Prodrugs

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A Bis(dipyridophenazine)(2-(2-pyridyl)pyrimidine-4-carboxylic acid)ruthenium(II) Complex with Anticancer Action upon Photodeprotection**

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Abstract: *Improving the selectivity of anticancer drugs towards cancer cells is one of the main goals of drug optimization; the prodrug strategy has been one of the most promising. A light-triggered prodrug strategy is presented as an efficient approach for controlling cytotoxicity of the substitutionally inert cytotoxic complex $[Ru(dppz)_2(CppH)](PF_6)_2$ (**C1**; CppH = 2-(2-pyridyl)pyrimidine-4-carboxylic acid; dppz = dipyrido[3,2-a:2′,3′-c]phenazine). Attachment of a photolabile 3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl (DMNPB) ester ("photocaging") makes the otherwise active complex **C1** innocuous to both cancerous (HeLa and U2OS) and non-cancerous (MRC-5) cells. The cytotoxic action can be successfully unleashed in living cells upon light illumination (350 nm), reaching similar level of activity as the parent cytotoxic compound **C1**. This is the first substitutionally inert cytotoxic metal complex to be used as a light-triggered prodrug candidate.*

Platinum- and ruthenium-based cytotoxic compounds are by far the most explored metal-based anticancer agents.[1] For the majority of such metal complexes, their anticancer activity originates from the presence of a labile ligand and/or a redox-active metal center.[2] (Organo)metallic complexes can, however, also exert anticancer activity in their inert intact form.[3] The best examples are the substitutionally inert Ru^{II} scaffolds, which have been found to not only act as potent kinase inhibitors, but also as effective cytotoxic compounds.[2c,e,4] The mechanism of action for cytotoxic Ru^{II} polypyridyl compounds is believed to be a complex function of inherent physicochemical and pharmacological properties. As interest in substitutionally inert metal complexes as anti-cancer drug candidates has only recently regained momentum,[3a,4a,b,d,5] in most cases only limited information is available on their precise mode of action and metabolic activity. Moreover, a scarcity of structure–activity relationship (SAR) studies focusing on these aspects also implies that biochemical understanding on most of these systems is still premature.[4a–c,6]

Nonetheless, targeting the cytotoxic aspects of one such coordinatively saturated and substitutionally inert Ru^{II} complex, $[Ru(dppz)_2(CppH)]^{2+}$ (**C1**; Scheme 1), we have demonstrated that this particular bis(dppz) complex exerts its cytotoxic action by disrupting the mitochondrial function.[7] Through correlations from the detailed SAR studies, we could deduce that structural alterations in this lead prototype can significantly diminish its cytotoxic potency.[8] Furthermore, with no decomposition of the complex in human plasma, it was concluded that the intact Ru^{II} complex is responsible for the cytotoxic activity.[7]

Taking the above findings into consideration, we explored the potential of light-triggered prodrug strategy for tuning the intracellular cytotoxic activity of the complex, while retaining the structural integrity of the active parent complex. Such molecules, which are rendered inactive through covalent modification with a photocleavable moiety but can regain biological activity upon light exposure, are commonly referred to as "photocaged" compounds.[9] Light-activatable pro-moieties allow the modulation of the release and activity of a "photocaged" drug as a function of the wavelength, duration, intensity, or location of illumination.[10] Whilst photochemical control of activity (also referred to as "photocaging/uncaging") has been widely explored with organic drugs, application of this concept to metal coordination complexes has been surprisingly limited.[9,11] Of note, metal complexes were previously used in combination with light to trigger biological activity.[3a,11,12] Specific to substitutionally inert Ru^{II} complexes, photoactivation to date has primarily revolved around the studies exploring their capacity to undergo photoinduced ligand exchange/expulsion, DNA binding, DNA cleavage, and cytotoxic effects.[3a,4a–c,13] To the best of our knowledge, this work is the first example of a light-triggered structurally inert metallo-prodrug candidate, with

