

Chapter 2

Experimental Section

2.1 Materials and Instrumentation

All the synthesis starting materials were from commercially available resources, and all the solvents were of at least analytical grade. Fast atom bombardment (FAB) mass spectra were acquired on a Finnigan Mat 95 mass spectrometer. Electrospray ionization (ESI) mass spectrometry experiments were performed on a Waters Micromass Q-ToF Premier quadrupole time-of-flight tandem mass spectrometer or on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). ^1H NMR spectra were recorded on DPX 400 M and 300 M Bruker FT-NMR spectrometers with tetramethylsilane as the reference. UV–Vis spectra were obtained on a Perkin-Elmer Lambda 19 UV–Vis spectrophotometer. Steady-state emission spectra were performed on a SPEX 1681 Fluorolog-3 spectrophotometer. Elemental analysis was performed by the Institute of Chemistry at the Chinese Academy of Science, Beijing. For MTT and protein assays, the absorbance was quantified using a Perkin-Elmer Fusion Reader (Packard BioScience Company). Fluorescence images were examined in an Axiovert 200 (Carl Zeiss) and in an Axio Vision Rel. 4.5 imaging system (Carl Zeiss). Dynamic light scattering (DLS) was recorded on a Malvern ZetaSizer 3000HSA. TEM analysis was performed on an FEI Tecnai G2 20 S-TWIN scanning transmission electron microscope. Most of the experimental procedures are based on our previous reports [1, 2].

2.2 X-Ray Crystallography

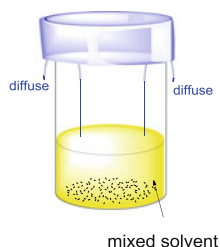
2.2.1 Crystal Growth

There are three common methods to grow single crystals: liquid–liquid diffusion, evaporation of mixed solvents, and vapor–liquid diffusion.

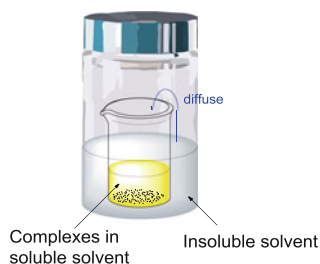
1. **Liquid-liquid diffusion** (a) Dissolve the compound with soluble solvent. (b) Slowly add the insoluble solvent. (c) Seal the bottle with a cap and then a parafilm.
2. **Evaporation of mixed solvent** (a) Dissolve the compounds into a well soluble, volatile solvent which is mixed with less soluble, less volatile solvent. (b) The bottle can be sealed with a cap or a plastic wrap to slow down the evaporation rate so as to obtain larger crystals.
3. **Vapor-liquid diffusion** (a) Place insoluble, volatile solvent (e.g., Et₂O, hexane) into a large bottle. (b) Then place a smaller tube/bottle containing the compounds, dissolved in a soluble, less volatile solvent. (c) Seal the bottle with a cap and then parafilm.



Liquid-liquid diffusion



Evaporation of mixed solvent



Vapor-liquid diffusion

2.2.2 Single Crystal Analysis

Computing program: SHELXS97 [3].

Geometry: All estimated standard deviations (esds) (except the esd in the dihedral angle between two l.s. planes) were estimated using the full covariance matrix. The cell esds are taken into account individually in the estimation of esds in distances, angles, and torsion angles; correlations between esds in cell structure report parameters were only used when they were defined by crystal symmetry. An approximate (isotropic) treatment of cell esds was used for estimating esds involving l.s. planes.

Refinement: Refinement of F^2 against ALL reflections. The weighted R -factor wR and goodness-of-fit S were based on F^2 , and conventional R -factors R were based on F , with F set to zero for negative F^2 . The threshold expression of $F^2 > 2\sigma(F^2)$ was used only for calculating R -factors(gt) and is not relevant to the choice of reflections for refinement. R -factors based on F are statistically about twice as large as those based on F^2 , and R -factors based on ALL data will be even larger.

