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Platinum(IV) cisplatin derivative *trans*, *cis*, *cis*-bis(heptanoato)amine(cyclohexylamine)dichloridoplatinum(IV) has an enhanced therapeutic index compared to cisplatin for the treatment of non-small cell lung cancer

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ABSTRACT

Lung cancer is the leading cause of cancer-related death in the United States, and non-small cell lung cancer (NSCLC) the most common type. Platinum (Pt) anticancer agents, such as cisplatin, remain a mainstay in the clinic; however, these agents are not tumor-specific and, thus, the patient experiences negative side-effects. We here prepare *trans*, *cis*, *cis*-bis(heptanoato)amine(cyclohexylamine)dichloridoplatinum(IV) and demonstrate that it is greater than 50-fold more toxic toward NSCLC cells than is cisplatin. Furthermore, it has a much improved therapeutic index. This Pt(IV) complex binds to DNA in a manner similar to that of cisplatin, and can be incorporated into mesoporous silica nanoparticles for fine-controlled release and the targeting of tumors.

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1. Introduction

Cis-diamminedichloroplatinum(II), cisplatin, shown in Fig. 1, was the first platinum (Pt)-based drug used as an anti-proliferative agent and is renowned for its high level and broad spectrum of anticancer activity [1]. Since Rosenberg discovered the anticancer activity of cisplatin in the 1960s, a large number of anticancer Pt(II) drugs have been found [2], but only a few of them are used in clinical therapy [3]. However, side-effects of cisplatin and related Pt(II) complexes limit their clinical usefulness. Cisplatin along with other Pt(II) complexes can easily bind with thiol groups on proteins in plasma, leading to deactivation of cisplatin and severe negative side effects [2,4]. Furthermore, as cisplatin is highly toxic toward rapidly dividing healthy cells, severe dose-limiting side-effects and acquired or intrinsic resistance to cisplatin are frequently observed; thus, its application is limited [5,6].

To overcome these drawbacks, a new generation of platinum agents, Pt(IV) octahedral compounds, have been developed [7–12]. The binding of cisplatin to nuclear DNA elicits its biologic effect, although it also interacts with RNA, proteins and enzymes containing sulfur functional groups and mitochondria [13,14]. Pt(IV) complexes have lower ligand exchange rates than Pt(II) complexes and must be "activated" via bio-reduction to their Pt(II) counterparts before interacting with DNA and other biological targets; thus, they are considered to be promising prodrugs of biologically active Pt(II) complexes. The biological reducing agent ascorbic acid is frequently cited in connection with the reduction of Pt(IV) complexes to Pt(II) [15–18]. Ascorbic acid is a two electron reducing agent present in blood plasma with a concentration of 50–150 μM and the cytosol at approximately 1 mM [19,20]. Previously, it was shown that *cis*, *trans*, *cis*-$[PtCl_2(OH)_2(NH_3)_2]$ (oxoplatin) and its carboxylate-modified analog, *cis*, *trans*, *cis*-$[PtCl_2(OH)(O_2CCH_2CH_2CO_2H)(NH_3)_2]$, both Pt(IV) complexes, can be reduced by ascorbic acid and bind to plasmid pBR322 DNA [15]. Blatter and co-workers revealed that ascorbic acid-reduced species of iproplatin and oxoplatin, both Pt(IV) complexes, can bind to and unwind closed circular PM2 DNA [18]. Furthermore, the octahedrally coordinated Pt(IV) complexes allow for more flexibility in structure modifications than Pt(II) complexes, including the potential for axial ligand modification to enhance their ability to enter tumor cells.

In this work, the cytotoxicity of a lipophilic Pt(IV) complex, *trans*, *cis*, *cis*-bis(heptanoato)amine(cyclohexylamine)dichloridoplatinum(IV), referred to in text as **5** and shown in Fig. 1, with a

