Gold (III) Porphyrin Complexes Induce Apoptosis and Cell Cycle Arrest and Inhibit Tumor Growth in Colon Cancer

Shuiping Tu, MD, PhD[1]; Raymond Wai-Yin Sun, PhD[2]; Marie C. M. Lin, PhD[2]; Jian Tao Cui, MD[1]; Bing Zou, MD, PhD[1]; Qing Gu, PhD[1]; Hsiang-Fu Kung, PhD[3]; Chi-Ming Che, PhD[2]; and Benjamin C. Y. Wong, MD, PhD[1]

BACKGROUND: Gold (III) compounds have exhibited favorable antitumor properties both in vitro and in vivo. In a previous study, the authors reported that the novel gold (III) complex 1a (gold 1a) exhibited strong cytotoxicity in some tumor cell lines. In the current study, the effect of gold 1a was investigated on colon cancer cells. **METHODS:** The cytotoxicity of gold 1a was determined by using the 3-(4,5-dimethyl-2-thihazyl)-2,5-diphenyl-2H-tetrazolium bromide method. Flow cytometry was used to detect apoptosis and cell cycle. The expression of protein was evaluated by Western blot assay. Tumor growth in vivo was evaluated in nude mice. **RESULTS:** Gold 1a exhibited marked cytotoxic effects in vitro to human colon cancer, and the concentration of drug required to inhibit cell growth by 50% compared with control (IC_{50}) values ranged from 0.2 μM to 3.4 μM, which represented 8.7-fold to 20.8-fold greater potency than that of cisplatin. Gold 1a significantly induced apoptosis and cell cycle arrest and cleaved caspase 3, caspase 7, and poly(ADP-ribose) polymerase; released cytochrome C, and up-regulated *p53, p21, p27,* and *Bax*. In vivo, intraperitoneal injection of gold 1a at doses of 1.5 mg/kg and 3.0 mg/kg significantly inhibited tumor cell proliferation, induced apoptosis, and suppressed colon cancer tumor growth. An acute toxicology study indicated that gold 1a at effective antitumor concentrations did not cause any toxic side effects in mice. **CONCLUSIONS:** The current results suggested that gold 1a may be a new potential therapeutic drug for colon cancer. **Cancer 2009;115:4459–69. © 2009 American Cancer Society.**

KEY WORDS: gold (III), complexes, apoptosis, cell cycle arrest, colon cancer.

Colon cancer is 1 of the leading causes of cancer death in the world. Although early stage colon cancer is cured with surgery, advanced-stage colon cancer often recurs and becomes fatal, even in patients who receive combination chemotherapy.[1] Cisplatin, which is 1 of the most widely used metal anticancer drugs with a well understood mechanism of action, has been recognized as 1 of the effective agents against colon cancer.[2] However, cisplatin induces drug resistance, and its side effects still are major limitations to its

Corresponding author: Benjamin C. Y. Wong, MD, PhD, Department of Medicine, The University of Hong Kong, Hong Kong, China; Fax: (011) 852-29049443; bcywong@hku.hk

[1]Department of Medicine, the University of Hong Kong, Hong Kong, China; [2]Department of Chemistry and Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis, the University of Hong Kong, Hong Kong, China; [3]Li Ka Shing Institute of Health Sciences and Center for Emerging Infectious Diseases, the Chinese University of Hong Kong, Hong Kong, China

Received: November 11, 2008; **Revised:** February 13, 2009; **Accepted:** February 27, 2009

Published online July 1, 2009 in Wiley InterScience (www.interscience.wiley.com)

DOI: 10.1002/cncr.24514, www.interscience.wiley.com

clinical use.[3] Thus, there is an urgent need for the development of new anticancer drug candidates with cytotoxicity that differs from that of cisplatin and can be given to patients who respond poorly to cisplatin.

Gold (III) compounds have a long history of medicinal uses, such as treatment for rheumatoid arthritis.[4] In recent years, Gold (III) compounds have received special attention as promising candidates for new anticancer drugs.[5-7] Gold (III) compounds exhibit strong cytotoxic activity in vitro against tumor cells and have tumor-inhibiting properties in vivo.[8-11] However, currently, some available gold (III) compounds exhibit poor solubility or are effective only at relatively high doses.[12] We recently synthesized a series of gold (III) mesotetraarylporphyrins that are stable against demetallation in physiologic conditions.[13] The porphyrin ligands in these compounds can stabilize the gold (III) center and carry the metal to their cellular targets. One of these new gold (III) compounds, gold 1a, exhibits 30-fold to 100-fold stronger cytotoxicity than cisplatin in several human cancer cell lines, including a cisplatin-resistant human nasopharyngeal carcinoma cell line (CNE-1), and in liver cancer.[13-16]

Although it has been demonstrated that various gold (III) compounds have favorable anticancer properties,[8-11] their activities against human colon cancer remain to be studied. Furthermore, the mechanism by which gold (III) compounds inhibits tumor growth remains largely unknown.[5] In the current study, we explored the antitumor effect of gold 1a and its mechanism against colon cancer. In this study, we demonstrate that gold 1a exhibits strong cytotoxicity and induction of apoptosis and inhibits tumor growth in vivo in colon cancer. The results indicate that gold 1a may be a promising chemotherapeutic drug for patients with colon cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human colon cancer cell lines SW1116, Colo 205, CRL-238, CCL-2134, and HCT-15 (American Type Culture Collection, Rockville, Md) were maintained in RPMI-1640 medium (GIBCO BRL, Grand Island, NY) that contained 10% fetal calf serum (GIBCO BRL), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (BioWhittaker, Walkersville, Md). All cell lines were maintained at 37°C in a humidified incubator with an atmosphere of 5% carbon dioxide.

Drugs and the 3-(4,5-Dimethyl-2-Thihazyl)-2,5-Diphenyl-2H-Tetrazolium Bromide Assay

Gold 1a was synthesized and purified as described previously and was dissolved in dimethyl sulfoxide (DMSO).[13] Cells were treated with different concentrations of drugs for 72 hours. One hundred microliters of 3-(4,5-dimethyl-2-thihazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) stock solution (1 mg/mL) were added into each well, and the cells were incubated again at 37°C for 4 hours. Cytotoxicity was measured by using the MTT assay.[17] The ratio of absorbance in the treated cells relative to that in the control cells was calculated and is expressed as the percentage of cell death.

4′-6-Diamidino-2-Phenylindole Staining

Cells were fixed with 4% formalin/phosphate-buffered saline (PBS), stained with 10 μg/mL 4′-6-diamidino-2-phenylindole (Sigma Chemical Company, St. Louis, Mo), and observed under a Zeiss Axioscope fluorescence microscope. Cells that revealed cytoplasmic and nuclear shrinkage, chromatin condensation, or nuclear fragmentation were defined as apoptotic cells. In the determination of the apoptotic index and to evaluate the appearance of aberrant nuclei, $\geq$300 cells per field were counted.