2.3 Stability Testing

2.3.1 UV-Vis Absorption Measurements

2.3.1.1 Beer-Lambert Law

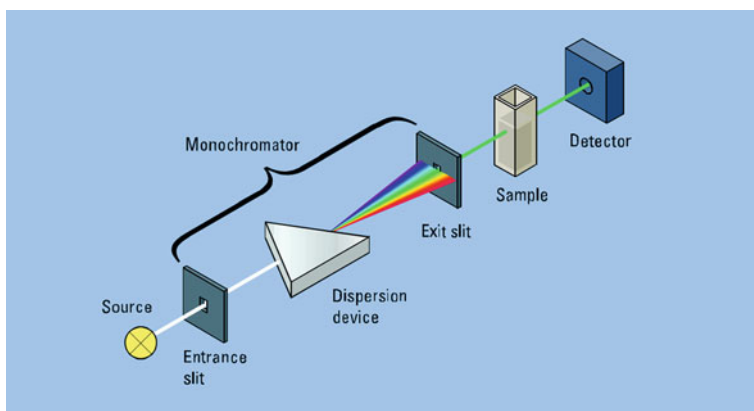
The law states that the absorbance is proportional to the concentration and path length of the sample.

$$A = -\lg T = -\lg \frac{I}{I_0} = \epsilon bc$$

where A is absorbance with no unit; T is Transmittance; I is intensity of the light; ϵ is molar absorptivity in $\text{L mol}^{-1} \text{cm}^{-1}$; b is the path length of the sample in cm; and c is the concentration in mol L^{-1} .

2.3.1.2 Instrumentation

Figure [4] depicts the working principle of the UV-Vis absorption instrument. Full range light goes through the monochromator to get light of a fixed wavelength to go through the sample solution; the unabsorbed light is recorded by the detector.



2.3.2 ESI-MS Measurements

Electrospray ionization mass spectrometry utilizes soft electrospray (where a high voltage is applied) to disperse and ionize samples in solution. The molecular weight of the sample can be quite high since multicharge can be easily produced (the m/z

ranges from 200 to 2000). Similar to UV–Vis absorption, the ion intensity is proportional to concentration in a given range; therefore, ESI-MS can be applied to analyze reactions both qualitatively and quantitatively.

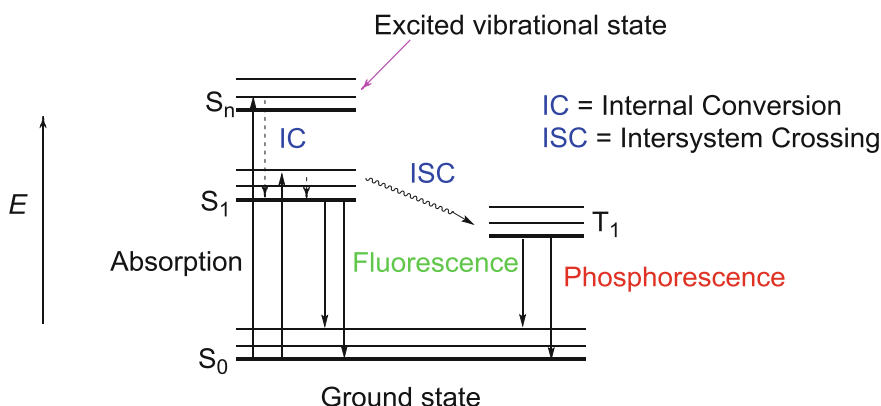
2.3.3 ^1H NMR Measurements

UV–Vis changes can indicate the reactivity of the complexes in solution, and ESI-MS can reveal the possible reaction product(s) qualitatively (though in some cases can be quantitatively). ^1H NMR is a method that can give the reaction product both qualitatively and quantitatively. When preparing the sample reactions to mimic the biological processes, a buffer solution (phosphate buffer) should be applied. For reactions that contain air-sensitive components (e.g., thiol-containing peptides/proteins), it is necessary to degas the sample solutions by bubbling N_2 or Ar gas.

