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A Concerted Redox- and Light-Activated Agent for Controlled Multimodal Therapy against Hypoxic Cancer Cells

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1. Introduction

Selective activation of a prodrug at a target site is a promising molecular design strategy to boost therapeutic efficacy and alleviate systemic toxicity.[i] In relation to the design of anticancer drugs, tumor microenvironment characteristics such as high levels of thiols,[ii] elevated reactive oxygen species (ROS),[iii] and overexpression of enzymes[iv] have been extensively exploited as specific stimuli for tumor-targeted therapy. Among them, hypoxia represents a compelling tumor therapeutic target because of its major contribution to tumor progression, resistance, and recurrence and its unique prevalence compared to healthy tissues.[v] Tumor hypoxia originates from the rapid proliferation of cancer cells and reliance on oxidative metabolism for energy production, as well as the unsound, erratic neovasculature. In such a hypoxic tumor microenvironment, intramolecular reductases such as nitroreductase, azoreductase, and DT-diaphorase are overexpressed.[vi]

Typically, molecular engineering that confers targeting hypoxia to a prodrug involves the use of a classic substrate, such as nitrobenzene,[vii] nitroimidazole,[viii] quinone,[ix] or azobenzene[x] which can undergo a selective enzymatic reduction in hypoxic tissues. The plethora of hypoxia-responsive prodrug examples reported early on lacked reporting mechanisms such as photoluminescence or photoacoustic signals and thus cannot give diagnostic feedback. Smart prodrugs that provide a combination of diagnostics and therapy, “theranostic agents”, are of recent origin and have received a surge of research interest.[i] In recent years, various hypoxia-responsive theranostic prodrugs have been reported, and in general, they act through the release of an organic fluorophore and a biologically active species such as a chemotherapeutic drug or photodynamic agent (which kills cells via photoactivated conversion of $O_2$ to $^1O_2$) upon the reduction of the conjugated enzyme substrate motif. However, almost all the reported molecular theranostic prodrugs hitherto have involved only a mono-therapeutic mode. Considering the complexity, diversity, and heterogeneity of hypoxic cancer tissues, prodrugs that integrate multimodal therapy are highly desired to overwhelm the capability of hypoxic cancer cells to resist death.[x]
In this regard, photoactive molecules are appealing and offer promise for providing an effective tandem therapy that is less subject to cross-resistance with existing pharmaceuticals. Such photosensitizers exert therapeutic efficacy via excited states which often have significantly different properties compared to traditional chemotherapeutics.\(^{[11]}\)

Photoactive Ru(II)-based complexes have attracted significant attention in recent years because they have excellent redox and photophysical properties and an associated broad range of light-driven anticancer bioactivities, from photodynamic therapy (PDT) and photochemotherapy (PCT) to photoredox catalysis. Of note is the recent discovery that excited-state Ru(II) polypyridyl complexes are capable of catalyzing oxidation of nicotinamide adenine dinucleotide (NADH) by oxygen.\(^{[12]}\) NADH is an essential coenzyme that participates in over 400 intracellular redox reactions\(^{[13]}\) and is involved in maintaining the redox balance and neutralizing ROS damage in cells. Targeted photoredox catalysis for NADH oxidation can readily tilt the redox balance and elicit cell death.\(^{[4a,14]}\) In addition to these, many Ru(II) complexes have relatively short retention times in living organisms, which can be advantageous for photocatalyzed anticancer therapy. Indeed, the Ru(II) polypyridyl complex, TLD-1433, is currently in clinical trials for PDT anticancer therapy.\(^{[15]}\)

Nitrogen mustards (NM) containing chloroethylamine groups were among the first chemotherapeutic anticancer agents, the classic example being chlorambucil. They are powerful in triggering persistent DNA damage due to their alkylation of nucleophilic sites, especially guanine N7 in DNA, thus forming intra-/inter-strand DNA as well as DNA-protein crosslinks. Unfortunately, most patients become refractory to NM treatment after long-term chemotherapy due to the emergence of resistance. Interest in NMs has also waned because of their significant systemic toxicity. This situation can be rectified by rerouting the drug to mitochondria to bypass the complicated interlocking repair mechanisms for nuclear DNA and thus circumvent resistance, and also by caging the agent in a prodrug to reduce off-target toxicity.\(^{[16]}\) Indeed, molecular re-engineering of the anticancer agents is needed to introduce novel mechanisms of action to combat resistant cancers effectively.

Herein we have designed a novel hypoxia-activated, Ru(II)-based, and mitochondria-accumulated multimodal theranostic prodrug RuAzNM (Scheme 1). The prodrug’s phosphorescence is quenched through photoinduced electron transfer (PeT) to the azo group, and the NM is stabilized and deactivated by the electron-withdrawing effects on the nitrogen lone pair of both the electron-deficient azo group and Ru(II). Moreover, due to its quenched excited state, the prodrug is non-photoactive in both PDT and photoredox catalysis. However, when the prodrug is exposed to hypoxia in tumor cells, it accumulates in mitochondria. Meanwhile, the azo group is reduced selectively by the reductases in hypoxic cells in an oxygen-sensitive manner to their corresponding primary amine products. This gives rise to two new species in hypoxia: the aniline mustard and the parent polypyridyl complex RuNH₂. The aniline mustard is an active

Scheme 1. Chemical structures of RuAzNM and related fragments, and schematics for the proposed mechanism of action.
alkylation agent which can exert a chemotherapeutic influence on hypoxic cancer cells by triggering ROS generation and damaging mtDNA. Now, in the absence of the PeT effect from the azo group, the Ru(II) center can exhibit phosphorescence and be used to probe hypoxia in cells. In addition, RuNH₂ is highly active in PDT and photoredox catalysis due to: 1) the lifetime of its excited state which is dramatically lengthened in hypoxia; 2) its more favorable excited-state photoredox potential to drive the photocatalytic cycle; 3) its superior binding affinity toward NADH over the prodrg. Hence, accompanied by an emission-directed photoradiation, multimodal therapeutic effects are selectively implemented in hypoxic cells and lead to a ROS outburst, NADH and ATP deprivation, mitochondrial membrane potential loss, mtDNA depletion, and eventually apoptosis.