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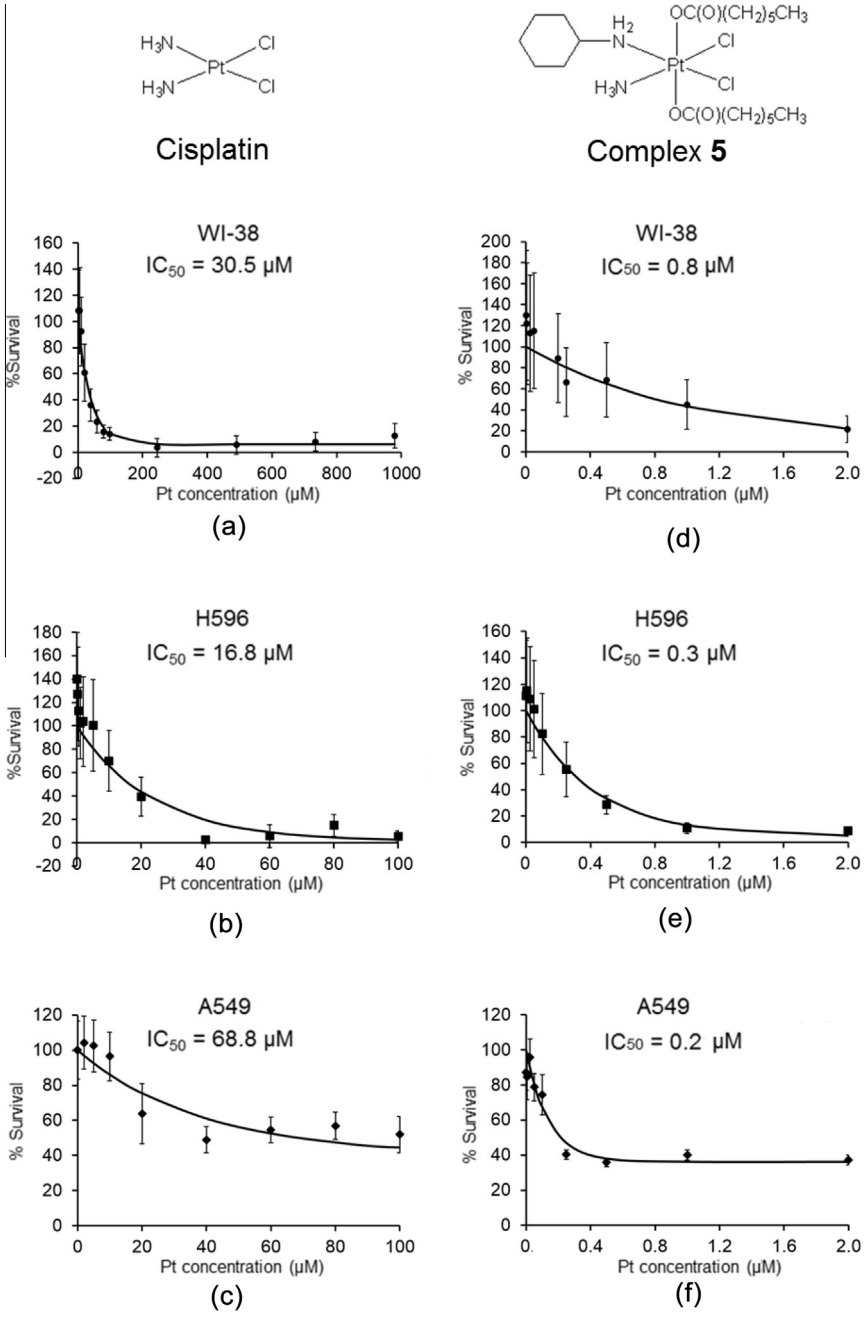

Fig. 1. Percent cell survival versus platinum (Pt) concentration in normal human lung fibroblast cell line, WI-38, and non-small cell lung cancer (NSCLC) cell lines, H596 and A549. The cells were exposed to cisplatin or complex **5** for 24 h before measurement.