Flow Cytometry

Cells were collected, fixed with ice-cold 70% ethanol in PBS. then incubated with 100 μL of RNase I (1 mg/mL) and 100 μL of propidium iodide (400 μg/mL) at 37°C for 30 minutes. Samples were analyzed by flow cytometry (Coulter, Luton, United Kingdom). The cell-cycle phase distribution was determined from a resultant DNA histogram using Multicycle AV software (Phoenix Flow Systems, San Diego, Calif).

Western Blot Analysis

Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5; 250 mM NaCl; 0.1% NP40; and 5 mM ethylene glycol tetraacetic acid containing 50 mM sodium fluoride, 60 mM β-glycerol-phosphate, 0.5 mM sodium vanadate, and 0.1 mM phenyl methyl sulfonyl fluoride).

Protein samples were electrophoresed in a 10% denaturing sodium dodecyl sulfate gel and transferred to Immobilon-P membranes (Millipore, Bedford, Mass). The blots were incubated with different primary antibodies, reacted with a peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), and finally observed by enhanced chemiluminescence (Amersham, Piscataway, NJ).

Terminal Deoxynucleotidyltransferase-Mediated Deoxyuridine Triphosphate-Digoxigenin Nick-End Labeling Assay

Tumor xenografts were excised and fixed in formalin immediately after resection. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labeling was carried out using the In ApoAlert DNA Fragmentation Assay Kit (Clontech Corporation, Palo Alto, Calif).[17] Apoptotic cells exhibited strong, green nuclear fluorescence.

The percentage of apoptotic cells was assessed in 10 randomly selected fields and was viewed at ×40 magnification. The apoptotic index was calculated as the number of apoptotic cells/total number of nucleated cells × 100%.

Immunohistochemistry

The expression of Ki-67 in tumor tissues was detected with the Dako LSAB+ Kit (Dako, Carpenteria, Calif) according to manufacturer's instructions. Briefly, slides were boiled in 10 mM citrate buffer (pH 6.0) (Bio Genex, San Ramon, Calif), for antigen retrieval. Then, the sections were incubated with anti-Ki-67 antibody (1:100 dilution; Dako) followed by biotinylated anti-immunoglobulin G antibody and StreptAB-complex/HRP (Dako). Sections were counterstained with hematoxylin and were evaluated independently by 2 investigators. Ki-67 staining was recorded as the ratio of positively stained cells to all tumor cells in 5 areas at 200-fold magnification.

Effect of Gold 1a on Tumor Growth In Vivo

Female Balb/C nude mice ages 5 weeks to 6 weeks were obtained from the Animal Laboratory Unit at the University of Hong Kong (Hong Kong, China). Tumors were established by the subcutaneous injection of 1×10^6 Colo 205 cells/0.2 mL PBS into the left flank of the mice. Tumor size was determined by measuring the 2 greatest perpendicular dimensions with a caliper every 3 days. When tumor size reached to 80 mm^3 to 100 mm^3, the mice were divided and treated with 1 of the following: no treatment, gold 1a (1.5 mg/kg or 3 mg/kg once per week for 3 weeks intraperitoneally) and cisplatin (3 mg/kg once per week for 3 weeks intraperitoneally; Pharmacia & Upjohn Inc., Bridgewater, NJ). Tumor volume was estimated by using the equation $V = 4/3\pi \times a/2 \times (b/2)2$, where a was the greatest dimension, and b was the perpendicular dimension.[18] All experiments included 4 or 5 mice per group, and each was repeated 3 times. The percentage inhibition was calculated as (1 − [mean size of tumor tests/mean sizes of tumor controls]) × 100%.

Acute Toxicity Test

The test was conducted at the Center for Drug Safety Evaluation and Research (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China). Seventy mice from an imprinting control region (ICR) strain (35 males and 35 females; weight, 18 g to 22 g) were chosen and divided into 6 groups for single intraperitoneal injections of gold 1a at doses of 3.0 mg/kg, 3.2 mg/kg, 4.0 mg/kg, 5.0 mg/kg, and 6.0 mg/kg, and a vehicle control also was included. The health status of the mice was observed for 14 days after injection. On Day 15, all mice were killed, and different organs, including the heart, lung, liver, kidney, intestine, spleen, stomach, testes, and ovaries, were examined.

Statistical Analysis

The mean and standard error were calculated from the raw data and then subjected to the Student t test. P values $<.05$ were regarded as statistically significant. The Kaplan-Meier method was used to analyze the survival of mice injected at different doses of gold 1a in an acute toxicity experiment.

RESULTS

Gold 1a Exhibits Strong Cytotoxicity on Colon Cancer Cells

First, we examined the cytotoxic effects of different doses of gold 1a on colon cancer cells in vitro using the MTT assay. The inhibitory concentrations (concentration of the

Table 1. The 50% Inhibitory Concentration of Gold (III) Complex 1a on Colon Cancer Cell Lines*

Cell Lines	Mean ± SEM, μM† Gold 1a‡	Cisplatin‡	IC_{50} Ratio, Cisplatin/Gold 1a
SW1116	0.20 ± 0.02	1.74 ± 0.27	8.7
Colo 205	0.27 ± 0.02	5.62 ± 0.95	20.8
CRL-238	1.41 ± 0.20	19.37 ± 0.22	13.7
CCL-2134	0.65 ± 0.13	9.71 ± 0.63	13.6
HCT-15	0.86 ± 0.15	9.75 ± 0.81	11.3
HCT-15A2	3.43 ± 0.46	31.62 ± 1.64	9.3

SEM indicates standard error of the mean; Gold 1a, gold (III) complex 1a; IC_{50}, concentration of the drug required to inhibit cell growth by 50% compared with control.

*The cytotoxicity of gold 1a and cisplatin was determined for 48 hours using the 3-(4,5-dimethyl-2-thihazyl)-2,5-diphenyl-2H-tetrazolium bromide method.

† Data represent the mean of 3 independent experiments.

‡ $P < .01$ for gold 1a versus cisplatin.

drug required to inhibit cell growth by 50% compared with control [IC_{50} values]) were evaluated from the dose dependence of surviving cells 48 hour after exposure to gold 1a and cisplatin (as a positive control). Gold 1a demonstrated significant cytotoxic activity with IC_{50} values that ranged from 0.20 μM to 3.4 μM. The anticancer potency of gold 1a was much higher than that of cisplatin. The IC_{50} of gold 1a was 8.7-fold to 20.8-fold lower than that of cisplatin. Furthermore, gold (III) exhibited 9-fold greater cytotoxicity in the multidrug-resistant cell line HCT-15A compared with cisplatin (Table 1).

