Photo-induced telomeric DNA damages in human cancer cells

Justin Weynand,[1,2] Harikleia Episkopou,[3] Gabriel Le Berre,[3] Martin Gillard,[1] Jérôme Dejeu,[2] Anabelle Decottignies,*,[3] Eric Defrancq,*,[2] and Benjamin Elias*,[1]

1 Université catholique de Louvain (UCLouvain), Institut de la Matière Condensée et des Nanosciences (IMCN), Molecular Chemistry, Materials and Catalysis (MOST), Place Louis Pasteur 1, bte L4.01.02, B-1348 Louvain-la-Neuve, Belgium

2 Université Grenoble-Alpes (UGA), Département de Chimie Moléculaire, UMR CNRS 5250, CS 40700, 38058 Grenoble, France

3 Université catholique de Louvain (UCLouvain), Genetic and Epigenetic Alterations of Genomes, de Duve Institute, Avenue Hippocrate 75, 1200 Brussels, Belgium

Abstract: *Herein we report on the study of novel dinuclear ruthenium (II) complexes designed to target and to photo-react with G-quadruplex telomeric DNA. Upon irradiation, complexes efficiently generate guanine radical cation sites as photo-oxidation products. The compounds also display efficient cell penetration with localization to the nucleus and show strong photocytotoxicity toward osteosarcoma cells. Thanks to a microscopic-based telomere dysfunction assay, which allows the direct visualization of DNA damage in cells, we brought the first evidence of forming photo-oxidative damages at telomeres in cellulo. This emphasizes interesting prospects for the development of future cancer phototherapies.*

In recent years, non-canonical DNA secondary structures have attracted much attention. Among them, G-quadruplex (G4) DNA is particularly appealing due to its multiple biological involvements whose discovery spans continuesly.[1-4] The most documented importance of these quadruple helical structures is their prevalence in human telomeric DNA. The telomeric sequences are

composed of hundreds of $^{3'}$TTAGGG$^{5'}$ repeats and play a crucial role in the development of cancer. The single-stranded overhang of telomeric DNA can potentially fold up to ten consecutive G4s linked by TTA spacers. After each cell division, they are shortened and once a limit is reached (i.e. the Hayflick limit), the cell enters in senescence.[5, 6] However, this situation is almost never reached in cancer cells due to telomere maintenance processes.[7, 8] This telomeric length retention leads to cancer cell immortality. As G4 structures are believed to regulate telomere maintenance mechanisms, a large number of ligands have been designed to target these G-quadruplex architectures with a view of developing new cancer treatments.[9, 10] Most of G4 ligands have been built from a rigid aromatic core able to interact with the G-quartet through π-stacking and decorated with substituents (often positively charged) that can interact with G-quadruplex grooves and/or loops, leading to improved affinity for G4s as well as selectivity over duplex DNA.[11-14]

Recently, smart G4 ligands were reported to trigger a subsequent reaction event in addition to interact selectively with G-quadruplex versus duplex DNA. This approach is of great interest to better control the activity and to limit off-target side effects of the ligands. Thereby, the covalent modification of G-quadruplex architecture has been achieved through different strategies including G4-metalation,[15, 16] G4-alkylation,[17] G4-scission[18, 19] and G4-oxidation.[20] This latter is particularly relevant as guanine is the nucleobase more prone to oxidation. In this context, a number of compounds including porphyrins, naphthalene, perylene diimides derivatives, metal complexes that can photo-oxidize G-quadruplex structures have been reported.[19, 21-25] However, none of them was shown to induce G-driven telomeric DNA lesions in cellulo. Herein, we report on the design of new smart G4 ligands based on Ru(II) complexes and combining i) high affinity towards multimeric G4 structures of the telomeric sequence and ii) photoreactivity to induce oxidative DNA damages. Thanks to Telomere Dysfunction-Induced Foci (TIF) experiments, we demonstrate that some complexes, with high affinity for G-quadruplex telomeric DNA, a suitable cell penetration and a photocytotoxic activity, are able to photo-induce DNA damages in the telomeric region of cancer cells. To the best of our knowledge, the present study demonstrates for the first time the

