Journal of Inorganic Biochemistry 193 (2019) 133–142

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

A cisplatin-based platinum(IV) prodrug containing a glutathione s-transferase inhibitor to reverse cisplatin-resistance in non-small cell lung cancer

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ARTICLE INFO

Keywords:
Pt(IV) prodrug
Glutathione s-transferase
Cisplatin resistance
Non-small cell lung cancer

ABSTRACT

A Pt(IV) prodrug of cisplatin containing a glutathione s-transferase (GSTs) inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX), complex 1, was designed and studied aiming to overcome cisplatin-resistance and reduce its toxicity by inhibiting GSTs overexpressed in cancer cells. The complex could be reduced to release its active Pt(II) species and axial ligand in the presence of ascorbic acid. In cytotoxicity study, complex 1 showed more potent anticancer activity than cisplatin and NBDHEX against all the tested cancer cells, especially toward cisplatin resistant A549/DDP cells with a resistance factor value of 0.37. By effectively inhibiting GSTs, complex 1 was found to be able to promote higher platinum uptake and cause more severe DNA damage in both A549 cells and A549/DDP cells as compared with cisplatin. Further mechanism study indicated that it could trigger cell death via an apoptotic pathway. In vivo tests on A549 xenograft tumor mice model showed that complex 1 presented higher tumor inhibiting rate and lower toxicity than cisplatin as well. In all, the Pt(IV) prodrug has potential to be developed as an anticancer agent.

1. Introduction

Platinum-based anticancer drugs such as cisplatin, carboplatin and oxaliplatin have been world widely used in the chemotherapy for a variety of solid tumors (Fig. 1) [1]. Application of these classical Pt(II) drugs in clinic has greatly improved the treatment efficacy of many cancers, such as testicular cancer, ovarian cancer and bladder cancer [2]. However, the severe dose-limiting side effects and intrinsic or acquired cisplatin-resistance have greatly limited their clinical uses [3]. Much study indicates that glutathione (GSH) and glutathione S-transferase (GSTs), over-expressed in most of cancer cells, are chief culprits blamed for the side effects and drug resistance of cisplatin [4–6]. Particularly, GSTs is a class of phase II enzyme to catalyze the conjugation of GSH with electrophilic compounds such as cisplatin to form GS-platinum adducts. The resulting adducts are usually water soluble in favor of the elimination of cisplatin by multidrug resistance-associated protein transporter that leads to the decreased intracellular accumulation of platinum and cisplatin-resistance [7,8]. Moreover, the GS-platinum adducts can be metabolized to a nephrotoxicant by γ-glutamyl transpeptidase and a cysteine s-conjugate β-lyase, and nephrotoxicity is the main reason for patients to discontinue treatment [9]. Therefore, inhibition of GSTs activity is a potential way to overcome cisplatin-resistance and reduce adverse side effects of cisplatin [10].

In contrast to a square-planar Pt(II) complex, an octahedral Pt(IV) complex with extra two axial ligands can provide a way to tune its biological properties such as lipophilicity, selectivity and redox stability [11,12]. Significantly, Pt(IV) complex can be activated by biological reducing agents like GSH or ascorbic acid to release active Pt(II) species and axial ligands [13,14]. And the activation of Pt(IV) complex is accompanied with the consumption of GSH, that is beneficial to overcome cisplatin-resistance and reduce side effects. Thus, Pt(IV) complexes are widely explored and expected to become the next generation platinum-based anticancer drug [15,16].

It is known that the number of patients suffering from lung cancers is now rapidly rising, which has been one of the leading causes of cancer death in many parts of the world [17,18]. At present, cisplatin-based adjuvant chemotherapy is still the first-line drug for non-small cell lung cancer (NSCLC) which accounts for nearly 85% of all cases of lung cancer worldwide. However, the application of cisplatin in the treatment of NSCLC is also limited by side effects and cisplatin-resistance [19,20]. In view of the high level of GSTs in lung cancer cells, especially in cisplatin-resistant cancer cells [21,22], a Pt(IV) complex

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https://doi.org/10.1016/j.jinorgbio.2019.01.014
Received 27 November 2018; Received in revised form 25 January 2019; Accepted 27 January 2019
Available online 29 January 2019
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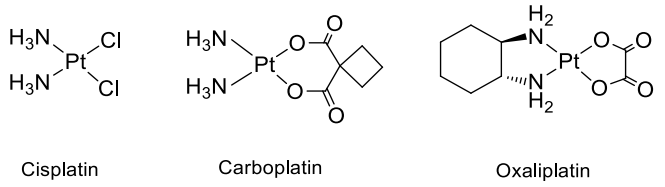


Fig. 1. Chemical structures of cisplatin, carboplatin and oxaliplatin.

derived from cisplatin containing a GSTs inhibitor, 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) [23,24], has been designed and prepared as a multifunctional Pt(IV) prodrug to overcome cisplatin resistance, which is expected to own the inhibitory ability of GSTs and the antineoplastic potential to treat with lung cancer. Ethacrynic acid (EA), a diuretic in clinic applications with the capacity of GSTs inhibition, had been attached to Pt(IV) centre [25,26], its ability to sensitize cancer cells to cytotoxic substances was discovered to be associated with dieresis. However, the long-term utility of EA was compromised by dose-limiting toxicities related to diuresis and subsequent fluid imbalance, which had limited the clinical efficacy of EA as a chemosensitizer [27]. Moreover, EA is often actively extruded from cells by specific export pumps [28]. Since NBDHEX (not a substrate of export pumps) as a novel GSTs inhibitor is stronger than EA in inhibiting GSTs catalytic activity, and its action mode is quite different from that of EA, it is of significance to design a new Pt(IV) prodrug using NBDHEX.

2. Results and discussion

2.1. Synthesis and characterization

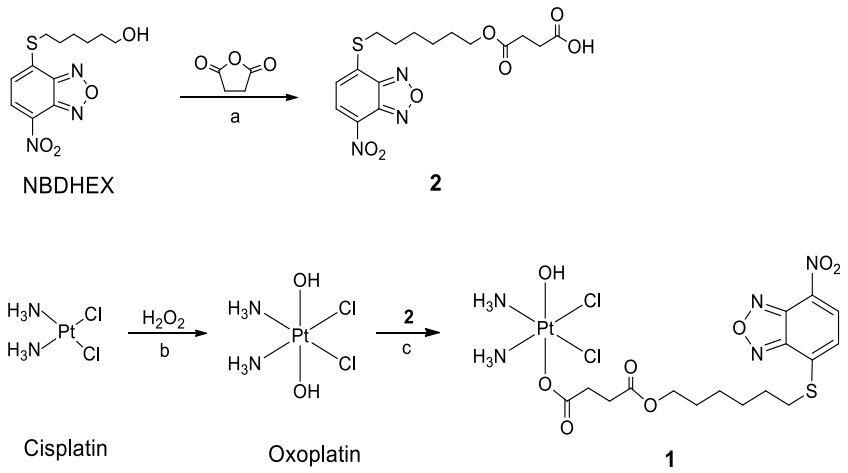
As the ester linkage was more susceptible to acid and esterase, it could be cleaved to release active biological species in cancer cells, NBDHEX as a GSTs targeting moiety was modified by reacting with succinic anhydride to generate compound **2** (Scheme 1) that was characterized by ESI-MS and ^{1}H NMR. As shown in Fig. S1, compound **2** gives two pseudo-molecular ion peaks at m/z = 396.08 and 793.18, assigned to the $[M - H]^-$ or $[2M - H]^-$ peak, respectively. In the ^{1}H NMR spectrum ($CDCl_3$), the multiple-peak of alkyl groups in the resulting succinic acid could be obviously observed at 2.61–2.73 ppm.

