Molecular Reaction Mechanisms of Combination Treatments of Low-Dose Cisplatin with Radiotherapy and Photodynamic Therapy

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Abstract: Combination of low-dose cisplatin with radiotherapy or photodynamic therapy (PDT) is a novel cancer treatment. Using time-resolved femtosecond laser spectroscopy, we reveal the molecular mechanisms of the combinations of cisplatin with radiotherapy and PDT using indocyanine green (ICG) excited at 800 nm. DNA damage measurements confirm that electron-transfer reactions of cisplatin with electrons generated in ionizing radiation and with the ICG singlet excited state in PDT are responsible for the cytotoxic enhancements.

Time-resolved femtosecond laser spectroscopy is a direct technique for real-time observations of molecular reactions.[1,2] In particular, femtosecond laser spectroscopy was predicted to be a powerful technique for studies of photochemical reactions in medicine, for example, for photodynamic therapy (PDT[a]) of cancer.[2] This technique has recently been employed to study the molecular mechanisms of action of anticancer drugs for chemotherapy,[3] radiotherapy,[4] and PDT.[5,6]

Cisplatin (CDDP) is one of the most widely used anticancer drugs.[7,8] It is particularly effective in treating testicular and ovarian cancer and increasingly used against cervical, head/neck bladder, and small-cell lung cancer. Despite its great success, CDDP has some significant limitations: severe toxic side effects and both intrinsic and acquired resistance. Such outstanding problems have even prompted the suggestion to stop the development of new platinum-based anticancer drugs.[9] Over the past 35 years, there have been many attempts to overcome these limitations and to broaden the range of treatable tumors by designing and testing various CDDP-like complexes. Over 3000 CDDP analogues have been synthesized and screened for anticancer activity, with no more than 30 compounds entering clinical trials and only one approved by the FDA: oxaliplatin, for the treatment of colorectal cancer.[8] Thus, the search for new anticancer drugs by traditional methods has proven to be a difficult and inefficient task. Alternatively, a biochemical modulation of the mechanisms of action of CDDP was proposed to circumvent the resistance and to improve the antitumor activity.[10] However, this strategy requires the knowledge of the precise mechanism of action of these anticancer drugs. Although it is known that the cytotoxicity of CDDP arises from its capacity to damage DNA,[7−10] the mechanism of CDDP reacting with DNA at the molecular level is unknown.[10−12] Prior to the binding of CDDP to DNA, the bonds between chlorine atoms and the Pt must be broken in one step or two steps, ending with the cis-$Pt(NH_3)_2$ unit binding to DNA. However, the binding dynamics and kinetics of CDDP to DNA, particularly the mechanism of how the cis-$Pt(NH_3)_2$ radical is generated, are not well understood. It is long known that halogenated molecules have very efficient dissociative attachment reactions with low-energy electrons to produce a halogen anion and a neutral radical.[13] And it has been observed that the presence of NH_3 can cause a large enhancement in electron-induced reactions of halogenated molecules.[14,15] CDDP ($Pt(NH_3)_2Cl_2$), a small molecule in which two NH_3 groups and two chlorine atoms are bound to a Pt atom, should have the most efficient dissociative attachment reactions with weakly bound electrons. Using time-resolved femtosecond laser spectroscopy, we have recently revealed an extremely high reactivity of CDDP with electrons produced in UV/ionizing radiation and an electron-transfer (ET) mechanism for the CDDP−DNA interaction, leading to the formation of the cis-$Pt(NH_3)_2$ radical.[3]

