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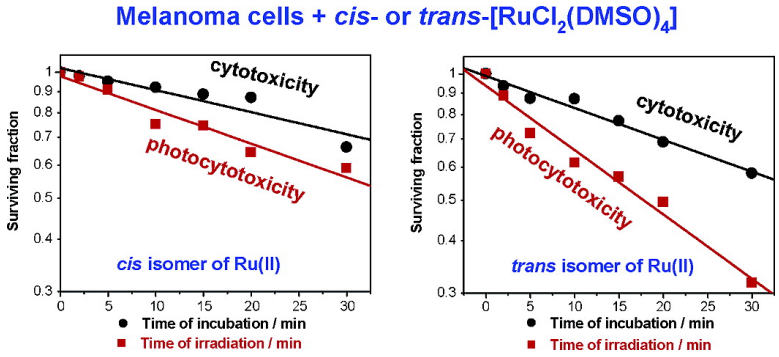

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Light-Induced Anticancer Activity of $[RuCl_2(DMSO)_4]$ Complexes

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The cytotoxicity and photocytotoxicity of *trans*-$[RuCl_2(DMSO)_4]$ and *cis*-$[RuCl_2(DMSO)_4]$ complexes was tested in two melanoma cell lines, human (SK-MEL 188) and mouse (S91). The trans isomer was found to be more effective for cell growth inhibition than its cis analogue both in the presence and in the absence of illumination. However, the antiproliferative activity of both isomers was significantly enhanced after irradiation with UVA light in comparison with their activity observed in the dark. The influence of light on the reaction of both ruthenium(II) isomers with the single-stranded hexanucleotide $d(T_2GGT_2)$, chosen as a model system for DNA, was also studied using chromatography and mass spectrometry techniques. The photochemical reaction of the ruthenium(II) complexes with the oligonucleotide $d(T_2GGT_2)$ resulted in the formation of $Ru(G-N7)_2$ adducts, which was not observed in the same time scale in thermal reactions. The initial short irradiation of the inert cis isomer was found to facilitate the covalent adduct formation with $d(T_2GGT_2)$ in the secondary thermal reactions and with a rate comparable to that found for the trans isomer, which is ca. 5−10 times more reactive in the dark.

Introduction

The development of more efficient anticancer drugs with better selectivity and diminished toxic side effects is currently an area of intense research. One of the approaches involves the use of different type carriers to improve the delivery of drugs exclusively to tumor cells (targeting strategy).[1] Another strategy is based on administration of prodrug, which can be selectively activated in tumor tissue.[2,3] In this respect, the use of light-inducible reactions offers a unique possibility of initiating the desired activity only in selected target tissue. The design of photoactive drugs is a new approach in chemotherapy that has been extensively studied during the past decade.[4] Excitation of photoactive molecules can induced different types of photoreactions including direct activation of pharmaceuticals or their indirect photodynamic action.[4] The latter so-called photodynamic effect is used in photodynamic therapy (PDT), which is one of the more active research fields in anticancer therapy.[5−7] The basic idea of PDT is to administer a photosensitizer with tumor-localizing properties followed by light activation in a site-specific manner. The excited photosensitizer then undergoes various reactions, among which electron transfer and energy transfer are the most important ones. Radicals and singlet oxygen produced in these processes are the dominant species responsible for damage of malignant cells. Alternatively, light can be used for activation of prodrugs at the target site, for example, by photoinduced reduction or ligand substitution reaction whereby a metal complex would either release a biologically active molecule or bind to nucleic acids or protein active sites.[2,4]

Some photoactive metallodrugs have recently been developed. A special interest has been focused on photoactive Pt(IV) analogues of the anticancer drug cisplatin ($[PtCl_2(NH_3)_2]$), such as Pt(IV)-diiododiamine and Pt(IV)-diazidediam(m)ine compounds.[8−12] When activated by visible light, these compounds form highly reactive Pt(II) species, which bind rapidly to nucleotides (5′-GMP, d(GpG)) and DNA forming cisplatin−nucleotide cross-links. Ruthenium(II) complexes with polypyridine ligands represent a second type of metallodrug that can be photoactivated. It has been found that some of these compounds can inhibit gene transcription by direct photoinduced electron transfer between the oxidizing complex (containing π-deficient ligands) and DNA with a concomitant formation of covalent photoadducts with DNA.[13−16]

The ruthenium(II) complexes studied in this work, *trans*-$[RuCl_2(DMSO)_4]$ and *cis*-$[RuCl_2(DMSO)_4]$, have both been shown to exhibit antiproliferative activity in several experimental tumors such as P388 leukemia, Lewis lung carcinoma, B16 melanoma, and MCa mammary carcinoma. It has been found that both isomers tend to localize to a higher extent in the tumor than in normal tissue and show significant antimetastatic activity and lower toxicity for normal proliferating tissue in comparison to cisplatin.[17−19] However the equitoxic doses of the ruthenium complexes are much higher than cisplatin (e.g., for the treatment of P388 leukemia ca. 80 or 1300 times higher doses are needed for the trans and cis isomer, respectively).[17] The therapeutic effects have been attributed to the ability of both isomers to form stable adducts with DNA, cross-links between adjacent guanines being the dominant ones.[20,21] The cis