Oxoplatin (*cis*, *cis*, *trans*-diammine-dichlorido-dihydroxido-platinum (IV)), a Pt(IV) precursor of cisplatin containing two axial hydroxyl groups confers the ability to attach functional or targeting groups. Oxoplatin was obtained through the oxidation of cisplatin by hydrogen peroxide in water. In brief, treatment of a yellow-orange suspension of cisplatin in water with excess H_2O_2 at 60 °C gave rise to the target complex [29].

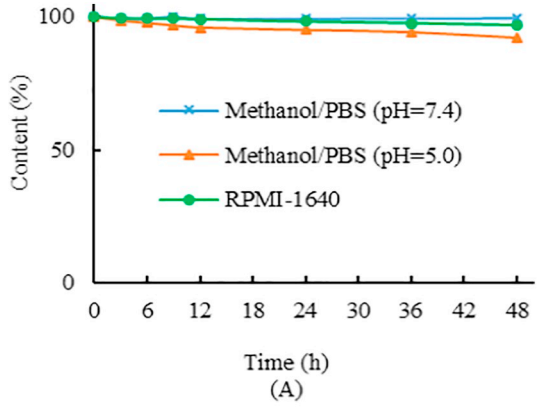
The reaction of oxoplatin and compound **2** in a 1:1.1 M ratio in DMF afforded complex **1** which was characterized by ESI-MS, ^{1}H, ^{13}C and ^{195}Pt NMR spectra together with elemental analysis. As shown in Fig. S2, the pseudo-molecular ion peaks $[M - H]^-$ (m/z = 712.08) and $[M + Cl]^-$ (m/z = 748.06) were found, accompanied with isotopic peaks derived from platinum atom in the mass spectra. In addition to the peaks of compound **2** in the ^{1}H NMR spectrum, multiply peaks appeared at 5.82–6.03 ppm (DMSO-d_6) were assigned to the two amino groups of complex **1**. The ^{195}Pt NMR signal of complex **1** was found at 1024.67 ppm, which provided information on the oxidation state of the metal atom and the nature of the coordinated ligands. The multiplet peaks arising from ^1J (^{14}N-^{195}Pt) coupling and temperature have influences on the linewidth, which was observed in the ^{195}Pt NMR spectrum. Moreover, the purity of complex **1** was detected by HPLC technique (Fig. S3), which could meet the requirement for further study.

2.2. Stability and released ability

It has been reported that Pt(IV) complexes are essentially chemical inert and can only perform anticancer activity after reduced by biological reducing agents such as ascorbic acid (VC) and GSH to release toxic Pt(II) species and the axial ligands [30]. Thus, HPLC technique was used to study the stability and released ability of complex **1**. As illustrated in Fig. 2, complex **1** was stable both in methanol/phosphate buffered saline (PBS, pH = 7.4) solution (v:v = 1:4) and in cell culture medium RPMI-1640 at room temperature for 48 h, but a little decomposition was observed in methanol/PBS (pH = 5.0) solution (v:v = 1:4). It was noted that a small amount of NBDHEX was released from complex **1** or compound **2** under the slightly acidic environment (Fig. S4), that means the ester linkage will be broken under acidic condition. As proved in many researches, the tumor acidic microenvironment and abundant esterases in the cells would make the ester linkage more prone to breakage. Complex **1** gradually began to decompose and release compound **2** in the presence of VC (Fig. S5). It was noted that cisplatin moiety was not observed due to its weak chromophore under the ultraviolet detecting condition in HPLC chromatograms. The results confirmed that complex **1** can be reduced by a biomolecular agent to release the active Pt(II) species and the axial ligand. Dyson et al. ever reported a Pt(IV) complex, named as ethacraplatin containing two molecules of EA, attempting to overcome cisplatin-resistance by inhibiting GSTs [25], but it was found that ethacraplatin could not readily release a Pt(II) species to exert the cytotoxic effect, consistent with our recent study on an axially bi-modified Pt(IV) complex that is much more difficult to be reduced than a mono-modified Pt(IV) complex [31].



Scheme 1. Synthesis of complex **1**. a) DCM, reflux; b) H_2O, 60 °C; c) TBTU, TEA, DMF, 50 °C.



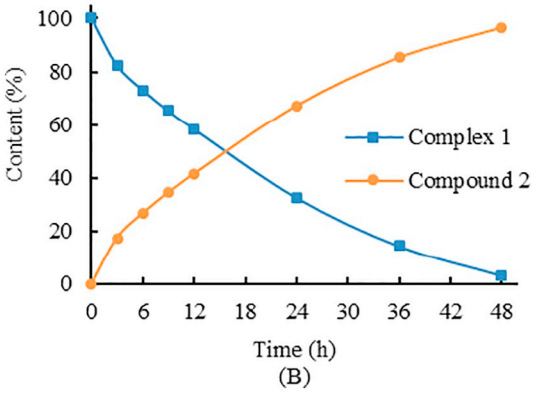


Fig. 2. (A) Stability of complex **1** in RPMI-1640 and PBS under different pH values, and (B) reduction behavior of complex **1** treated with VC (0.5 mg/mL).

2.3. In vitro anticancer activity

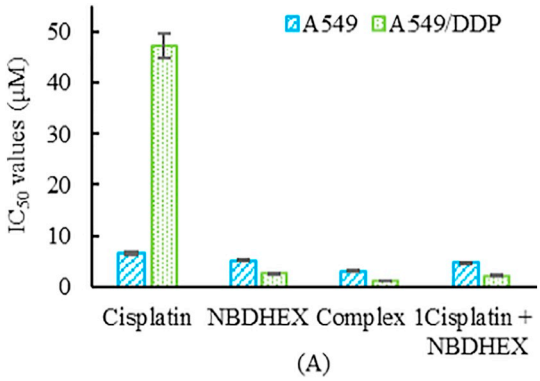
Cytotoxicity of complex **1** was initially screened against human liver (HepG2), colorectal (HCT-116), breast (MCF-7), ovarian (A2780) cancer cells and normal liver cells (LO2) by MTT assays, in which cisplatin and NBDHEX as well as a physical mixture of cisplatin and NBDHEX were used as positive controls. As listed in Table 1, complex **1** presented stronger cytotoxicity in all the tested cancer cells than positive compounds including its mother Pt(II) drug and NBDHEX, and even stronger than the equimolar mixture of cisplatin and NBDHEX against these cancer cells except A2780 cells. It was noted that complex **1** toward LO2 cells was much more toxic than cisplatin, but less than NBDHEX as well as their physical mixture, this may be due to the fact that liver is one of the main metabolic organs expressing higher level of GSTs [32]. The results mean that conjugation of NBDHEX to one axial position of the Pt(IV) complex can enhance the antitumor activity of the resulting hybrid compound.

Upon the above finding, complex **1** was further evaluated against non-small cell lung cancer cells (A549) and its cisplatin resistant counterpart (A549/DDP). As shown in Table 2 and Fig. 3A, complex **1** exhibited the stronger inhibitory activity toward A549 cells, two-fold as strong as cisplatin. As expected, complex **1** turned out to be the most sensitive to A549/DDP cells among the tested samples. Its cytotoxicity toward A549/DDP cells was nearly 42 times more potent than that of

Table 2
IC_{50} values of complex **1** and positive compounds toward A549 and its cisplatin-resistant cells A549/DDP for 72 h.

