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ARTICLE

Phloroglucinols Inhibit Chemical Mediators and Xanthine Oxidase, and Protect Cisplatin-Induced Cell Death by Reducing Reactive Oxygen Species in Normal Human Urothelial and Bladder Cancer Cells

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Phloroglucinols, garcinielliptones HA-HE (**1**−**5**), and C (**6**) were studied in vitro for their inhibitory effects on chemical mediators released from mast cells, neutrophils, and macrophages. Compound **6** revealed significant inhibitory effect on release of lysozyme from rat neutrophils stimulated with formyl-Met-Leu-Phe (fMLP)/cytochalasin B (CB). Compounds **3**, **4**, and **6** showed significant inhibitory effects on superoxide anion generation in rat neutrophils stimulated with (fMLP)/(CB), while compounds **1** and **5** revealed inhibitory effects on tumor necrosis factor-α (TNF-α) formation in macrophages stimulated with lipopolysaccharide (LPS). Compounds **1** and **3**−**6** showed inhibitory effects on xanthine oxidase (XO) and could inhibit the DNA breakage caused by $O_2^{-\bullet}$. Treatment of NTUB1 with 2 to 60 μM compound **3** and 5 μM cisplatin and SV-HUC1 with 9 to 60 μM **3** and 5 μM cisplatin, respectively, resulted in an increase of viability of cells. These results indicated that compounds **1** and **3**−**6** showed anti-inflammatory effects and antioxidant activities. Compound **3** mediates through the suppression of XO activity and reduction of reactive oxygen species (ROS), and protection of subsequent cell death.

KEYWORDS: Phloroglucinol; garcinielliptones HA-HE and anti-inflammatory; antioxidant; NTUB1; SV-HUC1

INTRODUCTION

Phloroglucinols are a major class of secondary metabolites of natural products. They have been investigated widely for its biological activity (*1*). In previous papers, we isolated several new terpenoids and phloroglucinols from *Garcinia subelliptica* Merr. (Clusiaceae) and reported their anti-inflammatory activity, cytotoxicity, and DNA strand-scission (*2*−*4*). However, phloroglucinol derivatives isolated from *Hypericum papuanum* Ridley (Clusiaceae) showed antioxidant activity (*5*). A phloroglucinol, hyperielliptone HB isolated from *Hypericum geminiflorum* revealed significant inhibition of oxidative DNA damage and an inhibitory effect on xanthine oxidase (XO) activity (*6*).

Mast cells and neutrophils, stimulated with various inducers may contribute to inflammatory disorders. Following the activation of macrophages, TNF-α and NO were generated in response to lipopolysaccharide (LPS) (*7*, *8*). NO plays a central role in macrophage-induced cytotoxicity, and excess of NO may induce pathophysiological septic shock (*9*). TNF-α is an important pro-inflammatory cytokine with immune and inflammatory functions and is generally considered as a principal mediator of septic shock. The overexpression of TNF-α is associated with autoimmune diseases such as rheumatoid arthritis (*10*).

XO is a key enzyme that catalyzes the oxidation of xanthine and hypoxanthine into uric acid and displays a vital role in causing hyperuricemia and gout (*11*). Allopurinol is a clinically used XO inhibitor in the treatment of gout. However, because of unwanted side effects of allopurinol, such as hepatitis, nephropathy, and allergic reaction, research for new alternatives with enhanced therapeutic activity and fewer side effects is necessary. In addition, superoxide anion radicals generated by XO are involved in various diseases, such as hepatitis, inflammation, and carcinogenesis (*12*). Thus, the research for novel XO inhibitors would be useful not only to treat gout but also to treat various diseases associated with the increase of superoxide anion radicals.