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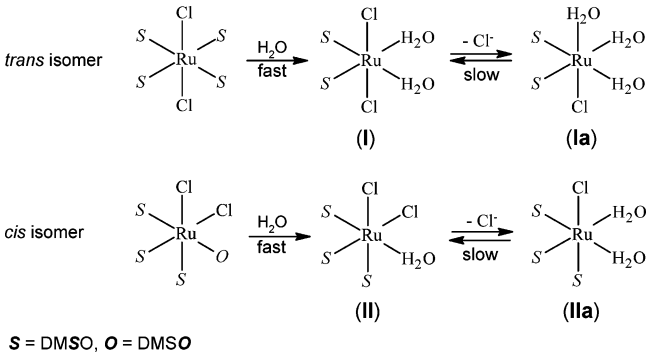
Scheme 1



isomer is more inert to substitution reaction and its reactivity toward selected nucleosides, nucleotides, and DNA is much lower than that for the trans isomer,[20,22−24] an effect that is claimed to be responsible for its lower anticancer activity.[17−19]

In this study, we explore whether the use of light (UVA) can enhance the antiproliferative effect of both isomers on human (SK-MEL 188) and mouse (S91) melanoma cell lines. Our preliminary studies have shown that both isomers are photoactive in aqueous solution. The compounds undergo gradual photodissociation of DMSO and Cl^- ligands, and the use of light was shown to enhance their antiproliferative activity.[25,26] We here further investigate the effect of light on cytotoxicity of both ruthenium complexes on melanoma cells. To find a possible explanation of the observed photocytotoxic effects, we also study the influence of light on reaction of *trans*- and *cis*-[$RuCl_2(DMSO)_4$] with d(T_2GGT_2) oligonucleotide as a model system of DNA. The direct photochemical reactions as well as the light-induced secondary thermal reactions with the oligonucleotide were studied by a combination of HPLC and MALDI-TOF techniques. The study shows that in light-induced reactions of both ruthenium(II) isomers with the oligonucleotide d(T_2GGT_2) adducts of the type Ru(G−N7)$_2$ are formed as a result of both direct photochemical reaction and secondary thermal processes.

Results and Discussion

Anticancer Effect of *cis*- and *trans*-Ruthenium(II) Complexes. Both the cis and trans isomers of the ruthenium(II) complex [$RuCl_2(DMSO)_4$] immediately release two or one DMSO ligand when dissolved in water giving rise to formation of *trans*,*cis*,*cis*-[$RuCl_2(DMSO)_2(H_2O)_2$] (**I**) and *cis*,*fac*-[$RuCl_2(DMSO)_3(H_2O)$] (**II**), respectively (Scheme 1).[27,28] The cellular growth inhibition by the complexes **I** and **II** was tested in the dark on two melanoma cell lines, SK-MEL 188 and S91, to determine the sublethal dose. The effects were assessed after 40 h, revealing a long-term cell response. As can be seen from Figure 1, there is no difference in surviving fraction between mouse (S91) and human (SK-MEL 188) melanoma cells treated with ruthenium(II) complexes at low concentrations. A sublethal dose of 10^{-7} M for both ruthenium(II) isomers was chosen for further experiments. At higher concentrations (above 1 μM), the cytotoxic effect was more pronounced for **I**, however. The higher potency of the trans isomer (**I**) in comparison with the cis analogue (**II**) has been previously observed for other experimental tumor systems.[17−19] The antiproliferative effect of **I** and **II** increases continuously with increasing time of incubation (see Figure 2). The mean (± SD) times of incubation in the dark required to inhibit S91 cell growth by 50% as compared to untreated control (IC_{50}) are 35.2 ± 4.3 and 51.1 ± 9.5 min for **I** and **II**, respectively. Similar growth inhibition was observed for the SK-MEL 188 cell line (for IC_{50} values see Table S1 in Supporting Information).

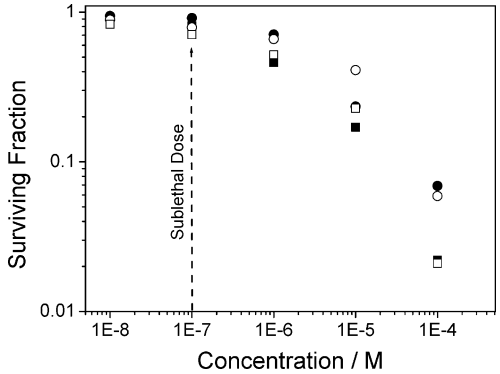


Figure 1. Surviving fraction of SK-MEL 188 cells treated with increasing concentration of the studied ruthenium complexes **I** (■) or **II** (●) and S91 cells treated with **I** (□) or **II** (○).

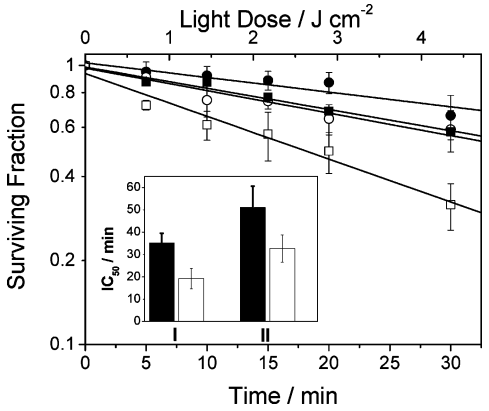


Figure 2. Effect of UVA on the growth inhibition of S91 cells in the presence of the sublethal dose (0.1 μM) of **I** and **II** complexes. The cells were either incubated entirely in the dark with **I** (■) or **II** (●) or illuminated in the presence of **I** (□) or **II** (○). Time corresponds to time of incubation for filled symbols or time of irradiation for opened symbols. The inset shows the mean (± SD) times of incubation required for both isomers (**I**, **II**) to inhibit cell growth by 50% relative to untreated control (IC_{50}) without (filled bars) or with (opened bars) irradiation.

