Chalcone Derivatives Inhibit Human Platelet Aggregation and Inhibit Growth in Human Bladder Cancer Cells

Chien-Ming Wu,a,b Kai-Wei Lin,a Chi-Hung Teng,a A-Mei Huang,c Yu-Chian Chen,d Ming-Hong Yen,a,b Wen-Bin Wu,e Yeong-Shiau Pu,f and Chun-Nan Lin*a,g

a Faculty of Pharmacy, College of Pharmacy, Kaohsiung Medical University; b Institute of Biochemistry, College of Medicine, Kaohsiung Medical University; c Faculty of Fragrance and Cosmetics, School of Pharmacy, Kaohsiung Medical University; Kaohsiung 80708, Taiwan; d Department of Physical Medicine and Rehabilitation, Yuan’s General Hospital; Kaohsiung 80249, Taiwan; e Department of Biological Science and Technology, School of Medicine, China Medical University; Taichung 40402, Taiwan; f School of Medicine, Fu-Jen Catholic University; New Taipei City 24205, Taiwan; and g Department of Urology, College of Medicine, National Taiwan University; Taipei 10051, Taiwan.

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In an effort to develop potent cyclooxygenase-1 (COX-1) inhibitors used as anticancer agent, a series of 2,5′-dimethoxychalcones was screened to evaluate their antiplatelet effect on human washed platelets suspension. Compound 2 exhibited potent inhibition of human washed platelet aggregation induced by collagen, significantly inhibited collagen- and arachidonic acid-induced thromboxane B₂ release, and revealed inhibitory effect on COX-1 activity. Molecular docking studies showed that 1, 2, and 4 were bound in the active site of COX-1. These indicated that the antiplatelet effect of these compounds were mainly mediated through the suppression of COX-1 activity and reduced the thromboxane formation. To investigate the mechanistic action of COX-1 inhibitor enhanced the cytotoxic effect against human bladder cancer cells, NTUB1, we assessed the cytotoxic effect of 2 against NTUB1. Treatment of NTUB1 cells with various concentrations of 2 led to a concentration-dependent increase of cell death and decrease of reactive oxygen species levels. The flow-cytometric analysis showed that 2 induced a G1 phase cell cycle arrest but did not accompany an appreciable sub-G1 phase in NTUB1 cells. In addition, compound 2 increased p21 and p27 expressions and did not inhibit the expression of COX-1 in NTUB1 cells. Our results suggested that 2 enhanced cell growth inhibition or antiproliferative activity in NTUB1 cells through G1 arrest by COX-1 independent mechanism.

Key words antiplatelet; chalcone; cyclooxygenase-1 (COX-1); G1 cell cycle arrest; cytotoxicity; bladder cancer

Natural and synthetic chalcones have been reported to exhibit a diverse range of pharmacological activities including antitumor, anti-inflammatory, antiplasmodial, immunosuppressant, and antioxidant property.[1] Previous studies showed that natural or synthetic chalcones with an inhibitory effect on arachidonic acid (AA)-induced platelet aggregation in washed rabbit platelets and secondary aggregation induced by epinephrine in human platelet-rich-plasma (PRP) are mainly mediated through the suppression of cyclooxygenase (COX) activity and reduced thromboxane formation or owing to inhibition of thromboxane synthase leading to the reduction of thromboxane formation.[2-4] In turn, we have also synthesized a series of 2,5′-dimethoxychalcone derivatives and reported as G2/M arrest-mediated apoptosis-inducing agents and inhibitors of nitric oxide production in rat macrophage.[5]

During the past few years, COX (COX-1, COX-2) was discussed as new targets for several types of cancer, including breast cancer. COX-2 proteins increase new blood vessel formation and proliferation.[6] COX-1 selective inhibitors may also have therapeutic value, as it was shown that COX-1 is overexpressed in some ovarian cancer cells, where it stimulates angiogenesis.[7] Most commercially available nonsteroidal anti-inflammatory drugs used as inhibiting COX-2 have shown anti-proliferative and anti-angiogenic properties.

