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Thioguanine Restoration through Type I Photosensitization-Superoxide Oxidation-Glutathione Reduction Cycles

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UVA-induced deleterious effect of thiopurine prodrugs including azathioprine, 6-mercaptopurine and 6-thioguanine (6-TG) increases the risk of cancer development due to the incorporation of 6-TG in patients' DNA. The catalytic mechanism by which thiobases act as a sustained oxidant producer has yet to be explored, especially through the Type I electron transfer pathway that produces superoxide radicals ($O_2^{\cdot-}$). Under Fenton-like conditions $O_2^{\cdot-}$ radicals convert to extremely reactive hydroxyl radicals (·OH), thus carrying even higher risk of biological damage than that induced by the well-studied type II reaction. By monitoring 6-TG/UVA-induced photochemistry in mass spectra and superoxide radicals ($O_2^{\cdot-}$) via nitro blue tetrazolium (NBT) reduction, this work provides two new findings: (1) In the presence of reduced glutathione (GSH), the production of $O_2^{\cdot-}$ via the type I reaction is enhanced 10-fold. 6-TG thiyl radicals are identified as the primary intermediate formed in the reaction of 6-TG with $O_2^{\cdot-}$. The restoration of 6-TG and concurrent generation of $O_2^{\cdot-}$ occur via a 3-step-cycle: 6-TG type I photosensitization, $O_2^{\cdot-}$ oxidation and GSH reduction. (2) In the absence of GSH, 6-TG thiyl radicals undergo oxygen addition and sulfur dioxide removal to form carbon radicals (C6) which further convert to thioether by reacting with 6-TG molecules. These findings help explain not only thiol-regulation in a biological system but chemoprevention of cancer.

Introduction

Thiol residues in DNA and proteins are important targets of reactive oxygen species (ROS), whereas the reversible reactions play a key role in regulation of cell signaling pathways.[1, 2] The strong nucleophilic character of thiols results from the lowest oxidation state of sulfur atom whose redox properties[3-7] and biological significance[8, 9] have been extensively studied for decades. In contrast to the thorough investigation of thiols as antioxidants, limited effort has gone to understand their self-initiated oxidation reactions, in which thiols act as a potential producer of oxygen radicals. Thiopurine prodrugs are sulfur-substituted nucleobases (thiobases) and have been used for the treatment of cancer and inflammatory disorders for decades. The long-term medication with thiopurines carries the risk of cancer development due to the incorporation of 6-thioguanine (6-TG) in patients' DNA.[10-12] Thiopurine-induced skin cancer was accelerated by patients' exposure to sunlight,[13] suggesting an involvement of photooxidation in this adverse effect.[14-20] Unlike guanine DNA base, 6-TG is a UVA-sensitive chromophore. Corral, Crespo-Hernandez and Gonzalez et al. reported that thiobases possess high intersystem crossing rate constants and near-unity triplet yields due to the thionation that stabilizes sulfur electronic excitation.[21] The main deactivation pathway of excited 6-TG involves the sequence of ultrafast transitions of $S_2 \rightarrow S_1 \rightarrow T_2 \rightarrow T_1$.[22] These studies provide a theoretical basis for the two possible decay paths of triplet 6-TG (Equation 1): electron transfer (Type I, Equation 2) and energy transfer (Type II, Equation 3). Type II reaction produces singlet oxygen ($^1O_2$) by energy transfer from triplet 6-TG (^{3}TG) to ground state oxygen ($^3O_2$). The high yields of triplet states in 6-TG nucleotides[23, 24] are in line with our quantum yield value of 0.55 for $^1O_2$ production.[25] The products formed in Type II reactions were reported to be guanine sulfinate G^{SO2} and guanine sulfonate G^{SO3},[17, 26] as well as G^{SOOH} as a primary intermediate, followed by successive formation of G^{SO2}, G^{SO4} and G^{SO3}.[27] Despite progress in unraveling this mechanism, we still do not understand the sustainable cytotoxicity of thiopurines.

