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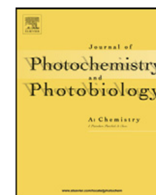

Quantification of thiopurine/UVA-induced singlet oxygen production

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ABSTRACT

Thiopurines were examined for their ability to produce singlet oxygen ($^1O_2$) with UVA light. The target compounds were three thiopurine prodrugs, azathioprine (Aza), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), and their S-methylated derivatives of 6-methylmercaptopurine (me6-MP) and 6-methylthioguanine (me6-TG). Our results showed that these thiopurines were efficient $^1O_2$ sensitizers under UVA irradiation but rapidly lost their photoactivities for $^1O_2$ production over time by a self-sensitized photooxidation of sulfur atoms in the presence of oxygen and UVA light. The initial quantum yields of $^1O_2$ production were determined to be in the range of 0.3–0.6 in aqueous solutions. Substitution of a hydrogen atom with a nitroimidazole or methyl group at S decreased the efficacy of photosensitized $^1O_2$ production as found for Aza, me6-MP and me6-TG. $^1O_2$-induced formation of 8-oxo-7,8-dihydro-2′-dexyguanosine (8-oxodGuo) was assessed by incubation of 6-methylthiopurine/UVA-treated calf thymus DNA with human repair enzyme 8-oxodGuo DNA glycosylase (hOGG1), followed by apurinic (AP) site determination. Because more 8-oxodGuo was formed in Tris D_2O than in Tris H_2O, $^1O_2$ is implicated as a key species in the reaction. These findings provided quantitative information on the photosensitization efficacy of thiopurines and to some extent revealed the correlations between photoactivity and phototoxicity.

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1. Introduction

Although thiopurine prodrugs, such as azathioprine (Aza), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), have been widely used in the treatment of cancer and inflammatory conditions and in the therapy of organ transplant patients for five decades, [1,2] the long-term use of thiopurines is frequently associated with malignancy, such as acute myeloid leukemia and skin cancer [3–5]. This adverse effect is known to be phototoxic, often manifested as a severe sunburn [6] and associated with thiopurine/UVA-initiated production of reactive oxygen species (ROS) [3,7–9]. However, it has been difficult to quantitatively correlate the photoactivity of thiopurines to oxidative DNA damage due to the limited information regarding their photosensitization efficacy.

As prodrugs, Aza is cleaved to 6-MP, which in turn is metabolized to 6-thioguanine nucleotides (6-TGN) that can be incorporated into DNA of patients taking Aza [8,9]. Thiopurines undergo enzymatic metabolism as well. One of the major pathways is initiated by thiopurine methyltransferase (TPMT), which converts 6-MP to 6-methylmercaptopurine (me6-MP) and 6-TG to 6-methylthioguanine (me6-TG). The structures and metabolism of these compounds are shown in Scheme 1. Unlike normal DNA bases, thiopurine DNA bases are strong UVA (315–400 nm, covering 90% of solar UV irradiation) chromophores. The less energetic UVA radiation can induces DNA damage through the absorption of light by sensitizers. A sensitizer may then react with DNA via electron or hydrogen abstraction to generate radicals (type I) or by energy transfer with oxygen (type II) to produce singlet oxygen ($^1O_2$). The oxidative damage of DNA by UVA radiation in cells and human skin has been reviewed, [10] indicating $^1O_2$-initiated formation of 8-oxo-7,8-dihydro-2′-dexyguanosine (8-oxodGuo). It was reported that Aza-treated DNA contained 6-TG that was both the production source [11] and target site [12] of reactive oxygen species (ROS) including $^1O_2$ [13]. Cooke and co-workers demonstrated the *in vivo* formation of 8-oxodGuo and alkali-labile sites in cells treated with biologically relevant doses of Aza and UVA, indicating the involvement of $^1O_2$ in oxidative DNA damage [14]. Very recently we reported the direct observation of $^1O_2$ production upon UVA irradiation of 6-thioguanines in aqueous solutions with quantum yield values ranging from 0.49 to 0.58 [15]. Obviously, 6-TG as well as other thiopurine metabolites such as 6-methylthiopurines can be an endogenous source of ROS in a biological system under UVA irradiation. An abrupt increase in ROS could cause oxidative stress and produce mutagenic DNA lesions [16,17]. Currently, the knowledge regarding the photosensitization efficacy of thiopurines is limited, which contrasts with the extensive studies of sulfide oxidation by $^1O_2$ [18,19] and self-photosensitized oxidation of thioketones [20,21].

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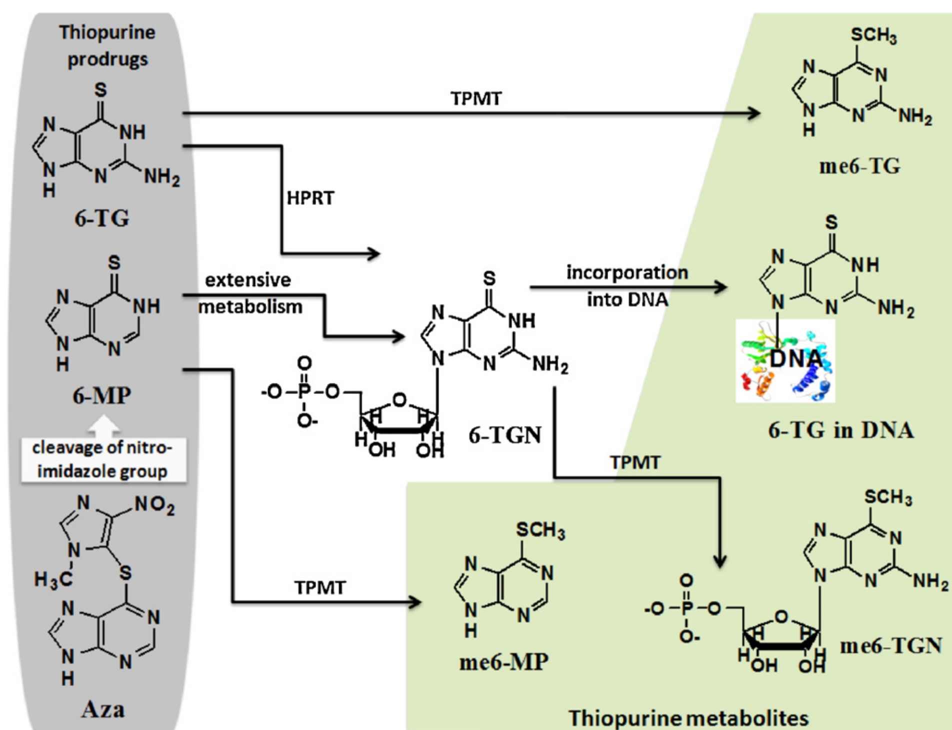