	IC_{50} (μM)			
	Complex **1**	Cisplatin	NBDHEX	Mixture[b]
A549	3.05 ± 0.09	6.48 ± 0.25	5.20 ± 0.21	4.65 ± 0.21
A549/DDP	1.13 ± 0.08	47.15 ± 2.18	2.55 ± 0.17	2.12 ± 0.11
RF[a]	0.37	7.27	0.49	0.46

[a] Resistance factor = IC_{50} (cisplatin-resistance cancer cells)/IC_{50} (cisplatin-sensitive cancer cells).
[b] Equimolar mixture of cisplatin and NBDHEX.



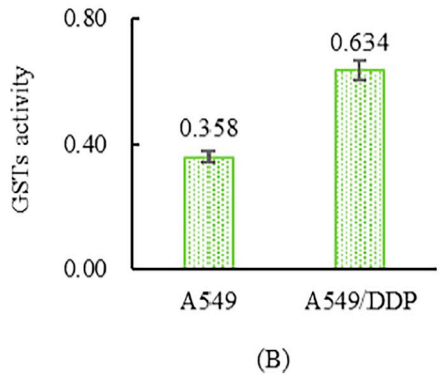


Fig. 3. (A) Statistic analysis of the IC_{50} values of complex **1** and positive compounds toward A549 and its cisplatin-resistant cells A549/DDP for 72 h. (B) GSTs activity ((nmol/min/10^4 cell)) in A549 and A549/DDP cancer cells.

Table 1
IC_{50} values of complex **1** and positive compounds against several human cancer cell lines.[a]

Compound	IC_{50} (μM)				
	HepG2	HCT-116	MCF-7	A2780	LO2
1	2.35 ± 0.09	2.82 ± 0.06	5.51 ± 0.29	2.71 ± 0.02	2.77 ± 0.13
Cisplatin	5.52 ± 0.22	10.70 ± 0.11	10.23 ± 0.62	3.80 ± 0.13	9.02 ± 0.15
NBDHEX	6.97 ± 0.44	6.16 ± 0.25	11.25 ± 0.71	2.99 ± 0.02	1.34 ± 0.02
Mixture[b]	3.72 ± 0.07	4.38 ± 0.18	9.16 ± 0.93	2.06 ± 0.04	1.06 ± 0.03

[a] IC_{50} is the drug concentration effective at inhibiting 50% of the cell growth measured by the MTT assay after 72 h of drug exposure expressed as the mean value ± standard deviation of three independent experiments.
[b] Equimolar mixture of cisplatin and NBDHEX.

cisplatin, and even two times greater than NBDHEX and the mixture. Moreover, complex **1** showed the lowest RF value of 0.37, while cisplatin had a RF value of 7.27, indicating that complex **1** can remarkably overcome cisplatin resistance in non-small cell lung cancer cells. The data confirmed that complex **1** was more sensitive to inhibit cisplatin-resistant A549/DDP cells than cisplatin-sensitive A549 cells. This would be partly due to the axial NBDHEX moiety of complex **1**, because NBDHEX could effectively inhibit GSTs that is highly expressed in A549/DDP cells (Fig. 3B). Although the GSTs inhibitor NBDHEX exhibited strong inhibitory activity against both A549 and A549/DDP cancer cells with a RF value of 0.49, its IC_{50} values were still higher than those of complex **1**. The above results demonstrated that complex **1** had the potential ability to overcome cisplatin- resistance. Not long ago, Dyson and his coworkers just revealed a Pt(IV) complex containing a EA moiety that was designed to act as an efficient dual-action GST-inhibiting anticancer Pt(IV) prodrug. Despite their compound with a similar structure to complex **1** showed remarkably potent cytotoxicity toward cisplatin-sensitive A2780 cancer cells, it did not show an acceptable resistance factor while compared with its IC_{50} value against cisplatin-resistant cancer cells A2780/cisR [26].

2.4. GSTs inhibitory ability

The residual activity of GSTs extracted from lung cancer cells was spectrophotometrically assayed by measuring the conjugation rate of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH as a function of time, because GSTs could catalyze the reaction of CDNB with GSH to form GS-DNB adducts with the maximum absorption wavelength at 340 nm [33]. As shown in Fig. 4, both NBDHEX and its succinic acid derivative compound **2** possessed dose-dependent ability to inhibit GSTs, surprisingly, the later was more effective than the former. Satisfactorily, complex **1** had almost the same GSTs inhibitory ability as compound **2**. The data indicated that the strategy of conjugating NBDHEX with the Pt (IV) moiety derived from cisplatin *via* a succinate linker not only maintained the inhibitory activity of NBDHEX toward GSTs, but also performed better anticancer activity.

2.5. Cellular uptake and DNA platination

It has been demonstrated that the reduced intracellular platinum accumulation is an important factor of cisplatin-resistance [6]. So the cellular uptake and DNA platination of cisplatin and complex **1** at different concentrations in A549 and A549/DDP cells were analyzed by ICP-MS. As shown in Fig. 5 and Table S1, in cisplatin-sensitive A549 cells, the cellular uptake of cisplatin and complex **1** was dose-dependent, and the platinum accumulation of complex **1** was 6.3 fold greater than that of cisplatin at a concentration of 10 μM and 2.6 fold at a concentration of 20 μM. Meanwhile, the DNA platination caused by complex **1** was 11.9 fold greater than that of cisplatin at a concentration of 10 μM and 5.1 fold at a concentration of 20 μM. In cisplatin-resistant

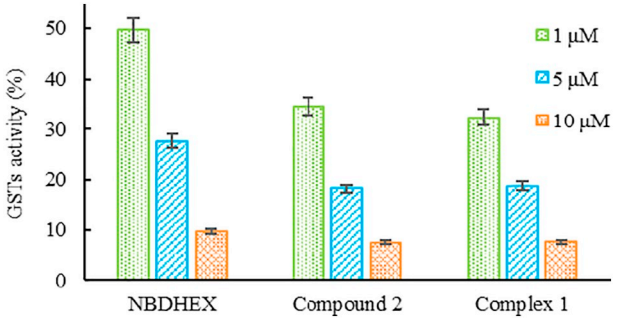


Fig. 4. Residual activity of GSTs under the treatment of NBDHEX, compound **2** and complex **1**.

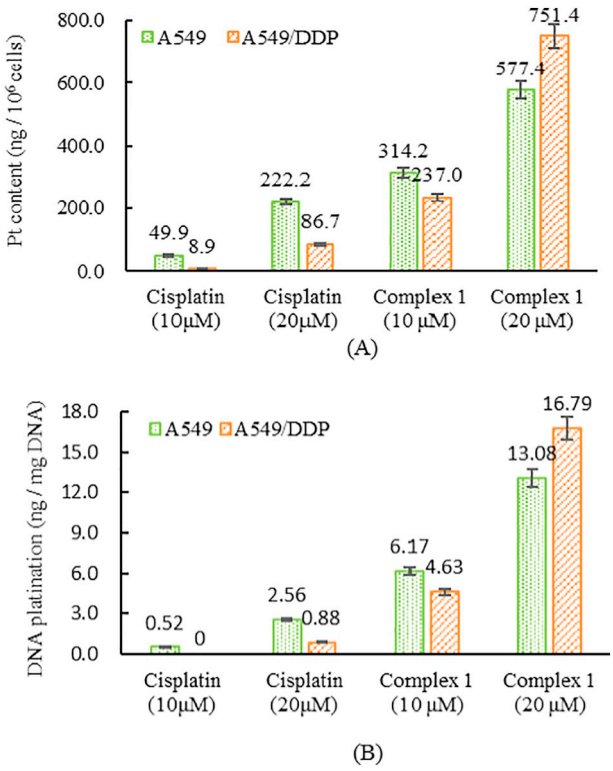


Fig. 5. (A) Cellular uptake and (B) DNA platination of cisplatin and complex **1** at different concentrations in cisplatin-sensitive A549 and cisplatin-resistant A549/DDP cells after 12 h of incubation. Results are expressed as the mean ± SD for three independent experiments.

