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Direct Observation and Quantitative Characterization of Singlet Oxygen in Aqueous Solution upon UVA Excitation of 6-Thioguanines

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Abstract

The incorporation of 6-thioguanine (6-TG) into DNA increases the risk of $^1\text{O}_2$ -initiated skin cancer. We herein provide the first report on quantitative characterization of the photoactivity of 6-thioguanines including 6-TG and 6-thioguanosine. Time-resolved singlet oxygen luminescence was observed directly for the first time after UVA irradiation of 6-thioguanes in both CHCN_3 and aqueous solutions. Their photosensitization was characterized by the quantum yield of singlet oxygen production, showing a dramatic decrease over time from initial 0.49–0.58 to zero. Experiments performed on both 6-TG and 6-thioguanosine did not show any significant difference in the quantum yield of singlet oxygen production, indicating that there was no potential participation of 7H- and 9H- tautomers. Our findings provide a primary basis for a better understanding of molecular events of thiopurine drugs in biological systems.

Introduction

The photophysics and photochemistry of thiopurine DNA bases are far less understood than those of normal DNA bases although some of them, such as azathioprine (Aza), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) have been used as cancer therapeutic and immunosuppressive agents for five decades.^{1,2} For normal single nucleotides, nonradiative decay occurs efficiently at subpicosecond timescale.^{3,4} This process is believed to enhance the photostability of DNA bases. The electronic properties of purines can, however, be greatly altered when oxygen atoms are replaced by sulfur. Unlike normal DNA bases, the formation yields of triplet states in thiocarbonyl compounds were found to be very high, e.g., 0.9 for thiouracils in H_2O , 0.99 for 6-thiopurine in THF,⁶ 0.77 and 0.68 for 4-thiouridine in CH_3CN , and a unity value for 4-thiothymidine in an ionic liquid.⁹ The interaction between triplet state and molecular oxygen can lead to the formation of singlet oxygen ($^1\text{O}_2$) via energy transfer and/or radicals (such as superoxide and hydroxyl radicals) via electron transfer processes. An understanding of the photoinduced activities of thiopurines became even more pronounced when researchers recognized the deleterious side effects with the use of thiopurine drugs. As a prodrug, Aza is cleaved to 6-MP that in turn is metabolized to 6-TG nucleotides (6-TGN). 6-TG is also directly converted to 6-TGN by hypoxanthine phosphoribosyltransferase (HPRT). 6-TGN is a precursor of DNA synthesis and 6-TG becomes incorporated into DNA.^{10,11} Their structures and metabolism are shown in scheme 1. The incorporation of 6-TG into DNA increased the risk of acute myeloid leukemia and skin cancer in thiopurine-treated patients.^{12–15} Accumulated evidence indicated that sunshine exposure and oxygen were the major contributors to this deleterious side effect.

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It was, however, only until recently that $^1\text{O}_2$ was acknowledged as a major risk factor for skin cancer from the patients with Aza treatment.^{16–18} Unlike normal DNA bases, 6-thioguanines have strong electronic transition in UVA region (315–400 nm). DNA substitution by 6-TG permits the absorption of UVA light, and subsequently introduces photoactive sites into DNA. An extensive interest has therefore been generated to elucidate the roles of thiopurines in skin sensitivity.^{12,19–21} Currently, researchers rely on indirect methods such as $^1\text{O}_2$ trapping or biological damage tests to identify the formation of reactive oxygen species (ROS) from thiopurines. Direct evidence and quantitative information regarding the production of $^1\text{O}_2$ is not available although this knowledge is crucial in elucidation of DNA damage mechanism. The extreme instability of thiopurines toward light and molecular oxygen complicates their physiochemical characterization.