2. Results and Discussion

2.1. Synthesis and Characterization of Prodrug RuAzNM and Product RuNH₂

The prodrug RuAzNM and the potential corresponding phototoxic product RuNH₂ were synthesized as racemic mixtures by reacting the precursor complex rac-[Ru(dip)₂Cl₂] (dip = 4,7-diphenyl-1,10-phenanthroline) with the chelating bipyridyl ligand, bpyAzNM or bpyNH₂ (shown in Scheme S1). The complexes were isolated as their ClO₄⁻ salts, purified and characterized by electrospray mass spectrometry (ES-MS), high-resolution mass spectrometry (HRMS), NMR (¹H, ¹³C, correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC)), and high-performance liquid chromatography (HPLC). The complexes were subjected to ion-exchange chromatography and transformed into Cl⁻ salts for biological studies. Details of the characterizations are in Figures S1–S23 (Supporting Information). Single crystals of bpyAzNM were obtained by slow diffusion of diethyl ether vapor into a cold saturated dichloromethane solution. The crystallographic data are in Tables S1 and S2 (Supporting Information).

2.2. Photophysical Properties and Redox Potentials

As shown in Figure S7 (Supporting Information), the bpy moiety and the phenylamine scaffold proximal to the NM motif are linked via a trans-azo bridge. They form a large conjugated system whereby there is strong π-π electronic communication between the N21 lone pair on the NM motif, the azo group, and the distal N=N chelation site (N1, N8). The photophysical properties of RuAzNM, RuNH₂, and bpyAzNM are documented in Table S3 (Supporting Information). The absorption spectra of the compounds are presented in Figure S24 (Supporting Information), and the emission spectra in Figure 1A. As anticipated, the prodrug is non-emissive, whereas RuNH₂ has an emission maximum at 618 nm. As shown in Figure S25 (Supporting Information), the long-lived emission lifetime (τ = 164 ns) at ambient conditions that characterizes RuNH₂ suggests that phosphorescence is the origin of the emission. Notably, the lifetime is significantly elongated from nanoseconds to microseconds (τ = 1.51 μs) in oxygen-free conditions.

Although it is well known that trans-to-cis isomerization of azo double bonds can readily be induced by short wavelength UVA radiation, only the reverse cis-to-trans conversion usually occurs by longer wavelength irradiation with blue light.[15] In the present case this was confirmed for RuAzNM (Figure S26, Supporting Information).

Cyclic and linear sweep voltammograms (CVs) of conjugated complex RuAzNM, reduction product RuNH₂, and functionalized bipyridyl ligand bpyAzNM are shown in Figure S27 (Supporting Information), and their corresponding redox potentials versus the saturated calomel electrode (SCE) are summarized in Table S4 (Supporting Information). The evident anodic shift of the half-wave potential for the first oxidation of RuAzNM (+1.21 V) compared to RuNH₂ (+1.06 V) can be ascribed to the introduction of the electron-deficient azo group. In addition, the remarkable anodic shift of the half-wave potential for the first reduction of RuAzNM (−0.87 V) compared to RuNH₂ (−1.35 V) can be explained by the lower-lying LUMO derived from the bpyAzNM ligand. By comparing the first reduction potential of RuAzNM (−0.87 V) with bpyAzNM (−1.22 V), it can be concluded that coordination to Ru(II) enhances the electron-deficient character of the azo group, making it thermodynamically more vulnerable to attack from reductases in cellulo. Indeed, this reduction potential is significantly higher than for free organic azo molecules that can be selectively reduced by hypoxic cells, such as methyl red (E²⁻¹ = −1.27 V) and disperse red (E²⁻¹ = −1.13 V).[36]

Density functional theory (DFT) computations on the Ru complexes (shown in Figure S28–S29, Supporting Information) revealed that the LUMO in RuAzNM resides primarily on the bpyAzNM ligand rather than the dip ligand which is consistent with the CV data. In addition, primary and minor contributions are made by the bpyAzNM ligand and Ru, respectively, to the composition of the HOMO. Therefore, the first oxidation of RuAzNM (E₁/₂ = +1.21 V, Table S4, Supporting Information) observed electrochemically, is dominated by oxidation of the chelated-ligand (bpyAzNM¹⁰¹⁰), rather than oxidation of ruthenium (Ru¹³/₂). Since the empirical positive potentials for microsomal enzyme substrate azo dyes lie in the range of +1 to +1.5 V,[19] reducing the azo moiety in RuAzNM by the enzyme system appears to be favorable. RuNH₂ has different MO distribution patterns, with the HOMO residing on both bpyNH₂ and Ru, and the LUMO of the dip scaffold, suggesting the mixed ligand-to-ligand charge-transfer (LLCT)/metal-to-ligand charge-transfer (MLCT) nature of the low-lying energy level.