A549/DDP cells, the uptake of cisplatin was dramatically decreased, but the uptake of complex **1** was still higher than that of cisplatin, which was 26.6 fold as much as that of cisplatin at 10 μM and 8.7 fold at 20 μM. Besides, the DNA platination in A549/DDP cells caused by cisplatin at 10 μM was not detected, but it was significantly increased under the treatment of complex **1**. The enhanced lipophilicity (log *P* values showed in Table S2) was probably one of the reasons for the increase of cellular uptake, while the dramatic increase of DNA platination was likely to owe to the inhibition of GSTs. The results exhibited that complex **1** owns the capacity of overcoming cisplatin-resistance, the enhanced platinum accumulation and DNA platination may be one of the determinants of platinum sensitivity. Obviously, introduction of a GSTs inhibitor to the Pt(IV) prodrug can significantly promote the accumulation of platinum and DNA platination, leading to the reversal of cisplatin-resistance of lung cancer cells.

2.6. Comet assay

Anticancer effects of platinum-based drugs are proved to be mediated by irreversible DNA damage. Comet assay, a gel electrophoresis–based method that can be used to measure DNA damage in individual eukaryotic cells, is versatile and relatively simple to perform [34]. If the negatively charged DNA was damaged, DNA supercoils were relaxed and broken ends were able to migrate to the anode. The comet head contains the high-molecular-weight DNA and the comet tail contains the leading ends of migrating fragments. As presented in Fig. 6, NBDHEX cannot cause DNA damage because its target is not DNA. Cisplatin could trigger distinct DNA damage in sensitive A549 cells as the DNA tails were found, while it was invalid in cisplatin-resistant cells. In contrast, both sensitive and resistant cells treated with complex **1** owned longer length of DNA tails, and the tails in A549/DDP cells were even longer than those of A549 cells, indicating that complex **1** caused more severe DNA damage especially in cisplatin-resistant cells

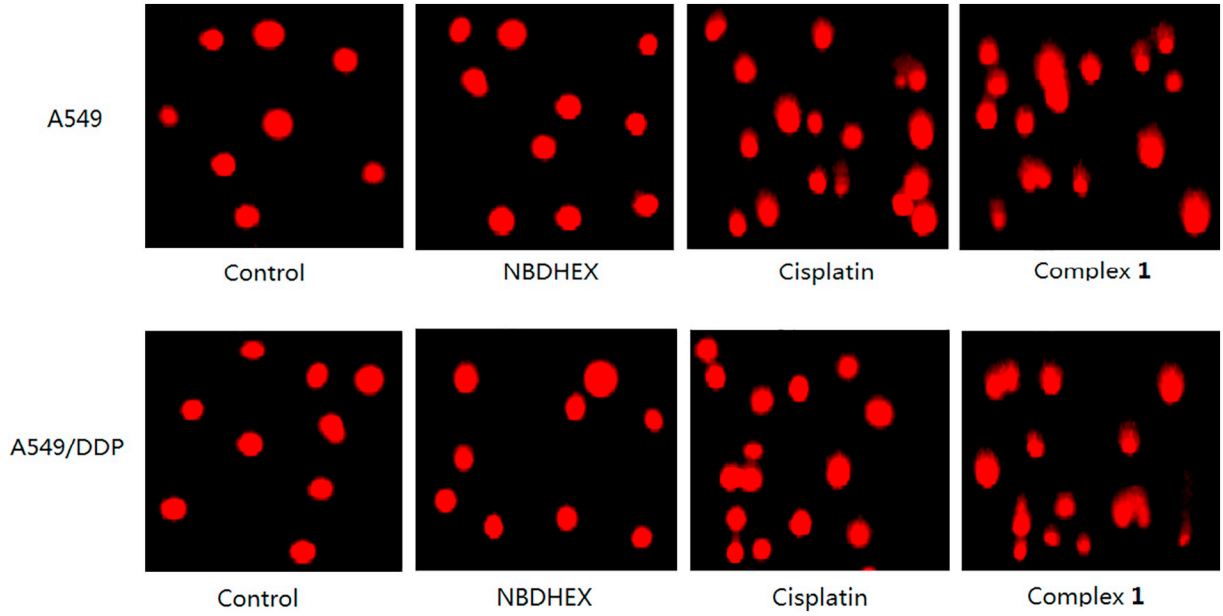


Fig. 6. Representative images of nuclei in comet assay. A549 and A549/DDP cells were treated with 20 μM of cisplatin or complex **1** for 12 h.

due to the enhancement of platinum accumulation. This result is consistent with the data obtained by MTT assay and DNA platination test.

2.7. Apoptosis study

Apoptotic analysis of complex **1** against A549 and A549/DDP cells was carried out by flow cytometric assay, cisplatin and NBDHEX were used as positive compounds. As shown in Figs. 7 and S6, few apoptotic or necrotic cells were present in both control panels; cisplatin achieved an apoptosis rate of 17.6% (5.1% early apoptosis, 12.5% late apoptosis) with a necrosis rate of 9.4% in sensitive A549 cells, while it was forceless in resistant A549/DDP cells (4.2% early apoptosis, 5.2% late apoptosis, 0.4% necrosis); however, the apoptosis rate under the treatment with complex **1** at the same concentration strongly increased to 76.5% (24.5% early apoptosis, 52.0% late apoptosis) in sensitive A549 cells with a necrosis rate of 3.2%, and 78.9% (29.5% early apoptosis, 49.4% late apoptosis) in resistant A549/DDP cells with a necrosis rate of 8.9%. It was noted that NBDHEX killed more A549 and A549/DDP cells than complex **1** mainly in the late stage of apoptosis, which may be related to the rapid response of cells toward NBDHEX. All the above results revealed that complex **1** could trigger lung cancer cell

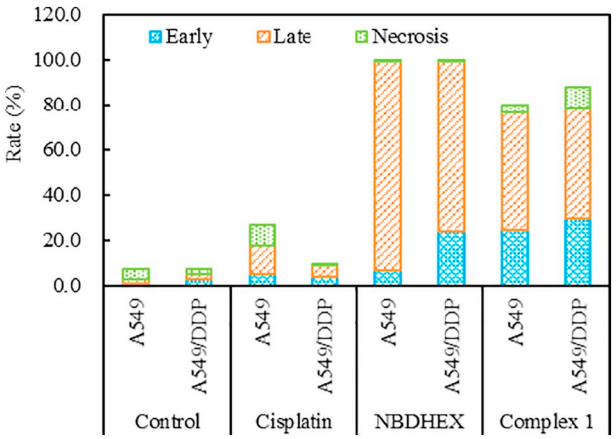


Fig. 7. Flow cytometric analysis of the distribution of A549 and A549/DDP cells untreated or treated with cisplatin, NBDHEX or complex **1** at a concentration of 20 μM for 24 h.

death *via* an apoptotic pathway and was more potent than cisplatin, especially in cisplatin-resistant cells.

2.8. Cell cycle arrest

Cell cycle arrest of complex **1** on A549 and A549/DDP cells was also analyzed by flow cytometric assay, cisplatin and NBDHEX were used as positive drugs. As shown in Figs. 8 and S7, cisplatin as well as NBDHEX inhibited the cell cycle mainly in S phase in both cell lines; while the cell cycle arrested by complex **1** was quite different in A549 and A549/DDP cells, the cell cycle of A549 was chiefly arrested at G2/M phase, but that of A549/DDP was primarily arrested at S phase. The results indicated that the cell cycle arrested by complex **1** in cisplatin sensitive lung cancer cells was different from that in cisplatin resistant cells.