2.4 Emission Measurements

2.4.1 Emission Spectra Measurements

When a complex absorbs a photon, it will go to the high energy level (S_n) and then go to the singlet S_1 state through internal conversion (IC). The excited electron in S_1 can either go to the lower triplet T_1 state through intersystem crossing (ISC), phosphoresce to singlet S_0 state, or go directly back to S_0 giving fluorescence. The following Jablonski energy diagram shows the generation of absorption, fluorescence, and phosphorescence.



To measure the emission quantum yield (the number of photons emitted divided by the number of photons absorbed), the reference compounds $[\text{Ru}(\text{bpy})_3](\text{PF}_6)_2$ (quantum yield of 0.062) together with the following equation were used:

$$\Phi_s = \Phi_r \times \left(\frac{B_r}{B_s} \right) \times \left(\frac{n_s}{n_r} \right)^2 \times \left(\frac{D_s}{D_r} \right)$$

where Φ_s and Φ_r are the quantum yields of the sample and reference complexes, respectively; $B = 1 - 10^{-AL}$ where A and L are the absorbance at the excitation wavelength and length of the optical path (unit cm), respectively; D_s and D_r are the integrated area of emission spectrum of sample and reference compounds, respectively.

Metal complexes usually emit phosphorescence with lifetime in the range of microseconds. Such long-lived excited states are quenched by air (oxygen); thus, the sample solution should be degassed (at least 5 freeze–pump–thaw cycles) before the measurement. Metal complexes can be highly emissive upon binding with biomolecules without degassing the solvents.

2.4.2 Emission Lifetime Measurements

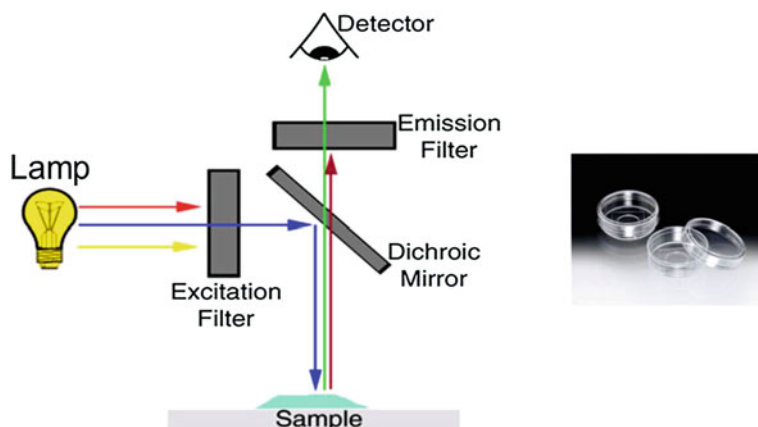
The samples were prepared in the same manner as the samples for emission quantum yield measurement. Once a sample was excited with a pulse laser, the intensity at a fixed wavelength was recorded with time processing. Then, the intensity decay curve was fitted following the first exponential decay function:

$$I_t = I_0 e^{-k(t-t_0)}$$

where I_t and I_0 are the emission intensity at time of t and t_0 ; $k = \frac{1}{t_0}$.

2.5 Fluorescence Microscopy

Fluorescence microscopy takes advantage of the fluorescence or phosphorescence of the compounds to generate images. Cell imaging analysis is quite useful to study the cellular localization and/or biotransformations of the compounds. Figure (left panel) [5] shows the working and basic principles of fluorescence microscopy. Samples are excited by light that passes through the excitation filter from the vertical direction; then, the emitted light goes through a dichroic mirror and emission filter and then detected by the naked eye or a camera. The right panel of the figure shows the glass bottom dishes that were used for cell imaging analysis.



The following table summarizes the emission/excitation filters on the Axiovert 200M used in this thesis.

