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Antitumor activity of diethynylfluorene derivatives of gold(I)

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ABSTRACT

A list of diethynylfluorenes and their gold(I) derivatives have been studied for their antitumor activity as a function of their structure–activity relationships. End-capping the fluoren-9-one unit with gold(I) moieties could significantly strengthen the cytotoxic activity in vitro on three human cancer cell lines with induction of reactive oxygen species generation on Hep3B hepatocellular carcinoma cells and exhibit attractive antitumor activity from in vivo nude mice Hep3B xenograft model with limited adverse effects on vital organs including liver and kidney.

1. Introduction

There is continuing research interest in the development of carbon-rich organic and organometallic compounds containing rigid, π-conjugated chains, due to their widespread and multifunctional applications in the field of materials science.[1–4] We and others have extensively studied a series of metal acetylide complexes and polymers.[5–9] However, little information is reported about the potential uses of these functional molecules from the biological and medicinal points of view, including any possible therapeutic actions, that could be complementary to organic compounds.[10–12] Medicinal inorganic chemistry began to develop as an up-and-coming scientific discipline after the serendipitous discovery of the antitumor activity followed by the successful clinical applications of cisplatin (CDDP).[13] Since then, this has stimulated considerable interest in searching for new metal complexes as modern therapeutics and diagnostic agents. However, in light of the undesirable side effects of CDDP in clinical uses, there have been extensive recent studies to develop new metal-based drugs with less drug resistance and fewer side effects.[14,15] According to the works by Che, Guo, Lippard, Sadler and others, there has been a growing interest in the chemistry community to investigate the anticancer activities of gold(I, III), iron(II), platinum(II), ruthenium(II, III) and vanadium(IV) complexes.[10–12,16–19] While many gold(III) complexes[20–25] including gold(III) porphyrins[26–29] and cyclometalated gold(III) complexes[30,31] have displayed interesting anticancer potencies, their medicinal applications have always been hampered by their poor stability in solution.[10–12,20,21] Examples of metal arylacetylide as antitumor agents are very scarce.[32] Upon ethynylation by suitable ligands, the water-soluble [Pt(terpy)(glycosylated arylacetylide)]$^+$ (terpy = terpyridine ligand) was shown to be at least eightfold more cytotoxic than [Pt(terpy)Cl]$^+$.[32]

In this contribution, a list of diethynylfluorenes and their gold(I) derivatives have been studied for the first time for their antitumor activity using Hep3B human hepatocellular carcinoma cells, SKHep-1 human hepatoma cells and MDA-MB-231 human breast cancer cells (Fig. 1).

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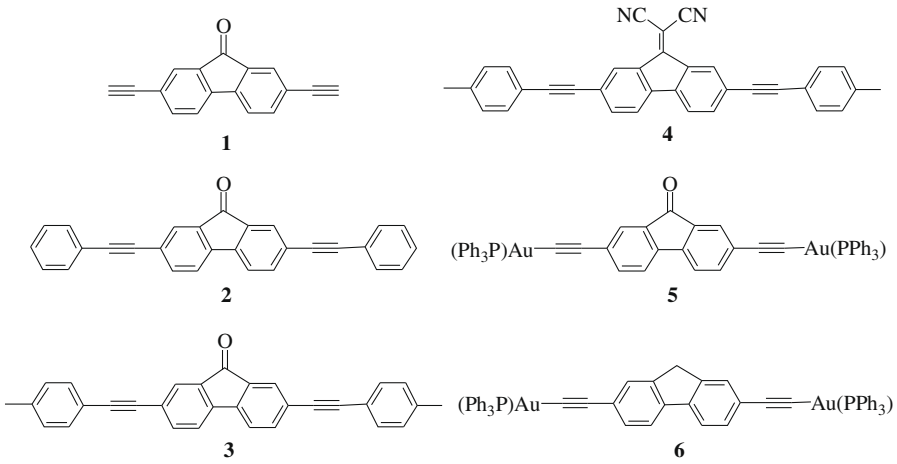


Figure 1. Chemical structures of organic diethynylfluorenes (**1**–**4**) and their gold(I) derivatives (**5** and **6**).

