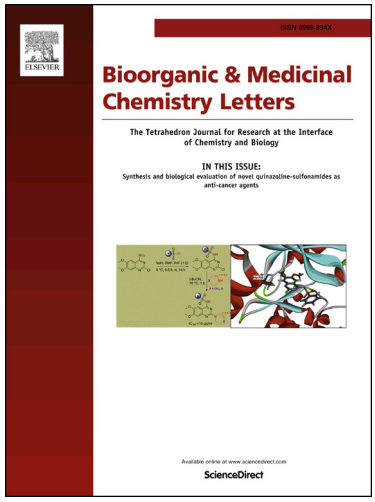

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The Synthesis, Structure-Toxicity Relationship of Cisplatin Derivatives for the Mechanism Research of Cisplatin-induced Nephrotoxicity

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Abstract:

Cisplatin is a widely used antineoplastic drug, while its nephrotoxicity limits the clinical application. Although several mechanisms contributing to nephrotoxicity have been reported, the direct protein targets are unclear. Herein we reported the synthesis of 29 cisplatin derivatives and the structure-toxicity relationship (STR) of these compounds with MTT assay in human renal proximal tubule cells (HK-2) and pig kidney epithelial cells (LLC-PK1). To the best of our knowledge, this study represented the first report regarding the structure-toxicity relationship (STR) of cisplatin derivatives. The potency of biotin-pyridine conjugated derivative **3** met the requirement for target identification, and the preliminary chemical proteomics results suggested that it is a promising tool for further target identification of cisplatin-induced nephrotoxicity.

Keywords: Cisplatin, nephrotoxicity, Structure-toxicity relationship (STR), biotin labeling, chemical proteomics, target identification

In recent years, target identification of small bioactive molecules received increasing attention for its important role in both academic and pharmaceutical

research.[1-4] For instance, the target identification of thalidomide teratogenicity [5, 6] offered a hint to the development of novel thalidomide drugs without teratogenic activity.

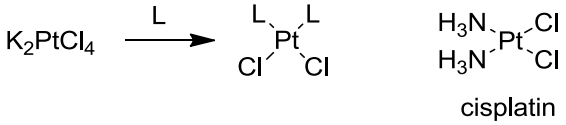
Cisplatin (*cis*-diamminedichloroplatinum (II), CDDP) is one of the most important anti-cancer drugs, which is widely used in the treatment of various solid tumors, e.g., germ cell tumor, head and neck cancer, ovarian cancer and bladder cancer. However, its clinical application is greatly hampered by its side effects, including nephrotoxicity, neurotoxicity, ototoxicity, etc.[7] Among them, nephrotoxicity is the most severe adverse effect of cisplatin for clinic patients.[8]

After decades of investigation, several mechanisms contributing to nephrotoxicity have been uncovered. DNA has conventionally been considered as the target, as cisplatin can crosslink with DNA, thereby interfering with DNA repair and causing DNA damage, and eventually inducing apoptosis in cancer cells.[9] Some cell death pathways were found to be involved in nephrotoxicity.[10] Cisplatin can also be transported into renal epithelial cells by some specific transporters, such as organic cation transporters (OCT) and copper transporter 1 (Ctr1), contributing to the accumulation of cisplatin in kidney cells.[11] Cisplatin can be metabolized to a reactive toxic thiol derivative through a biotransformation pathway that requires γ-glutamyl transpeptidase, aminopeptidase and renal dipeptidase.[12] Moreover, oxidative stress, inflammation and immunity are believed to be involved in nephrotoxicity as well.[13, 14]

Although many mechanistic studies of cisplatin-induced neurotoxicity have been reported previously, the direct protein targets are unclear. Among the frequently used protocols for target identification, biotin is a widely used tag. Therefore, a biotin-conjugated cisplatin derivative with comparable cisplatin-induced neurotoxicity will be of great help for target identification. In this study, we synthesized for the first time such a derivative as **3** (Scheme 2) on the basis of synthesis of a series of cisplatin derivatives and demonstrated that **3** might be a powerful tool for target identification based on the STR studies and pull-down assays.

