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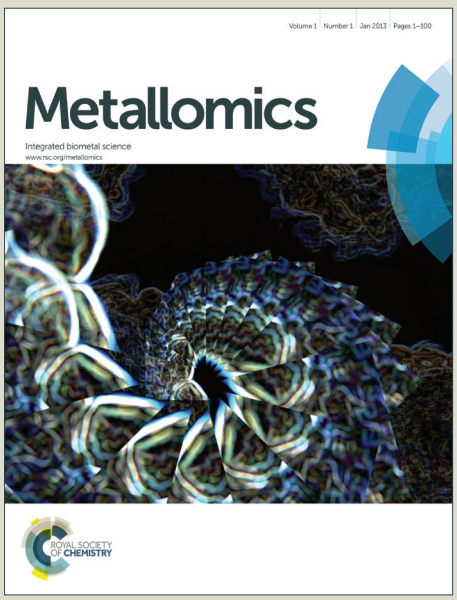

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Ruthenium polypyridyl complexes as inducer of ROS-mediated apoptosis in cancer cells by targeting thioredoxin reductase

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TrxR is a NADPH-dependent selenoenzyme upregulated in a number of cancers. It plays a pivotal role in cancer progression and represents an increasingly attractive target for anticancer drugs. The limitations of cisplatin in cancer treatment have motivated the extensive investigation to other metal complexes, especially ruthenium (Ru) complexes. In this study, we presented the *in vitro* biological evaluation of four Ru(II) polypridyl complexes with diimine ligands, namely, $[Ru(bpy)_3]^{2+}$ (**1**), $[Ru(phen)_3]^{2+}$ (**2**) , $[Ru(ip)_3]^{2+}$ (**3**), $[Ru(pip)_3]^{2+}$ (**4**) (bpy=2, 2'-bipyridine, phen=1,10-phenanthroline, ip=imidazole[4,5-f][1,10]phenanthroline, pip= 2-phenylimidazo[4,5-f][1,10]phenanthroline), and demonstrated that they exhibited antiproliferative activities against A375 human melanoma cells through inhibition of TrxR. As the planarity of the structure increases, their TrxR-inhibitory effects and *in vitro* anticancer activities were enhanced. Among them, complex **4** exhibited higher antiproliferative activity than cisplatin, and the TrxR-inhibitory potency of **4** was more effective than auranofin, a positive TrxR inhibitor. Complex **4** suppressed the cancer cell growth through induction of apoptosis as evidenced by accumulation of sub-G1 cell population, DNA fragmentation and nuclear condensation. Moreover, complex **4** was able to localize in mitochondria and therein induced ROS-dependent apoptosis by inhibition of TrxR activity. Activation of MAPKs, AKT, DNA damage-mediated p53 phosphorylation and inhibition of VEGFR signaling were also triggered in cells exposed to complex **4**. On the basis of this evidence, we suggest that Ru polypyridyl complexes could be developed as TrxR-targeted agents that demonstrate application potentials for treatments of cancers.

INTRODUCITON

With the improvement of people's living condition, cancer is a major public health problem in many parts of the world.[1] According to statistics, one in 4 deaths in the United State is due to cancer.[2] So it is urgent to seek strategy for the treatment of cancer. It is well cited that thioredoxin (Trx) system that consists of Trx, thioredoxin reductase (TrxR) and NADPH plays a vital role in regulating the intracellular signaling pathways and redox balance, making a new strategy for cancer treatment.[3] Some studies have found that thioredoxin system supports several processes crucial for antioxidant defense, cell function, redox-regulated signaling cascades and cell proliferation.[4-5] At the same time, TrxR is homodimeric selenoenzyme and necessary for activation and reduction of Trx, it catalyzes the NADPH-dependent reduction of Trx and several other oxidized cellular components.[6] What is more, TrxR is highly upregulated in a number of cancers, it can not only activate oxidized Trx to scavenge reactive oxygen species (ROS) but also play regulatory roles in a variety of cellular function through protein-protein interaction.[7] Besides, TrxR is characterized by broad substrate specificity and by easily accessible redox centers which are made up of cysteine-selenocysteine redox pair.[8] In the meanwhile, metal complexes could offer the facile construction of 3D architectures that tightly fill enzyme active sites, increasing selectivity and the possibility of facile coordination to protein residues that enhances enzyme inhibition.[9] Notably, the active site selenolate group which is reduced manifests a large tendency to react with "soft" metal ions, making TrxR a likely pharmacological target for a range of metallodrugs.[10-11]

As mentioned above, TrxR displays various cellular functions, thus it is not surprised that more and more research teams have been engaged in searching for TrxR inhibitors for cancer therapy. Previous studies have showed that, a lot of

therapeutically used drugs could inhibit TrxR in cancer cells.[3, 12] Aurothioglucose and auranofin are two gold compounds predominantly used in the treatment of rheumatoid arthritis, but studies have found that they could potently inhibit TrxR in a low dose and displayed no adverse side effects.[13-15] Meanwhile, motexafin gadolinium, a drug that has already been tested in phase Ⅲ clinical trials, could also inhibit TrxR to enhance cell apoptosis.[16-17] Moreover, cisplatin, one of the first therapeutically used anticancer drugs, was found to interact with the TrxR system and was identified as an efficient irreversible inhibitor of TrxR.[18-19] Besides the therapeutically used drugs, plenty of mammalian TrxR inhibitors have been discovered with anticancer activities, such as natural products, nonmetallic and metallic compounds.[12] Till now, many agents have been found to exhibit TrxR-inhibitory activities, such as 4-hydroxy-2-nonenal, flavonoids, curcumin and its analogs, quinines as well as isothiocyanates.[20-26] The nonmetallic TrxR inhibitors include anticancer alkylation agents, such as nitrosourea, nitrogen mustard, dinitrohalobenzene,[27-29] while the metallic compounds were mainly composed of gold compounds and platinum complexes.[9, 28, 30-38] For instance, studies have showed that Au(I) *N*-heterocyclic carbene complexes could selectively inhibit TrxR activity through binding of Au(I) to the C-terminal redox active center of TrxR.[39-43] Auranofin, a gold phosphine complex used in the treatment of rheumatoid arthritis, was also identified as a potent TrxR inhibitor by altering the intracellular redox status.[34, 44,45]

