Nucleus-enriched Ruthenium Polypyridine Complex Acts as a Potent Inhibitor to Suppress Triple-negative Breast Cancer Metastasis In vivo

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Article history:
Received 17 September 2018
Received in revised form 20 November 2018
Accepted 23 November 2018
Available online 29 November 2018

Keywords:
DPPZ-based Ru(II) complexes
triple-negative breast cancer
angiogenesis inhibitor
metastasis
DNA damage

Abstract
Polypyridine Ru(II) complexes have long been deemed to excellent antitumor agents that inhibit the proliferation of breast cancer cells. Nevertheless, their effects on the metastatic potency of breast cancer cells need further research. Herein, a class of polypyridine Ru(II) complexes coordinated with phenazine derivates (DPPZ) ([Ru(bpy)2(DPPZ-R)](ClO4)2, Ru(bpy)2DPPZ: R = -H, Ru(bpy)2BrDPPZ: R = -Br, Ru(bpy)2MDPPZ: R = -CH3, Ru(bpy)2BNDPPZ: R = -acene, Ru(bpy)2BEDPPZ: R = -NC6H4)) was synthesized by introducing different substituted groups to regulate the electron cloud density and planarity of the main ligands. Results indicated that this class of DPPZ-based Ru(II) complexes exhibited promising inhibitory effect against MDA-MB-231 triple-negative breast cancer cells, especially for Ru(bpy)2BEDPPZ, which is comparable with that of cisplatin. In addition, Ru(bpy)2BEDPPZ effectively inhibited the migration and invasion of MDA-MB-231 cells in vitro and suppressed focal adhesion and stress fiber formation. Moreover, it effectively blocked MDA-MB-231 cell metastasis in blood vessels and restrained angiogenesis formation in a zebrafish xenograft breast cancer model. Further studies showed that the mechanisms may involve DNA damage-mediated apoptosis probably due to Ru(bpy)2BEDPPZ, which was enriched in the cell nucleus and induced DNA damage. All these results suggested that the DPPZ-based Ru(II) complexes can act as potent anti-metastasis agents.

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1. Introduction
Breast cancer is a disease that significantly threatens women’s life. The triple-negative breast cancer (TNBC) is considered to be the most dangerous in the different types of breast cancer [1]. It is a common malignant tumor in China and has high degree of malignancy, incidence of recurrence, and metastasis [2]. The site of distant metastasis occurs mainly in the bone, lungs, liver, and brain [3]. Although methods such as endocrine and molecular targeted therapy are used in the treatment of TNBC, they are not effective. Currently, the commonly used treatment for TNBC is chemotherapy [4,5]. Anthracycline-based drugs, paclitaxel, and platinum antitumor drugs mainly target the DNA [6–8]. At the gene level, they affect the function of TNBC cells. However, they have no selectivity against normal cells, causing serious side effects to patients [9]. In TNBC, once metastasis develops in the surrounding tissue, the patient’s life is in danger [10]. Thus, developing drugs that can restrain TNBC metastasis and clarifying their mechanism are important [11].

Recently, Ru-based complexes have attracted considerable attention because of their high antitumor activity, low toxicity, and powerful DNA-binding affinity. Two Ru(II) complexes (NAMI-A and KP1019) have entered phase II clinical trials due to their potential antitumor behavior [12]. A number of evidence suggests that Ru(II) complexes have wonderful DNA binding properties [13,14]. DNA is considered as an accepted target for ruthenium complexes. Ru(II) complexes can enter into the cell nucleus and play a key role in biological activity [15]. Barton et al. reported that DPPZ-based Ru(II) complex ([Ru(DIP)2(dppz)]2+) with lipophilic DIP ligand facilitates complex cellular uptake into the cell cytoplasm but has low inhibition against different tumor cells as it cannot enter into the cell nucleus [16]. Moreover, Chao et al. found that a type of Ru(II) complex ([Ru(bpy)3(phen)DPPZ]2+) exhibits high antitumor activity against a series of tumor cell lines through accumulating in the nucleic region to bind DNA [17].