Scheme 1. Structures and metabolism of thiopurine prodrugs. Azathioprine (Aza) can convert to 6-mercaptopurine (6-MP) by cleavage of nitroimidazole group. Thiopurine prodrugs undergo extensive metabolism to 6-thioguanine (6-TG) nucleotides (6-TGN). 6-TG is also directly converted to 6-TGN by hypoxanthine phosphoribosyltransferase (HPRT). 6-TGN becomes incorporated into DNA. Thiopurine methyltransferase (TPMT) can convert 6-MP to 6-methylmercaptopurine (me6-MP) and 6-TG to 6-methylthioguanine (me6-TG).

The fact that thiopurines may be efficient endogenous sources for $^1O_2$ production in biological systems prompted us to determine systematically their photosensitization efficacy and the role of $^1O_2$ in 6-methylthiopurine/UVA-mediated oxidative DNA damage. Our results showed that three thiopurine prodrugs (Aza, 6-MP and 6-TG) and two S-methyl derivatives (me6-MP and me6-TG) were efficient $^1O_2$ sensitizers *in vitro* under UVA irradiation but rapidly lost their photoactivities for $^1O_2$ production over time by a self-sensitized photooxidation of sulfur atoms in the presence of oxygen and UVA light. DNA damage was quantified by using an aldehyde reactive probe (ARP, N′-aminooxymethylcarbonylhydrazino-D-biotin) that reacted with aldehyde groups present in the open ring form of apurinic or apyrimidinic (AP) sites. $^1O_2$-associated guanine oxidation was identified by incubation of treated DNA samples with 8-oxodGuo DNA glycosylase (hOGG1) prior to AP site determination. hOGG1 acts both as an N-glycosylase and an AP-lyase to release oxidized guanines from DNA to generate AP sites. We demonstrated that under our experimental conditions 6-methylthiopurine/UVA-induced DNA guanine base oxidation was mainly through type II ($^1O_2$) mechanism. These findings provide a primary basis for the quantitative understanding of phototoxicity of thiopurines in a biological system.

2. Experimental

2.1. 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%) was purchased from Strem Chemicals Inc. Azathioprine (Aza), 6-mercaptopurine (6-MP), 6-thioguanine (6-TG), S-methylmercaptopurine (me6-MP), S-methylthioguanine (me6-TG), sodium hydroxide, sodium azide (NaN_3), deuterium acetonitrile-d$_3$ (CD_3CN, 99.8% of D), deuterium oxide (D_2O, 99% of D), calf thymus DNA (D1501) and Tris(hydroxymethyl)aminomethine (>99.8%), were purchased from Sigma–Aldrich. Colorimetric Assay Kits for DNA Damage Quantification were purchased from Oxford Biomedical Research (Produc No. FR 09) or Dojindo (Product Code: DK02-12). The 8-oxodGuo DNA glycosylase (hOGG1, 1600 units/mL) was purchased from New England Biolabs Inc. Deionized water was obtained from a Nanopure Water System (Barnsted System, USA). A Q-switched Nd:YAG laser with pulse duration of 3–4 ns and a maximum energy of 7 mJ at 355 nm (Polaris II, Electro Scientific Industries, Inc.), equipped with a liquid N_2-cooled germanium photodetector (Applied Detector Corporation) was used for time-resolved $^1O_2$ 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 of 450–2000 nm (77250 1/8 m Monochromator and 77305 Grating, Newport Oriel Instruments), where the intensities in UVA range is below 15 W. A BioMate 3 UV–Vis spectrophotometer (Thermo Scientific) and a Cary 300 UV–Vis spectrophotometer (Varian, Inc.) were used for the measurements of absorbance and spectra. The determination of photooxidation products was performed using either 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. Thermo Labsystems Multiskan Ascent 354 from Thermo Labsystems was used for absorbance measurements for the microplate colorimetric assay. The production of $^1O_2$ was tested in CD_3CN and pH 10 NaOH/D_2O solutions. The measurements of absorption

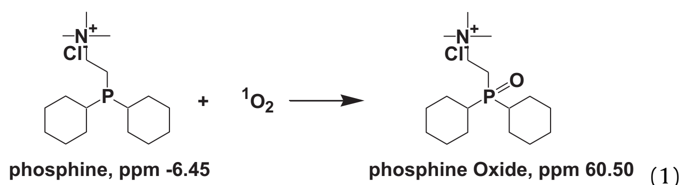
spectra and the oxidative DNA damage analyses were carried out in TE (50 mM Tris–HCl, 1 mM EDTA, pH 7.4) D_2O or H_2O buffer solutions. The stock solutions of 0.10 M H_2O_2, 0.10 M $FeCl_2·4H_2O$ and 10 K Units of SOD were prepared in TE buffer. DNA samples were also prepared using TE buffer at a final concentration of 0.10 mg/mL for oxidative DNA damage assays.