For determination of the influence of light on cytotoxicity of both ruthenium(II) complexes, the cell cultures were exposed to UVA light. Both ruthenium(II) complexes exhibit higher toxicity under illumination in comparison to their dark activity (compare Figure 2). The cytotoxic effect of complex **I** in the dark is similar to the cytotoxic effect of **II** with light. This suggests that irradiation of complex **II** could result in its conversion to trans analogue **I**, outside or inside the cells, and that the photoproduct(s) acts as a more efficient killing agent(s). The longer irradiation (higher dose of light) resulted in a stronger growth inhibition (compare Figure 2 and Table S1). The light dose necessary to kill 50% of cells (IC_{50}) was 3.16 ± 0.78 and 4.73 ± 0.88 J/cm^2 for **I** and **II**, respectively. Light alone caused small changes in the surviving fraction of the cells (data not shown).

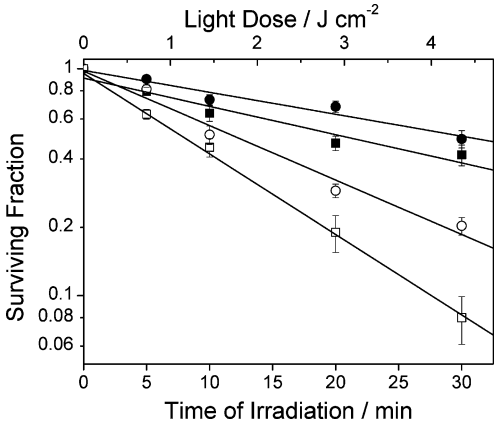


Figure 3. Photocytotoxic effect of ruthenium complexes after their preincubation with S91 cells prior irradiation (UVA) for 5 min, **I** (■), **II** (●), and for 30 min, **I** (□), **II** (○). Preincubation of cells with sublethal dose (0.1 μM) of both Ru complexes was followed by removal of excess of complexes before illumination.

The photocytotoxic effect resulting from light activation of ruthenium(II) complexes is more pronounced for compound **I**. The plausible explanation for that fact is that the higher efficiency arises not only from phototransformation of the complex itself but also from direct photochemical reactions between complex **I** and some components of cell.

To get more information about the nature of the phototoxic effect observed for both ruthenium(II) complexes, we preincubated cells with *trans*,*cis*,*cis*-[$RuCl_2(DMSO)_2(H_2O)_2$] (**I**) and *cis*,*fac*-[$RuCl_2(DMSO)_3(H_2O)$] (**II**) for 5 and 30 min, removed the excess of complex, and irradiated cells (for details see Materials and Methods section). For both ruthenium isomers, longer preincubation time resulted in a stronger growth inhibition (see Figure 3). The surviving fraction of the S91 cell culture was 50% after 11.7 ± 4.6 and 15.4 ± 4.6 min of irradiation for cells preincubated for 30 min with **I** and **II**, respectively. The antiproliferative activity increased twice in comparison with cell cultures preincubated only for 5 min. These results show that distribution of ruthenium(II) complexes is an important factor that directly influences the photocytotoxic effect.

Taken together, we show here that antiproliferative activity of both ruthenium complexes significantly increases upon irradiation. In all series of experiments done with or without illumination, the trans derivative (**I**) was more effective in killing melanoma cells than the cis isomer (**II**). The most substantial effect, up to 92% of cytotoxicity, occurred when **I** was preincubated with cells for 30 min and then irradiated for 30 min (compare Figure 3). Moreover, our findings indicate that complex **II** could be used as a prodrug (lower cytotoxicity) that after photoactivation exhibits a pronounced cytotoxic effect.

The eventual in vivo application of the studied systems will require overcoming the very limited tissue penetration by UVA light. This problem could be partially solved via application of the state-of-art lasers and fiber optics, which enable precise operation with a light beam and irradiation of very thin layers, one by one, to destroy the needed area. The studied systems may also be considered for the treatment of blood borne diseases, autoimmune diseases, leukemia, etc., by photopheresis since light penetration is much less of a problem in this method. And finally the new Ru(II) complexes can be designed via replacement of one or more DMSO or Cl^- molecules by appropriate ligands to access complex photoactivation with visible light.

