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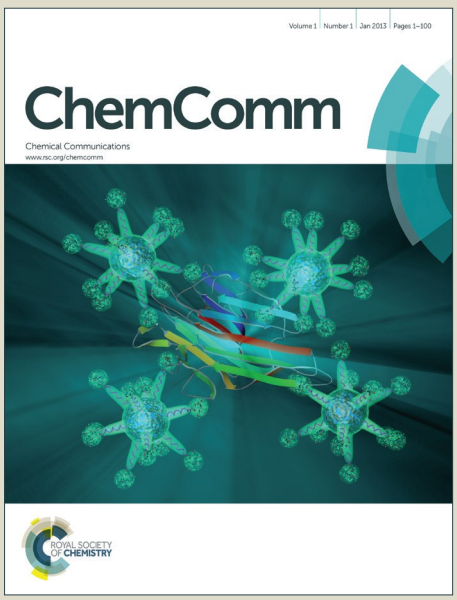

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ARTICLE TYPE

Selenium-containing ruthenium complex as cancer radiosensitizer, rational design and important role of ROS-mediated signalling

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A novel selenium-containing ruthenium complex Ru(phtpy)(phenSe)Cl(ClO$_4$) (phtpy = 4-phenyl-2,2':6',2''-terpyridine, phenSe = 2-selenicimidazole[4,5-f]1,10-phenanthroline) has been synthesized and found be able to enhance radiation-induced DNA damage through superoxide overproduction, which lead to G2/M arrest and apoptosis in cancer cells by activating ROS-mediated pathways.

More than 50% of diagnosed cancer patients receive radiotherapy, alone or in combination with other therapies worldwide[1]. Ionizing radiation (IR) as one of the primary treatments for various cancers is prized because of its unique advantages of being noninvasive and low systemic toxicity[2]. However, despite radiotherapy achieves varying degrees of success, many patients still suffer from recurrence and unexpected side effects[3]. Because the effect of radiotherapy is strongly limited by the radioresistance of cancer cells, the combination of radiotherapy with radiosensitizers as an experimental and clinical strategy has been established to reduce radioresistance[4]. In the past decades, radiosensitizers are widely used clinically and are considered to be able to improve the local-regional effects of radiotherapy[5]. Most radiosensitizers (such as cisplatin and carboplatin) could target DNA and thus sensitize cancer cells to radiation via enhancing DNA damage and inhibiting DNA repair process[6]. Therefore, based on this action mechanism to design new metal complexes is becoming a novel strategy for discovery of new anticancer drugs.

In the past decades, increasing number of metal-based complexes, especially platinum (Pt) complexes, were developed as radiosensitizers due to their DNA-binding property[7]. However, the application of Pt complexes was limited by serious toxic side effects, drug resistance and weak selectivity between tumour and normal tissues[8]. Ruthenium (Ru) complexes, possessing favourable properties suitable for flexible antitumor drug design[9], have been regarded as appropriate substitutes of Pt complexes[10]. Our previous work have proved that Ru complexes as a novel class of anticancer agents could induce DNA damage of cancer cells followed by triggering apoptosis or cell cycle arrest[11]. Studies have demonstrated photo-activated properties of Ru complexes[9, 12], which could be used as potential photodynamic therapy (PDT) agents. Inspired by these discoveries, we proposed that Ru complexes can probably be developed into

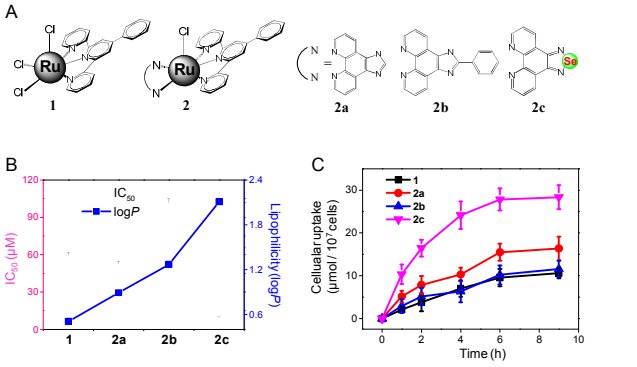

Figure.1 (A) Structure of Ru complexes. (B) IC$_{50}$ values and lipophilicity of the Ru complexes. A375 Cells were incubated with complexes for 72 h, and the IC$_{50}$ was determined by MTT assay. (C) Cellular uptake of complexes **1-2c** (10 μM) in A375 cells as determined by ICP-MS analysis.