The incidence of bladder cancer continues to increase yearly. In addition to transurethral resection, standard treatment for patients with superficial bladder cancer includes intravesical instillation of chemotherapies and/or immunotherapies. However, some patients do not response to the intravesical therapies. Additionally, it has serious and potential life-threatening side effects associated with their use.[8] In addition, COX-1 inhibitor, catechin exhibited significant cytotoxic effect against bladder cancer cells in vitro.[9] For developing the COX-1 inhibitor used as effective treating agent for patient suffering from bladder cancer, we studied the antiplatelet effect and cytotoxic effect against NTUB1 cells of previously reported chalcones, 1–7 (Fig. 1) and reported in the present paper.

MATERIALS AND METHODS

Chemistry Chalcones 1–7 have been synthesized and reported.[3]

Platelet Aggregation Collagen, aracidonic acid (AA), and indomethacin were purchased from Sigma Chemicals. Cisplatin was obtained from Pharmacia and Upjohn, Milan, Italy. All culture reagents were obtained from Gibco BRL. Type IV collagen was used as main inducer for platelet aggregation. For preparation of collagen, lyophilized type IV bovine collagen (100 mg, Sigma) was dissolved in acetic acid (25 mmol/L, 100 mL) and homogenized to obtained 1 mg/mL of collagen stock solution. For preparation of washed platelet suspension (PS), we collected blood from human volunteers and the procedure was carried out in accordance with approved by the animal welfare and ethics committees at institutional review board of Fu-Jen Catholic University. Briefly, blood was col-

* To whom correspondence should be addressed. e-mail: yen@kmu.edu.tw; linca@cc.kmu.edu.tw

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Thromboxane B2 (TXB2) Formation

Thromboxane A2 (TXA2) is rapidly hydrolyzed nonenzymatically to form TXB2. TXA2 release into supernatant during platelet aggregation was measured by thromboxane B2 EIA Kit (Cayman, Ann Arbor, MI, U.S.A.) according to the manufacturer’s protocol. Briefly, platelet suspension (4×10⁸ platelets/mL) was incubated with dimethyl sulfoxide (DMSO), compound or indomethacin for 3 min before adding of collagen or AA. At 6 min after the addition of agonists, indomethacin (50 µM), and 2 mM L-glutamine. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

COX-1 Activity

The reaction mixture (0.1 mL Tris–HCl, pH 8.0, 5 mM tryptophan, 8 mM hematin, test drugs, and 10 µg/mL of ram seminal vesicles (COX) was incubated for 3 min at 30°C. The reaction was initiated by adding 100 µM AA. The velocity of oxygen consumption in the reaction mixture was monitored continuously with a Clark-type oxygen electrode using a YSI biological oxygen monitor (Model 5300).

Molecular Docking and Dynamic Study

Docking experiments were performed using DS modeling 1.7 (Accelrys, San Diego, CA, U.S.A.). The coordinate for the X-ray crystal structure of the enzyme COX-1 was obtained from the Protein Data Bank (PDB ID: 1Q4G), crystal cell restriction was removed, and the hydrogens were added following whole structure typed in CHARMM force field. The scaffolds of recruited compounds were constructed under ChemOffice 2006 software (Cambridge Scientific Computing, Cambridge, MA, U.S.A.) and each compound was performed the minimization in MM2 force field. After the protein was defined as a receptor and the binding site was defined counting on the position of ligand, 2-(1,1′-biphenyl-4-yl)propanoic acid (BFL), in the crystal structure, the docking experiment on COX-1 was carried out. For using the LigandFit program the optimal binding orientation of each the recruited ligand was obtained after further minimized for 1000 iterations using the conjugate gradient method until a convergence of 0.001 kcal/mol-Å. The dock scores were gained and the remarkable compounds were chosen as the candidates for ligand–receptor complex dynamic cascade performance. Under the “Standard Dynamics Cascade” protocol, several consecutive simulations containing minimization, heating, equilibration, and production were executed for ligand–receptor complexes. Before this protocol running, the complex should be harmonic restrained. Two minimizations were carried out previously including “Steepest Descent” and “Adopted Basis NR” algorithms with the 0.1 and 0.0001 RMS gradients, respectively. Next, the whole system was heated to 310K with 2000 steps based on 0.001 picoseconds for each step. Equilibration was performed to stabilize the molecular system around the target temperature with 1000 steps for each 0.001 picoseconds per step. Finally, the complex was produced based on the constant-temperature, constant-volume ensemble (NVT). Subsequently the interaction energies were calculated based on Chemistry at Harvard Molecular mechanics (CHARMm) force field according to the following equation and the docking results were analyzed:

