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Gold(III) porphyrin complex is more potent than cisplatin in inhibiting growth of nasopharyngeal carcinoma *in vitro* and *in vivo*

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Nasopharyngeal carcinoma (NPC) is a common neoplasm in Southeastern Asia, and cisplatin-containing regimens for combinational chemotherapy are widely used for treating locally recurrent or metastatic diseases. However, resistance to cisplatin is not infrequently seen and its associated side effects may be life-threatening. In this report, another metallo-pharmaceutical agent gold(III) porphyrin complex [Au(TPP)]Cl was investigated in comparison to cisplatin for its *in vitro* and *in vivo* anticancer effects. Through induction of the intrinsic apoptosis pathway, [Au(TPP)]Cl exhibited 100-fold higher potency than cisplatin in killing NPC cells, including cisplatin-sensitive and cisplatin-resistant variants, and also an variant harboring the Epstein-Barr virus. In addition, a safety concentration window was demonstrated, allowing [Au(TPP)]Cl to kill tumors with minimal cytotoxicity to noncancerous cells. More importantly, weekly intraperitoneal injection of 3 mg/kg [Au(TPP)]Cl was more effective than the same dose of cisplatin in inducing tumor apoptosis *in vivo* and remarkably inhibited tumor growth in animals without any noticeable side effect. [Au(TPP)]Cl therefore is a promising chemotherapeutic agent that deserves further development as a novel drug for the treatment of advanced NPC, in particular, for cases with cisplatin-resistance.

Key words: Gold(III); porphyrin; nasopharyngeal carcinoma; cisplatin; neoplasm; chemotherapeutics

Nasopharyngeal carcinoma (NPC) is strongly associated with infection of Epstein-Barr virus (EBV),[1] and is a common neoplasm throughout Southeastern Asia, including Southern China, Hong Kong, Singapore and Taiwan, with a yearly incidence between 10 and 50 per 100,000.[2] Radiotherapy is effective against this tumor at the early stage. However, once the disease recurs or progresses, available treatment options are limited and in most cases chemotherapy is the only choice. Cisplatin is one of the most commonly used chemotherapeutic agents against NPC and is usually given in the format of combinational chemotherapy, concurrent chemo-radiation therapy, or adjuvant/neoadjuvant chemotherapy.[3] At least in combinational chemotherapy, cisplatin-containing regimens have response rates of 50–91%,[4,5] whereas the response rate for non-cisplatin-containing regimens was significantly poorer.[6,7] However, the effect of cisplatin is not universal and its side effects, such as cytotoxicity to peripheral blood cells and bone marrow, are sometimes life-threatening and not infrequently encountered.[8] Clinical trials employing other forms of combinational chemotherapy with or without concurrent irradiation for advanced NPC have demonstrated an improved progression-free and disease-free response rate. However, such treatment regimens are also frequently associated with significant short-term and long-term undesirable effects.[9–12] Novel approaches using cell-based immunotherapy have also been examined in human subjects with promising preliminary results.[13–15] Nevertheless, these modalities are still experimental. Newer chemotherapeutic agents that can improve overall survival with fewer side effects are in obvious need.

The success of cisplatin as an anticancer metallo-pharmaceutical drug has stimulated the use of metals, including gold, in anticancer medicine. Gold is known to exist in oxidation states ranging from −1 to +5 (*i.e.*, gold[-I] to gold[V]). Apart from gold(0) which is in the elemental and colloidal forms, gold(I) and gold(III) are the common forms of gold complexes known to exist in aqueous media.[16] Gold(I) complexes have long been used for the treatment of rheumatoid arthritis; notable examples include auranofin and myocrysin.[17] The discovery by Lorber and coworkers in 1979 that auranofin could inhibit *in vitro* proliferation of HeLa cervical cancer cells[18] had prompted extensive interest in searches for new pharmacological applications of gold, and a series of auranofin derivatives had since been evaluated for their *in vitro* and *in vivo* antitumor activities.[19] Unfortunately, these gold(I) complexes exhibited severe cardiotoxicity in animals, rendering them unfavorable for clinical use.[20,21] In contrast to gold(I) complexes, studies on gold(III) complexes are relatively sparse because of their high electrochemical potential rendering them to easily undergo decomposition to gold(I) or colloidal gold in physiological buffer solutions.[22] The poor solution stability of gold(III) complexes and the decomposition into gold(I) complexes conceivably may also lead to gold(I)-associated toxicity if used *in vivo*. Interestingly, when gold(III)-containing compounds are prepared in the form of gold(III) porphyrin complexes, such as [Au^{III}(tetraphenylporphyrin)]Cl_4 and its derivatives,[23,24] they could undergo reversible electrochemical reduction without decomposition.[25] It is likely that the porphyrin ring exerts strong chelating effect to avoid demetalation and provides a rigid ligand scaffold to stabilize the four-coordinate gold(III) by raising the kinetic barrier for reduction to two coordinate gold(I).[26] The use of gold(III) porphyrin complexes may thus avoid the gold(I)-induced cardiotoxicity and provide an advantage for clinical application.

We have previously reported that a series of gold(III) porphyrin complexes possess potent *in vitro* anti-cancer activity toward a va-

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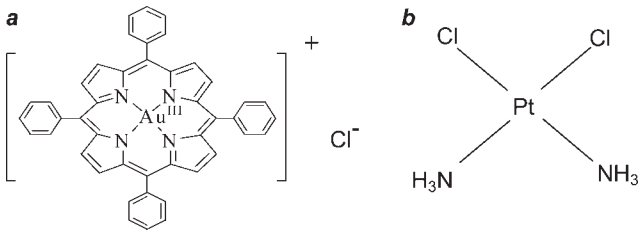

FIGURE 1 – Chemical structure of (*a*) gold(III) porphyrin [Au(TPP)]Cl, and (*b*) cisplatin.

riety of cancer cell lines, including NPC cells. Furthermore, these complexes are stable in the physiologically relevant buffer containing biological reducing agents without decomposition to gold(I) or gold(0).[27] We have also shown that the gold(III) tetraphenyl-porphyrin chloride ([Au(TPP)]Cl, Fig. 1) could noncovalently bind to DNA[28] and induce apoptosis of NPC cells *in vitro*.[29] These studies suggest that gold(III) porphyrins could be a promising anticancer drug for NPC treatment. However, several important issues still remain to be answered. First, because Epstein-Barr virus (EBV) genome is present in 90% of NPC samples from patients in endemic area[30] but none of the previously tested NPC cells contains EBV genomes, whether or not [Au(TPP)]Cl is also effective on EBV(+) NPC cells is yet to be determined. Second, safety issues such as the cytotoxicity to noncancerous cells have not been fully examined. Finally, the *in vivo* effect of gold(III) porphyrin complex on the kinetics of NPC tumor growth has not yet been documented. In this study, we compared the efficacy of gold(III) porphyrin complex [Au(TPP)]Cl with that of cisplatin in suppressing NPC tumor growth. Several NPC cell lines including cisplatin-sensitive, cisplatin-resistant variants and EBV-carrying cells were tested in parallel. Normal noncancerous cells were also investigated *in vitro* for the potential toxic effects of gold(III) porphyrin. The *in vivo* effect of [Au(TPP)]Cl was also examined in a tumor-bearing nude mouse model.