$$^0TG \xrightarrow{UVA} {}^1TG \xrightarrow{ISC} {}^3TG \quad (1)$$

$$^3TG + {}^3O_2 \xrightarrow{Type\ I\ \&\ e^-\ donors} {}^0TG + O_2^{\cdot-} \quad (2)$$

$$^3TG + {}^3O_2 \xrightarrow{Type\ II} {}^0TG + {}^1O_2 \quad (3)$$

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{Haber-Weiss} {}^{\cdot}OH + OH^- + O_2 \quad (4)$$

Due to the complex mechanism and large number of unstable intermediates, there have been remarkably few studies of the type I reaction that produces superoxide radicals ($O_2^{\cdot-}$) by electron transfer from ^{3}TG to $^3O_2$ in the presence of electron donors, e.g., 6-TG itself due to the strong reduction property of its sulfur atoms (equation 2). Dismutation of $O_2^{\cdot-}$ through Haber-Weiss chemistry generates extremely reactive hydroxyl radicals (·OH, equation 4),[28] thus carrying even higher risk of biological damage than that induced by Type II reactions (dark area in Scheme 1). The rate constants for equation 4 were determined to 0.50±0.09 M^{-1} s^{-1} at pH 0.5-3.5 and 0.13±0.07 M^{-1} s^{-1} at pH 7.0-9.9.[29] Alternatively, $O_2^{\cdot-}$ can serve as a source of ·OH by reducing Fe^{3+} to Fe^{2+}, thus permitting a Fenton reaction at much higher rate.[30] The sulfur-containing biological compounds react extremely rapidly with ·OH, with a second-order rate constant determined to be 1.47×10^{10} M^{-1} s^{-1} for GSH.[31] The

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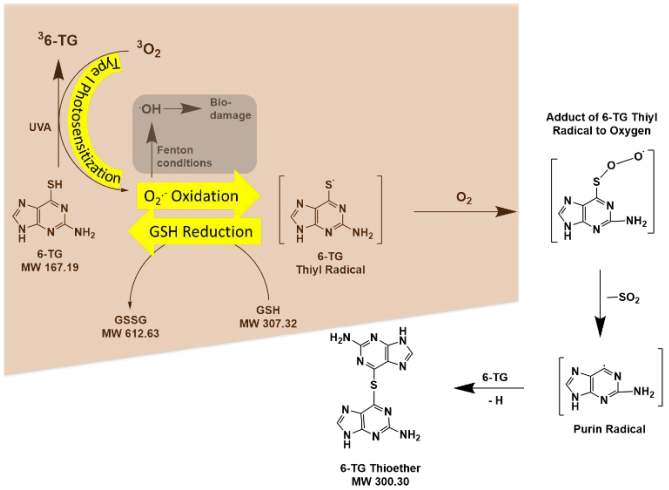
oxidation of GSH by ·OH would adversely affect its function of biological regulation.

Our previous work showed that thiopurines/UVA-induced type II sensitization was rapidly eliminated over time due to the oxidation of sulfur atoms,[32] while a biological regulation of 6-TG level must exist for its sustainable medication. Karran's group examined UVA-induced oxidation of 6-TG by $^1O_2$ in the presence of various thiols including N-acetylcystein, ß-mercaptoethanol, cysteine and reduced glutathione (GSH).[33] In all cases, these thiols significantly protected 6-TG from further oxidation. Decorti et al. reported that both cytotoxicity and $O_2^{\cdot-}$ production were enhanced by azathioprine in a concentration-dependent manner in the presence of N-acetylcysteine and glutathione-S-transferase-M1 genotype, respectively.[34] GSH has an intracellular concentration up to 10 mM. Liver cells normally maintain a ratio of GSH to its oxidized form – disulfide (GSSG) in excess of 100:1.[35] Given the high concentration of GSH in living organisms, we herein use 6-TG as a model compound to report two distinct mechanisms regarding Type I reaction: (1) In the presence of GSH, oxidized 6-TG is reduced to its original form via type I sensitization-$O_2^{\cdot-}$ oxidation-GSH reduction cycles, as highlighted in orange in Scheme 1; and (2) In the absence of GSH, 6-TG thiyl radicals undergo O_2 addition and SO_2 removal to form carbon radicals (C6) that convert to thioether by reacting with 6-TG molecules (Scheme 1).