Time-dependent DFT (TD-DFT) calculations provided further understanding of the singlet excited states (Sₘ, see Tables S5 and S6, Supporting Information). The one-photon absorption (OPA) spectra of RuNH₂ predicted by various approximations to the exchange-correlation function were compared (Figure S30, Supporting Information). The spectrum predicted by B3LYP[20] agreed well with the experiment; therefore, this functional was used in further calculations. The calculated OPA spectra are plotted in Figure 1B. For RuNH₂, the S₁ state is predicted to be a dark state (f = 0.0103). The first absorption peak, centered around 430 nm, contains excited states of varying character. However, all involve the electron density predominantly...
Figure 1. Photophysical properties and probe selectivity of the prodrug RuAzNM. A) Emission spectra of 10 μM bpyAzNM, RuNH2, and RuAzNM in chloroform at 298 K. B) Predicted time-dependent density functional theory (TD-DFT) one-photon absorption (OPA) spectra for RuNH2 (orange) and RuAzNM (cyan). C) HPLC analysis for RuAzNM (i), RuNH2 (ii), chloroform extracts of RuAzNM (10 μm) incubated with microsomes (0.25 mg mL−1) and NADPH (100 μm) under normoxia (iii), and hypoxia (iv). Inset: photo of the Ru solutions after the enzyme reaction with/without oxygen. D) Emission intensity enhancement-fold of RuAzNM (10 μm) at 618 nm in response to various bio-species in PBS (pH 7.4). E) 1O2 phosphorescence intensity versus optical density values of the indicated compounds at the excitation wavelength; inset: the 1O2 phosphorescence spectra of each compound at OD463nm = 0.2. F) DPBF consumption trajectories for the indicated compounds; inset: absorbance attenuation of DPBF at 418 nm in RuNH2-mediated photooxidation. G) EPR spectra of 1O2 trapped by TEMP in methanol solution under irradiation at 463 nm. H,I) UV–vis spectra of NADH (180 μm) after cumulative irradiation times during H,I) RuNH2-mediated (H) and RuAzNM-mediated (I) photocatalytic oxidation. J) EPR spectra of h2O2 trapped by TEMP in methanol solution under irradiation at 463 nm. K,L) The DFT energy-converged structures for NADH interacting with RuNH2 or RuAzNM at the bpy scaffold using the B3LYP exchange-correlation functional. The inserted arrows in (H), (I), and (J) show the directions of changes in the peaks.
residing on the dip ligands after photoexcitation. The only state which shows electron density residing on the bpyNH2 ligand is S93, which is significantly higher in energy than the lowest-energy peak. For RuAzNM, the S1 state at 549 nm is predicted to have a considerable oscillator strength (f = 0.6289), making it the brightest state of the 100 lowest-energy states. This state is predominantly described as bpyAzNM-based ligand-localized transfer (LLT) with a small MLCT contribution. The S2 and S4 states at 522 and 508 nm show largely bpyAzNM-based MLCT character. The excited states comprising the absorption peak centered at around 440 nm are mixed. For this absorption peak, states are observed in which the electron density moves from Ru/bpyAzNM to both dip ligands while observing a state with similar character to the aforementioned S1 state. This time a significantly higher contribution from Ru is observed.

2.3. Activation in Liver Microsomes

Given the distinctly different photophysical and electrochemical properties of the prodrug RuAzNM compared to the proposed active product, we then demonstrated the reactivity of the prodrug in hypoxic biological environments using rat liver microsomes which contain various reductase enzymes.[21] As shown in Figure S31, the enzymatic reaction requires NADPH as the hydride source to reduce the azo group. After adding NADPH as a cofactor, the prodrug was gradually reduced by liver microsomes in hypoxia with evident attenuation of the distinctive UV–vis absorption of the azo group above 500 nm. Such an absorbance change can also be observed by the naked eye (inset photos in Figure 1C). The Ru prodrug fragment appeared to be completely reduced to RuNH2 in hypoxia; azobenzene derivatives alone are generally reduced to the corresponding primary amine product by microsomal reductases, with the first step being a reversible oxygen-dependent process.[17,22]

As shown in Figure 1C, after incubation of prodrug RuAzNM with microsomes under hypoxia, a peak emerged with an HPLC retention time identical to RuNH2. In contrast, in normoxic conditions, the peak disappeared, implying that the generation of this component is oxygen-sensitive. Combined with the UV–vis change, this result strongly suggests the generation of RuNH2 as the enzymatic reaction product. This assumption was further evidenced by ES-MS analysis of the chloroform extract from the hypoxic enzyme reaction system (Figure S32, Supporting Information), where a peak at m/z = 468.4 for [RuNH2-2Cl]- (calculated m/z = 468.6) was observed. We were unable to detect the expected simultaneously generated aniline mustard species, but this is likely due to its very high reactivity and, thus, very short lifetime in this biological medium.[17,23]

Such a phenomenon highlights the importance of a prodrug strategy in maintaining the efficacy of highly active chemotherapeutic agents before arriving at their targets. The reduction of RuAzNM in the hypoxic microsomal enzyme system gave rise to a ca. 11-fold emission enhancement, with an emission spectrum analogous to that of RuNH2, indicative of the release of this as a photoactive product. This process was monitored by measuring the emission intensity recovery at 618 nm. The intensity trajectory shows that the reduction proceeded for 40 min before reaching a plateau (Figure S33, Supporting Information). To examine whether other bio-species would exert a similar influence on the phosphorescence response of the prodrug, a variety of different physiological ions, oxidants, reductants as well as amino acids were treated with the prodrug (Figure 1D). Importantly, these bio-species did not induce any significant phosphorescence response. This encouraging result underscores the specificity as well as apparent stability of the prodrug in complicated biological environments. Collectively, these promising results demonstrate that the prodrug can be selectively activated by hypoxic biological environments in a self-immolative (stimulated cascade) manner and unleash dually active species, suggesting that the proposed “hypoxia-controlled” design may have useful, practical applications.

2.4. Photosensitizer Properties and Photocatalysis

A series of experiments was conducted to determine 1O2 quantum yields (Φ1). In a direct method, 1O2 phosphorescence (∆Σg-(0,0) → 3Σg-(0,0)) at 1273 nm was recorded upon excitation of the compounds. As shown in Figure 1E, RuNH2 showed a steeper increase of 1O2 phosphorescence intensity than [Ru(bpy)3]2+ as the concentration increased, with a high quantum yield of Φ1 = 0.854. This value is higher than most Ru(II) complexes[24] while RuAzNM and bpyAzNM, in direct contrast, had no observable photosensitization activity (i.e., Φ1 = 0). It is not surprising that bpyAzNM is inactive because it lacks heavy atoms to enhance the spin-orbit coupling and intersystem crossing (ISC). RuAzNM containing a quiescent Ru(II) is strikingly different. These results were further corroborated by an indirect method using 1,3-diphenylisobenzofuran (DPBF) as the 1O2 indicator where RuNH2 gave Φ1 = 0.897 (Figure 1F). The 1O2 production was also verified by EPR measurements using TEMP as the spin trap. A set of characteristic peaks for TEMP-1O2 radicals was observed for RuNH2 in a methanol solution but only after irradiation at 463 nm for 5 min (Figure 1G). These results suggest that the PDT-inactive prodrug might elicit PDT efficacy in mildly hypoxic regions[9] by selectively releasing highly photoactive RuNH2, implying that controlled PDT is possible.