Effect of Light on the Reactivity of *cis*- and *trans*-Ruthenium(II) Complexes toward d(T_2GGT_2). The here described photocytotoxic effect could be the result of the influence of light on reaction of the studied ruthenium(II) complexes with various cell components such as cellular DNA, proteins, or others. Previous studies performed in the absence of light suggest that the cytotoxic effect might be due to interactions of *trans*- and *cis*-[$RuCl_2(DMSO)_4$] complexes with DNA.[20,21] In view of these reports, one of the possible mechanisms of the abovementioned photocytotoxicity could arise from enhancement of the reactivity of both isomers with cellular DNA upon irradiation. At least two different types of reactions can be considered, (i) direct photochemical reaction between ruthenium(II) complexes and cellular DNA and (ii) a light-induced secondary thermal reaction between the photoproducts of ruthenium(II) complexes and DNA. In the present study, the oligonucleotide d(T_2GGT_2) was chosen as a model system for DNA to study both types of reactions. This oligonucleotide provides a well defined binding site for the ruthenium center (i.e., the -GG- pair) and has been successfully used to study thermal interactions with both isomers.[24] The influence of light on the reactivity of both isomers toward the oligonucleotide was investigated by monitoring the progress of the reaction (HPLC technique), followed by identification of reaction products (HPCL or MALDI-TOF technique). For comparison, the most important information about the chemical and photochemical behavior of *trans*- and *cis*-[$RuCl_2(DMSO)_4$] complexes in aqueous solution is also given below.

Thermal Hydrolysis and Photolysis of *trans*- and *cis*-[$RuCl_2(DMSO)_4$] Complexes in Aqueous Solution. Chemical behavior of *trans*- and *cis*-[$RuCl_2(DMSO)_4$] isomers in aqueous solution has been previously studied[24,27,28] and is summarized in Scheme 1. According to the scheme, both isomers immediately after dissolution release DMSO ligands (two or one molecules) leading to the formation of *trans*,*cis*,*cis*-[$RuCl_2(DMSO)_2(H_2O)_2$] (**I**) and *cis*,*fac*-[$RuCl_2(DMSO)_3(H_2O)$] (**II**), respectively. Subsequent slow dissociation of one Cl^- ligand is observed for both isomers resulting in formation of *cis*,*fac*-[$RuCl(DMSO)_2(H_2O)_3]^+$ (**Ia**) and *fac*,*cis*-[$RuCl(DMSO)_3(H_2O)_2]^+$ (**IIa**), respectively (compare Scheme 1).[24] It has been suggested that on a longer time scale the second Cl^- ligand could be released in the case of the **Ia** complex.[24]

Both ruthenium complexes, that is *trans*,*cis*,*cis*-[$RuCl_2(DMSO)_2(H_2O)_2$] (**I**) and *cis*,*fac*-[$RuCl_2(DMSO)_3(H_2O)$] (**II**), are photoactive and when irradiated with UVA and visible light in aqueous solution undergo photoaquation processes.[25,26] The analysis of UV−vis, ^1H NMR, and electrochemical data[29] has shown that irradiation of *trans*,*cis*,*cis*-[$RuCl_2(DMSO)_2(H_2O)_2$] (**I**) and *cis*,*fac*-[$RuCl_2(DMSO)_3(H_2O)$] (**II**) complexes in the region of ligand field exited states leads to sequential release of DMSO and Cl^- ligands and coordination of water molecules. In the case of cis isomer (**II**) irradiation, the trans isomer (**I**) has been observed as an

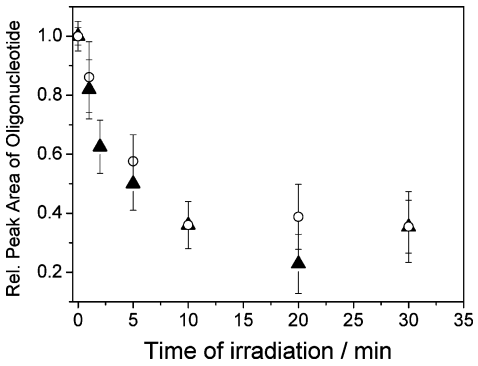


Figure 4. Normalized integrated HPLC peak area of unreacted oligonucleotide d(T_2GGT_2) for photochemical reactions with ruthenium(II) complexes **I** (○) and **II** (▲): [d(T_2GGT_2)] = 4×10^{-6} M; [**I**], [**II**] = 8×10^{-5} M; irradiation at $\lambda = 365$ nm, aqueous solution, room temperature. Error corresponds to SD of at least two independent experiments.

intermediate species. In comparison to thermal hydrolysis reaction, the photosubstitution process is much faster and more molecules of DMSO or Cl^- ligands or both are released leading to products with more labile coordination sites.

Photochemical Reactions of Ruthenium Complexes with d(T_2GGT_2). The direct photochemical reactions between the oligonucleotide d(T_2GGT_2) and *trans*,*cis*,*cis*-[$RuCl_2(DMSO)_2(H_2O)_2$] (**I**) or *cis*,*fac*-[$RuCl_2(DMSO)_3(H_2O)$] (**II**) complexes in aqueous solution were investigated after irradiation of the reaction mixture at $\lambda = 365$ nm from 1 to 30 min. The progress of the reactions was monitored by a HPLC technique. Recorded chromatograms indicated that for the reaction of both **I** and **II** complexes the intensity of the peak arising from free d(T_2GGT_2) decreased by 70% after 30 min of irradiation (compare Figure 4). However, even in the presence of a 20-fold excess of the ruthenium(II) complex over d(T_2GGT_2) a well-defined amount of oligonucleotide still remained unreacted. It must be emphasized that the thermal reaction of both ruthenium(II) complexes with d(T_2GGT_2) is slow enough ($t_{1/2} \geq 3$ h)[24] to not influence the photochemical reactions (a few minutes). The irradiation of d(T_2GGT_2) alone during 30 min did not cause any changes (as indicated by HPLC, results not shown).