2.2. Direct observation of $^1O_2$ upon 355 nm irradiation of thiopurines

Kinetics of $^1O_2$ phosphorescence was monitored at 1270 nm, as previously described.[15,22] Thiopurines were dissolved in either CD_3CN or pH 10 NaOH/D_2O solutions under dark to avoid light-induced oxidation. The absorbance of the samples was controlled to be in the range of 0.1–0.4 at an excitation wavelength of 355 nm, depending on the solubility of each thiopurine compound. First-order kinetic fitting of $^1O_2$ decay was calculated using Origin 6.1 program. $^1O_2$ decay curves were corrected from control experiments by using the same but N_2-saturated sample for pH 10 NaOH/D_2O solutions or air-saturated sample in the presence of 1.5 mM NaN_3 for CD_3CN solutions. Data points of the initial ~5 μs were not used due to electronic interference signals from the detector.

2.3. Φ_Δ measurement

Φ_Δ was determined in O_2-saturated pH 10 NaOH/D_2O solutions on a relative basis by steady-state trapping experiment using TCPP as a reference (Φ_Δ = 0.53 in weak alkaline solutions),[23] as previously reported.[15] A water-soluble phosphine, [2-(dicyclohexylphosphino)ethyl]trimethylammonium chloride, was used as an $^1O_2$ trap for both thiopurine and TCPP samples. The OD readings of thiopurines and TCPP at an excitation wavelength of 350 nm were matched. The Φ_Δ values of thiopurines were calculated based on the comparison of phosphine oxidation yields by thiopurines to those by TCPP, a reference sensitizer with known Φ_Δ.[23] An internal standard was not needed in determining the percent yield of phosphine oxides because the identical $^1O_2$ trapping conditions were applied to both thiopurines and TCPP samples. Control experiments in the dark and in the absence of thiopurines were also conducted to correct for any phosphine oxidation by heat or by ground-state oxygen molecules (Fig. S1 in Supporting Information). Thiopurines lose their photoactivities rapidly upon UVA irradiation in the presence of oxygen molecules. A 20 min-irradiation time led to a complete inhibition of thiopurine photoactivity while the photosensitization efficacy of TCPP was stable with irradiation time. The Φ_Δ measurements were based on the phosphine oxidation in a 20-min-irradiation period. Taking this into consideration, the initial Φ_Δ values from thiopurines were approximated by multiplication of an empirical factor of 2 (Eq. (2)). $\Phi_{\Delta,\text{thiopurine}}$ and $\Phi_{\Delta,\text{TCPP}}$ in Eq. (2) are the Φ_Δ from thiopurine and TCPP, respectively; and $\%_{\text{phosphine oxide by thiopurine}}$ and $\%_{\text{phosphine oxide by TCPP}}$ are the conversion yields of phosphine oxidation in the presence of thiopurine and TCPP, respectively.



(1)

$$\frac{\Phi_{\Delta,\text{thiopurine}}}{2\times\Phi_{\Delta,\text{TCPP}}}=\frac{\%_{\text{phosphineoxide by thiopurine}}}{\%_{\text{phosphineoxide by TCPP}}} \quad (2)$$

A brief description of trapping experiments is as follows. A mixture of 3.00 mL of 3.0–5.0 mM phosphine and 0.05–0.10 mM thiopurine with OD readings of 0.1–1.0 at a wavelength of 350 nm was added into a 1-cm quartz cuvette and irradiated using UVA light of 350 nm for 20 min followed by an immediate measurement of phosphine oxidation by ^{31}P NMR using a delay time of 3 s between pulses [23]. $^1O_2$ photooxidation of phosphine trap leads to the formation of a sole product of phosphine oxide (Eq. (1)). The peaks at δ-6.45 (s, 1P) and δ 60.50 (s, 1P) represent phosphine and phosphine oxide, respectively. The percent yields of phosphine oxide were controlled below 20% and calculated by comparison of the integrated ^{31}P NMR peaks of phosphine with those of phosphine oxide. The same trapping conditions were applied to the reference sensitizer TCPP. Control experiments in the dark and in the absence of thiopurines were also conducted to correct for any phosphine oxidation by heat or by ground-state oxygen molecules.

2.4. Preparation of 6-methylthiopurine/UVA-treated DNA samples

6-Methylthiopurine/UVA-treated DNA samples were prepared to test $^1O_2$-induced DNA damage, the absorbance of 6-methylthiopurines including me6-TG and me6-MP was controlled between 0.6 and 0.7 at a wavelength of 320 nm. 6-methylthiopurines and 1.00 mL of 0.10 mg/mL calf thymus DNA were irradiated at a wavelength of 320 nm in O_2-saturated TE/D_2O (50 mM Tris–HCl, 1 mM EDTA, pH 7.4) or TE/H_2O buffer solutions for 20 min, followed by incubation with hOGG1 and quantification of the number of AP sites generated in the DNA.

2.5. Incubation with hOGG1 to quantify $^1O_2$-induced guanine oxidation

To identify $^1O_2$-induced oxidative DNA damage, the treated DNA samples prepared above were incubated with hOGG1 prior to the quantification of AP sites using manufacture's protocol (New England Biolabs Inc.). Briefly, a 7.70 μL reaction containing 5.00 μL of 0.10 mg/mL DNA, 0.70 μL NEBuffer (10×) and 2.00 μL of hOGG1 (3.2 units) was incubated at 37 °C for 16 h to release oxidized guanines from double stranded DNA to generate AP sites. Next, 10.00 μL of ARP (aldehyde reactive probe) was added to the mixture and incubated at 37 °C for 1 h. The purification of ARP-labeled DNA was performed using either ethanol precipitation or DNA filtration tubes.