Ruthenium(Ru) complexes have emerged as leading players among the potential metal-based candidates for cancer treatments.[46] At present, two Ru(III) complexes, $[H_2im][trans\text{-}Ru(III)Cl_4(dmso\text{-}S)(Him)]$ (NAMI-A; Him=imidazole, dmso=dimethyl sulfoxide) and $[H_2ind][trans\text{-}Ru(III)Cl_4(Hind)_2]$ (KP1019; Hind = indazole) have entered clinical trials.[10] Many studies have demonstrated that, Ru complexes exerted their antiproliferative activities primarily through ligand exchange to interacting specifically with the classical target DNA and bind to different cellular proteins to induce cancer cell death.[45, 47] DNA-targeting therapy is based on the fact that malignant cells divide rapidly. A drawback of this strategy is that rapidly dividing healthy cells are affected as well, causing severe toxic side effects. This problem has prompted chemists to develop alternative strategies based on different targets. However, exceptions also existed with the examples of coordinatively saturated and substitutionally inert polypyridyl Ru(II) complexes which could specifically target mitochondria.[48] Moreover, many studies found that Ru complexes could inhibit TrxR activity owing to the appreciable "soft" character of Ru center. For instance, Mura et al demonstrated the inhibitory effects of Ru complexes on activity of rat cytosolic TrxR for the first time,[10] Casini et al reported a series of Ru(II)-arene compounds as inhibitors of TrxR,[49] Oehninger et al evaluated arene Ru(II) *N*-heterocyclic carbene complexes as organometallics interacting with TrxR.[50] Thus, the use of Ru complexes is a good choice for inhibition of TrxR. Furthermore, the above evidences suggest that inert polypyridyl Ru(II) complexes may exhibit their anticancer actions with the involvement of mitochondrial TrxR inhibition. Inspired by these findings, we decided to thoroughly investigate the *in vitro* anticancer mechanisms of Ru(II) polypridyl complexes with diimine ligands, namely, $[Ru(bpy)_3]^{2+}$ (**1**), $[Ru(phen)_3]^{2+}$ (**2**) , $[Ru(ip)_3]^{2+}$ (**3**), $[Ru(pip)_3]^{2+}$ (**4**) (bpy=2,2'-bipyridine, phen=1,10-phenanthroline, ip=imidazole[4,5-f][1,10]phenanthroline, pip=2-phenylimidazo[4,5-f][1,10]phenanthroline), and examined their interaction with TrxR. The results showed that, Ru complexes were able to anchor mitochondria and therein induce ROS-dependent apoptosis by inhibition of TrxR activity. Activation of MAPKs, AKT, DNA damage-mediated p53 phosphorylation and inhibition of VEGFR signaling were also triggered in cells exposed to Ru complexes. On the basis of this evidence, we suggest that Ru polypyridyl complexes could be developed as TrxR-targeted agents that demonstrate application potentials for treatments of cancers.

EXPERIMENTAL SECTIONS

General. In all experiments, organic solvents were analytical grade unless otherwise stated. $RuC1_3\ 3H_2O$, $NaClO_4$, cisplatin and ligands 2, 2'-bipyridine (bpy), 1,10-phenanthroline (phen), imidazole[4,5-f][1,10]phenanthroline (ip), 2-phenylimidazo[4,5-f][1,10]phenanthroline (pip) were purchased commercially and used without further purification. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dihydroethidium (DHE), 2',7'-dichlorofluorescin (DCF) and BCA assay kit were purchased from Sigma-Aldrich. Thioredoxin reductase (TrxR) activity kit was bought from Cayman Chemical. All of the antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA, USA). Mammalian genomic DNA extraction kit was purchased from Beyotime. Glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rs) activities assay kits were purchased from Beyotime Institute of Biotechnology. All aqueous solutions were prepared with doubly distilled water.

Elemental analysis were obtained on a EA2400II CHNS/O elemental analysis (USA), mass spectra were obtained on a ABI4000 Q TRAP liquid chromatography-mass spectrometer (ABI, USA).

Synthesis and characterization of the Ru(II) complexes. The complexes $Ru(diimine)_3(ClO_4)_2$ were prepared by literature methods by slight changes and got the same results as the reported literature.[51-54]

Cell culture and Cell viability assay. Melanoma cells (A375), breast adenocarcinoma cells (MCF-7), rat pheochromocytoma cells (PC-12), hepatocarcinoma cells (HepG-2), pulmonary carcinoma cells (A549) and human normal proximal tubular cells (HK-2) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM supplemented medium with fetal bovine serum(10%), penicillin (100 units/mL), and streptomycin (50 units/mL) at 37 ℃ in a humidified atmosphere of 5% CO_2 in a CO_2 incubator. The cell viability was determined by MTT assay which was carried out as described previously.[55]

Determination of thioredoxin reductase (TrxR), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rs) activities in A375 cells. To determine the activities of TrxR after different treatment in A375 cells, we used a Cayman's thioredoxin reductase assay kit as reported,[41] while the activities of GSH-Px and GSH-Rs after treatment with complex **4** were determined by the specific kits purchased from Beyotime Institute of Biotechnology as previous studied.[56]

Determination the inhibitory activities of complexes 1-4 on TrxR in A375 cell lysates. The inhibitory activities of complexes **1-4** on TrxR in A375 cell lysates were measured using a Cayman's thioredoxin reductase assay kit as our previous studies.[56-57] The inhibition of TrxR in A375 cell lysates by Ru(II) complexes was measured at 412 nm and expressed as a percentage of control.

Measurement of lipophilicity (lipo-hydro partition coefficient). The lipophilicity of complexes **1-4** was determined by using the "shake-flask" method previously reported.[48] The content of the Ru(II) complex in each phase was determined by ICP-AES analysis. Log*P* was calculated as the logarithmic ratio of the concentrations of the Ru(II) complex in the n-octanol phases and aqueous phases.

Interaction between Ru complexes and selenopeptide. The changes in the spectra of complex **4** after addition of selenopeptide (AGUVGAGLIK) were detected as previously reported.[58-59] Selenopeptide (20 μM) was added to the PBS solution (pH 7.4) of complex **4** (20 μM) and incubated for 12 h at 37 ℃.

Mitochondrial assay and DAPI staining. The intracellular localization of complex **4** in A357 cells was traced with the Mitotracker Green staining. Briefly, the cells were seeded in 2 cm glass vessels and cultured for 24 h. After additional culture with or without complex **4** for 5 h, the cells were stained with Mitotracker Green solution (finally concentration, 100 nM) and 1 μg/mL DAPI for 2 h and 30 min, respectively. After washing with PBS twice, the cells were examined under a fluorescence microscope.