In this study, we designed a class of polyppyridine Ru(II)-DPPZ complexes by introducing different substituent groups to regulate...
the electron cloud density of phenazine ligand. This process may improve molecular planarity and modify the terminal benzene ring to increase lipophilicity, thereby helping complexes enriched into the nucleus to enhance antitumor activity. We described the synthesis and the enhanced potency of this new series of polypyridine Ru(II)-DPPZ complexes to block the metastasis of breast cancer in vitro and in vivo. Further studies showed that this class of Ru(II) complexes may enter into breast cancer cells and localize at the nucleus, which ultimately may induce DNA damage to cause cell apoptosis [18,19]. Therefore, the Ru(II) complexes with low-toxicity can act as potential inhibitors against the metastasis of breast cancer for chemotherapy (Scheme 1).

2. Results and Discussion

2.1. In vitro Tumor Growth Inhibition of the Synthetic Ru(II) Complexes

To better assess the antitumor properties of Ru(II) complexes involving bidentate N-donor ligands with planar aromatic rings, we first evaluated the antiproliferative effects of these complexes on normal and some cancer cell lines (lung A549, breast MCF-7, liver HepG2, triple-negative breast MDA-MB-231, and HaCaT keratinocytes) and compared them with cisplatin. As shown in Table 1, the synthetic Ru(II) complexes emerged acceptable inhibition to various tumor cells after 72 h treatment, especially Ru(bpy)2BEDPPZ, which exhibited better antitumor activity than other complexes. The inhibitory effect (IC50) of Ru(bpy)2BEDPPZ against triple-negative breast MDA-MB-231 cell lines was approximately 17.2 μM, which was better than that of cisplatin (20.9 μM). Its construction is divalent platinum binds to two chlorine atoms and two ammonia molecules and it can bind to DNA and cause cross connection, thus damaging the function of DNA and inhibiting cell mitosis. It is a cellular non-specific drug. However, the ruthenium compound based on a phenazine ligand containing a large aromatic planar structure can better bind to DNA than cisplatin. The electric effect, planarity, and steric hindrance of substituted groups in ligands play key roles in antitumor activity of Ru(II) complexes [20]. Although for the complexes of the electron-donating group, with Ru(bpy)2BnDPPZ modified complexes (with IC50 of 32.5 μM) were better than Ru(bpy)2MDPPZ (with IC50 of 93.3 μM) and Ru(bpy)2BrDPPZ (with IC50 of 94.1 μM) modified one against A549 cells, the best one was Ru(bpy)2BEDPPZ substituted complexes (with IC50 of 20.0 μM). Unlike the IC50 value for different sized planar ligands of complexes to MDA-MB-231 cells, the complexes with high quantity of terminal benzene rings in the ligands had enhanced growth suppression to tumor cells [21–23]. Comprehensive analysis of these results showed that the complexes modified by the electron-donating group can enhance antitumor activities, suggesting that the introduction of a phenylethynyl group in the phenazine ligand can availably improve the growth inhibition of tumor cells by exhibiting certain sensitivity to MDA-MB-231 cells. On the basis of its promising in vitro activity, Ru(bpy)2BEDPPZ was selected for preliminary evaluation in further studies.

Table 1

| Comp.        | IC50/μM | logP<sub>ow</sub> |
|--------------|---------|-------------------|
|              | MDA-MB-231 | A549 | MCF-7 | HepG2 | HaCaT |
| Ru(bpy)2DPPZ | >100     | >100 | >100 | >100 | >100 | -1.87 |
| Ru(bpy)2BrDPPZ | >100 | 94.1 ± 4.2 | >100 | >100 | >100 | -1.58 |
| Ru(bpy)2MDPPZ | >100 | 93.3 ± 4.1 | 9.7 ± 6.1 | >100 | >100 | -2.17 |
| Ru(bpy)2BnDPPZ | >100 | 32.5 ± 1.6 | >100 | 23.0 ± 1.1 | >100 | -1.65 |
| Ru(bpy)2BEDPPZ | 17.2 ± 0.9 | 20.0 ± 0.7 | 74.9 ± 3.5 | 97.5 ± 4.4 | 47.2 ± 2.6 | -0.87 |
| Cis-platin    | 20.9 ± 1.9 | 21.1 ± 0.2 | 86.6 ± 5.9 | 15.5 ± 0.4 | 7.1 ± 0.5 | - |
2.2. Invasion and Migration of triple-negative breast cells inhibited by Ru(bpy)$_2$BEDPPZ

