An osmium-peroxo complex for photoactive therapy of hypoxic tumors

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Article

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Abstract

Its limited therapeutic effect on hypoxic and refractory solid tumors has hindered the practical application of photodynamic therapy (PDT). Herein, we report our investigation of an osmium-peroxo complex (**Os2**), which is inactive in the dark, but upon light irradiation, can release a peroxo ligand $O_2^{\cdot-}$, and is transformed into a cytotoxic osmium complex (**Os1**). The osmium-peroxo complex **Os2** produces $O_2^{\cdot-}$ under light irradiation even in the absence of oxygen, and retains its phototoxicity in hypoxic tumors. **Os1** is cytotoxic in the presence or absence of irradiation, behaves as a chemotherapeutic drug. The light-activated **Os2** induces distinct ferroptosis, which is mediated by GSH degradation, lipid peroxide accumulation and down-regulation of glutathione peroxidase 4 (GPX4). In addition, **Os2** causes photocatalytic oxidation of endogenous 1,4-dihydronicotinamide adenine dinucleotide (NADH) in living cancer cells, leading to ferroptosis. *In vivo* studies have confirmed that the **Os2** can effectively inhibit the growth of solid hypoxic tumors in mice. A new strategy is proposed for the treatment of hypoxic tumors with metal-based drugs.

Introduction

The hypoxic microenvironment of solid tumors caused by abnormal proliferation and vascularization of cells can greatly compromise the therapeutic effect of traditional photodynamic therapy (PDT),^[1] which usually requires O_2 for the production under light activation of reactive oxygen species (ROS) by a photosensitizer (PS).^[2] Many innovative methods have recently been introduced to solve the problem of tumor hypoxia. Some additives such as perfluorocarbons, artificial red blood cells and covalent organic frameworks (COF),^[3] carry O_2 directly to the tumor site, and others utilize unique *in situ* features of the tumor microenvironment to generate O_2 . These include CaO_2 and graphite phase-carbon nitride (gC_3N_4) carbonization^[4] or high H_2O_2 concentrations in tumor cells resulting from MnO_2 or H_2O_2 decomposition^[5], or even photocatalytic oxygen production by photosynthetic bacteria.^[6] The results however are still not ideal and there are numerous possible adverse side effects.^[7] In addition, *in vivo* studies have shown that enrichment of O_2 promotes proliferation and inhibits apoptosis of malignant cells, results which are not conducive to PDT.^[8] Therefore, improvement of the efficacy of phototherapy is necessary.

Photoactive chemotherapy (PACT) has the potential to overcome the limitations imposed by hypoxia. In PACT, metal complexes can be photoactivated in a controlled manner to produce cytotoxic substances.^[2a] For example, Chao et al. designed an iridium (III) complex that upon irradiation, produces free carbon radicals.^[9] Toxic gases such as NO ^[10] and CO ^[11], or ligand-centered cytotoxic substances can also be released by photoactivation.^[12] The superoxide radical ($O_2^{\cdot-}$) is one of the most toxic ROS and has been identified as a most useful oxidant for cancer treatment and an adjuvant in synergistic chemotherapy.^[13] Under the action of intracellular superoxide dismutase, $O_2^{\cdot-}$ can form H_2O_2 and O_2 . The accumulated H_2O_2 is further transformed into a hydroxyl radical ($OH\cdot$) with enhanced toxicity and reactivity, exacerbating oxidative damage of cancer cells and improving the anticancer effect.^[14] The design of a

photoactive compound that does not rely on O_2 but is photo-controlled to release ROS has significant application prospects in hypoxic tumor treatment.