2.9. Apoptosis-related protein analysis

To further investigate the apoptotic mechanisms of A549 and A549/DDP cells induced by complex **1**, western blot was carried out using cisplatin and NBDHEX as positive drugs. As shown in Fig. 9, in sensitive A549 cells, cisplatin induced cell apoptosis by down-regulating the expression of anti-apoptotic protein Bcl-2 (B-cell lymphoma-2), increasing the level of pro-apoptotic protein Bax (Bcl-2 associated X protein), activating caspase-3 to produce more cleaved type, cleaving and inactivating PARP (poly(ADP)ribose polymerase) which was associated with DNA repair; complex **1** also had the similar capacity which was more potential than that of cisplatin or NBDHEX. In resistant A549/DDP cells, although cisplatin could up-regulate Bax and cleaved caspase 3 modestly, it had lost the ability to reduce the expression of Bcl-2, of which the level was higher in the resistant cells than in the sensitive cells, and at the same time, it was useless to inactivate PARP; however, complex **1** still could remarkably down-regulate Bcl-2 and inactivate PARP to form more cleaved type. The results further confirmed that complex **1** was superior to cisplatin in the treatment of lung cancer, and owned the ability of overcoming cisplatin-resistance.

2.10. In vivo antitumor efficacy

In vivo antitumor efficacy was evaluated on A549 tumor-bearing nude mice. Mice were randomly divided into five groups: (1) physiological saline treated group (iv, once a week), (2) cisplatin treated

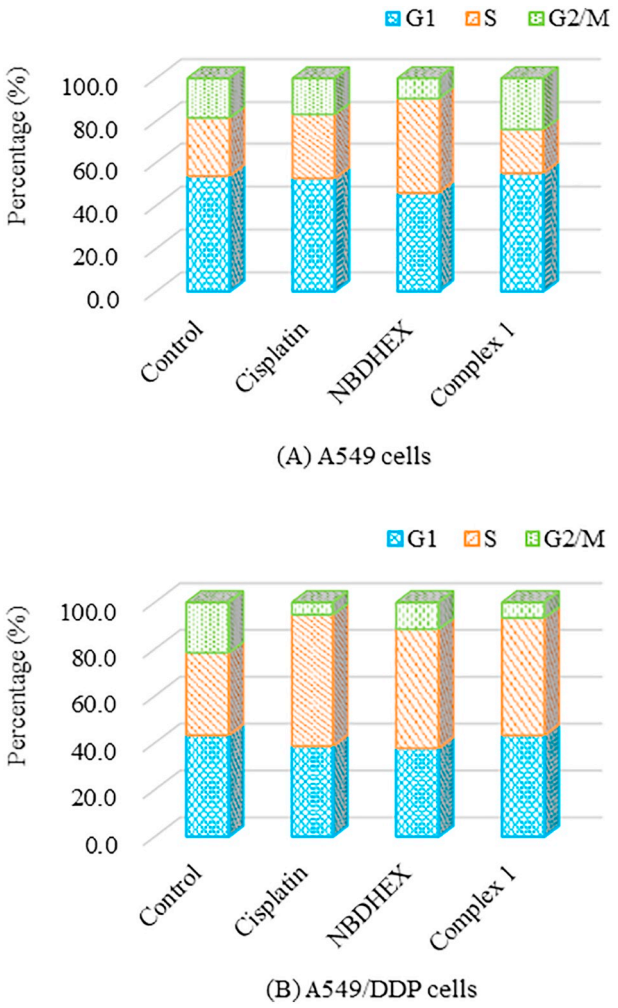


Fig. 8. Cell cycle arrest of (A) A549 and (B) A549/DDP cells. Cells were untreated or treated with cisplatin, NBDHEX or complex **1** at a concentration of 20 μM for 24 h.

group (5 mg/kg, iv, once a week), (3) NBDHEX treated group (5 mg/kg, po, every day for a week), (4) complex **1** treated group (5 mg/kg, iv, once a week), (5) physical mixture of cisplatin and NBDHEX with equal mass, and treated with above-mentioned formulations for 28 days in the entire observation period. As shown in Fig. 10, tumor volumes were measured every three days, and the tumor volume inhibitory rate of cisplatin, NBDHEX, complex **1** and the mixture was 50.2%, 48.4%, 52.5% and 49.7%, respectively. It was worth noting that at an equal mass dosage, complex **1** containing less platinum and GSTs inhibitor moiety than cisplatin and NBDHEX displayed better *in vivo* antitumor activity than that of other groups. Notably, complex **1** did not diminish animal body weight during the test period, suggesting that it had no obvious toxicity under the current treatment paradigm. In contrast, the mice body weight in other groups treated with cisplatin or NBDHEX decreased more than complex **1**. Histological analysis of the tumor and primary tissues also proved high efficacy and low side effects of complex **1** compared with the other samples. As shown in Fig. 11, the most obvious side effect of cisplatin was kidney injury, while that of NBDHEX was liver damage. However, less nephrotoxicity or little hepatotoxicity was found in the group treated with complex **1**. Moreover, alveolar spaces were observed enlarged in the group treated with NBDHEX or complex **1**, meaning that complex **1** has the advantage for the treatment of lung cancer. In all, complex **1** can effectively inhibit tumor growth and has low toxicity *in vivo*, leading to a favorable outcome in the treatment of NSCLC superior to cisplatin and NBDHEX in the animal experiment.

3. Conclusions

Despite cisplatin has been acting as the first-line drug for the treatment of NSCLC, it is severely limited by side effects and drug resistance. In this study, complex **1** as a Pt(IV) prodrug of cisplatin containing NBDHEX (a GSTs inhibitor) was deliberately designed, prepared and studied upon an idea to overcome cisplatin resistance and reduce its toxicity by inhibiting GSTs overexpressed in most of cancer cells. Complex **1** could be reduced to release its active Pt(II) species and axial ligand under the reduction of ascorbic acid. Its cytotoxicity against A549 cells was stronger than that of cisplatin, especially toward cisplatin-resistant A549/DDP cells with a resistance factor value of 0.37. By inhibiting GSTs, the promotion of platinum uptake and DNA platination, more severe DNA damage were observed in cancer cells treated with complex **1**, resulting in the reversal of cisplatin-resistance in lung cancer cells. Complex **1** could trigger cell death *via* an apoptotic pathway and arrested the cell cycle of sensitive A549 cells at G2/M phase, while mainly arrested the cell cycle of resistant A549/DDP cells at S phase. Different from cisplatin, complex **1** could down-regulate Bcl-2 and upregulate cleaved PARP in the cisplatin resistant cells. *In vivo* tests on A549 xenograft tumor mice model showed that complex **1** not only had the highest tumor inhibiting rate compared with cisplatin, NBDHEX and their physical mixture, but also exhibited least toxic

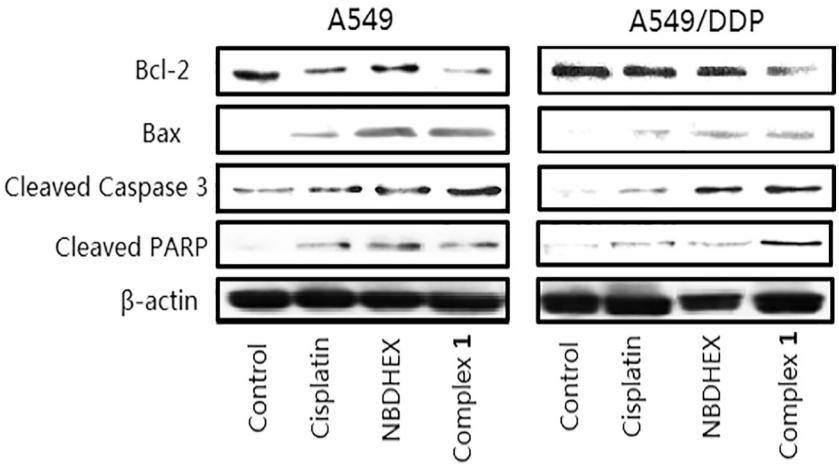


Fig. 9. Western blot analysis of the mitochondrial related apoptotic proteins and PARP which is associated with DNA repair. Cells were untreated or treated with cisplatin, NBDHEX or complex **1** at a concentration of 20 μM for 12 h.