Considering that metastasis is a severe risk in TNBC treatment, it is important to search for effective drugs that can inhibit it [24]. To investigate the anti-metastatic activity of the complexes, wound healing assay was performed to estimate cell migration and repair ability because the healing degree of scratches can reflect the inhibition effect of drugs on the migration ability of tumor cells. Fig. 1A shows an distinct reduction in the distance of wound closure without drug treatment for 72 h, but wound closure was suppressed with the addition of Ru(bpy)$_2$BEDPPZ. When the dose of Ru(bpy)$_2$BEDPPZ was increased to 4 μM, the wound-healing rate further decreased, which suggested that Ru(bpy)$_2$BEDPPZ inhibited migration in a dose-dependent manner.

Moreover, the invasion of MDA-MB-231 cells blocked by Ru(bpy)$_2$BEDPPZ was studied using FITC-gelatin assay. The quantity of black area (degraded area) in the FITC-gelatin showed the invasion capability of tumor cells, because highly invasive MDA-MB-231 cells can release matrix metalloproteinases to degrade the FITC-gelatin; that is, high numbers of black area indicated high invasiveness of MDA-MB-231 cells [25]. Fig. 1C shows a high number of black holes in the FITC-gelatin without drug treatment, which suggested that MDA-MB-231 exerted high invasive capability. With 5 μM of Ru(bpy)$_2$BEDPPZ treatment, the invasive capability of MDA-MB-231 cells was markedly suppressed, and the quantity of black holes in FITC-gelatin was decreased. These results suggested that the migration and invasion of MDA-MB-231 cells were effectively inhibited by this class of Ru(II) complexes [26].

The possible mechanism of anti-metastasis by Ru(bpy)$_2$BEDPPZ in vitro was studied (Fig. 2). Here, filamentous actin bundles and FA protein points were observed using paxillin [27], a focal adhesion-associated adaptor protein, suggesting cell adhesion ability on the surface [28]. Without treatment with Ru(bpy)$_2$BEDPPZ, a good deal of focal adhesions around the edge of MDA-MB-231 cells were observed. Nevertheless, after Ru(bpy)$_2$BEDPPZ treatment, it is found that a decreased quantity of paxillins in MDA-MB-231 cells with increasing concentration of Ru(bpy)$_2$BEDPPZ, and the cytoskeletal structure became loose. The stress fibers were also inhibited, after Ru(bpy)$_2$BEDPPZ treatment, as represented by F-actin with red fluorescence. The results indicated that Ru(bpy)$_2$BEDPPZ might suppress focal adhesions and stress fibers to inhibit the migration and invasion of MDA-MB-231 cells [29].

2.3. Suppression of Breast Cancer Growth and Metastasis in vivo

To detect the anticancer activity of the complexes in vivo, we treated the zebrafish breast cancer model with Ru(bpy)$_2$BEDPPZ. The model was constructed by using transgenic zebrafish (fli1:EGFP), and quantification breast cancer cells were microinjected into the zebrafish near the subintestinal vessel (SIV) area, in which blood vessels were labeled with green fluorescence and MDA-MB-231 cells with blue fluorescence [30,31]. As shown in Fig. 3, without drug treatment, a spot of blue breast...
cancer cells was distributed near the SIV area at 0 h. When the time increased to 72 h, an increasing number of MDA-MB-231 cells were observed, and some cells invaded into neighboring tissues and spread into the blood vessels of zebrafish tail, which suggested that MDA-MB-231 cells possessed the ability for infinite proliferation and blood vessel invasion [32]. However, with Ru(bpy)$_2$BEDPPZ (5 μM) treatment...
for 72 h, the number of MDA-MB-231 cells was notably reduced compared with the control group, which implied that Ru(bpy)2BEDPPZ can effectively suppress the proliferation of TNBC cells in zebrafish. Moreover, scarce MDA-MB-231 cells were found in the blood vessel of zebrafish, indicating that Ru(bpy)2BEDPPZ can suppressed the metastasis of MDA-MB-231 cells in zebrafish xenografts. Moreover, the total area of SIV in the Ru(bpy)2BEDPPZ treatment group zebrafish was obviously less than that in the control group, which indicated that Ru(bpy)2BEDPPZ exhibited a certain inhibitory effect on tumor angiogenesis. The above results suggested that Ru(bpy)2BEDPPZ can suppress the proliferation and metastasis of MDA-MB-231 cells in vivo and can be developed to become a potential candidate to block TNBC metastasis.