NADH is an important coenzyme that controls a multitude of intracellular redox reactions. It is known that the selective photocatalysis of NADH oxidation by photoexcited states can subdue cancer growth.[12a,14] Here we explored whether the Ru(II) complexes can induce NADH photo-oxidation. The experimental excited-state redox potentials (vs SCE) for the Ru(II) complexes are documented in Table S7 (Supporting Information), with the theoretical potentials (vs SCE) listed in Table S8 (Supporting Information). RuNH2* possesses excellent redox activity (E(M*/M) = +0.65 V, E(M*/M) = −0.94 V in Table S7, Supporting Information) toward NADH (E(NAD+/NADH) = −0.36 V) and O2 (E(O2/H2O2) = −0.37 V) providing a strong thermodynamic driving force for the catalytic cycle. The theoretical redox activity of RuNH2* is reported with respect to the singlet state S1 (E(M*/M) = +0.90 V, E(M*/M) = −0.76 V) and triplet state T1 (E(M*/M) = +0.64 V, E(M*/M) = −0.50 V), which supports both the oxidative and the reductive quenching cycles in the catalysis. The theoretical potentials for the excited state RuAzNM* are predicted to be E(M*/M) = +0.92 V, E(M*/M) = −0.36 V for S1, and E(M*/M) = +0.21 V, E(M*/M) = −0.09 V for T1.
S1 and significantly unfavorable for T1. This stems from a larger stabilization of the triplet species for RuAzNM (see Tables S9 +

**2.5. DFT Modeling**

To elucidate the above photophysical properties further and gain more insights into the molecular interactions, DFT calculations were performed. Specifically, the molecular interaction between NADH and triplet excited states of the Ru complexes was investigated. For RuNH2, the binding mode where NADH clamps the bpyNH2 ligand by a π–π interaction was preferred, with ca. 15 kcal mol−1 more stable than clamping around the dip ligand. For RuAzNM, the energies for binding at the dip or bpyAzNM ligands increase to around ~20 kcal mol−1, almost 30 kcal mol−1 larger than the binding energy at bpy in RuNH2. This result suggests that the binding energy is most favored for RuNH2 at bpyNH2 compared to RuAzNM (Figure 1K,L, Figure S39 and Table S12, Supporting Information). The higher affinity of RuNH2 for NADH facilitates higher single-electron-transfer (SET) efficiency between the excited-state Ru species and NADH.

**2.6. Lifetimes of Excited States**

The lifetimes of excited states contribute to photocatalytic performance as well. A short-lived lifetime could impede the catalytic efficacy when it is shorter than the diffusion lower limit of ca. 10−9 s. Ultrafast transient electronic absorption was used to elucidate the nature and decay of the excited state manifold of the Ru(II) complexes. As shown in Figure 2A,B, the excited state dynamics for RuAzNM persist for much less than 1 ns. The residual trace signals from 420 to 600 nm at later times (>1 ns) remain

= +0.34 V for T1. Hence, unlike RuNH2*, the S1 and T1 states of RuAzNM* can only drive the reductive quenching cycle by a similar driving force to RuNH2*, as the overall potential for the oxidative quenching cycle is essentially at equilibrium for S1 and significantly unfavorable for T1. This stems from a larger stabilization of the triplet species for RuAzNM (see Tables S9 and S10, Supporting Information) when compared to RuNH2, ultimately lowering the reductant strength of this species, and inhibiting the oxidative quenching cycle. The geometric structures of both Ru(II) complexes in the T1 state are shown in Figure S34 (Supporting Information). As can be seen, the spin density in the T1 state is different for both complexes, being entirely localized on Ru and one of the dip ligands for RuNH2 but on the bpyAzNM ligand for RuAzNM. Furthermore, the subsequent ground-state Ru(I) intermediate of RuAzNM in the catlytic cycle shows a significantly weaker reductive driving force (E(M/M−) = −0.90 V) than that of RuNH2 (E(M/M−) = −1.35 V). Collectively, RuNH2 shows more favorable redox potentials to drive the catlytic cycle.

The photocatalytic NADH/NAD+ conversion was monitored by the evolution of UV–vis absorption spectra during the photoirradiation in which the intensity of the characteristic NADH peak at 339 nm decreased as NADH was converted to NAD+. As expected, under irradiation at 463 nm, RuNH2 triggered a drastic decrease in absorption at 339 nm (Figure 1H) with a turnover frequency (TOF) = 78 h−1, while RuAzNM caused only a small consumption of NADH (Figure 1I) with TOF = 9.6 h−1. The highest observed TOF for RuNH2 was 102.5 h−1 (Table S11, Supporting Information) which is comparable to that reported for the Ir(III) [Ir(typy)(pq)Cl]+ (typy = 4"-(p-tolyl)-2,2′,6′,2″-terpyridine, pq = 3-phenylisouquinoline; TOF = 100.4 h−1), [24] much higher than Ir(III) half-sandwich catalyst [(η5-Cp2)(η6-ppy)]+ (TOF = 0.38 h−1).[25] and [Ru(bpy)3]2+ (TOF = 3.62 h−1).[26] A plot of ln(absorbance at 339 nm) versus time revealed that the reaction follows pseudo-first-order kinetics (Figure S35). The rate of the photocatalytic reaction in Figure 1H,I is ca. 30-fold higher for RuNH2 (8.19×10−2 min−1) compared to RuAzNM (2.72×10−3 min−1) (shown in Figure S36, Supporting Information). As a consequence of the difference in catalytic activity, the yield of H2O2 after 25 min irradiation was much higher for RuNH2 than RuAzNM (inset photos in Figure 1H,I). An array of control experiments (Figure S37, Supporting Information) revealed that RuNH2, light, and O2 are indispensable for the sustainable generation of NAD+ in the above photocatalysis. In addition, RuNH2 is robust enough to endure long-term irradiation, while RuAzNM underwent a limited extent of hypochromicity caused by photoisomerization of the azo group. These results demonstrate that the prodrug is nearly quiescent toward NADH photo-oxidation, whereas RuNH2 is an efficient catalyst, implying that the phototherapeutic effects are controlled by hypoxia.