We have investigated an osmium-peroxo complex (**Os2**), whose structure significantly improves the thermal stability of the osmium-peroxo moiety.^[15, 16] Upon photoactivation with 465 nm light, **Os2** releases $O_2^{\bullet -}$ even in severely hypoxic conditions (1% O_2), and is transformed into the cytotoxic **Os1** in a Cl^- containing PBS solvent (**Figure 1a**), thus maintaining useful photoactivation efficacy in hypoxia. **Os1** displays toxicity in both light and dark, contributing to a synergistic effect of chemotherapy and photoactive chemotherapy of **Os2** under light irradiation. We also found that irradiation of **Os2** induces distinct ferroptosis mediated by GSH degradation, lipid peroxide accumulation and GPX4 down-regulation. Furthermore, **Os2** can photocatalytically oxidize endogenous NADH in living cancer cells, triggering ferroptosis. *In vivo* studies confirm that **Os2** effectively inhibits the growth of solid hypoxic tumors in mice. Thus this work is developing a new process to release $O_2^{\bullet -}$ for the treatment of hypoxic tumor cells through the mechanism of ferroptosis.

Results

Photoactivation property

The synthesis of the osmium-peroxo complex **Os2** was based on our previously reported method,^[16] and the characterization data can be found in **Figures S1-S3** in the Supporting Information (SI). **Os2** is highly stable in phosphate-buffered saline (PBS) at pH = 7.4 or in Dulbecco's Modified Eagle Medium (DMEM) cell culture media in the dark at room temperature (rt) (**Figures S4 and S5**), and is also stable in the presence of reducing agents such as NADH, GSH, or Cys (**Figure S5**). However, with 465 nm light irradiation, the solution of **Os2** undergoes a gradual color change from brown to light pink (**Figure 1b**, insets), which led us to investigate this transformation in detail. High-resolution mass spectrometry (HRMS) measurements were performed to identify the photolytic products, and a prominent peak was observed at $m/z = 1175.2351$ (**Figure S6**). The mass and isotope distribution in this ion correspond well to **Os1**. The formation of **Os1** was further evidenced by HPLC analysis. As shown in **Figure 1c** (blue line), the retention time of the new peak is consistent with that of the independently synthesized **Os1** (**Figures S7-S11, Tables S1-S2**. See also SI for the synthesis and characterization of **Os1**). These results show that **Os1** is one of the main photoproducts of **Os2**.

ROS detection

Due to the dissociation of O_2 unit from the metal center of **Os2** under light irradiation, we sought to verify that the O_2 unit is released in the form of a superoxide anion ($O_2^{\bullet -}$). We first tried to detect $O_2^{\bullet -}$ by using the non-fluorescent dihydrorhodamine 123 (DHR123) probe,^[17] which can react with $O_2^{\bullet -}$ and emit strong green fluorescence around 526 nm. As shown in **Figure 2a** and **Figure S12**, when irradiated at 465 nm (13 mW/cm²), the solution of **Os2** showed an increasing emission at 526 nm, suggesting the generation of

$O_2^{\cdot-}$. We also simulated the Fenton reaction process.^[18a] When **Os2** (15 μ M) and a small amount of superoxide dismutase (SOD) were added into a PBS solution containing Fe^{2+} , the absorbance of methylene blue probe (MB, 5 μ g/mL) at 665 nm decreased with a degradation rate of 70.26 % (**Figure S13**). On the contrary, in control experiments using only **Os2** and **Os2**+SOD, respectively, no changes were observed in the characteristic UV-Vis absorption band of MB. On the basis of these findings and published data, we propose the mechanism in **Figure 2f**. Under the action of SOD enzyme, $O_2^{\cdot-}$ disproportionately generates H_2O_2 and O_2 . The $OH\cdot$ is generated from H_2O_2 under the action of Fe^{2+} , and then reacts with MB, thus decreasing its absorption. These findings confirmed that **Os2** can produce $O_2^{\cdot-}$ under light irradiation.