2.4. Angiogenesis Inhibited by Ru(bpy)2BEDPPZ in vitro and in vivo

In general, angiogenesis formation act a pivotal part in tumor proliferation and metastasis, some researchers have been studied that the application of Nanoparticles to the inhibition or synergistic treatment tumor through inhibit endothelial tubes formation [33,34], such as nanodiamond, mesoporous silica nanoparticles, etc. [35,36]. Besides, the vascular endothelial growth factor (VEGF) is one of the most critical factor inducing tumor vessel formation [37,38]. As tumor growth requires angiogenesis to provide adequate oxygen and nutrients, inhibiting angiogenesis formation is considered as an important way to overcome breast cancer. Angiogenesis inhibitors are widely used because of their safety and ability to block new blood vessel formation in tumors [39].

Given that tube formation indicates angiogenesis progression, we also demonstrated whether Ru(bpy)2BEDPPZ affects HUVECs’ angiogenic effect in vitro by using tube formation assay. As shown in Fig. 4A, without drug interference, HUVECs incubated with VEGF (200 ng/mL) established tubular structures on Matrigel. Then, we treated HUVECs with Ru(bpy)2BEDPPZ in the presence of VEGF and found that the tube formation of these cells was inhibited notably compared with control cells. With increasing Ru(bpy)2BEDPPZ treatment dose, the matrix-induced tube formation of HUVECs was suppressed, indicating its dose-dependent effects [40,41]. Moreover, comprehensive analysis of the tube number, mean node number, and total length of tube suggested that Ru(bpy)2BEDPPZ is responsible for the inhibitory effect of VEGF on the matrix-induced tube formation of HUVECs. These observations indicated that Ru(bpy)2BEDPPZ can suppress the angiogenesis in vitro.

Furthermore, the inhibitory effect of Ru(bpy)2BEDPPZ against angiogenesis formation was evaluated in zebrafish model. It is found that Ru(bpy)2BEDPPZ equally induces distinct defects in ISV formation.
To examine in detail the inhibitory effects of Ru(bpy)$_2$BEDPPZ on ISV formation in vivo, the Tg(flil1a:EGFP) transgenic embryos at 12 h post-fertilization (hpf) were used to treat with various concentrations (1.25, 2.5 and 5.0 $\mu$M) of Ru(bpy)$_2$BEDPPZ, owing to GFP expressing under the control of an endothelial-specific promoter at this time point. Given that this moment coincides with the construction of the dorsoventral axis, the analysis concentrated only on the inhibition of Ru(bpy)$_2$BEDPPZ on angiogenesis. After treatment with Ru(bpy)$_2$BEDPPZ, ISV was visualized at 48 hpf. In this in vivo angiogenesis model, 5.0 $\mu$M of Ru(bpy)$_2$BEDPPZ more effectively inhibited ISV formation than PTK787, which is a commonly used angiogenesis inhibitor. The above results show that angiogenesis formation were inhibited with this type of Ru(II) complex to block breast cancer cell metastasis in vitro and in vivo [42].