partition coefficient ($\log P_{o/w}$) of 4.10 [21], was synthesized and tested against WI-38 normal human lung fibroblast cells, and H596 and A549 human non-small cell lung cancer (NSCLC) cells. Lung cancer is the leading cause of cancer-related death in the United States, and non-small cell lung cancer (NSCLC) the most common type. Pt anticancer agents, such as cisplatin, remain a mainstay in the clinic for the treatment of this disease [22]. It was previously shown that **5** is antiproliferative toward A2780 ovarian and HCT116 colon carcinoma cell lines with half maximal inhibitory concentration (IC_{50}) values of 0.39 ± 0.01 nM and 1.65 ± 0.25 nM, respectively [21]. To further investigate the mechanism of action of this Pt(IV) complex, we used pBR322 DNA as a capture agent for the reduction product of **5** by ascorbic acid, and investigated the mobility of the platinated DNA forms in an agarose gel. The lipophilic nature of **5** allows for its encapsulation in mesoporous silica MCM-41 nanoparticles [23,24], to better target tumors. We previously demonstrated that mesoporous silica MCM-41 nanoparticles accumulate in ovarian and NSCLC tumors after intraperitoneal and intravenous (i.v.) injection, respectively [25,26]. Thus, we tested the loading and release of complex **5** from this type of nanoparticle.

2. Materials and methods

2.1. Materials

Cisplatin, tetraethylammonium chloride (Et_4NCl), dimethylacetamide (DMA), Dowex 50-W-X8H^+ cation exchange resin and cyclohexylamine were from Sigma–Aldrich (St. Louis, MO);

heptanoic anhydride was from Acros Organics (Morris Plains, NJ); A549, H596 and WI-38 cells were from American Type Culture Collection (Manassas, VA); CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) solution was from Promega (Madison, WI); and pBR322 DNA was from New England Biolabs (Ipswich, MA).

2.2. Synthesis of $[PtCl_3NH_3]^-$ (**1**)

All four Pt intermediates were prepared using literature methods [27], with some modifications. To cisplatin (0.468 g, 1.56 mmol) and tetraethylammonium chloride (Et_4NCl, 0.332 g, 1.88 mmol) was added 40 mL of fresh reagent grade DMA. The solution was stirred while heating at 100 ± 2 °C for 6 h in a round-bottom flask, while a slow stream of N_2 gas was introduced to the solution by a gas dispersion tube for purging. Fresh DMA was added to maintain the volume and temperature control, but the volume of the reaction mixture was allowed to be reduced to approximately 10 mL by the end of the reaction. After the orange solution obtained was cooled to room temperature (r.t.), it was poured into 90 mL of 1/1 v/v hexane/ethyl acetate and was stored at −15 °C overnight. The clear solution was decanted and discarded and an orange oil was obtained when it was warmed to r.t. The orange oil was dissolved with 8 mL of water and the mixture was allowed to stand for 30 min at r.t. to allow complete precipitation of a small amount of unreacted cisplatin. The mixture was then filtered via vacuum filtration; the filtrate contained $Et_4N[PtCl_3NH_3]$. The filtrate solution was stirred with 2.0 g of rinsed Dowex 50-W-X8H^+ cation exchange resin for 1 h, the resin was filtered via vacuum filtration, and the volume of the filtrate reduced to approximately 5 mL at r.t. To the yellow/orange filtrate, 0.6 mL of saturated KCl solution was added and the mixture stored and left to evaporate in the refrigerator (~7 °C) to slowly obtain orange crystals. Orange crystals collected: 162.9 mg (29.2% yield). Mass spectrometry (MS) (1:9 H_2O:MeOH) calculated molecular mass of H_3Cl_3KNPt: 357.57 g/mol, observed average molecular mass: 357.51 g/mol.

2.3. Synthesis of cis-[PtClIachNH$_3$] (ach is cyclohexylamine) (**2**)

A solution of NaI (0.324 g, 2.16 mmol) in 0.54 mL of H_2O was added to a stirred solution of complex **1** (0.406 g, 1.27 mmol) in 1.8 mL of H_2O in the dark, followed by 149 μL of cyclohexylamine. The mixture was then stirred at r.t. for 4 h. The yellow precipitate was collected via vacuum filtration and washed with approximately 3 mL of water and then approximately 3 mL of ethanol. The solid was suspended in approximately 3 mL of acetone and stirred in H_2O for 0.5 h, the suspension centrifuged and the light yellow clear supernatant removed and discarded. This procedure was repeated three times before the product was dried *in vacuo*. Pale yellow powder was collected: 345.6 mg (57.3% yield). MS (acetonitrile with NaI) calculated molecular mass of $C_6H_{16}ClIN_2Pt$: 473.64 g/mol, observed average molecular mass: 473.66 g/mol. ^{1}H NMR (300 MHz, DMF-d_7), δ (ppm) = 1.10 (1H, m), 1.31 (4H, m), 1.60 (1H, m), 1.74 (2H, m), 2.43 (2H, m), 2.95 (1H, m), 4.21 (3H, b), 4.96 (2H, b).