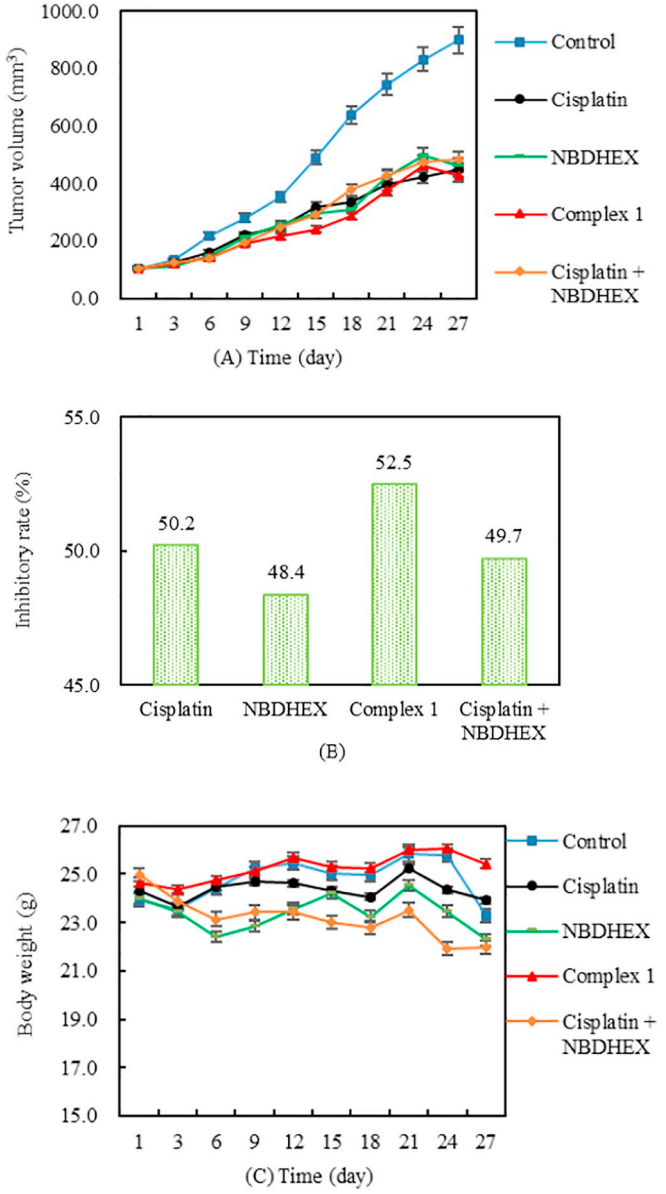


Fig. 10. (A) Tumor volume in each group during the observation period; (B) Tumor volume inhibitory rate in different treatment groups; (C) mice body weight in each group during the observation period.

among the testes samples. In short, complex **1** owning the GSTs inhibitory ability presented potent ability for the treatment of NSCLC, which has potential to be developed as an anticancer agent.

4. Materials and methods

4.1. Materials and instrument

All chemicals and solvents were of analytical reagent grade and used without further purification, unless noted specifically. Cisplatin was synthesized in our lab. All antibodies used in this study were purchased from Cell Signaling Technology (CST). Mass spectra were measured by an Agilent 6224 ESI/TOF MS instrument. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on a Bruker 300 MHz or 500 MHz spectrometer. ^{195}Pt NMR spectra were measured in DMSO-d_6 with a Bruker 600 MHz spectrometer. Elemental analyses of C, H, and N used a Vario MICRO CHNOS elemental analyzer (Elementar). Waters 1525 HPLC equipment was used to study the purity, stability and releasing ability of the compound. Platinum contents were determined by Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Optima 5300DV, PerkinElmer, USA). Cell cycle and apoptosis experiments were measured by flow cytometry (FAC Scan, Becton Dickenson) and analyzed by Cell Quest software.

4.2. Synthesis of 4-((6-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)thio)hexyl)oxy)-4-oxobutanoic acid (2)

Succinic anhydride (300.0 mg, 3.0 mmol) was added to a solution of NBDHEX (300.0 mg, 1.0 mmol) in anhydrous chloroform (40 mL) and stirred at refluxing for 6 h. Then, the reaction mixture was washed with saturated $NaHCO_3$ solution and pure water, then dried over sodium sulfate. The filtrate was concentrated under reduced pressure. Column chromatography (DCM:Methanol = 10:1, v:v) gave compound **2** as a yellow solid in a yield of 86% (342.1 mg). ESI-MS: calcd m/z = 397.40, found $[M-H]^-$ = 396.08. ^{1}H NMR (ppm, 300 MHz, $CDCl_3$): δ 8.43 (d, J = 9.0 Hz, 1H), δ 7.18 (d, J = 6.0 Hz, 1H), δ 4.14 (t, J = 7.5 Hz, 2H), δ 3.30 (t, J = 7.5 Hz, 2H), δ 2.67 (4H), δ 1.95–1.82 (m, 2H), δ 1.75–1.67 (m, 2H), δ 1.67–1.53 (m, 2H), δ 1.53–1.42 (m, 2H).

4.3. Synthesis of oxoplatin

Hydrogen peroxide (30 wt%, 30 mL) was added drop wise to a round bottom flask containing cisplatin (500.0 mg, 1.67 mmol). The reaction mixture was heated to 60 °C for 12 h. The bright yellow solution was kept at room temperature in the dark for overnight to allow crystallization of the product. Yellow crystals were separated by filtration, washed with cold water, and dried to get 485.0 mg of oxoplatin, yield 87%.

4.4. Synthesis of complex 1

A solution of **2** (218.6 mg, 0.55 mmol) and TBTU (176.0 mg, 0.55 mmol) in 7 mL of DMF dry was stirred at 40 °C under N_2 atmosphere. After 15 min, TEA (65 μL) was added and the reaction was stirred for 15 min. Oxoplatin (167.0 mg, 0.50 mmol) was then added and the reaction mixture was stirred at 50 °C for 24 h. The solvent was then removed by evaporation under reduced pressure. Column chromatography separation (ethyl acetate:methanol = 1:1, v:v) gave products as a yellow solid. Yield: 75.0 mg (21%). ESI-MS: calcd m/z $[M-H]^-$ = 712.0360, $[M+Cl]^-$ = 748.0121 found $[M-H]^-$ = 712.0870, $[M+Cl]^-$ = 748.0648. ^{1}H NMR (ppm, 500 MHz, DMSO-d_6): δ 8.60 (d, J = 10.0 Hz, 1H), δ 7.55 (d, J = 10.0 Hz, 1H), δ 5.93 (−NH_3, 6H), δ 4.04 (t, J = 7.5 Hz, 2H), δ 3.40 (t, J = 7.5 Hz, 2H), δ 2.47 (s, 4H), δ 1.85–1.77 (m, 2H), δ 1.68–1.57 (m, 2H), δ 1.56–1.47 (m, 2H), δ 1.45–1.37 (m, 2H). ^{13}C NMR (ppm, 125 MHz, DMSO-d_6): δ 173.27, 172.03, 149.10, 142.58, 139.95, 132.23, 132.02, 122.12, 63.74, 30.54, 28.70, 28.65, 27.88, 27.73, 27.26, 24.76. ^{195}Pt NMR (129 MHz, DMSO-d_6) δ = 1024.67 ppm. Anal. Calcd (%) for $C_{16}H_{25}Cl_2N_5O_8PtS$: C 26.94, H 3.53, N 9.82. Found: C 26.75, H 3.48, N 9.77.