To gain more insight into the nature of ROS, we made electron spin resonance (ESR) measurements using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and (2,2,6,6-tetramethylpiperidine)oxyl (TEMPO) as spin traps. Specifically, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)^[18] was used to trap $O_2^{\cdot-}$ generated by **Os2** under 465 nm light irradiation. After the mixture containing DMPO and **Os2** was exposed to light irradiation, several signal peaks of DMPO-OOH appeared in the 3440 – 3480 G (**Figure 2b**), which explained the generation of $O_2^{\cdot-}$. In order to study whether **Os2** produces 1O_2 under light irradiation, we used 2,2,6,6-tetramethylpiperidine (TEMP) to measure 1O_2 generation.^[19] As shown in **Figure 2c**, a three-line signal with the intensity of 1:1:1 between 3480 and 3530 G was observed in the ESR spectrum of **Os2** and TEMP mixing solution under light irradiation. A similar signal was observed in the presence of **Os1** (the light product of **Os2**). These ESR results suggest that **Os2** can generate both $O_2^{\cdot-}$ and 1O_2 under light irradiation.

In addition, 9,10-anthracenediyl-bis-(methylene) dimalonic acid (ABDA) was further used to detect the 1O_2 quantum yields of **Os1** and **Os2** under light irradiation. As shown in **Figures 2d** and **S14**, the characteristic absorption peak of ABDA at about 378 nm decreased gradually as the irradiation time increased. The 1O_2 quantum yields of **Os1** and **Os2** were calculated as 0.044 and 0.045 by comparison with $Ru(bpy)_3^{2+}$ (**Figure S14** and **Table S3**).

The above experiments were all conducted under aerobic condition. Next, we studied the production of ROS in the absence of oxygen (hypoxia, deaeration with nitrogen). As shown in **Figure S15**, the emission spectra showed enhancing green fluorescence in the presence of **Os2** and DHR123 probe. This result proves that **Os2** also produces $O_2^{\cdot-}$ under hypoxia. We further measured whether 1O_2 generation under hypoxia. As shown in **Figure S16**, the absorption of ABDA in the presence of both **Os1** and **Os2** failed to change obviously under hypoxia, confirming that no 1O_2 generation occurred under hypoxia. However, the absorption of ABDA was reduced in the presence of **Os2** + SOD. This is due to **Os2** produces $O_2^{\cdot-}$ under hypoxia, and that $O_2^{\cdot-}$ disproportionately generates H_2O_2 and O_2 under the action of the SOD enzyme, the O_2 being available for 1O_2 generation by the photoproduct **Os1** (**Figure 2f**). However, **Os1** is unable to produce any ROSs under hypoxia (**Figure S16**).

Generation of cellular ROS

We studied the photo-induced ROS generation ability of **Os2** in living HeLa cells. Dihydroethidium (DHE), hydroxyphenyl fluorescein (HPF) and singlet oxygen sensor green (SOSG) were used as probes to monitor the production of $O_2^{\cdot-}$, $OH^\bullet$ and 1O_2 , respectively.^[18a,b] The detection mechanisms of these probes for various free radicals are shown in **Figure 3a**. Confocal microscopy showed that when **Os2**-incubated HeLa cells were exposed to light under normoxia (20% O_2 , 465 nm, 13 mW/cm², 1 h), the fluorescence signal in DHE-stained cells is significantly enhanced (**Figure 3b,c**). This confirms the ability of **Os2** to release $O_2^{\cdot-}$ in cells under light irradiation. Under hypoxia (1% O_2), the fluorescence intensity after light irradiation was similar to that obtained under normoxia, indicating that the process of $O_2^{\cdot-}$ generation from **Os2** is independent of O_2 . This is different from the mechanism of O_2 -dependent type I photodynamic mechanism.