Since the efficacy of these phototherapeutic effects requires an adequate supply of oxygen, which is limited in hypoxic environments in tumors, such treatment modalities might not lead to the efficient eradication of cancer cells in extreme hypoxia. To combat this problem, our design includes a concomitant chemotherapeutic effect provided by the selective release of the conjugated aniline mustard in hypoxic microenvironments. Considering the complexity, diversity, and heterogeneity of hypoxic cancer tissues, tandem therapy is usually beneficial to overwhelm the capability of cancer cells to resist death. [30]

Fortunately, in an N2 atmosphere, the redox reaction between hemoprotein cyt c (Fe3+) and NADH is significantly accelerated by RuNH2-mediated photocatalysis under irradiation at 463 nm (Figure 1J). The control experiments highlighted that NADH, RuNH2, and light are all necessary for the reduction of cyt c (Fe3+) to cyt c (Fe2+) (Figure S38, Supporting Information). It is noteworthy that under ambient conditions, NADH (50 µM) alone reduced Fe3+-cyt c (10 µM) very slowly. The redox reaction was slightly accelerated by irradiation with a low TOF (2.8 h−1). After further adding RuNH2 (0.5 µM), the reaction rate rose significantly, with TOF increasing to 178 h−1. Since the NAD+/NADH redox pair serves as the primary source and carrier of electrons in cell metabolism that is highly associated with electron transport chain (ETC) function and plays a vital role in maintaining homeostasis,[26] the introduction of such photocatalysis severely disturbs the redox microenvironment and causes cell dysfunction, even cell death.[4a,27] On the other hand, cyt c resides in the mitochondrial intermembrane space and shuttles electrons between complex III and complex IV. In the presence of irreversible mitochondrial impairment, it translocates to the cytosol and initiates caspase-dependent apoptosis.[28] Such photocatalytic treatment triggers direct cell responses and creates a second line of attack that combats the resistance of hypoxic cancer cells.
constant up to the instrument limit of 3 ns, which can be assigned to azo photoisomerization. By contrast, RuNH₂ exhibits little change within the upper limit of 3 ns. The fs-TA spectra of RuNH₂ (Figure 2C) consist of two excited state absorption (ESA) signals and one ground state bleaching (GSB) signal. Taking TD-DFT and UV-vis spectrum into consideration, the strong GSB at 470 nm can be ascribed to MLCT/LLCT involving dip⁻ ← Ru/bpyNH₂ transitions. The intense ESA at 350 nm and broad ESA at 600 nm can be ascribed to dip⁻ absorption.[31] The kinetics of the signals at early times (< 10 ps) can be described as a combination of ISC, vibrational cooling, and internal conversion.[32] Very minor spectral changes can be observed after 10 ps because of the long-lived triplet excited state. The charge recombination process can be described as a dual-component decay from the triplet excited state to Ru/bpyNH₂, consistent with the biexponential fitting of the emission decay in Figure S25 (Supporting Information). For RuAzNM (Figure 2D) at early times (< 0.5 ps), the spectrum comprises two ESA signals for dip⁻, one below 400 nm and one above 580 nm, which resembles RuNH₂, and two GSB peaks at 458 nm (dip⁻ ← Ru/bpyAzNM transition) and 523 nm (bpyAzNM⁻ ← bpyAzNM/Ru transition). However, at later times (0.5 to 10 ps), a shoulder emerges at the high energy ESA peak. Meanwhile, the low energy ESA peak redshifts, accompanied by a decrease at 458 nm, an increase at ca. 523 nm, and the appearance of a quasi-isosbestic point at ca. 475 nm. These phenomena can be explained by an electron transfer from dip⁻ to bpyAzNM⁻ with a time constant of 0.47 ps (Figure 2E). At even later times (10 ps to 3 ns),
the spectrum comprises two redshifted and broadened ESA signals for bpyAzNM•− at below 430 nm and above 600 nm, and one GSB peak at ca. 523 nm (bpyAzNM•− ↔ Ru/bpyAzNM transition). The charge recombination decays at 375, 523, and 650 nm after 10 ps are very similar. All can be described as a dual-component non-irradiative decay from the bpyAzNM•− excited state to Ru/bpyAzNM with time a constant of 375 ps (60%) and 42 ps (40%), respectively (Figure 2F). Based on these analyses, the proposed kinetic models for the two Ru(II) complexes are depicted in Figure 2G,H. After photoirradiation of RuAzNM, excited states were quickly funneled (0.47 ps) to the low-lying bpyAzNM-based LLT/MLCT excited state wherein the ultra-fast conformational change around the N=N bond quickly dissipates energy within picoseconds before full interaction with other substrates. In other words, such distinct excited-state kinetics endowed by the azo group help to materialize the proposed “controlled” multimodal therapeutic effect.

2.7. Solution Stability and Lipophilicity

A series of solution studies was carried out in preparation for the biological experiments. Solutions of the compounds (10 µM) in RPMI-1640 at 310 K were monitored for 12 h by UV–vis spectroscopy. Whereas the absorbance of the ligand bpyAzNM decreased, indicative of a reaction with components of the medium, both RuAzNM and RuNH2 appeared to be highly stable (Figure S40, Supporting Information).

