Using estrone and pregnenolone as starting materials, some steroidal copper complexes were synthesized by the condensation of steroid ketones with thiosemicarbazide or diazanyl pyridine and then complexation of steroidal thiosemicarbazones or steroidal diazanyl pyridines with Cu (II). The complexes were characterized by IR, NMR, and HRMS. The synthesized compounds were screened for their cytotoxicity against HeLa, Bel-7404, and 293T cell lines in vitro. The results show that all steroidal copper (II) complexes display obvious antiproliferative activity against the tested cancer cells. The IC_{50} values of complexes 5 and 12 against Bel-7404 (human liver carcinoma) are 5.0 and 7.0 μM.

1. Introduction

Metal-based antitumor drugs play a relevant role in antiblastic chemotherapy [1, 2]. Cisplatin is regarded as one of the most effective drugs [3–8], even if severe toxicities and drug resistance phenomena limit its clinical use [9]. Therefore, in recent years, there has been a rapid expansion in research and development of novel metal-based anticancer drugs in order to improve clinical effectiveness, reduce general toxicity, and broaden the spectrum of activity [10–12].

Copper (Cu) is a transition metal that can exist in oxidised and reduced states. This allows it to participate in redox and catalytic chemistry, making it a suitable cofactor for a diverse range of enzymes and molecules. Cu deficiency or toxicity is implicated in a variety of pathological conditions.

Steroid hormones play an important role in the biochemistry of many cancers; a number of steroidal complexes connected to a metal pharmacophore had been designed and synthesized by many research groups, and their physiological activities were evaluated [13, 14].

Thiosemicarbazones have received considerable attention since the discovery of their cytotoxic activity against cancer cells and bacteriostatic effects [15]. As the disruption of copper homeostasis is a pathological feature of cancer cells, copper complexes had been investigated for their potential applications as anticancer drugs [16]. Cu complexes of thiosemicarbazone (TSC) compounds had been explored as antimalarial, antifungal, antinociceptive, and antibacterial agents [17–19]. Cu complexes of bis(thiosemicarbazones) (CuII(btsc)s) had also been investigated as metallodrugs and diagnostic agents [20]. More recently, Adsule et al. [13] investigated the bioactivity of some new steroidal thiosemicarbazones Cu (II) metal complexes and discovered that some compounds had better antineoplastic activity.

In the present study, some steroidal copper complexes were synthesized by the condensation of steroidal ketones with thiosemicarbazide or diazanyl pyridine and then complexation of steroidal thiosemicarbazones or steroidal diazanyl pyridines with Cu (II). The synthesized compounds were screened for their cytotoxicity against HeLa, Bel-7404, and 293T cell lines in vitro.
2. Materials and Methods

2.1. Materials. The sterols were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. All chemicals and solvents are of analytical grade from commercial sources. All solvents were used without further purification unless otherwise specified.

2.2. Instrumentation and Methods. Melting points were determined on an X4 apparatus (Beijing Tech Instrument Co. Ltd., Beijing, China) and were uncorrected. The $^1$H and $^{13}$C NMR spectra were recorded in CDCl$_3$ on a Bruker AV-600 spectrometer at working frequencies of 600 and 150 MHz and a Bruker AV-300 spectrometer at working frequencies of 300 and 75 MHz, respectively. Chemical shifts are expressed in parts per million (δ) values and coupling constants (J) in Hertz. Infrared spectra were measured with a Thermo Scientific Nicolet IS-10 Spectrophotometer (Thermo Scientific, USA). HREIMS was measured on an Agilent 6210 TOFMS instrument (Agilent Technologies, USA). The cell proliferation assay was undertaken by a MTT method using 96-well plates on a MLLITSKAN MK3 analysis spectrometer (Thermo Scientific, Shanghai, China).

Compounds I ($L^1$) and 2 ($L^2$) were prepared according to the method of [21].