2.5. Ru(bpy)$_2$BEDPPZ Cellular Localization

To investigate the potential antitumor mechanism of the Ru(II) complex, cellular localization of Ru(bpy)$_2$BEDPPZ in MDA-MB-231 cells at different times was investigated, and the results are shown in Fig. 5. The intracellular localization of Ru(bpy)$_2$BEDPPZ with red phosphorescence was slightly distributed in the cell nucleus and primarily enriched in the cell cytoplasm, after treatment for 6 h, which showed a mismatch with DAPI. However, with the increase of time, Ru(bpy)$_2$BEDPPZ entered into the cell nucleus. At 24 h, a small quantity of Ru(bpy)$_2$BEDPPZ was discovered in the cell cytoplasm surrounding the cell nucleus (blue phosphorescence) of the MDA-MB-231 cells. At 72 h, however, Ru(bpy)$_2$BEDPPZ was only gathered in the cell nucleus, but perfectly matched with DAPI. The common target for Ru(II) complex to inhibit tumor cells is DNA. Thus, the localization of Ru(bpy)$_2$BEDPPZ in the cell nucleus may play a key role in inhibiting breast cancer metastasis.

2.6. Growth Inhibition of Ru(bpy)$_2$BEDPPZ by DNA Damage-mediated Apoptosis Induction

As Ru(bpy)$_2$BEDPPZ emitted strong red fluorescence, which would interfere the PI signal, we used TUNEL assay to detect the apoptosis induction properties of 5 in MDA-MB-231 cells. Fig. 6 shows that the number of apoptotic cells was obviously decreased by upon the addition of Ru(bpy)$_2$BEDPPZ. Without drug treatment, a small number of apoptotic cells were observed. However, with 20 $\mu$M of Ru(bpy)$_2$BEDPPZ treatment, many apoptotic cells were observed. The apoptotic cells with fragmented DNA can be detected by TUNEL. We posited that Ru(bpy)$_2$BEDPPZ may induce apoptosis of MDA-MB-231 by inducing DNA damage.
In order to further confirm the DNA damage induced by Ru(bpy)2BEDPPZ, we performed comet assay. Moreover, we also detected the induction of DSBs in MDA-MB-231 cells stained with DSB biomarker γH2AX by neutral comet assays and confocal immunofluorescence assays. As indicated by the statistical increase in olive tail moment, Ru(bpy)2BEDPPZ extremely increased the generation of endogenous DSBs in MDA-MB-231 cells (Fig. 7A). The percentages of γH2AX focal positive cells were obviously increased in MDA-MB-231 cells treated with different concentrations of Ru(bpy)2BEDPPZ (0, 5 and 10 μM). These results indicated that Ru(bpy)2BEDPPZ mainly induced apoptosis of MDA-MB-231 cells through DNA damage, which led to endogenous DSBs [43].

3. Conclusions

In this work, we designed and synthesized a class of polypyrrole Ru-DPPZ derivatives by microwave-assisted synthesis technology [44,45]. This class of Ru(II) complexes exerted certain inhibition effects against TNBC cells, especially Ru(bpy)2BEDPPZ, which displayed commendable inhibitory activity against the proliferation, migration, and invasion of triple-negative breast cancer MDA-MB-231 cells. The structure-activity relationship analysis of these results suggested that the increased number of aromatic planar rings in ligands can effectively enhance antitumor activity and provided a guiding significance for further optimization of Ru(II) complexes [46,47]. The results of our mechanistic study showed that Ru(bpy)2BEDPPZ was enriched in the cell nucleus to induce DNA damage, leading to tumor cell apoptosis. Further evaluation of in vivo activity indicated that low concentrations of Ru(bpy)2BEDPPZ effectively inhibited the proliferation and metastasis of MDA-MB-231 cells in zebrafish xenografts. In summary, our study showed that the introduction of a phenylthiophenyl group in Ru(II) complexes can effectively enhance antitumor activity, which provided a guiding significance for optimizing antitumor drugs.