Material and methods

Chemical and reagents

Analytical grade organic solvents and autoclaved double–distilled deionized water were used throughout the experiments. All chemicals unless otherwise stated were purchased from Sigma-Aldrich Chemical Co. (St Louis, MI). The starting material $K[Au^{III}Cl_4]$ was from Heraeus (Dormagen, Germany). Electrophoresis grade acrylamide, polyvinylidene difluoride (PVDF) membranes (Immobilon-P) and the enhanced chemiluminescence (ECL) detection system were purchased from Amersham Biosciences (Orsay, France). All cell culture reagents were obtained from Invitrogen Corp. (CA).

Preparation of gold(III) porphyrin complex [Au(TPP)]Cl

The synthesis of [Au(TPP)]Cl was conducted under a nitrogen atmosphere using standard Schlenk technique.[27] Briefly, to a mixture of $K[Au^{III}Cl_4]$ (0.5 mM) and sodium acetate (2.5 mM) heated at 80°C in acetic acid (20 mL), a solution of free tetraphenylporphyrin (TPP, 0.4 mM) in 10 mL acetic acid was added, and the mixture was refluxed for 2 hr. After removal of solvent by vacuum, the residue was dissolved in CH_2Cl_2, and was washed twice with water to remove any unreacted $K[Au^{III}Cl_4]$ and sodium acetate, followed by chromatographic purification using neutral 90-alumina with CH_2Cl_2/MeOH as an eluant. The product gold(III) porphyrin was obtained in 70% yield after ion exchange using aqueous LiCl. UV-vis (CH_2Cl_2) λ_{max}/nm (log): 409 (5.68), 521 (4.73). ^1H NMR ($CDCl_3$): δ 9.28 (s, 8H), 7.89 (m, 8H), 8.24 (d, 8H) 7.89 (m, 4H). m/z = 809. Yield 70%. Anal. Calcd. for $C_{44}H_{28}N_4ClAu$ (%): C, 62.53; H, 3.34; N, 6.63. Found: C, 62.50; H, 3.61; N, 6.74. Because of its hydrophobic nature, [Au(TPP)]Cl was dissolved in PET (a mixture of polyethylene glycol, absolute ethanol and TWEEN80) for experiments.

Cell lines and cell culture

Human NPC cell lines (SUNE1, and its cisplatin resistant-variant CNE2) were derived from poorly differentiated NPC in Chinese patients.[31] C666-1 cells are derived from an NPC xenograft of Southern Chinese origin with undifferentiated NPC, and constitutively express EBV latent antigens and thus resemble the EBV latency II pattern.[32] Human lung fibroblast cell line CCD-19Lu was obtained from American Type Culture Collection (Rockville, MD). Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coat obtained from the Hong Kong Red Cross Blood Transfusion Service, and were isolated as previously described.[13] SUNE1, CNE2, C666-1 and PBMCs were maintained in the complete RPMI-1640 media. CCD-19Lu was maintained in complete Earle's minimum essential medium. Complete media were supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.

In vitro *drug treatment and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay*

A total of 5×10^3 cells/well of SUNE1, CNE2 or C666-1 resuspended in 100 μL complete media were plated in microtitre plates and incubated at 37°C. After 24 hr, the media were replaced with serum-free media for incubation for additional 24 hr. Serially-diluted [Au(TPP)]Cl, cisplatin or PET vehicle control were added into culture wells. Cells were subsequently incubated at 37°C for 48 hr and subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the Mosmann method[33] with modifications. Briefly, 10 μL MTT solution (Cell Proliferation Kit I, Roche, Mannheim, Germany) were added to each well at the end of 48 hr treatment and were further incubated for 4 hr, followed by adding 100 μL solubilization solution. The microtitre plates were then maintained in a dark, humidified chamber overnight. The formation of formazan was measured with a microtitre plate reader at 550 nm and the percentages of cell viability were determined by $(OD_{Vehichle} - OD_{Drug})/OD_{Vehicle} \times 100\%$ where OD represents optical density.

Annexin V-FITC and propidium iodide staining for assessment of cellular apoptosis

CNE2 cells or SUNE1 cells (1×10^5 cells/mL) were cultured in 60-mm tissue culture dishes. After 24 hr incubation, 0.25 μM [Au(TPP)]Cl, cisplatin or PET vehicle control was added to the dishes for additional 12 hr, 24 hr, 36 hr and 48hr. Staining was performed using the Annexin V-FITC apoptosis detection kit (BD PharMingen, CA, USA). After cell harvesting and washing, cells were resuspended in binding buffer, and Annexin V-FITC and propidium iodide (PI) were added to a final concentration of 1 μg/mL and the cells were incubated at room temperature in the dark for 15 min. Annexin V and PI staining of the cells were subsequently detected by flow cytometry (FACSCalibur, Becton Dickinson, Palo Alto, CA).

Western blot analyses

CNE2 cells (2×10^6) were treated with 0.1, 0.25 and 0.5 μM [Au(TPP)]Cl or PET vehicle control for 24 hr. After washing, cells were lysed in 100 μL RIPA buffer on ice, and the lysates were harvested after centrifugation. The cellular protein content was quantified by the *DC* Protein Assay (Bio-Rad, Richmond, CA, USA). In separate experiments, CNE2 cells were treated with 0.25 μM [Au(TPP)]Cl for 4 hr, 8 hr, 15 hr and 24 hr, and followed by cell lysis and protein extraction. Protein was also extracted from tu-

mor-bearing mice by homogenizing tumor with cell lysis buffer (Cell Signaling, MA) with PMSF on ice. Proteins were fractionated by SDS-PAGE electrophoresis and then blotted onto PVDF membranes. The PVDF membranes were preblocked at room temperature for 3 hr in Tris-buffered saline/0.1% Tween-20 (TBS/T) containing 10% nonfat milk, followed by incubation at 4°C overnight with primary rabbit anti-Bcl-2 antibody (1:1,000, Cell Signaling), rabbit anti-caspase 9 antibody (1:1,000, Cell Signaling) or goat anti-human actin antibody (1:1,000, Santa Cruz Biotech, CA) in TBS/T containing 5% nonfat milk. After washing, membranes were incubated with the respective peroxidase-conjugated secondary antibody for 1 hr. Cleaved forms of caspase-9 (35 kD and 37 kD) were also detected by the anti-caspase-9 antibody. Detection was performed using the chemiluminescence procedure (ECL Plus, Amersham Bioscience, Orsay, France) according to the manufacturer's instructions.