2.6. Quantification of AP sites using a colorimetric assay

The biotin–avidin-peroxidase assay was performed according to the manufacturer's protocols (Dojindo or Oxford Biomedical Research). Briefly, an aliquot of the diluted ARP-labeled DNA (60.00 μL) was added with 100 μL DNA binding solution to each well of a microwell plate. The samples were incubated overnight at room temperature. After the solution was discarded, the microplate was washed five times with diluted washing buffer. An aliquot of freshly diluted streptavidin-HRP solution (100–150 μL depending on the protocol used) was added and the microplate was placed on a plate shaker at 100 rpm for 1 h. After the excess streptavidin–HRP solution was discarded, the plate was washed five times with diluted wash buffer. An aliquot of substrate solution (100.00 μL) was added and incubated for 1 h at 37 °C for color development. The absorbance was taken at 650 nm on a microplate spectrophotometer. The number of AP sites in samples was quantified by comparing the standard ARP-tagged DNA conjugates with 0–40 ARP/105 bp DNA standard.

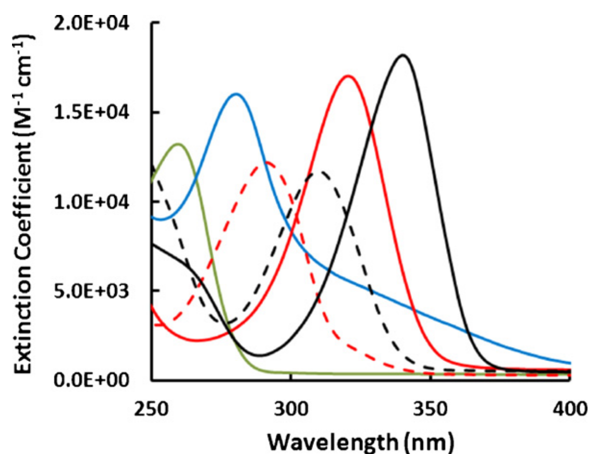


Fig. 1. Extinction coefficient spectra measured at ambient temperature in 50 mM TE buffer (pH 7.4) solutions for adenine (green solid line), Aza (blue solid line), 6-MP (red solid line), me6-MP (red dot line), 6-TG (black solid line) and me6-TG (black dot line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results

3.1. Spectroscopic properties of thiopurine drugs

The absorption spectra of thiopurines were collected in pH 7.4 TE buffer. Fig. 1 shows the changes in extinction coefficients. Unlike normal DNA bases (e.g., adenine in Fig. 1) which absorb little at wavelength longer than 300 nm, thiopurines absorb in the UVA region although their absorption maximum are often in the UVB. The extinction coefficient at maximum wavelengths for the thiopurines studied here were determined (Table 1). Our data are comparable to those from the literature, e.g., $1.34 \times 10^4\ M^{-1}\ cm^{-1}$ for adenine at 261 nm,[24] $1.73 \times 10^4\ M^{-1}\ cm^{-1}$ at 280.5 nm for Aza and $2.07 \times 10^4\ M^{-1}\ cm^{-1}$ at 321.6 nm for 6-MP in phosphate buffer at pH 7.4.[25]

3.2. Production of $^1O_2$ upon UVA irradiation of thiopurines

Thiopurines were examined for their ability to produce $^1O_2$ by time-resolved laser and steady-state trapping experiments. In general, $^1O_2$ detection at 1270 nm can be challenging due to the weak luminescence signal. Variations such as quenching, limited solubility, aggregation, formation of particles and electronic interference from the detector may adversely affect the measurement. Thiopurines are fairly soluble in polar organic solvents such as CH_3CN and aqueous solutions at basic pH due to the possible deprotonation of thiols or amines. The pK_a values for thiopurine drugs were reported to be in the range of 7.7–8.5 [26,27]. We have shown that upon UVA irradiation of thioguanines, no remarkable difference in Φ_Δ was observed in pH 7.4 TE/D_2O buffer and pH 10 NaOH/D_2O solutions although thiol deprotonation may occur at pH 10 [15]. Kinetics of $^1O_2$ luminescence as well as trapping experiments were therefore conducted in either air-saturated CD_3CN or pH 10 NaOH/D_2O solutions for accurate results. The data in Fig. 2 were assigned to $^1O_2$ phosphorescence because both kinetics and intensities of $^1O_2$ signals were sensitive to the concentrations of NaN_3 in the solution. NaN_3 is a well-known efficient $^1O_2$ quencher that physically reacts with $^1O_2$ at a rate constant of $5.0 \times 10^8\ M^{-1}\ s^{-1}$ in water [28]. Fig. 2a–e indicate that azide ions quench not only the lifetime of $^1O_2$ but also the initial intensity of $^1O_2$ luminescence. A decrease in initial $^1O_2$ intensity could be attributed to the reactions of azide ions with excited triplet states of a sensitizer, although the quenching of triplet states might be far less efficient than that of $^1O_2$ [29,30]. For comparison, the quenching rate constants by NaN_3 are $1.3 \times 10^7\ M^{-1}\ s^{-1}$ for triplet states of aluminum tetrasulphonated phthalocyanine and $4.4 \times 10^8\ M^{-1}\ s^{-1}$ for $^1O_2$ [30]. The kinetic decay was exponential with observed 1st-order solvent deactivation rate constants of $^1O_2$ (k_d) in CD_3CN equal to $9.3 \times 10^3\ s^{-1}$ for Aza, $5.5 \times 10^3\ s^{-1}$ for 6-MP, $5.6 \times 10^3\ s^{-1}$ for 6-TG,[15] $8.2 \times 10^3\ s^{-1}$ for me6-MP and $9.3 \times 10^3\ s^{-1}$ for me6-TG, which are comparable to literature values ranging from $2.3 \times 10^3\ s^{-1}$[31] to $9.3 \times 10^3\ s^{-1}$[32] in CD_3CN. The kinetic simulation is shown in the insertions of Fig. 2a–e. 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 as a control. The production of $^1O_2$ was further confirmed by steady-state trapping experiments using a phosphine, [2-(dicyclohexylphosphino)ethyl]trimethylammonium chloride, as an $^1O_2$ trap (see Φ_Δ measurements).