2.4. Synthesis of cis-[PtCl$_2$achNH$_3$] (**3**)

To a stirred suspension of complex **2** (0.307 g, 0.648 mmol) in 3.83 mL of H_2O in the dark was added $AgNO_3$ (0.182 g, 1.07 mmol). The mixture was then stirred at r.t. for 5 h and tested for Ag^+: 20 μL of reaction mixture was taken and centrifuged, and the colorless supernatant added to a NaCl solution prepared by adding 10 pieces of NaCl crystal to 1.0 mL of H_2O, and no precipitate formed. Another several pieces of NaCl crystals were added and no precipitate was observed, which confirmed a negative test. After the negative test was obtained, 82 mg of decolorizing carbon was added to the reaction mixture and stirred for 0.5 h at r.t. and then centrifuged. The clear supernatant was obtained and 1.92 mL of HCl (37%) was added. The mixture was left to stand overnight at r.t. and the pale yellow precipitate was collected via vacuum filtration and washed with 5 mL of H_2O, ethanol and ether, and then dried *in vacuo*. Pale yellow product collected: 156.1 mg (63.3% yield). MS (acetonitrile with NaI) calculated molecular mass of $C_6H_{16}Cl_2N_2Pt$: 382.19 g/mol, observed average molecular mass: 382.25 g/mol. ^{1}H NMR (300 MHz, DMF-d_7), δ (ppm) = 1.10 (1H, m), 1.27 (4H, m), 1.58 (1H, m), 1.73 (2H, m), 2.42 (2H, m), 2.93 (1H, m), 4.21 (3H, b), 4.89 (2H, b).

2.5. Synthesis of cis, trans, cis-[PtCl$_2$(OH)$_2$achNH$_3$] (**4**) and then cis, trans, cis-[PtCl$_2$(OC(O)(CH$_2$)$_5$CH$_3$)$_2$achNH$_3$] (**5**)

A suspension of complex **3** (0.141 g, 0.37 mmol) in 1.40 mL of H_2O was stirred at r.t. for 3 h. The suspension was heated to 70 °C before 365 μL of H_2O_2 (30%) was added and stirred for 2 h. The reaction mixture was then cooled at r.t. and let stand overnight before it was cooled in an ice bath for 0.5 h. The precipitate was collected via vacuum filtration. The product was washed with 1.5 mL of H_2O, ethanol and then diethyl ether. A very pale yellow/off-white powder obtained: 85.7 mg (55.8% yield). Complex **5** was then prepared in a similar manner as [Pt(*cis*-1,4-dach)-*trans*-(acetate)$_2$Cl$_2$] [28,29]. To a suspension of **4** (75 mg, 0.18 mmol) in 15 mL acetonitrile was added heptanoic anhydride (0.714 mL, 15-fold). The reaction mixture was refluxed for 15 h and the clear yellow solution obtained was evaporated to dryness under reduced pressure. The resulting yellow residue was redissolved in approximately 3 mL of acetone and centrifuged. The supernatant was saved and evaporated to a minimum volume and kept in the refrigerator (~7 °C). The pale yellow precipitate obtained was isolated via centrifugation, resuspended using 1 mL of diethyl ether, collected via vacuum filtration and washed with approximately 5 mL of diethyl ether. The product was dried *in vacuo*. Off-white powder obtained: 63.1 mg (53.8% yield). MS (acetonitrile with NaI) calculated molecular mass of $C_{20}H_{42}N_2O_4Cl_2Pt$: 640.54 g/mol, observed average molecular mass: 640.46 g/mol. ^{1}H NMR (300 MHz, DMF-d_7), δ (ppm) = 0.85 (6H, m), 1.22 (21H, m), 1.60 (1H, m), 1.72 (2H, m), 2.17 (2H, m), 2.25 (4H, m), 3.00 (1H, m), 7.04 (3H, b), 7.60 (2H, b).