2. Results and discussion

2.1. Synthetic chemistry

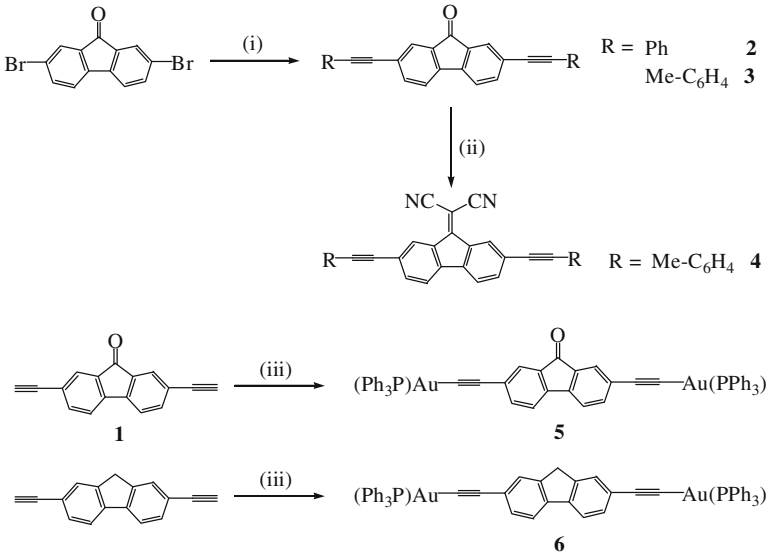
Scheme 1 summarizes the reaction steps leading to the target compounds in this study. Organic model compounds **2** and **3** were obtained by the Sonogashira type coupling between 2,7-dibromofluorenes and the appropriate 1-alkyne in good yields.[33] The dicyano-substituted compound **4** was prepared from **3** by thermal reaction with malononitrile in DMSO. The digold(I) bis(acetylide) complexes were synthesized from the base-catalyzed dehydrohalogenation between $Au(PPh_3)Cl$ and **1** or 2,7-diethynylfluorene in the presence of NaOH in MeOH.[34] All the compounds were obtained as air-stable solids in high purity and found to be soluble in chlorinated solvents such as CH_2Cl_2 and DMSO.

2.2. Biology

As shown in Table 1, the mean cytotoxicity at 50 μM towards these three human carcinoma cell lines follows the trend **5** ≫ CDDP > **1** > **2** > **3** but the values are quite comparable among the less effective compounds **3**, **4** and **6** within statistical limits. When only non-metallic compounds are considered, some preliminary structure–activity relationship could be observed. The presence of phenyl and tolyl end groups could significantly reduce the cytotoxic activity of **2**–**4** relative to the free alkyne **1**. The effects of electron-withdrawing carbonyl and dicyanomethylene groups on the cytotoxicity of organic diethynylfluorene do not differ very much. However, non-metallic diethynylfluorenes **1**–**4** did not show better cytotoxicity than our positive reference, CDDP, at 50 μM.

We also looked into the gold(I) congener of **1** (i.e., **5**) and its parent species without the carbonyl unit (**6**). Compound **5** showed superior cytotoxicity on the three human cancer cell lines tested. To ascertain our assumption, we also tested the possible biological activity of the non-carbonyl derivative of gold(I). As anticipated, **6** was nearly non-cytotoxic at 50 μM, akin to the organic pair (i.e., **3** vs **4**). Substitution of the carbonyl group in **5** with a CH_2 fragment in **6** would completely diminish its cytotoxicity, suggesting the key role played by the C=O group in designing the most effective agent.

Since complex **5** has the best potential to be further developed to be anticancer regimen, we tried to determine its IC_{50} against the three human cancer cell lines using sulforhodamine B assay.[35] Its mean IC_{50} was found to be ~1.7 μM for Hep3B, ~4.7 μM for SKHep-1 and ~5.1 μM for MDA-MB-231 after 48 h of incubation (Table 1). Compound **5** is 7–9-fold more cytotoxic than CDDP,



Scheme 1. Reagents and conditions: (i) phenylacetylene or tolylacetylene, $Pd(OAc)_2$, PPh_3, CuI, iPr_2NH; (ii) $CH_2(CN)_2$, DMSO, 110 °C; (iii) $Au(PPh_3)Cl$, NaOH, MeOH.