3.3. Φ_Δ upon UVA irradiation of thiopurine drugs

Φ_Δ was determined according to previously established method using TCPP as a reference [15] and a water-soluble phosphine, [2-(dicyclohexylphosphino)ethyl]trimethylammonium chloride as a $^1O_2$ acceptor. Phosphine oxidation was monitored by ^{31}P NMR and calculated by comparison of the integrated ^{31}P NMR peaks of phosphine with those of phosphine oxides [23]. The Φ_Δ values from thiopurine compounds were determined after irradiation for 20 min at an excitation wavelength of 350 nm. The phosphine oxidation was insignificant after 20 min irradiation due to the rapid deactivation of thiopurine photoactivity. Examples of NMR spectra are shown in Supporting Information. The values of Φ_Δ were calculated according to Eq. (2) and summarized in Table 2. Φ_Δ values for all of the thiopurines studied in this work fall in the range of 0.3–0.6.

3.4. Oxidation of guanines and formation of AP sites in calf thymus DNA

Studies have shown that $^1O_2$ can react with guanine nucleobase to form 8-oxodGuo [16,33–37]. The hOGG1, an 8-oxodGuo DNA glycosylase that acts both as a N-glycosylase and an AP-lyase, was used to release oxidized guanines from double stranded DNA to generate the AP sites. The AP-lyase activity cleaves 3′ to the AP site leaving a 5′ phosphate and a 3′-phospho-α, β-unsaturated

Table 1
Wavelength maximum (λ_{max}) and extinction coefficient maximum (ε_{max} at λ_{max}) determined from electronic absorption spectra of adenine and thiopurines at ambient temperature in pH 10 NaOH solutions.

Compound	λ_{max} (nm)	ε_{max} ($M^{-1}\ cm^{-1}$)
Adenine	260.0	1.3×10^4
Aza	274.0	1.5×10^4
Me6-MP	292.0	1.2×10^4
Me6-TG	310.0	1.2×10^4
6-MP	321.0	1.7×10^4
6-TG	340.0	2.1×10^4 [15]

Table 2
Φ_Δ obtained upon UVA irradiation of thiopurines.

Compound	$\lambda_{irradiation}$ (nm)	Φ_Δ
Aza	350	0.30 ± 0.03
6-MP	350	0.52 ± 0.05
6-TG	334	0.58 ± 0.08 [15]
Me6-MP	350	0.36 ± 0.05
Me6-TG	350	0.46 ± 0.05