Gold 1a Induces Apoptosis in Colon Cancer Cells

Treatment with gold 1a (1 μM and 3 μM) induced marked morphologic changes, including cytoplasmic vacuoles in HCT-15 cells (Fig. 1A) and cellular fragmentation and nuclear shrinkage 24 hours and 48 hours after treatment (Fig. 1B). There was a significant increase in the percentage of apoptotic cells (Fig. 1B). Treatment with cisplatin (3 μM) induced similar morphologic changes (Figs. 1A and 1B). Similar changes were observed in Colo 205 colon cancer cells that were treated with gold 1a and cisplatin (data not shown). Quantitative analysis indicated that the percentage of apoptotic cells was significantly higher in the gold 1a-treated mice than in the cisplatin-treated and DMSO-treated (control) mice (Fig. 1C). We also detected apoptosis by using fluorescence-activated cell sorter analysis. Gold 1a induced apoptosis in a dose-dependent manner in colon cancer cells. The frequency of cells at sub-G_1 phase in HCT-15 cells that were treated with 1 μM and 3 μM gold 1a for 48 hours was 11% and 37.2%, respectively, compared with the control group (4.5%) (Fig. 2A). Twenty percent to 45% of cells were undergoing apoptotic cell death when they were treated with 3 μM gold 1a for 48 hours in 4 colon cancer cell lines that we examined (Fig. 2B). Moreover, the effect of gold 1a on apoptosis was stronger than that of cisplatin (Fig. 2B). Treatment with gold 1a consistently resulted in the expression of several markers of apoptosis, such as caspase 3, caspase 7, and poly(ADP-ribose) polymerase (PARP) cleavage, and in the release of mitochondrial cytochrome C into the cytosol (Fig. 2C). Overall, these results confirmed the proapoptotic activity of gold 1a in vitro.

Gold 1a Causes Cell Cycle Arrest in G_0/G_1 Phase

When HCT-15 cells were treated with 1 μM gold 1a for 48 hours, the frequency of cells in G_0/G_1 phase (56.1%) was increased compared with that in the control group (44.2%), indicating a significant increase in the number of apoptotic cells (sub-G_1 cells, 11%) compared with the control group (4.5%) (Fig. 2A). To avoid the effect of dead cells on cell cycle phase, we treated HCT-15 cell at a low dose of gold 1a (0.1 μM) to reduce the induction of cell death; however, significant inhibition of cell proliferation was retained (data not shown). When HCT-15 cells were treated with 0.1 μM gold 1a, the number of cells in G_0/G_1 phases began to increase at 12 hours and was more significant at 24 hours, 48 hours, 72 hours, and 96 hours after treatment. The results from a typical experiment are shown in Figure 3A. Similar results were observed in Colo 205 cells (data not shown). These data indicated that gold 1a induces cell cycle arrest, resulting in the inhibition of cancer cell growth. In additional, the reduced cell growth also may be attributable to the apoptotic cell death observed with high doses of gold 1a (Fig. 2A).

Gold 1a Induces Expression of Cell Cycle-Related and Apoptosis-Related Protein

Western blot analysis indicated that gold 1a up-regulated the expression of $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and Bax proteins in HCT-15 cells and decreased the expression of