2.3. Synthesis

2.3.1. General Procedure for Preparation of Steroidal Thiosemicarbazones. Steroidal ketone (0.38 mmol) was dissolved in 40 mL 95% ethanol. After the solution was heated to 65 °C, a few drops of glacial acetic acid were added to adjust pH to 3–5, and thiosemicarbazide (1.70 mmol) was added. The mixture was stirred at 60–70 °C for 6 h (the progress of the reaction was monitored by TLC, $V_{ethyl acetate} : V_{petroleum ether} = 1:2$). Then, the reaction was terminated and majority of solvent was evaporated under reduced pressure. Suitable amount of water was added to the reaction mixture, and the product was extracted with CH$_2$Cl$_2$. The combined extract was washed with saturated NaHCO$_3$ solution, water, and saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The resulting residue was chromatographed on a column of silica gel with mixture of petroleum ether/ethyl acetate (1:1) to give steroidal diazanyl pyridine.

3β-Hydroxyoestrone-17-(2'-diazanyl)pyridine (9, $L^9$). Yellow solid, Yield: 56.0%; m.p. 269–271°C; IR (KBr) ν/cm$^{-1}$: 3361, 2935, 1601, 1576, 1444, 995, 871, 768; $^1$H NMR (600 MHz, DMSO) δ: 0.85 (3H, s, 18-CH$_3$), 2.36–2.30 (2H, m, C6-H and C9-H), 2.54 (1H, dd, J = 18.6, 9.0, C6-H), 2.70–2.69 (2H, m, C16-H), 6.45 (1H, d, J = 2.4, C4-H), 6.52 (1H, dd, J = 8.4, 2.4, C2-H), 6.67 (1H, t, J = 6.0, 5'-Py-H), 7.06 (1H, d, J = 8.4, C1-H), 7.07 (1H, d, J = 8.4, 3'-Py-H), 7.26 (1H, td, J = 8.4, 14', 4'-Py-H), 8.04 (1H, d, J = 3.6, 6'-Py-H), 8.98 (1H, s, -OH), 9.04 (1H, s, -OH); $^{13}$C NMR (150 MHz, DMSO) δ: 17.3 (C8), 23.0 (Cl1), 26.1 (C2), 26.9 (15'-C), 29.2 (7-C), 33.4 (6-C), 38.0 (12-C), 40.1 (8-C), 43.8 (9-C), 44.2 (13-C), 52.2 (14-C), 106.4 (3'-Py-C), 112.8 (2-C), 114.3 (4-C), 115.0 (5'-Py-C), 126.1 (1-C), 130.3 (10-C), 137.2 (4'-Py-C), 137.6 (5-C), 147.5 (6'-Py-C), 155.0 (3-C), 158.3 (2'-Py-C), 162.9 (17-C); HREIMS: [M+H]$^+$ 362.2250 (calcd for C$_{23}$H$_{38}$N$_2$O$_5$S, 362.2232).

3β-Hydroxyprogrenolone-20-(2'-diazanyl)pyridine (10, $L^{10}$). Yellow solid, Yield: 78.8%; m.p. 234–235°C; IR (KBr) ν/cm$^{-1}$: 3406, 2932, 1599, 1574, 1442, 838, 768; $^1$H NMR (600 MHz, DMSO) δ: 0.55 (3H, s, 18-CH$_3$), 0.94 (3H, s, 19-CH$_3$), 1.89 (3H, s, 20-CH$_3$), 3.35–3.20 (1H, m, C3-NH), 4.64 (1H, br s, NH), 5.27 (1H, s, C6-H), 6.69 (1H, t, J = 6.6, 5'-pyridine-H), 7.06 (1H, d, J = 7.2, 3'-pyridine-H), 7.57 (1H, t, J = 7.2, 4'-pyridine-H), 8.05 (1H, d, J = 6.6, 6'-pyridine-H), 9.07 (s, s, -OH); HREIMS: m/z 408.3024 [M+H]$^+$ (calcd for C$_{26}$H$_{36}$N$_2$O$_4$, 408.3015).

2.3.3. General Procedure for Preparation of Copper Complexes. Steroidal ligand (0.1 mmol) and 0.1 mmol CuCl$_2$-2H$_2$O were added to 8 mL of methanol. The mixture was stirred for 5 hour at 70 °C. The reaction was terminated when large
precipitant emerged. The resulting suspension was filtered, washed with ethyl acetate and water, and dried in a desiccator over phosphorus pentoxide to give target products.