radiosensitizers, since the X-ray possesses much higher energy than visible light, might activate these Ru complexes as well. Selenium (Se) is a necessary trace element with potential anticancer activities [13,14]. Organic Se, especially selenoheterocyclic compounds, has attracted more and more attention for their unique pharmacological activities[15]. Our previous studies have indicated that selenoheterocyclic compounds could effectively induce DNA damage and apoptosis of cancer cells[16]. We also showed that selenocompounds could effectively enhance the anticancer efficacy of X-ray through activation of diversified ROS-mediated signaling pathways [17]. Base on the interesting physical and biochemical characteristic and therapeutic advantages of Se, we attempt to improve the anticancer activities and radiosensitization of Ru complexes by introducing seleno-ligands. Therefore, in this study, a novel class of Ru complexes, Ru(phtpy)Cl$_3$ (**1**), Ru(phtpy)(ip)Cl(ClO$_4$) (**2a**), Ru(phtpy)(pip)Cl(ClO$_4$) (**2b**) and Ru(phtpy)(phenSe)Cl(ClO$_4$) (**2c**) (phtpy = 4-phenyl-2,2':6',2''-terpyridine, ip = imidazole[4,5-f]1,10-phenanthroline , pip = 2- phenylimidazole[4,5-f]1,10-phenanthroline and phenSe = 2-selenicimidazole[4,5-f]1,10-phenanthroline) have been synthesized (**Fig. 1A**) and their anticancer activities and radiosensitization effects against human melanoma A375 cells were also examined as well. Among these complexes, the Se-containing one, **2c**, possessed potent anticancer activity and radiosensitization effects. The studies on

Table 1. Growth inhibition of **2c** and **2c**-radiation treatment on A375 and HK-2 cells [a]

Complex	IC_{50} (μM)					
	A375	A375 + IR[b]	SER[c]	HK-2	HK-2 + IR	SER[d]
2c	9.7 ± 2.9	4.4 ± 1.4	2.2	110.9 ± 4.4	99.4 ± 5.9	1.1
Cisplatin	7.5 ± 1.3	4.9 ± 2.2	1.5	10.4 ± 2.2	7.3 ± 2.3	1.4

[a] Cell viability was determined by MTT assay after treatment for 72 h.
[b] The dose of IR (X-ray) is 8 Gy.
[c] SER (sensitivity enhancement ratio) = IC_{50} (A375)/IC_{50} (A375 + IR).
[d] SER = IC_{50} (HK-2)/IC_{50} (HK-2 + IR).

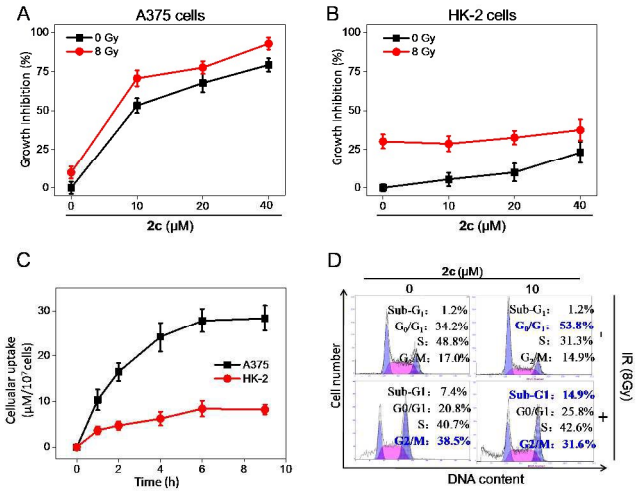

Fig. 2 Relationship between radiosensitization effects and cellular uptake of complex **2c**. (A) Growth inhibition of different treatments on A375 cells. Cells were exposed different treatments for 72 h, and the cell growth inhibition was determined by MTT assay. (B) Growth inhibition of different treatments on HK-2 cells (72 h). (C) Cellular uptake of complex **2c** (10 μM) in A375 and HK-2 cells as determined by ICP-MS. (D) Flow cytometric analysis of A375 cells treated for 24 h.

the action mechanisms revealed that, **2c** sensitized A375 cells to radiation by enhancing radiation-induced ROS-mediated DNA damage and downstream signalling pathways, eventually resulted in G2/M arrest and apoptosis.