4. Materials and Methods

4.1. Chemicals

All the chemicals and solvents were purchased from commercial vendors. Ruthenium(III) chloride hydrate was obtained from Mitswuha Chemicals. The ligand dipyridyl[3,2-α:2,3'-c]phenazine (DPPZ), 7-bromo-dipyridyl[3,2-α:2,3'-c]phenazine (7-Br-DPPZ), 6-Methyl-1,2-phenylene-amine (6-CH3-DPPZ) and Benzo[i]dipyridyl[3,2-α:2,3'-c]phenazine (DPPN) were synthesized by using the similar method in the literature. Using an Anton Paar monowave 300 microwave reactor we synthesized these complexes. The 1H NMR and 13C NMR spectra were recorded in a dimethyl sulfoxide (DMSO-d6) solution detected by a Bruker DRX2500 spectrometer at room temperature. Both C0 and Cw were tested by the electronic absorption spectra were recorded with an Shimadzu UV-2550, and the partition coefficient (P0/w) for the complex was calculated according to the equation: P0/w = A0/Aw. The images of wound-healing assay and comet assay were captured by Leica DMI8 Inverted Microscope (Leica, Germany). Cellular localization and immunofluorescence experiments were performed with an ZEISS LSM 800 Confocal Laser Scanning Microscope (Zeiss, Germany).

4.2. Synthesis and Characterization

4.2.1. Synthesis of [Ru(bpy)3DPPZ](ClO4)2 ([Ru(bpy)3DPPZ])

According to the literature, Ru(bpy)3DPPZ was synthesized by using similar method but with a few modifications. A mixture of cis-[Ru(bpy)3Cl2]2+ (103.9 mg, 0.2 mmol) and DPPZ (56.4 mg, 0.2 mmol) were heated at reflux for 6 h under N2 atmosphere in 30 mL of 9:1 glycol/water mixture. The solution was cooled to room temperature before the reaction mixture was diluted with 80 mL of water. Then the mixture was filtered, and excess sodium perchlorate was added to give a orange solid. Using cooled water and ether to filter and wash the crude products, and purified by flash silica gel column chromatography to afford a red powder, yields 67%. ESI-MS (in CH3CN, m/z): 348.0 ([M-2ClO4–]2+), 795.1 ([M-ClO4–]3+). 1H NMR (500 MHz, DMSO-d6): δ 9.64 (dd, J = 8.2, 1.2 Hz, 2H), 8.89 (dd, J = 14.6, 8.2 Hz, 4H), 8.53 (dd, J = 6.5, 3.4 Hz, 2H), 8.25 (ddd, J = 9.3, 6.8, 1.0 3 Hz, 4H), 8.21 (dd, J = 6.6, 3.4 Hz, 2H), 8.15 (td, J = 8.0, 1.3 Hz, 2H), 8.04 (dd, J = 8.2, 5.4 Hz, 2H), 7.85 (d, J = 5.2 Hz, 2H), 7.79 (d, J = 5.4 Hz, 2H), 7.65–75.75 (3m, 2H), 7.43–7.38 (m, 2H). 13C NMR (400 MHz, DMSO-d6): δ 155.64 (s), 155.35 (s), 152.28 (s), 150.79 (s), 149.19 (s), 140.78 (s), 139.01 (s), 136.94 (s), 136.83 (s), 132.11 (s), 131.45 (s), 129.05 (s), 128.28 (s), 126.78 (s), 123.36 (s), 123.25 (s).

4.2.2. Synthesis of [Ru(bpy)3Br]7-Br-DPPZ][ClO4]2 ([Ru(bpy)3Br]7-Br-DPPZ)

Ru(bpy)3BrDPPZ was synthesized as the same method, but with 7-Br-DPPZ (72 mg, 0.2 mmol), yields 82%. ESI-MS (in CH3CN, m/z): 387.9 ([M-2ClO4–]2+), 875.0 ([M-ClO4–]3+). 1H NMR (500 MHz, DMSO-d6): δ 9.56 (ddd, J = 14.6, 8.2, 1.2 Hz, 2H), 8.89 (dd, J = 14.2, 8.3 Hz, 4H), 8.70 (d, J = 2.2 Hz, 1H), 8.44 (d, J = 9.1 Hz, 1H), 8.32–8.20 (m, 5H), 8.16 (ddd, J = 8.1, 2.8, 1.4 Hz, 2H), 8.07–8.00 (m, 2H), 7.85 (d, J = 5.5 Hz, 2H), 7.82–7.77 (m, 2H), 7.64–7.60 (m, 2H), 7.41 (ddd, J = 7.0, 5.7, 1.2 Hz, 2H). 13C NMR (500 MHz, DMSO-d6): δ 157.25 (s), 156.98 (s), 154.25 (s), 154.11 (s), 152.41 (s), 151.90 (s), 151.03 (s).
4.2.3. Synthesis of [Ru(bpy)$_2$(7-CH$_3$-DPPZ)](ClO$_4$)$_2$ (Ru(bpy)$_2$MDPPZ)

Ru(bpy)$_2$MDPPZ was synthesized as the same method, but with 7-CH$_3$-DPPZ (59.2, 0.2 mmol), yields 85%.