The possibility that 6-TG may act as an endogenous $^1\text{O}_2$ sensitizer and provide a source of DNA damage prompted us to explore quantitatively their photosensitization ability. Each base has a number of tautomers, formed by permuting hydrogen atoms among the set of heteroatoms.⁴ Tautomerism complicates the interpretation of photophysical experiments because electronic structure can differ dramatically for individual tautomers. Therefore, it is essential to determine what tautomers are actually present in a given experimental condition. It is more likely that 6-TG contains a mixture of 7H- and 9H- tautomers, while in 6-thioguanosine the N9-position is substituted with a sugar group. The experiments were performed on both 6-TG and 6-thioguanosine to test the potential participation of tautomers in the production of $^1\text{O}_2$. The structures of 7H- and 9H-tautomers and 6-thioguanosine are given in scheme 2. Our results showed that both 6-TG and 6-thioguanosine were indeed efficient $^1\text{O}_2$ sensitizers under UVA irradiation. The deactivation of 6-thioguanines occurred rapidly over time in the presence of oxygen and light. We herein provide a first report of direct observation and quantitative characterization of $^1\text{O}_2$ production from 6-thioguanines. To some extent, our results explain the prevalence of photoactivity with thiopurine dose.

Materials and Methods

Materials and instrumentation

Reagents and solvents were obtained commercially and used without further purification. *meso*-Tetra(4-carboxylphenyl) porphine (TCPP) was purchased from Frontier Scientific, Inc., [2-(dicyclohexyl phosphino) ethyl]trimethyl ammonium chloride (> 98%) from Strem Chemicals, Inc., hydrochloric acid (37.5%) from Fisher Scientific, 6-thioguanine (6-TG), 6-thioguanosine, Tris(hydroxymethyl)aminomethine (> 99.8%), Ethylenedinitrilo)tetraacetic acid, disodium salt dihydrate (EDTA, > 99%), sodium hydroxide, sodium azide (NaN_3), deuterium acetonitrile- d_3 (CD_3CN , 99.8% of D) and deuterium oxide (D_2O , 99% of D) from Sigma-Aldrich. Deionized water was obtained from a Nanopure Water (Barnsted System, USA). A Q-switched Nd:YAG laser with pulse duration of 3–4 ns and a maximum energy of 30 mJ at 532 nm (Polaris II, Electro Scientific Industries, Inc.), and a liquid N_2 -cooled germanium photodetector (Applied Detector Corporation) were used for time-resolved $^1\text{O}_2$ luminescence measurements. Steady-state photooxidation was conducted in oxygen-saturated solution using a 150 W Xenon lamp (6255 Xenon lamp housed in 66907 Arc Lamp Source, Newport Oriel Instruments) equipped with an IR blocking filter (59042, Newport Oriel Instruments) and a monochromator with primary wavelength region 450–2000 nm (77250 1/8 m Monochromator and 77305 Grating, Newport Oriel Instruments), where the intensities in UVA range is below 15 W. Other instruments employed in this research include a BioMate 3 UV-Vis spectrophotometer (Thermo Scientific) and a Cary 300 UV-Vis spectrophotometer (Varian, Inc.) for taking absorbance and spectra. The determination of photooxidation products was done on a 300 MHz Bruker Spectrospin FT-NMR or a Varian Vnmrs 500 MHz NMR. All of the measurements were carried out at ambient temperature. Samples were protected from light when not being irradiated.

Direct observation of $^1\text{O}_2$ after 355 nm irradiation of 6-thioguanines

Kinetics of $^1\text{O}_2$ luminescence at 1270 nm was monitored by time-resolved Nd:YAG laser equipped with a low temperature cooled Ge detector, as previously described.^{22–25} 6-thioguanines were dissolved in pH 7.4 D₂O Tris buffer, pH 10 NaOH D₂O or CD₃CN solutions under dark, to avoid light-induced oxidation. The absorbances of the samples were controlled to be in the range of 0.1–0.4 at excitation wavelength of 355 nm. 1st-order kinetic fitting of $^1\text{O}_2$ decay was calculated using Origin 6.1 program. $^1\text{O}_2$ decay curves were corrected from control experiments by using the same but N₂-saturated sample or the air-saturated sample in the presence of 1.5 mM NaN₃. Data points of the initial ~10 μs were not used due to electronic interference signals from the detector.