Flow cytometric analysis. In this study, we used flow cytometry to analysis the cell cycle distribution of complex **4** as previously described.[60] Cell cycle distribution was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in the cell cycle pattern. For each experiment, 10,000 events per sample were recorded.

Fluorometric measurement of caspase activity. Caspase activity in A375 cells of complex **4** was determined by using Caspase activity Kit (BD Biosciences) as previously described.[61] The specific caspase substrates include caspase-3, caspase-8 and caspase-9. Relative caspase activity was expressed as percentage of control (as 100%).

Measurement of ROS generation. The intracellular ROS generation levels in A375 cells by complex **4** were measured by DHE and DCF-DA assay as reported.[56, 62] ROS generation was measured by the fluorescence intensity on a Tecan SAFIRE fluorescence reader, the excitation and emission wavelengths were 300 and 610 nm for DHE, 488 and 525 nm for DCF-DA, respectively. Relative DHE and DCF fluorescence intensity of treated cells was expressed as percentage of control (as 100%).

DHE staining was carried out as follow. The cells were seeded in 2 cm glass vessels and cultured for 24 h. After additional culture with or without complex **4** for 2 h, the cells were stained with DHE solution (finally concentration, 100 nM) for 20 min. After washing with PBS twice, the cells were examined under a fluorescence microscope.

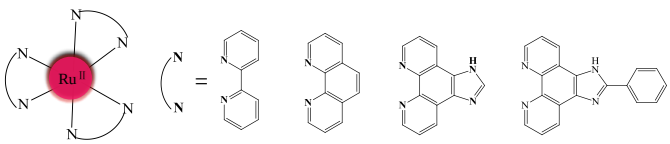
Protective effects of GSH or NAC. The cells were pretreated with 2 mM GSH or 1 mM NAC for 2 h prior to the addition of complex **4**. The protective effects of GSH or NAC on cell death was then examined by MTT assay.

Western blot analysis. To examine the expression levels of proteins which were related to different signaling pathways after treatment with complex **4**, we use Western blot analysis as our previous studies.[61]

Statistical analysis. All the data were expressed as mean ± standard deviation (SD). Differences between two groups were analyzed by two-tailed Student's test. One-way analysis of variance (ANOVA) was used in multiple group comparisons. These analyses were carried out by SPSS 12.0. Difference with $P < 0.05$ (*) or $P < 0.01$ (**) was considered statistically significant.

RESULTS AND DISCUSSION

Synthesis and cytotoxic effects of Ru(II) polypyridyl complexes. In the present study, tris(diimine) Ru(II) complexes **1-4**, namely, $[Ru(bpy)_3]^{2+}$ (**1**), $[Ru(phen)_3]^{2+}$ (**2**), $[Ru(ip)_3]^{2+}$ (**3**), $[Ru(pip)_3]^{2+}$ (**4**), were prepared as previously reported with slightly modification and got the same results (**Scheme 1**).[51-54] They have been characterized by elemental analysis and ESI-MS (**Table S1**). Among these complexes, we increased the plane of their chemical structures to examine the effects on their anticancer and TrxR-inhibitory activities.



Scheme 1. Structures of ruthenium polypyridyl complexes studied in this work.

Table 1. Cytotoxic effects on human cancer and normal cell lines (IC_{50}), TrxR-inhibitory activities in A375 cells (EC_{50}) and lipophilicity (log*P*) of ruthenium(II) polypyridyl complexes **1-4**.

Complexes	IC_{50} (μM)						EC_{50} (μM)	
	A375	MCF-7	PC-12	HepG-2	A549	HK-2	A375	log*P*
1	183.1	341.5	>400	388.4	179.4	—	>100	-0.41
2	153.9	268.0	257.1	>400	157.2	—	40.2	-0.33
3	137.9	91.5	16.9	77.1	177.3	72.4	20.7	0.42
4	0.9	66.0	53.0	225.8	>400	212.7	2.7	2.67
Cisplatin	7.3	29.7	32.3	13.6	18.8	10.3	—	—

When we evaluate the usefulness of a agent as a pharmacological drug, the balance between the therapeutic potential and toxic side effect of a compound should be seriously considered.[46] The triggering of cytotoxic effects by the Ru(II) polypyridyl complexes were investigated in various tumor cell lines, namely, A375 melanoma cells, MCF-7 breast adenocarcinoma cells, PC-12 rat pheochromocytoma cells, HepG-2 hepatocarcinoma cells and A549 pulmonary carcinoma cells. As a control, the toxicity of the complexes was also tested toward HK-2 human normal proximal tubular cells. As shown in **Table 1**, complexes **1** and **2** showed only slight effect on the tested cancer cells, while complex **3** was more effective than complexes **1** and **2** and lower cytotoxicity on normal cells than cisplatin. Notably, **4** was found to be the most active one toward cancer cells, and less toxic against HK-2 normal cells. The different sensitivity of the tested cancer cells to the synthetic Ru complexes should be due to their diversified cellular protein expression profiles. Among them, A375 melanoma cells exhibited the highest sensitivity to the complexes. Therefore, this cell line was used for further investigation on the underlying mechanisms accounting for their anticancer actions.

The lipophilicity of a compound is well known to have a strong influence on its toxicity.[41-42] Therefore, the lipophilicity (log*P*) of the synthetic complexes was tested. As shown in **Table 1**, with the increase in the plane of the chemical structures of the ligands, the lipophilicity of the corresponding complexes was enhanced. The lipophilicity and the anticancer efficacy of the complexes were positively correlated in A375 cells. The increase in the lipophilicity enhanced their cellular uptake, and thus improved their anticancer efficacy (**Figure S1**). For instance, complex **4** exhibited higher lipophilicity (log*P*=2.67) and lower IC_{50} value (0.9 μM) in A375 cells. The above findings indicate that, the increase in planarity of the ligands could effectively enhance the anticancer action of Ru complexes.