The products of initial photochemical reactions of both isomers (**I**, **II**) with the oligonucleotide d(T_2GGT_2) were analyzed by a MALDI-TOF technique since those were not detectable by HPLC under the employed experimental conditions (for sample preparation see Materials and Methods section). The influence from thermal reactions with d(T_2GGT_2) were neglected since those do not occur in the same time scale as that applied for irradiation. The mass spectra obtained for irradiated samples (Figure 5 parts b and c for cis and trans complexes, respectively) were similar to those obtained during thermal reaction of **I** or **II** with d(T_2GGT_2)[30] (Figure 5d). As shown in our previous study,[24] attachment of ruthenium to oligonucleotide can be confirmed by analysis of the isotopic fingerprints of ruthenium superimposed on the oligonucleotide isotopic profile (compare Figure 6). In each spectrum, the most abundant ion was characterized by *m/z* ca. 1811.3 and was attributed to $[T_2GGT_2 - H]^-$ (compare Figure 6). The most abundant ions of ruthenated oligonucleotide presented in the spectra were attributed to $[T_2GGT_2\{Ru\} - 3H]^-$ (*m/z* ca. 1911.3), $[T_2GGT_2\{Ru(H_2O)(DMSO)_2\} - 3H]^-$ (*m/z* ca. 2084.4), $[T_2GGT_2\{RuCl(DMSO)\} - 3H + Na]^-$ (or $[T_2GGT_2\{Ru\} - 3H + AA]^-$, where AA is the matrix component anthranilic acid) (*m/z* ca. 2049.3), and $[T_2GGT_2\{Ru(DMSO)\} - 3H]^-$ (*m/z* ca. 1990.4). All these ions were presented in the mass spectra of the reaction mixture obtained in photochemical as well as in thermal reactions of **I** and **II** with the oligonucleotide. The formation of the ruthenium−oligonucleotide adducts upon irradiation was found to be much more efficient for the trans isomer in comparison with the cis analogue. Several other ions, with much lower relative intensities, can also be assigned to ruthenated oligonucleotide assuming an in-source fragmentation in which chloride, water, or DMSO ligands are lost from the ionized reaction products, while H^+ are replaced by Na^+ or H_3O^+. Additionally, (in all spectra) (Figures 5a−d and 6) other contributions from ions, such as $[T_2GGT_2 - 2H + H_3O]^-$ (*m/z* ca. 1829.3), $[T_2GGT_2 - 2H + Na]^-$ (*m/z* ca. 1833.3), $[T_2GGT_2 - 2H + K]^-$ (*m/z* ca. 1849.3), and $[T_2GGT_2 - 3H + Na + K]^-$ (*m/z* ca. 1871.3), were also observed.

In summary, the MALDI-TOF data provide the direct evidence that Ru(G−N7)$_2$ adducts are formed as a result of photochemical reactions of the cis and trans isomers with d(T_2GGT_2). All mass spectra were recorded from

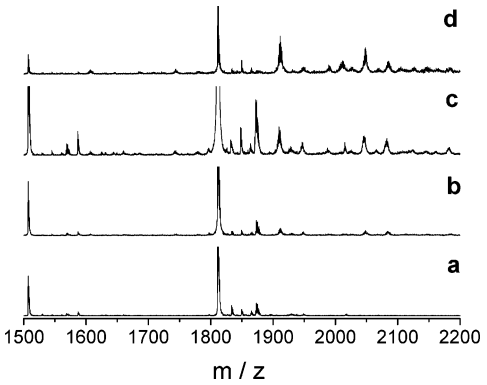


Figure 5. Selected regions of mass spectra recorded by MALDI-TOF after 3 min irradiation at $\lambda = 365$ nm of (a) d(T_2GGT_2), (b) d(T_2GGT_2) in the presence of **II**, (c) d(T_2GGT_2) in the presence of **I**, and (d) thermal reaction between **I** and d(T_2GGT_2) (the analyzed sample was collected after conversion of at least 70% of free oligonucleotide into reaction products).

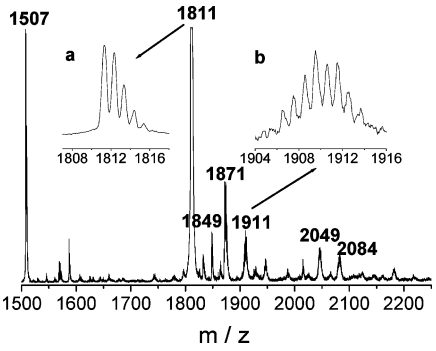


Figure 6. MALDI-TOF mass spectrum of products formed in the photochemical reaction (3 min irradiation at $\lambda = 365$ nm) of **I** with d(T_2GGT_2) in aqueous solution at room temperature. The inset shows the high-resolution MALDI-TOF mass spectrum of selected peaks: (a) $[T_2GGT_2 - H]^-$; (b) $[T_2GGT_2\{Ru\} - 3H]^-$.