Quantum yield of $^1\text{O}_2$ production (Φ_Δ)

Φ_Δ was determined in air-saturated pH 7.4 D₂O Tris buffer and pH 10 NaOH D₂O solutions on a relative basis by steady-state photolysis using TCPP as a reference. A Φ_Δ value of 0.53 for TCPP at pH 8–10 has been determined by our group.²⁴ A water-soluble phosphine, [2-(dicyclohexylphosphino)ethyl]trimethylammonium chloride, was used as a $^1\text{O}_2$ trap. A mixture of 3.00 mL of 6-thioguanines (OD = 0.1–1.0, 0.05–0.10 mM) and a phosphine (3.0–5.0 mM) trap were added into a 1-cm quartz cuvette and irradiated under UVA light for 20 minutes, followed by an immediate measurement of phosphine oxidation by ^{31}P NMR. $^1\text{O}_2$ photooxidation of phosphine trap leads to the formation of a sole product phosphonate (see scheme 3 for chemical structures). The peaks at δ –6.45 (s, 1P) and δ 60.50 (s, 1P) represent phosphine and phosphonate, respectively. The percent yields of phosphonates were controlled below 15% and calculated by comparison of the integrated ^{31}P NMR peaks of phosphine with those of phosphonate. The same trapping experiment was conducted for reference sensitizer TCPP under identical conditions. The absorbance of 6-thioguanines and TCPP at excitation wavelength of 350 nm was controlled the same. Control experiments in dark as well as in the absence of 6-thioguanines were carried out to ensure that there was no phosphine oxidation by heat or by ground-state oxygen molecules. Φ_Δ was calculated according to the equation in scheme 3.

Results and Discussion

Spectroscopic properties of 6-thioguanines

6-thioguanosine is fairly soluble in aqueous solutions. The solubility of 6-TG in pure water is limited but can be largely enhanced at higher pH solutions due to the deprotonation of thiols or amines. The absorption spectra of 6-thioguanines were conducted in both pH 7.4 Tris buffer and pH 10 NaOH solutions. Figure 1 shows the changes in the extinction coefficient with absorbed light energy. Unlike normal DNA bases (e.g., guanine in figure 1) that absorb little UVA light (315–400 nm), 6-thioguanines have strong electronic transition in UVA region. The extinction coefficients at their maximum absorption wavelengths were calculated and summarized in table 1. The extinction coefficients for both 6-TG and 6-thioguanosine are in the order of $10^4 \text{ M}^{-1} \text{ cm}^{-1}$. 6-thioguanines in pH 7.4 Tris buffer absorb furthest to red at 340 nm. Apparently, incorporation of 6-TG into DNA introduces UVA sensitive sites into DNA.

Direct observation of $^1\text{O}_2$ luminescence at 1270 nm

Kinetic decay of $^1\text{O}_2$ luminescence was observed directly for the first time at 1270 nm in air-saturated pH 7.4 D₂O Tris buffer, pH 10 NaOH D₂O and CD₃CN solutions upon irradiation of 6-thioguanines at 355 nm, using previously reported experimental setup.^{22–25} Examples are shown in figure 2. The decay traces were assigned to $^1\text{O}_2$ luminescence because both kinetics and intensities of $^1\text{O}_2$ signals were sensitive to the concentrations of NaN₃ (figures 2a) or molecular oxygen (figure 2b) in the solutions. NaN₃ is a well-known

efficient $^1\text{O}_2$ quencher that reacts with $^1\text{O}_2$ at a rate constant of $5.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ in water.²⁶ Figures 2a indicates that azide ions quench not only the lifetime of $^1\text{O}_2$ but also the initial intensity of $^1\text{O}_2$ luminescence, which can be explained by its reactions with both $^1\text{O}_2$ and excited states of a sensitizer.²⁷ The kinetic simulation is shown in the insertions of figure 2. 6-thioguanines belong to the analogue of sulfides that are readily oxidized by $^1\text{O}_2$ ²⁸ at a magnitude of rate constants 10^6 – $10^7 \text{ M}^{-1} \text{ s}^{-1}$.²⁹ The rapid deactivation of 6-TG in the presence of oxygen molecules and light (short dash line in figure 2b) was due to the efficient self-quenching of $^1\text{O}_2$, resulting in the oxidation of sulfur atoms. The total rate constants (k_d) of $^1\text{O}_2$ removal were measured to be $1.0 \times 10^4 \text{ s}^{-1}$ in pH 7.4 D_2O Tris buffer, $5.6 \times 10^3 \text{ s}^{-1}$ in CH_3CN and $7.2 \times 10^4 \text{ s}^{-1}$ in pH 10 NaOH D_2O solutions, which are comparable with literature values of $1.5 \times 10^4 \text{ s}^{-1}$ in D_2O ,³⁰ $2.3 \times 10^3 \text{ s}^{-1}$ in CH_3CN ³¹ and $4.4 \times 10^4 \text{ s}^{-1}$ in pH 10 NaOH D_2O ²⁴ within acceptable error limits respectively. Our results were also supported by the report that 6-TG in DNA was both the production source²⁰ and target site³² of ROS under UVA irradiation. For accurate calculations, decay traces were corrected for the background from other rapid events synchronized with laser pulses such as electronic interference from the detector, by using the same sample but in the presence of NaN_3 or a N_2 -saturated sample as a control.