As part of a continuing investigation on phloroglucinols with anti-inflammatory and antioxidant activities, we report the

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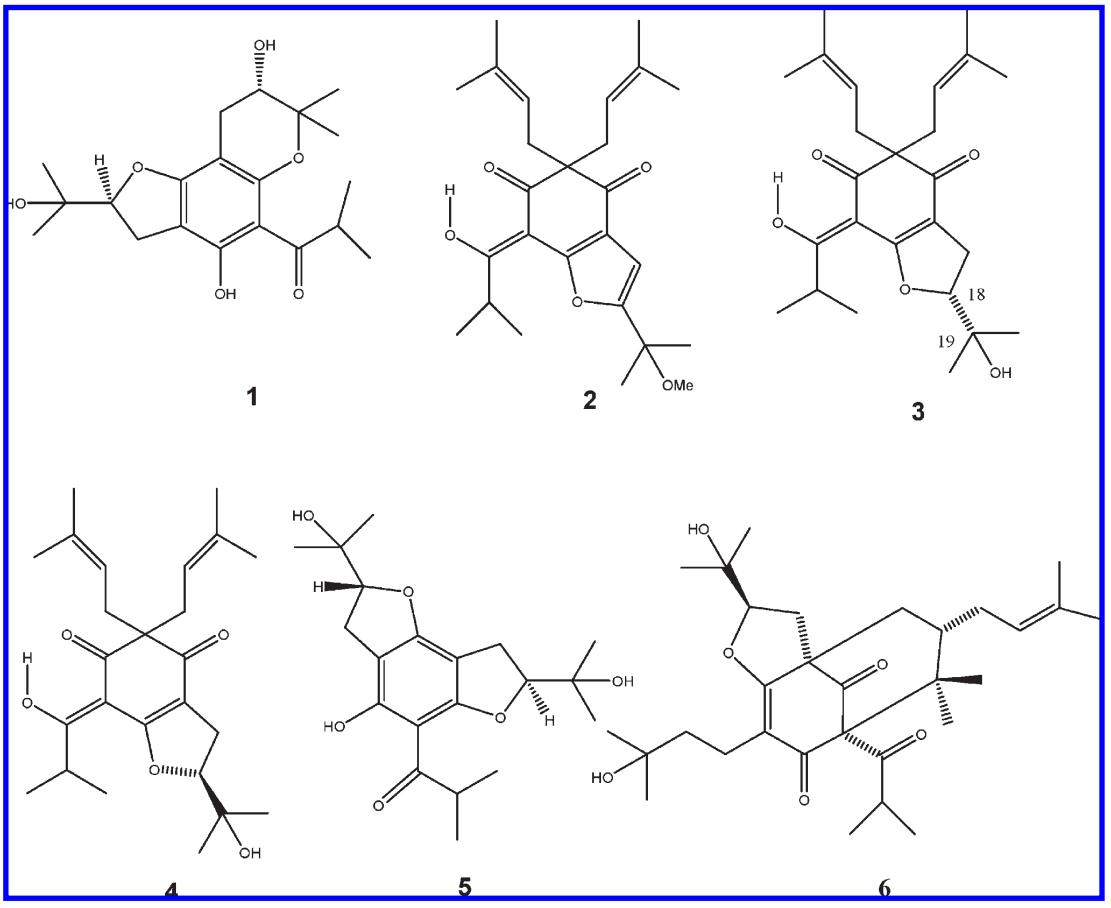

Figure 1. Structures of phloroglucinols, garcinielliptones HA-HE (**1**−**5**), and C (**6**).

anti-inflammatory and antioxidant activities of phlorogucinols, garcinielliptones HA-HE (**1**−**5**), and C (**6**) (**Figure 1**), isolated form *Garcinia subelliptica* in the present article.

MATERIALS AND METHODS

Chemicals. Garcinielliptone HA-HE (**1**−**5**) and C (**6**) were isolated from *Garcinia subelliptica* (*2*,*3*). Compound 48/80, histamine, formyl-Met-Leu-Phe (fMLP), mepacrine, trifluoperazine, heparin, bovine serum albumin, phenolphthalein-β-D-glucuronidase, *O*-phthadialdehyde, cytochrome *c*, superoxide dismutase (type I, from bovine liver), bacterial LPS (*Escherichia coli*, serotype O111: B4), and L-NAME were obtained from Sigma-Alderich (St. Louis, MO). Hanks' balanced salt solution (HBSS) was obtained form Gibco Lab, Grand Island, NY. Dextran T 500 was purchased from pharmacia LKB, Taipei, Taiwan. Dimethyl sulfoxide (DMSO) was obtained from Merck. Cisplatin was obtained from Pharmacia & Upjohn, Milan, Italy. All culture reagents were obtained from Gibco BRL.