In this study, complex **1** was synthesized by mixing equal quantities of $RuCl_3$ and phtpy ligand into ethanol to reflux at 85 □ for 4 h. Complex **2a-2c** were synthesized by refluxing the same quantity of **1** and corresponding ligand in ethanol for 6 h under N_2 atmosphere, followed by purifying by neutral alumina column chromatography with methylbenzene and acetonitrile as eluent. The chemical structure and the purity of the synthetic complexes were characterized and confirmed by ESI mass spectrometry, ^1H NMR spectroscopy and elemental analysis (**Fig. S1-S5**).

To examine the effects of Se on the biological application of Ru complexes, firstly, MTT assay was applied to assess the anticancer activities of the synthetic complexes. As shown in **Fig. 1B**, complex **1** exhibited slight growth inhibition on A375 cells after a 72-h treatment, with IC_{50} value at 59.6 μM. Meanwhile, **2a** demonstrated higher anticancer activity after coordination with **ip** ligand (IC_{50}=52.4 μM), suggesting the introduction of **ip** analogs could enhance the anticancer activities of Ru-phtpy system. Though complex **2a-2c** shared similar chemical structure, their anticancer efficacy was totally different. Complex **2b** with the **pip** ligand did not exhibit effective suppression on the growth of A375 cells, which may be due to its poor solubility as a result of

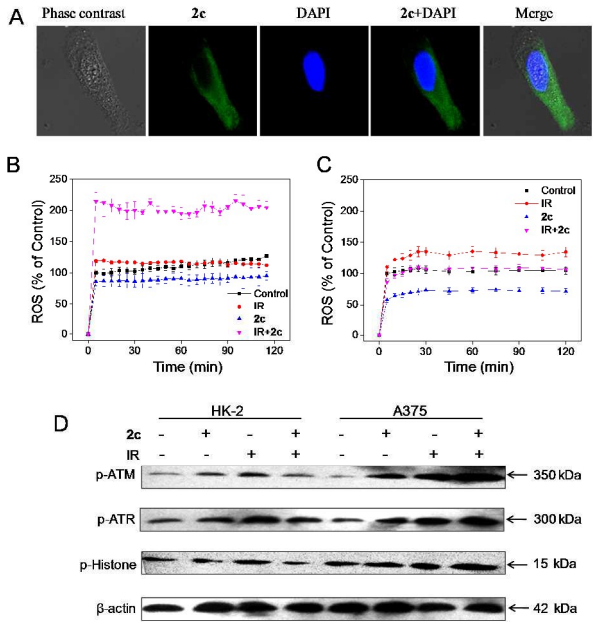

Fig. 3 ROS-mediated DNA damage induced by complex **2c** and X-ray. (A) Cellular location of complex **2c** in A375 cells. (B) Changes of intracellular ROS level induced by different treatments in A375 cells. (C) Changes of intracellular ROS level induced by different treatments in HK-2 cells. (D) Western blot analysis for the expression of p-ATM, p-ATR and p-Histone. β-actin was used as loading control. The concentration of **2c** was 10 μM, and the dose of radiation was 8 Gy.

the introduction of hydrophobic phenyl group. Moreover, complex **2c** with Se on the **ip** ligand shown a great enhancement of antiproliferative activities towards A375 cells (IC_{50}=9.7 μM), indicating that the introduction of Se into Ru complexes could improve their antitumor activities. Previously, Barton and co-workers have evidenced the cellular uptake and anticancer activity of complex were affected by their lipophilicity [18]. Therefore, we measured the partition coefficient (log*P*) and cellular uptake of **1-2c** to determine their relationship with the anticancer efficacy. As shown in **Fig. 1B** and **C**, the cellular uptake of complexes **1**, **2a** and **2c** was well correlated with their partition coefficients. However, complex **2b** with high log*P* showed lowest anticancer activity and low cellular uptake, which may due to its poor solubility in the aqueous cell culture condition. Among these complexes, **2c** displayed the highest log*P*, highest cellular uptake and anticancer activity. These results suggest that, the introduction of Se into Ru complexes could effectively increase their lipophilicity, thus enhance the cellular uptake and anticancer efficacy.

