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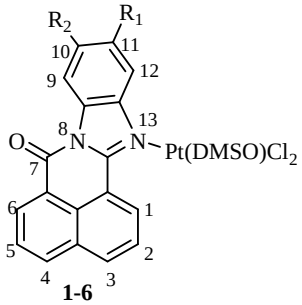
Graphical abstract



1-6

1: $R_1 = R_2 = H$
2: $R_1 = R_2 = Cl$
3: $R_1 = R_2 = CH_3$
4. $R_1 = Cl$, $R_2 = H$
5. $R_1 = F$, $R_2 = H$
6. $R_1 = H$, $R_2 = CF_3$

5: IC_{50}=2.36 μM

Design, synthesis and biological evaluation of naphthalenebenzimidizole platinum (II) complexes as potential antitumor agents

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Abstract

A serial of naphthalenebenzimidizole-Pt complexes **1**–**6** were designed and synthesized as antitumor agents. *In vitro* antitumor assay results showed that complexes **1**–**6** exhibited moderate to high antiproliferative activity against Hela, HepG2, SKOV-3, NCI-H460, BEL-7404 and A549 cancer cell lines, while they displayed obvious sensitivity and selectivity against SMMC-7721 and U251 cell lines and low toxicity against normal HL-7702 cells, in comparison with cisplatin. *In vivo* antitumor assay results indicated that complex **1** and **5** exhibited important *in vivo* antiproliferative activity in the NCI-460 and SMMC-7721 models, in comparison with cisplatin, respectively. Complexes **1** and **5** exhibited better antiproliferative activity against A549CDDP and SKOV3CDDP cell lines than cisplatin, with IC_{50} values of 6.98±0.47μM, 5.62±0.88μM and 13.13±2.11μM, 5.30±0.33μM, respectively, while they displayed potential antiproliferation against A549 and SKOV3 cell lines, with IC_{50} values of 7.32±0.51μM, 5.19±0.49μM and 14.92±0.11μM, 12.19±0.92μM, indicating the introduction of naphthalenebenzimidizole into platinum-metal system may overcome the resistance. Mechanistic studies showed that the representative complexes **1** and **5** exerted the antitumor effect mainly by the obvious covalent binding with DNA and the upregulation of the expression level of intracellular topo I, showing different action mechanism from cisplatin.

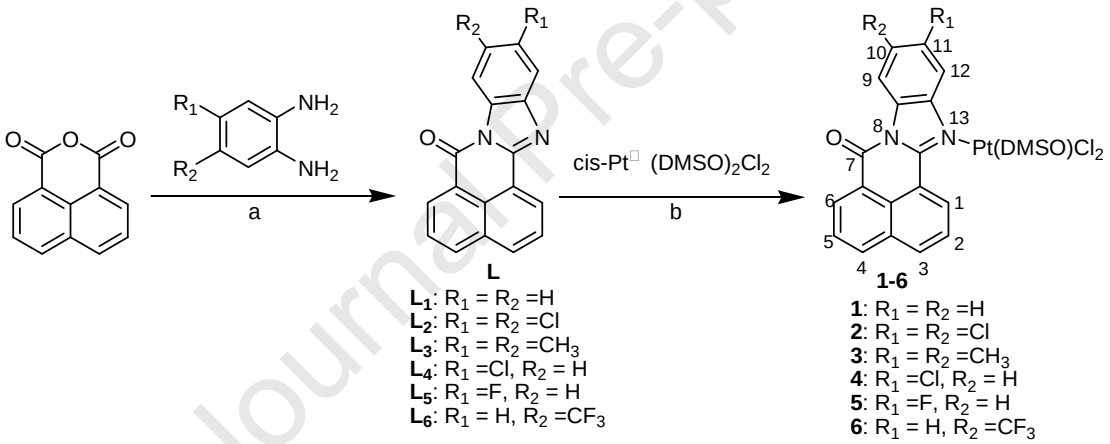
Keywords: naphthalenebenzimidizole-Pt complexes; antitumor activity; action mechanism; covalent DNA binding; topo I inhibition

1. Introduction.

Cisplatin (CDDP) has been approved as anticancer drugs by Food and Drug Administration (FDA) and widely used for the treatment of a variety of cancers including testicular, ovarian, lung, cervical, and bladder carcinomas in the clinical[1,2]. However, CDDP has been also found obvious clinical drawbacks containing drug resistance, side effects and high toxicity in the clinical applications[3,4]. Therefore, development of novel and more effective platinum-metal antitumor drugs for overcoming the above mentioned shortcomings have greatly attracted bioinorganic chemists' interest and become a hot research topic in bioinorganic chemistry. To overcome these shortcomings, a great deal of effort has been devoted to the design and synthesis of novel and effective platinum based drugs in the past two decades[3-5]. The classic strategy has been also tried to introduce the functional and potent antiproliferative active ligands into platinum-based compounds and proved to be an effective method[3,4].

Naphthalimides, which generally contain a coplanar, generally л-deficient aromatic system and basic side chains, are known as potential DNA and topoisomerase(topo) targeted antitumor agents and exhibit potential antiproliferative activity against several cancers lines including breast, hepatocellular, bladder and glioma carcinomas[6-16]. As one of the most widely studied naphthalimides, amonafide exhibits excellent antitumor activity in clinical phase II breast cancer trials and exerts the antitumor effect via binding DNA with intercalation and poisoning Topoisomerase[10,17-19], indicating that screening for antitumor agents based on the active pharmacal core naphthalimide may be a reasonable and feasible strategy, though it finally fails in clinical phase III trials[10,17]. So the naphthalimide derivatives naphthalenebenzimidizoles were herein selected as active ligands to introduce into platinum-based compounds to screening for high-efficiency and low-toxic new antitumor complexes. Since that both of naphthalimide derivatives[10,17] and platinum-based compounds[3,4] usually exhibit potential antiproliferative activity, it is expected that the combination of naphthalimide core and platinum-metal system may lead to better antitumor activity. In addition, naphthalimide aromatic moiety with multiple binding sites is able to coordinate with various kinds of biologically important species such as DNA, proteins, enzymes, biothiols and reactive oxygen species (ROS) etc[9]. Moreover, multiple mechanisms have been proposed to improve the cytotoxicy and cellular resistance of cisplatin and its analogues in preclinical models[3-5,20,21]. Furthermore, previous work had proved that