ability of metal complexes to photo-induce telomeric damages in cancer cells. The synthesis of six dinuclear complexes **1a-c** and **2a-c** (**Figure 1 A; Scheme S1** and **Figures S1-15**), bearing either 1,10-phenanthroline (phen) or 1,4,5,8-tetraazaphenanthrene (TAP) ancillary ligands, and a previously reported[26] bridging phenanthroimidazole ligand with flexible polyether linkers, was achieved through the coordination of $[Ru(phen)_2Cl_2]$ or $[Ru(TAP)_2Cl_2]$ to the desired bridging ligand. The use of dinuclear compounds compared to mononuclear compounds was driven towards designing systems with higher affinities for multimeric G quadruplexes. Ru(II) complexes bearing at least two TAP ligands are well known to trigger PET (Photo-induced Electron Transfer - type I photoreaction) with guanine upon light irradiation, inducing subsequent strand cleavage and photo-adduct formation.[27, 28] Recently, a TAP containing dinuclear Ru(II) compound also proved to induce phototoxicity in hypoxic regions of melanoma cancer spheroids.[29]

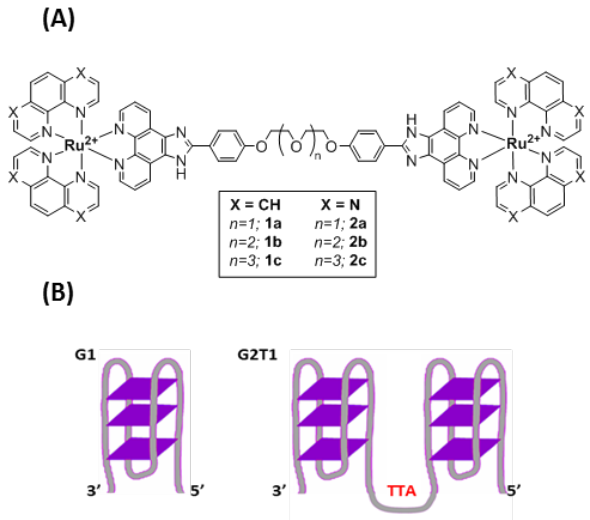

Figure 1. (A) Structures of Ru(II) complexes **1a-c** and **2a-c** (B) Schematic representation of the G1 and G2T1 model G-quadruplex DNA structures. G1: 3'-$A(GGGTTA)_3GGG$-5'; G2T1: 3'-$A(GGGTTA)_7GGG$-5'.

All complexes **1a-c** and **2a-c** exhibited ground-state absorption spectra and steady-state photoluminescence typical of charge transfer states occurring in Ru(II) polypyridyl complexes (**Table S1** and **Figures S16-17**). As anticipated, almost no difference was observed for the photo-

physical data of complexes bearing different polyether-lengths linkers. In cyclic voltammetry, complexes **1a-c, 2a-c** display successive one-electron reductions of the ancillary phen or TAP moieties (**Table S2** and **Figures S18-23**). From the estimated reduction potentials at the excited state, complexes **2a-c** exhibit stronger photo-oxidizing potentials than compounds **1a-c**. Therefore, the photoreactivity of the complexes towards 2'-deoxyguanosine 5'-monophosphate (dGMP) has been investigated thanks to luminescence quenching experiments. As expected, the oxidation potential of dGMP being close to 1.10 V vs. Ag/AgCl,[30] no luminescence quenching was observed with complexes **1a-c**. In contrast, the addition of dGMP to a solution of complexes **2a-c**, leads to an efficient luminescence quenching (**Figure S24**), with a high quenching rate constant ($3.1x10^9$ $M^{-1}s^{-1}$, $1.4x10^9$ $M^{-1}s^{-1}$ and $1.1x10^9$ $M^{-1}s^{-1}$ for **2a-c**, respectively). In agreement with the electrochemical data and the literature, this luminescence quenching can safely be ascribed to the photo-reduction of the complexes **2a-c** by a guanine moiety, leading to the generation of guanine radical cation (equation 1).