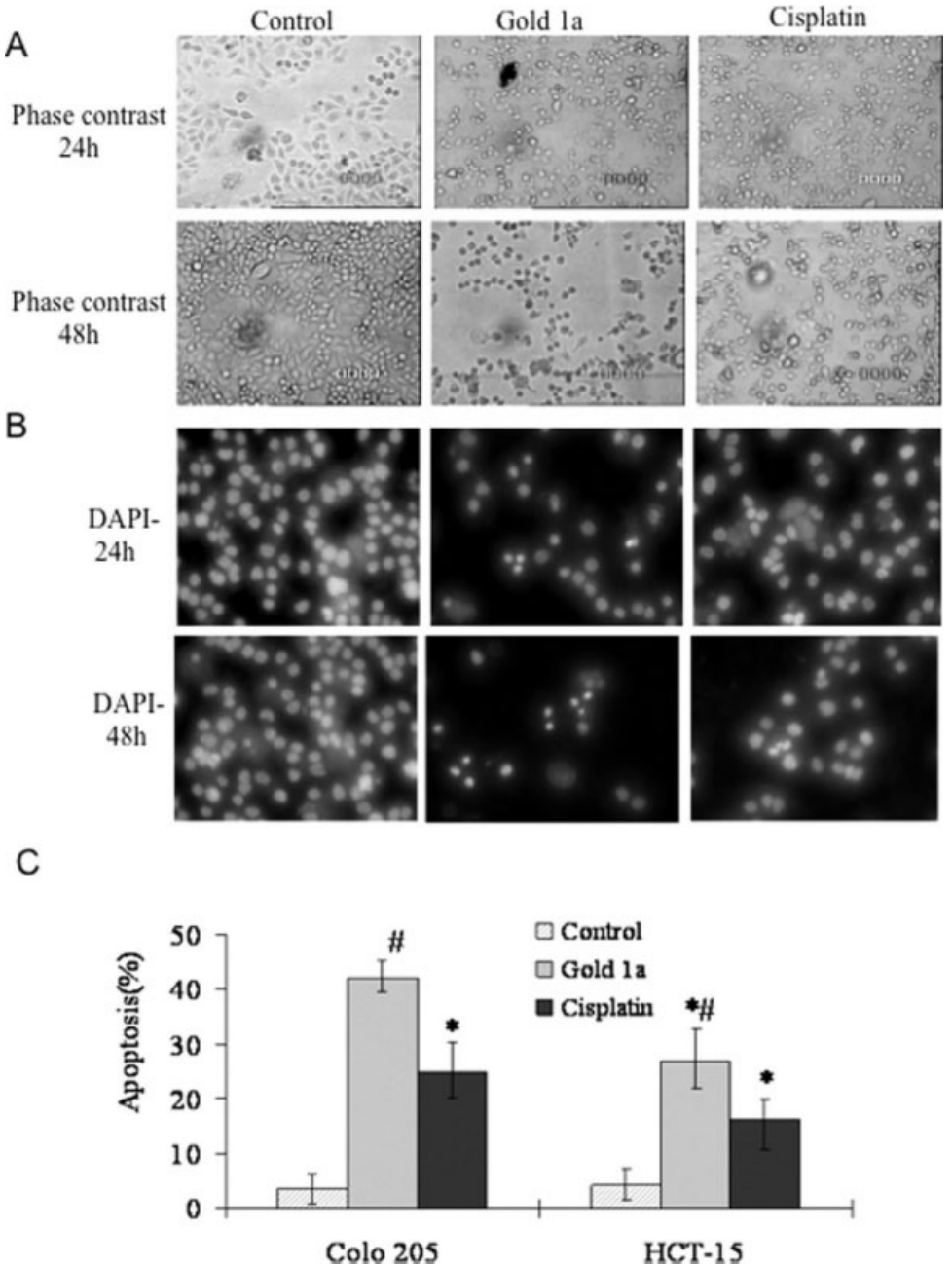

FIGURE 1. Gold (III) complex 1a (gold 1a) exhibited cytotoxicity and induced apoptosis in colon cancer. This figure illustrates the morphologic changes that were observed in cells treated with gold 1a and cisplatin at a dose of 3 μM for 24 hours (24h) and for 48h. Cells were either (A) photographed with a phase-contrast microscope or (B) stained with 4′-6-diamidino-2-phenylindole (DAPI) and photographed under fluorescence microscopy (original magnification, ×200 in A, ×400 in B). (C) These charts illustrate the quantification of cells with condensed DNA in HCT-15 cells treated with 3 μM gold 1a and cisplatin for 48 hours. Data represent the mean of 3 independent experiments. On average, 150 to 300 cells were counted for each determination. An asterisk indicates $P < .01$ versus the control group; pound sign, $P < .05$ versus the cisplatin group.

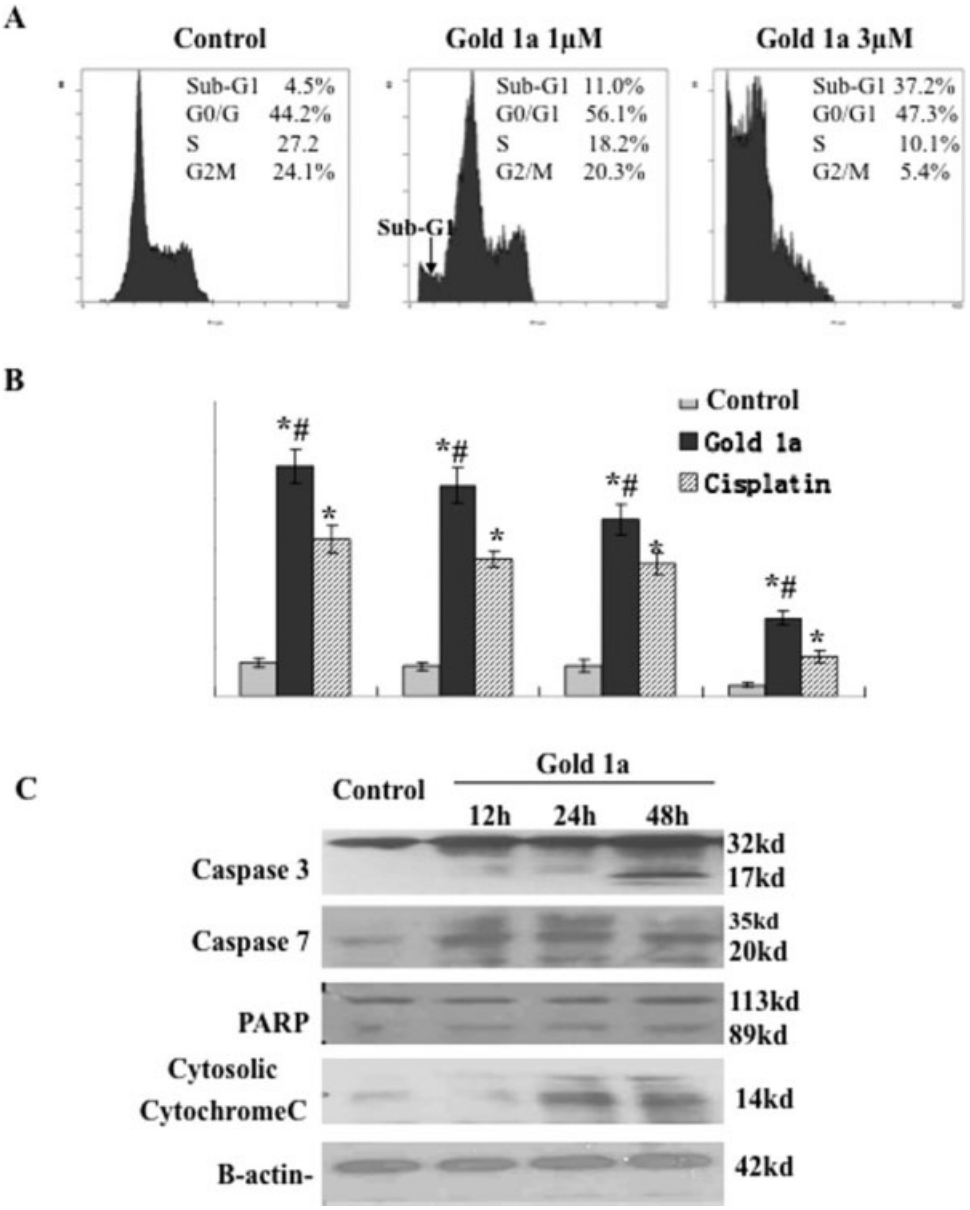

FIGURE 2. Gold (III) complex 1A (gold 1a) induced apoptosis in colon cancer cells. (A,B) These charts illustrate the quantification of apoptosis by flow cytometric fluorescence activated cell sorter analysis. (A) HCT-15 colon caner cells were treated with 1 μM and 3 μM gold 1a for 48 hours. (B) The indicated colon cancer cell lines were treated with 3 μM gold 1a and cisplatin for 48 hours. The data are expressed as the mean ± standard error of the mean from 3 independent experiments. An asterisk indicates $P < .01$ versus the control group; pound sign, $P < .05$ versus the cisplatin group. (C) Gold 1a induced cleavage of caspase 3, caspase 7, and poly(ADP-ribose) polymerase (PARP) and release of mitochondrial cytochrome C in HCT-15 cells that were treated with 3 μM gold 1a for the times indicated (12 hours [12h], 24h, and 48h). Protein expression was detected by Western blot analysis.

bcl-xl, whereas there was no change in the levels of bcl-2 protein (Fig. 3B). Levels of p53 protein began to rise 12 hours after treatment, reached the maximum level after 24 hours, and decreased thereafter (Fig. 3B). The $p21^{WAF1/CIP1}$ and Bax levels were slightly increased in all time intervals (12 hours, 24 hours, and 48 hours) compared with the levels in DMSO-treated cells. An increase in $p27^{KIP1}$ protein level occurred at later intervals (48 hours and beyond). The bcl-xl level decreased after 24 hours but then recovered at 48 hours after treatment (Fig. 3B). Similar results also were observed in Colo 205 cells (data not shown). These results indicate that gold 1a