2.6. Cytotoxicity of cisplatin and the complex **5** against A549, H596 and WI-38 cell lines

The studies involving human NSCLC cells, A549 and H596, and human lung fibroblast cells, WI-38, were carried out under standard conditions in a humidified, 37 °C, 5% CO_2 atmosphere incubator. The culture medium used for A549 and H596 cells was Roswell Park Memorial Institute (RPMI) containing 10% fetal calf serum (FCS), 100 μg/mL streptomycin, 100 IU/mL penicillin and 2.0 mM L-glutamine. The culture medium used for WI-38 cells was Minimum Essential Medium (MEM) containing 10% FCS, 100 μg/mL streptomycin, 100 IU/mL penicillin and 2.0 mM L-glutamine. Solutions of cisplatin and complex **5** at various concentrations were prepared. Solid cisplatin was dissolved in 0.9% sodium chloride and allowed to equilibrate in the dark for 24 h before being diluted with medium and used to treat cells. Complex **5** was first dissolved in a small amount of DMSO and then diluted using medium. Two control groups were included; the first being medium alone and the second being medium plus cells without treatment of cisplatin or **5**. Ten replicates were done for each concentration and each control group. The cells were seeded at 5×10^4 cells/mL (100 μL/well) in 96-well plates and allowed to grow for 24 h, after which

time the medium was removed and replaced with 100 μL of medium containing Pt complex. After an exposure time of 24 h, the medium containing the Pt complex was removed and replaced with 100 μL of fresh medium. To each well, 20 μL of CellTiter 96® AQueous One (MTS) solution was added. After 2 h incubation with the MTS solution, the absorbance was read at 490 nm using a Synergy™ H1 hybrid multi-mode microplate reader (BioTek). The percent survival of cells treated by each concentration of Pt drug was calculated using the following equation:

$$\% \text{ survival} = 100 \times \left[\frac{A_d - A_m}{A_c - A_m}\right] \quad (1)$$

where A_d is the absorbance of cells treated with Pt drug, A_m is the absorbance of medium alone and A_c is the absorbance of cells without treatment. The percent (%) survival data was fit to an exponential regression using Microsoft Excel Solver, and IC_{50} values determined.

2.7. DNA binding studies

The DNA binding studies were done in a total volume of 20 μL containing 3.85 μM base pairs pBR322 DNA and 10 mM pH 7.4 HEPES buffer (with DMSO when testing **5**). The final concentration of cisplatin and Pt(IV) complex, **5**, was in the range of 1.0–6.0 μM and 20–100 μM, respectively. Ascorbic acid was used as the reducing agent for **5**. The complex was first dissolved in DMSO followed by the addition of HEPES buffer and ascorbic acid to give an aqueous solution containing 250 μM of ascorbic acid and 2.5% DMSO v/v and was incubated for 72 h at 37 °C in sealed Eppendorf tubes. Then, pBR322 DNA stock solutions were added. The values of *r*, where *r* = [Pt]/[DNA-bp], in the reaction solutions are given in the figure captions. The samples were further incubated at 37 °C for 24 h in sealed Eppendorf tubes. After incubation, 2.5 μL of a loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose was added to each sample. An 8 μL volume of each solution was loaded onto a 1.0% (w/v) agarose gel in 1× TBE (0.090 M trisborate and 0.002 M ethylenediaminetetraacetic acid) buffer, pH 8.0, and electrophoresis was carried out at 70 V for 1.5 h. The gel was then exposed to a 5×10^{-5}% solution of ethidium bromide for 30 min, washed (destained) in distilled water for 15 min, and its image captured using a ChemiDoc MP imaging system (Bio-Rad). The DNA binding studies with cisplatin were done in the same manner, but no DMSO or ascorbic acid were used in sample preparation and only one control group (DNA alone) was included. The *r* values are given in the figure caption.