Because it is known that $O_2^{\cdot-}$ can produce $OH^\bullet$ in the presence of intracellular SOD enzyme and Fe^{2+} ions,^[18a] we further studied the generation of $OH^\bullet$ in the cells by HPF staining. Confocal imaging showed that **Os2** can produce $OH^\bullet$ in HeLa cells under normoxia or hypoxia (**Figures 3b, 3c**). In addition, we also compared the ability of 1O_2 generation of **Os2** and the photoproduct **Os1** by using a SOSG probe, and found that under hypoxia, **Os2** has more advantages in producing 1O_2 than **Os1**. The result showed that the green fluorescence in the **Os2** treated cells is strong under both normoxia and hypoxia. However, the fluorescence in the **Os1** treated cells was much weaker under hypoxia than that observed under normoxia (**Figure S17**). These results are consistent with those in **Figure 2f**, showing that **Os2** firstly induces the release of $O_2^{\cdot-}$ and production of **Os1**, accompanied by the formation of O_2 (as an oxygen source for **Os1**) and $OH^\bullet$ by a Fenton reaction. Finally 1O_2 is generated by the photoproduct, **Os1**. These data all show that **Os2** may be useful as a new effective drug for photoactive treatment of hypoxic tumor cells.

Phototoxicity *in vitro*

The dark- and photo-cytotoxicities of **Os2** and **Os1** against HeLa cells were determined with an MTT method.^[19] As showed in **Figure 4** and **Table 1**, **Os2** had low dark-cytotoxicity and high photo-cytotoxicity under both normoxia and hypoxia (**Figures 4a, 4c**). The $(IC_{50})_{\text{dark}}$ values, determined with only dark incubation, were 89.2 and >100 μM , respectively (**Table 1**). The $(IC_{50})_{\text{Light}}$ values of **Os2**, determined by photoirradiation under normoxia or hypoxia were 1.23 and 5.86 μM , respectively. The photo-cytotoxicity under hypoxia can be explained in terms of the release of $O_2^{\cdot-}$ by **Os2** and light irradiation, followed by the production of O_2 , which may be used by the light product **Os1** to generate 1O_2 , and $OH^\bullet$ with stronger toxicity *via* the Fenton reaction. In contrast, the light product (**Os1**) shows high dark-cytotoxicity and photo-cytotoxicity under both normoxia and hypoxia (**Figures 4b, 4d**). The $(IC_{50})_{\text{dark}}$ values of **Os1** under normoxia and hypoxia are 8.12 and 9.95 μM , and the $(IC_{50})_{\text{Light}}$ values under normoxia and hypoxia are 1.31 and 7.51 μM , respectively (**Table 1**). This result suggests that **Os1** is high photo- and dark- cytotoxic, and can be seen as a chemotherapeutic drug. However, with hypoxia, due to the lack of oxygen, **Os1**

shows no obvious photodynamic effect. The PI value of **Os2** (>18) under hypoxia is much higher than that of **Os1** (1.3).

Staining with alcein acetoxymethyl ester (Calcein-AM) or propidium iodide (PI) were also used to distinguish living cells (green) from dead cells (red). As shown in **Figure S18**, the dark group of **Os2** in normoxia and hypoxia shows strong green fluorescence (living cells) but no red fluorescence (dead cells), and the control group in the dark or under light irradiation also shows no dead cells. In contrast, the light group of **Os2** shows weak green fluorescence and strong red fluorescence, indicating that **Os2** leads to a large number of dead cells under light irradiation.

Table 1. The dark- and photo-IC₅₀ values of **Os2** and **Os1** against HeLa cells under normoxia and hypoxia.

Compounds	Dark [μ M]	Light ^[c] [μ M]	PI ^[d]
Os2 ^[a]	89.2±2.6	1.23±0.09	72.5
Os2 ^[b]	100	5.86±0.19	18
Os1 ^[a]	8.12±0.26	1.31±0.05	6.2
Os1 ^[b]	9.95±0.14	7.51±0.35	1.3

[a] under normoxia (20% O₂); [b] under hypoxia (1% O₂); [c] photoirradiation was imposed (465 nm, 13 mW/cm², 1 h) after 8 h of the complexes incubation; [d] photocytotoxicity index, the ratio of (IC₅₀)_{dark}/(IC₅₀)_{Light}.