Tumor implantation in nude mice and in vivo *drug treatment*

Four-week-old female BALB/c AnN-nu mice (nude mice) were obtained from the Laboratory Animal Unit, the University of Hong Kong. All animal experiments were performed with approval from the Committee on the Use of Live Animals for Teaching and Research, The University of Hong Kong. Tumor cells (5×10^6) resuspended in RPMI were implanted by subcutaneous injection on the right flank of the mice. When tumors were approximately 50–100 mm^3 in size, animals were randomly separated into 3 groups to receive treatment of weekly intraperitoneal injection of PET vehicle control, [Au(TPP)]Cl or cisplatin for 4 times, as indicated in individual experiment. Volume of the tumor and the weight of mice were measured every 3–4 days. Tumor volume was calculated by the formula: abc/2 in which a, represents tumor length; b, the width; and c, tumor thickness, as measured with a caliper and expressed in millimeter. After 24 days, the mice were sacrificed; tumors, hearts, spleens, livers and kidneys were harvested, fixed in 4% paraformaldehyde and paraffin-embedded for further analysis.

TUNEL assay

TUNEL was performed using the *In-Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostic, Germany) according to manufacturer's instruction. Briefly, paraffin sections were dewaxed and rehydrated. Tissue sections were incubated for 30 min at 37°C with proteinase K solution, followed by permeabilization with 0.1% Triton X-100, 0.1% sodium citrate for 8 min. Sections were rinsed with PBS and incubated with TUNEL reaction mixture in a humidified chamber for 1 hr at 37°C in dark. Positive control was prepared by incubating the permeabilized-sections at room temperature for 10 min with DNase I solution (Invitrogen Corp, CA), followed by incubation with TUNEL reaction mixture. Negative control was prepared by incubating the permeabilized-sections with TUNEL reaction mixture without the terminal transferase. After washing, sections were mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories) and were observed under a fluorescence microscope (Nikon eclipse E600, Japan). Electronic images were captured and saved to a computer using the software ACT-1 (Nikon Corp., Japan).

Statistical analysis

All datasets were tested for normal distribution using Kolmogorov-Smirnov test with $p > 0.05$. Statistical significance test was then performed using the parametric Student's *t*-test with significance set at $p < 0.05$.

Results

[Au(TPP)]Cl is 100-fold more potent than cisplatin in inducing NPC cell death in vitro

After exposure to various concentrations of [Au(TPP)]Cl or cisplatin for 48 hr, the survival of NPC cells was assessed by MTT assay. Results revealed that [Au(TPP)]Cl was more potent than cisplatin by at least 2-log in reducing the viability of NPC cells, including cisplatin-sensitive SUNE1 cells, cisplatin-resistant CNE2 cells, and EBV-harboring C666-1 cells (Figs. 2*a–c*). The concentration of [Au(TPP)]Cl causing 50% reduction in cell viability (*i.e.*, IC_{50}) was 0.12 ± 0.02, 0.14 ± 0.01 and 0.11 ± 0.02 μM respectively. In contrast, the IC_{50} of cisplatin was at least 100 times higher for any of these cell lines when compared with [Au(TPP)]Cl (Table I). Similar observations were made when the drug exposure time was extended to 5 days (data not shown). These results indicate that [Au(TPP)]Cl is at least 100-folds more potent than cisplatin in reducing the viability of various variants of NPC cells.

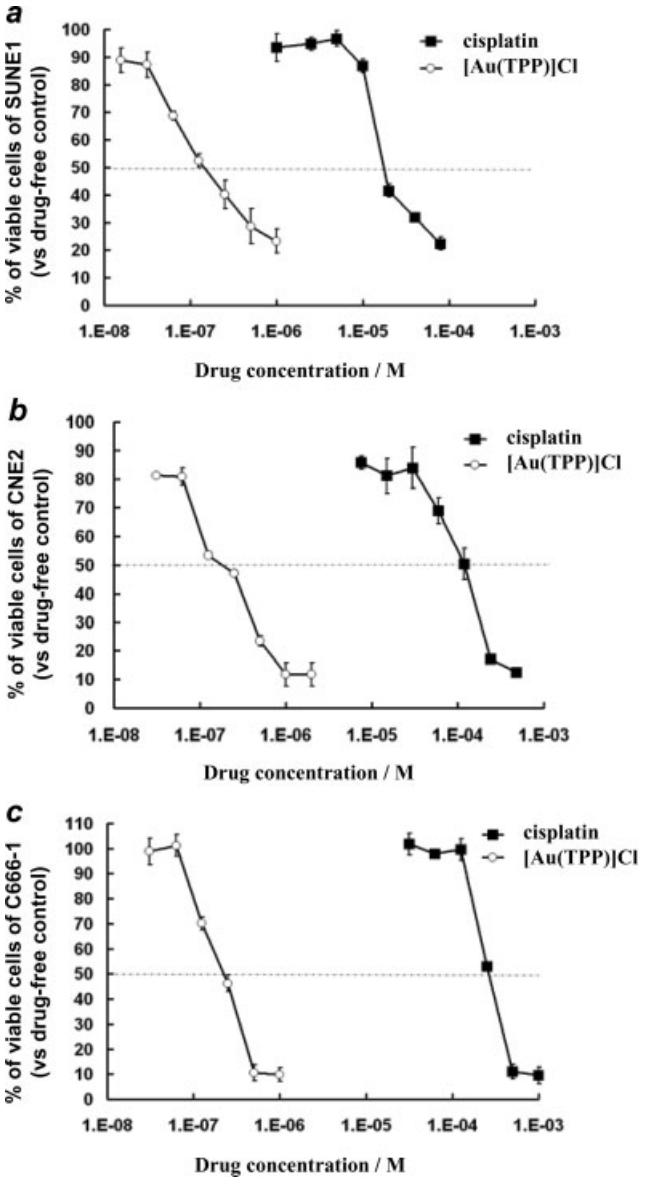

Figure 2 – Drug sensitivity profiles of gold(III) porphyrin complex and cisplatin towards nasopharyngeal carcinoma cells (*a*) SUNE1, (*b*) CNE2 and (*c*) C666-1. Cells were treated with different concentrations of [Au(TPP)]Cl and cisplatin for 48 hr and cell viability was examined by MTT assay. The viability of treated cells was compared with that of cells treated with vehicle control and expressed as percentage of viable cells. Data are expressed as mean ± standard error (SE) from 3 independent experiments.