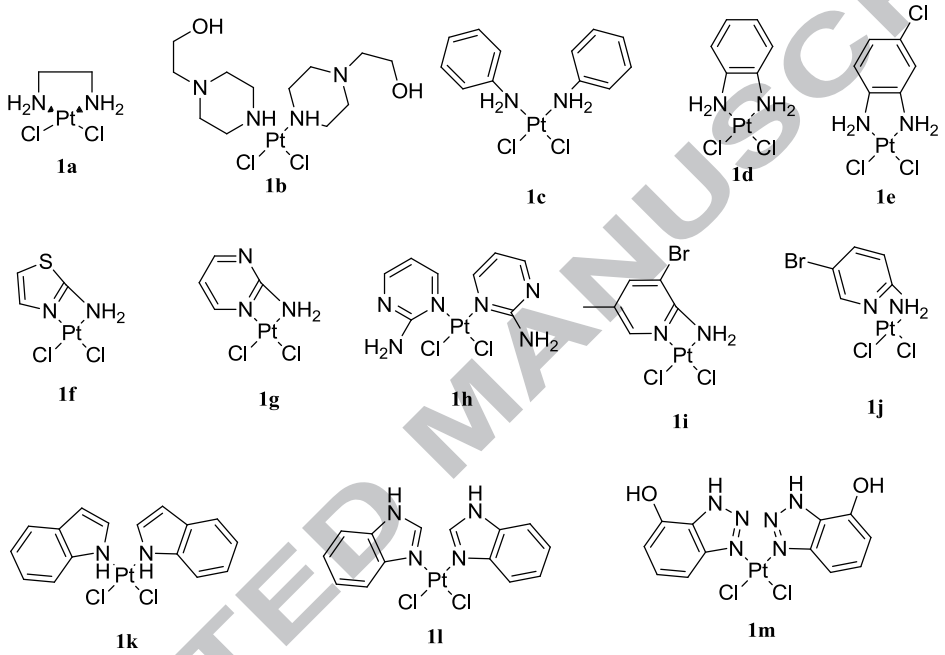
As illustrated in Scheme 1 and Figure 1, 29 cisplatin derivatives were prepared in parallel through the reaction of various ligands with K_2PtCl_4 by reported methods

(Scheme1).[15,16]



Scheme 1. Synthetic scheme for cisplatin derivatives. L denotes ligand.

Group 1: Cisplatin derivatives **1a-1m** in the first round screening



Group 2: Cisplatin derivatives **2a-2o** in the second round screening

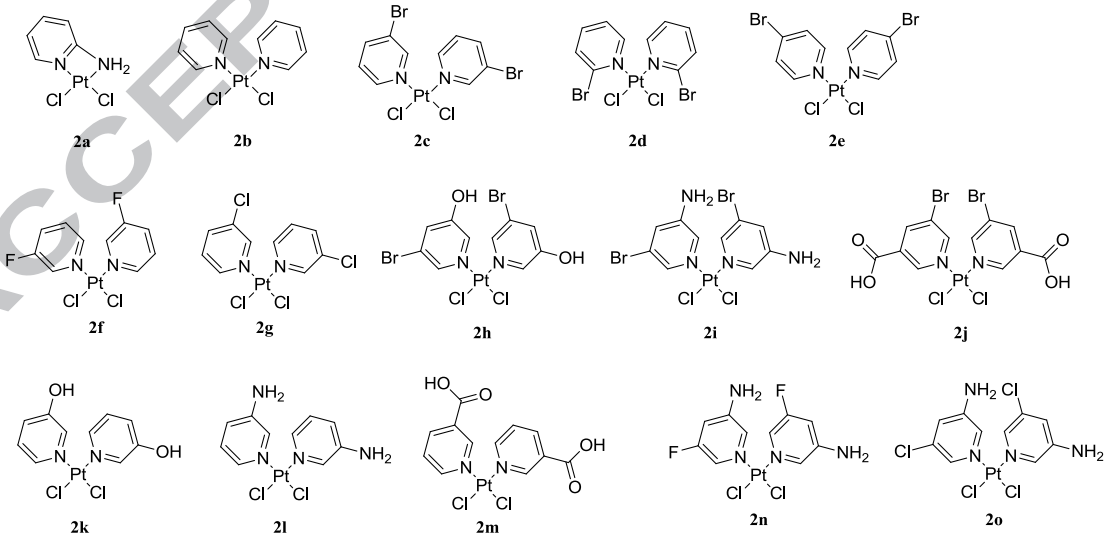


Figure 1. Structure of cisplatin derivatives **1a-1m** and **2a-2o**

The renal epithelial toxicity of these compounds was evaluated in human renal

proximal tubule cells (HK-2) and pig kidney epithelial cells (LLC-PK1) by methyl thiazolyl tetrazolium assay (MTT).[17] The results are summarized in Table 1.

In the first round screening, compounds **1a** (aliphatic amine), **1b** (alicylic amine), **1c-1e** (aniline) and **1f-1m** (heteroarylic amine) were selected based on the structure-diversity exploration of ligand. The toxicity study showed that compounds **1d**, **1i** and **1j** exhibited comparable renal epithelial toxicity to cisplatin, indicating pyridine is a promising ligand for further study, given the easy availability and promising potency.