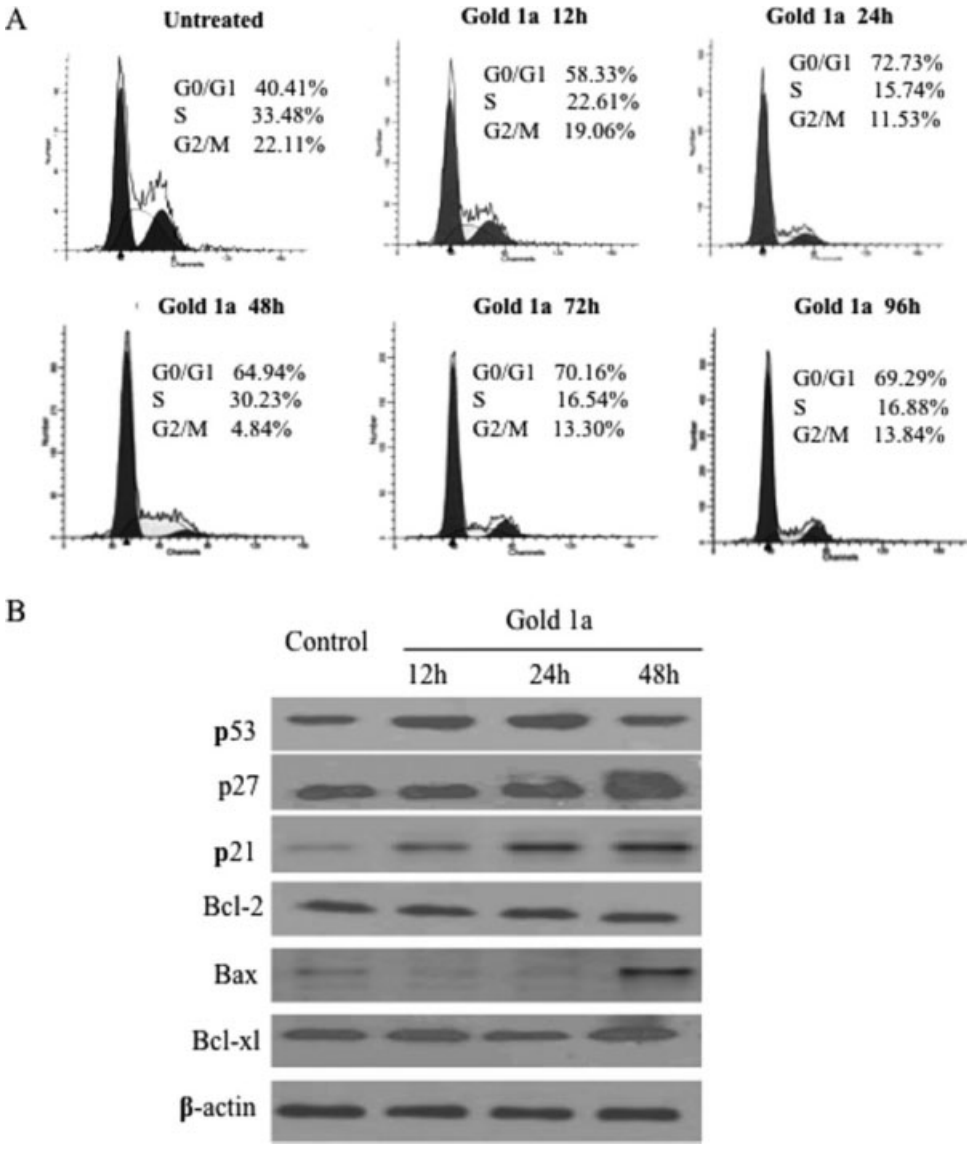

FIGURE 3. Gold (III) complex 1a (gold 1a) caused cell cycle arrest in G_0/G_1 phase. (A) HCT-15 cells were treated with 0.1 μM gold 1a for 12 hours, 24 hours, 48 hours, 72 hours, and 96 hours. Cells were subjected to flow cytometric fluorescence activated cell sorter analysis. Cell cycle phase distribution was calculated from the resultant DAN histogram using Multicycle AV software (Phoenix Flow Systems, San Diego, Calif). The results were obtained from representation of 3 independent experiments. (B) Gold 1a induced the expression of cell cycle-related and apoptosis-related genes. HCT-15 cells were treated with gold 1a for 12 hours, 24 hours, and 48 hours. Protein expression was detected by Western blot analysis.

induces cell cycle arrest and apoptosis by regulating the expression of p53, p21, p27, and Bax.

Gold 1a Inhibits Tumor Growth In Vivo

We investigated the in vivo effect of gold 1a on established subcutaneous tumors. Because Colo 205 cells have stronger capacity for tumorigenesis, we chose them for in vivo study. Figure 4A indicates that gold 1a (1.5 mg/kg per week and 3.0 mg/kg once per week for 3 weeks) significantly inhibited the growth of tumor xenografts compared with the control group. The antitumor efficiency of gold 1a was similar or better than that of the clinically used cisplatin (Fig. 4A). The hematoxylin and eosin staining revealed that mice that received gold 1a and cisplatin treatment exhibited larger areas of dead cells in the tumor tissue compared with mice in the control group (data not shown). Consistent with the in vitro results,