constructing complexes that displayed different DNA-binding modes was one of three viable options in the design of new platinum drugs[22]. It was thus to expect that the introduction of naphthalimides ligands into platinum-metal system may lead to the change of the action mechanism including the DNA-binding modes and even multiple mechanisms, which may consequently result to the improvement of the side effects and drug resistance. So a series of naphthalenebenzimidizole-platinum (II) complexes were designed and synthesized in the present work (Scheme 1). However, to the best of our knowledge, naphthalenebenzimidizole-platinum (II) complexes have not been described in the literature. The *in vitro* and *in vivo* antitumor activities and *in vitro* cytotoxity against normal cell lines, as well as the *in vitro* antiproliferative activity against CDDP-resistant strain cell line were also evaluated. Furthermore, the covalent DNA-binding and topo I inhibition assays were carried out to evaluate the action mechanism.



Scheme 1. General synthetic route of naphthalenebenzimidizole platinum (II) complexes **1**–**6**. (a) glacial acetic acid, 120 ^{o}C, 5h; (b) chloroform/methanol, 60 ^{o}C, 6h.

2. Results and Discussion

2.1. Synthesis

The ligands (naphthalenebenzimidizoles) $\mathbf{L_1}$–$\mathbf{L_6}$ and complexes (**1**–**6**) were synthesized as described in Scheme 1. The ligands $\mathbf{L_1}$–$\mathbf{L_6}$ used in this study was conveniently synthesized in moderate yields by the combination of 1,8-naphthalic anhydride with o-phenylenediamine/substituted o-phenylenediamine at the 120 ^{o}C for 5h, in the presence of glacial acetic acid, respectively, according to the previous literature[23,24]. The naphthalenebenzimidizole platinum (II) complexes **1**–**6** were then prepared in moderate yields by

the treatment of $\mathbf{L_1}$–$\mathbf{L_6}$ with cis-Pt(DMSO)$_2$Cl$_2$ at the 60 ^{o}C for 10h, in the presence of the mixture of chloroform/methanol (v/v=1:1), respectively. The structures of complexes **1**–**6** were determined by ^{1}H NMR spectroscopy, high-resolution mass spectrometry (HR-MS), elemental analysis, and single-crystal X-ray diffraction analysis (Part 1 in supplmentary materials).

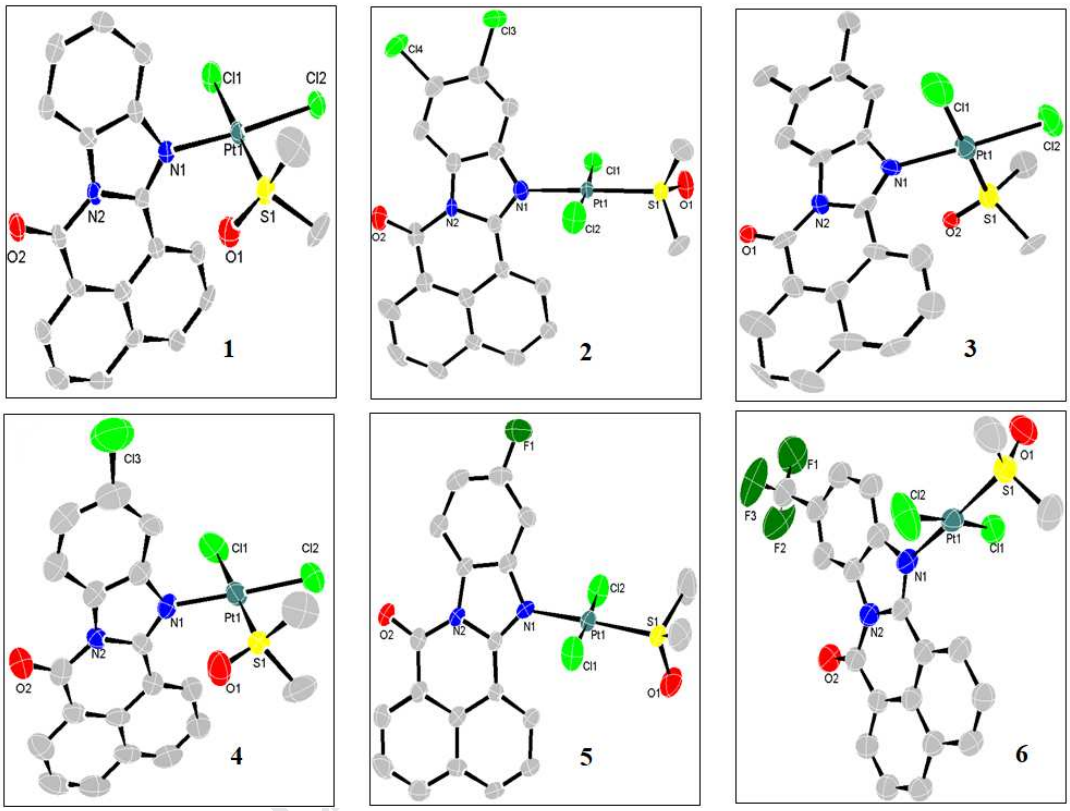


Fig. 1. The crystal structures of complexes **1**–**6**.

2.2. Crystal structures of complexes 1–6

The crystal structures of complexes **1**–**6** were determined by the X-ray diffraction and the crystallographic data were summarized in the Supporting Information (Tables S2-1–S2-12). As shown in Fig. 1, the ligands ($\mathbf{L_1}$–$\mathbf{L_6}$) and cis-Pt(DMSO)Cl$_2$ formed as monodentate coordination complexes, respectively. The Pt-N bond lengths for complexes **1**–**6** were found to be 2.041(10)Å, 2.063(15)Å, 2.056(10)Å, 2.041(8)Å, 2.052(9) Å and 2.029(8) Å, respectively, which were within the normal range.