Mast Cell Degranulation, Neutrophil Degranulation, Superoxide Anion Generation, Macrophage Culture and Drug Treatment, and NO Determination. The inhibitory assays for the chemical mediator induced by various stimulants in mast cells, neutrophils, and RAW 264.7 cells were performed by the methods as described in the literature (*13*).

Assay of XO Activity. The XO activity with xanthine as the substrate was measured at 25 °C, according to the protocol of Kong and others (*14*), with modifications. The assay mixture consisting of 50 μL of test solution, 60 μL of 70 mM phosphate buffer (pH 7.5), and 30 μL of enzyme solution (0.1 units/mL in 70 mM phosphate buffer (pH 7.5) was prepared immediately before use. After preincubation at 25 °C for 15 min, the reaction was initiated by the addition of 60 mL of substrate solution (150 μM xanthine in the same buffer). The reaction was monitored for 5 min at 295 nm. The XO activity was expressed as micromoles of uric acid per minute.

Lineweaver−Burk Plots. To determine the mode of inhibition by the active compound from the plant, Lineweaver−Burk Plot analysis was performed. This kinetics study was carried out in the absence and presence of active compounds with varying concentrations of xanthine as substrate. The initial rates were 0.5 and 3 min. The inhibition constant (*Ki*) was determined from the slopes of the Lineweaver−Burk Plot for competitive inhibition and intercept on vertical axis for noncompetitive inhibition (*15*).

Inhibition of Oxidative DNA Damage. A mixture of supercoiled plasmid pBR322 DNA (1 μg/μL) and XA (2 mM)/XO (0.7 U/mL) in 10 mM phosphate buffer (pH 7.4) was incubated for 20 min with 500 μM SOD, quercetin, and **1**−**6** in a total volume of 20 μL in a 1.5 mL microcentrifuge tube at 37 °C. Quercetin was used as the positive control. After incubating for 20 min, a 15 μL aliquot of the mixture was loaded into 1.0% agarose gel containing ethidium bromide (0.05 μg/mL) in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer. The electrophoresis was carried out for 30 min at 100 V, then the gels were illuminated with UV light and photographed. Plasmid DNA subjected to electrophoresis without SOD, quercetin, and **1**−**6** served as the control. The gel electrophoretic mobility of the various forms of DNA was compared with the control (*16*).

Cell Culture and MTT Assay for Cell Viability. NTUB1 human eurthelial carcinoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin-G, 100 μg/mL streptomycin, and 2 mM L-glutamine. SV-HUC1 immortalized normal human urothelial cell line was obtained from American type Culture Collection (Rockville, USA) and maintained in F12 medium supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin-G, 100 μg/mL streptomycin, and 2 mM L-glutamine. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

For evaluating the cytotoxic effect of **3** with cisplatin, a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) assay was performed (*17*). Briefly, the cells were plated at a density of 1800 cells/well in 96-well plates and incubated at 37 °C overnight before drug exposure. Cells were then cultured in the presence of graded concentrations of **3** with or without 5 μM cisplatin

(Pharmacia & Upjohn) at 37 °C for 72 h. At the end of the culture period, 50 μL of MTT (2 mg/mL in PB) was added to each well and allowed to react for 3 h. Following centrifugation of plates at 1000*g* for 10 min, media were removed, and 150 μL DMSO was added to each well. The proportions of surviving cells were determined by absorbance spectrometry at 540 nm using an MRX (DYNEXCO) microplate reader. The cell viability was expressed as a percentage of the viable cells from the control culture conditions. The IC_{50} values of each group were calculated by the median-effect analysis and presented as the mean ± standard deviation (SD).