Scheme 1. 6-TG/UVA-induced Type I sensitization-$O_2^{\cdot-}$ oxidation-GSH reduction cycles in the presence (highlighted in orange) and absence of GSH, with the middle dark area showing the formation of ·OH under Fenton-like conditions

Results and discussion

Production of $O_2^{\cdot-}$

6-TG has an absorption band between 300 and 360 nm with a maximum wavelength at 340 nm in 50 mM pH 7.4 Tris-HCl buffer solution, while the UVA absorption of GSH at this wavelength is minor (Figure 1). The concentration of 6-TG was determined by its absorption at 340 nm using an extinction coefficient of 2.1×10^4 $M^{-1}\cdot cm^{-1}$.[25] The reducing nature of sulfur atoms in 6-TG makes it a strong electron donor. In type I reaction, a triplet sensitizer (^{3}TG) reacts directly with an electron donor substrate (TG) to produce a sensitizer radical anion ($TG^{\bullet-}$) and a substrate radical cation ($TG^{\bullet+}$). $O_2^{\cdot-}$ is then generated by the direct electron transfer from $TG^{\bullet-}$ to $^3O_2$. The amount of $O_2^{\cdot-}$ produced was monitored by $O_2^{\cdot-}$ reduction of Nitro blue tetrazolium (NBT) via two-electron addition to a purple-colored monoformazan (MF^+) that has an extinction coefficient of 1.7×10^4 M^{-1} cm^{-1} at 545 nm.[36] Under our experimental conditions, the concentration of NBT was kept ten to one hundred times higher than that of $O_2^{\cdot-}$ accumulated. Figure 2 shows that $O_2^{\cdot-}$ was produced upon irradiation of 6-TG at 365 nm in air-saturated 50 mM pH 7.5 Tris buffer solutions. The generation of $O_2^{\cdot-}$ was significantly intensified by GSH (black line and red line) for the initial irradiation period of 1.5 hours, and then reached a plateau for a longer irradiation time, indicating that the oxidized 6-TG is restored to its original form but finally converts to photo-inactive products with the consumption of GSH (Also see mass spectra below.). The formation of $O_2^{\cdot-}$ in the presence (black line) and absence (blue line) of GSH demonstrates a GSH-initiated lasting photoactivity. NaN_3 is a well-known $^1O_2$ quencher. Within the acceptable experimental error range, NaN_3 showed little effect on $O_2^{\cdot-}$ production (compare black line and red line), thus ruling out the possibility of $^1O_2$ as a precursor of $O_2^{\cdot-}$. To examine whether NBT was reduced by $O_2^{\cdot-}$, superoxide dismutase (SOD) was added to the sample of 6-TG, GSH and NBT (black line). The observation that SOD completely inhibited the reduction (compare black line and orange line) suggested that NBT was reduced by $O_2^{\cdot-}$. Under our experimental conditions, the dark controls of all samples did not show any effect on NBT reduction (not shown in Figure 2). These observations also are in line with the literature in which replacing an oxygen atom at the 6-position of guanine with sulfur greatly enhanced the formation of $O_2^{\cdot-}$,[37] and $^1O_2$.[38] Based on Figure 2, the accumulated $O_2^{\cdot-}$ is estimated to be 2.2 µM after 1-hour irradiation and 3.7 µM after 2-hour irradiation in the absence of GSH, but 25 µM after1-hour irradiation and 35 µM after 2-hour irradiation in the presence of GSH. 6-TG/UVA-induced $O_2^{\cdot-}$ production was enhanced *ca.* 10-fold by the presence of GSH (11 fold after 1-hour irradiation and 9.5 fold after 2-hour irradiation).