2.3. Stability and solubility studies of complexes 1–6

The stability of complexes **1–6** in 10 mM Tris-HCl buffer solution (Tris, 80%DMSO, pH 7.4) was monitored by the high performance liquid chromatography (HPLC) assay, according to the literature[25]. As shown in Fig. S2-1(in supplmentary materials), for complexes **1–6**, there were

chiefly two peaks (one for DMSO and the other for complexes **1–6** in the HPLC spectra for 0h, 24h and 48h, and these peaks in HPLC showed no obvious change within these periods. The results indicated that these six complexes were stable in DMSO under the physiological conditions.

2.4. *In vitro* antiproliferative activity

The *in vitro* antiproliferative activity of ligands $\mathbf{L_1}$–$\mathbf{L_6}$ and complexes **1**−**6** were evaluated against the Hela (human cervical cancer cells), HepG2 (human liver cancer cells), SKOV3 (human ovarian cancer cells), NCI-H460 (human large cell lung cancer cells), BEL-7404 (Human liver cancer cells), A549 (human lung cancer cells), SMMC-7721 (human liver cancer cells), U251 (human glioma cells) and HL-7702 (human liver normal cells) cell lines, using the methylthiazoltetrazolium (MTT) assay[25,26]. For comparison, cisplatin was used as positive control.

Table 1. [α]IC_{50} values of complexes **1**−**6** towards the selected tumor cell lines.

Entry	IC_{50}（μM）					
	Hela	HepG-2	NCI-H460	BEL-7404	SMMC-7721	U251
$\mathbf{L_1}$	87.97±1.32	20.86±0.37	13.31±0.82	>100	>100	>100
$\mathbf{L_2}$	>100	>100	>100	>100	>100	>100
$\mathbf{L_3}$	>100	>100	>100	>100	>100	89.31±0.48
$\mathbf{L_4}$	>100	>100	>100	>100	>100	>100
$\mathbf{L_5}$	>100	18.73±.084	11.58±0.37	>100	>100	90.09±0.56
$\mathbf{L_6}$	>100	52.05±1.56	>100	>100	>100	>100
1	9.24±1.35	18.17±1.26	17.98±0.42	78.30±1.32	6.85±1.41	11.61±1.19
2	>100	>100	>100	>100	5.46±0.76	19.00±0.39
3	9.37±0.72	17.01±0.67	>100	41.99±0.76	4.92±1.55	9.80±0.84
4	>100	>100	>100	>100	4.99±1.64	11.44±0.57
5	>100	24.87±0.37	13.62±0.72	>100	2.36±1.44	8.37±0.94
6	19.09±0.13	16.33±0.98	>100	>100	7.33±0.89	8.75±1.34
Cisplatin	9.45±0.25	10.28±0.77	20.36 ± 0.50	10.08 ±0.52	27.43±0.35	40.6±0.77

[α]IC_{50} values are presented as mean ± SD (standard error of the mean) from three repeating experiments.

As shown in Table 1 and Table S3-1, the complexes **1**−**6** exhibited obvious sensitivity and selectivity against SMMC-7721 and U251 cell lines, while they displayed lower cytotoxicity on the HL-7702 normal cell line, compared with cisplatin, indicating that complexes **1**−**6** may be good candidates for antitumor agents. By the comparison of the antiproliferative activity of $\mathbf{L_1}$

with **1**, $\mathbf{L_2}$ with **2**, $\mathbf{L_3}$ with **3**, $\mathbf{L_4}$ with **4**, $\mathbf{L_5}$ with **5**, $\mathbf{L_6}$ with **6**, respectively, it could be found that the complexes **1**−**6** exhibited increased antiproliferative activity than their corresponding ligands against the selected cancer cell lines, implying that synergistic effect of complexes could improve the antiproliferative activity. In the Hela line assay, complexes **1** and **2** exhibited equal antiproliferation to cisplatin (IC_{50} = 9.45±0.25 μM), with IC_{50} values of 9.24±1.35 and 9.37±0.7 μM, respectively. In the SKOV-3 and A549 cell line assay (Table S3-1), complex **6** showed better antiproliferative activity than cisplatin (IC_{50} =16.32±1.37μM and 6.37±1.03μM), with IC_{50} of 12.19±0.92 μM and 5.19±0.49μM, respectively. In the NCI-H460 cell line assay, complexes **1** and **6** exhibited higher antiproliferation than cisplatin (IC_{50} = 20.36±0.50 μM), with IC_{50} of 17.98±0.42 and 13.62±0.72 μM, respectively. It was important noting that complexes **1**−**6** exhibited better antiproliferation than cisplatin against SMMC-7721 and U251 cell lines, with IC_{50} values in the range of 2.36−11.61 μM, indicating that the introduction of naphthalenebenzimidizole ligands into platinum-metal system may lead to sensitivity and selectivity against SMMC-7721 and U251 cell lines.

The structure-activity relationship study for complexes **1**−**6** was then performed. The substituents in benzimidazole moiety were found to have important and irregular influence on their antiproliferative activity. By the comparison of complex **1** with **2**, **3** and **6**, respectively, it could be found that the introduction of two methyl, two chlorine and trifluoromethyl groups in benzimidazole moiety increased the cytotoxity against the normal cell line HL-7702, implying that the introduction of substituents in 12-position (R_2) may increase the toxicity. In addition, by the comparison of complex **1** with **4**, **1** and **5**, respectively, it could be concluded that the introduction of fluorine and trifluoromethyl groups in benzimidazole moiety could increase the antiproliferation against SMMC-7721 and U251 cell lines. Moreover, by the comparison of complex **1** with **3** and **4**, respectively, it should be found that the introduction of one chlorine and two methyl groups in moiety may decrease the scope of antiproliferation.

2.5. *In vivo* antiproliferative activity

In vivo antiproliferation assay was performed to further evaluate the antitumor activity of complex **1** and **5**. The NCI-460 and SMMC-7721 xenograft models were chosen in this study, and cisplatin was used as the positive control. The SPF BALB/c nude mice were randomly divided

into three groups including the vehicle control group, the experimental group, and the positive control group.

As shown in Fig. 2, complex **1** at the dose of 4 mg/kg exhibited important inhibition on NCI-460 tumour growth, and the relative tumor increment rates (T/C) were found to be 55.9%, based on the tumor volume. In comparison, cisplatin at the 2 mg/kg dose showed a lower T/C value of 29.9%. The tumors were harvested and weighted on day 14, and the inhibitory rates on the growth of tumor weight were then determined. As shown in Fig. 2C, complex **1** (at the 4 mg/kg dose) displayed obvious antitumor activity in the NCI-460 model with an inhibitory rate of 39.7%, lower than that of cisplatin (63.2%, $P < 0.01$). Further investigation showed that no significant change in body weight and no other adverse effects were observed among the mice treated with complex **1**, suggesting that **1** exhibited no significant toxicity to the mice within the 13-day period of treatment (Fig. 2B).