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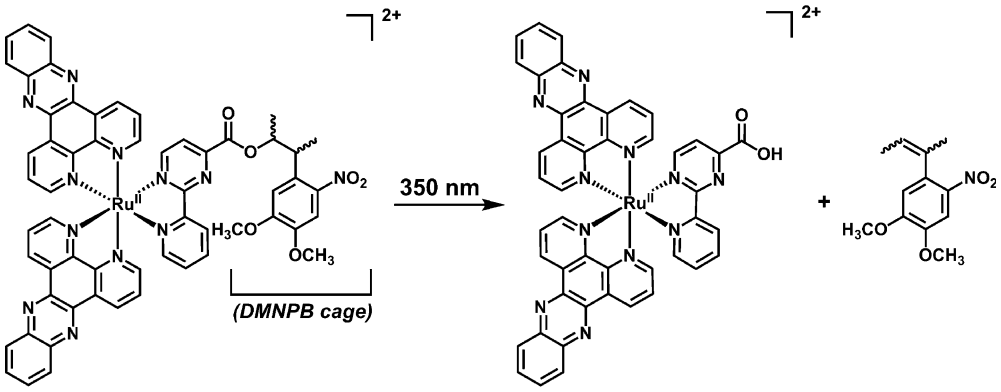

Scheme 1. Chemical structures of the active bis(dppz)Ru^{II} complex **C1** and its photolabile protected version **C2** (isolated as racemic mixtures of hexafluorophosphate salts). The DMNPB group is released upon irradiation at 350 nm.[14] CppH = 2-(2-pyridyl)pyrimidine-4-carboxylic acid; dppz = dipyrido[3,2-*a*:2′,3′-c]phenazine.

no "caged" variants of any substitutionally inert cytotoxic metal complex been previously constructed.

For the proof-of-principle design of a light-activatable Ru^{II} prodrug candidate, inspiration was drawn from the recent structure–activity analysis on the lead prototype. The results pointed towards the presence of the carboxylate functionality on the pyrimidine ring as being essential for cytotoxic activity of the complex, making it an ideal site for attachment of a photocleavable moiety. 3-(4,5-Dimethoxy-2-nitrophenyl)-2-butyl (DMNPB) ester was used as the photocleavable moiety for derivatization of the carboxylic handle (Scheme 1).[14] This *ortho*-nitrophenyl chromophore has previously been used for efficient photocontrolled release of L-glutamate, a neurotransmitter, and for phototriggered cell adhesion of "caged" RGD peptides, at near-UV wavelengths (ca. 360 nm).[15] The synthesis of the aryl butyl esterified Ru^{II} pro-moiety (**C2**) followed the route shown in the Supporting Information, Scheme S1. The identity of **C2** was confirmed by ^{1}H NMR spectroscopy and mass spectrometry, and its purity was determined by elemental analysis (see the Supporting Information for more details).

The hydrolytic stability of **C2** was evaluated by monitoring a sample of this compound in phosphate-buffered saline (PBS) at 25 °C in the dark. Over a time period of 24 h, aliquots were examined by HPLC for the release of **C1**; formation of about 7% **C1** was observed over the time of experiment. Photolytic stability of **C2** was also assessed prior to performing the cytotoxicity experiments. The complex solution in PBS (pH 7.2) was irradiated with 350 nm UV-A radiation, and changes in the UV/Vis absorption spectra were monitored over time (Figure 1). Upon light irradiation, the metal-to-ligand charge transfer (MLCT) band centered at 478 nm is hypsochromically shifted to a broad MLCT band centered at 451 nm. With time, a clear isosbestic point at 467 nm is observed along with increasing absorption intensity in the 250–400 nm region. As the photolytic reaction proceeds, a shoulder at about 318 nm that is characteristic of the free complex **C1** also appears, reflecting its photorelease from **C2**. Furthermore, removal of DMNPB from the prodrug candidate **C2** to release **C1** could also be easily confirmed by UPLC-MS (Supporting Information, Figure S2). With time, gradual disappearance, upon light irradiation, of the peak corresponding to **C2** (t_R = 2.6 min) was seen along with appearance of the peak for free **C1** (t_R = 2.3 min); the observed spectral changes are in agreement with the expected photolytic reaction. After 20 min of light irradiation (5.16 J cm^{-2}), an almost quantitative amount of **C1** ($\geq$ 92%) was photoreleased (Supporting Information, Figure S3), as estimated from UPLC analysis (see the Supporting Information for more details). The calculated quantum yield for the photorelease of **C1**, as determined by comparison with 1-(2-nitrophenyl)ethyl phosphate (Φ = 54%),[16] was found to be 3.8%, indicating a modest photolytic efficiency (Supporting Information, Figure S4).