Table 1
(Upper) Percentage of survival cells after treating with different diethynylfluorenes and their gold(I) derivatives at 50 μM after 48 h of incubation

	1	2	3	4	5	6	CDDP
Hep3B	78.1 ± 4.6	85.0 ± 2.5	94.9 ± 3.1	99.8 ± 2.4	4.6 ± 1.8	96.7 ± 4.1	39.5 ± 3.0
MDA-MB-231	81.4 ± 3.4	90.3 ± 1.6	97.5 ± 1.4	103.6 ± 3.7	9.1 ± 2.5	101.6 ± 2.7	72.7 ± 3.8
SKHep-1	77.6 ± 5.1	84.8 ± 5.3	92.2 ± 2.5	101.0 ± 4.7	5.8 ± 0.5	99.8 ± 5.4	44.0 ± 1.6
		Hep3B		MDA-MB-231			SKHep-1
5		1.7 ± 1.4		5.1 ± 2.6			4.7 ± 1.5
CDDP		38 ± 4.2		78 ± 4.6			41 ± 2.8

CDDP was used as a positive reference. Three independent experiments were performed and similar results were obtained. Results represent the mean ± SD from one representative experiment. (Lower) IC_{50} values of **5** against three human carcinoma cell lines as determined by sulforhodamine B assay expressed in micro-molarity [μM] after 48 h of incubation. CDDP was used as a positive reference. Three independent experiments were performed and similar results were obtained. Results represent the mean ± standard derivation from one representative experiment.

and IC_{50} values of **5** are 8–22-fold smaller than the reference standard.

The in vitro data enhance our tendency to investigate its in vivo antitumor activity. Athymic nude mice xenografted with human Hep3B carcinoma cells were employed as a tool for this purpose. Mice with Hep3B cancer cells xenografted of average tumor volume about 200 mm^3 received intraperitioneal injection daily of either 50 μL of buffer vehicle or equal volume of **5** at 2.5 mg kg^{-1} for nine consecutive days. As shown in Figure 2, the mean tumor volume of **5**-treated mice began to reduce on day 6, and on day 10, their tumor size returned to the initial mean tumor volume while there was more than a triple increment for vehicle control group. A general increase in body weight was observed from both vehicle treated mice and **5**-treated mice (Fig. 3).

In order to determine whether **5** has any possible toxicology effects in the internal vital organs of mice under the current therapeutic protocol, autopsies of vital organs including livers and kidneys were examined for their anatomy. We found that no observable necrotic features were found from autopsy samples of all the 10 mice (Fig. 4). Further plasma liver functional enzymes analysis including alanine aminotransferase (ALT) and asparate aminotransferase (AST) showed normal liver functions from these 10 mice as well (Table 2).

We also prepared the tumor section for histochemistry study. However, after the embedding procedure, we found that the tissues were completely damaged because of the extensive necrosis caused by **5**, which provided direct evidence for the therapeutic effect of **5**.

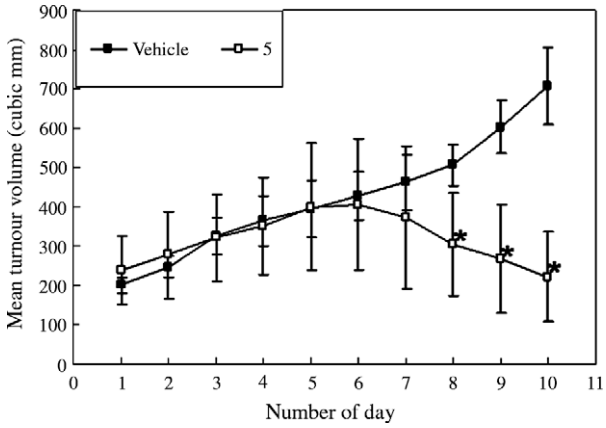


Figure 2. Representative results showing the changes of Hep3B tumor volume (mm^3) against number of day of mice treated with vehicle or **5** for 9 consecutive days from day 1 to day 9. Intraperitoneal injection started for both groups when mean tumor volume of mice reached ~200 mm^3. A total of 10 mice were randomly divided into two groups. On day 10, after measuring individual tumor volume, all mice were sacrificed for plasma and vital organs collection. Results are shown as mean ± standard derivation. Asterisk (*) indicates that there is a statistical significance between the considered group with vehicle control (P <0.05).