\[ E_{\text{interaction}} = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{receptor}}) \]

where \( E \) is energy and the complex contain ligand and receptor.

Cell Culture and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT) Assay for Cell Viability

NTUB1, a human bladder carcinoma cell was 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. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For evaluating the cytotoxic effect of cisplatin (positive control), and tested compounds 1 and 2, respectively, a modified MTT (Sigma Chemical Co.) assay was performed. 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 10 µM cisplatin (positive control), tested compounds, 1 and 2, respectively at 37°C for 72 h. At the end of the culture period, 50 µL of MTT [2 mg/mL in phosphate buffered saline (PBS)] was added to each well and allowed to react for 3h. Following centrifugation of plates at 1000×g for 10min, media were removed and 150 µL DMSO were added to each well. The proportions of surviving cells were determined by absorbance spectrometry at 540nm using MRX (DYNEXCO) microplate reader. The cell viability was expressed as a percentage to the viable cells of control culture condition. The IC₅₀ values of each group were calculated by the median-effect analysis and presented as mean±standard error.
deviation (S.D.).

**Quantitative Analysis of Intracellular Reactive Oxygen Species (ROS)** Production of ROS was analyzed by flow cytometry as described previously. Briefly, cells were plated and treated as indicated conditions. 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; Molecular Probes, Eugene, OR, U.S.A.) was added to the treated cells 30 min prior harvest. The cells were collected by trypsinization and washed with PBS. The green fluorescence of intracellular 2′,7′-dichlorofluorescein (DCF) was then analyzed immediately by FACSscan flow cytometer with a 525 nm bandpass filter (Becton Dickinson).

**Flow Cytometry Analysis** DNA content was determined following propidium iodide (PI) staining of cells as previously described. Briefly, 8 × 10$^5$ cells were plated and treated with 10 μM cisplatin and various concentrations of 2 for 24 h, respectively. These cells were harvested by trypsinization, washed with 1× PBS, and fixed in ice-cold MeOH at -20°C. After overnight incubation, the cells were washed with PBS, and incubated with 50 μg/mL propidium iodide (Sigma Chemical Co.) and 50 μg/mL RNase A (Sigma Chemical Co.) in PBS at room temperature for 30 min. The fractions of cells in each phase of cell cycle were analyzed using FACSscan flow cytometer and Cell Quest software (Becton Dickinson).

**Western Blot Analysis** Cells were harvested by trypsinization and resuspended with suitable amount of PBS adjusted with the cell numbers. The cells were mixed with equal volume of 2× sample buffer and boiled for 10 min twice to denature the proteins. Cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, U.S.A.) using a semi-dry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in TBST buffer (100 mM Tris–HCl (pH 7.5) 150 mM NaCl, and 0.1% Tween-20). The membranes were incubated with specific antibodies at 4°C overnight. The membranes were washed with TBST buffer and incubated with secondary antibody at room temperature for another 1 h. Signals were detected by chemiluminescence ECL reagent after TBST wash and visualized on FUJI SuperRX film.

