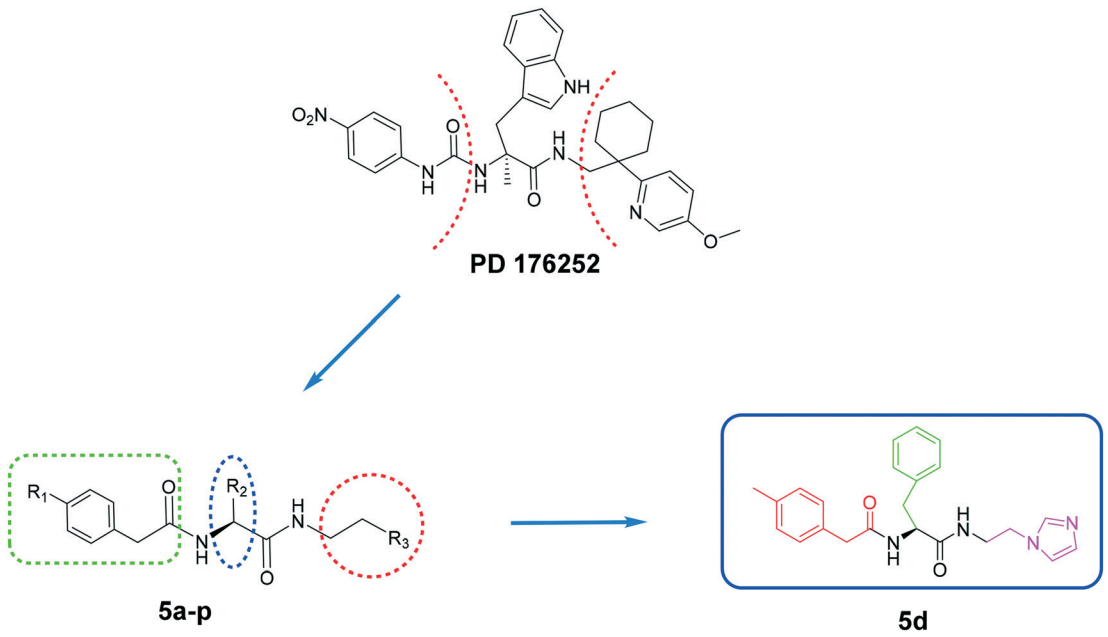
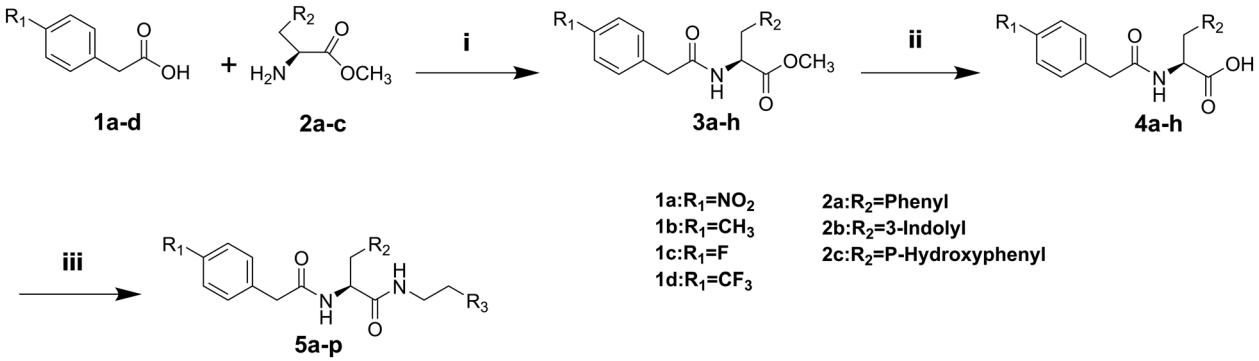


Fig. 1 The general design strategy.



Scheme 1 Synthesis of compounds **5a**–**p**. Reagents and conditions: (i) EDCI, HoBt, TEA, DCM, rt., 24 h; (ii) Na_2CO_3/CH_3OH, 3–5 h; HCl/H_2O; (iii) EDCI, HoBt, TEA, DCM, rt., 24 h.

powerful potency than PD176252 and maintained the level of protection in cells treated with cisplatin in a concentration-dependent manner (Fig. 3).

It is not hard to find that the methyl group, as an electron-donating group, gives better protection. However, the analogues become more active after the indole ring is replaced by a benzene ring or *p*-hydroxyphenyl moiety. Moreover, good results can be obtained while using 1-ethyl-1*H*-imidazole or 4-ethylmorpholine, both of which are simple and facile to obtain, instead of the rightmost fragment of PD176252.