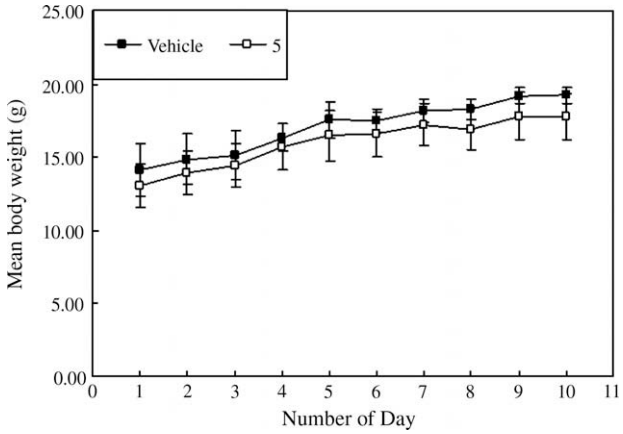


Figure 3. Changes of body weight (grams) against number of day of mice treated with vehicle and **5** at 2.5 mg kg^{-1} for 9 consecutive days. Results are shown as mean ± standard derivation. N = 5 from each group.

Understanding the underlying mechanistic working principle is an important aspect for development of novel therapeutic agent. In our case, we investigated that the gold(I) complex **5** could induce a significant generation of intracellular reactive oxygen species (ROS), as detected by using the 2′,7′-dichlorofluorescein diacetate probe, after incubation for 24 h at 10 μM with Hep3B human hepatocellualr carcinoma cells. Preliminary data suggested that the presence of gold in **5** could be involved in the generation of ROS as **1** was unable to stimulate the production of ROS significantly. We believe that generation of ROS in **5** is essential for its cytotoxicity since co-incubation with catalase could partially reverse the generation of intracellular ROS as well as cytotoxic activity against Hep3B human carcinoma cells (Fig. 5).[36]

As further pre-incubation of the Hep3B cancer cells with a pan-caspase inhibitor, zVADfmk at 20 μM, could not reverse the cytotoxicity effect of **5** at 10 μM after a further incubation for 24 h, it seems that caspase family members do not play a significant role in **5**-induced Hep3B cell death (data not shown). DNA is one of the major targets for anticancer drugs, and binding of metallodrugs to DNA has been extensively studied.[29,37–40] Intuitively, similar interactions may be involved in the presence of gold center in the present case.

We also examined the relative cellular uptake of **5** and **6**. Using Hep3B cells as a demonstration model and by means of ICP-MS studies, our results revealed that good uptakes of gold were demonstrated in both **5** and **6**. The gold uptake by **5** was about 8 to 18 times more than **6** (from four trials), on average 11 times more, per μg of protein. Thus, a much higher uptake of **5** was observed in the cells relative to that for **6** and this is apparently a manifestation of the role of the keto group in **5** to account for its better cytotoxic effect.