Statistical Analysis. Data were expressed as the means ± SD. Statistical analyses were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the Student's *t*-test method for two-group comparison. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

The anti-inflammatory effect of **1**−**6** (**Figure 1**) was studied in vitro for their inhibitory effect on chemical mediators released from mast cells, neutrophils, and macrophages. Compound **6** showed significant inhibitory effect on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP/CB in a concentration dependent manner with % inhibition of 48.5 ± 3.7 at 30 μM and IC_{50} value of 27.4 ± 5.8 μM, respectively (**Figure 2**), while **1**−**5** had no significant inhibitory effect on mast cell and neutrophil degranulation (data not shown). Compound **6** with a bicycle [3. 3. 1] and previously reported phloroglucinols, garcinielliptin oxide, and garcinielliptone F (*4*) with a bicycle [4. 3. 0] and [3. 3. 1] nonane moieties exhibited significant inhibitory effects on the release of β-glucuroniase and lysozyme from rat neutrophil (**Figure 2**), while phloroglucinols such **1**−**5** had no effect on the release of β-glucuronidase and lysozyme from the rat neutrophils in response to fMLP/CB. It indicated that phloroglucinol with a bicyclo [3. 3. 1] or [4. 3. 0] nonane skeleton enhanced the inhibitory effect on the release of chemical mediators from rat neutrophil response to fMLP/CB. Compounds **3**, **4**, and **6** showed significant inhibitory effects in a concentration-dependent manner on superoxide anion formation in neutrophils stimulated with fMLP/CB with IC_{50} values of 5.8 ± 4.1, 16.6 ± 2.2, and 11.5 ± 0.6 μM, respectively (**Figure 3**), while having no significant inhibitory effect on superoxide anion formation in neutrophil response to phorbol myristate acetate (PMA) (data not shown). This clearly indicated that methylation of C-19-OH such as **2** shows a slight inhibitory effect on superoxide anion formation in neutrophil culture medium in response to PMA with a % inhibition of 48.6 ± 12.1 at 3 μM and attenuated the inhibitory effect on superoxide anion generation in neutrophil response to fMLP/CB. But an α-oriented chemical bond between C-18 and C-19 such as **3** enhanced the inhibitory effect. The observation that **3**, **4**, and **6** had no appreciable effect on PMA-induced response suggests the involvement of a PMA-independent signaling pathway (*18*).

TNF-α is an important pro-inflammatory cytokine with immune and inflammatory functions and is generally considered as principal mediator of septic shock. As shown in **Figure 4**, compounds **1**, **5**, and the positive control, genistein, revealed potent inhibitory effects on TNF-α formation in RAW 264.7 cells stimulated with LPS in a concentration-dependent manner with IC_{50} values of 4.8 ± 1.9, 23.7 ± 3.0, and 18.7 ± 3.6 μM, respectively while **2**−**4** and **6** did not reveal an inhibitory effect on TNF-α formation. Compound **1**, exhibited stronger inhibitory effect than the positive control, genistein, and may be used for septic shock. The results showed that phloroglucinol with two furan rings or a pyran and furan rings fused on a benzene ring significantly enhanced the inhibitory effect on the formation of TNF-α in the culture medium of RAW 264.7 cell response to LPS.

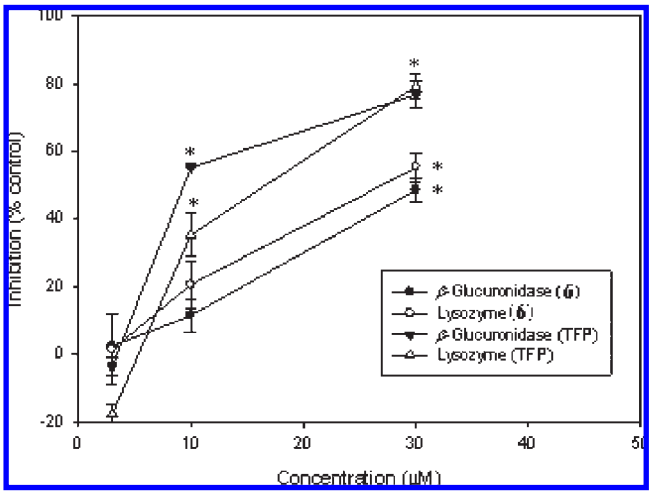

Figure 2. Inhibitory effects of **6** and TFP on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP (1 μM)/CB (5 μg/mL). Data are presented as the means ± SD (n = 3−6). Trifluoperazine (TFP) was used as a positive control. * $p < 0.01$ compared to the control value.