Oxidation of thiocarbonyl chromophores contributes toward their instability and reactivity. Similar to guanine, thioguanine has thioketone and enethiol tautomers in addition to the 7H- and 9H-amino tautomers. Since the initial report by Gattermann and Schulze,³³ the stability of several thioketones toward oxygen in the presence or absence of light has been investigated by several groups. The oxidation mechanisms of sulfides by $^1\text{O}_2$ have also been extensively examined,^{28,34} suggesting a persulfoxide as a primary key intermediate.²⁸ Ramamurthy and co-workers identified $^1\text{O}_2$ as oxidizing species in light-induced oxidation of thioketones.^{35,36} The photooxidation of diaryl, aryl alkyl, and dialkyl thioketones by $^1\text{O}_2$ generated via self-sensitization or other independent methods yielded the corresponding ketone and sulfine in varying amounts.³⁶ Recently Karran's group revealed that under UVA irradiation Aza-treated DNA contained 6-TG that was both production source²⁰ and target site³² of ROS. They identified the formation of $^1\text{O}_2$ ¹⁸ and its photooxidation products as guanine sulphinate (G^{SO_2}),³⁷ guanine-6-sulphonate (G^{SO_3}) and guanine-6-thioguanine.^{16,18} Both $^1\text{O}_2$ ^{16,18,38–40} and G^{SO_3} ^{16,18} have been proven to play a key role in mutagenic oxidative DNA damage. Apparently, $^1\text{O}_2$ -initiated photooxidation of sulfur atoms in thioguanines led to not only deactivation of the compounds in photosensitization ability but also various intermediates and products that might be harmful to biological systems. The phototoxicity of those intermediates and final products, however, has not been fully understood and requires further investigations.

Determination of Φ_Δ

Φ_Δ is an important measure of photosensitization efficacy. It is usually determined on a relative basis that requires a reference. A well developed sensitizer, 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin (TCPP) was selected for this purpose. Φ_Δ value of TCPP has been determined to be 0.53 in alkaline solutions of pH 8–10.²⁴ A time-resolved $^1\text{O}_2$ measurement is awkward to quantify photosensitization ability of 6-thioguanines due to the weak signals and rapid deactivation of 6-thioguanines. Φ_Δ were therefore measured by steady-state photolysis using a water-soluble phosphine, [2-(dicyclohexylphosphino)ethyl]trimethylammonium chloride as a $^1\text{O}_2$ acceptor. It has been reported that the photooxidation of phosphines by $^1\text{O}_2$ leads to the formation of either phosphonate or a mixture of phosphonate and phosphinate.^{41,42} A sole product, phosphonate was observed and monitored by ^{31}P NMR as previously reported (figure 3). $^1\text{O}_2$ photooxidation reaction of phosphine is shown in scheme 3. The percent yields of phosphine oxides were calculated according to the equation in scheme 3 (see discussion

below), by comparison of the integrated ^{31}P NMR peaks of phosphine δ -6.45 (s, 1P), with those of phosphonate δ 60.50 (s, 1P). Control experiments in the absence of 6-thiopurines as well as in dark did not show any increase in the conversion yield of phosphines to phosphonates although the initial phosphonate peaks were observed due to the slow phosphine oxidation by triplet oxygen in air (see examples in figure 4). Thus the formation of phosphine oxidation by heat or by ground-state oxygen molecules could be neglected under our experimental conditions.