TABLE I – IC_{50}[1] OF [Au(TPP)]Cl AND CISPLATIN FOR REDUCING VIABILITY OF VARIOUS NASOPHARYNGEAL CARCINOMA CELL LINES AND NON-CANCER CELLS

Drugs	NPC cells			Non-cancer cells	
	SUNE1	CNE2	C666-1	CCD19Lu	PBMC
[Au(TPP)]Cl	0.12 ± 0.02[2]	0.14 ± 0.01	0.11 ± 0.02	1.90 ± 0.20	2.50 ± 0.40
Cisplatin	16.50 ± 1.04	89.30 ± 5.21	185.21 ± 15.23	24.00 ± 3.79	22.50 ± 2.02

[1]IC_{50} represents the concentration causing 50% inhibition of cell growth.–[2]Data are expressed as mean ± standard error in μM and are results from 3 independent experiments.

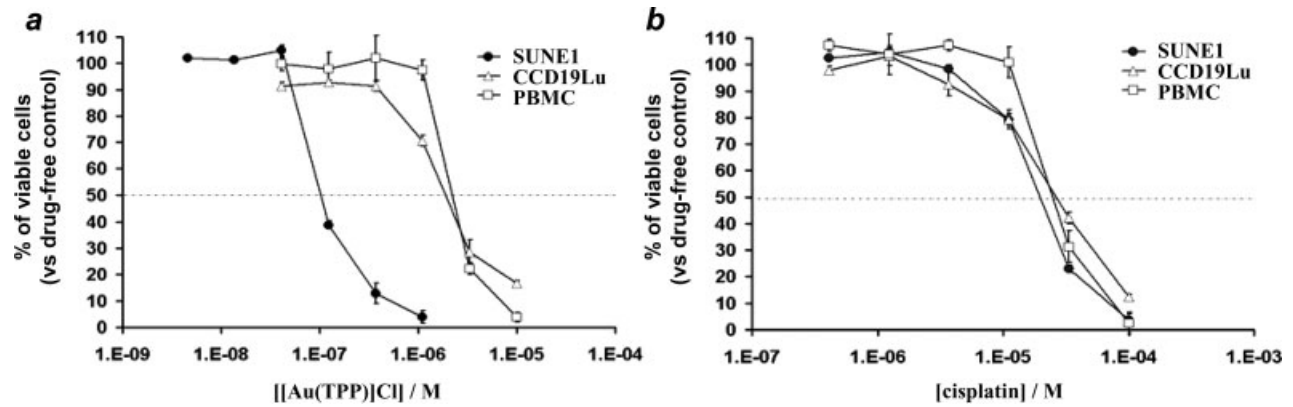

FIGURE 3 – A safety concentration window for gold(III) porphyrin complex exists, allowing selective antitumor activities without remarkable killing of normal cells. Viability of SUNE1 cells, CCD19-Lu lung fibroblast cells and freshly isolated peripheral blood mononuclear cells (PBMCs) after treatment with various concentrations of (*a*) [Au(TPP)]Cl or (*b*) cisplatin for 48 hr was examined by MTT assay. The viability of treated cells was compared with that of cells treated with vehicle control and expressed as percentage of viable cells. Data are expressed as mean ± SE from 3 independent experiments.

A safety concentration window for [Au(TPP)]Cl, but not cisplatin, exists for its tumoricidal effects without significant cytotoxicity to noncancerous cells

To compare the cytotoxic effect of [Au(TPP)]Cl on NPC cells and normal/noncancerous cells, SUNE1 cells, peripheral blood mononuclear cells (PBMCs) from healthy individuals and CCD19-Lu cells (a fibroblast cell line derived from normal lung) were treated with various concentrations of [Au(TPP)]Cl for 48 hr and the cell viability was determined. Results by MTT assays showed that when used from 0.1 μM to 1 μM, [Au(TPP)]Cl reduced the SUNE1 survival to 50% to < 10%, while maintaining the survival of the majority (70%–100%) of PBMCs and CCD19-Lu cells (Fig. 3*a*). The IC_{50} of [Au(TPP)]Cl for PBMCs and CCD19Lu were 2.50 ± 0.40 μM and 1.90 ± 0.20 μM, respectively, at least 15-fold higher than that of the cancer cells SUNE1 (0.12 ± 0.20 μM, Table I). Therefore, a reasonable safety concentration window 0.1–1 μM indeed exists for [Au(TPP)]Cl to efficiently kill tumor cells while preserving the survival of most of the normal or noncancerous cells. On the contrary, cisplatin reduced the survival of SUNE1 cells as well as PBMCs and CCD19-Lu cells with the same kinetics (Fig. 3*b*). Notably, the IC_{50} of cisplatin for cisplatin-resistant CNE2 cells (89.30 ± 5.21 μM) and EBV-harboring C666-1 cells (185.21 ± 15.23 μM) were actually significantly higher than that for PBMCs (24 ± 3.79 μM) or fibroblasts (22.5 ± 2.02 μM, Table I), suggesting that appreciable killing of tumor cells by cisplatin was inevitably associated with significant death of normal and noncancerous cells, at least *in vitro*.