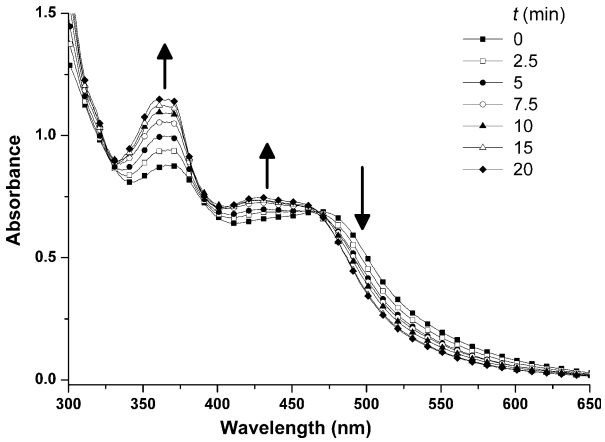

Figure 1. Changes in absorption spectra of **C2** (50 μM in PBS, pH 7.2) as observed upon irradiation at 350 nm. Arrows indicate the direction of change in absorbance with increasing periods of irradiation.

Having confirmed the light-triggered removal of DMNPB from the prodrug candidate **C2** to release **C1**, the cytotoxic evaluations were performed on cervical cancer (HeLa), bone cancer (U2OS), and non-cancerous lung fibroblast (MRC-5) cell lines. Resazurin based fluorometric assay was employed for the cytotoxicity assessment of the prodrug candidate in the dark and upon 350 nm light irradiation (Table 1). Different concentrations of prodrug candidate **C2** administered for 4 h in the dark to both HeLa and U2OS cells, followed by incubation at 37 °C in fresh cell culture media for additional 48 h, were found to be non-toxic up to the highest complex concentration (100 μM) examined in this study. Similar cytotoxic response was also observed on MRC-5 cells upon

Table 1: Cytotoxic activity data (IC_{50}) for **C1**, **C2**, DMPNB (**4**), and cisplatin against human cervical carcinoma (HeLa), human osteosarcoma (U2OS), and non-cancerous lung fibroblast (MRC-5) cell lines.

	IC_{50} [μM]						
	HeLa			U2OS			MRC-5
	4 h (dark)	4 h (+ UV-A)[a]	48 h (dark)	4 h (dark)	4 h (+ UV-A)[a]	48 h (dark)	48 h (dark)
C2	>100	17.0±0.8	85.8±5.8	>100	17.2±3.8	>100	85.3±0.2
C1	16.0±0.1	5.9±1.7	10.0±1.3[7]	30.5±1.1	13.5±2.1	13.5±2.5[7]	15.1±2.2[7]
DMPNB (**4**)	>100	>100	>100	>100	>100	>100	>100
cisplatin	9.8±4.5	12.7±3.6	9.9±0.9	26.8±1.9	32.6±5.1	11.8±1.7[7]	8.5±0.9

[a] 10 min UV-A irradiation (350 nm, 2.58 J cm^{-2}).

incubation with **C2**. Interestingly, even extending the treatment up to 48 h for prodrug candidate **C2** did not induce any cytotoxicity towards U2OS cells up to the highest dosed complex concentration (100 μM), also causing no significant change in its cytotoxic action towards HeLa and MRC-5 cells ($IC_{50} \approx 85$ μM). That prodrug candidate **C2** shows a promising lack of cytotoxicity toward both cells lines, in the dark, is in stark contrast with the cytotoxic activity data obtained for the active Ru^{II} complex, **C1**, for which the IC_{50} values against HeLa, U2OS, and MRC-5 cell lines were determined to be 10.0 μM, 13.5 μM, and 15.1 μM, respectively. The parent cytotoxic complex **C1** losing its activity upon covalent modification reflects similar findings previously reported by us.[7,8] For example, the benzylic and ethyl ester derivatives of **C1** showed 3–4 times less activity on HeLa cells than the parent complex.[8]

The effect of light irradiation on the cytotoxic action of the prodrug candidate **C2** was examined on HeLa and U2OS cells. The cells were first incubated with **C2** in the dark for 4 h, before suspension in fresh cell culture media followed by irradiation at 350 nm for 10 min (2.58 J cm^{-2}) and incubation for additional 48 h. As anticipated, light irradiation of HeLa and U2OS cells exposed to prodrug **C2** restored the cytotoxic effect (Table 1). An IC_{50} value of ca. 17.0 μM was obtained when cells were subjected to light exposure. Notably, the cytotoxicity level re-attained by the pro-moiety **C2** upon light irradiation is in excellent agreement with the cytotoxic response observed for **C1**, in the dark, toward HeLa cells ($IC_{50} = 16.0$ μM) under similar conditions, with an almost twofold increase in cytotoxicity against U2OS cells (Table 1). Control light irradiation experiments performed on HeLa and U2OS cells either in the absence of the complex or after incubation with the photolabile group DMNPB showed no toxic effect on the cells, ruling out their possible contributions to the elevated cytotoxic effects observed upon light activation.

