

# Reduction in Copper Uptake and Inhibition of Prostate Cancer Cell Proliferation by Novel Steroid-based Compounds

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**Abstract.** *Background/Aim:* Knockdown of human copper transporter 1 has been associated with reduction in copper uptake and suppression of prostate cancer cell proliferation and tumor growth. This study evaluated the effects of steroid-based compounds on copper uptake and proliferation of prostate cancer cells based on their anticancer activity and previous docking analysis of steroid-based copper transporter 1 inhibitors. *Materials and Methods:* We synthesized several new steroid-based compounds and used <sup>64</sup>Cu uptake assay and copper quantification assay with inductively coupled plasma mass spectrometry to study their effects on the cellular copper uptake by prostate cancer cells. Additionally, we used CCK-8 cell proliferation assay to study their effects on the proliferation of prostate cancer cells. *Results:* Significant reduction in cellular copper uptake was observed in the prostate cancer cells treated with these new steroid-based compounds. Moreover, proliferation of prostate cancer cells was suppressed by treatment with the steroid-based compound 6, which had the strongest copper uptake inhibition activity. *Conclusion:* Reduction in copper uptake and inhibition of cell proliferation were demonstrated in prostate cancer cells treated with the new steroid-based compounds synthesized in this study. Steroid-based copper transporter 1 inhibitors may become novel anticancer drugs for targeted anti-copper therapy of prostate cancer and other copper hypermetabolic cancers.

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*Key Words:* Prostate cancer, steroid-based compounds, anticancer drugs, copper, human copper transporter 1.

Prostate cancer is one of the leading causes of death in men worldwide (1). There are continuous efforts in developing effective drugs for the treatment of patients with castration-resistant prostate cancer lesions in order to improve overall survival of the patients (2-4). Steroids is a class of polycyclic molecules that play an important role in regulating normal physiological processes and pathophysiological responses in combating various diseases (5). Many steroidal derivatives exhibit strong anticancer activity (6, 7). Kadioglu *et al.* have conducted a docking analysis of steroid-based copper transporter 1 inhibitors (8).

Copper is a transitional metal, which fluctuates between oxidized (cupric, Cu<sup>2+</sup>) and reduced states (cuprous, Cu<sup>+</sup>) in living organisms. Human copper transporter 1 (hCtr1) mediates cellular copper uptake and knock-down of hCtr1 was associated with reduction in cellular copper uptake and suppression of prostate cancer cell proliferation (9). Based on the docking analysis of steroid-based copper transporter 1 inhibitors by Kadioglu *et al.* (8), we designed and synthesized several new steroid-based compounds and tested their effects on cellular copper uptake and the proliferation of prostate cancer cells for their potential use in the treatment of copper-hypermetabolic prostate cancers.

## Materials and Methods

*Reagents and chemical synthesis of steroid derivatives.* All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Steroid-based compounds were designed according to the docking analysis of steroid-based copper transporter 1 inhibitors by Kadioglu *et al.* (8) and synthesized using the method shown in Figure 1. <sup>1</sup>H, <sup>13</sup>C NMR spectra were recorded on a Varian 400 MHz spectrometer. Chemical shifts ( $\delta$  values) were reported in ppm and coupling values in Hz. Mass spectrometry (MS) was recorded using the Agilent 6540 UHD Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS Systems (Agilent Technologies, Santa Clara, CA, USA).