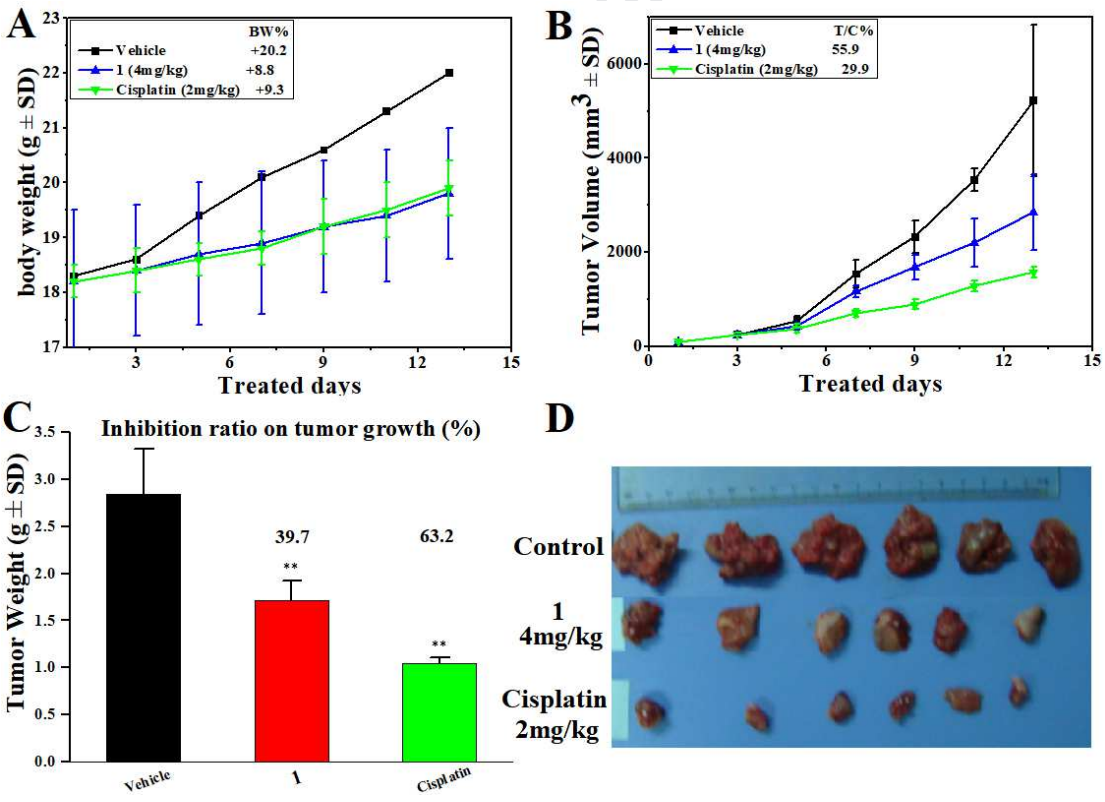


Fig. 2. *In vivo* anticancer activity of **1** in the NCI-460 xenograft model. (A) Effect of **1** (at the doses of 4 mg/kg), cisplatin (at the dose of 2 mg/kg), or vehicle (5% DMSO in saline, v/v) on tumor growth. Tumor growth was monitored by the mean tumor volume (mm^3) ± SD (n = 6) and calculated as the relative tumor increment rate (T/C, %). (B) Body weight change of the mice treated with **1**. (C) Tumor weight of the mice. The tumors were collected in the mice at day 14.

The "**" signs represent $p < 0.01$ (versus the vehicle control group). (D) Photographs of the harvested tumors from the mice.

Fig. S3-1 (in supplmentary materials) showed that complex **5** exhibited evident inhibition on SMMC-7721 tumour growth, with the relative tumor increment rates (T/C) of 20.99% (at 10 mg/kg dose) and 46.24% (at 4 mg/kg dose). In comparison, T/C value of cisplatin at the 2 mg/kg dose was found to be 41.31%. It was important to note that complex **5** (at the 10 mg/kg dose) exhibited potent antitumor activity in the SMMC-7721 model with the inhibitory rate of 64.63%, much higher than that of cisplatin (35.85%, $P < 0.05$). In addition, no significant changes in body weight were observed among the mice treated with complex **5**. This result showed that complex **5** may be good candidate for antitumor drug.

2.6. Drug resistance trait experiment

According to the MTT results in Table 1 and Table S3-1, complexes **1** and **5** showed the most potential antiproliferation sensitivity and selectivity activity against these cancer cell lines and lower cytotoxicity on the HL-7702 normal cell line. Therefore, complexes **1** and **5** were selected as representative compounds for further studies. In order to investigate the drug resistance of representative complexes **1** and **5**, the antiproliferative activity of the complexes **1** and **5** were evaluated against the cisplatin-resistant strain A549CDDP and SKOV3CDDP cell lines by MTT[26], using cisplatin as positive control.

As shown in Table S3-1, the IC_{50} of cisplatin against A549 and SKOV3 cell lines were found to be 6.37±1.03μM and 16.32±1.37μM, while that of cisplatin against A549CDDP and SKOV3CDDP cell lines was determined to be 23.01±0.46μM and 53.68±1.85 (more than three times of that in A549 and SKOV3 assay), indicating that cisplatin exhibited obvious and serious drug resistance. It was worth noting that the IC_{50} of complexes **1** and **5** against A549CDDP and SKOV3CDDP were similar with (or lower than) that against A549 and SKOV3, respectively, demonstrating that complexes **1** and **5** exhibited no obvious drug resistance. The antitumor mechanisms of complexes **1** and **5** were then investigated.

