Synthesis of Renoprotective Chalcone Analogues That Protect Against Cisplatin-induced Cytotoxicity in LLC-PK1 Cells

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Kidneys have major roles for balancing electrolytes, maintaining pH homeostasis, and controlling blood pressure. In particular, kidneys also remove excess of waste products, medication, and endogenous toxic waste. Therefore, kidneys are major targets for toxic effects of various chemical agents or medications. In a recent survey performed on critical patients, nephrotoxicity caused by medication is responsible for about 25% of hospital admissions for acute kidney injury (AKI).[1–4]

Cisplatin (*cis*-dichlorodiammine platinum(II), CDDP) is one of the most potent anticancer drugs for the treatment of epithelial malignancies including stomach, neck, head, bladder, testicular, ovarian, and lung cancers.[5] However, the utility of cisplatin is often limited, because of its toxicities inducing cytotoxicity, gastrotoxicity, myelosuppression, and allergic reactions.[6] Among toxicities of cisplatin, the main dose-limiting side effect is its severe nephrotoxicity.[7–10]

Cisplatin accumulates in the kidney to a greater degree via transport than in other organs.[10] Accumulated cisplatin in kidney cells activates the mitogen-activated protein kinases (MAPK) cascade and molecular responses that are involved in the typical stress response.[11,12] The mechanisms that lead to cisplatin-induced AKI involve oxidative stress, inflammation, and renal cell apoptosis.[3,13–15]

Several naturally occurring compounds that protect against cisplatin-induced kidney damage have been identified.[16,17] The kidney cell protection effects of sulforaphanes are mediated by inhibition of the apoptosis pathway via the c-Jun N-terminal kinase (JNK)-p53-caspase apoptotic cascade.[18] Ginsenosides from *Panax ginseng* protect against cisplatin-induced nephropathy in renal tubular epithelial cells.[19] In our previous research on the synthesis of bioactive constituents from natural resources, we found that chalcone derivatives had significant anti-inflammatory effects and renoprotective effect against cisplatin-induced damage in kidney cells.[20]

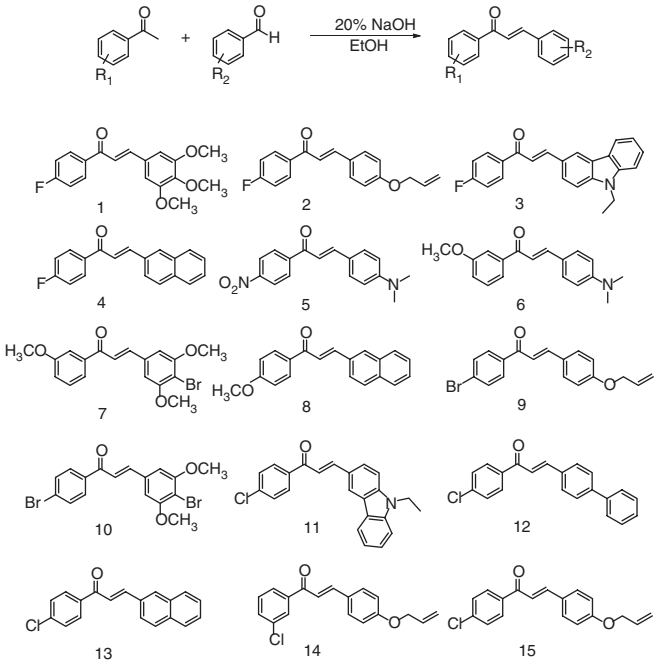
In our previous research, potent chalcone derivatives exhibited slight cytotoxicity at high concentration in LLC-PK1 cells.[20] Therefore, we tried to develop chalcone derivatives with no adversary effects at high concentration. We synthesized novel chalcone derivatives to evaluate their potential renoprotective effects. Chalcone analogues (**1–15**) were prepared by Claisen-Schmidt condensation reaction using various acetophenone and benzaldehyde in the presence of 20% NaOH and EtOH solutions (Scheme 1).[20–23]

A reaction mixture of benzaldehyde and acetophenone in EtOH treated with NaOH solution was refluxed overnight. The crude chalcone derivatives were purified by either silica gel column chromatography or recrystallization with EtOH.

The synthesized chalcone derivatives were analyzed using ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and liquid chromatography-mass spectrometry (LC-MS). The newly synthesized chalcone derivatives have methoxy-, halogenated-, or nitro-substitution groups that have electron-donating or electron-withdrawing properties. We assumed that the properties of these substitution groups could affect on the renoprotective and cytotoxic effects in LLC-PK1 cells.