The *in vitro* radiosensitization of **2c** against A375 and HK-2 cells was examined by MTT assay using cisplatin as a positive control. The cells were incubated with different concentrations of complex **2c** or cisplatin for 6 h, followed by radiation at a dose of 8 Gy, then cells were cultured for another 66 h before examining their cell viability. As shown in **Table. 1**, **2c** effectively sensitized A375 cells to radiation with a sensitivity enhancement ratio (SER) at 2.2, which was much higher than that of cisplatin (SER=1.5). Moreover, for the human normal cell line (HK-2 human kidney cells), complex **2c** demonstrated low cytotoxicity toward HK-2 cells (IC_{50}=110.9 μM), which was about 10 times

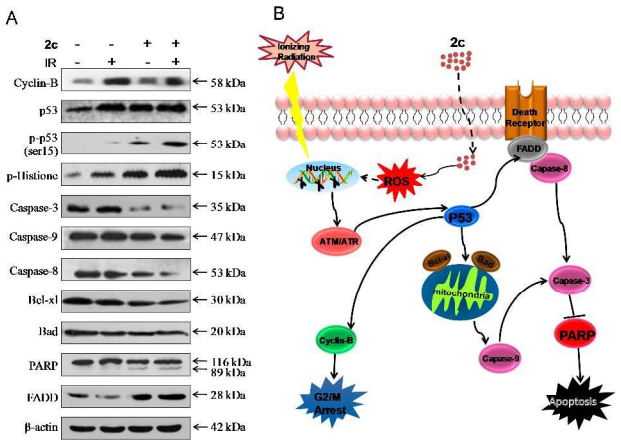

Fig. 4 Signalling pathways triggered by complex **2c** and X-ray. (A) Western blot analysis of the expression of related proteins. β-actin was sued as loading control. The concentration of **2c** was 10 μM, and the dose of radiation was 8 Gy. (B) The main signalling pathways accounting for the radiosensitization effects of complex **2c**.

lower than that of cisplatin (IC_{50}=10.4 μM). The SER value of **2c** (1.1) was also lower than that of cisplatin (1.4), which demonstrate the higher selectivity of the synthetic complexes.

Studies were also carried out to examine the reason accounting for the different selectivity and radiosensitization effects of **2c** between cancer and normal cells. As shown in **Fig. 2A**, the combined treatment of **2c** and radiation was more cytotoxic to A375 cells than **2c** or X-ray alone. A remarkable decrease in cell numbers and change in cell morphology (such as cell shrinkage and cell rounding) were observed in the cells received the combined treatment (**Fig. S6**). In contrast, **2c** alone only showed slight growth inhibition on HK-2 cells, and it didn't enhanced the cytotoxicity of X-ray toward the cells (**Fig. 2B**). The different effects of **2c** on cancer and normal cells could be due to the difference in cellular uptake. Consistent with this hypothesis, we found that, the uptake of **2c** in A375 cells was much higher than that in HK-2 cells (**Fig. 2C**), which contributes to the higher growth inhibition and radiosensitization.

Flow cytometric analysis was performed to examine the action modes of radiosensitization induced by Ru complexes. As shown in **Fig. 2D**, **2c** and radiation co-treatment induced G2/M arrest in A375 cells, as reflected by the increase of the percentage of cells at G2/M phase (co-treatment at 31.6 % vs. control at 17.0%). In addition, **2c** enhanced the radiation-induced cell apoptosis, as evidenced by the increase in Sub-G1 phase from 7.4% to 14.9 % (co-treatment). These results suggested that **2c**-radiation co-treatment could induce disruption of cell-cycle progression and apoptotic cell death.