2.7 Action mechanism

2.7.1. Covalent DNA binding assayed by HR-MS

To better evaluate whether DNA is the potential target of complexes **1–6**, the HR-MS with high accuracy was used for determining the interaction of complexes with DNA. Previous work has indicated that cisplatin exerted the antitumor effect mainly by covalent binding with the N-7 atom of guanosine monophosphate (GMP) in DNA[5,27]. As shown in Fig. 3, cisplatin could be hydrolyzed into mono- and diaqua- products under the physiological conditions, respectively. By the nucleophilic substitution reaction of these mono- and diaqua- products with GMP, respectively, monoadducts was subsequently obtained and finally to offer bisadduct[5,27]. So GMP was herein selected as a DNA model to study the interaction between the complexes with DNA. In this part, complexes **1** and **5** were chose as representative compounds.

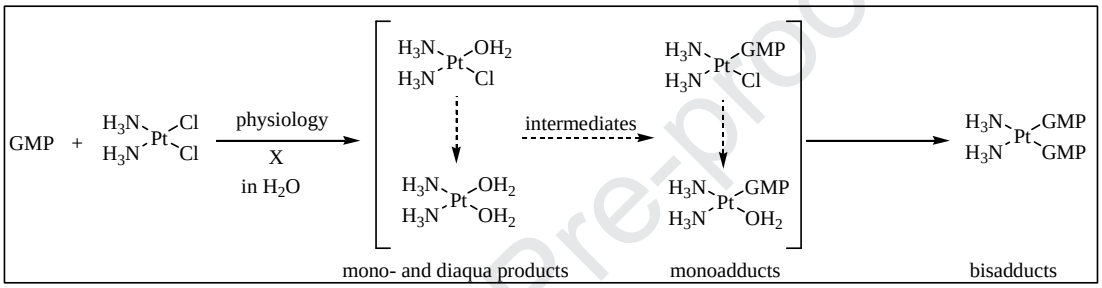


Fig. 3. A variety of adducts generated by the interaction of cisplatin and guanosine

As shown in Fig. 4, there were mainly four peaks in the HR-MS of the mixture of complex **1** with GMP for 1h, 6h, 12h and 24h, respectively. The peaks at 596.0530 and 657.0394 were attributed to the complexes of $[\mathbf{1}–Cl+H_2O]^+$ and $[\mathbf{1}–Cl+DMSO]^+$, respectively, whereas the peaks at 956.9932 and 1210.459 were attributed to that of $[\mathbf{1}–Cl+GMP+H_2O]^+$ and $[\mathbf{1}–DMSO–2Cl+2GMP+H_2O]^+$. The peak intensity of $[\mathbf{1}–Cl+H_2O]^+$ increased with time and stabilized in about 12h. The peak intensity of $[\mathbf{1}–Cl+DMSO]^+$ decreased with time and stabilized in about 12h. However, the peaks of $[\mathbf{1}–Cl+GMP+H_2O]^+$ and $[\mathbf{1}–DMSO–2Cl+2GMP+H_2O]^+$ occurred within 1h and its intensity decreased with time. Evidently, there was an important competitive relationship among these four complexes. In fact, due to the important departure ability of chlorine group, complex **1** may covalently bond with H_2O, DMSO and GMP. The covalent binding ability should be in the order H_2O > DMSO > GMP. These result demonstrated that complex **1** exhibited obvious covalent binding ability with GMP to offer mono- and bis- adducts, similar with that of cisplatin[5,27].