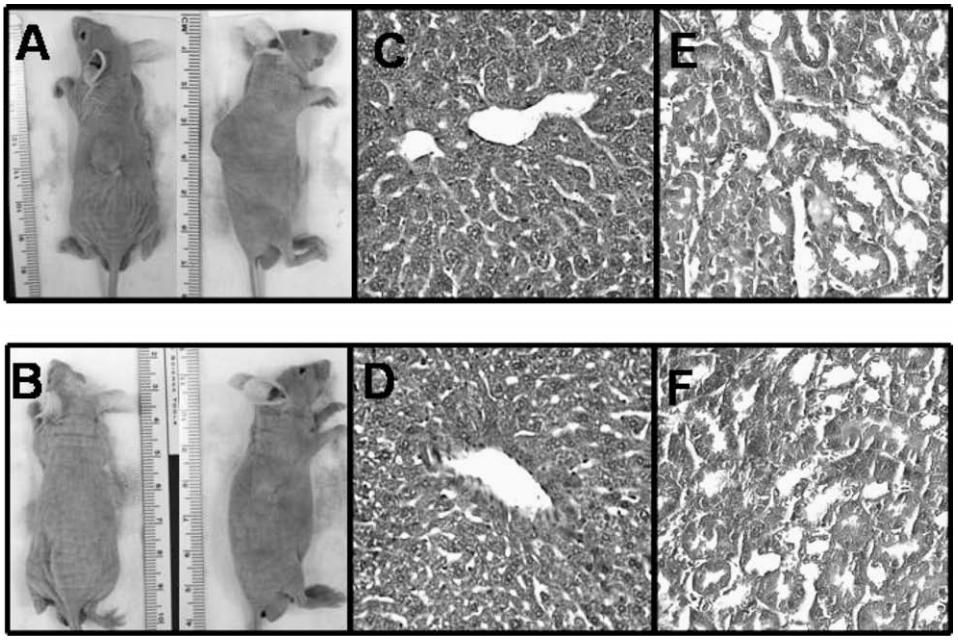


Figure 4. (A and B) Representative Hep3B xenografted nude mice treated with vehicle (A) or **5** (B) at 2.5 mg kg^{-1} for 9 consecutive days. The tumor size of Hep3B from A is much bigger than that of B. (C and D) Histochemistry study of liver section from sacrificed mice of A and B to observe any possible liver toxicity. No necrotic tissue and damage was discovered. (E and F) Histochemistry study of kidney section from sacrificed mice of A and B to observe any possible kidney toxicity. No necrotic tissue and damage was discovered.

Table 2
Plasma liver enzyme assays for vehicle control and **5**-treated Hep3B xenografted athymic nude mice

	Vehicle	**5**	Reference
ALT	49.6 ± 5.3	46.6 ± 7.2	28–132
AST	78.2 ± 29.6	89.6 ± 20.6	59–246

$N = 5$ for both vehicle control group and **5**-treated group. Enzymatic levels were determined by the IDEXX laboratories machine using its veterinary biochemistry assay kits and expressed as units per liter. Results represent the mean ± standard derivation.

3. Conclusions

In summary, this contribution revealed that the dinuclear gold(I) species containing a fluorenone group **5** was shown to possess a significant biological activity both in vitro and in vivo. This compound is of significance in that it exhibits noticeably potent cytotoxic activity on human cancer cells and provides an additional example of metal arylacetylide complexes with impressive antitumor activities, which are still very scarce. It also highlights the prospect of gold(I) acetylides as another promising lead for anticancer metallodrug development. The generation of ROS from the carbonyl group of the central ligand spacer is believed to be essential for the cytotoxicity of **5**. This is suggested from consideration of structure–activity relationships between compounds tested, and supported by an experiment using catalase. Our findings also provide a platform of organic skeleton for conjugation with other metals to synthesize a variety of organometallic derivatives for other biological applications.

4. Experimental

4.1. General

All reactions were carried out under nitrogen atmosphere with the use of standard Schlenk techniques, but no special precautions were taken to exclude oxygen during work-up. Solvents were predried and distilled from appropriate drying agents. All chemicals, unless otherwise stated, were obtained from commercial sources and used as received. Preparative TLC was performed on 0.7 mm silica plates (Merck Kieselgel 60 GF_{254}) prepared in our laboratory. The starting materials 2,7-bis(trimethylsilylethynyl)fluorene,[41–43] 2,7-diethynylfluorene,[41–43] 2,7-bis(trimethylethynyl)fluoren-9-one,[41] and 2,7-diethynylfluoren-9-one (**1**)[41] were prepared by the literature methods. Infrared spectra were recorded as KBr pellets using a Perkin Elmer Paragon 1000 PC or Nicolet Magna 550 Series II FTIR spectrometer. NMR spectra were measured in appropriate solvents on a JEOL EX270 or a Varian INOVA 400 MHz FT-NMR spectrometer, with ^{1}H NMR chemical shifts quoted relative to the TMS standard. Electron impact (EI) and fast atom bombardment (FAB) mass spectra were recorded on a Finnigan MAT SSQ710 mass spectrometer.