Ubiquitous thiols have posed a major threat to the survival of most metal-based catalysts,[33] as well as NMs[34] in vitro. As such, the stability of the compounds toward 1 mM GSH was studied by HPLC (Figure S41, Supporting Information). Under the conditions used, the azo group in bpyAzNM and RuAzNM was not reduced by GSH. Surprisingly, bpyAzNM reacted with GSH even after a very short incubation time (<10 min, 310 K). This implies that the electron-withdrawing effect is not strong enough to prevent the NM from forming an aziridinium intermediate ion. Importantly, no new species were detected by HPLC from reactions of GSH with RuAzNM nor RuNH2, implying their inertness. Compared with the bpyAzNM bidentate ligand alone, coordination to Ru(II) further influences the availability of the electron lone pair on the nitrogen, as is evident from the changes in the 1H NMR chemical shifts of the CH2 units in the chloroethyl group (Figures S3 and S18, Supporting Information). The major reactivity of NMs is their ability to alkylate DNA bases leading to DNA impairment. However, bpyAzNM, RuNH2, and RuAzNM (50 µM) were all unreactive toward guanine (100 µM), a model nucleobase, as monitored by HPLC, even after 12 h incubation (Figure S42, Supporting Information).

Hence our strategy for the design of the prodrug appears to be successful.

The lipophilicity of drugs often correlates with cellular uptake, biodistribution, and even toxicity.[35] Here we determined logP (partition coefficient) and K (capacity factor) values (Figure S43, Supporting Information). Unlike [Ru(bpy)]3+, (−ve logP), all three compounds have lipophilic (+ve logP), with RuAzNM possessing the highest logP and K values.

2.8. Uptake, Distribution, and Response to Hypoxia in Cells

Time-dependent cellular uptake studies revealed that the accumulation of RuAzNM by HepG2 liver cells increased with the time of incubation (Figure S44, Supporting Information). After 6 h incubation with 10 µM RuAzNM, the internalized Ru content rose to ca. 8 ng/10⁶ cells which is more than double that for RuNH2 under identical conditions. This might be a result of the higher lipophilicity of RuAzNM. To study the cellular uptake mechanism, HepG2 cells with various pretreatments were incubated with RuAzNM. As shown in Figure S45, neither endocytosis inhibitors nor metabolic inhibitors significantly affected the cellular accumulation of RuAzNM; the only factor that reduced the accumulation was a lower incubation temperature, suggesting that the complex was internalized into HepG2 cells via passive diffusion. Many cancer drugs are preferentially active against highly proliferating cells, but cancer cells in hypoxic regions are quiescent in metabolism, which leads to suppressed active transport and, thus, reduced therapeutic efficacy.[36] From this perspective, passive diffusion suffers much less from the microenvironment change and is probably a preferred way for a prodrug to access hypoxic regions. To study the response to hypoxic cells, a cover slide was positioned over adhered cells on a confocal dish to induce oxygen deprivation. As shown in Figure 3A, by placing a coverslip over attached cells that had been preloaded with RuAzNM and incubated for 2 h to deplete oxygen in cells, only cells beneath the coverslip restored the phosphorescence. In contrast, those beyond the coverslip triggered no appreciable emission recovery, indicating that the prodrug can be reduced selectively by the oxygen-starved cells. Based on this, we incubated HepG2 cells with the prodrug under different hypoxic conditions to study whether it can be used as a probe to gauge hypoxia. As shown in Figure S46 (Supporting Information), the phosphorescence dramatically faded as the O2 level was elevated from 1% to 5% and disappeared at 21%. These combined results reveal the diagnostic utility of the prodrug toward hypoxic cells. Next, the intracellular distribution of the prodrug was studied. Interestingly, co-localization confocal imaging revealed that RuAzNM accumulated in mitochondria with a Pearson’s correlation coefficient of 0.75 (Figure 3B). This result was corroborated by ICP-MS measurements (Figure S47, Supporting Information); 72.5% of internalized Ru in cells was found in the mitochondria. This preferential mitochondrial accumulation can be ascribed to the lipophilic cationic RuAzNM. Since mitochondria harbor Fe²⁺/cyt c and mtDNA, both of which are highly important for cell fate, such an intracellular distribution enables the prodrug to be located adjacent to these vital components and deliver effective phototherapeutic and chemotherapeutic outcomes.