ESI-MS (in CH$_3$CN, m/z): 28

4.2.4. Synthesis of [Ru(bpy)$_2$(DPPN)](ClO$_4$)$_2$ (Ru(bpy)$_2$BnDPPZ)

Ru(bpy)$_2$BnDPPZ was synthesized as the same method, but with DPPN (66.4 mg, 0.2 mmol), yields 51%.

ESI-MS (in CH$_3$CN, m/z): 4

4.3. Cell Culture

Human breast cancer MDA-MB-231 and MCF-7 cells, human lung adenocarcinoma A549 cells, hepatocellular carcinoma HepG2 and the immortalized human normal epidermal HaCaT cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were cultured by Dulbecco’s modified Eagle medium (DMEM) containing fetal bovine serum (BSA; 10%, HyClone), streptomycin (50 units/mL) and penicillin (100 units/mL).

4.4. MTT Assay

Seeding cells in 96-well tissue culture plates (5 × 10$^4$ cells per well 24 h) and treating with different concentration complex (0, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μM) for 72 h. Then, MTT stock solution (20 μL/well, 5 mg/mL) was added, companied with incubation for another 4 h. Then, aspirated the medium and replaced with 150 μL/well of DMSO, it was measured at 490 nm using a microplate spectrophotometer (Thermo Scientific Multiskan GO).

4.5. Ru(bpy)$_2$BEDPPZ Wound-Healing Assay

MDA-MB-231 cells (1 × 10$^5$ cells/well) were seeded in a 6-well plates with two marked lines on the back. When the cells covered >85% of the culture plate, using a tip (200 μL pipet) to scratch a line on the plate and orthogonal to the mark. Then, treatment with Ru(bpy)$_2$BEDPPZ in different concentrations (0, 2 and 4 μM) in DMEM without FBS and observe migrating cells in the same visual field every 24 h. Based on 10 fields of view for each cell type, the average migration rate and the cell trajectory were computed through Slidebook and Excel software.

4.6. Fluorescein isothiocyanate (FITC)-Conjugated Gelatin Invasion Assay

According to the manufacturer’s instructions (Invitrogen), cells (2 × 10$^5$ per well) treating with Ru(bpy)$_2$BEDPPZ (0 and 5 μM) were plated onto the FITC-gelatin-coated coverslips and incubated (37 °C, 24 h). Using laser confocal microscope evaluated and photographed the FITC-gelatin degradation status [48].

4.7. Immunofluorescence

Adding cell suspension culture on 1 cm diameter cover glass which in six-well (Fisher Scientific, Pittsburgh, PA). Then, using 4% formaldehyde solution fixation for 25 min, and cover glass was cultured with these reagents: 5% BSA for 30 min, 1:100 rabbit monoclonal Anti-Paxillin antibody (ab32115) for 60 min, 1:200 Alexa Fluor 488 -conjugated Affinipure Goat Anti-Rabbit IgG (H + L) (proteintech) for 30 min, 1: 100 Rhodamine phalloidin (Amanita phalloides, cytoskeleton- (for F-actin) for 30 min, and stained with DAPI (0.5 μg/mL, 8 min). Using PBS wash two times in each incubation step, then it were transferred onto a microscopic slide, which observed under a laser confocal microscope.

4.8. Tube Formation Assay

Dissolving matrigel (4 °C, 24 h) and it were coated with prechilled 96-well plates (50 μL/well, 37 °C and stay at least 60 min). Then, the unsolid fluid were removed, HUVECs at the density of 3 × 10$^4$ were cultured in DMEM with VEGF (200 ng/mL) containing the different concentrations of Ru(bpy)$_2$BEDPPZ (0, 5 and 10 μM) for 6 h. Analyze and capture these images [49].