$$[Ru^*\text{-}Ru]^{4+} + G \rightarrow [Ru\text{-}Ru]^{3+} + G^{\bullet+} \qquad \text{(equation. 1)}$$

Then, the capacity of complexes **1a-c** and **2a-c** to interact with G4 model structures G1 ($^{3'}$TT(GGGATT)$_3$GGG$^{5'}$) and G2T1 ($^{5'}$TAGGG(TTAGGG)$_7{}^{3'}$) (**Figure 1 B**) was investigated in sodium and potassium buffer by circular dichroism (CD) and bio-layer interferometry (BLI) analysis. Upon addition of complexes **1a-c** and **2a-c**, no major change was observed in the CD spectra at room temperature, neither with G1 nor G2T1, in sodium or potassium buffer, suggesting that the complexes did not induce any major structural changes at 20 °C (**Figures S25-28**). CD melting assays were then performed in both Na^+ and K^+ containing buffers to assess the thermal stabilization of the quadruplex structures G1 and G2T1 when interacting with complexes **1a-c** and **2a-c**. The melting temperature of G1 and G2T1 was recorded upon addition of the complexes **1a-c** and **2a-c** with a 2:1 ratio of Ru(II) metal centre with respect to each G-quadruplex structures (**Table S3** and Figures **S29-32**). The addition of **1a-c** and **2a-c** induced a significant thermal

stabilization of the dimeric G2T1 that was more pronounced in Na^+ containing buffer. A very low stabilization –or even a destabilization– was also observed with the monomeric structure G1. However, it should be mentioned that the stabilization imparted by the ligand would be naturally more pronounced in the intrinsically less stable oligonucleotides (*i.e.* the dimeric G2T1) [ref], the direct comparison of Δ*T*m values cannot provide straightforward information on the binding affinity.[31]

Thus, to gain insight into the binding affinity of the complexes for the different DNA G4s, bio-layer interferometry (BLI) experiments were carried out. This technique indeed allows the study of the affinity independently of the stability of the oligonucleotides. The kinetic and thermodynamic parameters of the interaction towards **G2T1**, **G1** and duplex hairpin DNA **HP GC** ($^{3'}(GC)_4TTTT(GC)_4{}^{5'}$) as a control, both in sodium and potassium buffer (**Table 1**, **S4** and **Figures S33-44**) were determined. All complexes display high affinity in the nanomolar range for the dimeric and monomeric G4 structures, in both buffers. In comparison with the previously reported mononuclear Ru(II) complexes, for which the equilibrium dissociation constants (K_D) values were in the micromolar range, the new dinuclear complexes show a much higher affinity towards G-quadruplex DNA.[24] For all complexes, the affinity proved to be slightly stronger for the dimeric quadruplex **G2T1** than for the monomeric structure G1. We also noticed that the length of the polyether linkers mildly impacts the binding affinity with dimeric and monomeric G-quadruplex DNA.

Table 1. Dissociation constant (K_D, nM)[a] of the interaction of complexes 1a-c and 2a-c with DNA structures G2T1, G1 and HP GC determined by BLI experiments.