Cytotoxicity assay

During the discovery of a drug or a lead compound, there is a high safety requirement for targets; therefore, this protection must be considered. In this case, the effect of different concentrations of **5d**, **5m** and PD176252 on the viability of HK2 cells by the MTT assay was used to study cell cytotoxicity (Fig. 4). PD176252 reduced the viability of HK2 cells in a dose-dependent manner, while **5d** and **5m** did not show any significant cytotoxicity. This result was obtained by using a low concentration to about 60 times the concentration of the dosage. In this condition, the analogues **5d** and **5m** modified by the PD176252 structure can indeed provide better safety than the control.

Anti-inflammatory activity by real-time PCR

Proinflammatory mediators (TNF-α, IL-6 and MCP-1) play important roles in cisplatin-induced cell injury. Their expression level in HK2 cells is a crucial indicator for the degree of

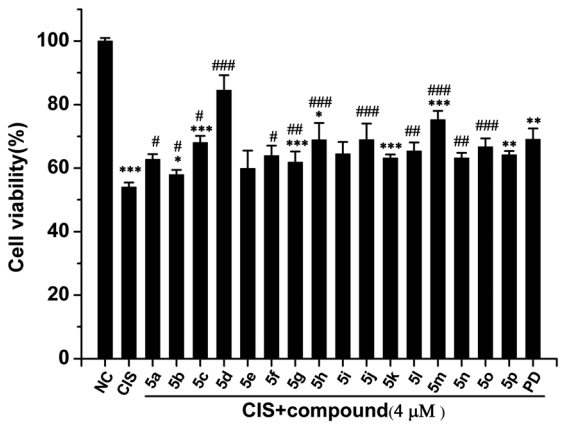


Fig. 2 Effect of compounds on cell viability with cisplatin treatment. Compounds and PD176252 (4 μM) restored cell viability in cisplatin-treated HK2 cells (ATCC, USA). HK2 cells were pre-cultured for 24 h, the cells were then treated with the indicated concentrations of compounds for 6 h, and then exposed to 20 μM cisplatin for 24 h. The results are shown as means ± SD (n = 3) for at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the cisplatin-stimulated group. CIS, cisplatin; compounds, **5a**–**p**; PD, PD 176252.

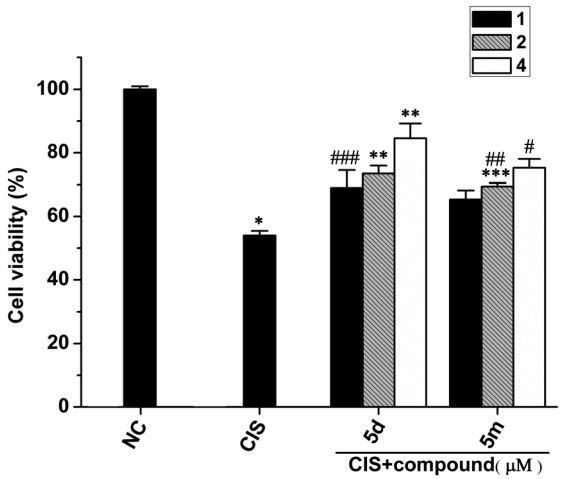


Fig. 3 Effect of different concentrations of **5d** or **5m** on cell viability with cisplatin treatment. **5d** and **5m** restored cell viability in cisplatin-treated HK2 cells (ATCC, USA). HK2 cells were pre-cultured for 24 h, the cells were then treated with the indicated concentrations of compounds for 6 h, and then exposed to 20 μM cisplatin for 24 h. The results are shown as means ± SD (n = 3) of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with cisplatin-stimulated group. CIS, cisplatin.

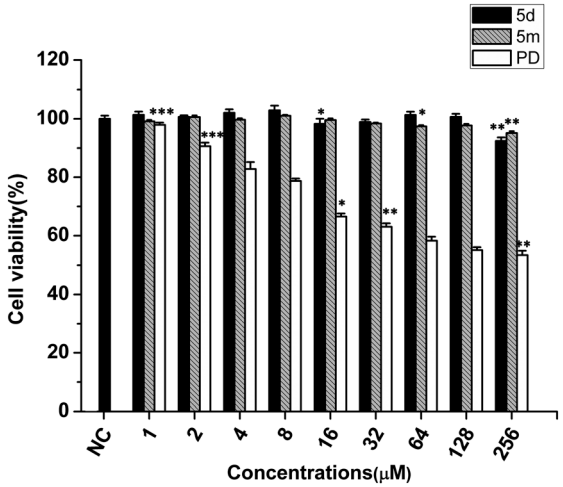


Fig. 4 Cytotoxicity of different concentrations of **5d**, **5m** or PD 176252 on HK2 cells. **5d** and **5m** reduce the viability of HK2 cells (ATCC, USA). HK2 cells were pre-cultured for 24 h, the cells were then treated with the indicated concentrations of compounds for 24 h. The results are shown as means ± SD (n = 3) of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control group.