To compare with potency chalcone derivatives, the renoprotective potential of the 15 synthesized chalcone derivatives (**1–15**) was evaluated using 25 μM of cisplatin-treated LLC-PK1 cells and followed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Figure 1, compounds (**1**, **4**, **8**, **11**, **14**, and **15**)

†† These authors contributed equally to this work.



Scheme 1. Synthesis of chalcone analogues and their structures.

ameliorated the reduced cell viability in a dose-dependent manner (Figure 1). However, chalcone derivatives (**7** and **10**) with 3-bromo-2,4-dimethoxy group showed no renoprotective effects and cytotoxicity in high concentration. Compounds (**1** and **8**) with 3,4,5-trimethoxy or naphthalene group showed relatively high potency in renoprotective effects. Chalcone derivatives (**11**, **14**, and **15**) with chloro-substitution group showed potent renoprotective effects among other substitution group. Notably, among 4-allyloxy phenyl derivative (**14** and **15**), except from bromo-substitution (**7**) which show lower potency, the derivatives with meta and para chloro-substitution exhibited relatively high potency in renoprotective effects compared to other derivatives with cabazolyl group (**11**) (Figure 1(a)).

Of these chalcone derivatives, compound **15** ameliorated cisplatin-induced nephrotoxicity to 92.0 and 98.3% of the control value at 50 and 125 μM, respectively. In particular, compound **15** exhibited no cytotoxicity at higher than 125 μM. In addition, microscopic image analysis shows that compound **15** treated cells have high renoprotective effects compared with cisplatin-treated cells (Figure 1(b)).

Consequently, we conclude that of the 15 synthesized chalcone derivatives, compound **15** is the most potent compound that protects against cisplatin-induced nephrotoxicity (Figure 1).

The renoprotective potential of synthetic or naturally occurring compounds is related to their antioxidant, antiapoptotic, and anti-inflammatory effects.[16,18,20] An image-based cytometric assay was performed to verify the antiapoptotic potential of compound **15**; Figure 2(a) presents representative images. Compound **15** significantly inhibited the percentage of apoptotic cells: compared with the cisplatin-treated group, significant inhibition of 30 and 85%

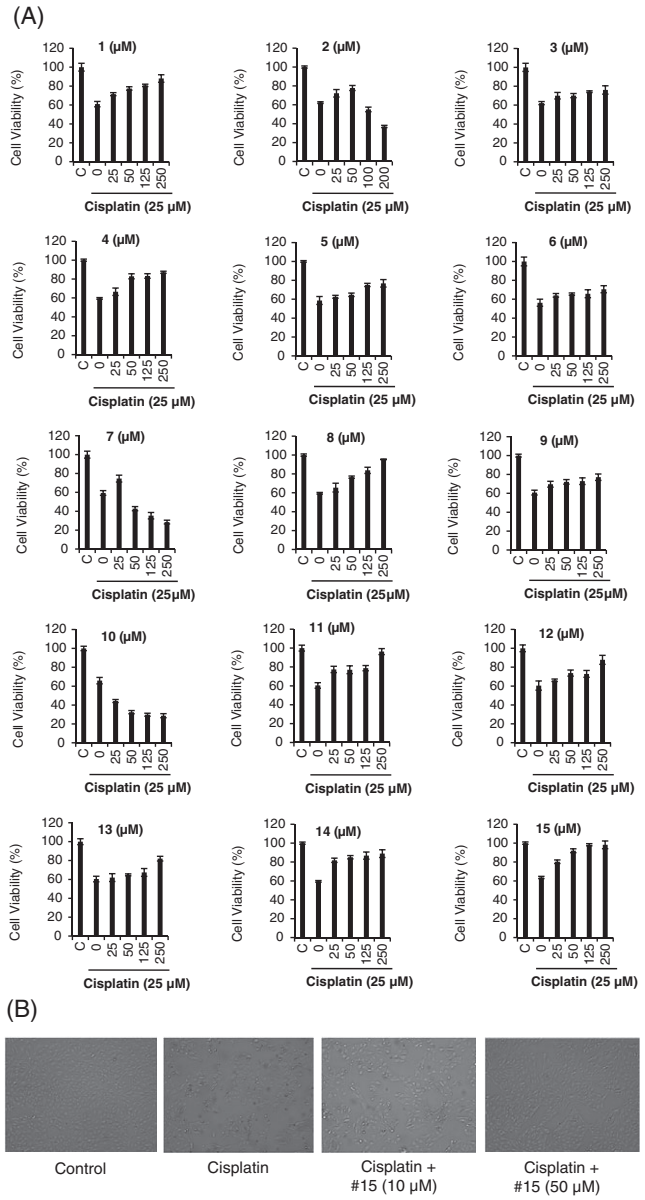


Figure 1. Comparison in the protective effect of synthesized 15 chalcones derivatives on cisplatin-induced renal cell damage. (a) Protection assay with compounds (**1–15**) in cisplatin-induced (25 μM) LLC-PK1 cells. (b) Representative microscopic image of the renoprotective effect of compound **15** following treatment with 25 μM cisplatin.