[Au(TPP)]Cl is more potent than cisplatin in inducing apoptosis of NPC cells by down-regulating Bcl-2 expression and up-regulating the activated caspase 9

To investigate the mechanism(s) of tumor death, NPC cells were treated with 0.25 μM [Au(TPP)]Cl for 48 hr and cellular apoptosis was assessed by Annex-V/PI staining at serial time points. This concentration (0.25 μM) was chosen arbitrarily from the safety window (0.1–1 μM, Fig. 3*a*), and the same concentration of cisplatin was used in parallel for comparison. Results showed that the percentage of Annexin V(+) PI(−) cells (*i.e.*, early apoptotic cells) of C666-1, SUNE1 and CNE2 was increased over time. In remarkable contrast, treatment with the same concentration of cisplatin or vehicle control had no effect at all (Figs. 4*a–c*). Interestingly, maximal killing by [Au(TPP)]Cl was observed at 36 hr posttreatment for both cisplatin-sensitive SUNE1 and cisplatin-resistant variant CNE2, indicating that both types of cells responded to [Au(TPP)]Cl with a similar sensitivity (Figs. 4*b* and 4*c*). In separate experiments, [Au(TPP)]Cl-treated CNE2 cells were lysed and proteins were extracted for western blotting. Consistent with the findings from apoptosis assay, time-dependent up-regulation of the expression of both 35 kDa and 37 kDa activated caspase 9 and also the reduction of the expression of antiapoptotic protein Bcl-2 were observed (Fig. 4*d*). In addition, after 24 hr treatment of CNE2 cells with 0.1, 0.25 and 0.5 μM [Au(TPP)]Cl, the expression of Bcl-2 protein was reduced while the expression of activated caspase 9 was up-regulated in a dose-dependent manner (Fig. 4*e*). Similar results were obtained from using SUNE1 cells (data not shown).

Intraperitoneal delivery of 3 mg/kg/week [Au(TPP)]Cl effectively inhibits growth of implanted NPC cells in tumor-bearing mice

To examine the *in vivo* effect of [Au(TPP)]Cl, SUNE1 cells were implanted into nude mice and [Au(TPP)]Cl (1.5, 3 and 6 mg/kg) was subsequently administered intraperitoneally at weekly intervals for 4 weeks. Tumor volume and body weight of the animals were evaluated every 3 to 4 days. Results demonstrated that injection of 3 mg/kg/wk [Au(TPP)]Cl significantly inhibited the SUNE1 tumor growth, whereas 1.5 mg/kg/wk was significantly less effective (Fig. 5*a*). Furthermore, injection of 6 mg/kg/wk [Au(TPP)]Cl was actually lethal and caused death in 7 of 10 animals after the 1st injection and death of the rest after the 2nd injection (Figs. 5*a* and 5*b*). Regular body-weight measurement showed that mice receiving either 1.5 or 3 mg/kg/wk [Au(TPP)]Cl had no significant weight loss (Fig. 5*b*). In separate experiments to compare the effectiveness of [Au(TPP)]Cl with cisplatin, we found

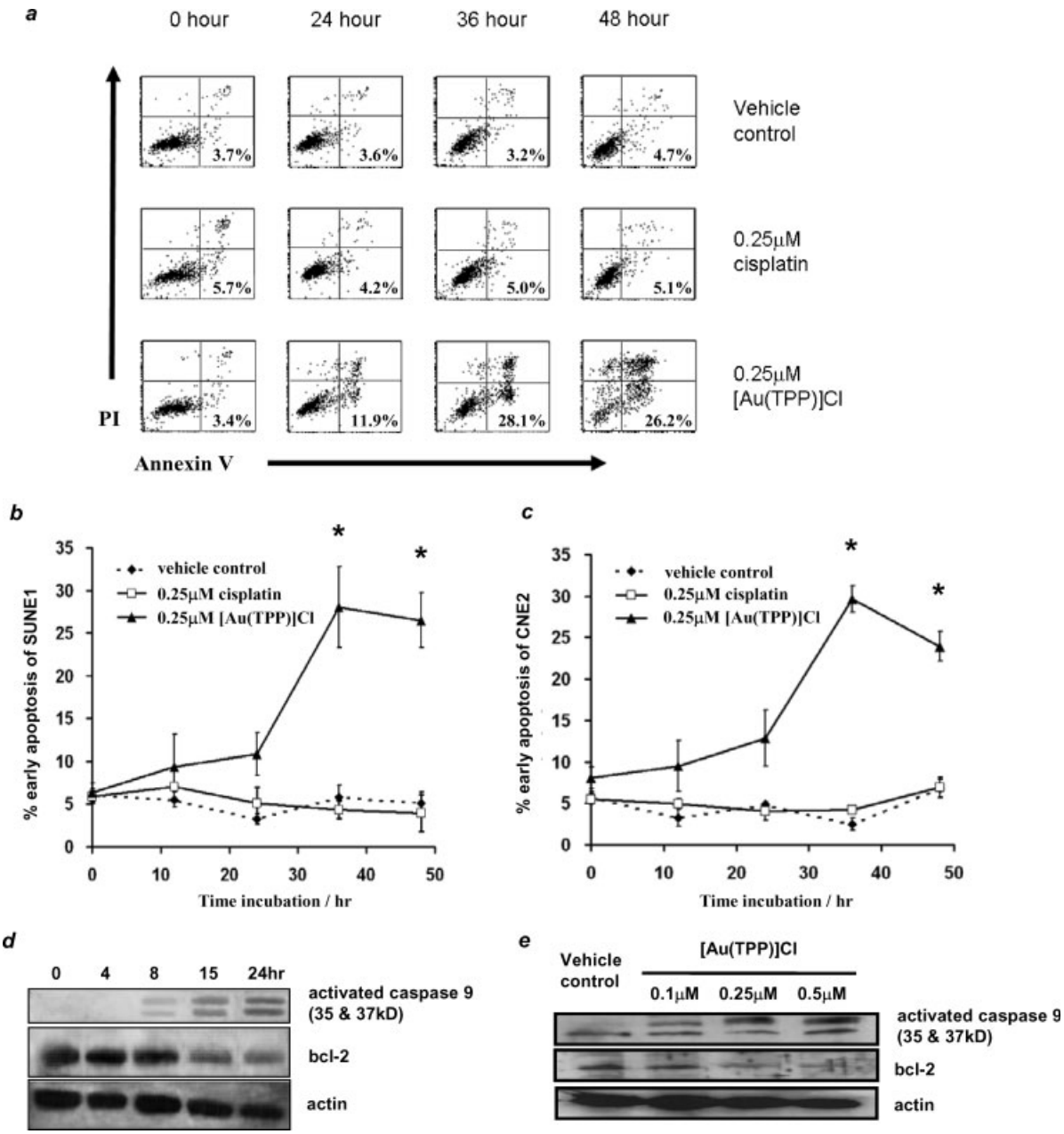

Figure 4 – Gold(III) porphyrin complex induces apoptosis of NPC cells through intrinsic apoptotic pathway. (*a*) C666-1 cells were treated with 0.25 μM [Au(TPP)]Cl, cisplatin or vehicle control for 0 hr to 48 hr and cell viability was assessed by Annexin V-FITC and propidium iodide (PI) staining. (*b*) SUNE1 and (*c*) CNE2 cells were similarly treated and apoptosis was evaluated as described in *a*. Annexin V(+) PI (−) early apoptotic cells are expressed as mean ± SE ($n = 3$). In *b* and *c*, * represents $p < 0.05$ when compared with vehicle control group. (*d*) Western blot analysis for the expression of activated caspase 9 and Bcl-2 in CNE2 cells treated with 0.25 μM [Au(TPP)]Cl for the indicated time periods. Data are representative of 3 independent experiments. (*e*) Western blot analysis for the expression of activated caspase 9 and Bcl-2 in CNE2 cells treated with [Au(TPP)]Cl at the indicated concentration for 24 hr. Data are representative of 3 independent experiments.

that treatment with 3 mg/kg/wk [Au(TPP)]Cl consistently and significantly inhibited the *in vivo* growth of implanted NPC cells, but treatment with the same concentration of cisplatin did not lead to any reduction in tumor volume (Figs. 5*c–e*).