nephrotoxicity.[11] To assess whether **5d**, **5m** and PD176252 reduced kidney damage by the inhibition of inflammatory responses, the mRNA expression of TNF-α, IL-6 and MCP-1 was examined in HK2 cells using real-time PCR. Cisplatin used alone significantly increased the expression of inflammatory response compared with normal saline-treated cells, which is shown in Fig. 5, and the phenomenon is the same as that reported in the literature.[35–38] Additionally, real-time PCR showed that **5d**, **5m** and PD176252 suppressed the levels of TNF-α, IL-6 and MCP-1. Perhaps, an important reason for the effective protection of **5d** and **5m** in HK2 cells with cisplatin is that the gene transcription and mRNA stabilization of proinflammatory mediators were interdicted.

Anti-inflammatory activity by enzyme-linked immuno-sorbent assay (ELISA)

The anti-inflammatory effect of the synthesized compounds was further evaluated by ELISA analysis. From the results in Fig. 6, it can be seen that **5d** and PD176252 showed

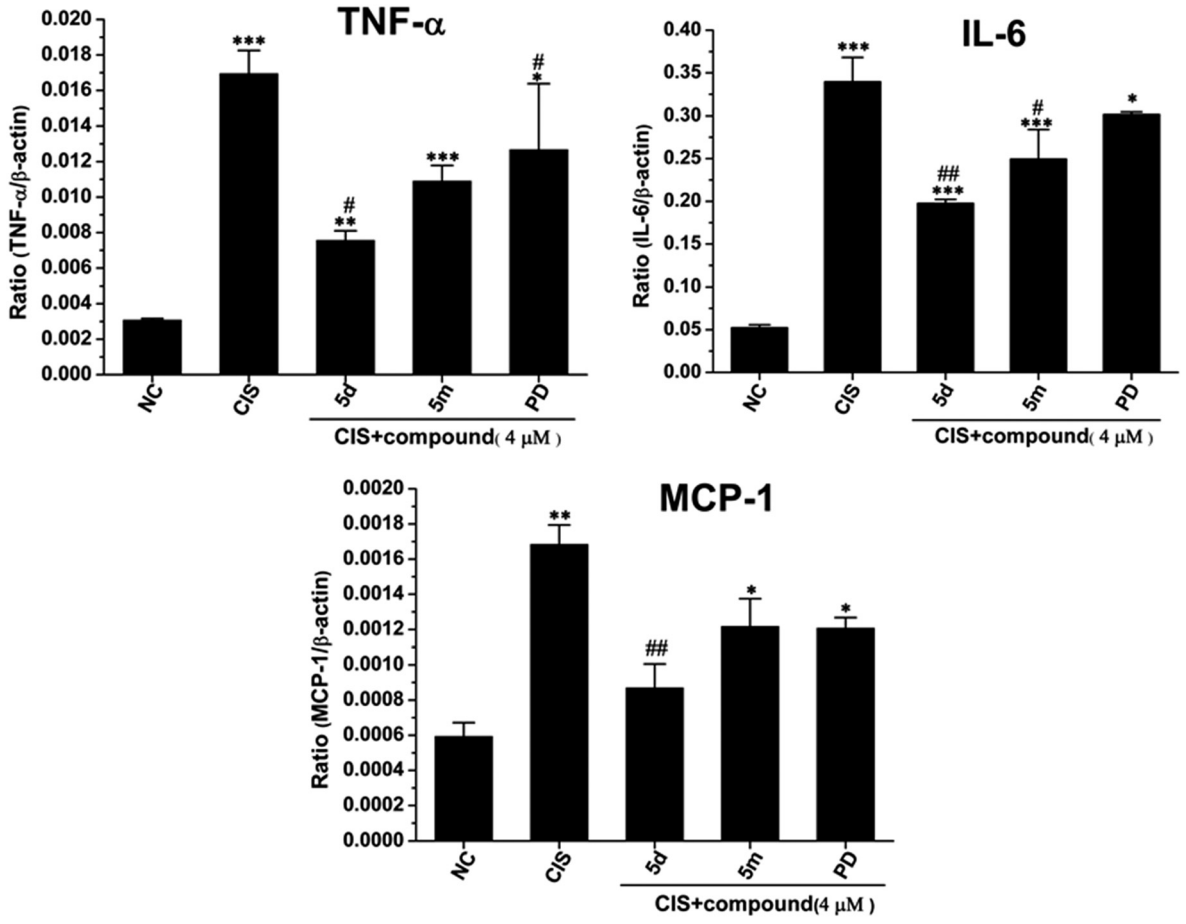


Fig. 5 Effect of **5d** or **5m** for inflammatory response in HK2 cells with cisplatin treatment. Real-time PCR in HK2 cells (ATCC, USA). The results demonstrate that the treatment of compounds reduced cisplatin-induced mRNA levels of TNF-α, IL-6, and MCP-1. Data represent the mean ± SEM for 3–4 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the cisplatin-treated group. CIS, cisplatin; PD, PD176252.