*Copper coordinating ability test.* Copper coordinating ability of steroid-based compounds was assessed by measurement of free

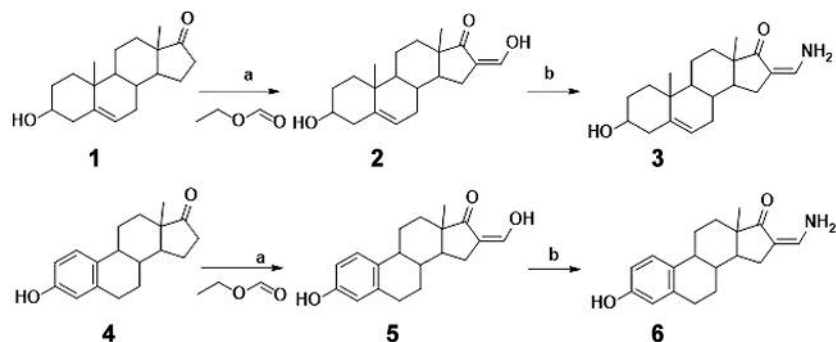


Figure 1. Scheme for synthesis of steroid-based compounds. Reagents and conditions: a) NaOEt,  $\text{CH}_2\text{Cl}_2$ , room temperature, b) reflux, 2 h  $\text{NH}_3\cdot\text{H}_2\text{O}$ .

copper ions in the supernatant after incubation of steroid-based compounds with a solution of  $\text{CuCl}_2$  using a copper assay kit (Sigma-Aldrich). Copper chelators, clioquinol (CQ), and tetrathiomolybdate (TM) were used as control. A mixture of 50  $\mu\text{M}$  of steroid-based compounds, CQ, or TM (dissolved in a solution with DMSO) and 50  $\mu\text{M}$  of  $\text{CuCl}_2$  was mixed at a ratio of 1:1 or 2:1 and incubated for 1 h, 12 h, and 24 h, at 37°C and 100°C, respectively. After the incubation, 10  $\mu\text{l}$  of the mixture was collected and diluted 1,000 times with water. After centrifugation at 10,000 rpm for 5 min, copper ions in the clear supernatant were measured colorimetrically (359 nm) using a copper assay kit (Sigma-Aldrich). Copper complexes of steroid-based compounds, CQ or TM in the precipitate after centrifugation were analyzed by MS.

**Cellular  $^{64}\text{Cu}$  uptake assay.** The radioactive  $^{64}\text{CuCl}_2$  was purchased from Washington University (St Louis, MO, USA), which was produced via  $^{64}\text{Ni}(p,n)^{64}\text{Cu}$  on a biomedical cyclotron and supplied in the form of  $^{64}\text{CuCl}_2$  in 0.1 M HCl solution. The specific activity of  $^{64}\text{Cu}$  was  $6.9 \pm 2.5$  Ci/ $\mu\text{mol}$ . The PC-3 prostate cancer cells purchased from ATCC (Manassas, VA, USA) were cultured in 24-well-plates in a density of  $1.4 \times 10^5$  cells/well overnight. Then, the medium was removed and fresh medium containing 10 and 50  $\mu\text{M}$  of steroid-based compounds was added to the wells, with the cells of the control group incubated with cell culture medium only. After 24 h incubation, the medium was removed and fresh medium containing steroid-based compounds was added, along with 10  $\mu\text{l}$  of  $^{64}\text{CuCl}_2$  (~9  $\mu\text{Ci}$ ), to the wells and incubated for another 12 h. After washing the cells with phosphate-buffered saline (PBS), 1 ml of 1M NaOH was added to wells and incubated in the room temperature for 10 min. Then, the solution was transferred to a tube for  $\gamma$  counting of radioactivity using PerkinElmer 2480 Wizard<sup>2</sup> Automatic  $\gamma$  Counter. The protein concentration of each well was determined by using the Pierce<sup>TM</sup> BCA protein assay Kit (Thermo scientific, Rockford, IL, USA). The cellular  $^{64}\text{Cu}$  uptake was recorded as cpm/ $\mu\text{g}$  protein. A significant decrease in  $^{64}\text{Cu}$  activity in the cells incubated with steroid-based compounds compared with the  $^{64}\text{Cu}$  activity of cells incubated with medium only was interpreted as the cellular copper uptake reduction effect of the tested steroid-based compounds.