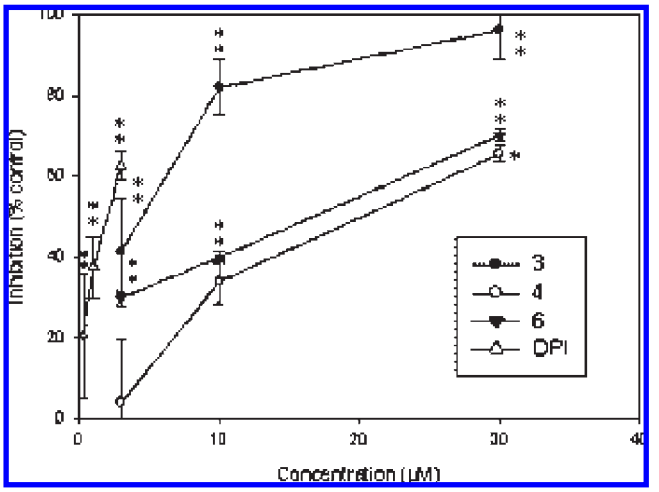

Figure 3. Inhibitory effects of **3**, **4**, **6**, and DPI (diphenylene iodonium chloride) on superoxide anion generation in rat neutrophils stimulated with fMLP (0.3 μM)/CB (5 μg/mL). Data are presented as the means ± SD (n = 3−6). DPI was used as the positive control. * $p < 0.05$ and ** $p < 0.01$ compared to the control value.

The antioxidant activities of **1**−**6** and allopurinol (positive control), a drug clinically prescribed in the clinic for gout treatment, on xanthine oxidase (XO) were studied in vitro. As shown in **Figure 5**, compounds **1**, **3**−**6**, and allopurinol inhibited XO activity in a concentration-dependent manner with IC_{50} values of 31.7 ± 0.4, 16.6 ± 0.2, 172.6 ± 8.9, 104.3 ± 1.9, 59.9 ± 1.1, and 2.0 ± 0.7 μM, respectively. Compound **3** was identified as a stronger XO inhibitor than those of the same chemical skeleton such as **2** and **4**. It indicated that a free OH group attached at C-19 and the α-oriented chemical bond between C-18 and C-19 also enhanced the activity of XO inhibition. For xanthine oxidase assays performed as a function of different concentrations of xanthine in the absence or presence of **1** and **3**, respectively, Lineweaver−Burk transformation of the data are shown in **Figure 6**. The data indicate that **1** and **3** act as noncompetitive and mixed inhibitors of xanthine oxidase, respectively. It revealed that **1** could be bound to free enzyme but that **3** could be bound to free enzyme and the enzyme−substrate

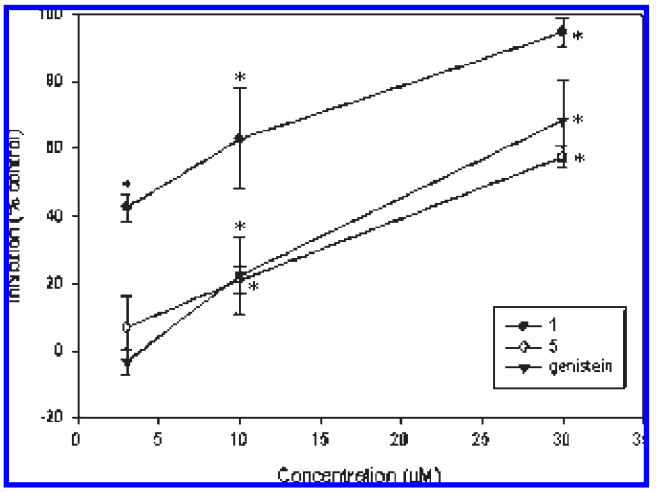

Figure 4. Inhibitory effects of **1**, **5**, and genistein on the formation of TNF-α in the culture media of RAW 264.7 cells in response to LPS (1 μg/mL). Data are presented as the means ± SD ($n = 3-6$). Genistein was used as the positive control. * $p < 0.05$ compared to the control value.