A novel approach to overcome the above limitations associated with CDDP is the combination of low-dose CDDP with radiotherapy or photodynamic therapy to achieve a sufficient therapeutic efficacy. For example, a combination of ionizing X-ray radiation with CDDP has been shown to lead to the enhancement of DNA damage.[16] Moreover, some researchers have recently observed that the combination of CDDP with a PDT drug, e.g., photofrin activated at 630 nm (ref 17) or indocyanine green (ICG) at 805 nm (ref 18), led to significant enhancements in cytotoxic and apoptoxic death of cancer cells. However, the molecular mechanism for the enhancements is unknown. This approach may be of particular significance, since PDT itself is emerging as a novel clinical approach. It uses lasers and drugs (photosensitizers) for the treatment of various tumors and other nonmalignant conditions, such as age-related macular degeneration, and has great potential for applications in regenerative medicine.[19−21] PDT has potential advantages over surgery and other therapies; it is comparatively noninvasive, can be targeted accurately, and has fewer side effects. A few photosensitizers have been approved for clinical use. However, existing photosensitizers are activated at the wavelength range 630−690 nm, at which the tissue penetration depth of light is only 2−4 mm and the PDT effective depth is very limited.[22] Thus, existing PDT drugs are unsuitable for treatment of deep tumors. One of the current research focuses is to develop PDT drugs sensitive to NIR light at wavelengths of 800−1000 nm to increase the penetration depth, which has been considered as an important factor in enhancing the clinical efficacy of PDT.[22] ICG is a promising NIR photosensitizer, but its PDT efficiency is very limited. In addition, the conventional PDT requires molecular oxygen and involves the photochemical generation of the singlet oxygen species to kill the target cells or destroy the vascular endothelial cells leading to tumor ischemia.[19−22] This requirement of oxygen can greatly limit the potential of PDT for solid tumors, where hypoxic regions may exist. Thus, the combination of low-dose cisplatin with PDT using ICG photoexcited at 800 nm may lead to the development of an efficient therapy for treatment of many cancers.

In this Letter, we present our results of real-time femtosecond laser spectroscopic observations of the reactions of cisplatin with the photosensitizer ICG activated at the NIR wavelength of 800 nm. Moreover, since the cytotoxicity of cisplatin as an effective anticancer drug arises from its capacity to damage DNA, we also measure the effects of the molecular reaction mechanism on the resultant DNA damage in the combination therapies of cisplatin with UV/ionizing radiation and with PDT by ICG.

The experimental details for static absorption and femtosecond laser transient absorption spectroscopic and DNA damage

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[a] Abbreviations: PDT, photodynamic therapy; ICG, indocyanine green; CDDP, cisplatin; ET, electron transfer; SC, supercoiled; OC, open circle; SSB, single-strand breaks; DSB, double-strand breaks; CL, cross-links.

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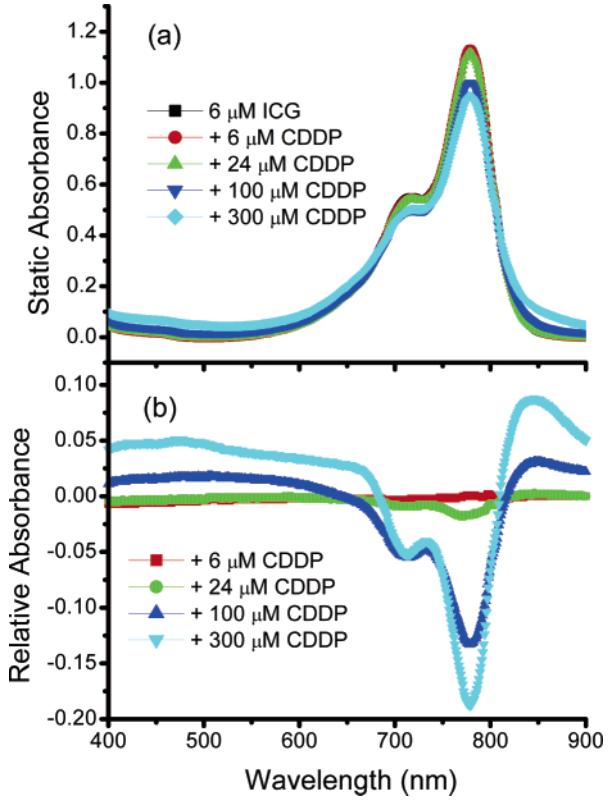

Figure 1. (a) Absorption spectra of 6 μM ICG and of the mixtures of 6 μM ICG with various concentrations of CDDP (6, 24, 100, and 300 μM) measured at 1 h after the mixing and (b) absorption difference spectra of the mixtures relative to the spectrum of 6 μM ICG.