Ru(II) complexes inhibit cancer cell growth by targeting mitochondrial TrxR. TrxR together with Trx and NADPH constitutes a critical system for maintaining the cellular redox state, making it an attractive target for antitumor drug development.[12] Moreover, it was reported that complexes could trigger cell death through the inhibition of TrxR, thus suppressed the cancer cell progression.[41] Although polypyridyl Ru(II) complexes were less "soft" than the gold(I) or platinum(II) centers, they might still manifest an appreciable affinity for the selenol groups of the TrxR active site.[49] Inspired by these findings, we decided to investigate whether the synthetic Ru(II) complexes could act as a mammalian TrxR inhibitor. As shown in **Table 1**, complexes **1-4** showed differential inhibition on TrxR activities in A375 cells. Their TrxR-inhibitory activities enhanced with the increase of the planarity of the ligands. Another striking result we found was that, the inhibition of the complexes on TrxR was positively correlated with their cytotoxicity against A375 cell lines. These results suggest that the planarity of the structure of the ligands exhibits a positive effect on the lipophilicity and anticancer actions of the Ru complexes. What is more, TrxR system could be the target of the synthetic Ru complexes against A375 cancer cells.

Moreover, the TrxR-inhibitory activities of the complexes were also examined in cells-based system by comparing with auranofin (AF), a gold phosphine complex widely used as positive TrxR inhibitor.[57] As shown in **Figure 1A**, under the same concentrations, the synthetic complexes exhibited differential inhibitory effects on TrxR. Especially, complex **4** was more effective than the positive control auranofin. As glutathione peroxidase (GSH-Px) is also one of the main intracellular selenoenzymes that could keep the cell redox balance,[30] the activity of GSH-Px in A375 cells exposed to complex **4** was also examined. The intracellular GSH-Px activity was not distinctly affected by complex **4** (**Figure S2A**). At the same time, the activity of glutathione reductase (GSH-Rs) which is the structurally and functionally similar to TrxR[58] was also examined in A375 cells exposed to **4**. The intracellular GSH-Rs activity showed no significant difference after treatment with complex 4 **(Figure S2B)**. These results suggest that complex **4** displays selectivity among TrxR, GSH-Px and GSH-Rs.

Moreover, we also examined the expression levels of Trx and TrxR in A375 cells in response to complex **4** by Western blotting. As shown in **Figure 1B**, treatment with **4** down-regulated the expression levels of Trx and TrxR in A375 cells. This result further confirmed the potency of Ru complexes as TrxR inhibitor. Furthermore, we also examined the inhibitory kinetics of the complexes on TrxR by adding the complexes to A375 cell lysates. As shown in **Figure 2**, treatment with complexes **1-4** resulted in dose- and time-dependent reduction in TrxR activities, with complex **4** exhibited the most efficient inhibition.

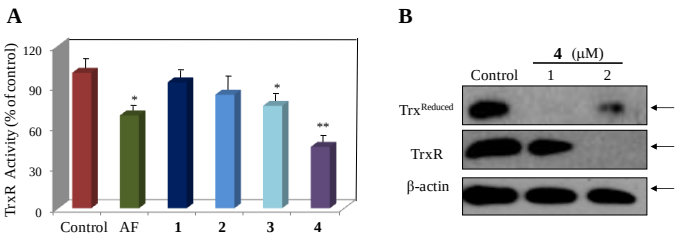


Figure 1. Ruthenium(II) complexes suppress the cancer cell progression by inhibiting TrxR. (A) TrxR activities in A375 cells after treatment with AF and complexes **1-4**, the final concentration of different treatments is 4 μM. Significant difference between treatment and control groups is indicated at $P < 0.05$ (*) or $P < 0.01$ (**) levels. (B) Western blot analysis of the expression levels of TrxR and redox thioredoxin in A375 cells after treatment with complex **4**. Equal loading was confirmed by analysis of β-actin in the protein extracts. All results shown here are representative of three independent experiments with similar results.

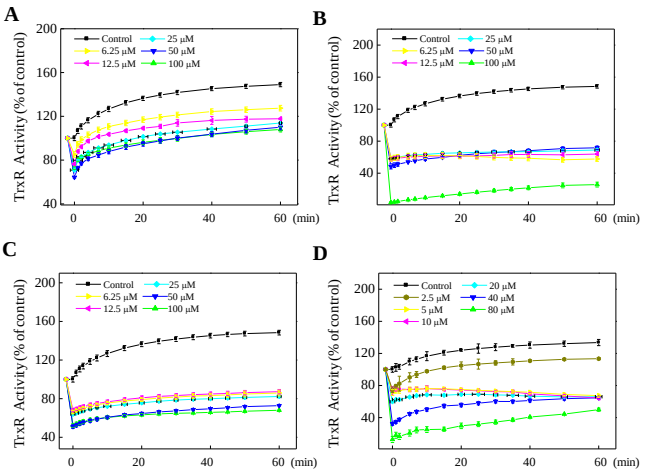


Figure 2. Inhibitory activities of complexes 1-4 on TrxR in A375 cell lysates. (A) **1**, (B) **2**, (C) **3**, (D) **4**.

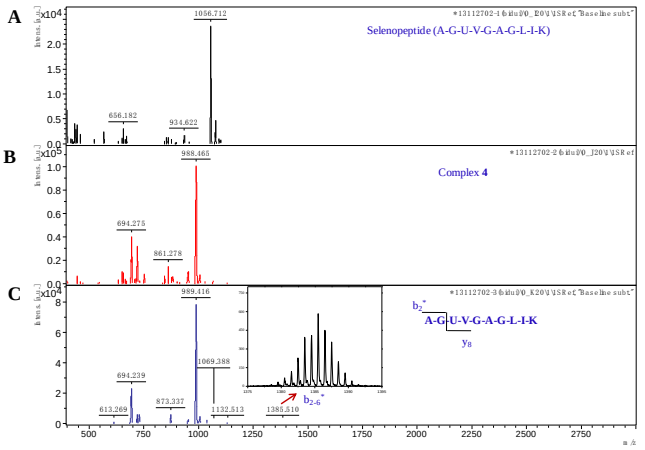


Figure 3. Interaction of the ruthenium(II) complexes with TrxR. MALDI-TOF-MS spectrum of the (A) Selenopeptide. (B) Complex **4**. (C) Complex **4** and selenopeptide. Molecular ion at m/z 1385 formed by ionization of a 1:1 mixture of complex **4** with the selenopeptide fragment H-UVGA-OH after 12 h of incubation at 37 ℃. Signal groups marked with an asterisk (*) stem from species that contain complex **4**.