**Cellular copper quantification by ICP-MS.** Effects of steroid-based compounds on cellular copper uptake were also assessed by quantification of copper ions with inductively coupled plasma mass

spectrometry (ICP-MS). PC-3 prostate cancer cells ( $1 \times 10^6$  cells/well) were incubated with  $\text{CuCl}_2$  (50  $\mu\text{M}$ ), steroid-based compounds, or steroid-based compounds plus  $\text{CuCl}_2$  (50  $\mu\text{M}$ ) for 24 h. At the end of incubation, the medium was removed, and the wells were washed three times with PBS. After digesting cells with trypsin solution (0.3 ml trypsin/well) in room temperature for 10 min and washing them with PBS, the cell digests were transferred to a centrifuge tube and centrifuged (900 rpm, 5 min). After removal of the supernatant, 0.2 ml Aquaregia was added to the centrifuge tube. Copper ions of the cellular content in the precipitate were quantified by ICP-MS using a 7700 series ICP-MS instrument (Agilent technologies).

**CCK-8 cell proliferation assay.** Effect of steroid-based compounds on cell proliferation was assessed with CCK-8 (cell counting kit-8) assay by following the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). Briefly, PC-3 cells on a 96-well microplate ( $1 \times 10^4$  cells/well) were inoculated and cultured overnight. The cells were incubated for 24, 48 or 72 h, in fresh cell culture medium containing steroid-based compounds at the noted concentrations (50 or 100  $\mu\text{M}$ ). Steroid-based compounds were dissolved in a normal saline solution containing 0.05% DMSO, and sterilized by filtration with a 0.22  $\mu\text{m}$  filter prior to adding onto cell culture wells. At the end of incubation, CCK8 assay solution was added to each well and cell numbers were calculated. The cell numbers at the time of the CCK8 assay were recorded as percentages of the cell numbers initially inoculated. The experiment was conducted in triplicate for each time point and repeated three times. The inhibition concentration 50% ( $\text{IC}_{50}$ ) values were also calculated.

**Statistical analysis.** The data are expressed as mean $\pm$ SD. Two-tailed, unpaired Student's test was used to compare the cellular  $^{64}\text{Cu}$  uptake after treatment with steroid derivative compound 6 and without treatment. The same test was also used to test the copper ion concentrations in PC-3 cells after treatment with or without steroid derivative compound 6. *p*-Values less than 0.05 were considered statistically significant.

## Results

**Chemical synthesis and characterization of steroid-based compounds.** Several steroid-based compounds were synthesized using the method shown in Figure 1. Steroid-based compound

**3** and other compounds (compounds **2** and **5**) were obtained from the reaction of (+)-Dehydroisoandrosterone (**1**) or Estrone (**4**) with ethyl formate, and then with further reaction with  $\text{NH}_4\text{OH}$  to obtain compounds **3** and **6**. All the steroid-based compounds obtained were of high yield and purity.

*(Z)*-3-hydroxy-16-(hydroxymethylene)-10,13-dimethyl-1,2,3,4,7,8,9,10,11,12,13,14,15,16-tetradecahydro-17H-cyclopenta[a]phenanthren-17-one (steroid-based compound **2**). A total of 2.36 g (8.19 mmol) of (+)-Dehydroandrosterone and ethyl formate (9.1 g, 121 mmol) were dissolved in  $\text{CH}_2\text{Cl}_2$  at room temperature.  $\text{NaOEt}$  (5.58 g, 82.0 mmol) was then added into this solution and stirred overnight. Then, the solvent was removed under vacuum. Water was used to dissolve them again and 2 M of  $\text{HCl}$  was added to acidify them. A precipitate was formed, which was washed with water followed by water evaporation to obtain the product of compound **2** (2.08 g, 80.2%).