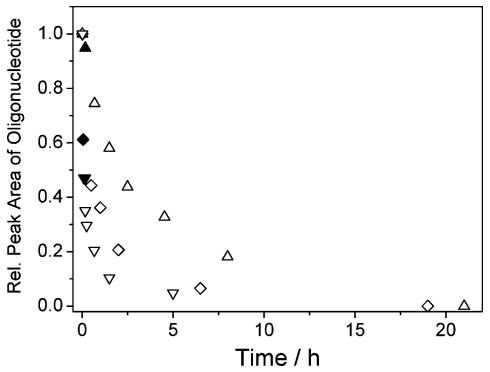


Figure 7. Changes of $d(T_2GGT_2)$ concentration with time observed for the secondary thermal reactions of $d(T_2GGT_2)$ with **II** after initial irradiation of the reaction mixture at $\lambda =$ 365 nm for 1 (△), 2 (◇), and 5 min (▽). The second point of each experiment, denoted by filled symbols, refers to decreasing of oligonucleotide concentration just after irradiation. $[d(T_2GGT_2)] = 4 \times 10^{-6}$ M; [**II**] $= 8 \times 10^{-5}$ M; $T = 37$ °C.

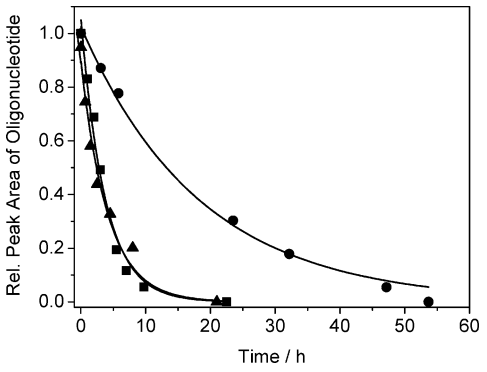


Figure 8. Changes of $d(T_2GGT_2)$ concentration with time observed for the thermal reaction of $d(T_2GGT_2)$ with **I** (■), **II** (●), and **II** preceded by 1 min irradiation at $\lambda = 365$ nm (▲). $[d(T_2GGT_2)] = 4 \times 10^{-6}$ M; [**I**], [**II**] $= 8 \times 10^{-5}$ M; $T = 37$ °C. Solid lines represent fits of experimental data to single-exponential functions.

400 to 3000 Da, and in this range, we did not find any additional ions that were exclusively present in irradiated samples. However, we cannot exclude that other photochemical products at lower concentration or at masses below 400 Da could be formed upon irradiation.

Light-Induced Secondary Thermal Reactions of Ruthenium Complexes with $d(T_2GGT_2)$. Our previous studies[24] have shown that the thermal reaction of $d(T_2GGT_2)$ with the cis isomer (*cis*,*fac*-$[RuCl_2(DMSO)_3(H_2O)]$, **II**) in freshly prepared aqueous solution is ca. 5 times slower in comparison with the corresponding reaction with the trans analogue (*trans*,*cis*,*cis*-$[RuCl_2(DMSO)_2(H_2O)_2]$, **I**). In view of the photochemical properties of the cis isomer (**II**),[26,29] we therefore investigated whether the thermal reaction between relatively inert complex **II** and the oligonucleotide $d(T_2GGT_2)$ could be accelerated by light.

The direct photochemical reaction of **II** with the oligonucleotide $d(T_2GGT_2)$ (vide supra) was found to be followed by much slower secondary thermal reactions, which took place between the oligonucleotide and the photoproducts of **II**. The first process resulted in a rapid initial reduction of the observed peak area of the free oligonucleotide to ca. 90%, 61%, and 47% upon irradiation of the reaction mixture for 1, 2, and 5 min, respectively (compare Figure 7). The subsequent thermal reactions, which occurred during next few hours, were examined by observation of further decrease of the peak from free oligonucleotide and concomitant build-up of the peaks from products. The reaction rate was estimated from the plots of integrated peak area of unreacted oligonucleotide vs time (compare Figure 8). Irradiation of the reaction mixture of complex **II** and $d(T_2GGT_2)$) for 1 min was enough to reach a product formation level similar to that found for complex **I** in the dark reaction after 20 h (compare Figure 8). The experiment performed with excess chloride ($[Cl^-] = 0.2$ M) shows that the light-induced acceleration is similar for the reaction between the cis isomer of ruthenium(II) complex and $d(T_2GGT_2)$ also under these conditions (results not shown).

The chromatograms obtained for the photoinduced secondary thermal reaction of **II** with $d(T_2GGT_2)$ show that the same products are being formed as those observed for the thermal reaction (results not shown), all in agreement with our previous study.[24]

Under the employed reaction conditions, the initial photoreaction transforms the inert cis ruthenium isomer into reactive photoproducts, which can bind to the oligonucleotide and hence show(s) higher reactivity in the reaction with oligonucleotide. Moreover, photolysis of the cis isomer leads to the formation of ruthenium species that ruthenate the oligonucleotide with a rate similar to or higher than that observed for the trans analogue in the dark reaction.