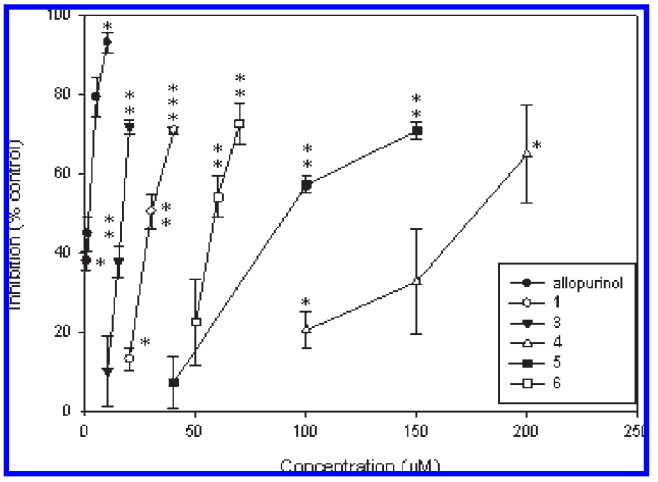

Figure 5. Dose-dependent inhibition of XO by **1**, **3**−**6**, and allopurinol. Data are presented as the means ± SEM, $n = 3-6$. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control value.

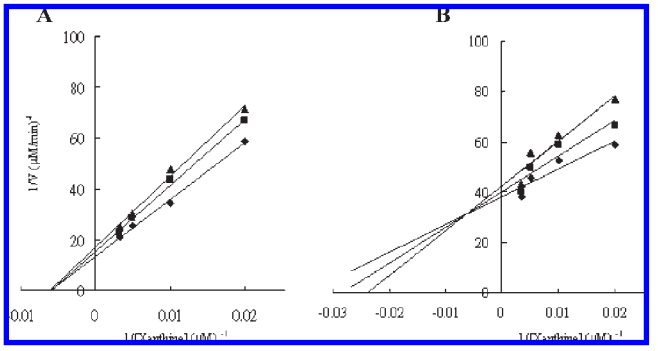

Figure 6. Linweaver−Burk plot of XO inhibition of **1** (**A**) and **3** (**B**) with various concentrations of xanthine. Linweaver−Burk transformed data were plotted and followed by line regression of the points. Data represent the average of triplicate experiments.

complex. Biochemically, enzyme inhibition is associated with hydrogen binding of phenolic hydroxyls and/or carbonyls of the substrate with the amide carbonyls and/or amino groups in the

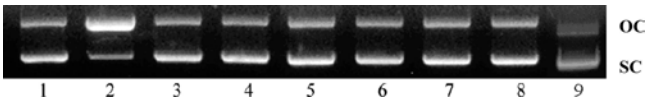

Figure 7. Inhibition of DNA strand breaks induced by $O_2^{-\bullet}$ (generated by XA/XO) in the presence of **1** and **3**−**6** studied by gel electrophoresis. Supercoiled plasmid pBR322 DNA (500 ng) in phosphate buffer (pH 7.4) solution was incubated for 20 min with XA/XO acting as the control. Lane 1, DNA (without XA/XO); lane 2, control; lane 3, control + SOD (300 μM); lane 4, control + quercetin (300 μM) serving as the positive control; lane 5, control + **1** (300 μM); lane 6, control + **3** (300 μM); lane 7, control + **4** (300 μM); lane 8, control + **5** (300 μM); lane 9, control + **6** (300 μM).

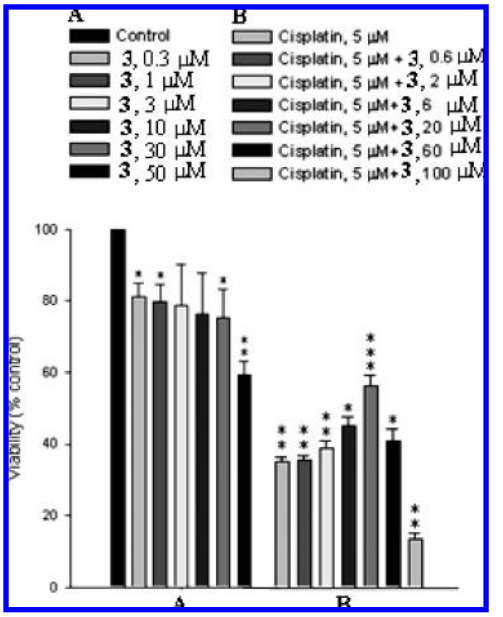

Figure 8. Compound **3** (**A**) or **3** cotreated with cisplatin (**B**) induced NTUB1 cell death. Cell viability was assessed by the MTT assay 72 h after treating by different concentrations of **3** or 5 μM cisplatin cotreated with different concentrations of **3**. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control value.

peptide chain of the enzyme (*19*). Therefore, in addition to the chemical skeleton and conformation of the compound, the phenolic hydroxyl, alcoholic hydroxyl, and carbonyl may interfere with interaction between the enzyme and enzyme−substrate complex.