$^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  10.77 (s, 1H), 7.33 (s, 1H), 5.26 (s, 1H), 4.61 (s, 1H), 3.21 (s, 1H), 1.05-2.20 (m, 15H), 0.96-0.99 (m, 5H), 0.74 (s, 3H).  $^{13}\text{C}$  NMR (100 MHz, DMSO)  $\delta$  209.06, 150.62, 141.88, 120.50, 113.42, 70.37, 50.28, 49.92, 47.72, 42.63, 37.24, 36.69, 31.81, 31.70, 30.95, 30.67, 24.78, 20.42, 19.57, 14.48. MS(ESI+)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{28}\text{O}_3$  [M + H]<sup>+</sup> 317.2, found 317.3. HRMS (EI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{28}\text{O}_3$  [M + H]<sup>+</sup> 317.2072, found 317.2810.

*(Z)*-16-(aminomethylene)-3-hydroxy-10,13-dimethyl-1,2,3,4,7,8,9,10,11,12,13,14,15,16-tetradecahydro-17H-cyclopenta[a]phenanthren-17-one (steroid-based compound **3**). In 325 mg of steroid derivative compound **2** (1.03 mmol) dissolved in 10 ml of ethanol, 10 ml of ammonium hydroxide was added slowly. After reflux for 30 min, a precipitate begun to form. Then, reflux was performed for another 2 h, followed by recrystallization to obtain product **3** (105 mg, 32.3%).

$^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  6.38 (s, 1H), 5.27 (s, 1H), 4.62 (s, 1H), 3.32 (s, 1H), 1.00-2.41 (m, 16H), 0.70-1.00 (m, 7H).  $^{13}\text{C}$  NMR (100 MHz, DMSO)  $\delta$  206.52, 141.95, 140.99, 120.62, 105.08, 70.40, 50.80, 50.50, 47.18, 42.62, 37.24, 36.73, 32.11, 31.82, 30.94, 25.40, 20.50, 19.56, 14.91. MS(ESI+)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{29}\text{NO}_2$  [M + H]<sup>+</sup> 316.2, found 316.3. HRMS (EI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{29}\text{NO}_2$  [M + H]<sup>+</sup> 316.2232, found 316.2971.

*(Z)*-3-hydroxy-16-(hydroxymethylene)-13-methyl-6,7,8,9,11,12,13,14,15,16-decahydro-17H-cyclopenta[a]phenanthren-17-one (steroid-based compound **5**). Steroid-based compound **5** was produced using a similar procedure as steroid-based compound **2**. Estrone (2.2 g, 8.14 mmol), ethyl formate (9.03 g, 122 mmol) and  $\text{NaOEt}$  (5.54 g, 81.4 mmol) were used to produce compound **5** (2.05 g, 84.4%).  $^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  10.67 (d,  $J=7.3$  Hz, 1H), 9.01 (s, 1H), 7.38 (d,  $J=5.1$  Hz, 1H), 7.03 (d,  $J=8.5$  Hz, 1H),

6.57-6.38 (m, 2H), 2.83-2.61 (m, 2H), 1.72-2.39 (m, 5H), 1.56-1.19 (m, 5H), 0.79 (s, 2H).  $^{13}\text{C}$  NMR (100 MHz, DMSO)  $\delta$  209.05, 155.40, 151.11, 137.52, 130.49, 126.34, 115.38, 113.46, 113.18, 48.84, 48.19, 43.91, 37.91, 31.87, 29.46, 26.72, 26.09, 24.48, 14.82.

MS(ESI+)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{22}\text{O}_3$  [M + H]<sup>+</sup> 299.2, found 299.2. HRMS (EI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{28}\text{O}_3$  [M + H]<sup>+</sup> 299.1602, found 299.1775.

*(Z)*-16-(aminomethylene)-3-hydroxy-13-methyl-6,7,8,9,11,12,13,14,15,16-decahydro-17H-cyclopenta[a]phenanthren-17-one (steroid-based compound **6**). Steroid-based compound **6** was obtained in a similar method as steroid-based compound **3**. 343 mg of steroid derivative compound **5** (1.15 mmol) was dissolved in ethanol, and 10 ml of ammonium hydroxide was added. After reflux and recrystallization, the product of compound **6** (125 mg, 36.5%) was obtained.