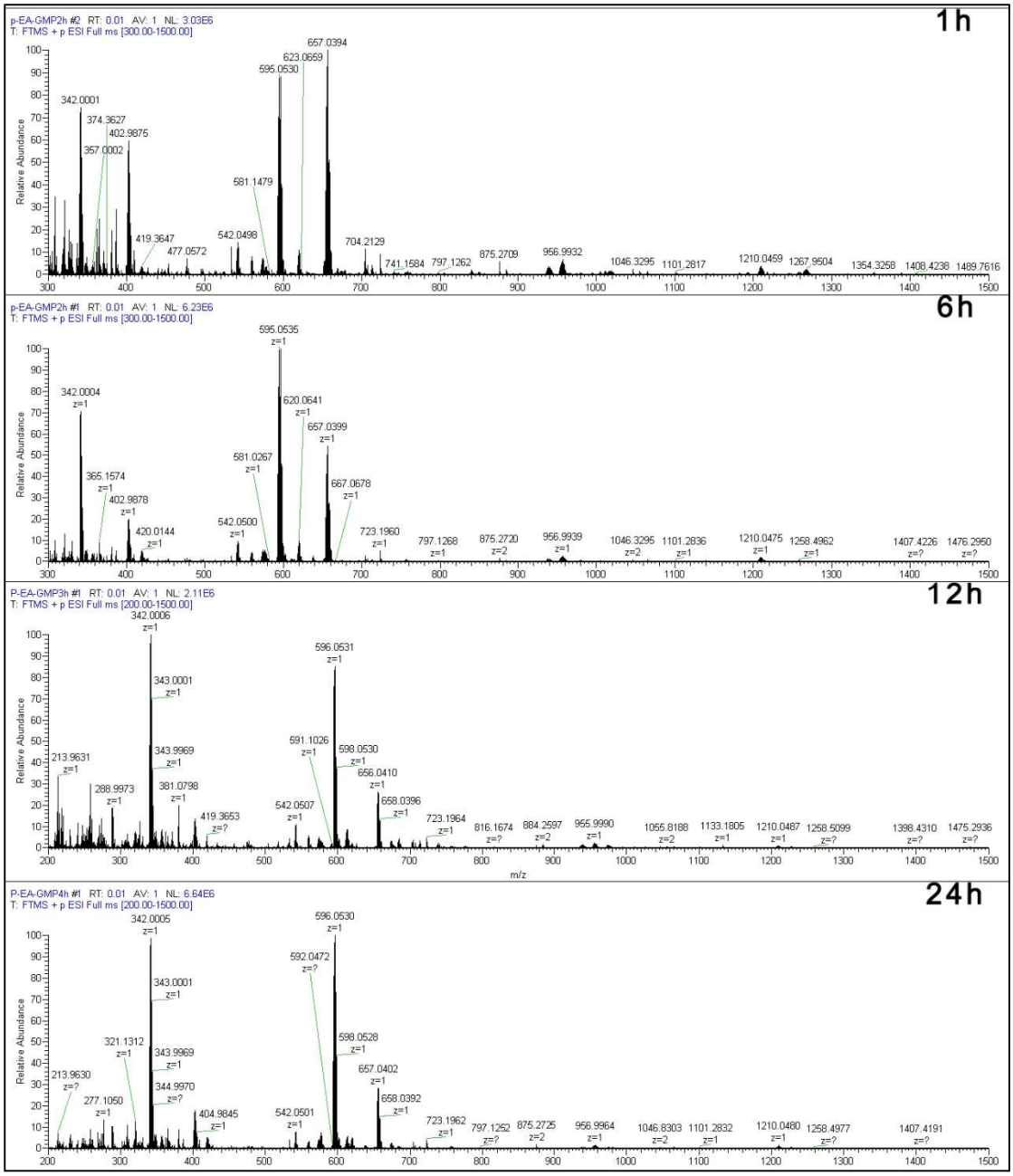


Fig. 4. HR-MS of the mixture of **1** with GMP for 1h, 6h, 12h and 24h.

The HR-MS of the mixture of complex **5** with GMP (Fig. S4-1, in supplmentary materials) for 1h, 6h, 12h and 24h was similar with that of complex **1**. There were four main peaks at 614.0428[**5**–Cl+H_2O]$^+$, 675.0298[**5**–Cl+DMSO]$^+$, 957.9574[**5**–Cl+GMP]$^+$ and 974.9836 [**5**–Cl+GMP+H_2O]$^+$ in the HR-MS, respectively, indicating that complex **5** displayed obvious covalent binding ability with GMP to offer monoadducts, similar with that of complex **1** and cisplatin[5,27]. Fig. S4-1 also demonstrated that the covalent binding ability order of complex **5** to H_2O, DMSO and GMP was similar with that of complex **1**, namely H_2O > DMSO > GMP.

2.6.2 Verification experiment of topoisomerase I target

2.6.2.1 Topoisomerase I assayed by gel electrophoresis

It is generally accepted that the main targets of naphthalimide derivative are DNA binding and topoisomerase inhibition[6-16]. Moreover, naphthalimide exhibits a similar structure with the classic topoisomerase I inhibitor camptothecin (Fig. 5). The interaction of complexes **1**–**6** with topo I was thus assayed by gel electrophoresis.

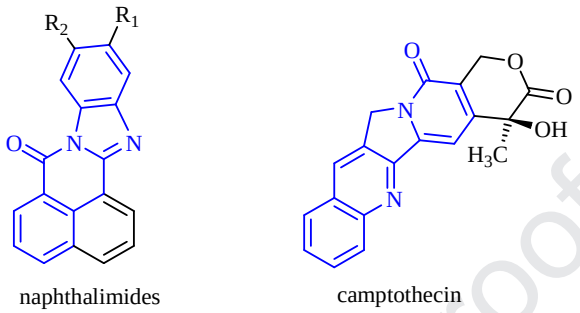


Fig. 5. The chemical structures of naphthalimides and camptothecin.

As shown in Fig. S5-1 (in supplmentary materials), complexes **1**–**6** exhibited obvious *in vitro* inhibition on topo I. It was worth noting that complexes **1** and **5** completely inhibited the catalytic function of topo I (0.1U/L) at the concentration of 40μM, while complexes **2**–**4** gained that effect at 80μM. It was well known that camptothecin was a classical topo I poison with IC_{50} values of about 20μM[28]. By the comparison of the topo I inhibition concentrations values of camptothecin (T, Fig. S5-1) with complexes **1** and **5** (20μM), respectively, it could be concluded that complexes **1** and **5** showed similar *in vitro* inhibition activity with camptothecin. These results indicated that complexes **1** and **5** may exert the antitumor activity by the inhibition of topo I at molecular level.

It is well known that topoisomerase I inhibitors could be divided into topoisomerase I poisons and suppressors. Suppressors prevent or reverse topoisomerase I-DNA complex formation, which may lead to the upregulation topo I in cancer cells, while poisons act specifically at the level of the topoisomerase I-DNA complex and stimulate DNA cleavage[29,30]. To further study whether complexes **1** and **5** have important effect on the expression level of intracellular topo I, the treatment of topo I in SMMC-7721 cells with complexes **1** and **5** were then investigated by western blot assay. As shown in Fig. 6, the treatment of SMMC-7721 cells with complexes **1** and **5** lead to evident increment in the expression level of topo I, compared to the control, while complex **5** could upregulate the expression level of intracellular topo I in a concentration-dependent manner. This result confirmed that in addition to covalent binding to

DNA, complexes **1** and **5** may also exert the antitumor activity by the cellular upregulation of topo I, demonstrating that the antitumor mechanism of complexes **1** and **5** was indeed different from that of cisplatin.

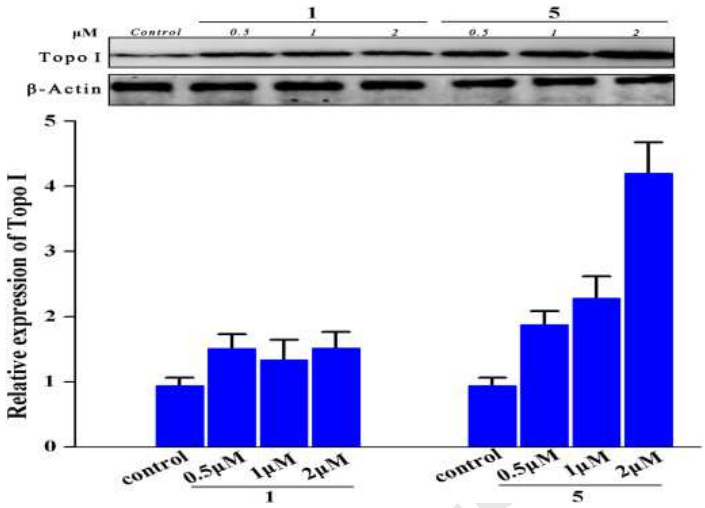


Fig. 6. The expression of topo I in SMMC-7721 cells with **1** and **5**.