4.2. ICP-MS analysis

The amount of gold uptake was compared among the two samples (**5** and **6**) and the control (vehicle) using ICP-MS [ELAN DRC II ICP-MS (PerkinElmer, Waltman, MA, USA)]. Briefly, after incubating the Hep3B cells with these compounds at 50 μM for 2 h, Hep3B cells were subsequently washed twice with physiological buffered saline and total cellular protein was extracted by lysis buffer. The protein concentration was determined by Bradford's assay. The protein samples were diluted, filtered and injected right away for semi-quantitative analysis of gold.

4.3. Syntheses

The syntheses of compounds followed the procedures reported recently.[33,34]

4.4. Cancer cell culture

The human cancer cells Hep3B (HB-8064), MDA-MB-231 (HTB-26) and SKHep-1 (HTB-52) were obtained from American Type of

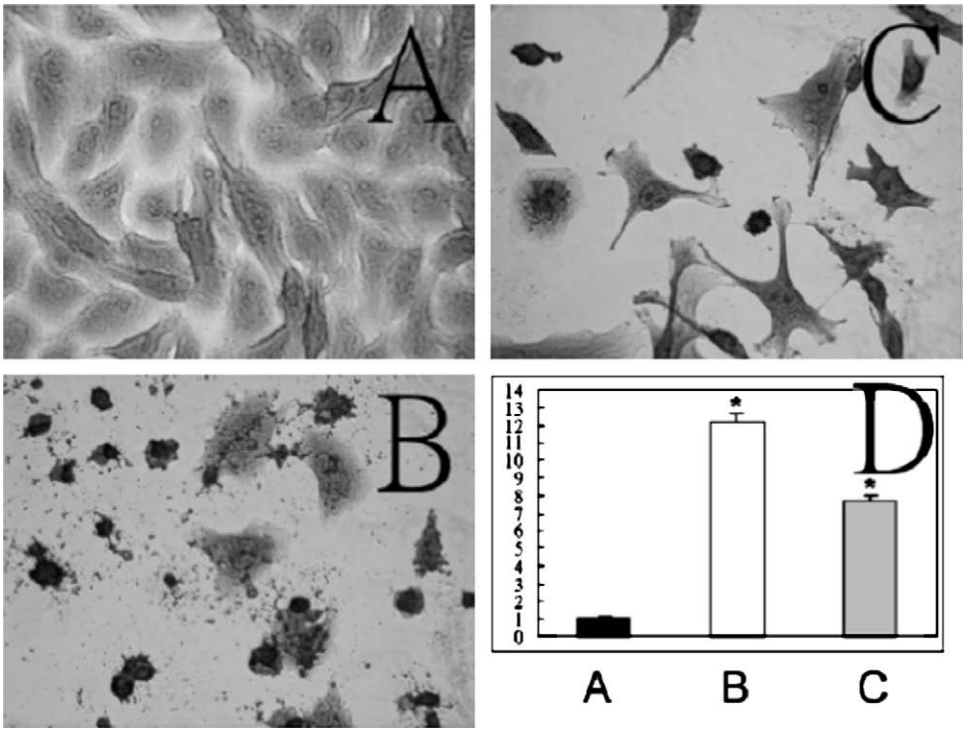


Figure 5. Human liver cancer cells Hep3B were treated with (A) vehicle control, (B) **5** at 10 μM for 24 h, (C) **5** at 10 μM and catalase 1000 units for 24 h and stained with sulforhodamine B. Compound **5** induced significant morphology changes including cell rounding, bubbling of cell membrane and shrinkage. Co-incubation of **5** with catalase could preserve some cancer cells from **5-**induced cell death. (D) Relative folds of generation of ROS as detected by 2′,7′-dichlorofluorescein diacetate from different treatment of Hep3B cancer cells where 'vehicle control' is defined as '1'. Results are shown as mean ± SD from three independent experiments. Asterisk (*) indicates that there is a statistical significance between the considered group with vehicle control (P <0.05).