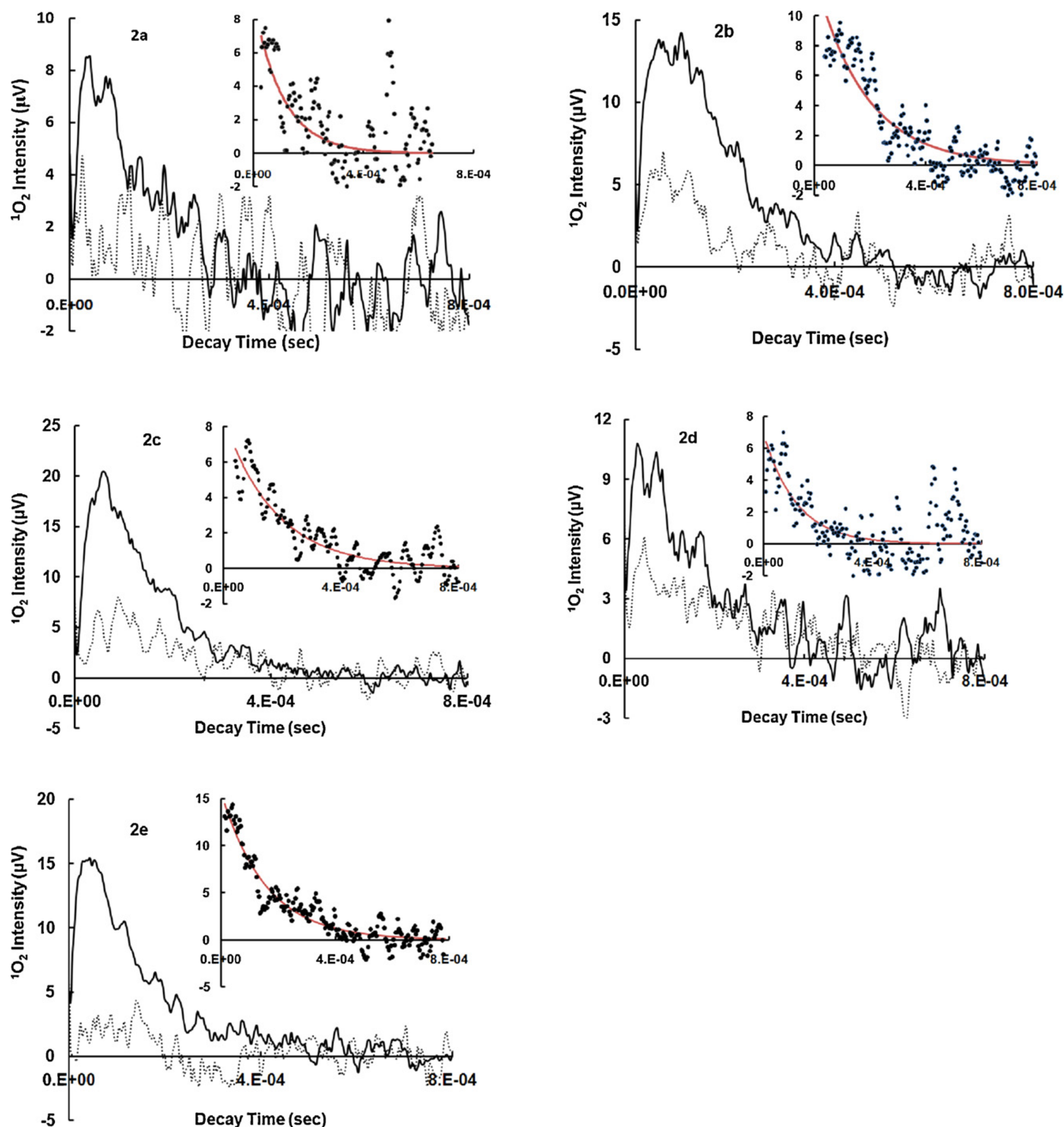


Fig. 2. Time-resolved $^1O_2$ phosphorescence recorded at 1270 nm upon pulsed-irradiation of thiopurines at 355 nm. a–e: $^1O_2$ decay in air-saturated CD_3CN solutions in the absence (solid line) and presence of 1.5 mM NaN_3 (dot line), insertion: 1st-order kinetic fitting of $^1O_2$ decay after correction with the same sample but in the presence of NaN_3 as a control, dots: experimental data and red line: theoretical simulation. Each decay curve is an average of data points obtained from 2 to 5 laser pulses. a: Aza at $OD_{355\,nm}$ = 0.21, b: 6-MP at $OD_{355\,nm}$ = 0.19, c: 6-TG at $OD_{355\,nm}$ = 0.19, d: me6-MP at $OD_{355\,nm}$ = 0.15, e: me6-TG at $OD_{355\,nm}$ = 0.07.

aldehyde [38]. The dependence of AP site formation on $^1O_2$ was studied in both H_2O and D_2O of 50 mM TE buffer (pH 7.4) solutions where $^1O_2$ has different lifetimes, e.g., 67 μs in D_2O [39] and 4 μs in H_2O [40]. Examples are shown in Fig. 3. There was a 3–4 fold higher generation of AP sites for me6-TG in D_2O as compared to me6-TG in H_2O (columns 5 and 6 in Fig. 3). Similar results were obtained for me6-MP with a higher generation of AP sites for me6-MP in D_2O as compared to me6-MP in H_2O (columns 9 and 10 in Fig. 3). Control experiments for DNA/me6-TG and DNA/me6-MP without hOGG1 incubation showed similar results for the direct generation of AP sites for D_2O (columns 3 for me6-TG and 7 for me6-MP in Fig. 3) and H_2O (columns 4 for me6-TG and 8 for me6-MP in Fig. 3), which was likely due to direct strand breakage induced by ROS [41]. Our results are in agreement with literature reports showing $^1O_2$ reaction with DNA could form strand breaks at guanine residues [10,42]. No oxidative DNA damage was observed from the control DNA samples in the absence of 6-methylthiopurines (columns 1 and 2 in Fig. 3). These data clearly indicated that $^1O_2$ was the dominate species in 6-methylthiopurine/UVA-induced guanine oxidation.

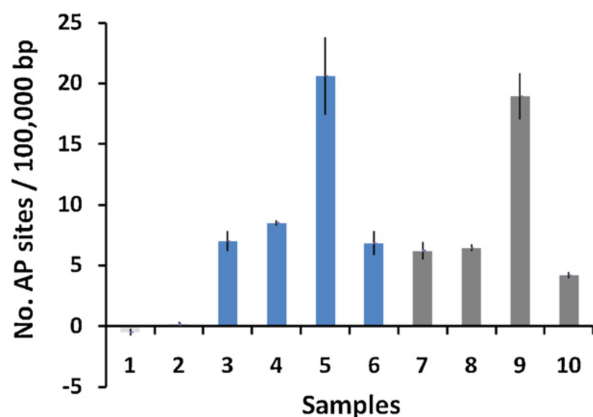


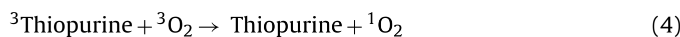
Fig. 3. The effect of D_2O and H_2O on AP site formation upon UVA (~10 W lamp) irradiation of 0.10 mg/mL DNA in the presence of 6-methylthiopurines. The data represent the mean of three to six repeating experiments in D_2O or H_2O of 50 mM TE buffer (pH 7.4) solutions. 1. DNA in D_2O, 2. DNA in H_2O, 3. DNA and me6-TG (0.05 mM) in D_2O, 4. DNA and me6-TG (0.05 mM) in H_2O, 5. DNA and me6-TG in D_2O prior incubation with hOGG1, 6. DNA and me6-TG (0.05 mM) in H_2O prior incubation with hOGG1, 7. DNA and me6-MP (0.15 mM) in D_2O, 8. DNA and me6-MP (0.15 mM) in H_2O, 9. DNA and me6-MP (0.15 mM) in D_2O prior incubation with hOGG1, 10. DNA and me6-MP (0.15 mM) in H_2O prior incubation with hOGG1.

4. Discussion

4.1. $^1O_2$ production and detection after UVA irradiation of thiopurines

It was identified as early as the 1960s that both UV- and X-ray-sensitivity of *E. coli* was enhanced when an appreciable proportion of normal DNA bases were substituted by thioguanines [43]. In the 1980s, Moore and his coworkers demonstrated that free 6-MP and Aza were photodynamically active and underwent light-induced reactions that required molecular oxygen [44,45]. Their observations on the formation of triplet states and reactions with the quenchers of ROS suggested the formation of $^1O_2$ as well as $O_2^{\bullet-}$ from these compounds. It was only recently acknowledged that $^1O_2$ was a major risk factor for skin cancer for patients treated with Aza [14,46,47]. We report herein the direct observation of $^1O_2$ luminescence at 1270 nm upon UVA irradiation of thiopurines (Fig. 2). Thiopurines are strong UVA chromophores with extinction coefficients up to $10^4\,M^{-1}\,cm^{-1}$, as shown in Fig. 1 and Table 1. The general mechanisms of thiopurine-induced $^1O_2$ photosensitization are presented in Eqs. (3) and (4). Upon UVA irradiation, thiopurines are activated to the excited singlet state (^{1}Thiopurine) which undergoes an intersystem crossing (ISC) to the excited triplet state (^{3}Thiopurine) (Eq. (3)). The triplet energy of thiopurines is quenched efficiently by ground state oxygen ($^3O_2$) to generate $^1O_2$ (type II, Eq. (4)). Triplet thiopurines may also lose energy via electron transfer to $^3O_2$ to produce $O_2^{\bullet-}$ or other ROS. This process is normally favored in the presence of electron donors (e^- donor, Eq. (5)).