was observed with 10 and 50 μM compound **15**, respectively (Figure 2(b)). Next, we used Western blot analysis to measure the expression of inflammatory signal proteins, including p-JNK, p-p38, p-ERK, and cleaved caspase-3, in LLC-PK1 cells. Upregulated phosphorylation of JNK, p38, and ERK by cisplatin treatment was decreased markedly after co-treatment with compound **15** (Figure 3). The results demonstrate that blocking the MAPKs-caspase-3 signaling cascade plays a critical role in mediating the protective effect of compound **15** against cisplatin-induced nephrotoxicity.

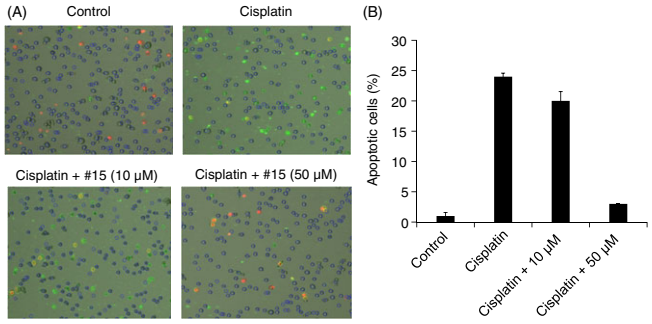


Figure 2. Effects of compound **15** on apoptosis in LLC-PK1 cells. (a) Representative images of apoptosis detection. (b) Percentage of annexin V-positive-stained apoptotic cells. Dead and apoptotic cells were stained with red and green fluorescence. Apoptosis was determined using a Tali image-based cytometer.

To evaluate the effect of compound **15** on the anticancer activity of cisplatin, the human cervical cancer HeLa cells were treated with the compound **15** and cisplatin alone or in combination. Co-treatment with compound **15** at the dose up to 50 μM did not attenuate the cytotoxic properties of cisplatin (Figure 4). No obvious anticancer effect of compound **15** alone was observed. Our findings suggest that compound **15** with cisplatin may alleviate the nephrotoxicity without compromising therapeutic efficiency of cisplatin.

Chalcone derivatives **1**, **4**, **8**, **11**, **14**, and **15** have therapeutic potential for protecting against anticancer drug-induced nephrotoxicity. The chalcone analogues with meta or para-chloro phenyl and 4-allyloxy phenyl group (**14** and **15**) had highly potent effects in the kidney cell protection assay, while the chalcone derivatives (**7** and **10**) with 3-bromo-2,4-dimethoxy phenyl group had no protective effects. Compound **15** had no cytotoxic effects on LLC-PK1 cells, even at high concentrations (250 μM). Compound **15** exhibited the strongest renoprotective effects by blocking the MAPKs-caspase-3 signaling cascade.

In anticancer activity, cisplatin stimulated cancer cell signal pathways, including ATR, p53, p73, and MAPK, and culminate the activation of apoptosis.[24] Compound **15** may selectively block MAPK signal pathway activated by cisplatin. Therefore, co-treatment of cisplatin with compound **15** exhibited no attenuating anticancer effects in the human cervical cancer cells.

MAPK signal pathways are all known to play roles in modulating nephrotoxicity, caused by cisplatin. Stimulation of these signal pathways induced apoptosis in kidney cells. (E)-3-(2-bromo-4,5-dimethoxyphenyl)-1-(4-bromophenyl) prop-2-en-1-one[19] showed potent renoprotective effects at 25 μM by blocking MAPK-p53-caspase-3 signal pathway. However, high dose of this compound showed decreasing renoprotective effects.

Compound **15** is therefore a relatively potent chemical agent that has a significant effect on kidney protection in cell culture. Compound **15** might ultimately lead to the development of chemotherapeutic agents.