$^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  9.12 (s, 1H), 7.98 (s, 1H), 7.23-6.87 (m, 2H), 6.65-6.53 (m, 2H), 2.93-2.77 (m, 1H), 2.47-2.12 (m, 3H), 2.06-1.80 (m, 2H), 1.65-1.18 (m, 5H), 0.88 (d,  $J=14.8$  Hz, 3H).  $^{13}\text{C}$  NMR (100 MHz, DMSO)  $\delta$  206.80, 154.87, 141.12, 137.25, 130.47, 125.99, 115.02, 112.81, 104.81, 49.35, 47.33, 43.74, 39.29, 38.87, 37.58, 31.83, 29.11, 26.55, 25.83, 24.67, 14.85.

MS(ESI+)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{23}\text{NO}_3$  [M + H]<sup>+</sup> 297.2, found 297.2. HRMS (EI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{23}\text{NO}_3$  [M + H]<sup>+</sup> 298.1762 found 298.2479.

*Absence of copper coordinating capability of steroid derivative compounds.* Steroid-based compounds were evaluated for their direct copper coordinating ability to determine whether these steroid-based compounds have strong copper binding affinity similar to copper chelators CQ or TM. No significant change in free copper ions was detected in the  $\text{CuCl}_2$  solution mixed with steroid-based compounds **3** and **6**, in a 1:1 ratio of ligand to copper concentration (Table I) or a 2:1 ratio of ligand to copper concentration. In contrast, a reduction in free copper was observed in the  $\text{CuCl}_2$  solution mixed with copper chelator CQ or TM (Table I and Table II). Two CQ removed about one copper ion, while one TM removed one copper in these solutions at all conditions, indicating that CQ and TM chelated copper in a ratio of 2:1 and 1:1, respectively. This was also shown by MS, with the following peaks of copper chelates with CQ ( $\text{CuCQ}_2$ , calcd for  $\text{C}_{18}\text{H}_{10}\text{Cl}_2\text{I}_2\text{N}_2\text{O}_2\text{Cu}$  [M + H]<sup>+</sup> 672.7507) and TM ( $\text{CuTM}$ , calcd for  $\text{H}_8\text{N}_2\text{S}_4\text{MoCu}$  [M + H]<sup>+</sup> 260.2769). No peaks of copper complexes were found in the  $\text{CuCl}_2$  solution mixed with compound **3** or **6**. These findings suggest that the steroid-based compounds synthesized in this study do not have high copper binding affinity of copper chelators such as CQ and TM.

Table I. Free copper concentration after incubation of 25  $\mu\text{M}$  of copper and 25  $\mu\text{M}$  of steroid-based compounds or copper chelators (ligands:copper=1:1, volume) at 37°C for 1, 12, and 24 h. Copper concentration in solution of  $\text{CuCl}_2$  mixed with compounds **3** and **6** was similar to the copper concentration in the  $\text{CuCl}_2$  solution mixed with dimethylsulfoxide (DMSO) of the control. CQ removed about half of copper ions, while TM removed almost all copper ions in the solution.

Mixture	1 h	12 h	24 h
Compound <b>3</b> : $\text{CuCl}_2$	23.8 $\pm$ 0.56	24.1 $\pm$ 0.85	25.0 $\pm$ 0.56
Compound <b>6</b> : $\text{CuCl}_2$	24.1 $\pm$ 0.39	24.0 $\pm$ 1.0	24.2 $\pm$ 1.08
CQ : $\text{CuCl}_2$	13.1 $\pm$ 0.43	12.8 $\pm$ 0.59	12.4 $\pm$ 0.16
TM : $\text{CuCl}_2$	6.17 $\pm$ 0.51	4.60 $\pm$ 0.45	3.25 $\pm$ 0.28
DMSO : $\text{CuCl}_2$	24.8 $\pm$ 0.34	24.6 $\pm$ 0.49	25.8 $\pm$ 0.14