2.8. Incorporation of complex **5** into mesoporous silica (MCM-41)

Mesoporous silica (MCM-41) nanoparticles were prepared as previously described [26]. The nanoparticles were approximately 400 nm. To a solution containing 1 mg of complex **5** in 200 μL ethanol was added 5 mg of MCM-41. The mixture was stirred vigorously at r.t. for 24 h and then centrifuged at 1000*g* for 10 min. The clear supernatant was discarded, and the pellet washed twice with ethanol and once with PBS, and then dried *in vacuo*. The dried sample was digested with hydrofluoric acid at 80 °C for 16 h, then evaporated to dryness, and further digested in 70% nitric acid for 2 h at 70 °C, before being analyzed using a NexION 300D (Perkin Elmer) inductively coupled plasma-mass spectrometer (ICP-MS). To study release, **5**-containing MCM-41 was added to PBS, pH 7.4, and the mixture vortexed at r.t. for 24 h. After this time, it was centrifuged at 1000*g* for 10 min, the supernatant removed and the pellet dried *in vacuo*. The dried sample was digested as previously described and analyzed using ICP-MS.

3. Results

Lipophilic Pt(IV) complex **5** was synthesized and characterized using ^{1}H NMR and mass spectrometry. The toxicities of cisplatin and **5** toward human NSCLC cells (A549 and H596) and human lung fibroblast cells (WI-38) were then investigated. Cells were exposed to cisplatin or complex **5** for 24 h. In Fig. 1, % survival is plotted against Pt concentration, and the IC_{50} values listed. Complex **5** is more toxic toward all three cell lines than is cisplatin. The IC_{50} values of complex **5** toward H596 and A549 cells are 0.3 μM and 0.2 μM, respectively, while those of cisplatin are 16.8 μM and 68.8 μM, respectively. The IC_{50} values of complex **5** and cisplatin toward WI-38 cells are 0.8 μM and 30.5 μM, respectively.

The mechanism of action of complex **5** was investigated through agarose gel electrophoresis. Fig. 2(a) shows the image of an agarose gel of pBR322 DNA treated with cisplatin in pH 7.4 buffer (Lane 2–8) for 24 h at 37 °C. When *r* increases, the mobility of Form I DNA decreases and then, at higher *r* values, it increases. This is a typical binding pattern for cisplatin to Form I DNA [30]. Fig. 2(b) shows an image of an agarose gel of pBR322 DNA treated with **5** (after reduction with ascorbic acid) in pH 7.4 buffer (Lane 4–7) for 24 h at 37 °C. Plasmid pBR322 DNA was used to capture cisplatin and the Pt(II) reduction products; DNA binding affects the mobilities of the DNA in the gel. The pBR322 DNA has both closed and nicked circular forms in each individual lane; the faster migrating Form I is the supercoiled closed circular form of DNA and the slower migrating Form II is the nicked circular form. Pt complex **5** was first dissolved in a small amount of DMSO and then diluted using aqueous buffer; the final concentration of DMSO in each sample was no more than 2.5% (v/v). To examine and exclude possible effects of the reducing agents and DMSO on the plasmid DNA, three control groups were run in the gel with complex **5**. Lane 1 contains only DNA, lane 2 contains DNA and 2.5% DMSO and lane 3 contains DNA and the reducing agent ascorbic acid. In Fig. 2(b), with higher *r* value, the mobility of Form I DNA decreases, revealing binding of reduced **5** to Form I DNA.

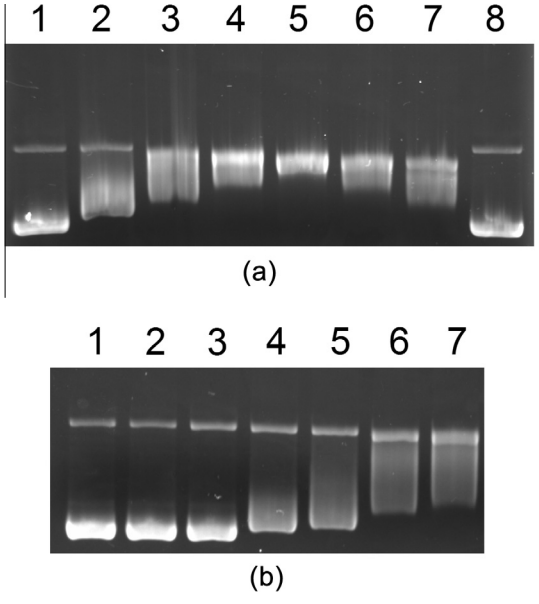

Fig. 2. Ethidium bromide-stained agarose gels of pBR322 DNA (38.5 μM). The pBR322 DNA was in the presence of cisplatin (a) or ascorbic acid-reduced complex **5** (b) for 24 h. The fastest migrating band (bottom) is covalently closed circular Form I DNA, while the slowest migrating band (top) is nicked circular Form II DNA. Lane and *r* ([compound]/[DNA-bp]) are: (a) Cisplatin – 1, 0; 2, 0.03; 3, 0.04; 4, 0.05; 5, 0.08; 6, 0.10; 7, 0.13; and 8, 0. (b) Complex **5** – 1, 0; 2, 0 (DMSO); 3, 0 (DMSO + ascorbic acid); 4, 0.52; 5, 1.04; 6, 2.08; and 7, 2.60.