[CuLCl₂]⁺ (Compound 5). Compound 5 is a mixture of (S)- and (R)-configuration isomer (5-S: 5-R = 1.7:1, ¹H NMR data). Gray yellow solid, Yield: 55%; m.p. 245–247°C; IR (KBr) ν/cm⁻¹: 3441, 1604, 1541, 1409, 1452, 1280, 811, 616; ¹H NMR (300 MHz, DMSO) δ: 0.81 (s, 1.07H, 18-CH₂–R), 0.86 (s, 1.71H, 18-CH₂–S), 6.44 (s, 1H, C4-H), 6.50 (d, J = 4.5, C2-H), 7.05 (d, J = 4.5, C1-H), 0.77 (s, 0.19H, -NH₂–R), 8.00 (s, 0.31H, -NH₂–S), 8.65 (s, 0.36H, -NH₂–S), 8.85 (s, 0.25H, -NH₂–R), 9.03 (s, 1H, -OH), 10.30 (s, 0.34H, -NH₂–S), 10.64 (s, 0.20H, -NH₂–R).

[CuLCl₂]⁻ (Compound 6). Compound 6 is a mixture of (S)- and (R)-configuration isomer (6-S: 6-R = 1.5:1, ¹H NMR data). Gray solid, Yield: 66.7%; m.p. 189–190°C; IR (KBr) ν/cm⁻¹: 3416, 2927, 1604, 1534, 1496, 1447, 1776, 816, 751; ¹H NMR (600 MHz, DMSO) δ: 0.85 (s, 1.85H, 18-CH₂–S), 1.01 (s, 1.25H, 18-CH₂–R), 2.21 (s, 3H, COCH₃), 6.79 (s, 1H, C4-H), 6.83 (d, J = 6.0, C2-H), 7.29 (s, J = 6.0, Cl–H), 7.73 (s, 0.38H, -NH₂–S), 8.04 (s, 0.45H, -NH₂–S), 8.68 (s, 0.48H, -NH₂–R), 8.84 (s, 0.33H, -NH₂–R), 9.023 (s, 1H, -OH), 10.31 (s, 0.40H, -NH₂–S), 10.63 (s, 0.33H, -NH₂–R); ¹³C NMR (150 MHz, DMSO) δ: 16.5 (18-C), 20.6 (CH₂CO), 22.3 (11-C), 26.1 (15-C), 26.4 (14-C), 28.6 (7-C), 28.8 (6-C), 33.5 (12-C), 37.1 (8-C), 37.5 (8-C), 43.1 (9-C), 44.6 (13-C), 51.6 (14-C), 118.6 (2-C), 121.2 (4-C), 126.0 (1-C), 136.8 (5-C), 137.4 (10-C), 148.0 (3-C), 154.7 (17-C), 169.1 (COCH₃), 170.1 (C=S), 171.3 (C=S).

[CuLCl₂]⁻ (Compound 7). Compound 7 is a mixture of (S)- and (R)-configuration isomer (7-S: 7-R = 1:1.5; ¹H NMR data). Gray solid, Yield: 60.2%; m.p. 189–191°C; IR (KBr) ν/cm⁻¹: 3319, 1604, 1531, 1434, 1369, 1290, 1050, 953; ¹H NMR (600 MHz, DMSO) δ: 0.51 (s, 1.2H, 18-CH₂–S), 0.68 (s, 1.8H, 18-CH₂–R), 0.91 (s, 3H, 19-CH₃–S), 1.96 (s, 1.2H, 21-CH₃–S), 2.00 (s, 1.8H, 21-CH₃–R), 3.23–3.12 (m, 1H, C3–H), 4.61 (s, 1H, OH), 5.24 (s, 1H, C6–H), 7.75 (s, 0.6H, -NH₂–R), 8.01 (s, 0.4H, -NH₂–S), 8.73 (s, 0.4H, -NH₂–S), 8.95 (s, 0.6H, -NH₂–R), 10.29 (s, 0.4H, -NH₂–S), 10.89 (s, 0.6H, -NH₂–R); ¹³C NMR (150 MHz, DMSO) δ: 12.7 (19-C), 18.7 (18-C), 20.3 (11-C), 20.8 (21-C), 21.9 (15-C), 23.7 (16-C), 27.0 (2-C), 31.0 (8-C), 31.7 (7-C), 35.8 (1-C), 36.6 (10-C), 37.4 (4-C), 41.9 (12-C), 43.0 (13-C), 49.2 (9-C), 55.7 (17-C), 58.2 (14-C), 69.7 (3-C), 121.7 (6-C), 139.2 (5-C), 140.9 (20-C), 169.5 (C=S).