Name	Excitation filter (nm)	Dichroic mirror (nm)	Emission filter (nm)
New_1	340	–	510
DAPI	365	395	445 \pm 25
New_2	450 \pm 20	–	>580
FITC	470 \pm 20	510	>515
Rhodamine	546/12	580	>590

2.6 Cell Culture and Cytotoxicity Studies

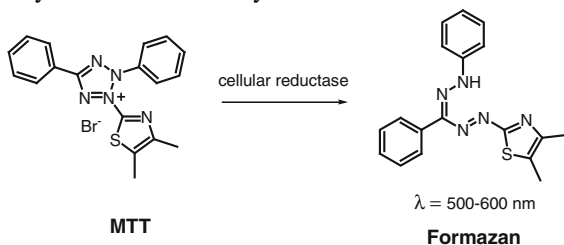
2.6.1 Cell Subculture

Subculture of cells comprises the following steps: (1) removal of the medium; (2) washing the cell monolayer once or twice with PBS; (3) addition of trypsin–EDTA (rotating the dish to cover the entire surface of the dish); (4) incubation of the cells in a 37 °C incubator or at room temperature for 2–10 min; and (5) addition of medium containing FBS to deactivate trypsin. (Centrifuging was generally used to remove all the trypsin.)

2.6.2 MTT Assay

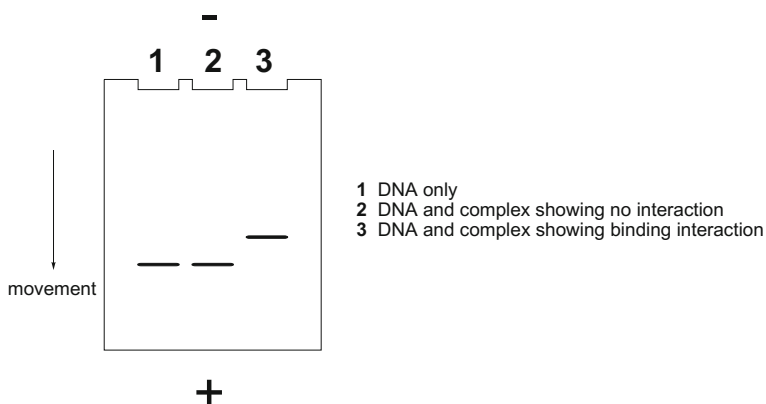
Cytotoxicity was evaluated by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [6]. MTT is a cell permeable agent that can

be reduced by intracellular reductases to form insoluble formazan. The reduced product can be dissolved in solvents, such as DMSO, a solution of sodium dodecyl sulfate (SDS) in diluted HCl or acidified ethanol. Formazan (purple) has a distinguishable absorption band at 500–600 nm that can be quantified by spectrophotometry so that the viability of cells can be determined.



2.7 Gel Mobility Shift Assay

The gel mobility shift assay is a method often used to investigate the interaction of metal complexes with DNA (or other biomolecules). Agarose gels are commonly utilized to resolve DNA fragments. In general, mixtures of DNA and complexes containing bromophenol blue are resolved by 2 % (w/v) agarose gel electrophoresis in Tris–acetate–EDTA (TAE) buffer. After the electrophoresis is finished (the blue dye runs to the end of the gel), the gel is stained with an ethidium bromide (EB) solution, and the fluorescence is visualized by UV transillumination.



2.8 Spectroscopic Binding Studies

2.8.1 Determination of DNA-Binding Constants

DNA-binding constants were determined by absorption titration experiments [7]. The absorption spectra of a series of solutions containing a fixed concentration of complex and varied concentrations of DNA were assessed. The binding constant was determined using the Scatchard equations

$$[\text{DNA}]/\Delta\epsilon_{\text{ap}} = [\text{DNA}]/\Delta\epsilon + 1/(\Delta\epsilon \times K_{\text{b}}),$$

where $\Delta\epsilon_{\text{ap}} = |\epsilon_{\text{A}} - \epsilon_{\text{F}}|$ ($\epsilon_{\text{A}} = A_{\text{obs}}/[\text{complex}]$) and $\Delta\epsilon = |\epsilon_{\text{B}} - \epsilon_{\text{F}}|$ (ϵ_{B} and ϵ_{F} are extinction coefficients of the DNA-bound and DNA-unbound complex, respectively).