[Au(TPP)]Cl induces tumor apoptosis in vivo *through induction of intrinsic apoptotic pathway while preserving cell survival in major vital organs*

After 4 weekly injections of 3 mg/kg [Au(TPP)]Cl or cisplatin, mice were sacrificed on day 3 postfinal treatment (*i.e.*, day 24 after the first injection) and tumors were excised and sectioned for TUNEL staining. Results clearly showed that injection of cisplatin (3 mg/kg/wk) did not induce any significant tumor cell death, as revealed by virtually no increase in the number of TUNEL(+) cells in the implanted SUNE1 (Figs. 6*a* and *b*), CNE2 or C666-1 tumors (Fig. 6*b*). In contrast, treatment with 3 mg/kg/wk [Au(TPP)]Cl resulted in a significantly increased number of TUNEL(+) cells in all implanted tumors when compared to mice treated with vehicle control (Figs. 6*a* and *b*). Western blot analysis of protein extracted from the C666-1 tumors treated by [Au(TPP)]Cl showed reduced expression of Bcl-2 and pro-caspase 9, and also increased expression of activated caspase-9, compared

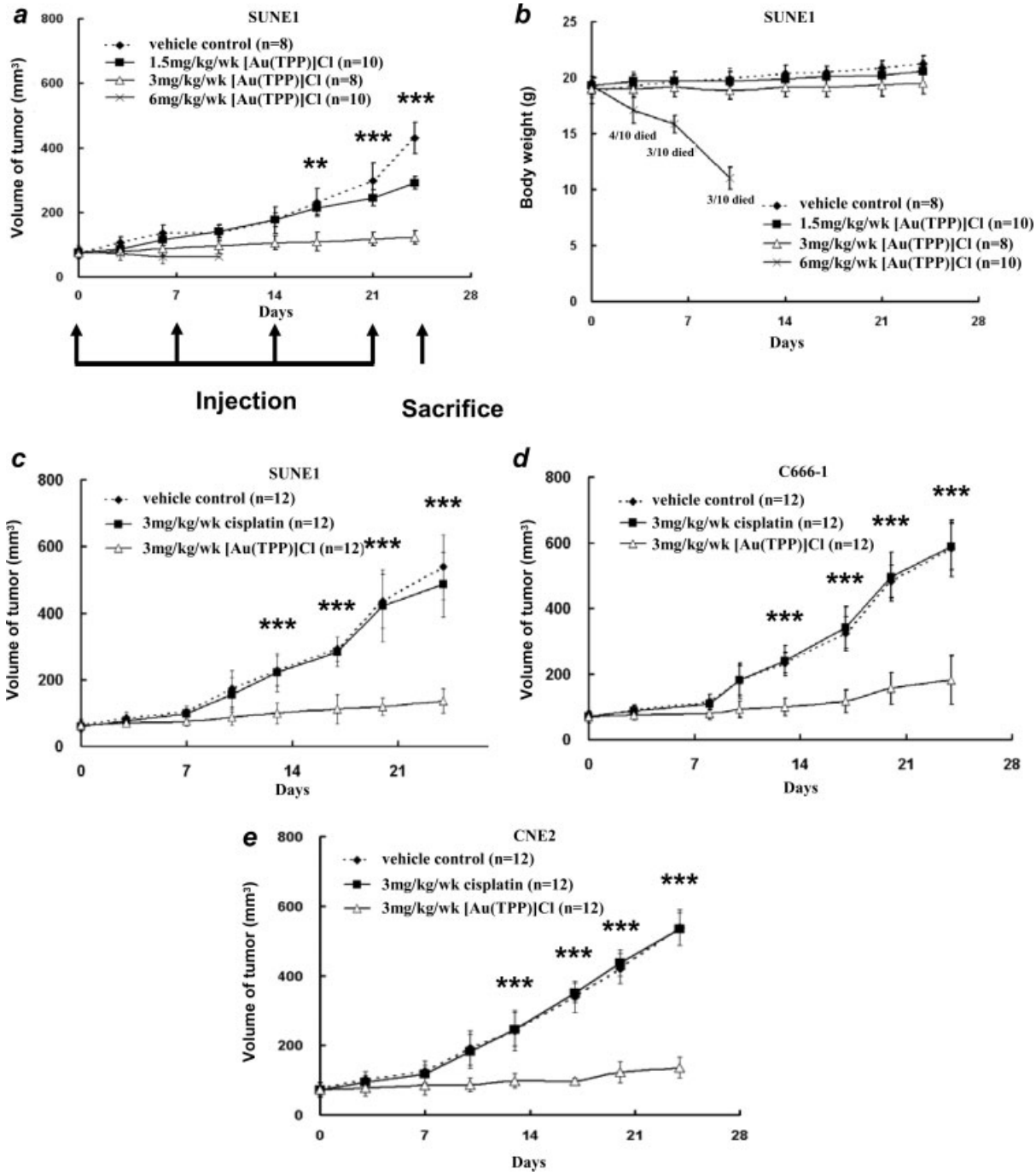

Figure 5 – Intraperitoneal administration of 3 mg/kg/week [Au(TPP)]Cl, but not cisplatin, inhibits NPC growth in tumor-bearing animals. SUNE1-implanted nude mice were given weekly injection of 1.5, 3 and 6 mg/kg gold(III) porphyrin for 4 injections on day 0, 7, 14 and 21; (*a*) the tumor volume and (*b*) body weight were evaluated before sacrifice on day 24. The tumor volume of (*c*) SUNE1, (*d*) C666-1 or (*e*) CNE2 cells implanted in nude mice treated with 3 mg/kg/wk [Au(TPP)]Cl, 3 mg/kg/wk cisplatin or vehicle control were measured in parallel. Data are expressed as mean ± SE; ** and *** represent $p < 0.01$ and $p < 0.005$, respectively, when compared with vehicle control group in all panels.