4.5. Cell lines and cell culture

Human liver (HepG2), colorectal (HCT-116), breast (MCF-7), ovaries (A2780), lung (A549), cisplatin-resistant lung cancer cells (A549/DDP) and normal liver cells (LO2) were purchased from Jiangsu KeyGen BioTECH Co. Ltd. Cancer cells were incubated carefully in RPMI-1640 medium or Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and ampicillin sodium (100 μg/mL) in an atmosphere of 5% CO_2 and 95% air at 37 °C. Moreover, A549/DDP cells were cultured and screened in RPMI-1640 supplemented with 10% FBS, streptomycin (100 μg/mL), ampicillin sodium (100 μg/mL) and 800 ng/mL cisplatin before use.

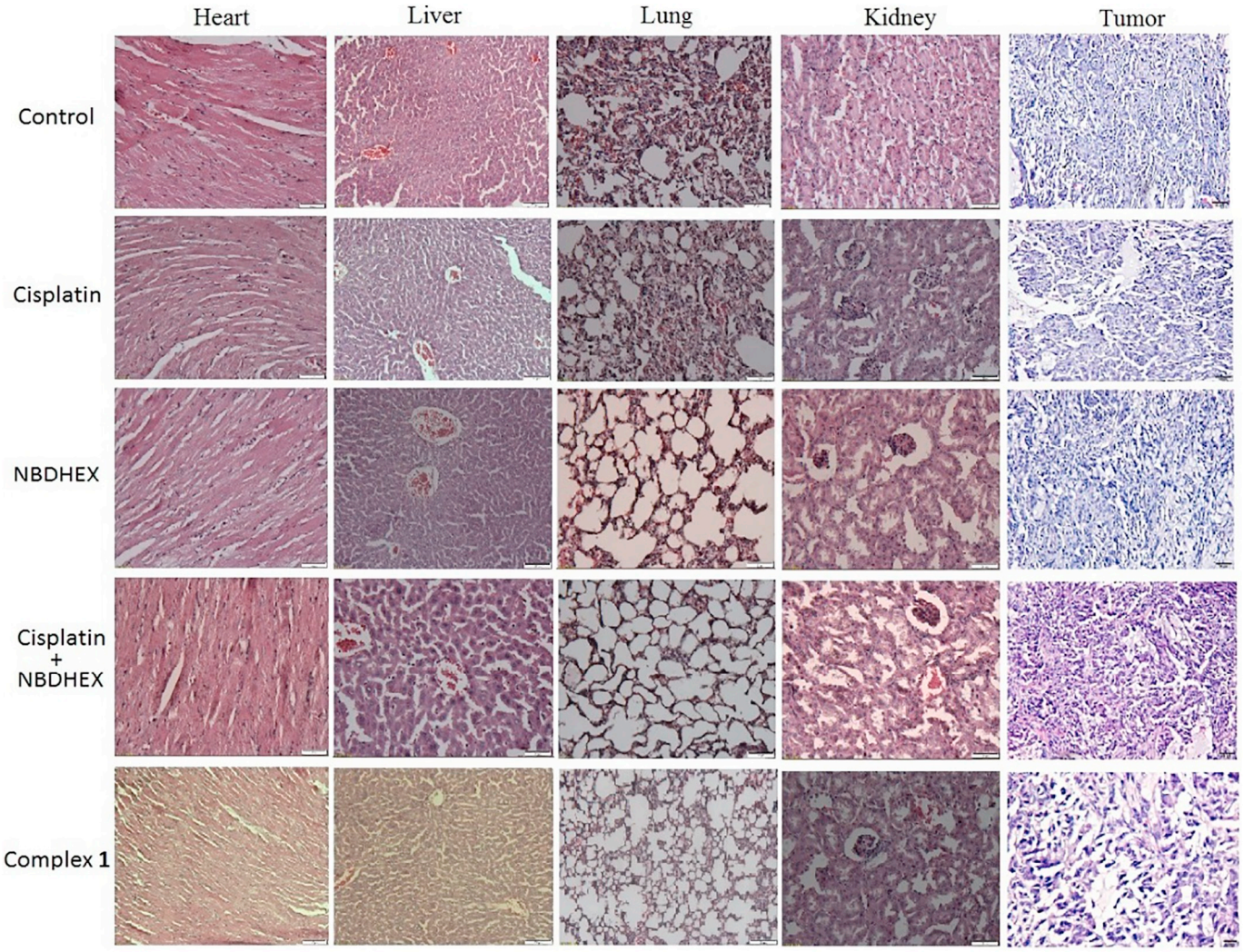


Fig. 11. H&E staining of the tumor and primary tissues in different treatment group.

4.6. Cell sensitivity to compounds

In vitro anticancer activity of complex **1** was evaluated by MTT assays, using cisplatin, carboplatin, oxaliplatin, NBDHEX and the physical mixture of cisplatin and NBDHEX as the positive drugs. Cells were grown in 96 well plates at 6000 cells per well in a final volume of 200 μL of culture medium and cultured in an incubator (5% CO_2, 37 °C) until the cells reached 70–80% confluency. Samples were dissolved by DMF and diluted with medium to various concentrations (the final concentration of DMF was less than 0.4%). After being incubated at 37 °C for 72 h, cells were stained with MTT (5 mg/mL) for another 4 h and dissolved with 150 μL of DMSO. The UV absorption intensity was detected with an ELISA reader at 490 nm. IC_{50} values were calculated by SPSS software after three parallel experiments and all experiments were performed in triplicate.

4.7. HPLC analyses on the stability and releasing ability

The stability of complex **1** (0.5 mg/mL) in methanol/PBS (pH = 7.4 and 5.0) solution (v:v = 1:4) and cell culture medium RPMI-1640 at room temperature was investigated by HPLC chromatograms at different times (0, 3, 6, 9, 12, 24, 36 and 48 h). Reduction of complex **1** was carried out by adding ascorbic acid into the solution at a final concentration of 0.5 mg/mL and examined by HPLC. Reversed phase HPLC was implemented on a 250 × 4.5 mm ODS column and the HPLC profiles were recorded on UV detection at 280 nm. Mobile phase consisted of ACN/water (v:v = 80:20), flow rate was 1.0 mL/min. Samples were taken for HPLC analysis after filtered by 0.45 μm filter.

4.8. GSTs activity

GSTs activity was spectrophotometrically assayed at 340 nm by measuring the rate of CDNB conjugation with GSH as a function of time at 25 °C, as reported previously. The assay mixture contained 1 mM GSH, 1 mM CDNB and 0.1 mM EDTA in 1 mL (final volume) of 0.1 M potassium phosphate buffer, pH 6.5. In brief, NBDHEX, compound **2** and complex **1** at different concentrations (1, 5, 10 μM) were added to GSTs which was extracted from cancer cells and mixed well. After incubation at room temperature for 5 min, CDNB and GSH were added and quickly mixed well. Absorbance was measured at 340 nm for 5 min. Prior to each experiment, the baseline of the UV spectrometer was corrected by replacing GSTs solution with phosphate buffer. All experiments were performed in triplicate.