2.8.2 Binding with Proteins

The binding of complexes with protein (e.g., BSA) was determined by fluorescence quenching experiments [8]. The emission spectrum of a solution containing a fixed concentration of protein and varied concentrations of complex was recorded at a suitable excitation wavelength. The binding constant was determined using the following equation:

$$\log[(I_0 - I)/I] = \log K + n \log [Q]$$

where I_0 and I are the fluorescence intensities of the initial protein without complex and the protein after adding the complex, respectively; $[Q]$ is complex (quencher) concentration.

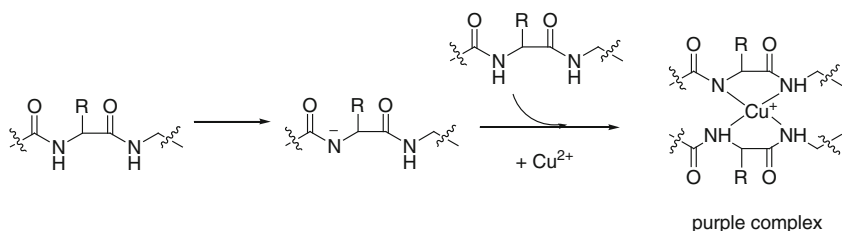
2.9 Transfection

Transfection is a biological technology to introduce cell-impermeant nucleic acids to the intracellular environment. The widely used vehicles to transfect nucleic acids are nanocarriers, including liposomes and cationic polymers. Lipofectamine 2000, (LP2000) developed by Invitrogen, contains a cationic liposome formulation. It can readily form liposomes that are capable of carrying anionic nucleic acids and can recognize anionic cell membranes.

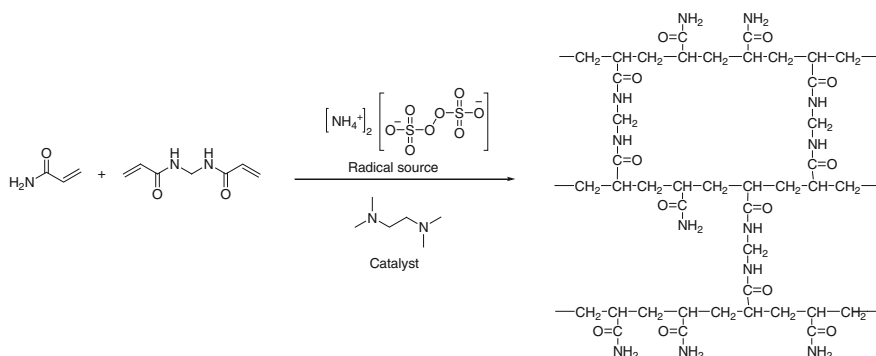
2.10 Western Blot

Western blot is an efficient and standard method to analyze or detect a specific protein based on electrophoretic resolution of proteins. The procedures can generally be divided into the following steps:

1. **Cell lysate from cell culture.** The cells treated with drugs are washed 2–3 times with PBS and treated with RIPA lysis buffer (1 % Triton X-100, 10 % deoxycholate, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % SDS, 0.1 mM phenylmethanesulfonyl fluoride [PMSF, serine protease inhibitor], 10 $\mu\text{g/mL}$ leupeptin [cysteine, serine and threonine protease inhibitor], and 10 $\mu\text{g mL}^{-1}$ aprotinin [trypsin inhibitor]) on ice for 5–10 min. Then, the sample is centrifuged at 13,500 rpm for 10 min, and the supernatant is collected.
2. **Determination of protein concentration.** The protein concentration was quantified by DC Protein Assay (Bio-Rad). The color change is based on the below reaction: The protein reduces Cu^{2+} to Cu^+ that can be reoxidized by Mo^{VI} in Folin reagent, forming molybdenum blue. The protein concentration can then be measured by the absorbance at 595 nm.