Based on the strong inhibition of TrxR by the synthetic Ru complexes, mass spectrometry was used to identify their possible interactions. According to the previous study, we used the selenocysteine-containing model peptide Ala-Gly-Sec-Val-Gly-Ala-Gly-Leu-Ile-Lys (AGUVGAGLIK) to check for the binding of the complexes to selenocysteine present in the active site of TrxR.[58] As shown in **Figure 3**, after incubation of the selenopeptide with complex **4** for 12 h, the signals of the selenopeptide completely vanished, as accompanied by the formation of a new molecular ions at m/z 1385, which corresponds to complex **4** attached to the selenopeptide fragment (H-UVGA-OH), which suggests that Ru complexes might form a covalent adduct with the nascent selenol of the active site in the peptide. To confirm this hypothesis, we added the selenopeptide to complex **4** under physiological conditions (at 37 ℃ in PBS buffer) to check the photophysical changes of complex **4**, which led to a new absorption band centered at 497 nm and the bands of complex **4** disappeared concomitantly (**Figure S3A**). Furthermore, no fluorescence was found, when **4** was incubated with selenopeptide (**Figure S3B**). These results demonstrate that complex **4** interacts with TrxR to suppress the cancer cell progression.

From the previous study, we proposed that the interactions of complex **4** with TrxR may occur in several steps. Firstly, complex **4** inhibited TrxR directly and produced modified TrxR, triggering the inactivation of TrxR. This step would have an effect on the protein expression of TrxR and Trx. Secondly, modification of TrxR resulted in enhanced ROS accumulation, which could attack the biomolecules inside the cancer cells. Finally, the complex **4** formed conjugate with protein selenothiol. In the results of Western blotting, we found that, the expression levels of Trx and TrxR in A375 cells in response to complex **4** were down-regulated, which was consistent with previous study showing that the inhibition of TrxR activity and its protein expression were related.[23, 63] Taken together, complex **4** exhibited strong inhibition on TrxR by targeting selenothiol at the C-terminus of TrxR.

TrxR is abundant in mitochondria as well as in cytoplasm in higher eukaryotic cells. Mitochondrial TrxR functions to protect mitochondria from oxidative stress, where reactive oxidative species are mainly generated. In addition, cytosolic TrxR acts to maintain redox balance of cytosol.[64] To further characterize the effects of Ru complexes on TrxR system, confocal microscopy was employed to examine cellular localization of complex **4** in the A375 cells. Moreover, Pierroz et al have synthesized a series of Ru(II) polypyridyl complexes which could target the mitochondria of cancer cells and induce mitochondrial mediated apoptosis,[48] Jin-Quan Wang et al have found mitochondria were the primary target in the induction of apoptosis by chiral ruthenium(II) polypyridyl complexes in cancer cells.[65] Thus, Mitotracker Green was used to probe mitochondria, while DAPI was used to image cell nucleus. As showed in **Figure 4A**, the well merge of green and red fluorescence indicated the localization of complex **4** in mitochondria. These results suggest that mitochondria could be the intracellular targeting organell of Ru complexes. Mitochondria are pivotal in controlling cell growth and death. It could not only disrupt electron transport, triphosphate production and cellular redox potential, but also release apoptogenic factors into cytosol to activate downstream caspase family proteases.[66] Therefore, in this study, we also examined the effects of complex **4** on the status of mitochondria. As shown in **Figure 4B**, the mitochondrial network in the healthy A375 cells was extensively interconnected and appeared filamentous extended throughout the cytoplasm. However, in response to treatments of complex **4**, large-scale mitochondrial fragmentation and release of mitochondrial contents into cytosol were observed in a dose-dependent manner. There results demonstrate the involvement of mitochondria in the anticancer action of Ru complexes.

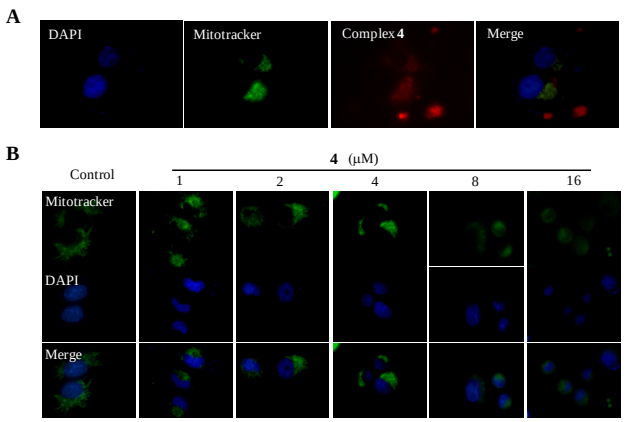


Figure 4. Ru(II) complexes inhibit cancer cell growth by targeting mitochondrial TrxR. (A) Fluorescence confocal microscopy images of A375 cells incubated with complex **4** (8 μM) for 7 h, Mitotracker Green for 2 h and DAPI for 0.5 h. (B) Fluorescence confocal microscopy images of A375 cells incubated with different concentration of complex **4** for 7 h, Mitotracker Green for 2 h and DAPI for 0.5 h.

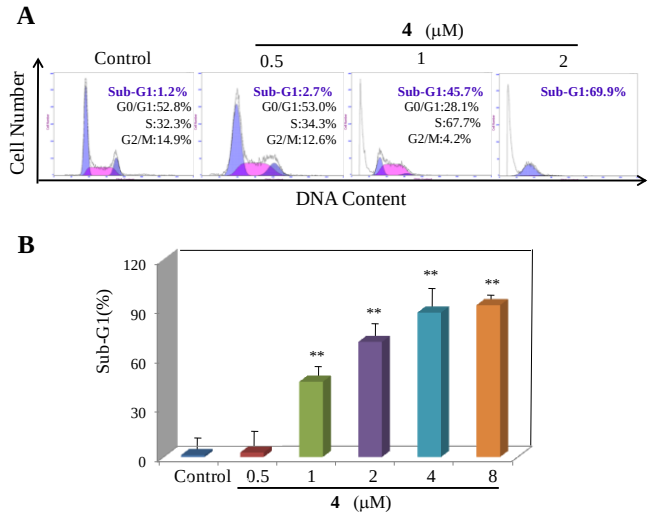


Figure 5. Complex 4 induces cancer cell apoptosis. (A) Quantitative analysis of apoptotic cell death induced by complex **4** by PI-flow cytometric analysis in A375 cells. (B) Effects of complex **4** concentration on apoptosis cell death in A375 cells. Significant difference between treatment and control groups is indicated at $P < 0.05$ (*) or $P < 0.01$ (**) levels.