4.9. Cellular uptake and DNA platination

A549 or A549/DDP cells were cultured in 6-well plates until the cells reached about 90% confluence. Complex **1** or cisplatin was added at a concentration of 10 μM or 20 μM. After incubated for 12 h, cells were collected and washed three times with cold PBS, followed by

centrifugation for 10 min and resuspension in 1 mL PBS. Then, 100 μL suspended cells were taken to measure the cell density. The rest of the cells were spun down and digested at 65 °C in 200 μL 65% HNO_3 for 24 h. The concentrations of platinum were detected by ICP-MS and all experiments were performed in triplicate. For the measurement of Pt concentration in cellular DNA in A549 and A549/DDP cells, cellular DNA was isolated using Genomic DNA Mini Preparation Kit (KeyGEN, China) according to the manufacturer's instructions. Following determination of concentration, the DNA in solution was digested with 70% HNO_3 and Pt content in DNA was analyzed using ICP-MS. The DNA-Pt adduct was represented as ng of platinum per mg of DNA.

4.10. Measurement of log P

The log *P* values were obtained by using the shake-flask method and UV–vis spectroscopy. Stock solutions of **1**, **2** and NBDHEX were prepared in n-octanol that was pre-saturated with 0.9% NaCl (*w/v*) solution. Stock solutions of cisplatin were prepared in 0.9% NaCl (w/v) solution that was presaturated with n-octanol. Subsequently, the stock solution was added to an equal volume of n-octanol or 0.9% NaCl (w/v) solution. The heterogeneous mixture was shaken vigorously for 24 h before centrifuging to achieve phase separation. The concentrations of the compounds in the organic and aqueous phases were then determined using UV absorbance spectroscopy. log *P* was defined as the logarithm of the ratio of the concentrations of the compound in the organic and aqueous phases.

4.11. Comet assay

A549 or A549/DDP cells were plated into 6-well culture plates and treated with cisplatin, NBDHEX or complex **1** at a concentration of 20 μM for 24 h. Cells were harvested, mixed with molten LM Agarose (Trevigen) and immediately pipetted onto Comet Slide (Trevigen). Slides were immobilized at 4 °C for 10 min, and immersed in prechilled Lysis buffer at 4 °C for 30 min. Then the slides were immersed in alkaline unwinding solution (1 mM EDTA, 200 mM NaOH) for 40 min at room temperature. Electrophoresis was done using alkaline electrophoresis solution (1 mM EDTA, 200 mM NaOH) at 25 V for 30 min. Finally, slides were washed with water and stained with propidium iodide (PI) and visualized by microscopy.

4.12. Cell apoptosis

Cancer cells were plated into 6-well culture plates and cultured in 5% CO_2 at 37 °C overnight. Complex **1**, cisplatin or NBDHEX was added at a concentration of 20 μM. After 24 h, cells were digested with trypsin and washed twice with cold PBS, then collected by centrifugation (2000 rpm, 5 min). Cell apoptosis was determined by flow cytometry using an Annexin V-FITC/PI Apoptosis Detection Kit (Keygen, China) according to the manufacturer's protocol. Detailed operation as follows: cells were stained with 5 μL Annexin V-FITC for 5 min in Annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). PI was added to cells with 5 μL before incubated at room temperature for 15 min and fluorescence was measured by flow cytometer.

4.13. Cell cycle measurement

A549 and A549/DDP cells were transferred into 6-well plates with a density of 10,000 per well, and cultured overnight at 37 °C. Then, 20 μM of the tested compounds were incubated with cells for 24 h. All adherent and floating cells were collected and washed twice with PBS. Then, the cells were fixed with 70% ethyl alcohol at 4 °C for 24 h. After that, fixed cells were washed with PBS. After being centrifuged, cells were stained with 50 mg/mL PI solution containing 100 mg/mL RNase at 37 °C for 0.5 h. The sample (at least 1×10^4 cells) was measured by flow cytometry (FAC Scan, Becton Dickenson) using Cell Quest software and recording PI in the FL2 channel.

4.14. Western blot

After treatment with the indicated concentration (20 μM) of each sample for 12 h, proteins were extracted by lysis buffer. The concentration of protein was measured by the BCA (bicinchoninic acid) assay with a varioskan multimode microplate spectrophotometer (Thermo, Waltham, MA). Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred onto polyvinylidene difluoride (PVDF) Immobilon-P membrane (Bio-Rad) with a transblot apparatus (Bio-Rad). The blots, blocked with 5% nonfat milk in PBST (Tris-buffered saline plus 0.1% tween 20) for 1 h, were incubated with primary antibodies diluted in PBST overnight at 4 °C. The membrane was washed with PBST and incubated with IRDye 800 conjugated secondary antibody for 1 h at 37 °C, then washed with PBST twice and PBS once. Detection was performed by an odyssey scanning system (Li-COR, Lincoln, Nebraska).

4.15. In vivo antitumor efficacy

In vivo antitumor activity was investigated in nude mice. Five-week-old male nude mice (16–18 g) were purchased from Nanjing Yuanduan biotechnology company (China); tumors were induced by a subcutaneous injection in their back region with 10^7 cells in 0.1 mL of sterile PBS. Mice were randomly divided into five groups (No. 1–5), and started on the second day. When the tumors reached a volume of 100–150 mm^3 in all mice, the first group (No. 1) was injected with an equivalent volume of normal saline *via* a tail vein as the vehicle control mice. No. 2 was treated with cisplatin (dosed intravenously at 5 mg/kg once a week), No. 3 was treated with NBDHEX (dosed orally at 5 mg/kg for one week), No. 4 was treated with complex **1** (dosed intravenously at 5 mg/kg once a week), No. 5 was treated with cisplatin (dosed intravenously at 5 mg/kg once a week) in combination with NBDHEX (dosed orally at 5 mg/kg for one week). Cisplatin was dissolved in normal saline, NBDHEX and complex **1** were dissolved in a small amount of DMF, and then diluted with normal saline injection. Tumor volume and body weight were recorded every third day after drug treatment. All mice were sacrificed after 4 weeks of treatment and the tumor volumes were measured with electronic digital calipers and determined by measuring length (A) and width (B) to calculate volume ($V = AB^2/2$).

Abbreviations

Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma-2
CDNB	1-chloro-2,4-dinitrobenzene
DCM	dichloromethane
DMF	*N*,*N*-dimethylformamide
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
EA	ethacrynic acid
FBS	fetal bovine serum
GSTs	glutathione s-transferase
GSH	glutathione
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
NBDHEX	6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol
NSCLC	non-small cell lung cancer
PARP	poly(ADP)ribose polymerase
PBS	phosphate buffered saline
PI	propidium iodide
RPMI	Roswell Park Memorial Institute
TBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate
TEA	trimethylamine

Acknowledgments

We are grateful to the National Natural Science Foundation of China (No. 21571033) and the New Drug Creation Project of the National Science and Technology Major Foundation of China (Grant No. 2015ZX09101032) for financial aids to this work. Fundamental Research Funds for the Central Universities (Project 2242016K30020 and 2242017K41024) and Priority Academic Program Development of Jiangsu Higher Education Institutions for the construction of fundamental facilities are also appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2019.01.014.

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