Ferroptosis mechanism

It has been reported that the photo-chemical process of ROS generation could cause ferroptosis in cancer cells.^[2e, 20] This led us to consider if **Os2** can induce ferroptosis. GSH is closely related to ferroptosis,^[21] and we first detected the ability of **Os2** to consume GSH. As shown in **Figures 5a-5b**, with an increase of light irradiation time, the absorption at 412 nm decreased, indicating that **Os2** when irradiated, could consume GSH. We determined the GSH levels in the cells after treatment with **Os2**, and found that the GSH level in the irradiated group was significantly lower than that in the non-irradiated group (**Figure 5c**). Therefore, we concluded that **Os2** can consume cellular GSH under irradiation conditions.

Ferroptosis is a type of iron-dependent cell death caused by excessive lipid peroxidation. The main feature of ferroptosis is that after the inactivation of cell antioxidant capacity, phospholipids containing polyunsaturated fatty acids are peroxidated on the cell membrane, destroying the cell membrane and leading to ferroptosis.^[22] The antioxidant glutathione peroxidase 4 (GPX4) specifically catalyzes loss of oxidative activity in the lipid peroxides in a glutathione-dependent manner,^[23] and subsequent inhibition of GPX4 induces ferroptosis. GSH consumption can indirectly inhibit the expression of GPX4. We speculated that **Os2** could further inhibit the expression of GPX4 and we verified this hypothesis by

western blot analysis of GPX4. As shown in **Figures 5f-5g**, **Os2** fails to reduce the expression of GPX4 in the dark. Upon light irradiation, **Os2** significantly reduces the expression of GPX4, and the GSH consumption caused by ROS and the inhibition of GPX4 will further lead to the accumulation of lipid peroxides and induce ferroptosis. We used C11-BODIPY as a lipid peroxide probe with which to monitor intracellular accumulation of lipid peroxides (**Figures 5d, 5e**). Confocal microscopy showed that the fluorescence of HeLa cells treated with **Os2** was significantly enhanced after exposure to light, indicating a significant accumulation of lipid peroxides, which could be effectively inhibited by Ferrostatin-1 (Fer-1, a ferroptosis inhibitor). All the above results confirm that **Os2** induces ferroptosis as shown in **Figure 5h**.

NADH photocatalytic oxidation

As a cofactor, 1,4-dihydro-nicotinamide adenine dinucleotide (NADH) regulates the redox balance of cellular mitochondria, and plays an important role in regulating energy production. If NADH is oxidized to NAD^+ , it can destroy the whole respiratory chain and kill cells.^[24] We studied whether NADH can be oxidized by **Os2** under light irradiation, which could provide a photocatalytic oxidation pathway to kill cancer cells. The photocatalytic efficiency of **Os2** (20 μM) towards NADH (175 μM) was first determined by UV-Vis absorption spectroscopy. As shown in **Figure 6a**, the absorbance at 339 nm decreases and the absorbance at 259 nm increases gradually with increase of the irradiation time. In contrast, the absorption of the non-illuminated **Os2** group exhibits no obvious changes (**Figure S19**). This indicates that **Os2** could reduce the enzyme activity of NADH under light irradiation. We also calculated the NADH oxidation turnover number (TON) of **Os2** at 339 nm to evaluate its photocatalytic efficiency. The TON value of NADH oxidation by **Os2** under light irradiation is 3.808, which is 30-times higher than that of the non-illuminated group (**Figure 6b**). Similarly, **Os1** also shows a similar photocatalytic oxidation effect on NADH (**Figure S20**).