Induction of caspase-mediated apoptosis by complex 4. TrxR has been found to play a pivotal role in cancer progression by various signaling pathways.[12] Therefore, the search for novel TrxR inhibitors has represented as an increasingly attractive target for anticancer drugs design.[7] Generally, anticancer drugs inhibit the cancer cell proliferation through apoptosis, cycle arrest or a combination of these two modes. In this study, we have identified Ru complexes as novel TrxR inhibitor. However, the complexes inhibited cancer cell proliferation through TrxR-mediated pathways remain elusive. In order to clarify this problem, PI-flow cytometric analysis was used to examine the mode of cell death induced by the complex **4**. As shown in **Figure 5A** and **Figure S4**, exposure of A375 cells to different concentrations of complex **4** led to increase in the proportion of apoptotic cells in a dose-manner,

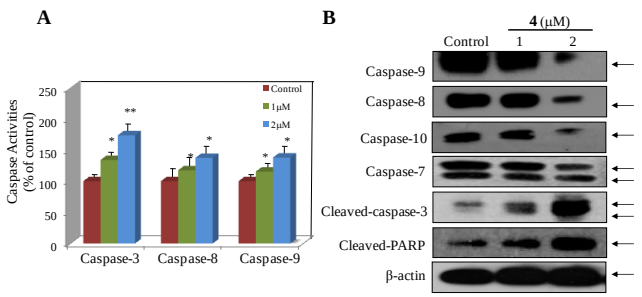


Figure 6. Induction of caspase-mediated apoptosis by complex 4 in A375 cells. (A) Caspase activities as measured by specific fluorescent substrates for caspase-3/8/9. Significant difference between treatment and control groups is indicated at $P < 0.05$ (*) and $P < 0.01$ (**) levels. (B) Western blot analysis the quantitative of caspases and PARP cleavage in the apoptosis induced by complex **4**. All results shown here are representative of three independent experiments.

as reflected by the increase of sub-G1 populations from 1.2% (control) to 92.2% (8 μM) (**Figure 5B**). These results indicated that apoptosis was the major mode of cell death induced by the synthetic Ru complexes. This finding is consistent with the previous studies, which showed compounds induced cancer cells apoptosis through TrxR-mediated pathways.[31, 67]

Several lines of evidence have indicated that caspases were important for initiation and execution of apoptosis.[33, 68] Generally, apoptosis could be initiated by two mechanisms, extrinsic and intrinsic pathways, both of them are dependent on the cleavage of caspases. In order to delineate the contributions of caspases to complex **4**-induced apoptosis, the activities of caspase-3, -8 and -9 were examined in treated cells. As shown in **Figure 6A**, exposure of A375 cells to complex **4** resulted in significant activation of the caspase family. These findings were further confirmed by Western blot analysis that demonstrated the cleavage of caspases-3, -8 and -9, which indicate the activation of both extrinsic and intrinsic apoptotic pathways by Ru complexes. Subsequently, complex **4**-induced caspase cleavage triggered the proteolytic cleavage of PARP that served as a biochemical marker of apoptosis (**Figure 6B**). Taking the above result together, we can come to a conclusion that, Ru complexes induced cancer cell apoptosis through TrxR-mediated signaling pathways.

Bcl-2 family proteins are involved in mitochondria-mediated apoptosis induced by complex 4. Bcl-2 family members have been described as the key regulators of mitochondrial outer membrane permeabilization that controls the release of apoptogenic proteins such as cytochrome *c*, AIF and Smac, and then triggers cell apoptosis.[66] Previously, Cattaruzza et al have demonstrated that gold(III) complexes induced mitochondria-mediated apoptosis in prostate cancer through regulation of Bcl-2 family proteins,[69] Andrew et al have reported that the TrxR inhibitor auranofin triggers apoptosis through a Bax/Bak-dependent process.[15] Due to the observation of TrxR-mediated mitochondrial dysfunction, we examined the effects of the complex **4** on the expression levels of Bcl-2 family proteins in A375 cells by Western boltting. As shown in **Figure 7A**, complex **4** down-regulated the expression levels of pro-survival proteins including Bcl-xL, Bcl-2 and Mcl-1. Meanwhile, it up-regulated the pro-apoptotic proteins

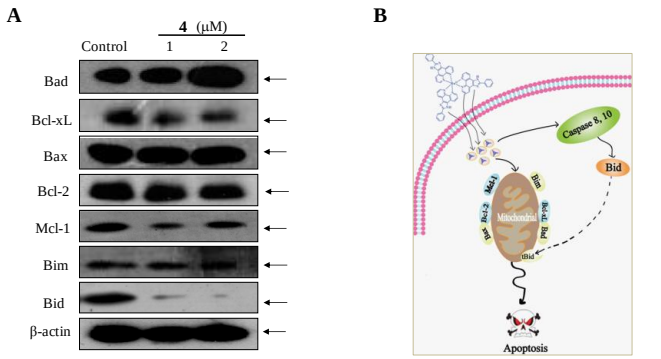


Figure 7. Bcl-2 family proteins are involved in mitochondria-mediated apoptosis induced by complex 4. (A) Western blot analysis of the expression levels Bcl-2 family proteins in A375 cells. Equal loading was confirmed by analysis of β-actin in the potein extracts. All results shown here are representative of three independent experiments with similar results. (B) Schematic illustration of Bcl-2 family proteins-mediated apoptosis induced by complex **4**.