$$\text{Thiopurine} \xrightarrow{\text{UVA}} {}^1\text{Thiopurine} \xrightarrow{\text{ISO}} {}^3\text{Thiopurine} \quad (3)$$



$$ {}^3\text{Thiopurine} + {}^3O_2 \rightarrow \text{Thiopurine} + {}^1O_2 \quad (4)$$

$$ {}^3\text{Thiopurine} + {}^3O_2 \xrightarrow{e^- \text{donor}} \text{Thiopurine} + O_2^{\cdot -} \quad (5)$$

$^1O_2$ luminescence at 1270 nm was observed after UVA irradiation of thiopurines in air-saturated CD_3CN or in pH 10 NaOH/D_2O solutions. The efficient quenching of signals by NaN_3 (Fig. 2a–e) indicated the formation of $^1O_2$, although the data is murky in some cases (e.g., Aza in Fig. 2a) due to the weak $^1O_2$ emission. The rapid inhibition of thiopurine photoactivity in the presence of oxygen molecules and light was due to the efficient quenching of $^1O_2$ by thiopurine itself, resulting in the oxidation of sulfur atoms. Thiopurines belong to the analogue of sulfides that are readily oxidized by $^1O_2$ [18] at a magnitude of rate constants 10^6–$10^7\,M^{-1}\,s^{-1}$ [48]. The formation of $^1O_2$ is also supported by steady-state trapping experiments using a phosphine as an $^1O_2$ acceptor (see discussion below). Our observation confirmed that thiopurines were both the production sources and target sites of $^1O_2$.

4.2. Φ_Δ as a measure of photosensitization efficacy for thiopurines in vitro

Φ_Δ is an important measure of photosensitization efficacy and usually determined on a relative basis that requires a reference. It is difficult to quantify the photosensitization ability for thiopurines using time-resolved $^1O_2$ measurement because of the weak emission signals and the rapid deactivation of photoactivity. Φ_Δ values reported in this paper were determined according to previously reported method by steady-state photolysis using a phosphine as a $^1O_2$ trap [15]. The bimolecular removal rate constants of $^1O_2$ by both sulfides [48] and phosphines [49] are at the same magnitude of 10^6–$10^7\,M^{-1}\,s^{-1}$. Under our experimental conditions, the concentrations of phosphine (3–5 mM) were controlled considerably higher than those of thiopurines (0.05–0.10 mM) to ensure that the majority of $^1O_2$ would react with the phosphine trap, while neglecting the quenching of $^1O_2$ by thiopurines. Phosphadioxirane is an intermediate as well as a powerful oxidant in the reaction of ortho-substituted arylphosphine with $^1O_2$ [50]. A high concentration of phosphine would also ensure that intermolecular reactions occurred only between phosphadioxirane (if there is any) and phosphines but not thiopurines. The percent yields of phosphine oxides were controlled below 20% to assure an efficient/steady trapping condition for all of the thiopurines as well as the TCPP reference.

Many of the biological molecules such as dinucleotide, [51] purine and pyrimidine bases, [52,53] were reported to be able to act as $^1O_2$ sensitizers. Unlike these biomolecules that contain normal DNA bases, sulfur atoms in thiopurines have two lone pair electrons and can be oxidized to sulfinate and sulfonate. The oxidation of sulfur atoms leads to the inhibition of thiopurine photoactivity. Φ_Δ values were calculated by comparing the conversion yield of phosphine oxides induced by thiopurine/UVA to that induced by a reference TCPP sensitizer. The Φ_Δ values obtained were in the range of 0.3–0.6 for all five thiopurines (Table 2), which was comparable to the literature values of some other thiocarbonyl compounds, e.g., 0.63 for 6-azauracil in CH_3CN upon UVB irradiation [54] and 0.50 for 4-thiothymidine in CH_3CN upon UVA irradiation [55]. Our data indicated that 6-TG ($\Phi_\Delta = 0.58$) [15] and 6-MP ($\Phi_\Delta = 0.52$) were the most efficient $^1O_2$ sensitizers among the thiopurines studied in this paper. Since the thiopurine ring is the reactive site for $^1O_2$ production, the frequency of collisions that result in the formation of $^1O_2$ is related to the probability that an $^3O_2$ molecule makes physical contact with the reactive site in its excited triplet state. The obviously larger steric effect of the nitroimidazole group in Aza (Scheme 1) could result in reduced collision frequencies and subsequently, a lower Φ_Δ value when compared to those from other thiopurines. Similar explanations might also, at least partially, apply to the lower Φ_Δ values from me6-MP and me6-TG than those from 6-MP and 6-TG, respectively. These findings provide a quantitative basis for understanding of molecular events in thiopurine/UVA-initiated DNA damage. Our results were supported by the triplet excitation nature of thiol-DNA or thiol-RNA bases as well. The photosensitized production of $^1O_2$ is actually the quenching process of a sensitizer's triplet state by ground oxygen. To some extent the formation yield of the triplet state can be a measure of $^1O_2$ production. In general, the formation yields of the triplet state in thiocarbonyl compounds was found to be very efficient, e.g., 0.9 for thiouracils in H_2O [56], 0.99 for

6-thiopurine in THF, [57] 0.7 [58] and 0.6 [59] for 4-thiouridine in CH_3CN, an unity for 4-thiothymidine in an ionic liquid, [60] and 0.8 for thioguanosine in aqueous solution [61], and was quenched by $^3O_2$ at a diffusing rate constant of $6.8 \times 10^9\,M^{-1}\,s^{-1}$ [62]. These measurements are in line with our Φ_Δ value of 0.58 for 6-TG [15].