The bimolecular removal rate constants of $^1\text{O}_2$ by both sulfides²⁹ and phosphines⁴¹ are at a same magnitude of 10^6 – $10^7 \text{ M}^{-1} \text{ s}^{-1}$. $^1\text{O}_2$ photooxidation of sulfur atoms in 6-thioguanines resulted in their deactivation, as shown in figure 2b. Under our experimental conditions, the concentrations of phosphine (3–5 mM) were controlled considerably higher than these of 6-thioguanines (0.05–0.10 mM) to ensure that the majority of $^1\text{O}_2$ would react with phosphine trap, while the quenching of $^1\text{O}_2$ by 6-thioguanines could be neglected. The percent yields of phosphonates were controlled below 15% to assure an efficient trapping condition. Dramatic decrease in photosensitization ability was evident by the trivial conversion of phosphine to phosphonate after 20 minutes irradiation at 350 nm as shown in figure 3. Taking this into account, the Φ_{Δ} calculated for 6-thioguanines was approximated by multiplication of a factor of 2 when using TCPP, a conventional, stable sensitizer under UVA irradiation, as a reference. Φ_{Δ} was calculated by comparing phosphonate yields from 6-thioguanines to that from TCPP reference. Φ_{Δ} values thus obtained fell in the range of 0.49–0.58 (see table 2). Our results are comparable to literature values of some other thiocarbonyl compounds, e.g., 0.63 for 6-azauracil in CH_3CN upon UVB irradiation⁴³ and 0.50 for 4-thiothymidine in CH_3CN upon UVA irradiation.⁴⁴

Guanine has keto and enol tautomers in addition to the 7H- and 9H-amino tautomers found in adenine. The substitution of (deoxy)ribose at N9 position removes a heteroatom from the set that can accept hydrogen, reducing the number of possible tautomers in the nucleosides and nucleotides. Both of 6-TG and 6-thioguanosine were tested in this study to clarify the potential participation of 7H- or 9H-tautomers in the production of $^1\text{O}_2$. As shown in table 2, no significant difference in Φ_{Δ} from pH 10 or pH 7.4 aqueous solutions was observed from both compounds. Our data ruled out the effect of 7H- and 9H-tautomers on photosensitization ability of 6-thioguanines.

The results are also supported by the triplet excitation nature of thiol-DNA or -RNA bases. The photosensitized production of $^1\text{O}_2$ is actually the quenching process of a sensitizer's triplet state by ground oxygen. To some extent the formation yield of triplet state can be a measure for $^1\text{O}_2$ production. In general, the formation yields of triplet state in thiocarbonyl compounds were found to be very efficient, e.g., 0.9 for thiouracils in H_2O , 0.99 for 6-thiopurine in THF, 0.77 and 0.68 for 4-thiouridine in CH_3CN . Very recently Crespo-Hernandez's group reported an unity triplet yield upon UVA excitation of 4-thiothymidine in an ionic liquid,⁹ and theoretically predicted its phosphorescence energy of 2.23 eV (557 nm), which is in good agreement with experimental emission maximum at 542 nm.⁴⁵ The energy difference between triplet and singlet states of molecular oxygen is 1270 nm or 0.98 eV. Obviously the triplet energy from thiol-DNA bases is high enough to excite molecular oxygen from triplet state to singlet state.

It is important to note that, *in vivo*, the levels of thioesters and thiols could be kept constantly in reduced forms in the presence of reductases. The possible regulation of thiopurine DNA bases *in vivo* could provide a constant phototoxicity site. Electron transfer between excited triplet thiopurines and molecular oxygen is another possible pathway that may generate superoxide radicals. Theoretical calculations and experimental studies indicated that the excited states of DNA bases might be reordered by solvation in terms of

quantum yield and lifetime.⁴ The long-term risk associated with thiopurines can also result indirectly from photoactive products and metabolites. These factors should be taken into considerations in elucidation of biological damage. Obviously these are the areas that require further investigations.