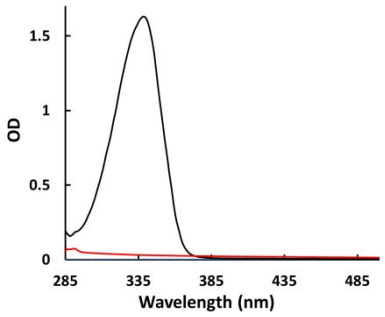


Figure 1. Absorption spectra of 7.5×10^{-5} M 6-TG (black line) and 0.1 mM GSH (red line) taken against 50 mM pH 7.4 Tris-HCl buffer solution as a blank.

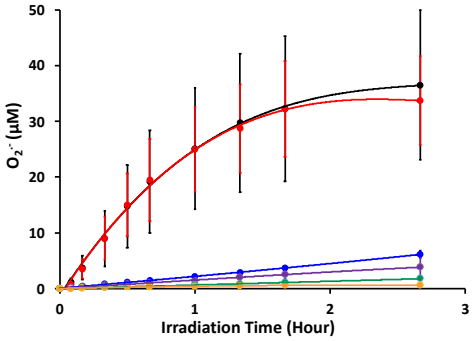


Figure 2. Time-dependent $O_2^{\cdot-}$ production monitored by NBT (0.32 mM) method upon UVA irradiation of 2.0×10^{-5} M 6-thioguanine (6-TG) at 365 nm in 50 mM pH 7.5 Tris-HCl buffer solutions in the presence and absence of 1.1×10^{-4} M GSH. The total volume of irradiated solution is 2.0 mL. Black Line: 6-TG, GSH and NBT; red line: 6-TG, GSH, NBT and NaN_3 (0.2 mM); blue line: 6-TG and NBT; purple line: GSH and NBT; green line: NBT only; orange line: 6-TG, GSH, NBT and SOD (1000 U). The error bars represent the standard deviations from triplicate measurements.

Mass spectra of 6-TG, GSH and their photosensitization products

The mass label and relative abundance of each peak in Figure 3 reveal how reactants and products relate to each other. The structure of each species is given in Scheme 1. 6-TG has a molecular weight of 167.19 that matches the peaks at M/Z 167.5-168.2 observed upon UVA irradiation of 6-TG for 0 (mass spectrum A) and 20 minutes (mass spectrum B) in the absence of GSH. Electron transfer from thiolate is known to be involved in the formation of thiyl radicals and their dimerization to disulfides.[39, 40] A growing peak at M/Z 300.3-301.2 is seen in the mass spectra of B, C and D upon UVA irradiation for 20, 40 and 80 minutes, respectively; in the absence of GSH, while 6-TG fades to zero at 40 minutes (mass spectrum C). This growing peak is assigned to 6-TG thioether (MW 300.30) formed via thiyl radical-oxygen adduct that converts to carbon radicals at the 6-position in the purine ring by losing SO_2 (Scheme 1). The carbon radical then reacts with 6-TG to generate thioether at M/Z 300.3-301.2.