with those from control mice and cisplatin-treated mice (Fig. 6*c*). Similar results were obtained from CNE2 and SUNE-1 tumor samples (data not shown). These findings indicate that [Au(TPP)]Cl could indeed induce *in vivo* tumor apoptosis, which is at least in part through the induction of the intrinsic apoptosis pathway. To examine if [Au(TPP)]Cl treatment has any cytotoxic effect on normal cells *in vivo*, vital organs such as liver, heart, spleen and kidney were harvested for TUNEL staining at the end of the experiment. As shown in Figure 6*d*, no significant cell death was observed in these organs, suggesting that 3 mg/kg/wk injection of [Au(TPP)]Cl could efficiently suppress tumor growth with little cytotoxic effect to normal cells of vital organs.

Discussion

The gold(III) ion in [Au(TPP)]Cl is iso-electronic to platinum (II) and likewise has a square-planar structure as in the case of

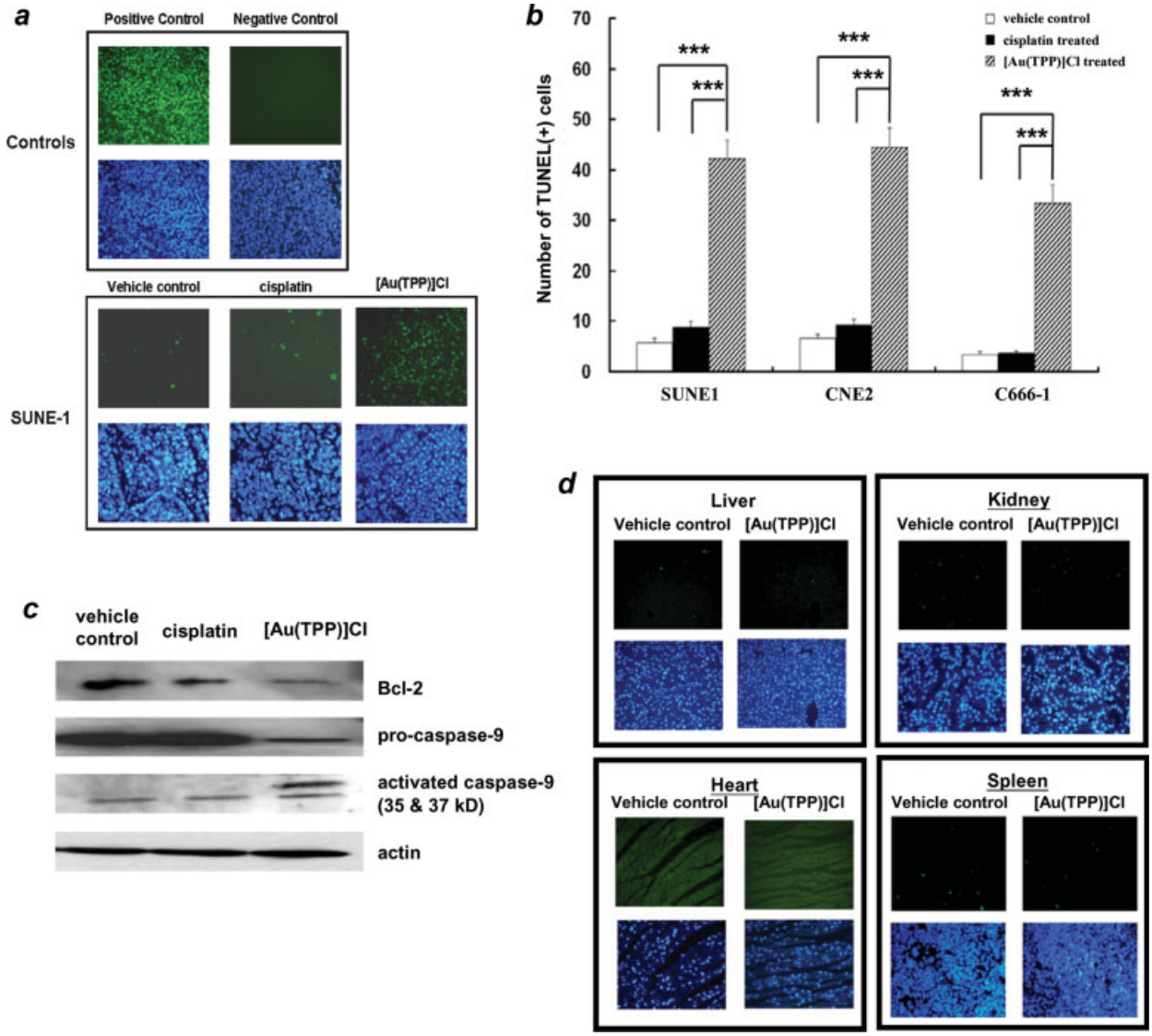

FIGURE 6 – *In vivo* treatment with [Au(TPP)]Cl induces apoptosis of the implanted NPC tumor cells without cytotoxic effects on major vital organs. (*a*) TUNEL staining of tumors excised from tumor-bearing mice after 4 injections of 3 mg/kg/wk [Au(TPP)]Cl, 3 mg/kg/wk cisplatin or vehicle control. DAPI counter-stain (blue color) showing cell nuclei for each section is shown below the TUNEL staining (fluorescent green) in each panel. Positive and negative controls for TUNEL staining were also shown. All images shown are at 400× magnification. (*b*) Number of TUNEL(+) cells from tumor sections of each treatment group was counted from 3 randomized fields at a magnification of 400×. Data shown are mean ± SE pooled from 3 independent tumor samples. *** represents $p < 0.005$. (*c*) Western blot analysis for the expression of Bcl-2, caspase-9 (activated and inactivated form) and actin of protein extracted from tumor in mice implanted with C666-1 and treated with 3 mg/kg/wk [Au(TPP)]Cl, 3 mg/kg/wk cisplatin or vehicle control. Data shown are representative of 3 independent experiments. (*d*) TUNEL staining of liver, kidney, heart and spleen from C666-1 tumor bearing mice after 4 injections of 3 mg/kg/wk [Au(TPP)]Cl or vehicle control. DAPI counter-stain showing cell nuclei for each section is shown below the TUNEL staining. Images shown (400×) are representative results from 3 independent samples.