ROS have been known to damage many biological macromolecules, with DNA being a significant target. The ability of **1**−**6** to inhibit the DNA damage caused by $O_2^{-\bullet}$generated by XA/XO) was investigated by the agarose gel electrophoresis method. As shown in **Figure 7**, lane 1 served as the control and has two different plasmid DNA forms, open circular and supercoiled DNA. However, the plasmid DNA was damaged in the presence of XA/XO (lane 2) with an increased proportion of the open circular form. Adding SOD (500 μM) to the XA/XO system inhibited the DNA damage completely (lane 3), confirming the role of $O_2^{-\bullet}$ in plasmid DNA damage. Quercetin and phloroglucinols **1** and **3**−**6** at the same treated concentration, 300 μM, significantly inhibited DNA scission (lane 4−9). The above result revealed that these compounds may inhibit the formation of ROS in cells and may protect cell death (*20*).

For further evaluation of the protective effect on cells, NTUB1 or SV-HUC1 immortalized normal human urothelial cells were treated with various concentrations of selective compound **3**, showing a potent inhibitory effect on the chemical mediator and XO, in the presence and absence of 5 μM cisplatin, and we

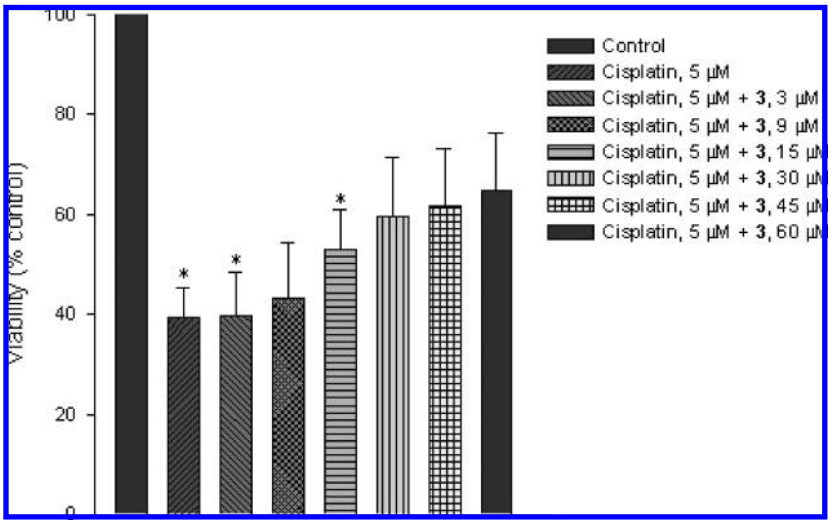

Figure 9. Cisplatin cotreated with **3** in SV-HUC1 cells. Cell viability was assessed by the MTT assay 72 h after treating with different concentrations of 3 and 5 μM cisplatin. *$p < 0.05$ compared to the control value.