Monoclonal antibody specific for β-actin was purchased from Novus Biologicals (Littleton, CO, U.S.A.). The antibody, p21, p27, β-tubulin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-COX-1 antibody was purchased from Gene Tex (Gene Tex, CA, U.S.A.).

**Data Analysis** Data were expressed as mean±S.D. or S.E. Statistical analysis were performed using the Bonferroni t-test method after ANOVA for multigroup comparison and the Stu-

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**Fig. 2. Effect of Various Synthetic Chalcones on Collagen-Induced Platelet Aggregation**

Human PS were preincubated with vehicle (DMSO) or the indicated concentrations of chalcones for 3 min and followed by the addition of collagen (10 μg/mL). Platelet aggregation was monitored by aggregometry. Data were expressed as the percentage of control (CT). (n=3–4). *p<0.05, **p<0.01 compared to control value, respectively.

**Fig. 3. Concentration-Dependent Effect of Selected Chalcones, (A) 1, (B) 2, (C) 3, and (D) 5, on Collagen-Induced Platelet Aggregation**

Human PS were preincubated with vehicle (DMSO) or the indicated concentrations of the selected chalcones for 3 min and followed by the addition of collagen (10 μg/mL). Platelet aggregation was monitored by aggregometry. Data were expressed as the percentage of control (n=2–4). *p<0.05, **p<0.01 compared to control value, respectively.
dent’s *t*-test method for two group comparison, with *p*<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

We have synthesized a series of chalcones and demonstrated that chalcones inhibited platelet aggregation of rabbit washed platelets and human platelet-rich plasma (PRP) induced by various inducers and cyclooxygenase (COX) activity. In addition, the prostate cancer cells (DU145 and LnCap) or bladder cancer cells treated with catechin, a COX-1 specific inhibitor, did exhibit a significant decrease in cellular proliferation. Thus, the effect of previously synthesized chalcones 1–7 on collagen-induced platelet aggregation was determined in washed platelets suspension (PS). Figure 2 revealed that 100 µM 1–5 had potent antiplatelet effect on collagen-induced aggregation while 50 µM 1–3 also significantly exhibited antiplatelet effect on collagen-induced aggregation.

As shown in Fig. 3A–D and 2 in Fig. 4, the antiplatelets of selective chalcones 1–3 and 5 on collagen-induced aggregation appeared to be concentrating dependent.

To investigate the possible mechanism of action of selective 2, we examined TXA2 formation using a TXB2 EIA kit. Figure 5 shown that collagen and AA induced an increase of TXB2 which was inhibited by nonselective COX inhibitor indomethacin (indo), indicating that thromboxane formation stimulated by these two inducers was COX-dependent. It was found that 2 alone did not affect the basal TXB2 level while significantly inhibited both collagen and AA-induced TXB2 formation during platelet aggregation suggesting that it can directly affect COX or thromboxane synthase activity. The collagen-induced aggregation was almost abolished by 200 µM 2 (Fig. 4) while the collagen-induced TXB2 formation was significantly reduced, by same concentration of 2 (Fig. 5). It suggested that other signaling components are also affected during platelet aggregation.

![Fig. 4. Effect of 2 on Collagen-Induced Platelet Aggregation](image)

Human PS was preincubated with vehicle (DMSO) or the indicated concentrations of 2 for 3 min and followed by the addition of collagen (10 µg/mL). Platelet aggregation was monitored by aggregometry. The representative traces from three independent experiments were shown. ∆T: changes in transmission.