Conclusion

By using photochemical techniques we were able to provide the first report of direct observation of $^1\text{O}_2$ luminescence at 1270 nm after UVA irradiation of 6-TG and 6-thioguanosine. Their photoactivity toward UVA light and molecular oxygen was evaluated by Φ_{Δ} in aqueous solutions. Our results showed that both 6-TG and 6-thioguanosine were efficient $^1\text{O}_2$ sensitizers with Φ_{Δ} values in the range of 0.49–0.58 but lost photoactivity rapidly over time. The effect of 7H- and 9H- tautomers on $^1\text{O}_2$ production was not observed. These findings provide a primary basis for quantitative understanding of molecular events of thiopurines in biological systems and may lead to novel design of antioxidants toward thiopurine drugs. This research has the potential to add new perspective into material science as well when DNA construction is used.

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Scheme 1.
Structures and metabolism of thiopurine prodrug incorporation into DNA

Scheme 2.

Structures of 6-TG 7H- and 9H-amino tautomers and 6-thioguanosine

Scheme 3.
Measurement and calculation of Φ_{Δ}

Figure 1.

Extinction coefficient spectra of 6-TG in pH 7.4 Tris buffer (solid black line) and in pH 10 NaOH (dash black line), 6-thioguanosine in pH 7.4 Tris buffer (solid red line) and pH 10 NaOH (dash red line), and guanine in pH 10 NaOH (blue line) solutions at ambient temperature

Figure 2.

Time-resolved $^1\text{O}_2$ luminescence recorded at 1270 nm upon pulsed-irradiation of 6-TG at 355 nm. 2a: $^1\text{O}_2$ decay in an air-saturated pH 7.4 D_2O Tris buffer solution in the absence (solid line) and presence of 1.5 mM NaN_3 (dot line); insertion: 1st-order kinetic fitting of $^1\text{O}_2$ decay after correction with a control in the presence of NaN_3 , dots: experimental data and red line: theoretical simulation. 2b: $^1\text{O}_2$ decay in an air-saturated pH 10 NaOH D_2O solution at time intervals of zero minute (solid line) and 3 minutes (short dash line), dot line: N_2 -saturated sample; insertion: 1st-order kinetic fitting of $^1\text{O}_2$ decay after correction with a N_2 -saturated control, dots: experimental data and red line: theoretical simulation.

Figure 3. ^{31}P NMR spectra of 9.0 μmol of phosphine and 0.15 μmol 6-TG in O_2 -saturated pH 10 D_2O solutions with irradiation time of 0 (3a), 20 min (3b), 60 min (3c) and 120 min (3d) at 350 nm

Figure 4.

^{31}P NMR spectra from control experiments, 4a: 9.0 μmol of phosphine in O_2 -saturated pH 10 D_2O solutions before irradiation, 4b: 20 min irradiation of 4a at 350 nm, 4c: 9.0 μmol of phosphine and 0.15 μmol 6-TG before irradiation, 4d: 4c kept in dark for 20 min

Table 1

Wavelength maximum ($\lambda_{\max}$) and extinction coefficient maximum ($\epsilon_{\max}$ at $\lambda_{\max}$) determined from electronic absorption spectra of guanine and 6-thioguanines at ambient temperature

Compound	Solvent	$\lambda_{\max}$, nm	$\epsilon_{\max}$, M ⁻¹ Cm ⁻¹
Guanine	pH 10 NaOH	273	1.1×10 ⁴
6-TG	pH 10 NaOH	337	1.8×10 ⁴
6-TG	pH 7.4 Tris buffer	340	2.1×10 ⁴
6-thioguanosine	pH 10 NaOH	320	1.8×10 ⁴
6-thioguanosine	pH 7.4 Tris buffer	342	2.0×10 ⁴

Table 2 Φ_{Δ} measured upon UVA irradiation of 6-thioguanines

Compound	Solvent	$\lambda_{\text{irradiation}}$, nm	Φ_{Δ}
6-TG	pH 10 NaOH	337	0.58 ± 0.08
6-TG	pH 7.4 Tris buffer	340	0.56 ± 0.18
6-thioguanosine	pH 10 NaOH	320	0.49 ± 0.09
6-thioguanosine	pH 7.4 Tris buffer	342	0.55 ± 0.08