DNA structures	Buffer	1a	1b	1c	2a	2b	2c
G2T1	Na^+	**326** ± 143	**166** ± 13	**124** ± 24	**102** ± 16	**221** ± 102	**212** ± 50
	K^+	**320** ± 137	**361** ± 170	**522** ± 304	**333** ± 147	**370** ± 129	**482** ±

G1	Na^+	**427** ± 42	**256** ± 35	**231** ± 125	**167** ± 41	**254** ± 45	**649** ± 182
	K^+	**549** ± 222	**448** ± 304	**404** ± 192	**524** ± 287	**501** ± 87	**1648** ± 893
HP GC	Na^+	**1600** ± 50	**1200** ± 252	**476** ± 23	**366** ± 50	**377** ± 38	**962** ± 139
	K^+	**1146** ± 200	**2662** ± 276	**3092** ± 826	**7607** ± 3643	**1410** ± 952	**943** ± 275

[a] Equilibrium dissociation constants deduced from the kinetic rate constants. The provided errors are standard deviations from the mean values. The running buffer was Tris-HCl 10 mM, NaCl or KCl 100 mM (pH 7.04) and 0.5% v/v surfactant.

Confocal microscopy was implemented to investigate the cellular uptake of the complexes in U2OS human osteosarcoma cells. As depicted in **Figure 2** (see also **Figure S45**), all complexes showed effective cell penetration. In the studied concentration range, differences in intracellular distribution were observed: while complex **1a** seems to localize mostly to the nucleus, complexes **1b-c** and **2a-c** localized to both the cytoplasm and the nucleus.

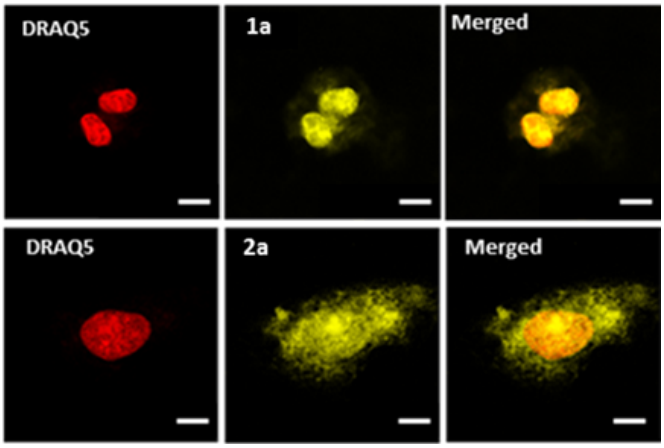

Figure 2. Fluorescence microscopy images of U2OS cells after incubation (1h30) with **1a** (10 µM) or **2a** (50 µM)* complex in DMEM buffer. From left to right: the nucleus in red, stained by DRAQ5; **1a** and **2a** luminescent complexes in yellow; merged images. Scale, 10 µM. * Due to the inherent quenching associated with TAP-containing complexes, a higher concentration was used for **2a**.

The capacity of the complexes to photo-induce cellular toxicity towards the U2OS cell line was then tested. Importantly, no cellular toxicity was observed when the different complexes were

added to the medium in the dark. In contrast, light irradiation led to a dramatic decrease of the survival rate of U2OS cells. IC_{50} values for each complex were within the micromolar range or lower (**Table 2** and **Figure S46**). The strong photo-cytotoxicity of reference complexes **1a-c** could be explained by the photo-sensitization of singlet oxygen, namely type II photo-oxidation as those types of Ru(II) compounds are studied for photodynamic therapy applications.[32, 33] However, due to the singlet oxygen diffusion, this process should lead to less localised damages. In contrast, **2a-c** complexes are likely to induce cell mortality by both type I and II photo-oxidation

Table 2. IC_{50} values (μM) for 1a-c and 2a-c in U2OS cells in the dark or upon light irradiation.

	1a	1b	1c	2a	2b	2c
Light	0.83	1.02	0.59	1.71	0.84	2.27
Dark	>10	>10	>10	>10	>10	>10

Cell viability studies. U2OS cells were incubated for 1h30 with complexes **1a-c** and **2a-c** before 30 minutes irradiation with blue LED light (405 nm at 15.7 Wm^{-2}). Tetrazolium salt-based cellular viability assays were performed 24h post-irradiation. Negative control: blue light irradiation without any Ru(II) complex shows no effect on cells viability.