In the second round screening, the substitute effects on the pyridine ring were first studied, with 2-aminopyridine (**2a**), unsubstituted pyridine (**2b**) or 3-bromide pyridine (**2c**) to determine whether the bromine or amino group was required. It could be seen in Table 1 that compound **2c** exhibited comparable activity to cisplatin, indicating that the bromine, but not the amino group, was essential for nephrotoxicity.

To further explore the effect of halogen position and halogen type on the pyridine ring, 4 compounds (**2d-2g**) were synthesized. The results indicated that the position of bromine greatly affected the activity of the compound, both the *ortho*- (**2d**) and *para*-position (**2e**) reduced the potency. Replacement of the bromine on the pyridine ring with fluorine (**2f**), or chloride (**2g**) groups yielded compounds with comparable potency and without clear STR trends.

As the aim of present study was to tag cisplatin derivatives with biotin, the compounds with different reactive groups, amino, hydroxyl and carboxyl attached to pyridine were assessed (**2h-2j**). It was noted that the type of reactive group had great impact on cell viability. Derivative with amino analogue (**2i**) was highly toxic to renal epithelial cell lines, producing an extremely high level of cell death. However, cells could tolerate very well the derivative with hydroxyl or carboxyl group (**2h**, **2j**), and compounds without bromine had little activity on the viability of these cells (**2k-2m**).

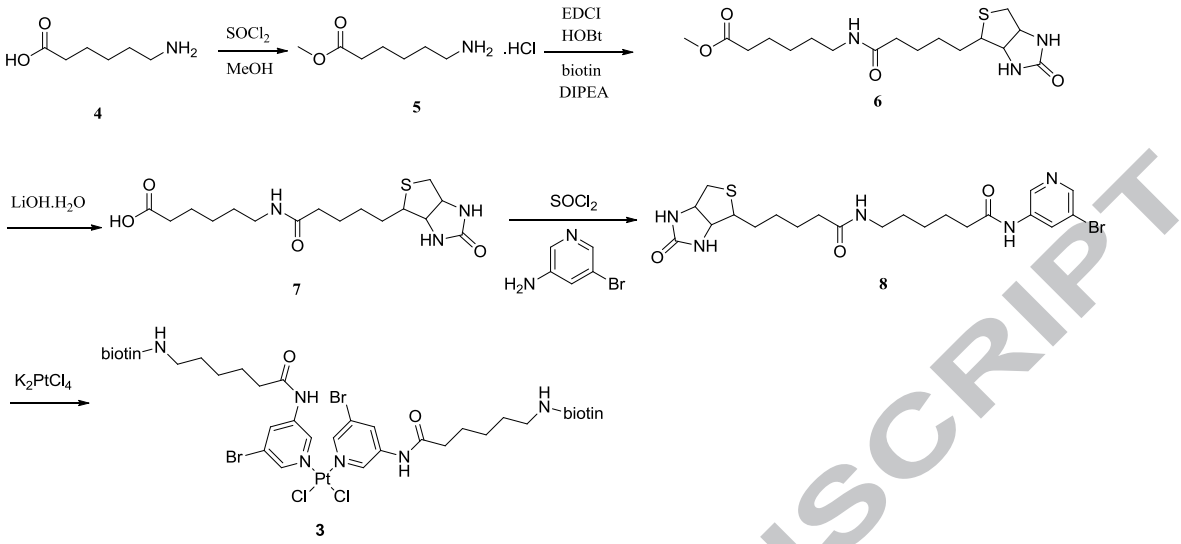
We also examined the antiproliferative effect of compounds with amino group and different halogens in renal epithelial cell lines (**2n, 2o**),and the activity was slightly reduced compared with their respective bromide analogues (**2i**).

Table 1. The nephrotoxicity profile of cisplatin and its analogues **1a-7a**, **2a-2o** and **3**.