2.9. (Photo-)cytotoxicity

The (photo-)cytotoxicity of RuAzNM, RuNH2, and bpyAzNM was determined in 2 human cancer cell lines (A549 lung, HepG2 liver) and 3 normal human cell lines (MRC-5 lung fibroblast, THL-3 immortalized liver, HEK-293 embryonic kidney). For A549 and HepG2, cytotoxicity in hypoxia (1% O2) was also determined. Cisplatin, 5-aminolevulinic acid (5-ALA), and
5-ALA-induced protoporphyrin IX (PpIX) were used as controls. The toxicity profiles are documented in Table S13 (Supporting Information). Overall, bpyAzNM did not show promise due to its poor stability and solubility. The clinical PDT prodrug, 5-ALA, was inactive (IC\textsubscript{50} > 200 µm) under the treatment regimens used in this study. Under normoxia, RuAzNM showed low toxicity toward A549 (41.1 µm), HepG2 (36.6 µm), MRC-5 (34.8 µm), THL-3 (60.5 µm), and HEK-293 (32.5 µm) cells, which is highly desirable for a prodrug. Light irradiation (465 nm, 12 J cm\textsuperscript{-2}) under normoxia barely potentiated the toxicity of prodrug RuAzNM, attributable to its suppressed PDT and photocatalytic activities. Although RuNH\textsubscript{2} showed similar low toxicity toward tested cell lines in the dark under normoxia, its toxicity increased significantly after irradiation, with a photocytotoxicity index (PI = IC\textsubscript{50,dark}/IC\textsubscript{50,light}) of 54 observed for HepG2 (1.3 µm) due to its excellent photoactivity. PpIX is highly active as a photosensitizer with the highest PI of 125. Under hypoxia, the dark toxicity of the compounds decreased significantly compared to normoxia (IC\textsubscript{50} values for A549 cells doubled for cisplatin), except that RuAzNM showed the reverse trend. Reduced toxicity may be caused by several factors, such as reduced cellular uptake due to low proliferation, enhanced detoxification in hypoxia, and shortage of oxygen to yield sufficient ROS to exert lethality.
Under irradiation in hypoxia, prodrug RuAzNM exhibited phototoxicity with higher PI values than RuNH2 in both tested cancerous cell lines, which may be ascribed not only to a difference in uptake, but also to the potential synergy between chemotherapy and phototherapeutic effects, since both led to enhanced ROS production in cells, as will be shown (vide infra). Strikingly, RuAzNM showed the strongest anticancer potency (IC50 = 2.3 µm) with light toward hypoxic HepG2 cells, ca. 10-fold more potent than cisplatin (IC50 = 24.5 µm), ca. 2-fold more potent than PpIX (IC50 = 6.0 µm), and 25-fold more toxic than towards THL-3 normal liver cells in normoxia (IC50 = 58.2 µm). In addition, RuAzNM showed similar phototherapeutic activity (IC50 = 3.4 µm) toward HepG2 multicellular spheroids (MCSs, diameter = 400 µm). The potency of RuAzNM is higher than that of the clinical drug cisplatin (IC50 = 24.7 µm) and PpIX (IC50 = 18.8 µm). These results show that RuAzNM holds promise as a potent hypoxia-responsive anticancer drug candidate.

2.10. Photocatalytic NADH Oxidation and ROS Generation

HepG2 cells were used for the subsequent studies since they are sensitive to the RuAzNM-mediated multimodal therapy. Next, in vitro ROS generation was studied. Consistent with the solution study, in normoxia, RuNH2-mediated photosensitization triggered a 4 to 7.5-fold increase in H2O2 level in a dose-dependent manner compared to the control (Figure 3C). By contrast, RuAzNM does not exhibit a significant increase in H2O2 generation (Figure S48, Supporting Information). These results indicate that the efficacy of photocatalytic NADH oxidation can be transferred from solution to an in vitro cell setting with the induction of an H2O2 outburst. In another mode of phototherapeutic effect in normoxia, enhanced singlet oxygen production was observed in RuNH2-mediated PDT, whereas RuAzNM was silent (Figure 3D). The produg RuAzNM triggered only a very weak ROS enhancement by DCFH-DA staining (Figure 3E), which was not significant on irradiation. This low-level ROS might be due to superoxide O2− generated by the azo group in the presence of both the microsomal enzyme system and O2.[37] as can be evidenced by the elevated O2− level compared to control in a staining experiment using dihydroethidium (DHE) as a fluorescent probe (Figure S51, Supporting Information). Interestingly, RuAzNM induced higher ROS generation in hypoxic cells even without light input (Figure 3E), which might be due to oxidative stress caused by the release of the aniline mustard.[38] Under irradiation, an outburst of excess ROS in hypoxic cells was observed (Figure 3E and Figure S51, Supporting Information), indicating that oxygen-independent photocatalysis disrupts homeostasis and elevates oxidative stress. To provide direct insight into oxidative stress, the NADH content of cells was quantified. As shown in Figure 3F, the released photoactive species RuNH2 triggered NADH consumption dose-dependently. A significant NADH decrease began to emerge when 2.5 µm RuNH2 was used. As a downstream redox reaction that requires the participation of NADH, the reduction of MTT in mitochondria forms formazan, which can reflect the NADH level in cellulo. As shown in Figure 3G, RuAzNM treatment did not affect formazan formation in normoxia. However, in hypoxia, formazan formation was partially blocked, and photoirradiation further enhanced the inhibition. Taken together, the prodrug is inherently non-phototoxic under normoxia, but is activated in vitro under hypoxia.

2.11. Effect on ATP and Mitochondrial Membrane Potential

When exposed to hypoxia, RuAzNM unleashes the chemotherapeutic aniline mustard, boosting ROS generation and releasing the highly phototoxic species, RuNH2. As the produg preferentially accumulates in mitochondria, its photoinduced damage would readily impair the delicate ETC in OXPHOS and result in decreased ATP production. This assumption was validated by measuring the ATP content in vitro. As shown in Figure 3H, cells in hypoxia lost nearly half of their ATP content when receiving the RuAzNM-mediated 2.5 µm phototherapeutic regimen. In contrast, in normoxic cells, RuAzNM did not have similar activity, and its administration had little effect on ATP production.

The significant decrease in ATP production strongly suggested mitochondrial malfunction. The mitochondrial membrane potential (ΔΨmt) is susceptible to ETC impairment and is deeply involved in mitochondrial regulation and cyt c release.[39] As shown in Figure 4A, ΔΨmt can be visualized by JC-10 staining, where ΔΨmt loss causes JC-10 to be transformed from red aggregation to green monomer in confocal imaging. RuAzNM treatment in normoxia did not trigger appreciable ΔΨmt loss. In hypoxia, ΔΨmt loss became evident with RuAzNM treatment in the dark and was exacerbated by a photoirradiation regimen.