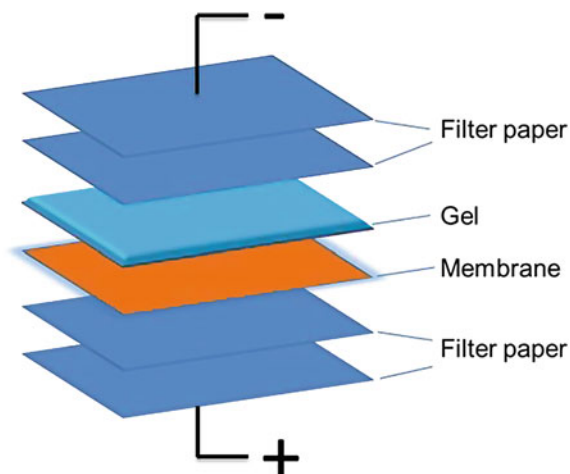


3. **Preparing samples for loading into gels.** Loading buffer (2 % SDS, 10 % 2-mercaptoethanol, 10 % glycerol, 0.002 % bromophenol blue in 62.5 mM Tris-HCl) is added to protein lysate which is incubated at 100 °C for 5 min. After cooling, the samples are centrifuged.
4. **PAGE gels.** SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used to resolve the proteins. The gel is based on the polymerization reaction



where ammonium persulphate (APs) is the radical source, and the reaction is catalyzed by N,N,N',N'-Tetramethylethylenediamine (TEMED). The separating gel is prepared in Tris, pH 8.8, whereas the stacking gel is prepared in Tris, pH 6.8.

5. **Sample loading and gel running.** The sample (20–40 μg) was loaded by special pipette tips that have a long sharp head. The sample volume was smaller than that of the well. After loading, the gels were run at a low voltage for ~ 15 min to condense the protein and then at a high voltage to condense the protein separation. A protein marker was used to estimate the size of the target protein bands.
6. **Protein transfer to membrane.** The protein on gel is transferred to polyvinylidene difluoride (PVDF) membrane by a sandwich of (–)/filter paper \times 2/gel/membrane/filter paper \times 2/(+). The membranes were activated in 100 % methanol for 1–2 min before the transferring.



7. **Block the membrane.** The membrane needs to be blocked to prevent the non-specific binding interactions of antibodies with the membrane. BSA (5 % w/v) in TBST was used to block the membranes overnight at 4 °C.
8. **Incubation with primary antibodies.** Primary antibodies were diluted in the blocking buffer (usually 1:1000 v/v) and then incubated with the membranes at room temperature for 1–2 h with gentle shaking.
9. **Incubation with secondary antibodies.** Before adding secondary antibodies, the membranes were washed at least three times in TBST (each for 10 min) to remove excess primary antibody. The secondary antibodies were diluted by 1:5000 (v/v) and incubated with the membranes at room temperature for 1–2 h with gentle shaking.
10. **Development by chemiluminescence.** Extra secondary antibodies were washed out by TBST (three times, each for 10 min). Then, the membranes were incubated with the chemiluminescence agent which can give luminescence only in the presence of secondary antibody (conjugated to horseradish peroxidase [HRP]). The signal was detected by photographic film.

2.11 Inductively Coupled Plasma Mass Spectrometry

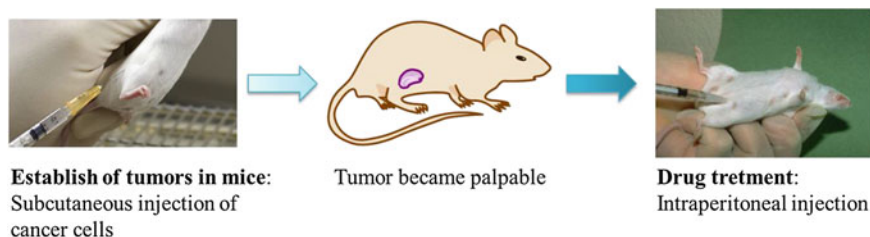
Inductively coupled plasma mass spectrometry (ICP-MS) is one kind of mass spectrometry for detecting metals (and some nonmetals) at ultralow concentrations (below ppb level). It is a powerful method that is particularly suitable for the measurement of cellular uptake and protein-bound metal contents. For ICP-MS analysis, the samples were fully digested before the assay. Nitric acid is usually used for digestion together with heating.