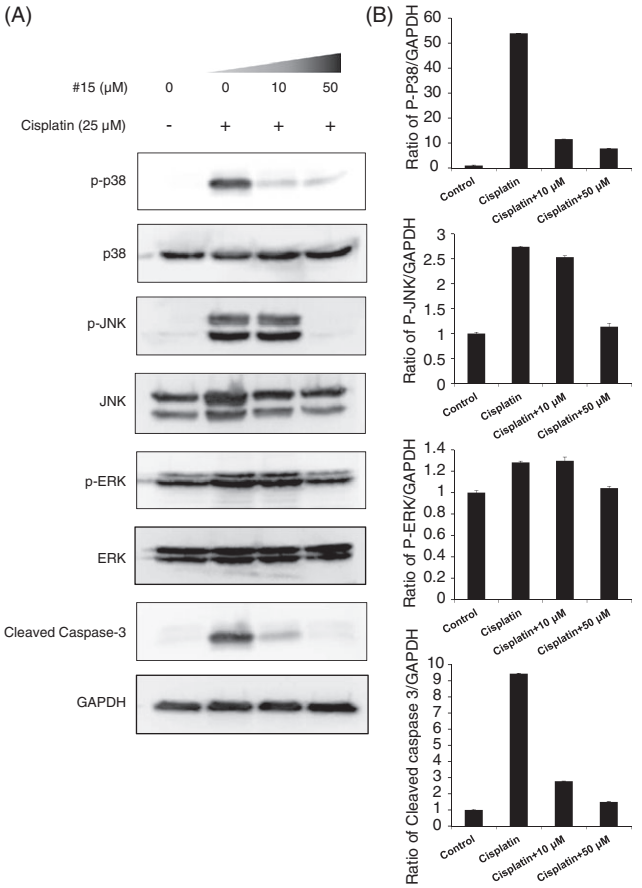


Figure 3. The MAPKs-caspase-3 signaling pathway mediates the protective effect of compound **15** against cytotoxicity in cultured LLC-PK1 cells. The Western blot analysis shows the levels of phosphorylated (p)-p38, p38, p-ERK, ERK, p-JNK, JNK, cleaved caspase-3, and GAPDH in LLC-PK1 cells treated with compound **9** with or without cisplatin at different concentrations for 24 h.

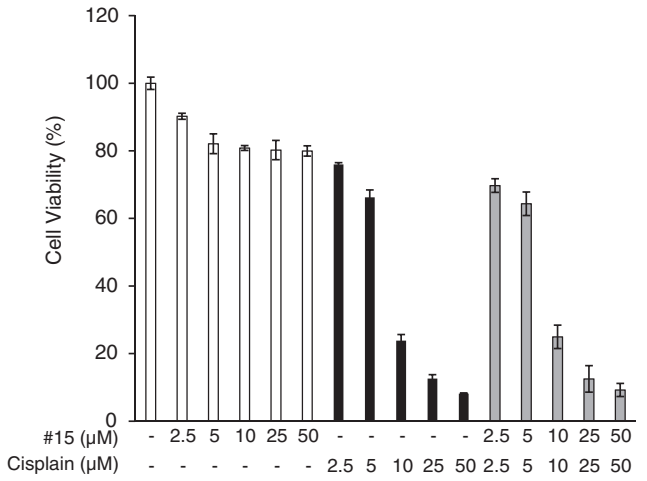


Figure 4. The effect of the combination treatment of compound **15** and cisplatin on the cell viability in HeLa cells. Cell viability was determined after 24 h culture by cell counting assay kit, and the results were expressed as percentage of viable cells.

Experimental

The Synthesis of (E)-3-(4-(allyloxy)phenyl)-1-(4-chlorophenyl)prop-2-en-1-one (15)**.** The reaction condition was followed the same procedure. 4-Chloroacetophenone (77.5 mg, 0.5 mmol) and 4-allyloxybenzaldehyde (81.5 mg, 0.5 mmol) were afforded to compound **15** (86 mg, 57.7%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.95 (dd, *J* = 6.8, 2 Hz, 2H), 7.78 (d, *J* = 15.6 Hz, 1H), 7.59 (d, *J* = 8.8 Hz, 2H), 7.47 (d, *J* = 8.8 Hz, 2H), 7.36 (d, *J* = 15.6 Hz, 1H), 6.95 (d, *J* = 8.8 Hz, 2H), 6.10–6.01 (m, 1H), 5.42 (dd, *J* = 14.0, 1.6 Hz, 1H), 5.32 (dd, *J* = 10.4, 1.2 Hz, 1H), 4.59 (dt, *J* = 5.2, 1.6 Hz, 2H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 189.2, 160.9, 145.2, 139.0, 136.8, 132.7, 132.0, 130.3, 129.8, 129.7, 128.9, 127.9, 127.6, 119.2, 118.1, 115.2, 115.0, 68.9. ESI-MS 299.1 [M + H].

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Supporting Information. Additional supporting information is available in the online version of this article.

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