The data obtained from the studies of the photochemical reaction of ruthenium(II) complexes with a model system of the DNA ($d(T_2GGT_2)$) suggest that the enhancement of antiproliferative activity of these compounds upon irradiation could be the result of their interactions with the nuclear DNA. This assumption is supported by the observation of the significantly elevated photocytotoxic effect in cell cultures preincubated with ruthenium(II) complexes prior to irradiation (compare Figure 3), a procedure that allows for accumulation of a higher concentration of the complex inside the cell. The interaction of ruthenium(II) complexes with cellular DNA can proceed via direct photochemical reactions or subsequent thermal reactions with photoproducts of ruthenium(II) complexes. The higher photocytotoxic effect observed for the trans isomer in comparison with its cis analogue can arise from more efficient photochemical reaction with DNA. In a model system, the irradiation of both complexes in the presence of oligonucleotide $d(T_2GGT_2)$ resulted in the formation of ruthenium−oligonucleotide adducts; however it was found to be more efficient for trans isomer. The proposed mechanism gives one possible explanation for the observed photocytotoxic effect, but we cannot exclude that other reaction pathways are involved, for example, redox processes or interactions with other cellular components.

Conclusions

The present work shows that the cytotoxic effect of two ruthenium(II) complexes, *trans*,*cis*,*cis*-$[RuCl_2(DMSO)_2(H_2O)_2]$ (**I**) and *cis*,*fac*-$[RuCl_2(DMSO)_3(H_2O)]$ (**II**), against melanoma cells (SK-MEL 188 and S91) is significantly increased by irradiation with UVA light. Two factors likely contribute to the increased anti-

proliferative activity after illumination, (i) phototransformation of the complex itself into more active species, which subsequently react with cellular components, and (ii) a direct photochemical reaction between the metal complexes and cellular components.

The direct photochemical reaction observed between complex **I** or **II** and the $d(T_2GGT_2)$ oligonucleotide, the latter chosen as a model system of the DNA, resulted in formation of the $Ru(G-N7)_2$ adducts. Moreover, it has been found that the initial short irradiation of the more inert cis isomer facilitates the covalent binding to the $d(T_2GGT_2)$, which occurred with a rate similar to that obtained in the thermal reaction for the trans isomer. These results suggest that the enhancement of the antiproliferative activity of both studied ruthenium complexes upon irradiation could arise from their interactions with nuclear DNA. The degradation of the free oligonucleotide $d(T_2GGT_2)$ is much faster on the photochemical pathway (minutes) than on the thermal one (hours).

The presented results indicate clearly a potential for the development of ruthenium prodrugs that can be photoactivated locally in the target tissue. As has been shown by our comparative studies of cytotoxic and photocytotoxic effect on melanoma cells (SK-MEL 188 and S91), the same result could be achieved on the photochemical pathway for much lower doses of ruthenium $[RuCl_2(DMSO)_4]$ complexes than for the thermal one. Moreover, site specific activation of prodrugs by a light beam operated with fiber optics and state-of-the-art lasers allows for much more selective therapeutic action. Both effects, higher selectivity and lower cytotoxic doses of the used prodrugs, in consequence should lead to much lower unwanted side effects.

Materials and Methods

Chemicals and Reagents. The ruthenium complexes, *trans*-$[RuCl_2(DMSO)_4]$ and *cis*-$[RuCl_2(DMSO)_4]$, were synthesized according to published procedures.[27,31] In all experiments, aqueous solutions of both complexes were freshly prepared before use. Further studies were performed assuming instant conversion of these parent complexes (*cis*- and *trans*-$[RuCl_2(DMSO)_4]$) to their corresponding aqua species, that is, *trans,cis,cis*-$[RuCl_2(DMSO)_2(H_2O)_2]$, **I** (from trans isomer), and *cis,fac*-$[RuCl_2(DMSO)_3(H_2O)]$, **II** (from cis analogue), upon dissolution.[27,28] The oligonucleotide $d(T_2GGT_2)$ was purchased from Scandinavian Gene Synthesis AB and used without further purification.

Experimental Procedures. HPLC analyses were performed on a LaChrom liquid chromatograph (Merck Hitachi) with D-7000 interface and D-7400 UV/vis detector set at 260 nm. Separation of unreacted oligonucleotide from reaction products was achieved under reverse phase conditions with a Vydac protein and peptide C18 column (100 mm × 4.6 mm i.d., 10 μm particle diameter) equipped with a guard. The column was thermostated at 30 °C by use of an L-7350 oven and cooling module (Merck Hitachi). Solutions of 0.10 M ammonium acetate, NH_4OAc (ACROS), adjusted to pH 6.0 with acetate acid, HOAc (Merck), **A**, and a 1:1 mixture of **A** and acetonitrile, CH_3CN (LabScan), **B**, were used as eluents. The separation was obtained in a low-pressure gradient system with a constant flow of 1 mL/min and a varying ratio **A**:**B**, typically from 88:12 to 65:35 over 17 min. A high-pressure mercury lamp (HBO, 200 W) was used as a source of light in the experiments on the photoreactivity of both ruthenium(II) complexes in the presence of oligonucleotide $d(T_2GGT_2)$. Irradiation at distinct wavelengths was achieved with interference filter $\lambda = 365$ nm. For irradiation of the wells containing cellular cultures, a Philips HB 311 lamp emitting UVA light (300−420 nm, $\lambda_{max} = 354$ nm) was used. The incident light dose on cells was evaluated by an UVX 36 digital radiometer (Ultra-Violet Products, California). Linear MALDI-TOF mass spectra were recorded on a time-of-flight reflectron mass spectrometer Bruker Reflex IV equipped with a nitrogen laser (337 nm, 4 ns pulses). The accelerating voltage was 20 kV for all samples, and the grid voltage was 18.45 kV. All spectra were collected in the negative mode.