3. Conclusions

In summary, we designed and synthesized a serial of naphthalenebenzimidizole-Pt complexes **1**–**6** and evaluated their antiproliferative activity against eight cancer cell lines (Hela, HepG2, SKOV3, NCI-H460, BEL-7404, SMMC-7721, U251, A549). We identified the six complexes exhibiting obvious *in vitro* sensitivity and selectivity against SMMC-7721 and U251 cell lines and low toxicity against normal HL-7702 cells, while complex **1** exhibited important *in vivo* antiproliferative activity in the NCI-460 model. Drug resistance experiment showed that representative complexes **1** and **5** exhibited no resistance on A549-CDDP, while cisplatin showed evident and serious resistance. Mechanistic studies showed that complexes **1** and **5** exerted their antitumor effect mainly by the covalent binding with DNA and the upregulation of the expression level of intracellular topo I, indicating that their targets were different from that of cisplatin. This work suggests that the introducing new ligands naphthalenebenzimidizole into platinum-metal system may change the anti-tumor mechanisms and thus to improve the antipoliferative activity, drug resistance and toxicity, well consistent with the previous literatures[31-34].

4. Experimental

4.1. Chemistry.

All chemicals of reagent grade were commercially available and used without further purification. NMR spectra were recorded on a BRUKER AVANCE AV 400/500 spectrometer using tetramethylsilane (TMS) as the internal standard. The mass spectra were collected on a BRUKER ESQUIRE HCT spectrometer. The GelRed nucleic acid stain was purchased from Biotium.

4.2 General synthetic procedure for complexes 1–6

The mixture of 1,8-naphthalene anhydride (1 mmol), substituented o-phenylenediamine (1mmol) and 20 mL glacial acetic acid was reacted at 120^{o}C for 6h, according to the literature[23,24]. The solution was cooled to room temperature and then filtered to offer yellow powders of **L(L$_1$–L$_6$)**. It was important to noted that Ligands **L$_1$–L$_3$** were then used without further purification, while the powders of **L$_4$–L$_6$** needed to be further purified by silica column chromatography with hexane/ethylacetate (v/v=5:1) as the eluent. The mixture of ligands **L(L$_1$–L$_6$)**(0.37mmol), cis-dichloride bis(dimethyl sulfoxide) platinum(II) (0.37mmol), 5mL methanol and 5mL dichloromethane was reacted at 60^{o}C for 48h. The hot solution was filtered and cooled to the room temperature. The cooled solution was filtered to offer yellow powder. Upon recrystallization from the mixed solution of methanol and chloroform (V:V=1:1), yellow crystals of the complex was obtained.

1: Yields 75.15%. ^{1}H NMR (400 MHz, d-DMSO) δ 8.72 (d, J = 8.3 Hz, 1H, H-Ar), 8.68 (d, J = 7.3 Hz, 1H, H-Ar), 8.51 (d, J = 8.2 Hz, 1H, H-Ar), 8.45 – 8.39 (m, 1H, H-Ar), 8.37 (d, J = 8.3 Hz, 1H, H-Ar), 7.95 – 7.85 (m, 3H, H-Ar), 7.53 – 7.45 (m, 2H, H-Ar). ESI-HRMS, calculated m/z for $[M–Cl+DMSO]^{+}$: 657.0408, found: 657.0385. Anal. Calc.(for $C_{20}H_{16}Cl_2N_2O_2PtS$) C 39.10; H 2.62; N 4.56%,Found. C 39.41; H 2.57; N 4.32%.

2: Yields 47.17%. ^{1}H NMR (400 MHz, d-DMSO) δ 8.63 (dd, J = 13.3, 5.6 Hz, 2H, H-Ar), 8.49 (d, J = 8.2 Hz, 1H, H-Ar), 8.47 – 8.25 (m, 2H, H-Ar), 8.01 – 7.73 (m, 2H, H-Ar), 7.65 – 7.55 (m, 1H, H-Ar). ESI-HRMS, calculated m/z for $[M+Cl]^{-}$: 716.8866, found: 716.8864. Anal. Calc.(for $C_{20}H_{14}Cl_4N_2O_2PtS$) C 35.16; H 2.07; N 4.10%,Found. C 35.14; H 2.09; N 4.12%.

3: Yields 60.46%. ^{1}H NMR (400 MHz, d-DMSO) δ 8.78 – 8.67 (m, 2H, H-Ar), 8.54 (d, J = 7.3 Hz, 1H, H-Ar), 8.38 (d, J = 7.5 Hz, 1H, H-Ar), 8.24 (s, 1H, H-Ar), 8.00 – 7.88 (m, 2H, H-Ar), 7.66 (s, 1H, H-Ar), 2.43 (s, 3H, CH_3), 2.39 (s, 3H, CH_3). ESI-HRMS,

calculated m/z for $[M–Cl+DMSO]^+$: 685.0721, found: 685.0704. Anal. Calc.(for $C_{22}H_{20}Cl_2N_2O_2PtS$) C 41.13; H 3.14; N 4.36%,Found. C 41.33; H 3.17; N 4.35%.

4: Yields 76.13%. ^{1}H NMR (400 MHz, d-DMSO) δ 8.71 (dd, J = 14.3, 7.4 Hz, 2H, H-Ar), 8.54 (d, J = 8.1 Hz, 1H, H-Ar), 8.49 – 8.31 (m, 2H, H-Ar), 8.11 – 7.77 (m, 3H, H-Ar), 7.51 (d, J = 8.3 Hz, 1H, H-Ar). ESI-HRMS, calculated m/z for $[M–Cl+DMSO]^+$: 691.0018, found: 690.9980. Anal. Calc.(for $C_{20}H_{15}Cl_3N_2O_2PtS$) C 37.02; H 2.33; N 4.32%,Found. C 37.04; H 2.30; N 4.32%.

5: Yields 72.14%. ^{1}H NMR (400 MHz, d-DMSO) δ 8.63 (dd, J = 13.3, 5.6 Hz, 2H, H-Ar), 8.49 (d, J = 8.2 Hz, 1H, H-Ar), 8.47 – 8.25 (m, 2H, H-Ar), 8.01 – 7.73 (m, 3H, H-Ar), 7.65 – 7.55 (m, 1H, H-Ar). ESI-HRMS, calculated m/z for $[M+Cl]^-$: 663.0125, found: 663.0032. Anal. Calc.(for $C_{20}H_{15}Cl_2FN_2O_2PtS$) C 37.99; H 2.39; N 4.43%, Found. C 37.92; H 2.38; N 4.42%.