2.12 Tube Formation Assay

Tube formation was followed by an In Vitro Angiogenesis Kit (Cayman Chemical). Briefly, the diluent buffer (10×) was mixed with ECMatrix solution at 0 °C. Then, the mixture was transferred to a 96-well plate (each sample containing 50 µL) and was incubated at 37 °C for 1 h to promote polymerization. Afterward, MS1 cells (5×10^4) in DMEM containing drugs/complexes are added to the top of the polymerized matrix and incubated for a given time, until the tubes in the control growth were formed. The cell viability was verified to ascertain all the drug-treated cells remained alive.

2.13 In Vivo Antitumor Study

The animals were cared for according to the guidelines of the Laboratory Animal Unit of the University of Hong Kong (HKU). All animal experiments were conducted under the guidelines approved by the Committee on the Use of Live Animals in Teaching and Research of HKU. The following figure is a summary of the key steps for the in vivo antitumor experiments.



Drug administration and preliminary toxicity studies. Drug administration methods were intraperitoneal injection (i.p.), intratumoral injection (i.t.), intravenous injection (i.v.), and subcutaneous injection (s.c.), and this method sometimes cannot be distinguished from i.t. if the tumor was planted by s.c. Preference was given to i.p. due to the ease of administration compared to the other systemic injection methods (e.g., i.v). I.t. is a kind of local injection method that is not commonly used.

The drugs/complexes were first dissolved in the excipient (most commonly PET with 60 % polyethylene glycol 400, 30 % ethanol, and 10 Tween-80) and then diluted with PBS (if using DMSO as excipient, it should be below 2 % and PET could be 10 %). The treatment dosage/frequency was carefully considered. The injection volume needed to be lower than 0.5 mL (usually 0.1–0.2 mL was used). Tumor-free mice were used to test the drug toxicity in order to determine the drug treatment dosage.

Establishment of tumors models. A sufficient number of cancer cells were prepared before the establishment of the tumors in mice. For each mouse, around $2-5 \times 10^6$ cells. After harvesting all the cancer cells, the medium and trypsin were removed by centrifugation, and the cells were immersed in PBS at 0 °C before injection. Then, the cancer cells in 0.1 mL PBS were injected (s.c.) into the right back flanks of the mice.

Drug treatment. Once the tumors became palpable (volumes $\sim 50 \text{ mm}^3$, 2–3 days after tumor inoculation), the mice were randomly divided into different treatment groups (5–7 mice for each group). The mice bearing tumors were injected with drug or vehicle control at the dosages/frequencies derived from the preliminary toxicity studies. The longest (*a*) and shortest (*b*) diameters were used to determine

the tumor volumes in the formula $V = 0.52 \times ab^2$. The body weight of the mice was recorded to track side effects.

Sacrifice of mice bearing tumors. According to the guidelines of HKU, the tumor volume must remain lower than 10 % of the body volume. After the tumors exceeded these limits, the mice were sacrificed by lethal anesthesia. The tumors were then collected and immersed in 10 % formalin (a dilution of ~40 % formaldehyde stock) in PBS. After 12 h, the tumors were resuspended in 70 % ethanol and stored at 4 °C.

Tumor growth inhibition was calculated as a ratio of tumor volume increases in the drug-treated group to that in the control group:

$$\text{Inhibition} = \left(1 - \frac{V_t - V_0}{V'_t - V'_0} \right) \times 100 \%$$

where V_t and V'_t are the tumor volumes of drug treatment and solvent control group, respectively. V_0 and V'_0 are the tumor volumes at day 0 of treatment in the drug treatment and solvent control groups, respectively.

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Reductase Inhibitors and Endoplasmic Reticulum
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