4.3. Thiopurine/UVA-induced oxidative DNA damage

Oxidative reactions within DNA commonly result in base modification. Among the four DNA bases, guanine is the most susceptible to various oxidants, with one of the major products being 8-oxodGuo [33–37] as well as others such as spiroiminodihydantoin from the reaction of guanine with $^1O_2$ were identified [10,33,63,64]. Studies by Foote's group on an organic-soluble guanosine derivative, 2′,3′,5′-*O*-(*tert*-butyldimethylsilyl)guanosine, led to the conclusion that an unstable endoperoxide was the primary adduct produced via the [4+2] cycloaddition of $^1O_2$ with the imidazole ring [36,65,66]. The chemical reaction rate constants (k_R) for all nucleosides but guanosine derivatives were too low to be accurately determined [67]. The k_R and total quenching rate constants (k_T) for the guanosine derivatives were measured to be $(4.8 \pm 0.5) \times 10^4\,M^{-1}\,s^{-1}$ and $(3.0 \pm 0.2) \times 10^6\,M^{-1}\,s^{-1}$ in 1,1,2-trichlorotrifluoroethane, respectively, while k_T for all other nucleoside derivatives were in the range of 6×10^3 to $6 \times 10^4\,M^{-1}\,s^{-1}$. These data indicated that guanine was the only reactive DNA base toward $^1O_2$. Further research showed that $^1O_2$ reacting with guanosine or deoxyguanosine part of nucleotides did not, by itself, cause DNA cleavage [68]. These results implied that the quantification of $^1O_2$-induced DNA damage should involve the identification of guanine oxidation. Incubation of damaged DNA with various repair enzymes is therefore needed to recognize the specific base lesions and generated strand breaks [69–72].

The histochemical and immunohistochemical methods have been reviewed to localize ROS-induced damage in tissues and cells [73]. Studies concluded that several kinds of human cancer tissues such as lung, [74] renal, [75] and colorectal carcinoma [76,77] showed the higher levels of DNA oxidation compared to their non-tumorous counterparts, based on 8-oxodGuo determination that might be induced among hydroxyl radical, $^1O_2$ or photodynamic action [73,78]. $^\bullet$OH is non-selective oxidant. The two main decomposition pathways of guanine moiety involve initial addition of $^\bullet$OH at C4 (∼60%) and C8 (∼25%) [79]. The involvement of $^1O_2$ in 8-oxodGuo formation upon UVA irradiation of 6-methylthiopurines was examined by performing the reactions in 50 mM TE (pH 7.4)/D_2O buffer. The lifetime of $^1O_2$ is ∼17 times longer in D_2O than in H_2O. Consequently, enhanced guanine oxidation should occur in D_2O if $^1O_2$ was involved in the reactions. This was indeed the case. The generation of 8-oxodGuo was examined based on AP site formation from DNA/6-methylthiopurines incubated with hOGG1. The AP sites were tagged with biotin residues and quantified using a biotin-avidin-peroxidase assay. UVA irradiation of DNA in the presence of thiopurines produced 3–4 fold more AP sites in 50 mM pH 7.4 Tris/D_2O than in 50 mM pH 7.4 Tris/H_2O (Fig. 3). These results suggest that $^1O_2$ is the primary intermediate in 6-methylthiopurine/UVA-induced production of ROS and is responsible for the production of 8-oxodGuo. 6-methylthiopurines exist as the major metabolites of thiopurine chemotherapy in a biological system. When these photoactive compounds occur in DNA, they tend to react close to their site of formation because ROS are highly unstable, thus inducing efficient biological damage. 6-methylthiopurine/UVA-initiated guanine oxidation confirmed the efficient production of $^1O_2$ and clarified the selective role of $^1O_2$ in oxidative DNA damage. It should be noted that the UVA/thiopurine-induced photosensitized formation of triplet states and ROS may be largely affected by their binding to DNA. The oxidation of DNA guanine bases in cells treated with Aza/UVA has been observed [14]. A comparative study of 6-TG incorporated into DNA and its free counterpart would reveal the effect of DNA helix on photosensitization. Apparently, this is a research area that requires further investigation.

It is important to note that various factors may affect the behavior of thiopurine drugs *in vitro* or in a biological system. Persulfoxide is the primary intermediate in the reactions between $^1O_2$ and sulfides [18]. This weakly bound species can behave as an oxidant to undergo various inter- and intra-molecular reactions, and has a tendency to convert to secondary intermediates or transfer an oxygen atom to a nucleophilic trap. The involvement of subsequent reactions between the persulfoxides and DNA is yet unknown. Moreover, levels of thiopurines *in vivo* can be kept constantly in reduced forms in the presence of proper reductases. The possible regulation of thiopurine DNA bases *in vivo* may provide a constant photoactivity site. 8-oxodGuo is readily subjected to further oxidation as well, which has become a point of interest [64,80]. Except for 8-oxodGuo, $^1O_2$ also reacts with DNA to form strand breaks [42]. Type I pathway may become efficient especially in the presence of electron donors. These factors should be taken into considerations in elucidation of thiopurine/UVA-induced biological damage.

5. Conclusion

In conclusion, by using photochemical techniques, $^1O_2$ luminescence at 1270 nm was observed directly upon UVA irradiation of thiopurine prodrugs and their S-methylated metabolites. The photoactivity of these compounds toward UVA light and molecular oxygen was systematically evaluated by Φ_Δ *in vitro*. Our results show that thiopurines are efficient $^1O_2$ sensitizers with the initial Φ_Δ values ranging from 0.3 to 0.6. S-methylation somewhat inhibits photosensitized production of $^1O_2$. Methylthiopurine/UVA-associated formation of 8-oxodGuo gave rise to the most oxidative DNA damage, subsequently indicating $^1O_2$ as the major ROS. The specific pathways through which thiopurine levels are regulated *in vivo* and transformed into products by light require further investigations. Our data provides primary basis for a better understanding of thiopurine photoactivity and its correlation to phototoxicity in a biological system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jphotochem.2011.09.001.

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