6: Yields 53.14%. ^{1}H NMR (400 MHz, d-DMSO) δ 8.77 – 8.48 (m, 4H, H-Ar), 8.40 (dd, J = 8.2, 4.4 Hz, 1H, H-Ar), 8.21 – 7.85 (m, 3H, H-Ar), 7.80 (d, J = 8.4 Hz, 1H, H-Ar). ESI-HRMS, calculated m/z for $[M–Cl+CH_3CH_2OH]^+$: 691.0540, found: 690.9979. Anal. Calc. (for $C_{21}H_{15}Cl_2F_3N_2O_2PtS$) C 36.96; H 2.22; N 4.11%,Found. C 37.01; H 2.23; N 4.08%.

4.3. *In vitro* antipoliferative activity

The cell lines were all obtained from the Institute of Biochemistry and Cell Biology, China Academy of Sciences. They were cultured in a humidified, 5% CO_2 atmosphere at 37°C and maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 mg/mL penicillin. Chemosensitivity was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, exponentially growing cells were seeded into 96-well plates and treated with the indicated concentrations of complexes for 48 h, and then 10 mL of MTT (10 mg/mL) was added. After incubation for 4 h at 37^{o}C, the purple formazan crystals (a reduced form of MTT) generated in viable cells were dissolved by adding 100 μL DMSO to each well. The plates were swirled gently for 10 min to dissolve the precipitate, and quantified by measuring the optical density of the plates at 490 nm using a plate reader (TECAN infinite M1000). Each

concentration was repeated in three wells and the same experimental conditions were maintained for all testing procedures. The MTT assays were repeated three times for each cell line.

4.4 *In vivo* antipoliferative activity

Nude mice received subcutaneous injection of 5×10^7 tumor cells in the right flank. When the xenograft tumor growth to the volume about 1000 mm^3, the mice were killed and the tumor tissue were cut into small pieces at about 1.5 mm^3 and then transplanted into the right flank of male nude mice. When the average tumors reached the volumes of 100−150 mm^3, the mice were randomly divided into solvent control and treatment groups (n = 6 (or 4) /group). The complexes at different doses (5% v/v DMSO/saline) were given twice a day. Cisplatin was given to mice by ip administration at a dosage of 2 mg/kg/per 2 days and used as a positive reference for comparison. Tumor size and body weight were monitored every 2 days. The animals were finally sacrificed for humane reasons, and the tumors were weighted and recorded. The tumor volumes were determined every 2 days by measuring length (l) and width (w) and calculating with the formula of $V = lw^2/2$ as described elsewhere. Meanwhile, body weight of mice was measured and taken as a parameter of systemic toxicity. The rate of tumor growth was calculated using a formula of (1 − TWt/TWc) × 100, where TWt is the tumor weight of comlexes treated mice and the TWc is the tumor weight of vehicle-treated animals.

4.5 DNA binding assay

Briefly, the Tris-NO_3 (pH7.4) solution of GMP(4μM) and the DMSO solution of complex **1**/**5** (2μM) were treated for 1h, 6h, 12h and 24h, respectively, and then mixtures were diluted to 10 times with methanol and determined by high-resolution mass spectrometry (BRUKER ESQUIREHCT).

4.6 Topo I inhibition[35]

Briefly, a mixture (20 μL) containing 0.25 μg of pBR322 DNA in relaxation buffer (10 mM Tris, 0.1 mM EDTA, 5 mM MgCl2, 50 mM KCl, plus 1 mM DTT, 0.01% bovine serum albumin (BSA), pH 7.5) was incubated with 1 unit of calf thymus Topisomerase I (topo I, from TaKaRa Biotechnology Co., Ltd., Dalian) in the absence or in the presence of complexes at different concentrations, for 30 min at 37 °C. Topo I was preincubated with

the complex for 15 min at 37 °C prior to the addition of plasmid pBR322 DNA. The reaction was terminated by the addition of 4 μL of 10 × loading buffer (0.9% sodium dodecyl sulfate (SDS), 0.05% bromophenol blue and 50% glycerol). The sample was then analyzed using a 0.8% agarose gel in TBE buffer. The gels were stained with gel red and visualized in the electrophoresis gel documentation and analysis system.

2.8. Western Blot Assay[36]

Tumour cells were collected after treatment with complex (10 μM) for 12 h and then lysed in ice-cold lysis buffer (1% sodium dodecyl sulfate in 25 mM pH 7.5 Tris–HCl, 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin and 10 mg/mL soybean trypsin inhibitor). Whole-cell lysates were centrifuged at 12,000×g for 5 min. Thereafter, the protein concentration was determined with a bicinchoninic acid protein assay kit (Beyotime Co, China). An aliquot of cell lysate (40–50 μg) was fractionated by SDS-PAGE on 12% polyacrylamide gels for 2 h and transferred to polyvinylidene difluoride membranes. After blocking with 5% non-fat dry milk in PBS-t for 1 h at room temperature, the membranes were incubated with topo I (topo I, from TaKaRa Biotechnology Co., Ltd., Dalian) overnight at 4^{o}C, washed with tris-buffered saline and Tween 20, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Proteins were detected by electrochemiluminescence, Thermo Fisher Scientific, USA) and analysed by Image J software.

4.9. Statistical Analysis

Data are expressed as mean ± SD for three different determinations. Statistical significance was analyzed by one-way ANOVA. Mean separations were performed using the least significant difference method. $P<0.05$was defined as statistically significant.

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Highlights

- A serial of naphthalenebenzimidizole-Pt complexes **1**–**6** were designed and synthesized as antitumor agents
- *In vitro* cytotoxic and noncancerous screening for all the targeted compounds.
- Complex **1** and **5** exhibited important inhibitory on tumor growth in NCI-H460 and SMMC-7721 xenograft mouse models, respectively.
- Complexes **1** and **5** showed obvious covalent binding with DNA.
- Complexes **1** and **5** upregulated of the expression level of extracellular and intracellular topo I.

Declaration of Interest Statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “Design, synthesis and biological evaluation of naphthalenebenzimidizole platinum (II) complexes as potential antitumor agents”.