Based on ICP-MS results, complex **5** can be loaded into and then released from mesoporous silica MCM-41 nanoparticles. After 24 h adsorption at r.t., MCM-41 contains 0.9% w/w of **5**. After 24 h in PBS at r.t., 95.9% of **5** is released from mesoporous silica MCM-41.

4. Discussion

Lung cancer is the leading cause of cancer-related death in the United States, and approximately 85–90% of lung cancer patients have NSCLC. NSCLC is extremely difficult to treat, the 5-year survival rate being 17% [31,32]. Complex **5** was synthesized following literature procedures [26–28], with some modifications. The synthesis and characterization of **5** and its intermediates are reported here in detail. It has been reported that complex **5** is highly lipophilic, with a $\log P_{o/w}$ of 4.10, and its high toxicity toward A2780 ovarian and HCT116 colon carcinoma cell lines has been demonstrated [21]. We tested and compared the toxicity of complex **5** and cisplatin toward human NSCLC cells, as well as normal human lung fibroblast cells (Fig. 1). As illustrated in Fig. 1(b)–(c) and (e)–(f), complex **5** is significantly more toxic toward both NSCLC cell lines (H596 and A549) than cisplatin. Complex **5** was again more toxic than cisplatin toward a normal human lung fibroblast cell line (WI-38); however, the therapeutic indexes (TI) of **5** compared to cisplatin were much improved. TI is defined as the ratio of the IC_{50} of normal cells to that of neoplastic cells [33]. In the case of H596 relative to WI-38, the TI is 1.82 for cisplatin and 2.63 for **5**. In the case of A549 relative to WI-38, the TI is 0.443 for cisplatin and 3.79 for **5**. Thus, complex **5** could be a candidate for use in the clinic.

Plasmid pBR322 DNA was used in agarose gel electrophoresis to investigate the mechanism of action of complex **5** in the presence of the biological relevant reducing agent ascorbic acid (Fig. 2). As shown in Fig. 2(a), the mobility of Form I DNA changed with increased *r*, which indicates that Pt(II) binds to the negatively supercoiled Form I DNA. The mobility of Form I DNA at first decreased, indicating Form I DNA became less negatively supercoiled due to the binding of Pt(II) to DNA. When *r* was greater than 0.08, Form I DNA became positively supercoiled and its mobility increased with *r*. In Fig. 2(b), with greater *r* value, the mobility of form I DNA decreases, revealing DNA binding of reduced complex **5** to Form I DNA in a pattern similar to that of cisplatin when *r* is less than 0.08.

The lipophilic complex **5** has the potential to be incorporated into nanocarriers to achieve enhanced tumor accumulation. Preliminary studies show that it can be incorporated into mesoporous silica MCM-41 nanoparticles at approximately 1% w/w, and most is released in pH 7.4 buffer after 24 h. These 400 nm MCM-41 nanoparticles were previously shown to accumulate in tumors after i.v. injection into mice [26]. In fact, approximately 4.5% of the initial dose of radiolabeled MCM-41 per gram of tissue accumulated in tumors after 24 h. Thus, complex **5**-loaded MCM-41 nanoparticles could be a promising drug delivery system for the treatment of NSCLC. Drug loading capacity may be further improved by modifying the MCM-41 nanoparticles.

5. Conclusions

Lung cancer, especially NSCLC, demonstrates high prevalence and high mortality, and effective treatment options are lacking. Thus, we prepared complex **5**, which has the potential to be used alone or with other therapeutic agents, both radio- and chemotherapeutic, and administered free or encapsulated in nanocarriers. This complex has a higher therapeutic index than cisplatin, and shares its mechanism.

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