[CuLCl₂]⁻ (Compound 8). Compound 8 is a mixture of (S)- and (R)-configuration isomer (8-S: 8-R = 1:3.1; ¹H NMR data). Gray solid, Yield: 75%; m.p. 163–165°C; IR (KBr) ν/cm⁻¹: 3424, 1723, 1596, 1534, 1432, 1364, 1040; ¹H NMR (600 MHz, DMSO) δ: 0.50 (s, 1.7H, 18-CH₂–S), 0.47 (s, 1.07H, 18-CH₂–R), 0.94 (s, 3H, 19-CH₃–S), 1.95 (s, 1.71H, 21-CH₃–S), 1.98 (s, 1.3H, 21-CH₃–R), 2.24 (s, 3H, COCH₃), 7.75 (s, 0.43H, -NH₂–R), 7.962 (s, 0.49H, -NH₂–R), 8.666 (s, 0.51H, -NH₂–R), 8.929 (s, 0.52H, -NH₂–S), 10.276 (s, 0.56H, -NH₂–R); ¹³C NMR (150 MHz, DMSO) δ: 12.7 (19-C), 18.7 (18-C), 20.3 (11-C), 20.8 (21-C), 21.9 (15-C), 23.7 (16-C), 27.0 (2-C), 31.0 (8-C), 31.1 (7-C), 35.8 (1-C), 36.6 (10-C), 37.4 (4-C), 41.9 (12-C), 43.0 (13-C), 49.2 (9-C), 55.7 (17-C), 58.2 (14-C), 69.7 (3-C), 121.7 (6-C), 139.2 (5-C), 140.9 (20-C), 169.9 (C=S).
Further that compound 7 is the mixture of (S)- and (R)-configuration isomers (7-S:7-R = 1:1.5, 1H NMR data) from the chemical shift of 18-CH₃ and 21-CH₃ (18-CH₃: 0.51 (1.2H, S-), 0.68 (1.8H, R-) ppm; 21-CH₃: 1.96 (1.2H, S-), 2.00 (1.8H, R-) ppm).

In order to investigate the effect of different ligand on the antiproliferative activity of complexes, 3β-hydroxyestrone-17-(2′-diazanyl)pyridine-Copper(II) 11 and 3β-Hydroxyprogrenolone-20-(2′-diazanyl) pyridine-Copper (II) 12 were synthesized according to Scheme 2. Ligands 9 and 10 were obtained as a (E)-configuration by reacting estrone or pregnenolone with 2-hydrazinopyridine. Furthermore, the reaction of compounds 9 and 10 with CuCl₂·2H₂O gave steroidal copper (Cu(II)) complexes 11 and 12 as (S)- and (R)-configuration, respectively. The structures of 11 and 12 were confirmed by analysis of IR, NMR, and HRMS.

3.2. Cytotoxic Activity In Vitro. The antiproliferative activities of all steroidal Cu(II) metal complexes were determined in vitro on Bel-7404 (human liver carcinoma), HeLa (human cervical carcinoma), and 293T (normal kidney epithelial) cell lines. The MTT method was used to assay the antiproliferative activity and cisplatin was used as a positive control. The results are summarized as IC₅₀ values in μM in Table 1.