Tris(diimine)–Ru^{II} complexes can produce $^1O_2$ upon light irradiation and therefore induce $^1O_2$-mediated DNA photocleavage, and can also exert phototoxicity.[4b,c,13a,17] In our case, the separately conducted measurements indeed confirmed the ability of **C1** to produce singlet oxygen (see Supporting Information) upon irradiation at 350 nm with the determined quantum yield (Φ) of 0.81 and 0.06 in acetonitrile and PBS (pH 7.2), respectively; the values indicating an extremely efficient singlet oxygen production in lipophilic environments. Given that the prodrug candidate **C2** only regains cytotoxic activity after light activation, it thus follows that any cytotoxic effect introduced on light irradiation of **C1** may have a role to play in the elevated cytotoxic potency of the prodrug candidate **C2**. That the photoinduced toxicity of **C1** contributes to the observed light-triggered increase in activity of **C2** is supported by the fact that light irradiation at 350 nm of HeLa and U2OS cells dosed with **C1** (4 h) further led to up to about threefold increase in cytotoxicity compared to those kept in the dark. Nevertheless, photolytic removal of DMNPB from **C1** still remains the critical first step in the case of **C2** regaining cytotoxicity upon photolysis. Thus, it is conceivable that as the complex **C1** is photoreleased from the prodrug candidate **C2**, the overall enhancement in exerted cytotoxicity in HeLa cells is a cumulative effect of the direct cytotoxic activity of **C1** and the activity, in part, potentially originating as a result of a cascade of photolytic reactions involving the RuN_6 coordination sphere of complexes **C1** and **C2**.

Furthermore, the effect of light irradiation on the cellular localization of the prodrug candidate **C2** was also probed qualitatively using confocal laser scanning microscopy (CLSM; Supporting Information, Figure S7). CLSM studies on HeLa cells treated with **C2** in the dark revealed a non-specific manner of localization (Supporting Information, Figure S7a). Intense luminescence signals were observed from the cytoplasmic regions as well as the cell nucleoli. Furthermore, a distinct sharp signal marking the periphery of the nuclear membrane was also observed, which persisted even after co-staining of cellular DNA with DAPI (4′,6-diamidino-2-phenylindole; Supporting Information, Figure S7c). For the cells exposed to light irradiation (350 nm), a relatively less-intense luminescence was observed in cells (Supporting Information, Figure S7b). Nevertheless, from the respective overlay image with DAPI staining (Supporting Information, Figure S7d), accumulation appeared to mainly occur in cytoplasmic organelles, with weak visual signs of red emission from the nucleus under the conditions used for the confocal microscopy experiments. However, a possibility that the polarity and water accessibility of bis(dppz)–Ru^{II} complexes in the cellular microenvironment is reflected in the decreased intensity of the emission signals, cannot be ruled out. This highlights the uncertainty on precise cellular accumulation of metal complexes as assessed by confocal microscopy. However, in case of **C1**, we could previously show that visually observed mitochondrial accumulation is highly

correlated to that determined using high-resolution continuum source atomic absorption spectrometry (HR-CS AAS).[7]

In conclusion, we report on an efficient approach for controlling cytotoxicity of a substitutionally inert cytotoxic Ru^{II} complex. Attaching an appropriate photolabile moiety to **C1** makes the otherwise active complex innocuous to both cancerous (HeLa and U2OS) and non-cancerous (MRC-5) cells. Furthermore, the cytotoxic action of the pro-moiety **C2** is controlled by light and can be regained upon illumination. With light-induced (350 nm) liberation of **C1** from the pro-moiety, cytotoxic action of the prodrug on the cancer cells is unleashed, reaching similar levels of cytotoxicity as for the original complex in absence of light. Though still at the prototype stage, this light-triggered prodrug strategy holds tremendous potential for designing more sophisticated prodrug systems, where properties are tailored to produce a controllable cytotoxic action in physiologically relevant optical window. Efforts towards this end are currently under investigation. It should be noted, however, that the UV-A light dosage (2.58 $J\,cm^{-2}$) used to induce cytotoxicity in **C2** is comparable to that frequently employed for other UV-A activated metal complexes.[11,12d,18]

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