In addition to the photo-toxicity experiments, the ability of the compounds to target and damage telomeric DNA was studied in U2OS cells. Telomere Dysfunction-Induced Foci (TIF) assays allow to monitor damaged telomeres in cells thanks to the combination of fluorescent in situ hybridization (FISH) - to detect telomeres- and immunofluorescence (IF) against a DNA damage marker, in this case 53BP1.[34] We selected two lead compounds for TIF analyses, namely **1a** and **2a**.[35] The bar chart of **Figure 3** displays the frequency of nuclei with at least two damaged telomeres (TIF) in the various depicted conditions. We found that, while light irradiation of U2OS cells pre-incubated with **1a** did not increase TIF frequency, a significant three-fold increase in the frequency of nuclei with >2 TIF was observed upon light irradiation for cells incubated with **2a**. Note that the presence of a low frequency of TIF in non-treated cells was expected as U2OS cells maintain their telomeres

with a telomerase-independent mechanism, dubbed ALT, characterized by replicative stress and low level of DNA damage at telomeres.[36] Importantly, TIF were not increased when **2a** was added in the dark. It can therefore be stated that complex **2a** is able to drastically increase the amount of telomeric damages thanks to its ability to photo-oxidize guanine residues. Light irradiation also resulted in the appearance of 53BP1 foci outside of telomeres (see Figure S47). This is consistent with the fact that, although G4 structures are prevalent at telomeres, they are also detected at other genomic loci, including oncogene promoters, gene bodies or 5' untranslated transcribed regions.[37]

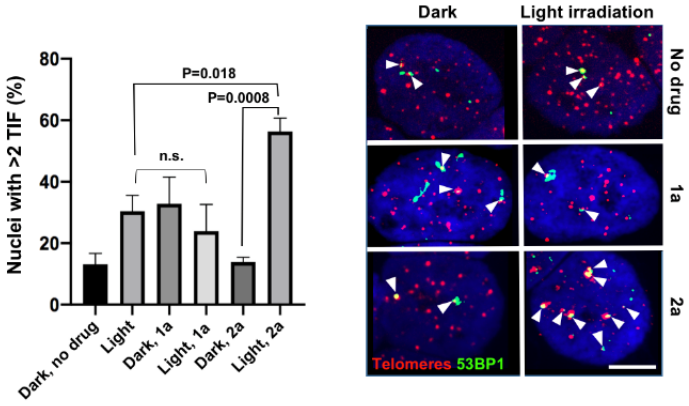

Figure 3. TIF experiments in U2OS cells. Left panel, bar chart of the frequency of nuclei showing at least two TIF (at least 50 nuclei per experiment). Mean ± SEM. Unpaired Student's t-tests were applied; n.s.: non-significant. Right panel, representative pictures from the FISH/IF experiments. Telomeres, detected by FISH, appear in red. 53BP1, a marker of DNA damage, detected by IF, appears in green. Arrowheads indicate TIF. Scale, 10 μM.

In conclusion, a series of new ruthenium(II) dinuclear complexes bearing 1,10-phenanthroline (phen) and 1,4,5,8-tetraazaphenthrene (TAP) ligands have been synthesized as new telomeric photo-reactive agents. Complexes **2a-c** are able to (i) strongly interact with telomeric DNA and (ii) photo-induce electron transfer with dGMP. All complexes were found to efficiently penetrate into the cells and to induce dramatic damages upon light irradiation, whereas no toxicity was observed

in the dark. Crucially, we evidenced the presence of photo-induced DNA lesions at the telomeres of U2OS osteosarcoma cells incubated with complex **2a**. These results emphasize the importance of direct PET with guanine and represent, to the best of our knowledge, the first in cellulo evidences of G-driven photo-oxidative damages at telomeric DNA of cancer cells. In the future, the synthesis of related photo-redox active structures with higher selectivity towards duplex DNA may provide new powerful approaches to target telomeres. This is currently under investigation.

Acknowledgements

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