HPLC Measurements. All reactions were performed in aqueous solution with the ruthenium complex in ca. 20-fold excess with respect to oligonucleotide and with typical reactant concentrations of $[Ru] = 8.0 \times 10^{-5}$ M and $[d(T_2GGT_2)] = 4.0 \times 10^{-6}$ M. The progress of all reactions with $d(T_2GGT_2)$ was monitored by following the time dependence of the integrated HPLC peak area of unreacted oligonucleotide. Photochemical reactions of **I** and **II** complexes with $d(T_2GGT_2)$ were studied by irradiation at $\lambda = 365$ nm of the reaction mixture for different time intervals and directly analyzed by HPLC measurements before the secondary thermal reactions started. No thermal reaction between both ruthenium complexes and $d(T_2GGT_2)$ has been observed for the same time scale as that for irradiated samples (ca. 30 min). The photoinitiation of reactions between **II** and $d(T_2GGT_2)$ was achieved by irradiation at $\lambda = 365$ nm of the reaction mixture during 1, 2, and 5 min followed by HPLC study of the thermal reaction at 37 °C. Thermal reactions of **I** and **II** complexes with $d(T_2GGT_2)$ were carried out at 37 °C.

MALDI-TOF Measurements. The products obtained in the photochemical and thermal reactions of $d(T_2GGT_2)$ with **I** or **II** were analyzed by MALDI-TOF mass spectrometry. In photochemical studies, the reaction mixture, typically $[Ru] = 14.0 \times 10^{-4}$ M and $[d(T_2GGT_2)] = 7.0 \times 10^{-5}$ M, was irradiated at $\lambda = 365$ nm for 3 min. The resulting sample was immediately frozen in liquid nitrogen and analyzed by use of mass spectrometry. No reaction between either ruthenium complexes and $d(T_2GGT_2)$ was observed within 3 min incubation without illumination. In the thermal reactions, the reaction mixture (the same concentrations as in photochemical reaction) was incubated at 37 °C until at least 70% of oligonucleotide was converted into products (typically a few hours) and afterward analyzed by mass spectrometry. The progress of the reaction was checked periodically by HPLC.

The MALDI matrix was a solution of nicotinic acid (2.46 mg, 0.02 mmol) and anthranilic acid (5.48 mg, 0.04 mmol) in a mixture of acetonitrile (100 μL) and water (50 μL) followed by addition of 50 μL of spermine (100 mM). In each analysis, 2 μL of analyte was mixed with 3 μL of the matrix solution. Aliquots of the resulting mixture (0.75 μL) were spotted on the MALDI plate and air-dried. All measured samples exhibited peaks at *m/z* 1811.3 and 1507.3 (assigned to $[T_2GGT_2 - H]^-$ and $[T_2GGT - H]^-$, respectively), which were used for molecular weight calibration of the instrument. The spectrometer resolution was set to 0.5 Da (peak width at half-weight), and reported *m/z* values correspond to the highest intensity peak observed for a given species.

Determination of Cell Growth Inhibition. Antiproliferative activity of the ruthenium(II) complexes, *trans*- and *cis*-$[RuCl_2(DMSO)_4]$, was tested in a cell culture system using two melanoma cell lines, human (SK-MEL 188) and mouse (S91). The cells were grown in F10 medium supplemented with 10% fetal calf serum (FCS) or RPMI 1640 with 5% FCS and antibiotics for SK-MEL 188 and S91 cells, respectively. Cells were cultured at 37 °C in 5% CO_2/95% air. All cell culture reagents were purchased from Gibco BRL, Life Technologies TM, Germany. For evaluation of cytotoxicity of both Ru complexes, cells were seeded in 96-well culture plates at a density of 10^4 cells/well and incubated for 8 h in culture medium. The medium was removed, and cells were treated with freshly prepared aqueous solutions of both ruthenium isomers at concentrations between 10^{-8}−10^{-4} M and incubated for 30 min in the dark. Then the ruthenium solution was removed, and both treated and untreated cultures were washed three times with fresh culture medium and left in the incubator for another 40 h. Afterward the surviving fraction

of the cells was determined by counting the number of cells in the treated and control cultures using the optical microscope (Hund Wiloverts Wetzlar, Helmunt Hund, Germany). The concentration of 10^{-7} M for both Ru complexes was chosen for evaluation of the influence of incubation time (2−30 min) on cell growth, and the same procedure was used as described above.

Photocytotoxic effect was assessed by addition of Ru(II) complexes at 10^{-7} M concentration to cell cultures and immediate irradiation with UVA (2.0 mW/cm^2) using a Philips HB311 lamp. Cells were irradiated for 2, 5, 10, 15, 20, and 30 min, which correspond to light doses of 0.29, 0.73, 1.45, 1.18, 2.90, and 4.35 J/cm^2, respectively. In another series of experiments, cells were preincubated with Ru compounds for 5 or 30 min and then the excess of the complex was removed, fresh medium was added, and subsequently the same light doses were applied. The surviving fraction of the cells was determined using the same procedure as described above.

All data points represent the mean value, and errors are SD of at least two independent experiments. Control and treated groups were statistically analyzed using Student's *t*-test ($P \leq 0.06$).

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Supporting Information Available: Table 1S containing all IC_{50} values for tested Ru(II) complexes without and with irradiation in human (SK-MEL 188) and mouse (S91) melanoma cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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