Compounds (Ligand)	**IC_{50} (μM)**		**Compounds** (Ligand)	**IC_{50} (μM)**	
	HK-2	**LLC-PK1**		**HK-2**	**LLC-PK1**
Cisplatin	17.0	15.8	**2b** (pyridine)	32.0	>100
1a(ethylenediamine)	55.2	7.75	**2c** (3-Br Pyridine)	12.9	24.4
1b (*N*-(2-Hydroxyethyl)piperazine)	> 100	>100	**2d** (2-Br pyridine)	64.0	37.2
1c (aniline)	36.6	>100	**2e** (4-Br pyridine)	19.5	>100
1d (2-NH_2 aniline**)**	20.9	16.6	**2f** (3-F pyridine)	28.3	36.1
1e (4-Cl-2-NH_2 aniline**)**	>100	43.1	**2g** (3-Cl pyridine)	10.1	9.40
1f (2-NH_2 thiazole**)**	>100	>100	**2h** (3-Br 5-OH pyridine)	64.0	>100
1g (2-pyrimidinamine**)**	>100	>100	**2i** (5-Br 3-NH_2 pyridine)	19.8	26.2
1h (2-pyrimidinamine dimer**)**	78.4	37.0	**2j (**5-Br 3-COOH pyridine)	>100	>100
1i (2-NH_2-3-Br-5-methyl pyridine**)**	28.0	8.19	**2k** (3-OH pyridine)	33.1	>100
1j (2-NH_2-5-Br-pyridine**)**	26.2	27.6	**2l** (3-NH_2 pyridine)	32.0	>100
1k (indole)	> 100	>100	**2m** (3-COOH pyridine)	>100	>100
1l (benzothiazole)	> 100	> 100	**2n** (3-F 5-NH_2 pyridine)	45.8	41.6
1m (HOBT)	> 100	49.4	**2o** (3-Cl 5-NH_2 pyridine)	34.6	36.3
2a (2-NH_2 pyridine**)**	> 100	>100	**8** （Ligand of **3**）	>100	>100
3 (biotinylated 3-Br 5-NH_2 pyridine)	44.8	53.2			

[a] The cytotoxic effects of various compounds on HK-2 and LLC-PK1 cells are determined by the MTT assay, and the results are expressed as the mean IC_{50} calculated from three independent experiments.

Based on the above STR studies, **2i** was selected for biotin labeling. As shown in Scheme 2, biotin-pyridine conjugated derivative **3** was synthesized *via* a 5-step synthesis route using **4** as starting material. Biotin was bound to 3-amino-5-bromopyridine with a 6-carbon amide linker.[18] Intermediates **5-7** and ligand **8** were prepared by reported methods [18, 19] with modifications presented in Scheme 2.[20-23]



Scheme 2. The synthesis of ligand **8** and cisplatin derivative **3**

The activity (Table 1) of the biotinylated cisplatin derivative **3** met the requirement for target identification. With derivatives **8** and **3**, the preliminary mechanism of cisplatin-induced nephrotoxicity was studied with the modified pull-down assay following the listed protocol.[24]

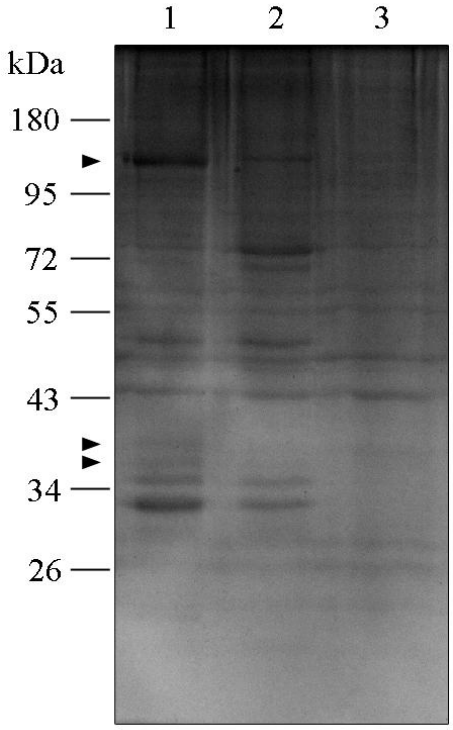


Figure 2. Proteins bind to biotinylated platinum compound in HeLa cell nuclear extracts. HeLa cell nuclear extracts were incubated with **3** (lane 1), **8** (lane 2) or DMSO (lane 3). **3**

specific/cisplatin related bands are indicated with solid arrow heads.

As shown in Figure 2, there are three specific bands for **3**, suggesting **3** has the potential for target identification.

In conclusion, a series of platinum derivatives were prepared and all the compounds were screened for cytotoxicity against HK-2 and LLC-PK1 cells. Among all the tested compounds, **2i** exhibited promising cytotoxicity and was used for biotinylation; the biotinylated compound **3** was able to inhibit HK-2 cell growth and bind some specific proteins. Based on the results of the present study, **3** may be prospectively used in target exploration of cisplatin-induced nephrotoxicity.

Acknowledgments

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Supplementary data

The list of experimental details and spectroscopic characterization of **1-8** are available online.