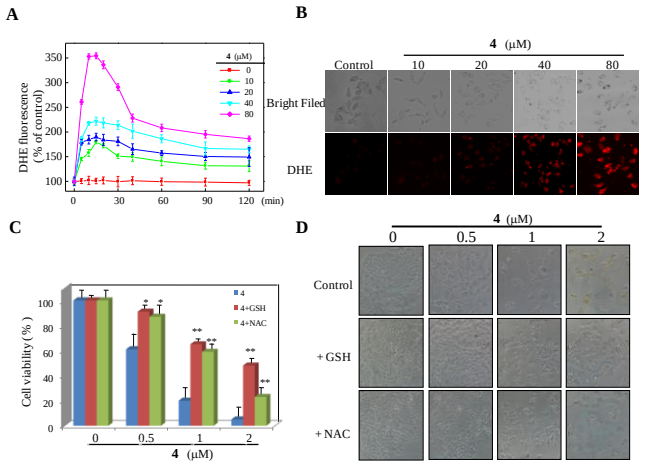


Figure 8. Important roles of ROS in cell apoptosis induced by complex 4. (A) Effects of concentration on intracellular ROS generation after treatment with complex **4** in A375 cells. A375 cells were treated with 10 μM DHE for 30 min. (B) Fluorescence confocal microscopy images of ROS generation in response to complex **4** treatment, as detected by DHE staining (magnification, 200×). (C) Protective effects of GSH or NAC induced growth inhibition in A375 cells. Bars with different characters are statistically different at $P < 0.05$ (*) and $P < 0.01$ (**) levels. (D) Protective effects of GSH or NAC on cell morphological changes (original magnification, 200). The cells were pretreated with GSH (2 mM) or NAC (1 mM) for 2 h followed by co-incubation with different concentrations of complex 4 for 72 h.

Bad, and little change on the expression levels of Bax and Bim was observed. The imbalance of Bad/Bcl-xL and Bax/Bcl-2 led to mitochondrial dysfunction and cleavage of caspase-9. Complex **4** also induced the truncation of Bid that acted as the downstream signal of caspase-8 and -10, and transduced the apoptotic signal from cell membrane to mitochondria (**Figure 7B**).

Important roles of ROS in cell apoptosis induced by complex 4. Reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide and hydroxyl radical, have been reported involved in the actions of many anticancer drugs through initiation of various apoptotic signaling pathways during chemotherapy.[70] Excess intracellular ROS could cause DNA damage and trigger p53, ATM/ATR, AKT and MAPKs signals activation.[71-74] In contrast, antioxidant enzymes like TrxR can disturb the balance of cellular ROS, even sweep away ROS.[4] Our previous studies have showed that, selenocystine enhanced the auranofin-induced apoptosis in MCF-7 human breast cells by inhibition of TrxR activity and increased ROS generation.[57] Cattaruzza et al also found that, gold(III)-dithiocarbamato derivatives exhibited their antitumor activities on prostate cancer cells and xenografts with the involvement of ROS generation.[33] Therefore, we examined the levels intracellular ROS generation in A375 cells after the induction of TrxR-mediated mitochondrial dysfunction by complex **4** by measuring DHE fluorescence intensity. As shown in **Figure 8A**, the cells treated with complex **4** greatly triggered the ROS generation in a time- and dose-dependent manner. Especially, when the cells were treated with 80 μM complex **4**, the production of ROS increased to 3.5 folds of control. These results were further confirmed by fluorescence confocal microscopy. DHE fluorescence probe can penetrate the cell membrane freely and could be oxidized by ROS, resulting in strong red fluorescence. As shown in **Figure 8B**, treatments of the cells with complex **4** led to the enhancement of cellular fluorescence, which indicated the overproduction of superoxide. In addition, we also used DCF-DA probe to detect the ROS level in cells after treatment with complex **4.** As shown in **Figure S5**, ROS overproduction was also found in A375 cells exposed to complex **4**. Moreover, ROS scavengers, glutathione (GSH) and N-acetylcysteine (NAC) were also used to verify the role of ROS in cells apoptosis. The results showed that, pretreatment of the cells with GSH (2 mM) and NAC (1 mM) effectively suppressed the cell death and morphological changes induced by complex **4** (**Figure 8C, D**). Taken together, our results suggest that, ROS plays an important role in cell apoptosis induced by complex **4**.

ROS triggers p53 phosphorylation. p53 is a tumor suppressor gene that plays a critical role in the cell apoptosis by regulating the transcription of a wide variety of genes involved in cell apoptosis, such as p21, Bax, Fas, PUMA and Bid.[75] Previous studies have showed that small molecule RITA induced p53-dependent apoptosis through inhibition of TrxR and overproduction of ROS.[76] In this study, we have demonstrated that complex **4** up-regulated the expression levels of Bax and down-regulated the expression levels of Bid in A375 cells (**Figure 7A**), which suggests the activation of p53 pathway by this complex. Therefore, we examined the expression levels of total and phosphorylation p53 in A375 cells treated with **4**. As shown in **Figure 9A**, treatment of the complex resulted in enhancements of total and phosphorylated p53 at Ser 15. Ser 139-Histone H2A.X, a maker of DNA damage, was also up-regulated in treated cells. Moreover, the protein levels of phospho-ATM was also up-regulated in response to complex **4**. These results demonstrate the involvement of ROS-activated p53 signaling pathway after inhibition of TrxR by Ru complexes.

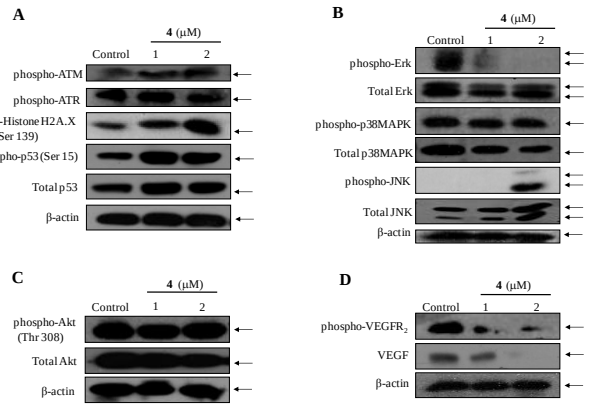


Figure 9. Stress-response signaling pathways activated by Ru complexes. Western blot analysis the effects of complex **4** on expression levels of different proteins in A375 cells. (A) ROS triggers p53 phosphorylation. (B) MAPKs pathway. (C) Phosphorylation Akt and total Akt. (D) Phosphorylation $VEGFR_2$, VEGF. Equal loading was confirmed by analysis of β-actin in the potein extracts. All results shown here are representative of three independent experiments with similar results.