Table II. Free copper concentration after incubation with 25  $\mu\text{M}$  of copper and 25  $\mu\text{M}$  of steroid-based compounds or copper chelators (ligands:copper=2:1, volume) at 37°C for 1, 12, and 24 h. Copper concentration in solution of  $\text{CuCl}_2$  mixed with compounds **3** and **6** was similar to the copper concentration in  $\text{CuCl}_2$  solution mixed with dimethyl sulfoxide (DMSO) as a control. In contrast, CQ and TM removed most of the free copper from the  $\text{CuCl}_2$  solutions at a ratio of ligand:copper=2:1 (volume).

Mixture	1 h	12 h	24 h
Compound <b>3</b> : $\text{CuCl}_2$	15.5 $\pm$ 1.20	16.3 $\pm$ 0.58	17.5 $\pm$ 0.67
Compound <b>6</b> : $\text{CuCl}_2$	16.1 $\pm$ 0.49	14.9 $\pm$ 0.59	16.0 $\pm$ 0.64
CQ : $\text{CuCl}_2$	1.53 $\pm$ 0.51	1.81 $\pm$ 1.98	0.56 $\pm$ 0.28
TM : $\text{CuCl}_2$	4.69 $\pm$ 0.51	4.60 $\pm$ 0.34	4.78 $\pm$ 1.15
DMSO : $\text{CuCl}_2$	16.6 $\pm$ 0.21	16.3 $\pm$ 0.39	17.9 $\pm$ 0.61

**Reduction in copper uptake by PC-3 cells treated with steroid-based compounds.** Significant reduction in  $^{64}\text{Cu}$  uptake was detected in the cells treated with 50  $\mu\text{M}$  of steroid-based compounds for 24 h *in vitro* by the cellular  $^{64}\text{Cu}$  uptake assay (Figure 2A). Among the steroid-based compounds tested (compound **2**, **3**, **5**, and **6**), compound **6** showed the strongest inhibitory effect on cellular  $^{64}\text{Cu}$  uptake by PC-3 prostate cancer cells. Cellular  $^{64}\text{Cu}$  uptake was decreased 50% after treatment of cells with 10  $\mu\text{M}$  of compound **6** and cellular  $^{64}\text{Cu}$  uptake was decreased 80% when the cells were treated with 50  $\mu\text{M}$  of compound **6**. Reduction in cellular copper uptake after treatment with steroid-based compounds was further confirmed by copper quantification using ICP-MS. Copper levels increased from 4.03 $\pm$ 0.33 to 7.55 $\pm$ 0.80  $\mu\text{g}/\text{mg}$  protein after 24 h incubation of cells with 50  $\mu\text{M}$  of  $\text{CuCl}_2$ . Compared with the copper level of 7.55 $\pm$ 0.80  $\mu\text{g}/\text{mg}$  protein in the cells incubated with  $\text{CuCl}_2$ , cellular copper level was significantly decreased to 6.21 $\pm$ 0.42 and 5.61 $\pm$ 1.36  $\mu\text{g}/\text{mg}$  protein ( $p < 0.05$ ) after 24 h incubation of cancer cells with 50  $\mu\text{M}$  of compound **6** plus

$\text{CuCl}_2$  or 100  $\mu\text{M}$  of compound **6** plus  $\text{CuCl}_2$  (Figure 2B). No significant difference in copper concentration was found between the PC-3 cells treated with 50  $\mu\text{M}$  of compound **6**, 100  $\mu\text{M}$  of compound **6**, or cell culture medium, individually (Figure 2B).

**Suppression of prostate cancer cell proliferation by steroid-based compounds.** Suppressed proliferation of PC-3 cells was observed following incubation of cells with steroid-based compound **6** for 24, 48, 72 h (Figure 3). The  $\text{IC}_{50}$  value of compound **6** was calculated at 104.27  $\mu\text{M}$ , compared with the  $\text{IC}_{50}$  value of 122.06  $\mu\text{M}$  for compound **2**, 179.63  $\mu\text{M}$  for compound **3**, >200  $\mu\text{M}$  for TM as a control.