measurements are given in Supporting Information. The static absorption spectra of 6 μM ICG and its mixtures with various CDDP concentrations are shown in Figure 1. The results show that the major absorption peaks of isolated ICG molecules decrease by up to 17% as the CDDP concentration increases to 300 μM and a new peak grows at the wavelength of ~845 nm. These results indicate the formation of ground-state ICG/CDDP complexes. Since our recent study has shown that CDDP is extremely efficient in dissociative electron attachment to electrons produced in ionizing radiation,[3] it is very reasonable to expect an electron transfer (ET) from the excited-state ICG* created by 800 nm single-photon excitation to CDDP in the complexes. Thus, our time-resolved femtosecond laser spectroscopic experiments were set to directly observe the following ET reactions:

$$[\mathrm{ICG/Pt(NH_3)_2Cl_2}] + h\nu \rightarrow [\mathrm{ICG^+/Pt(NH_3)_2Cl_2^{*-}}] \rightarrow \mathrm{ICG^+ + Pt(NH_3)_2Cl^\bullet + Cl^-}$$

$$[\mathrm{ICG/Pt(NH_3)_2Cl}] + h\nu \rightarrow [\mathrm{ICG^+/Pt(NH_3)_2Cl^{*-}}] \rightarrow \mathrm{ICG^+ + Pt(NH_3)_2^\bullet + Cl^-} \quad (1)$$

The transient absorption spectra of ICG and its mixtures with various CDDP concentrations are shown in Figure 2. The spectra for the mixtures are normalized to the initial peak of the spectrum for the pure ICG, since the introduction of CDDP leads to a slight decrease of the absorption of ICG at 800 nm, as shown in Figure 1. Without CDDP, ICG* shows two decay lifetimes: one is ~5.0 ps, and the other is a much longer lifetime. For ICG in pure water, the fluorescence lifetime of the singlet ICG* was calculated to be about 20 ps at ICG micromolar concentrations.[23] However, no time-resolved fluorescence spectra of ICG with a femtosecond (subpicosecond) time resolution have been reported. The decay lifetime of ~5.0 ps is short but is significantly longer than the timescales for vibration relaxation or salvation dynamics of molecules ranging from 100 fs to 1 ps.[24] Thus, the decay lifetime of ~5.0 ps is tentatively attributed to the lifetime of the singlet ICG* state. The results in Figure 2 show that the presence of CDDP mainly alters the decay dynamics of ICG* in the first 10 ps, while the slow decay dynamics is essentially unchanged. This indicates the ET reaction between the singlet ICG* and CDDP in the ICG/CDDP complexes. This assignment is also supported by the observation of similar changes in the fast decay dynamics of ICG* in ICG/O_2 complexes with millimolar concentrations of oxygen in the solutions. Compared with the latter, the reaction of ICG* with CDDP is much stronger than that with oxygen. These results indicate an efficient ET reaction between CDDP and ICG* in ICG/CDDP complexes. Similar to the reaction of CDDP with prehydrated electrons produced in UV (ionizing) radiation,[3] the ET reaction followed by CDDP dissociation results in the formation of $Pt(NH_3)_2Cl$ and $Pt(NH_3)_2$ radicals that then initiate significant DNA damage and cell death in the combination of PDT with CDDP.

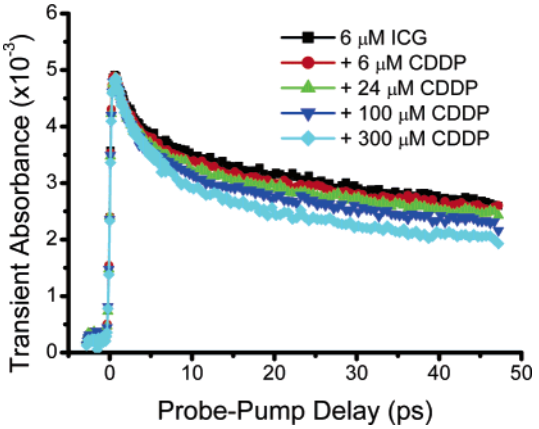

Figure 2. Femtosecond transient absorption spectra of 6 μM ICG with various CDDP concentrations pumped at 800 nm and probed at 600 nm for detection of the ET reaction between ICG* and CDDP. The spectra for the mixtures are normalized to the peak of the pure ICG spectrum.