cisplatin (Fig. 1). Based on MTT assay, [Au(TPP)]Cl indeed significantly reduces the survival of various NPC cells, including the cisplatin-resistant variants (Figs. 2*a–c*). It can also inhibit the growth of EBV(+) NPC cells (Fig. 2*c*). The *in vitro* tumoricidal effect of this gold(III) porphyrin complex against some NPC cells has been previously demonstrated.[27,29] However, none of the NPC cells tested in previous reports have expression of EBV genome or detectable residential virus after prolonged propagation in culture.[34,35] Given that EBV DNA is expressed in the majority of primary NPC biopsies and also in tumors found in Southern China,[36–38] results from the use of EBV(+) tumor cells and the demonstration of their responsiveness to [Au(TPP)]Cl become more clinically relevant and significant. Furthermore, our titration experiments also indicate a safety concentration window that allows [Au(TPP)]Cl to exert its cytotoxicity against tumor cells without significantly affecting the survival of PBMCs and fibroblasts (Fig. 3*a*). In contrast, such a window could not be demonstrated for cisplatin (Fig. 3*b*, Table I). In this regard, [Au(TPP)]Cl is indeed superior to cisplatin.

The *in vivo* effect of gold(III) porphyrin complexes against survival of cancer cells has recently attracted much attention but unfortunately has not yet been demonstrated. In the present study, we have confirmed the *in vivo* efficacy of [Au(TPP)]Cl against various types of NPC cells, thereby providing guidance in future trials on human subjects. The titration experiments have demonstrated that intraperitoneal injection of 3 mg/kg/wk

[Au(TPP)]Cl effectively suppresses NPC growth with minimal undesirable side-effects but 6 mg/kg/wk is lethal to the mice (Figs. 5*a–b*). As to cisplatin, similar titration experiments also showed that 6 mg/kg/wk caused > 50% animal death (data not shown). Indeed, the LD50 (dose causing 50% lethality) of cisplatin to mice has been reported to be 6 mg/kg by intraperitoneal route (Chemical Safety Information from Intergovernmental Organizations).[39] In another NPC-nude mouse model, a modest suppression (*e.g.*, 35% reduction in tumor volume) of the cisplatin-sensitive HONE-1 tumor growth was observed when 3 mg/kg cisplatin was administered twice a week over a period of 24 days.[40] Another report demonstrated that for the cisplatin-resistant C666-1 cells, a single dose of 8 mg/kg cisplatin also failed to inhibit tumor growth in a xenograft SCID mouse model.[41] In order to compare the *in vivo* efficacy of [Au(TPP)]Cl with cisplatin, we thus tested the anti-NPC effect of these 2 drugs at the same dose, and in line with the *in vitro* findings, 3 mg/kg/wk injection of [Au(TPP)]Cl is indeed more effective than the same dose of cisplatin in suppressing NPC growth (Figs. 5*c–e*), including cisplatin-resistant variants.

It has attracted much attention in recent years to find ways to reverse cisplatin-resistance of cancer cells, and unfortunately few measures could successfully do so, one of the rare examples being exogenous addition of Bcl-2 antisense oligodeoxynucleotides in the format of combinational therapy to inhibit the antiapoptotic machinery in C666-1 cells.[41] Recently, the combinational use of cisplatin with other novel classes of antitumor agents (*e.g.*, cetuximab) are being tested,[42] which is potentially associated with fewer side effects. In this context, it is likely and deserves further study that [Au(TPP)]Cl may also provide more clinical benefits than cisplatin in combination with other regimens, in addition to its use in monotherapy.

Our results also showed that both the *in vitro* and *in vivo* tumoricidal effect of [Au(TPP)]Cl is mediated, at least in part, by the induction of apoptosis through the intrinsic pathway. These observations are consistent with our previous report that by confocal microscopy, treatment with [Au(TPP)]Cl on cervical cancer HeLa cells results in apoptotic body formation and genomic DNA fragmentation, with no or little cellular necrosis.[27] The detailed mechanism(s) by which this gold(III) porphyrin complex triggers intrinsic cellular apoptosis is still unclear and remains to be investigated. We have previously reported that gold(III) porphyrin complexes can bind to DNA,[27,28] one of the major targets for other anticancer drugs,[43] and they are stable towards demetallation into free metal ion or the free base porphyrin components.[44] It is therefore likely that [Au(TPP)]Cl disturbs or dys-regulates the antiapoptotic machinery of the tumor cells by binding to DNA in the nucleus and/or mitochondria in its complex form, instead of in the form of free radicals. Moreover, we have also shown that gold(III) porphyrin complexes bind to DNA noncovalently,[28] which is different from that described for cisplatin.[45] Whether or not the difference in DNA binding characteristics may contribute to different biological consequences deserves further study. Alternatively, as the binding of gold(III) porphyrin complexes to some model proteins is stronger than to the DNA *in vitro*,[46] it is also likely that the binding of [Au(TPP)]Cl to other intracellular proteins may also contribute to the down-regulation of the antiapoptotic activities. Apart from apoptosis, a previous study has shown that cisplatin can induce senescence-like growth arrest in NPC cells.[47] However, this mechanism is unlikely to be significantly involved in [Au(TPP)]Cl-induced tumor cell death because senescence-associated beta-galactosidase activity was not observed in [Au(TPP)]Cl-treated NPC cells (Supporting Information). Whatever the case, it is worth noticing that the maximal tumoricidal effect of [Au(TPP)]Cl was observed at 36 hr for both cisplatin-sensitive and -resistant cells (Figs. 4*b* and 4*c*), suggesting a comparable sensitivity for both cell types to this compound, which again indicates the superiority of [Au(TPP)]Cl to cisplatin for potential clinical use.

In summary, we have demonstrated both *in vitro* and *in vivo* effects of the gold(III) porphyrin complex [Au(TPP)]Cl in inhibiting proliferation of cisplatin-sensitive, cisplatin-resistant, as well as EBV-carrying NPC cells through the induction of cellular apoptosis. To our knowledge, this is the first report to demonstrate the *in vivo* anticancer effect of a gold(III) porphyrin complex on the kinetics of nasopharyngeal carcinoma growth in a tumor-bearing mouse model. Of significance, the presence of a safety window for selective antitumor activities without major side effects has endowed gold(III) porphyrin complexes the potentiality of becoming a promising chemotherapeutic agent against NPC. Given its superior potency and fewer undesirable effects than cisplatin, the potential of [Au(TPP)]Cl in inhibiting the *in vivo* growth of cancers of other origins also warrants further investigation.

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