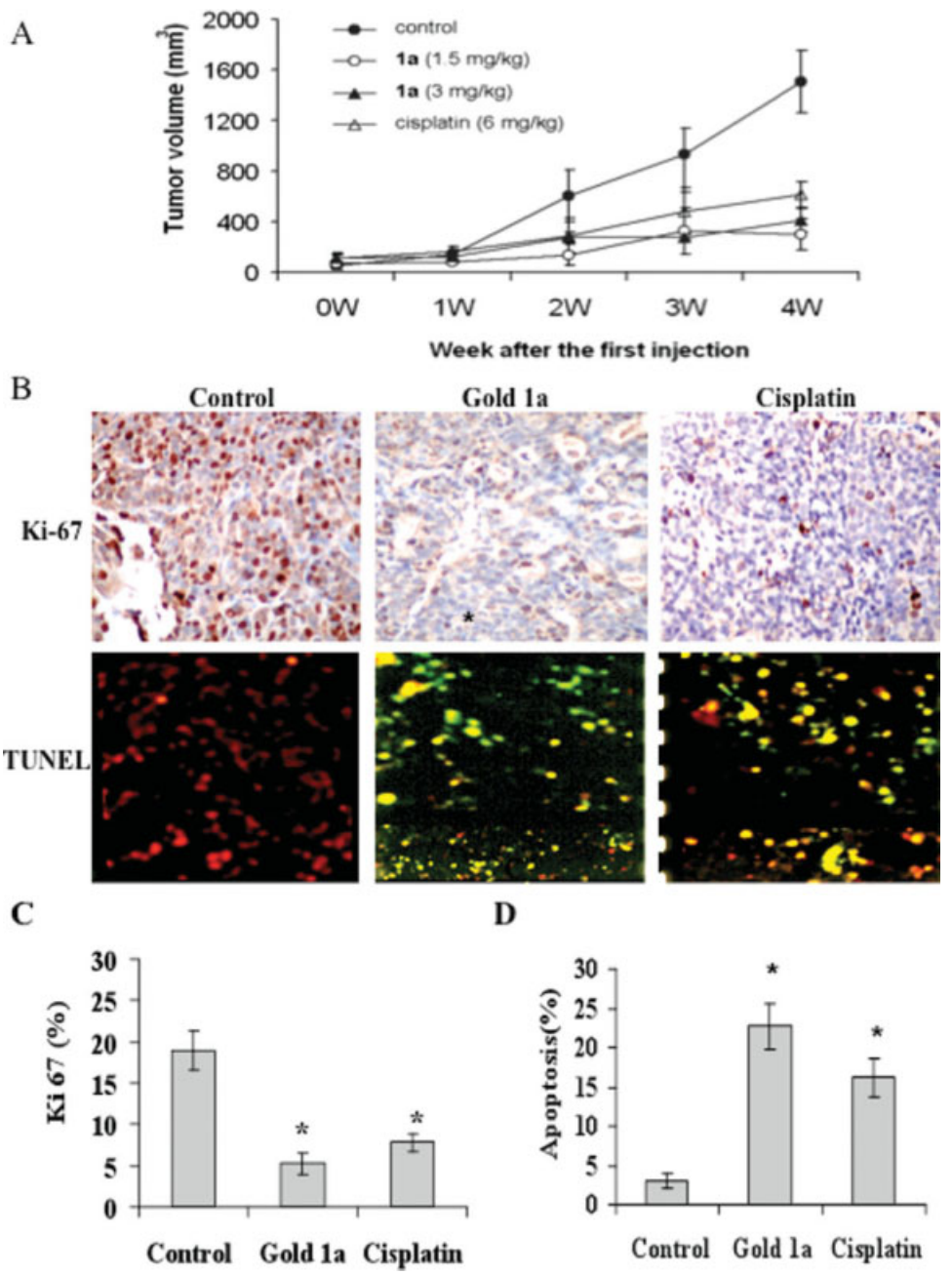

FIGURE 4. Gold (III) complex 1a (1a) inhibited xenograft growth of colon cancer in vivo. (A) These are colon cancer growth curves. Colo 205 cells were injected subcutaneously into nude mice. The mice were injected intraperitoneally with phosphate-buffered saline (control), gold 1a, or cisplatin once weekly for 3 weeks at the doses indicated, and tumor size was determined every week. Each point represents the mean tumor size (±standard error of the mean) of results obtained from 12 mice. W indicates week. (B) Gold 1a inhibited cell proliferation and induced apoptosis in tumor xenografts in vivo. Mice were treated with gold 1a and cisplatin as described previously. Tumor tissue sections were subjected to Ki-67 immunostaining to detect cell proliferation and to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labeling (TUNEL) to detect apoptotic cells. The images shown are representative fields of view from tumors after treatment. Ki-67-positive cells are stained brown, and apoptotic cells are stained green (original magnification, ×400). (C,D) These bar charts illustrate the quantification of (C) Ki-67-positive cells and (D) apoptotic cells in vivo. Data represent the mean ± standard error of the mean from 4 mice that were treated with gold 1a at a dose of 1.5 mg/kg. An asterisk indicates $P < .01$ compared with the control group.

the proportion of Ki-67-positive cells in tumors that were treated with gold 1a (5.3% ± 1.3%) was slightly lower than the proportion in tumors that were treated with cisplatin (6.7% ± 1.2%; $P > 0.05$) and significantly lower than the proportion in DMSO-treated tumors (18.9% ± 2.4%; $P < .01$) (Fig. 4B,C). It is noteworthy that tumors treated with gold 1a had a higher percentage of apoptotic cells (22.7% ± 2.9%) than the percentage in DMSO-treated tumors (3.2% ± 0.6%; $P < .01$) was similar to the percentage in cisplatin-treated tumors (18.7% ± 2.5%; $P > .05$) (Fig. 4B,C). These results suggest that the inhibition of tumor growth is caused by an increase in apoptosis and a decrease in cell proliferation induced by gold 1a in vivo.

Gold 1a At its Effective In Vivo Concentration Demonstrates Low Toxicity

Finally, we evaluated the safety of gold 1a in vivo. We did not observe any histologic changes in major organs, including the heart, lung, liver, kidney, intestine, spleen, stomach, testes, or ovaries, in mice that were treated with gold 1a (data not shown). We also evaluated the acute toxicity of gold 1a at serial doses ≥3.0 mg/kg (ie, 3.0 mg/kg, 3.2 mg/kg, 4.0 mg/kg, 5.0 mg/kg, and 6.25 mg/kg) (Fig. 5A,B). All mice survived during the observation period of 14 days with treatment of gold 1a at 3.0 mg/kg. However, 3 of 10 mice died after treatment of gold 1a for 8 to 10 days at a dose of 3.2 mg/kg. Increasing doses of gold 1a to 4.0 mg/kg, 5.0 mg/kg, and 6.25 mg/kg decreased body weight and increased the death rate of the treated mice. The 50% lethal dose value of gold 1a derived from these curves was identified as 4.4 mg/kg. After a single intraperitoneal injection of gold 1a, the body weight of mice decreased gradually after 2 days. For the mice that survived, their body weight was regained 5 days to 7 days after treatment. At the end of the observation period (Day 14), the dead or killed (at Day 15) mice were dissected and underwent histologic assessment of organ damage. No obvious damage in the major organs was observed. The results suggested that gold 1a exhibits low toxicity in vivo at its effective concentration.