To examine the role of the ET reaction mechanism on the biological effects induced by action of cisplatin combined with radiotherapy and PDT, we measured the ET-induced DNA damage. First, we measured the DNA damage induced by in situ generation of an extra tiny electron source in the DNA−cisplatin complex. With the same setup as for our femtosecond laser spectroscopic measurements,[3] the electron source was generated by focusing the UV light at 318 nm with a power of 150 μW into a volume of ~2×10^{-3} mm^3 in the stirring solution of 250 μL containing plasmid DNA. Agarose gel electrophoresis images of the DNA damage (single-strand breaks, double-strand breaks, intrastrand cross-links, and interstrand cross-links) induced by 50 μM cisplatin without and with the presence of the extra electron source are shown in Figure S1 in the Supporting Information. The populations of various DNA forms versus the electron source duration are plotted in Figure 3. For the control sample of plasmid DNA, 94.9% DNA is in the undamaged supercoiled (SC) form and 5.1% is in the open circle (OC) form attributed to single-strand breaks (SSB), in good agreement with those reported in the literature.[25] The result for the same amount of DNA incubated with 50 μM CDDP overnight in darkness shows a small but detectable difference from that for the control sample; the SC and OC (SSB) forms

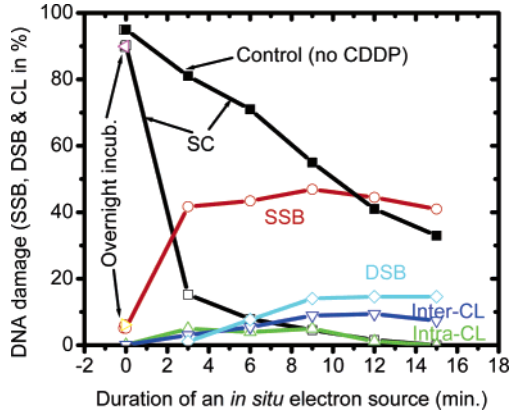

Figure 3. DNA damage induced by 50 μM CDDP enhanced by the presence of an in situ tiny electron source. Shown are populations of undamaged DNA (supercoiled, SC), single-strand breaks (SSB), double-strand breaks (DSB), and intrastrand (intra-CL) and interstrand cross-links (inter-CL) as a function of the duration of the tiny electron source. They were expressed in percentages with respect to the total DNA in the control sample, measured by agarose gel electrophoresis images shown in Figure S1. The solid squares are the data for DNA damage induced solely by the electron source without CDDP.

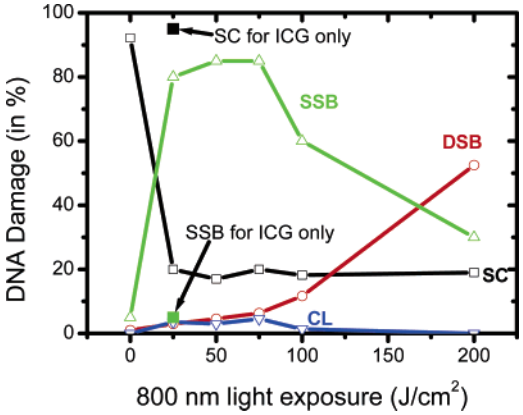

Figure 4. DNA damage induced by 5 μM CDDP and 20 μM ICG under laser irradiation at 800 nm. Shown are populations of undamaged DNA (SC), single-strand breaks (SSB), double-strand breaks (DSB), and cross-links (CL) as a function of the laser dose (J/cm^2), measured by agarose gel electrophoresis images (Figure S2). The solid square data points are for the DNA with ICG only at the light dose of 25 J/cm^2.