## Discussion

Copper is required for cell proliferation and plays a role in uncontrolled cancer cell proliferation (10, 11). Expression of hCtr1 was elevated in cancer cells to meet the increased demand of copper for the rapid growth of cancer cells (12). RNAi-mediated knockdown of hCtr1 was associated with reduction in  $^{64}\text{Cu}$  uptake by positron emission tomography/computed tomography imaging and growth inhibition of human prostate cancer xenograft tumors in mice (9). In 2015, Kadioglu *et al.* reported a docking analysis of steroid-based copper transporter 1 inhibitors (8). In this study, we designed and synthesized several new steroid-based compounds using cimetidine as the lead compound. Significant reduction in copper uptake by PC-3 prostate cancer cells treated with steroid-based compounds was demonstrated by the cellular  $^{64}\text{Cu}$  uptake assay (Figure 2A) and copper quantification by ICP-MS (Figure 2B). Steroid-based compound **6** showed the strongest reduction effect on cellular copper uptake among the new steroid-based compounds synthesized in this study. In contrast to the copper chelators CQ and TM, the steroid-based compounds synthesized in this study (compounds **3** and **6**) did not show high copper binding affinity as measured by the copper coordinating assay (Table I and Table II).

We also further tested the effects of compound **6** on the proliferation of prostate cancer cells. As expected, proliferation of PC-3 prostate cancer cells was suppressed after treatment with compound **6** (Figure 3). Copper chelators such as TM have been clinically tested as anti-copper cancer therapy; however, the clinical application of anti-copper cancer therapy with TM was hampered by the side effects of global copper depletion, such as bone marrow suppression manifested as anemia (13). The  $\text{IC}_{50}$  value of steroid-based compound **6** was calculated at 104.27  $\mu\text{M}$  compared with the  $\text{IC}_{50}$  value >200  $\mu\text{M}$  of TM. The findings of this study support further investigation of steroid-based compounds as potential hCtr1 inhibitors, compatible with the previous docking analysis of steroid-based copper transporter