2.12. Effect on Mitochondrial DNA

Since NMs are known to induce DNA crosslinking, the DNA crosslinking ability of the RuAzNM was studied by agarose gel electrophoresis. Sodium dithionite (SDT) solution was used to mimic the reducing environment of hypoxia.[40] As shown in Figure 4B, RuAzNM and SDT co-incubation led to inhibition of the electrophoretic mobility of the pBR322 DNA (Forms I, II, and III) in a dose-dependent manner in the range of 5 to 20 µm. In addition, the well-known antitumor crosslinking agent chlorambucil, was used as the positive control. RuAzNM, after reduction, exhibited comparable DNA crosslinking ability to chlorambucil at the same concentration. This result confirms the efficacious chemotherapeutic potency of RuAzNM in hypoxia.

mtDNA is located only in mitochondria and is a promising target for reversing cancer resistance.[41] To investigate the effect of RuAzNM-mediated multimodal therapy on mtDNA, PicoGreen was used as the staining reagent for mtDNA[42] in confocal imaging. As shown in Figure 4C and S53, cells stained with PicoGreen showed bright nuclear (nDNA) and punctate cytoplasmic (mtDNA) staining. The individual nucleoids were quantified to assess mtDNA depletion. As shown in Figure 4D, RuAzNM caused no significant change in the number of nucleoids in every HepG2 cell and, therefore, mtDNA in normoxia. When cells were treated with RuAzNM in hypoxia, the number fell, but not significantly. However, RuAzNM-mediated photoactivation in hypoxia results in significant mtDNA depletion.
2.13. Mechanism of Cell Death

Calcein AM/PI co-staining showed that the RuAzNM-mediated multimodal attack under hypoxia ultimately triggered irreversible damage to the cells and induced effective cell death (Figure 4E and S54). We also investigated the cell death mechanism by a 24-h MTT assay on co-incubation with various pathway inhibitors along the RuAzNM-mediated photoactivated pathway in hypoxia. As shown in Figure 4F, only in the presence of Z-VAD-fmk, a pan-caspase inhibitor, was the cell viability significantly elevated after photoirradiation. This result implies that apoptosis is likely to be a primary pathway for post-irradiation cell death. This phenomenon was corroborated by the annexin-V/PI co-staining assay (Figure S55), where early apoptosis was observed immediately after the irradiation.

2.14. Activity in Multicellular Spheroids (MCSs)

To demonstrate the theranostic utility of RuAzNM further, MCSs were used to mimic hypoxic tumors in a longer-term study. As shown in Figure 5A, it is apparent that after administering RuAzNM (10 µm) to the MCSs, hypoxia emerges on day 2 along the MCS growth timeline by the advent of a red-phosphorescent signal from the interior of the MCSs. This diagnostic result suggests that RuAzNM can penetrate MCS cores and release photoactive RuNH2 when hypoxia arises. The red-emission feedback suggested the optimum time to implement the photoactivation regimen subsequently (on day 2). The growth curves for the MCSs were plotted by measuring the MCS volumes from day 0 to day 7 (Figure S56, Supporting Information). Cisplatin (20 µm) and 5-ALA (200 µm) were used as the positive controls. As can be seen in Figure 5B, high-dose cisplatin administration demonstrated chronic growth inhibition activity after day 2. Interestingly, 5-ALA-mediated phototherapy resulted in growth delay, which is primarily due to its induced photoactivated damage to the outer proliferation zone of the MCSs. However, this damage was compensated through a 5-day recovery, probably due to its poor lethality toward the interior...
hypoxic quiescent zone. RuAzNM showed no apparent growth inhibition activity preceding its activation on day 2. Emission-induced photoradiation, split into two identical courses, allowed the generated RuNH$_2$ to diffuse outwards. Meanwhile, the MCSs replenish oxygen inwardly which maximizes the phototherapeutic outcome, and the MCSs started to shrink. Unlike 5-ALA, the damage is irreversible this time since it is a consequence of oxygen-independent multimodal therapy. Aside from its lower toxicity toward normal cells, RuAzNM-mediated therapeutic effects outperform high-dose cisplatin in inhibiting MCS growth, as shown by the post-treatment ATP cell content in Figure 5C. MCSs treated with RuAzNM showed significantly lower ATP levels in comparison with cisplatin. A similar trend was confirmed by staining MCSs with LIVE/DEADTM Kit (Thermo) to visualize directly live (green) and dead (red) cells in MCSs after the treatment regimen (Figure 5D).

3. Conclusion

We have developed a simple, versatile, and efficacious prodrug, RuAzNM, to combat hypoxic cancer cell resistance in a controlled multimodal therapeutic manner. The non-emissive pro-drug RuAzNM is stable, crosses membranes via passive diffusion, preferentially accumulates in mitochondria, and can be selectively reduced by hypoxic cells. Then it releases an aniline mustard which has chemotherapeutic effects, and the photoactive complex RuNH$_2$ which exerts a phototherapeutic influence. Unlike prodrug RuAzNM, for which the photophysical activity is caged by an azo group, the uncaged RuNH$_2$ is emissive and highly active, not only boosting $^{1}$O$_2$ and H$_2$O$_2$ generation in normoxia, but also accelerating redox reactions between NADH and Fe$^{3+}$-cyt c in hypoxia, so perturbing the cellular redox balance. The prodrug-mediated multimodal therapeutic effect in hypoxic HepG2 cells triggered a ROS outburst, NADH and ATP deprivation, mitochondrial membrane potential loss, and mtDNA depletion preceding the ultimate apoptosis, whereas it is essentially non-toxic toward normoxic cells, especially normal THL-3 human liver cells. Furthermore, the hypoxia-responsive theranostic utility of the prodrug was successfully demonstrated in a multicellular spheroid (MCS) model. The prodrug was efficacious in reporting hypoxia and inhibiting the proliferation of MCSs by multimodal therapy. These results suggest that RuAzNM is an efficacious smart
candidate prodrug with the potential for combating hypoxia resistance in cancer cells.

4. Statistical Analysis

Statistical analysis was performed with Origin 2021b. All biological experiments were performed at least twice, with triplicates in each experiment. Normality and homogeneity tests were conducted. In the case of normal distribution, the statistical comparison of results was checked with a Student’s t-test. The levels of significance were set at n.s. (no significant difference), * p < 0.05, ** p < 0.01, and *** p < 0.001. Representative results are shown herein, and data are presented as “mean ± standard deviation (SD)” with statistical significance. Statistical significances and sample sizes in all graphs are indicated in the corresponding Figure legends.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

aniline mustard, hypoxia, photoredox catalysis, ruthenium complexes, theranostics

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