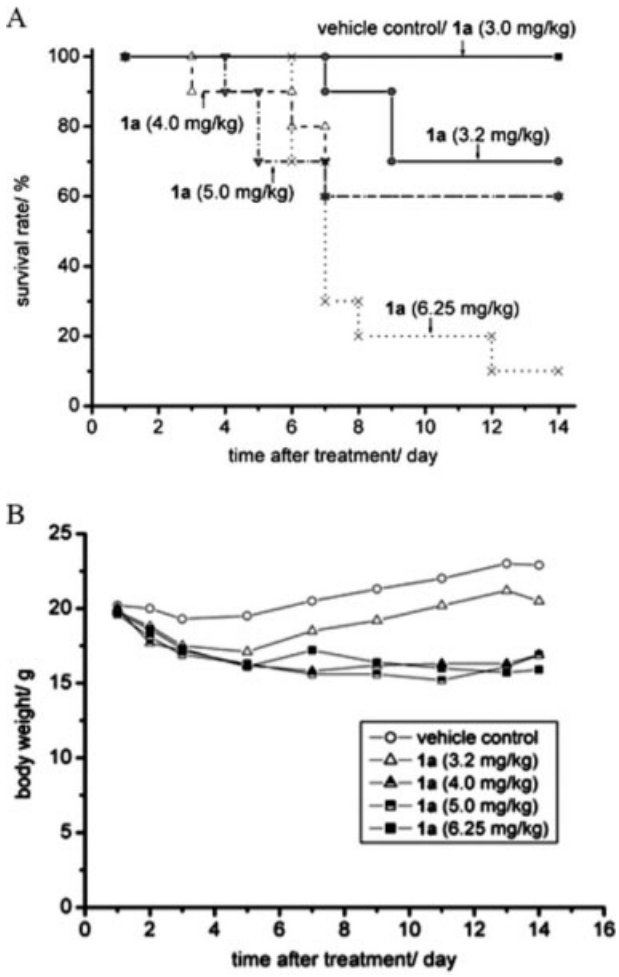

FIGURE 5. Gold (III) complex 1a (1a) exhibited lower toxicity at in vivo effective dosage. Mice were received gold 1a intraperitoneally at the indicated dose. Animals were monitored and bodies were weighed every day. (A) The survival of mice was analyzed using the Kaplan-Meier method, and no lethal toxicity was observed at effective concentrations of gold 1a in vivo. (B) This chart illustrates the changes in mouse body weight. Each point represents the mean weight of results obtained from 4 or 5 mice.

DISCUSSION

It has been known for a long time that gold (III) compounds possess antitumor activities.[4-7] However, most of the reported cytotoxic gold (III) complexes are unstable in physiologic conditions[19] and have marginal or moderate in vivo antitumor activity against human carcinoma xenografts.[6,12,20] An organogold (III) complex (2-[dimethylaminomethyl] phenyl phenyl gold [III] chloride) exhibits promising in vitro cytotoxicity in colon cancer cells, but it fails to inhibit in vivo tumor growth in HT-29 colon cancer xenografts.[21] In the current study, we observed that gold 1a exhibited significant cytotoxicity against different colon cell lines with 8.7-fold to 20.8-fold greater higher potency than that of cisplatin. Furthermore, gold 1a

displayed stronger cytotoxicity to multidrug-resistant colon cancer than cisplatin. More noteworthy, for the first time to our knowledge, we observed that gold 1a effectively inhibited colon tumor growth in vivo. These data reveal that gold 1a is a potent cytotoxic agent against colon cancer.

The mechanisms responsible for gold (III) complex antitumor activity still are largely unknown. It is believed generally that the cytotoxic effects of metal complexes are the consequences of direct damage to nuclear DNA.[13,22,23] However, some studies have demonstrated that gold (III) complex interactions with DNA are not as tight as platinum interactoins.[6,24] Gold (III) compounds inhibit DNA and RNA synthesis and have only minimal cross-resistance to cisplatin,[25] suggesting a different mechanism of action. Recent data suggest that the induction of apoptosis is a major mechanism of gold 1a.[14,15,21] Our result indicated that gold 1a exhibits strong induction of apoptosis in colon cancer by cleaving caspase 3, caspase 7, and PARP; releasing cytochrome C, and up-regulating Bax, suggesting that gold 1a induces apoptosis by a mitochondrial death pathway. The result is consistent with a recent report that gold 1a can induce apoptosis in nasopharyngeal cancer cell through a mitochondrial death pathway related to reactive oxygen species.[14] Microarray data revealed that gold 1a can up-regulate several genes that are involved in the induction of apoptosis.[15] Furthermore, we observed that gold 1a up-regulated the expression of p53, p27, p21, and Bax proteins in HCT-15 cells and decreased the expression of bcl-xl, whereas there was no change in the levels of bcl-2 or Bak protein. It is noteworthy that the up-regulation of p53 and p27 protein began to rise 12 hours after treatment, indicating that p53 and p21 may mediate the early apoptosis event induced by gold 1a. These data suggest that gold 1a can induce apoptosis by regulating the expression of apoptosis-related genes *p53*, *p21*, *p27*, and Bax.

Cell cycle checkpoints maintain genomic stability in eukaryotes in response to genotoxic stress. The induction of the cell cycle is a major mechanism of chemotherapeutic drugs and plays an important role in the antitumor activity of such drugs. It was reported that cisplatin caused G_2/M cell cycle arrest and inhibition of DNA synthesis in a panel of human cancer cell lines.[26] One study demonstrated that organogold (III) compounds II and III can increase cell numbers in G_2/M phase among ovarian cancer cells, similar to the action of cisplatin, while organogold (III) compound I caused cell cycle arrest in G_0/G_1 phase.[27] In the current study, we observed that gold 1a caused cell cycle arrest in colon cancer cells in G_0/G_1 phase. Our results are consistent with data indicating that gold 1a treatment decreased cell numbers at G_2/M phase and increased cell numbers at G_0/G_1 phase.[16] Therefore, gold 1a-induced cell cycle arrest is different from that induced by cisplatin, although recent data also demonstrated that G_1 arrest induction represents a critical determinant for cisplatin in G_1 checkpoint-retaining human cancer.[28] Furthermore, we observed that the up-regulation of p53, p21, and Bax began 12 hour after treatment, consistent with the time point of G_0/G_1phase arrest. The expression of p53 and p21 may play an early role in cell cycle arrest. The up-regulation of Bax and p27 began at 48 hours after treatment; at that time, the expression of p53 decreased, and p53, p21, and p27 played a pivotal role in cell cycle arrest and apoptosis.[29-32] Another study indicated that p53 provokes cell cycle arrest in G_0/G_1 phase by up-regulating p21 and Bax.[33] In the current study, p27 up-regulation may maintain cell cycle arrest and apoptosis in gold 1a-treated cells at a late treatment stage. Thus, our results have demonstrated a new mechanism by which gold 1a induced apoptosis and caused cell cycle arrest through the regulation of p53, p27, p21, and Bax.

In summary, gold 1a effectively inhibits cell proliferation, induces apoptosis, and inhibits tumor growth in colon cancer. Given the poor long-term survival and prospects for patients who have late-stage colon cancer and the lack of successful treatments at this stage, this highly effective gold 1a can provide crucial insights into the anticancer mechanism of colon cancer.

Conflict of Interest Disclosures

Supported by grants from the Areas of Excellence Scheme (AoE/P-10/01) administered by the University Grants Council (Hong Kong SAR, China) and the Gastroenterological Research Fund of University of Hong Kong, Hong Kong, China.

References

1. Chung KY, Saltz LB. Adjuvant therapy of colon cancer: current status and future directions. *Cancer J*. 2007;13:192-197.
2. Macdonald JS, Astrow AB. Adjuvant therapy of colon cancer. *Semin Oncol*. 2001;28:30-40.

3. Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat Rev*. 2007;33:9-23.
4. Messori L, Abbate F, Marcon G, et al. Gold(III) complexes as potential antitumor agents: solution chemistry and cytotoxic properties of some selected gold(III) compounds. *J Med Chem*. 2000;43:3541-3548.
5. Marcon G, Messori L, Orioli P. Gold (III) complexes as a new family of cytotoxic and antitumor agents. *Expert Rev Anticancer Ther*. 2002;2:337-346.
6. Marcon G, Carotti S, Coronnello M, et al. Gold (III) complexes with bipyridyl ligands: solution chemistry, cytotoxicity, and DNA binding properties. *J Med Chem*. 2002;45: 1672-1677.
7. Tiekink ER. Gold (III) derivatives for the treatment of cancer. *Crit Rev Oncol Hematol*. 2002;42:225-248.
8. Kean WF, Forestier F, Kassam Y, Buchanan WW, Rooney PJ. The history of gold (III)therapy in rheumatoid disease. *Semin Arthritis Rheum*. 1985;14:180-186.
9. Calamai P, Carotti S, Guerri A, et al. Cytotoxic effects of gold(III) complexes on established human tumor cell lines sensitive and resistant to cisplatin. *Anticancer Drug Des*. 1998;13:67-80.
10. Calamai P, Carotti S, Guerri A, et al. Biological properties of 2 gold(III) complexes: AuCl3(Hpm) and AuCl2(pm). *J Inorg Biochem*. 1997;66:103-109.
11. Cossu F, Matovic Z, Radanovic D, Ponticelli G. Cytotoxic activity of some gold(III) complexes. *Farmaco*. 1994;49:301-302.
12. Buckley RG, Elsome AM, Fricker SP, et al. Antitumor properties of some 2-[(dimethylamino)methyl]phenylgold(III) complexes. *J Med Chem*. 1996;39:5208-5214.
13. Che CM, Sun RW, Yu WY, Ko CB, Zhu N, Sun H. Gold (III) porphyrins as a new class of anticancer drugs: cytotoxicity, DNA binding and induction of apoptosis in human cervix epitheloid cancer cells. *Chem Commun (Camb)*. 2003;21:1718-1719.
14. Wang Y, He QY, Sun RW, Che CM, Chiu JF. Gold III porphyrin 1a induced apoptosis by mitochondrial death pathways related to reactive oxygen species. *Cancer Res*. 2005;65:11553-11564.
15. Lum CT, Yang ZF, Li HY, et al. Gold(III) compound is a novel chemocytotoxic agent for hepatocellular carcinoma. *Int J Cancer*. 2006;118:1527-1538.
16. Wang Y, He QY, Che CM, Chiu JF. Proteomic characterization of the cytotoxic mechanism of gold (III) porphyrin 1a, a potential anticancer drug. *Proteomics*. 2006;6:131-142.
17. Tu SP, Jiang XH, Lin MC, et al. Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer. *Cancer Res*. 2003;63:7724-7732.
18. Tu SP, Cui JT, Liston P, et al. Gene therapy for colon cancer by adeno-associated viral vector-mediated transfer of survivin Cys84Ala mutant. *Gastroenterology*. 2005;128:361-375.
19. Shaw CF. Gold-based therapeutic agents. *Chem Rev*. 1999; 99:2589-600.
20. Milacic V, Chen D, Ronconi L, Landis-Piwowar KR, Fregona, D, Dou QP. A novel anticancer gold(III) dithiocarbamate compound inhibits the activity of a purified 20S proteasome and 26S proteasome in human breast cancer cell cultures and xenografts. *Cancer Res*. 2006;66:10478-86.
21. Engman L, McNaughton M, Gajewska M, Kumar S, Birmingham A, Powis G. Thioredoxin reductase and cancer cell growth inhibition by organogold(III) compounds. *Anticancer Drugs*. 2006;17:539-544.
22. Drobnik J. Antitumor activity of platinum complexes. *Cancer Chemother Pharmacol*. 1983;10:145-149.
23. Mirabelli CK, Johnson RK, Sung CM, Faucette L, Muirhead K, Crooke ST. Evaluation of the in vivo antitumor activity and in vitro cytotoxic properties of auranofin, a coordinated gold (III)compound, in murine tumor models. *Cancer Res*. 1985;45:32-39.
24. Messori L, Orioli P, Tempi C, Marcon G. Interactions of selected gold(III) complexes with calf thymus DNA. *Biochem Biophys Res Commun*. 2001;281:352-360.
25. Ronconi L, Marzano C, Zanello P, et al. Gold (III) dithiocarbamate derivatives for the treatment of cancer: solution chemistry, DNA binding, and hemolytic properties. *J Med Chem*. 2006;49:1648-1657.
26. Mueller S, Schittenhelm M, Honecker F, et al. Cell-cycle progression and response of germ cell tumors to cisplatin in vitro. *Int J Oncol*. 2006;29:471-479.
27. Coronnello M, Mini E, Caciagli B, et al. Mechanisms of cytotoxicity of selected organogold(III) compounds. *J Med Chem*. 2005;48:6761-6765.
28. Un F. G1 arrest induction represents a critical determinant for cisplatin cytotoxicity in G1 checkpoint-retaining human cancers. *Anticancer Drugs*. 2007;18:411-417.
29. Aneja R, Ghaleb AM, Zhou J, Yang VW, Joshi HC. p53 And p21 determine the sensitivity of noscapine-induced apoptosis in colon cancer cells. *Cancer Res*. 2007;67:3862-3870.
30. Rother K, Kirschner R, Sanger K, Bohlig L, Mossner J, Engeland K. p53 Downregulates expression of the G1/S cell cycle phosphatase Cdc25A. *Oncogene*. 2007;26:1949-1953.
31. Lee B, Kim CH, Moon SK. Honokiol causes the p21WAF1-mediated G-phase arrest of the cell cycle through inducing p38 mitogen activated protein kinase in vascular smooth muscle cells. *FEBS Lett*. 2006;580:5177-5184.
32. Chen WJ, Chang CY, Lin JK. Induction of G1 phase arrest in MCF human breast cancer cells by pentagalloylglucose through the down-regulation of CDK4 and CDK2 activities and up-regulation of the CDK inhibitors p27(Kip) and p21(Cip). *Biochem Pharmacol*. 2003;65:1777-1785.
33. Hayward RL, Schornagel QC, Tente R, et al. Investigation of the role of Bax, p21/Waf1 and p53 as determinants of cellular responses in HCT116 colorectal cancer cells exposed to the novel cytotoxic ruthenium(II) organometallic agent, RM175. *Cancer Chemother Pharmacol*. 2005;55:577-583.