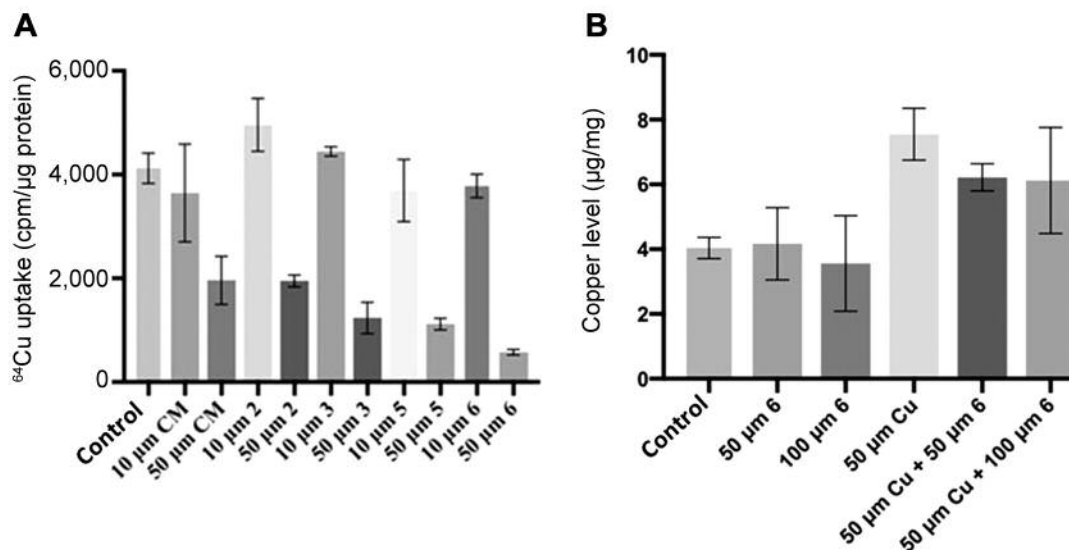


Figure 2. Reduction in copper uptake in cells treated with steroid-based compounds. A. Decreased  $^{64}\text{Cu}$  uptake was detected in PC-3 cells treated with 50  $\mu\text{M}$  of steroid-based compounds 2, 3, 5, or 6 as measured by the cellular  $^{64}\text{Cu}$  uptake assay. B. Reduced copper ions in PC-3 cells treated with compound 6 and  $\text{CuCl}_2$  by ICP-MS. Concentration of copper ions in PC-3 cells treated with 50  $\mu\text{M}$  of  $\text{CuCl}_2$  plus 50 or 100  $\mu\text{M}$  of compound 6 was significantly lower than the copper concentration of PC-3 cells treated with 50  $\mu\text{M}$  of  $\text{CuCl}_2$  ( $p < 0.05$ ), indicating a suppression of cellular copper uptake after treatment with compound 6. CM: Culture medium.

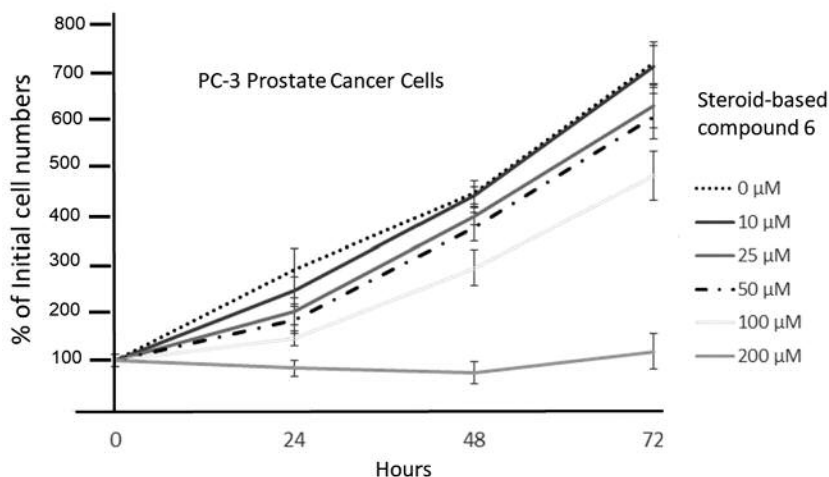


Figure 3. Proliferation of PC-3 cancer cells was inhibited after treatment with various concentrations of compound 6, in a time- and concentration-dependent manner.

1 inhibitors (8). Additional docking analysis of steroid-based compounds synthesized in this study, as well as binding affinity assay using hCtr1 molecules will be significant for determining whether steroid-based compounds are indeed hCtr1 inhibitors. Successful development of hCtr1 inhibitors will be highly significant for targeted anti-copper therapy of prostate cancer and other copper hypermetabolic cancers.

## Conclusion

Significant reduction in copper uptake was demonstrated by prostate cancer cells treated with the newly synthesized steroid-based compounds. Proliferation of prostate cancer cells was inhibited after treatment with steroid-based compound 6 that exhibited the strongest inhibitory activity on cellular

copper uptake of prostate cancer cells. The findings of this study provided strong evidence to support further investigation of steroid-based compounds as anti-prostate cancer agents targeting copper's role in cell proliferation. The steroid-based compounds hold potential for treatment of prostate cancer and other cancers that required copper for uncontrolled cell proliferation and tumor growth.

### Conflicts of Interest

The Authors declare that there are no conflicts of interest concerning this article.

### Authors' Contributions

Hypothesis and conceptual design of this study: FP; Synthesis and characterization of chemical compounds, and execution of experiments: FX; Data analysis and manuscript writing: FX and FP.

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