Stress-response signaling pathways activated by Ru complexes. MAPKs and PI3K/Akt pathways are major oxidative stress-sensitive signal transduction pathways in most cell types exposed to excess ROS.[72] MAPKs family, including Erk, p38 MAPK, JNK, plays important roles in regulation of cell fate in responses to stress conditions.[77] Among them, Erk could prevent cell apoptosis by blocking the cleavage of caspase and control the cell differentiation, proliferation and motility.[78] Previous studies have demonstrated that acrolein-mediated activation of MAPKs was mediated by the inhibitory of TrxR.[27] Due to the observation of superoxide overproduction in complex **4**-treated cells, we examined the expression and phosphorylation of MAPKs by Western blot analysis. As shown in **Figure 9B**, after treatment with complex **4**, the expression of phosphorylated Erk was weakened in a dose-dependent manner. Contrary to Erk, JNK and p38 MAPK promote cancers cell apoptosis during drug treatments.[79] The results of Western blot analysis revealed that complex **4** activated triggered the phosphorylation of JNK, but showed no significant effect on p38 MAPK. Akt is a crucial regulator of cell survival function in response to growth factor stimulations. Once activated, it could inhibit the cell apoptosis and promote the cell proliferation.[80] Under conditions where the apoptotic activity of p53 prevails, it is conceivable that the destruction of Akt plays a role in accelerating the apoptotic process.[81] In addition, Kralova and co-workers demonstrated inhibitory TrxR level mediated selenite-induced apoptosis as well as reducing Akt expression.[82] However, in this study, the results of Western blot analysis showed that complex **4** exhibited no significant effect on the expression of phosphorylation Akt (**Figure 9C**).

Studies have reported that vascular endothelial growth factor (VEGF) could prevent ceramide- and starvation-induced apoptosis by inhibiting JNK and activating p38 MAPK and Erk signals.[83] Previous studies have demonstrated that TrxR regulated the angiogenesis by increasing endothelial cell-derived vascular endothelial growth factor.[84] Therefore, Western blotting was employed to elucidate the crosstalk between VEGF and MAPKs pathways. As shown in **Figure 9D**, the expression levels of phospho-VEGFR2 and VEGF in cells exposed to complex **4** were suppressed in a dose-dependent manner. Taken together, these findings suggest that, inhibition of TrxR by Ru complexes result in cancer cell apoptosis through regulation of MAPKs and VEGFR signaling.

CONCLUSIONS

TrxR is a NADPH-dependent selenoenzyme upregulated in a number of cancers. It plays a pivotal role in cancer progression and represents an increasingly attractive target for anticancer drugs. The limitations of cisplatin in cancer treatment have motivated the extensive investigation to other metal complexes, especially Ru complexes. In this study, we presented the *in vitro* biological evaluation of four Ru(II) polypridyl complexes with diimine ligands, and demonstrated that they exhibited antiproliferative activities against A375 human melanoma cells through inhibition of TrxR. As the planarity of the structure increases, their TrxR-inhibitory effects and *in vitro* anticancer activities were enhanced. Among them, complex **4** exhibited higher antiproliferative activity than cisplatin, and the TrxR-inhibitory potency of **4** was more effective than auranofin, a positive TrxR inhibitor. Complex **4** suppressed the cancer cell growth through induction of apoptosis as evidenced by accumulation of sub-G1 cell population, DNA fragmentation and nuclear condensation. Moreover, complex **4** was able to localize in mitochondria and therein induced ROS-dependent apoptosis by inhibition of TrxR activity. On the basis of the results, the action mechanisms and the underlying signaling pathways of the synthetic Ru complexes were proposed in **Figure 10**. First of all, **4** inhibited the cancers growth by targeting TrxR. Because of the inhibition of TrxR, it was beneficial to the generation and accumulation of ROS. Secondly, the overproduction of ROS promotes phosphorylation of p38MAPK and JNK, and de-phosphorylation of Erk, causes DNA damage in A375 cells. Thirdly, complex **4**-induced DNA damage up-regulates and phosphorylates p53 protein. On the one hand, the activation of p53 induces the mitochondrial dysfunction through regulating the expression of Bcl-2 family proteins, and then triggered the mitochondrial release of apoptogenic factors and caused cleavage of caspase family proteases. Moreover, phosphorylation p53 enhanced the activation of caspase-8 and caspase-10, and the subsequent truncation of Bid, which in turn facilitates the mitochondrial function and ROS generation. Taken together, inhibition of TrxR by Ru complexes leads to ROS-mediated apoptosis through regulation of p53, MAPKs and VEGFR signaling. These results suggest that, Ru polypyridyl complexes could be developed as TrxR-targeted agents that demonstrate application potentials for treatments of cancers.

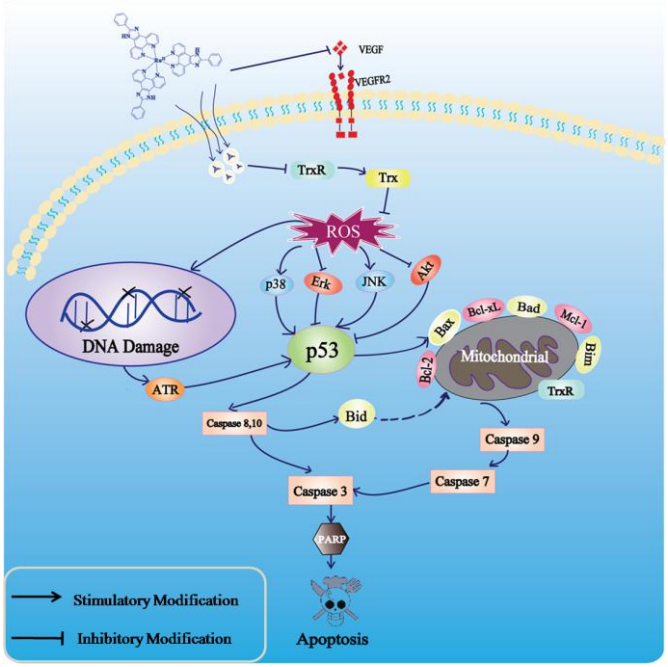


Figure 10. Proposed signaling pathway triggered by complex 4 in A375 cells.

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† Electronic Supplementary Information (ESI) available: Synthesis details, ESI-MS and elemental analysis of synthesized compounds, the relationship between cytotoxicity and lipophilicity of compounds **1-4**, enzymatic activities of GSH-Px and GSH-Rs in A375 cells after treatment with complex **4**, quantitative analysis of apoptotic cell death induced by **4** (4 μM and 8 μM) by flow cytometric analysis in A375 cells. UV/vis absorption and fluorescence emission spectra of complex **4** during its reaction with selenopepetide, effects of concentration on intracellular ROS generation after treatment with complex **4** in A375 cells. A375 cells are treated with 10 μM DCF-DA. See DOI: 10.1039/b000000x/.

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