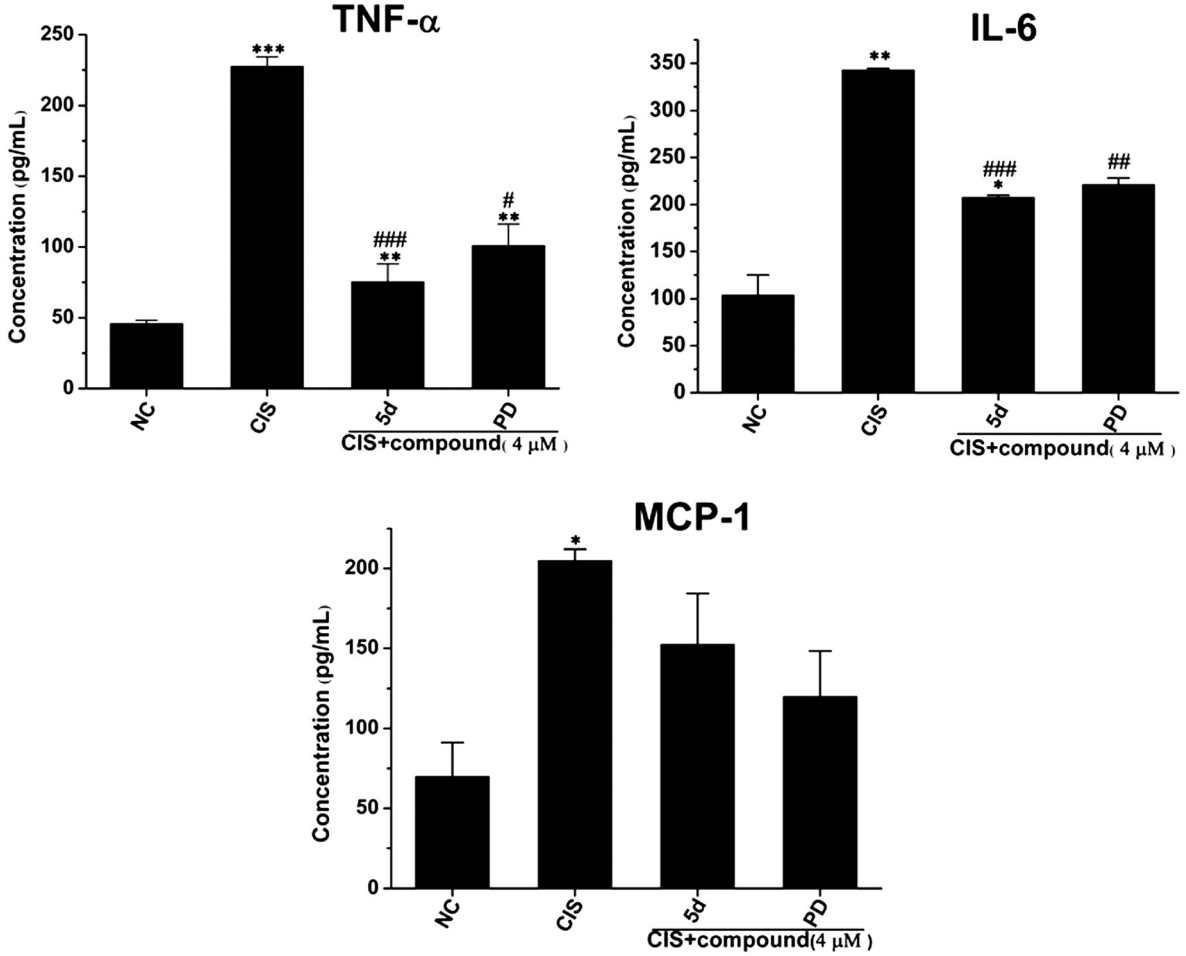


Fig. 6 Effect of **5d** or **5m** for inflammatory response in HK2 cells with cisplatin treatment. ELISA in HK2 cells (ATCC, USA). The results demonstrate that the treatment of compounds reduced cisplatin-induced mRNA levels of TNF-α, IL-6, and MCP-1. Data represent the mean ± SEM for 3–4 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to cisplatin-treated group. CIS, cisplatin; PD, PD 176252.

significant anti-inflammatory activity as evidenced by the decreased MCP-1, IL-6, and TNF-α expression levels. Moreover, **5d** was significantly effective than the PD agent in the abatement of TNF-α and MCP-1 levels.

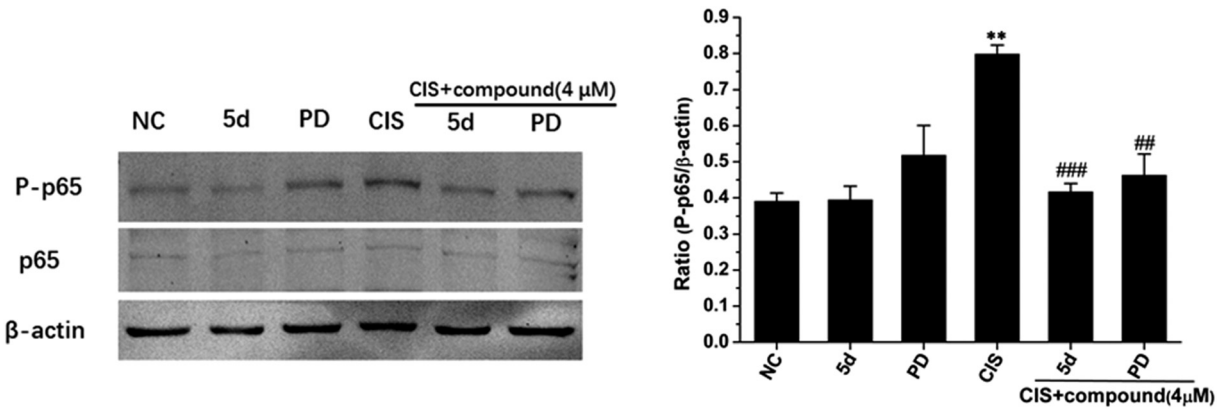


Fig. 7 Effect of **5d** and PD 176252 on cisplatin-induced phosphorylation of NF-κB p65. Results of western blot and quantitative data indicated that **5d** and PD176252 had an effect on the phosphorylation of p65 for cisplatin-treated HK2 cells (ATCC, USA). Data represent the mean ± SEM for 3–4 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the cisplatin-treated group. CIS, cisplatin; PD, PD 176252.

Compound 5d suppresses NF-κB activation

Nuclear factor κB (NF-κB) is a nuclear transcription factor that is critical for the production of proinflammatory cytokines.