4.9. Evaluation of the Angiogenic Effect In vivo

24 hpf transgenic Tg(fox1a:EGFP) zebrafish embryos were dechorionated ata petri dishing protease, then randomly distributed into 6-well microplate, treated with Ru(bpy)$_2$BEDPPZ (0, 1.25, 2.5 and 5 μM). After 48 h treatment at 28.5 °C, using fresh water to wash zebrafish larvae for two times and then transferred into the 96-well plate, the subintestinal vessel plexus (SIVs) of zebrafish larvae were observed and imaged under the fluorescence microscope. The number of SIVs of each zebrafish was measured via manual counting, average number of SIVs of zebrafish larvae was calculated in each group [50].
4.10. TUNEL Assay

Cells were fixed by using 4% p-formaldehyde in PBS for 30 min and permeabilized simultaneously with 0.3% Triton X-100 in PBS (25 °C, 8 min). Then, adding TUNEL reaction mixture (100 μL/well) into cells for 1 h and under dark condition incubate DAPI (0.5 μg/mL, 37 °C, 8 min). Using fluorescence microscope observe cell morphology.

4.11. Cellular Localization

MDA-MB-231 cells (5 × 10^6 cells/mL) were incubated with Ru(bpy)2BEDPPZ for 6, 24, 72 h at 37 °C. Then, PBS wash these cells two times, using 4% p-formaldehyde fixed for 30 min, after stained with DAPI (0.5 μg/mL, 8 min). Finally, using confocal laser microscope to observe the cell morphology.

4.12. Single Cell Gel Electrophoresis

MDA-MB-231 cells (5 × 10^6 cells/mL) were incubated with Ru(bpy)2BEDPPZ (0, 5 and 10 μM) for 72 h. According to 1:7 percentage mix with cell suspension and melted LM agarose. Pipetting the mixture (80 μL) to the slide (Comet Slide TM). Using prechilled lysis solution (4 °C 60 min) immerse the slide before refrigeration for 10 min, and it was wash PBS twice times. Next, using freshly prepared alkaline solution (6 g NaOH, 0.25 mM EDTA (2.5 mL), pH = 13) immerse for 20 min, and it was suffered alkaline solution for electrophoresis (25 V 30 min),when the DNA unwinding. After, using PBS wash the slide twice, DNA was stained with EB after observed under a fluorescence microscope.

4.13. Growth and Metastasis Inhibition of Ru(bpy)2BEDPPZ in vivo

MDA-MB-231 cells were labeled fluorescence by Dil and gathered at a density of 10^7 cells/mL. Then, cells (50–500 cells) were injected into the perivitelline space near the subintestinal vessels (SIVs) of the transgenic zebrafish (fltl:EGFP) embryos at 48 hpf to establish this zebrafish embryo breast cancer model. Juvenile zebrafish of breast cancer model (48 h old) were incubated in 6-well plates (10 fishes in every well) with 2 mL solutions without or with Ru(bpy)2BEDPPZ (0 and 5 μM) in aquaculture water. The inhibitory effect of Ru(bpy)2BEDPPZ in breast cancer zebrafish were captured pictures every 24 h with a fluorescence microscopy [32,51].

Acknowledgments

The authors acknowledge the National Natural Science Foundation of China (Grant Nos. 81572926, 81703349). The China Postdoctoral Science Foundation (Grant No. 2017M610576). The Provincial Major Scientific Research Projects in Universities of Guangdong Province (Grant No. 2014KZDXM053), The Science and Technology Project of Guangdong Province (Grant No. 2014A020121312), The Innovation Projects in Universities of Guangdong Province (2015CXXQ151); Tradition Chinese Medicine Bureau of Guangdong Province (20151265), the Innovation Team Projects in Universities of Guangdong Province (2016KCXTD018), the Projects of Guangzhou Key Laboratory of Construction and Application of New Drug Screening Model Systems (No.201805010006) and Key Laboratory of New Drug Discovery and Evaluation of ordinary universities of Guangdong province (No. 2017KSYS002).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2018.11.010.

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