From the data shown in Table 1, all steroidal copper (Cu(II)) complexes show an obvious antiproliferative activity...
against the tested cancer cells. The compounds 5–7 display a better activity to Bell-7404 and HeLa cells compared to that of cisplatin. Comparing the antiproliferative activity of steroidal thiosemicarbazone ligands with that of their copper (II) complexes, we can see that steroidal thiosemicarbazone copper (II) complexes show a better inhibiting activity compared to their homologous ligands (1 versus 5 and 2 versus 6). Particularly, complexes 5 and 7 show an excellent antiproliferative activity against Bell-7404 cells with the IC₅₀ values of 5.0 and 9.5 μM, and complexes 6 and 7 possess IC₅₀ values of 7.7 and 6.8 μM against HeLa cells.

Comparing compound 7 with compound 8, we can observe that after 3-hydroxyl group of 7 was converted into 3-acetoxy group (compound 8), the antiproliferative activity of the compound was remarkably decreased and the cytotoxicity to normal cells 293T was increased. The result shows that 3-hydroxyl of the compound to the antiproliferative activity plays an important role.

Unfortunately, these steroidal copper (Cu(II)) complexes to normal kidney epithelial cells (293T) show similar cytotoxicity except for compound 5 which exhibits a smaller activity to 293T cells compared to cisplatin (27 μM versus 10.3 μM).

4. Conclusion

In conclusion, using estrone and pregnenolone as starting materials, through different chemical methods, some steroidal copper (II) complexes were synthesized and characterized by IR, NMR, and HRMS. Their antiproliferative activities were assayed by MTT method. The results show that all steroidal copper (II) complexes display obvious antiproliferative activity against the tested cancer cells, and compounds 5–7 show better cytotoxicity compared to a positive control, cisplatin. Among them, complexes 5 and 12 show an excellent antiproliferative activity against Bell-7404 cells with the IC₅₀ values of 5.0 and 7.0 μM, and complexes 6 and 7 possess IC₅₀ values of 7.7 and 6.8 μM against HeLa cells. The result may be useful for the design of novel chemotherapeutic drugs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] B. Rosenberg, L. Van Camp, and T. Krigas, “Inhibition of cell division in Escherichia coli by electrolysis products from a platinum electrode,” Nature, vol. 205, no. 4972, pp. 698–699, 1965.
[2] B. Rosenberg, L. Van Camp, E. B. Grimley, and A. J. Thomson, “The inhibition of growth or cell division in Escherichia coli by different ion species of platinum(IV) complexes,” Journal of Biological Chemistry, vol. 242, no. 6, pp. 1347–1352, 1967.
[3] S. M. Shovata, F. Bettio, M. Mozzon et al., “Cisplatinum and transplatinum complexes with benzylmiminoether ligands; synthesis, characterization, structure-activity relationships, and in vitro and in vivo antitumor efficacy,” Journal of Medicinal Chemistry, vol. 50, no. 19, pp. 4775–4784, 2007.
[4] D. Kovala-Demertzis, A. Galani, N. Kourkoumelis, J. R. Miller, and M. A. Demertzis, “Synthesis, characterization, crystal structure and antiproliferative activity of platinum(II) complexes with 2-acetylpyridine-4-cyclohexyl-thiosemicarbazone,” Polyhedron, vol. 26, no. 12, pp. 2871–2879, 2007.
[5] X. Liu, H. Shen, H. Zhu, K. Cui, and S. Gou, “In vitro cytotoxicity study on platinum(II) complexes with epoxysuccinates as leaving groups,” Bioorganic and Medicinal Chemistry Letters, vol. 17, no. 14, pp. 3831–3834, 2007.
[6] J. Zhang and X. Zhao, “Synthesis, cytotoxicity and DNA-binding levels of ammine/proplyamine platinum(II) complexes with carboxylates,” European Journal of Medicinal Chemistry, vol. 42, no. 2, pp. 286–291, 2007.
[7] A. S. Abu-Surrah and M. Kettunen, “Platinum group antitumor chemistry: design and development of new anticancer drugs complementary to cisplatin,” Current Medicinal Chemistry, vol. 13, no. 11, pp. 1337–1357, 2006.
[8] G. Momokov, A. Bakalova, and M. Karaivanova, “Novel approaches towards development of non-classical platinum-based antineoplastic agents: design of platinum complexes characterized by an alternative DNA-binding pattern and/or tumor-targeted cytotoxicity,” Current Medicinal Chemistry, vol. 12, no. 19, pp. 2177–2191, 2005.
[9] K. Barabas, R. Milner, D. Lurie, and C. Adin, “Cisplatin: a review of toxicities and therapeutic applications,” Veterinary and Comparative Oncology, vol. 6, no. 1, pp. 1–18, 2008.
[10] J. Shao, Z.-Y. Ma, A. Li et al., “Thiosemicarbazone Cu(II) and Zn(II) complexes as potential anticancer agents: syntheses, crystal structure, DNA cleavage, cytotoxicity and apoptosis induction activity,” Journal of Inorganic Biochemistry, vol. 136, pp. 13–23, 2014.
[11] G. Tamasi, C. Bernini, G. Corbini et al., “Synthesis, spectroscopic and DFT structural characterization of two novel
ruthenium(III) oxicam complexes. In vivo evaluation of anti-inflammatory and gastric damaging activities,” *Journal of Inorganic Biochemistry*, vol. 134, pp. 25–35, 2014.