DNA has been regarded as the main target of X-ray and most metal-based anticancer drugs. In order to examine the role of DNA in the anticancer action of **2c**, firstly, we used the cell model to examine the cellular distribution of the complex by monitoring its autofluorescence. As shown in **Fig. 3A**, **2c** mainly located in cytoplasm, which suggesting that **2c** doesn't interact with DNA directly. In the cytoplasm, cellular proteins have been proposed to be favourable targets for cytotoxic metal complexes[19], Che and co-workers have discovered that metal complexes could inhibit some cellular proteins (such as TrxR) to cause the accumulation of reactive oxygen species (ROS) and DNA damage, resulting in cell arrest and apoptosis eventually[20]. Importantly, the X-ray-induced ROS generation has been identified as the major cause of DNA damage[4b]. Therefore, we measured the ROS level in A375 and HK-2 cells by examination of dihydroethidium (DHE) fluorescence intensity. As shown in **Fig. 3B,** the co-treatment remarkably increased the intercellular ROS generation in A375 cells to over 200% of control, but no significant change was observed in cells exposed to **2c** or X-ray alone. However, in HK-2 human normal cells, X-ray alone activated the intracellular ROS generation to about 130% of control group (**Fig. 3C**). However, the co-treatment of the cells with complex **2c** reduced the ROS generation to the control level, which was much lower than that in A375 cells. Furthermore, we found that, in A375 cells, the phosphorylation of ATM, ATR and Histone (**Fig. 3D**), three important biochemical hallmark of DNA damage[21], was more obvious than those in HK-2 cells after treated with **2c** and X-ray. These results suggest that, Se-containing Ru complexes enhance the anticancer effects of X-ray by triggering ROS-mediated DNA damage.

The chemical interaction between the complexes and X-ray were also examined by ESI-MS and ^{1}H NMR. As shown in **Fig. S7,** no significant change in the Mass spectra and chemical shift was observed in the complexes after radiation. The consistency of the UV-Vis spectra of the complexes before and after radiation further confirmed the stability of the synthetic complexes (**Fig. S8**). We also found that, the UV-Vis spectra of complex **2c** kept stable during incubation in aqueous solutions for 24 h (**Fig. S9**). Even with the presence of 50-500 μM H_2O_2, the UV-Vis spectra of complex **2c** didn't show change after 30 min (**Fig. S10**). The stability of this kind of synthetic complexes supports their future application in the chemo- and radio-therapy of cancers .

To further elucidate the signalling mechanisms contributing to the radiosensitization effects of **2c**, we measured the level of proteins related with the regulation of G2/M arrest and apoptosis. As shown in **Fig. 4A**, the co-treatment up-regulated the level of cyclin-B, a crucial cell cycle regulator necessary for the progression of the cells into and out of M phase of the cell cycle[22]. Meanwhile, the combined treatment also induced the proteolytic cleavage of PARP and obvious decrease in the expression levels of total Caspase-3,-8 and -9, indicating the proteolytic cleavage of these proteins, which confirmed the involvement of the extrinsic and mitochondria-mediated intrinsic apoptosis pathways in the co-treatment-induced apoptosis. As expected, the combined treatment also increased the expression of FADD and suppressed the expression of Bcl-xl, a pro-survival member of Bcl-2 family protein. The observation of ROS accumulation and activation of mitochondria-mediated apoptosis proves the induction of mitochondrial dysfunction by **2c**. Considerable evidence has pointed out that selenocompounds could induce ROS-mediated DNA damage and apoptosis through p53 signalling pathway[16a, 16b]. Interestingly, we found that **2c** triggered the elevation and phosphorylation of p53 at ser 15 site and histone. Taken together, these results indicate that, **2c** sensitizes cancer cells to X-ray by triggering ROS-mediated DNA damage and activation of p53 pathway (**Fig. 4B**).

In summary, this study provided a strategy for rational design of metal complex-based radiosensitizers by introducing Se into the complexes. The synthetic Se-containing Ru complexes were

able to enhance radiation-induced DNA damage through superoxide overproduction, which further result in G2/M arrest and apoptosis in cancer cells by activating ROS-mediated pathways.

Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis details, ESI-MS, ^{1}H NMR and UV spectrum analysis of synthesized compounds with/without ionizing radiation. See DOI: 10.1039/b000000x/.

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