take 89.8% and 6.2% of the total DNA, respectively, and the rest of about 4.0% became undetectable fragments. It follows that ~5.1% ± 0.5% of the total DNA was degraded because of the incubation with CDDP. These results are close to those observed for DNA in cells with similar cisplatin concentrations (4−96 μM), in which 0.5−5% of the total cellular DNA is degraded and causes cell death.[26] The more interesting results are those for the same DNA and cisplatin concentrations with various durations of the electron source. Besides the SC and OC forms of DNA, the linear form that moves slightly more quickly than the OC and the two bands that move more slowly than the OC were observed, indicating the formation of double strand breaks (DSB) and cross-links (intra-CL and inter-CL).[25] It is most striking that the activation of the tiny electron source for only 5 min, i.e., the deposition of a small light energy of only 4.5×10^{-2} J into the sample of a total volume of 250 μL, can even degrade ~90% of the total DNA (Figure 3). The initial DNA damage forms are SSB (up to 45% of the total DNA) and intra-CL (5%), while DSB and inter-CL increase up to ~15% and ~10%, respectively, with increasing duration of the electron source. The intra-CL disappeared at higher electron doses, which can be attributed to the conversion into DSB. The formation of DSB has been inferred from the genetic analysis of recombinational pathways for repair of cisplatin-induced DNA damage.[11,12,26] As the duration of the electron source increased to 12 min, nearly all the DNA was completely degraded with the presence of CDDP. Also shown in Figure 3 are the data (solid squares) for DNA damage induced solely by the electron source without CDDP. It can be seen that the damage is much milder; the undamaged DNA with a 15 min electron exposure without CDDP is about equivalent to that with only 1.5 min exposure with CDDP. We also observed that to obtain a similar result without the presence of an extra electron source, an increase of the cisplatin concentration for overnight incubation by 2 orders of magnitude, i.e., to >5000 μM, is required. These results demonstrate that the ET reaction of cisplatin is an extremely efficient process in inducing DNA damage.

The DNA damage induced by the combination therapy of 5 μM cisplatin with 20 μM ICG activated by an 800 nm laser is shown in Figure 4. It can be clearly seen that without the light activation or the presence of ICG, the DNA damage induced by CDDP is negligibly small at this concentration. In contrast, the photoactivation of ICG at 800 nm induces significant DNA damage immediately. Even this low cisplatin dose combined with a low light dose as small as 25 J/cm^2 leads to a large degradation of ~80% of the total DNA, mainly into single-strand breaks with small percentages in double-strand breaks and cross-links, whereas no DNA damage is observed for ICG only at this low light dose (solid squares in Figure 4). As the light dose increases to ≥100 J/cm^2, even the single-strand broken DNA and cross-linked DNA fragment significantly. When the light dose increases to 200 J/cm^2, nearly all the DNA is damaged to become small fragments. The significant DNA damage will unavoidably lead to cell death[7−12,26] and therefore enhances the cytotoxicity of cisplatin. These results thus offer a mechanistic understanding of the observed enhancement of low-dose cisplatin cytotoxicity by combination with PDT using ICG.[18] Note that this CDDP concentration is ~10 times lower than that required for chemotherapy with CDDP alone and the required light dose is also low compared with the typical doses (100−500 J/cm^2) used in conventional PDT.[22] These results show great promise for improving the cancer treatments by CDDP and for enhancing the clinical efficacy of PDT for cancer and other diseases.

The discovery of the extremely high efficiency of electron-transfer reactions of cisplatin can have great significance. It not only provides a mechanistic understanding of the cisplatin−DNA interaction in chemotherapy[3] but sheds light on the mechanisms underlying combinational therapies of cisplatin with radiotherapy and photodynamic therapy of cancers. It is expected that the findings of the extremely high reactivity of CDDP with electrons generated in aqueous environments under ionizing radiation, from the DNA guanine bases and from a photosensitizer, may have profound significance for understanding and improving the therapeutic efficacy of CDDP. The results imply that the therapeutic effect can be localized to a small cancer area by controlling the light irradiation, which is important to many treatments. The low dose and spatial specificity will certainly reduce the side effects of CDDP significantly. Furthermore, the novel PDT protocol combined with CDDP can also be effective for hypoxic tumors because of no requirement of the presence of oxygen, where the reactive radical is generated from the highly reactive CDDP after electron capture from the excited PDT agent. This will be especially beneficial for PDT of deep and solid tumors. The understanding and development

of new PDT that can be activated by single-photon absorption in the NIR are areas of intense interest and future direction in PDT for treatments of cancer and other diseases, including age-related macular degeneration.

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Supporting Information Available: Details on materials and methods; agarose gel electrophoresis images. This material is available free of charge via the Internet at http://pubs.acs.org.

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