[12] B. Božić, J. Rogan, D. Poleti, N. Trišović, B. Božić, and G. Ušćumić, “Synthesis, characterization and antiproliferative activity of transition metal complexes with 3-(4,5-diphenyl-1,3-oxazol-2-yl)propanoic acid (oxaprozin),” *Chemical and Pharmaceutical Bulletin*, vol. 60, no. 7, pp. 865–869, 2012.

[13] S. Adsule, S. Banerjee, F. Ahmed, S. Padhye, and F. H. Sarkar, “Hybrid anticancer agents: isothiocyanate-progesterone conjugates as chemotherapeutic agents and insights into their cytotoxicities,” *Bioorganic and Medicinal Chemistry Letters*, vol. 20, no. 3, pp. 1247–1251, 2010.

[14] G. A. G. Santos, A. P. Murray, C. A. Pujol, E. B. Damonte, and M. S. Maier, “Synthesis and antiviral activity of sulfated and acetylated derivatives of 2β,3α-dihydroxy-5α-cholestan,” *Steroids*, vol. 68, no. 2, pp. 125–132, 2003.

[15] H. Huang, Q. Chen, X. Ku et al., “A series of α-heterocyclic carboxaldehyde thiosemicarbazones inhibit topoisomerase IIA catalytic activity,” *Journal of Medicinal Chemistry*, vol. 53, no. 8, pp. 3048–3064, 2010.

[16] C. Duncan and A. R. White, “Copper complexes as therapeutic agents,” *Metalomics*, vol. 4, no. 2, pp. 127–138, 2012.

[17] D. X. West and A. E. Liberta, “Thiosemicarbazone complexes of copper(II): structural and biological studies,” *Coordination Chemistry Reviews*, vol. 123, no. 1-2, pp. 49–71, 1993.

[18] H. Beraldo and D. Gambino, “The wide pharmacological versatility of semicarbazones, thiosemicarbazones and their metal complexes,” *Mini-Reviews in Medicinal Chemistry*, vol. 4, no. 1, pp. 31–39, 2004.

[19] F. Tisato, C. Marzano, M. Porchia, M. Pellei, and C. Santini, “Copper in diseases and treatments, and copper-based anticancer strategies,” *Medicinal Research Reviews*, vol. 30, no. 4, pp. 708–749, 2010.

[20] J. L. Dearling, J. S. Lewis, G. E. Mullen, M. J. Welch, and P. J. Blower, “Copper bis(thiosemicarbazone) complexes as hypoxia imaging agents: structure-activity relationships,” *Journal of Biological Inorganic Chemistry*, vol. 7, no. 3, pp. 249–259, 2002.

[21] Y. Huang, E. Kong, C. Gan, Z. Liu, Q. Lin, and J. Cui, “Synthesis and antiproliferative activity of steroidal thiosemicarbazone platinum (Pt(III)) complexes,” *Bioinorganic Chemistry and Applications*, vol. 2015, Article ID 742592, 7 pages, 2015.

[22] C. Gan, J. Cui, S. Su et al., “Synthesis and antiproliferative activity of some steroidal thiosemicarbazones, semicarbazones and hydrozones,” *Steroids*, vol. 87, pp. 99–107, 2014.