![Fig. 5. Effect of 2 on (A) Collagen- and (B) AA-Induced TXB2 Formation](image)

Human PS (4×10⁵ cells/mL) was incubated with 2 (different concentrations as indicated in the figure) or indo (indomethacin) (10 µM) for 3 min and followed by the addition of collagen (10 µg/mL) (A) or AA (150 µM) (B). After 6 min, the reaction was terminated and TXB2 release into the supernatant was measured by TXB2 EIA kit. Data were expressed as TXB2 concentration and were mean±S.E. (*n*=3–5). ** *p*<0.01 compared to control value.
For study of the mechanism of antiplatelet activity of these compounds, the activity of fatty acid COX-1 from ram vesicular glands was measured in the presence of the tested compounds, indo (positive control), 1, 2, and 4, significantly inhibited the enzyme in a dose-dependent manner with an IC\textsubscript{50} values of 1.2±0.2, 29.1±7.0, 15.1±2.7, and 85.5±7.2 µM, respectively (Fig. 6). The above results suggested that the antiplatelet of 2 is probably mediated through the suppression of COX-1 activity and reduces thromboxane formation or owing to the inhibition of thromboxane synthase, leading to reduce thromboxane formation.\textsuperscript{15–17} Further experiments are needed to elucidate the detailed mechanism of action.

Docking studies were performed using DS modeling 1.7 (Accelrys, San Diego, CA, U.S.A.). A maximum of 10 docking studies were generated for 1, 2, and 4, with early termination of the process if the respective root mean square deviations (RMSDs) of the three highest ranked docking solutions were within 1.5 Å RMSD of one another. These RMSDs values exhibited that the docking experiments could be relied upon. In crystallization experiments, it was reported that alternative

![Fig. 6. Inhibitory Effects of Indomethacin (Positive Control), 1, 2, and 4 on COX-1 Activity](image)

Various concentrations of indomethacin, 1, 2, and 4 were preincubated with the enzyme at 30°C for 3 min before addition of AA to start the reaction. The data shown represent mean±S.D. performed in triplicate.*p<0.05 and **p<0.01 compared to control values.

![Fig. 7. Compound 1 Was Docked into the Active Site of COX-1 and Showed One Hydrogen Bond Presented as a Green Broken Line with SER530 (2.06 Å)](image)

Color images were converted into gray scale.

![Fig. 8. Compound 2 Was Docked into the Active Site of COX-1 and Showed Three Hydrogen Bonds Presented as a Green Broken Line with ARG120 (1.93 Å, 3.21 Å) and with TYR355 (2.57 Å), Respectively](image)

Color images were converted into gray scale.

![Fig. 9. Compound 4 Was Docked into the Active Site of COX-1 and Revealed One Hydrogen Bond Presented as a Green Broken Line with ARG120 (1.43 Å)](image)

Color images were converted into gray scale.
conformations of ARG120, TYR355, and SER530 have been previously observed in the COX crystal structures complexed with several inhibitors, highlighting the innate plasticity of the active site.18

Docking of selective compounds 1, 2, and 4 into the COX-1 active site, compound 1 is located at the apex of COX-1 channel. The CO of 1 displays H-bond with SER530 (2.06 Å) (Fig. 7). Compound 2 is lied on the internal side of the constriction, at the base of the funnel-shaped entrance to the active site (Fig. 8). The CO and OMe-2 of inhibitor form two H-bonds with ARG120 (1.93 Å) and TYR355 (2.57 Å), and a H-bond with ARG120 (3.21 Å), respectively. Compound 4 is located at the internal side of the constriction and reveals a H-bond (1.43 Å) with ARG120 (Fig. 9). Compound 2 is located on the base of the funnel-shaped entrance of COX-1 channel and blocks the approach of fatty acid substrate to the active site for its oxygenation. Compound 2 exhibited significant inhibitory effect on COX-1 activity. It may coincide with prediction obtained from docking result of 2.

Table 1. LigScore 2 and Jain Score of Selective Compounds 1, 2, and 4

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a) Average of LigScore 2 and Jain score.

Fig. 10. Antiproliferative Effects of Various Concentrations of 1 and 2 on NTUB1 Cells, Respectively

Cells were incubated with various concentrations of 1 (A) and various concentrations of 2 (B), and cell growth were determined after 72 h treatment. The data shown represent mean ± S.D. (n = 5) for one experiment. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the control values, respectively.