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16. Typical procedure for the synthesis of cisplatin analogues.

The solution of L was added dropwise to the water solution of K2PtCl4 at constant stirring. After the addition of the ligand, the solution was stirred for 2 days away from light. The precipitation was filtered, washed several times with ethyl ether and dried in vacuum desiccator.

17. Briefly, cells (5000/well) were seeded in 96-well plates and cultured for 24 hours, followed by treatment with the compounds for 48 h. 20 μL of 5 mg/mL MTT was added per well and incubated for another 4 h at 37°C. Then the supernatant was removed and 150 μL/well DMSO was added. The absorbance (OD) of each well was measured at 570 nm, using a Multiscan Mk3 elisa reader (Thermo Scientific).

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20. Thionyl chloride (1.4 equiv) was added dropwise to a stirred methanol suspension of amide linker 4 (1 equiv) at 0 ℃. The resulting solution was stirred overnight at room temperature. The solvent and thionyl chloride in excess were removed under reduced pressure.

21. HOBt (1.2 equiv), biotin (1.2 equiv), EDCI (1.2 equiv) and DIPEA (1 equiv) were dissolved in 100 mL DMF. Then added 5 (1 equiv) and DIPEA (3 equiv) in 50 mL DMF, and the solution was stirred overnight at room temperature. The reaction was diluted with 600 mL DCM, then washed with saturated solution of NaCl (3 × 80 mL), HCl 0.2 N (3 × 80 mL), saturated solution of NaHCO3 (1 × 80 mL), saturated solution of NaCl (1 × 80 mL). The solution was dried with magnesium sulfate and the remaining solvents were removed under reduced pressure.

22. Lithium hydroxide (4 equiv) solution was added to 6 (1 equiv) dissolved in methanol. The solution was heated to reflux overnight. The product 7 was precipitated as white solid. Then a hydrochloric acid solution (6 N) was added until the pH was 5. The product was filtered, and dried under vacuum.

23. Thionyl chloride (20 equiv) was added dropwise to dry solid 7 (1 equiv) at 0 ℃. The solution was stirred overnight at room temperature. The thionyl chloride in excess was removed under reduced pressure. Then the product was dissolved in DCM and added dropwise to 3-amino-5-bromopyridine (1 equiv) in DCM at 0 ℃. The resulting mixture was stirred overnight at room temperature. The product was filtered, washed with saturated solution of NaHCO3, and dried under vacuum.

24. HeLa cell nuclear extracts were prepared using KeyGEN nuclear and cytoplasmic protein

extraction kit according to the manuals. 200 μL nuclear extracts were incubated with 50 μM 3 (lane 1), 8 (lane 2) or same volume of DMSO (lane 3) on rotator at 4℃ for several hours. Then the extracts were incubated with 20 μL Pierce Streptavidin Agarose Resins(Thermo Fisher Scientific) on rotator at 4℃ overnight. The resins were washed twice with PBS and boiled in SDS-PAGE sample buffer. The samples were used for the analysis by SDS-PAGE, followed by Coomassie blue staining.

Graphical abstract

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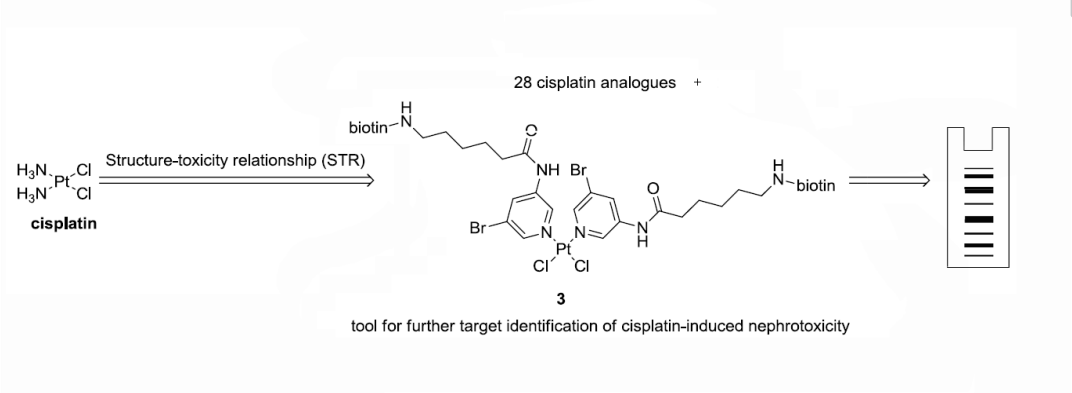

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