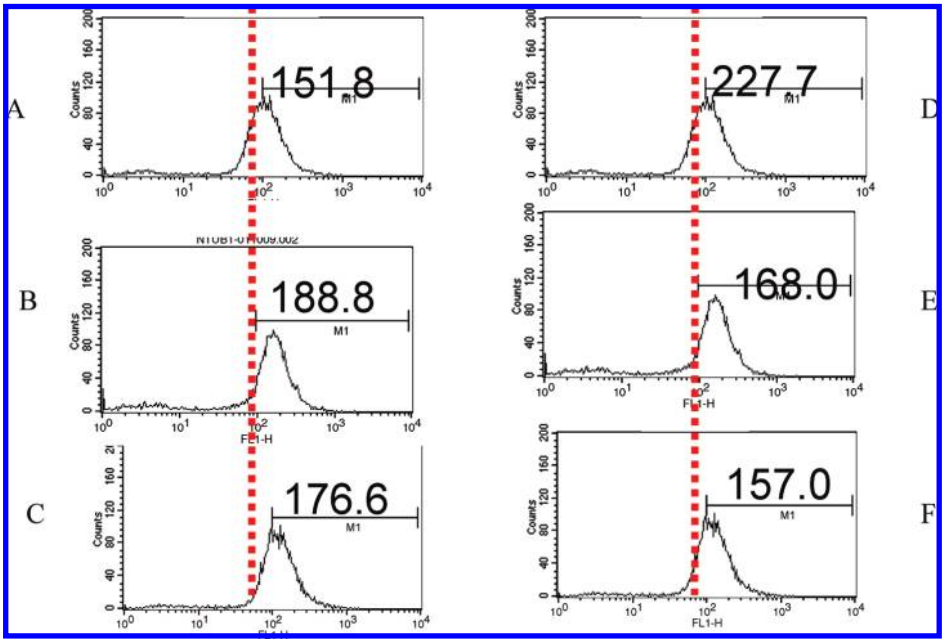

Figure 10. Effect of **3** and **3** cotreated with cisplatin in the production of ROS in NTUB1 cells. (**A**) Control; (**B**) 20 μM cisplatin; (**C**) 20 μM **3**; (**D**) 50 μM **3**; (**E**) 20 μM cisplatin + 20 μM **3**; (**F**) 20 μM cisplatin + 50 μM **3**, and 20 μM cisplatin cotreated with different concentrations of **3** for 24 h, and the amount of ROS was assayed by H_2DCFDA staining. Results were repeated by independent experiments.

determined the cell viabilities by the MTT assay. Compound **3** at 0.3 to 30 μM or 3 to 60 μM did significantly cause increased NTUB1 (**Figure 8**) or SV-HUC1 (data not shown) cell death in a concentration-dependent manner. Further evaluations showed that the viability of NTUB1 cells treated with **3** and 5 μM cisplatin was protected by **3** between 6 to 60 μM, while at 100 μM, it did not protect NTUB1 cells from cisplatin-induced cytotoxicity (**Figure 8**). Compound **3** exhibited weak cytotoxicity against SV-HUC1 cells (data not shown), while at 15 to 60 μM cotreated with 5 μM cisplatin, it significantly protected SV-HUC1 cell death induced by cisplatin (**Figure 9**).

Many studies have suggested that cisplatin stimulates cells to produce ROS (*21*). ROS have been shown as causative factors involved in many human degenerative diseases, and antioxidants have been found to have some degree of preventive and therapeutic effects on these disorders (*22*). Compounds **1**, **3**, and **6** significantly inhibited the DNA damage induced by $O_2^{-\bullet}$ in vitro and also the XO activity. This suggested that each of the three compounds or each of these compounds cotreated with cisplatin may inhibit the formation of ROS through the inhibitory effect on XO and protected cells through the diminishing of free radicals in NTUB1 and SV-HUC1 cells, respectively.

Now, it is clear that ROS have a cell signaling role in many biological systems, both in animals and plants. ROS induce programmed cell death or necrosis, induced or suppressed the expression of many genes, and activated cell signaling cascades, such as those involving mitogen-activated protein kinases (*20*). As shown in **Figure 10**, the amount of ROS produced by **3** cotreated with cisplatin in NTUB1 cells were less than that generated by cisplatin or **3** alone as determined by the fluorescent dye, H_2DCFDA, which preferentially detected intracellular ROS. The results showed that **3** mediates through its inhibitory effect on

XO and protected cells through the diminishing of ROS in NTUB1 cells in the presence of cisplatin, and prevents the following cell death. It also suggested that **3** may mediate through its inhibitory effect on XO and may protect cells through the diminishing in immortalized normal human urothelial cells (SV-HUC1) in the presence of cisplatin and prevent the following cell death.

In conclusion, the phloroglucinols showed significant anti-inflammatory and antioxidant activities. The protective effect of **3** cotreated with cisplatin is due to its significant inhibitory effect on XO activity and diminishing the formation of free radicals. Our findings suggested that these phloroglucinols may protect against radical stress by inhibiting ROS formation and may be used as a protective agent for diseases associated with increased amounts of ROS. It is valuable for further studies on the protective effect and the bioavailability of selective compound **3** in vivo.

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