[39] The IκB protein is phosphorylated to turn on transcriptions of inflammatory genes when cisplatin stimulates the activation of NF-κB. Herein, in Fig. 7, the result of western blots and quantitative data showed that compound **5d** significantly blocked the cisplatin-induced phosphorylation of NF-κB p65. It is a possible mechanism by which compound **5d** alleviated the cisplatin-induced inflammation. It is noteworthy that PD176252 displayed a certain level of toxicity, *i.e.*, it gently induced p65 phosphorylation; however, PD176252 significantly reduced the phosphorylation level of NF-κB when cisplatin was added.

Conclusions

In the *in vitro* experiments, PD176252 analogues, **5d** and **5m**, possessed better activity in alleviating cisplatin-induced nephrotoxicity through screening for a higher activity. A dose–response study of **5d** and **5m** demonstrated that they exhibited effective protection in a concentration-dependent manner. It is worth noting that the synthesized analogues have very low cytotoxicity. We further identified that **5d** and **5m** suppressed the inflammatory response *via* a NF-κB-dependent mechanism. In conclusion, this study demonstrated that compound **5d** protects against cisplatin-induced cell injury and inflammation. It may be further explored as a preventive agent for cancer patients treated with cisplatin.

Conflicts of interest

The authors declare no conflict of interest associated with this manuscript.

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