Culture Collection. They were cultured in RPMI-1640 medium with 5% fetal bovine serum (complete cell culture medium) at 37 °C in 5% CO_2 humidified incubator.

4.5. Sulforhodamine B assay for cell viability

Cancer cells were removed from 75 mL sterile cell culture flasks with trypsin and neutralized with fetal bovine serum. After washing with phosphate buffered saline and centrifugation, cancer cells were re-suspended in complete cell culture medium at a concentration of approximately 1×10^5 cells/mL and counted manually using a haematocytometer under an inverted microscope. Cancer cells seeded in the 96 wells microtitre plates for 24 h were prepared for the screening of the synthesized compounds. The compounds were dissolved in molecular biology grade DMSO at a stock concentration of 50 mM. CDDP was used as the positive reference compound and added at a starting concentration of 50 μM. Compounds were added at a starting concentration of 50 μM followed by a serial of twofold dilutions and incubated with cells for a further period of 48 h. Afterwards, the evaluation of possible anti-proliferative potential of our synthesized compounds was performed by the sulforhodamine B protein staining methods. Briefly, cancer cells were fixed with trichloroacetic acid, washed and stained with sulforhodamine B. Afterwards, cells were washed again with acetic acid and stained cells were dissolved in unbuffered Tris-base. Finally, optical absorptions were measured at 575 nm using a microplate reader (Victor V form Perkin Elmer, Life Sciences). The 50% inhibitory concentrations of compounds and CDDP were calculated from these experimental results.

4.6. Determination of ROS

Hep3B cancer cells were seeded at a concentration of approximately 1×10^5 cells/mL and counted manually using a haematocytometer under an inverted microscope. After 24 h, the culture medium was changed and incubated with compound **5** with or without catalase. After a further incubation of 24 h, the culture medium was changed and cells were incubated with 2′,7′-dichlorofluorescein diacetate (Molecular Probe) for a further of 30 min. Cancer cells were washed and collected. Cells were subsequently lysed and total cellular protein was collected while debris was discarded. Protein content was determined by using the Bradford reagent. The relative level of ROS from each of 20 μg of total protein sample was determined by measuring its fluorescence units at 515 nm after an excitation at 485 nm using a microplate reader.

4.7. Caspases inhibition assay

Hep3B cancer cells were seeded at a concentration of approximately 1×10^5 cells/mL and counted manually using a haematocytometer under an inverted microscope. After 24 h, the culture medium was changed and cells were pre-incubated with pan-caspase inhibitor (zVADfmk from Promega) for 2 h before adding compound **5**. After a further incubation of 24 h, cellular viability was determined by the sulforhodamine B protein staining methods as mentioned above.

4.8. In vivo athymic nude mice experiment

Eight weeks old athymic nude mice, weighing approximately 15–20 g, were purchased from the animal unit of The Chinese University of Hong Kong and maintained in a sterile facility, in accordance with the institutional guidelines on animal care, with the required consistent temperature and relative humidity. All the procedures were approved by the Animal Research Ethics Committee. Ten athymic nude mice were injected subcutaneously with the human hepatocellular carcinoma Hep3B cells. They were housed in a sterile condition. Tumor size was measured by the electronic

calliper daily. When tumor size reached a mean volume of about 200 mm^3 where tumor volume was calculated by the formula (length × width × width)/2, they were randomly divided into two groups. Compound at a concentration of 2.5 mg kg^{-1} body weight/day was administrated intraperitoneally for a continuous period of 9 days starting from day 1. Each group consisted of 5 mice. Body weight of each animal was also recorded. On day 10, mice were sacrificed and H and E staining of autopsy analysis including livers and kidneys from all the 10 animals were investigated for any possible significant toxicology effects including occurrence of any necrotic tissue. Whole blood was also collected and plasma liver enzymes including ALT and AST were measured by the Vet biochemistry assay kits for the IDEXX laboratories machine to determine whether there are any liver failure phenomena from both groups of mice by comparing with the normal control ranges.

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