The photocatalytic oxidation of NADH was also monitored by ^1H NMR in $\text{D}_2\text{O}/\text{CD}_3\text{OD}$ (1/3, v/v) at 298 K. As shown in **Figure 6c**, In the **Os2** and NADH illuminated group, new peaks from hydrogens on the nicotinamide ring of NAD^+ are observed at 6.13, 8.31, 8.55, 8.99, 9.36 and 9.58, but no new NAD^+ peak is observed in either the non-illuminated group or the non-**Os2** group (**Figure 6c**). Intuitively, this shows that **Os2** could oxidize NADH under light irradiation, transforming it into NAD^+ . Subsequently, we measured the NADH photocatalytic oxidation ratio at the cellular level using a NAD/NADH-GloTM method, which is a bioluminescence method for the detection of NAD^+ and NADH. As shown in **Figure 6d**, the chemical luminescence intensity of **Os2**-light group proved to be lower than that of other groups. These results show that **Os2** can effectively oxidize NADH at the cellular level by light irradiation, thereby killing cancer cells. As can be seen in **Figure 6e**, NADH can not only be converted to NAD^+ by interacting with Os1^* (the excited state of **Os1**), but can also be oxidized to NAD^+ by $\text{O}_2^{\cdot-}$ released from **Os2**. The down-regulation of NADH indirectly aids the reduction of oxidized glutathione (GSSG) to GSH by glutathione reductase (GR),^[25] resulting in the accumulation of lipid peroxides, and ultimately achieving the synergistic induction of ferroptosis.

Photoactive antitumor therapy *in vivo*

We studied the feasibility of photoactive therapy *in vivo* by Os2. Since Os2 is a small molecule and there is no specific targeted group, it can be administered intratumorally. As shown in Figure 7b, compared with the other three groups, the tumor growth in mice treated with Os2-light group was inhibited. The tumor size of the Os2-light group was the smallest in the four groups (Figure 7d), and the average tumor weight of the Os2-light group was significantly lower than that of the other groups (Figure 7c). The tumor tissues after final treatment were collected for histological assessment. The hematoxylin and eosin (H&E) staining showed obvious destruction of tumor tissues in the Os2-light group, while tumor tissues in the other three groups were not affected (Figure 7e).

In order to evaluate the biological safety of Os2, we first analyzed the H & E staining slices of the main organs of healthy mice i.v. injected with three times of the therapeutic dose (2.69 mg kg^{-1}). The results showed no obvious tissue damage in these slices (Figure S21). We also used Singapore wild-type zebrafish to test the biological safety of Os2 (Figure S22) with the green fluorescent protein (GFP) commonly used as a biomarker to visualize the physiological processes. After 5 days of incubation with Os2, the blood vessels of the zebrafish were apparently not damaged, showing good biocompatibility *in vivo*.

Conclusions

In summary, we have demonstrated that an osmium-peroxo complex (**Os2**) can release $\text{O}_2^{\cdot-}$ under light irradiation in the absence of O_2 , and at the same time is transformed into another active osmium complex (**Os1**), which exhibits both chemotherapeutic and photodynamic properties, thus maintaining good phototoxicity in hypoxic tumors. The osmium-peroxo complex **Os2** can induce ferroptosis, which is characterized by GSH degradation, GPX4 down-regulation and lipid peroxide accumulation. In addition, under light irradiation, the same osmium-peroxo complex oxidizes NADH into NAD^+ , further helping induction of ferroptosis. At the *in vivo* level, the osmium-peroxo complex achieves highly effective photoactive therapy of solid hypoxic tumors. This study reports the first example of a metal-peroxo complex for O_2 -independent photoactive therapy and provides a promising strategy for combating hypoxic tumors.

Declarations

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Author contributions

N. L., J. G., H. X., and P. Z. designed the study. Z. D. synthesized and characterized the complexes. N. L. and C. L. performed the experiments in vitro and in vivo. N. L., Z. D., J. G., H. X. and P. Z. analyzed the data and wrote the paper. All authors contributed to the general discussion.

Conflict of interests

The authors declare no conflict of interests.

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Figures

Figure 1

(a) Schematic diagram of light-mediated Os2 release of $\text{O}_2^{\bullet-}$ and Os1. (b) The UV-Visible spectral changes of Os2 (20 μM) in PBS solution (pH = 7.4, 1% DMSO) under light illumination (465 nm, 13 mW/cm²) at rt. (c) HPLC analysis of Os2 in PBS solution (1% DMSO) in the dark or after irradiating for 60 min. Mobile phase was CH₃CN and water (v/v 1:1). $\star$: Individual control of Os1.