Fig. 11. (A) Flow Cytometry Analysis of 2-Treated NTUB1 Cells

NTUB1 cells (3 × 10⁵ cells/6 cm dish) was treated with no compound (control), 10 μM cisplatin (positive control) and various concentrations of 2 for 24 h. At the time indicated, cells were stained with propidium iodide (PI), and DNA contents were analyzed by flow cytometry, apoptosis was measured by the accumulation of sub-G1 DNA contents in cells. The control cells were treated with vehicle. Values shown were means ± S.E. of the percentage of cells in individual phases of the cell cycle from three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the control values, respectively.

(B) Compound 2 Alteration of the Expression Cell Cycle-Involving Proteins

Cell cycle-related proteins p21 and p27 treated with vehicle control or 2 for 24 h. The Western blot analysis shown here are representation of three independent experiments with similar results. β-Tubulin were employed as a loading control.
In order to obtain the accuracy of the prediction, the docked poses were ranked using two different scoring functions namely Ligscore2 and Jain, since Ligscore2 can accurately predict the binding affinity between ligand molecules and their protein–receptors and Jain score is the sum of the contributions such as hydrophobic interaction, polar interaction, and degree of freedom in order to ensure the binding stability in the chalcone–receptor complex. For investigating the mechanistic action of the chalcone–receptor complex. In this study, the selective COX-1 inhibitor would significantly decrease the cell proliferation in NTUB1 cells, we examined the effect of 1 and 2 on the cell viability of NTUB1 using MTT assay. NTUB1 cells were treated with increasing dose of these compounds, respectively. As shown in Fig. 10, compounds 1 and 2 induced a decrease in viable formazan accumulation after 72h treatment with an IC50 values of 2.2±0.3 and 0.4±0.2 μM, respectively. Based on the above data together with previously reported IC50 values of 1 and 2 against other cancer cell lines. It suggested that NTUB1 cells must be sensitive to COX-1 inhibitor, 1 and 2.

ROS induce programmed cell death or necrosis, induce or suppress the expression of many genes, and activate cell signaling cascades. For investigating the mechanistic action of inducing cell death by COX-1 inhibitor, we selected 2 to determine whether it can stimulate ROS production in NTUB1 cells. Exposure of cells to various concentrations of 2 caused a significant decrease in fluorescence response compared to that of control value (data not shown). It suggested that 2 could decrease the amount of ROS generated in NTUB1 cells. The result indicated that the cell death induced by 2 did not associate with ROS.

Regulation of cell cycle progression in cancer cells was considered to be an effective mechanism for control of proliferation. Treatment of NTUB1 cells with 2 resulted in appreciable G1-phase arrest of cell cycle progression (Fig. 11A). The expression of cyclin-dependent kinase (CDK) inhibitors, p21 and p27, which regulate the expression of cells in the G1 phase were assessed. Protein levels of p21 and p27 were increased following treatment with 0.4 and 1.2 μM 2 for 24 h (Fig. 11B). The result suggested that the induction of G1 arrest by 2 may be associated with up-regulation of p21 and p27 protein expressions. Nonsteroidal antiinflammatory drug (NSAID) inhibits COX-1 and COX-2. The NSAID, sulindac sulfide and sulindac sulfone indicating significant inhibition of COX-1 and COX-2 activity cause growth inhibition and induce apoptosis in human prostate cancer cells by a COX-1 and COX-2 independent mechanism. Western blot analysis indicated that only moderate level of COX-1 expression in NTUB1 cells (data not shown). The exposure of various concentrations of 2 and indomethacin (positive control) to NTUB1 cells did not inhibit the COX-1 expression (data not shown). Therefore, compound 2 could cause growth inhibition and induce G1 arrest in NTUB1 cells by COX-1 independent mechanism. It needs to elucidate the detailed mechanism of selective compound 2 induced growth inhibition of NTUB1 cells.

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REFERENCES