Figure 2

(a) The emission spectra for monitoring O₂^{•-} generation by Os2 (10 μM) using DHR 123 probe (λ_{ex} = 500 nm) in normoxia at 298 K. (b) The ESR signal of O₂^{•-} trapped by DMPO after light irradiation in normoxia. (c) The ESR signal of 1O₂ trapped by TEMP after light irradiation in normoxia. (d) The UV-Vis absorption spectra of ABDA for monitoring of 1O₂ generated by Os2 under light irradiation in normoxia. (e) The plot of A₀-A of ABDA at 378 nm versus irradiation time in the presence of Os1 or Os2 in hypoxia or normoxia. (f) Schematic diagram of generating O₂^{•-}, 1O₂ and OH[•] by light-activating Os2. Light: 465 nm, 13 mW/cm².

Figure 3

(a) Schematic diagram of detection of O₂^{•-}, OH[•] and 1O₂ with DHE, HPF and SOSG probes, respectively. (b) Confocal microscope images of O₂^{•-}, OH[•] and 1O₂ in HeLa cells under normoxia (20% O₂) or hypoxia (1% O₂) probed by DHE, HPF and SOSG, respectively. (c) The average fluorescence intensities calculated

from the images in b. (** $P \leq 0.05$, *** $P \leq 0.001$). HeLa cells incubated with Os2 (20 μM) for 8 h, and then treated with 10 μM of DHE, HPF or SOSG for 30 min. DHE: $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 600 \pm 30 \text{ nm}$; HPF: $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 530 \pm 30 \text{ nm}$; SOSG: $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \pm 30 \text{ nm}$. Scale bar: 20 μm .

Figure 4

The viability of HeLa cells treated with different concentrations of Os2 or Os1 in the dark or upon light irradiation under normoxia (20% O_2) or hypoxia (1% O_2).

Figure 5

(a) Irradiation time-dependent GSH depletion by Os2 upon blue light irradiation and (b) the absorption at 412 nm was decreased by increasing the irradiation time. (c) The GSH levels in cells after different treatments. (d) The fluorescence images of lipid peroxides in the treated cells detected by C11-BODIPY probe. C11-BODIPY: $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 570 \pm 80 \text{ nm}$. (e) The average fluorescence intensities calculated from the images in (d). (f) Western blot analysis of GPX4 in HeLa cells after treatment with Os2 with or without light treatment. RSL3 is the positive control group. Light: 465 nm, 13 mW/cm². (g) The relative expression levels of GPX4 calculated from (f). (h) The process of ferroptosis in this photoactive antitumor therapy.

Figure 6

(a) Reaction of Os2 (20 μM) and NADH (175 μM) in PBS solution under blue light irradiation monitored by UV-Vis spectra at 298 K. (b) TON of Os2 under dark or irradiation conditions. (c) Photocatalytic oxidation of NADH (3.5 mM) by Os2 (0.25 mM) under dark or irradiation conditions monitored by ¹H NMR spectroscopy. Peaks associated with blue triangles represent NADH, those with red squares represent NAD⁺. (d) NADH concentrations in the treated HeLa cells. (e) Schematic diagram of the photocatalytic oxidation of NADH by Os2 and followed by induced ferroptosis. Light: 465 nm, 13 mW/cm².

Figure 7

(a) Schematic of the in vivo (HeLa tumor-bearing Balb/c mice) therapeutic protocol. Mice were irradiated by light (465 nm, 13 mW/cm²) for 60 min after i.t. injection with 25 μL of PBS containing 500 μM Os2. (b) Tumor growth curves after treatment. Error bars are standard errors calculated based on five mice/group. (c) Tumor weights of mice at day 16 after various treatments. (d) The digital photos of representative mice after various treatments. (e) H&E staining images of HeLa tumor tissues in mice after various treatments. *** $P \leq 0.001$.

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