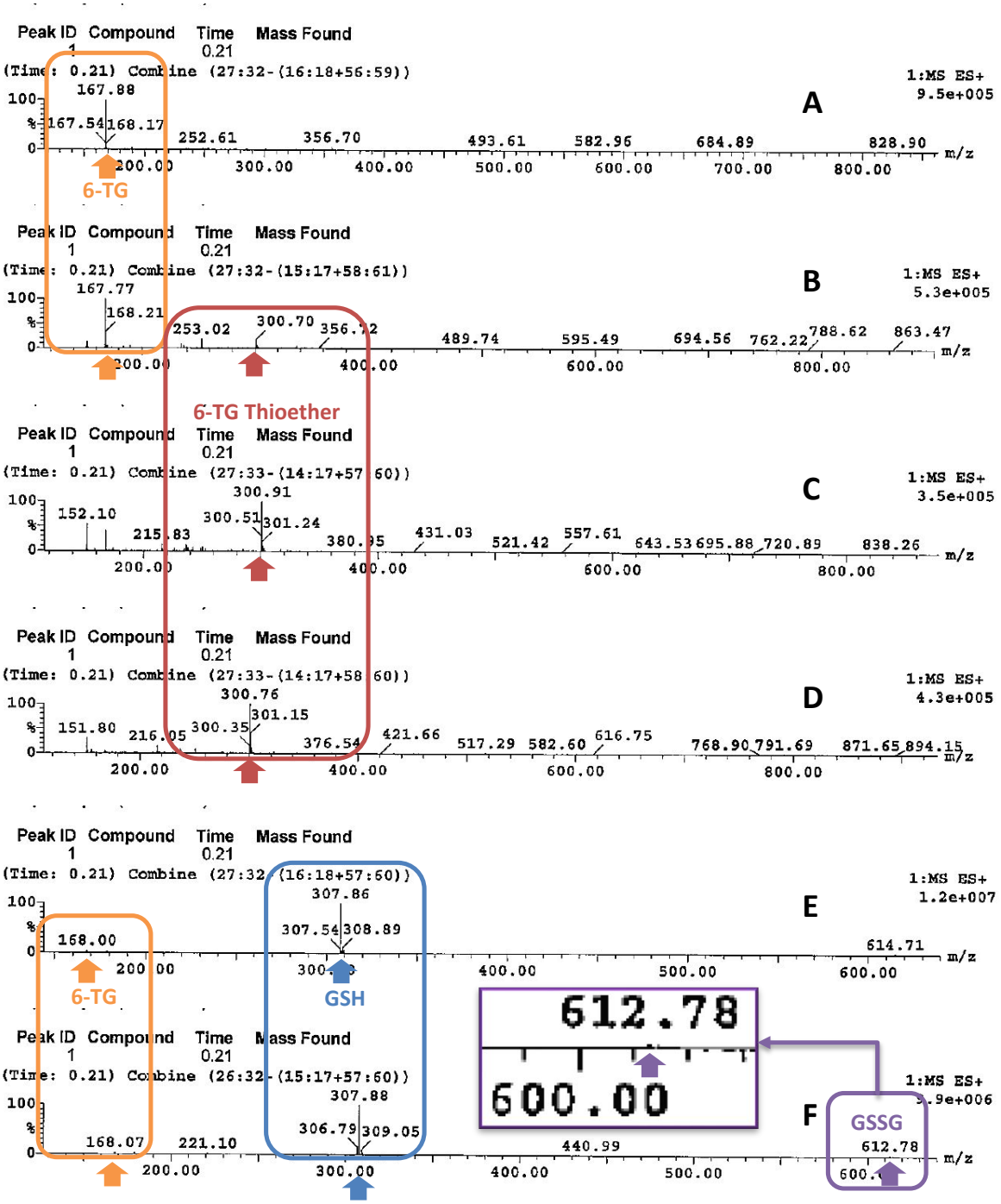


Figure 3. Mass spectra of 0.30 mM 6-TG in deionized water collected at 0.21 min under UVA irradiation at 365 nm in the absence of GSH for 0 min (A), 20 min (B), 40 min (C) and 80 min (D); and in the presence of 3.0 mM GSH for 0 min (E) and 80 min (F with an enlarged insertion for GSSG)

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Thiyl radicals play an important role in synthetic organic chemistry[40] and biological oxidation.[41, 42] However, their detection tends to be challenging and is usually based on the indirect method such as electron paramagnetic resonance (EPR) because of the short half-lives on an order of microseconds.[43] There are a number of possible decay pathways for thiyl radicals, e.g., formations of disulfides through self-recombination, thiylperoxyl radicals ($RSOO^{\cdot}$) by oxygen addition at diffusion-control rates, etc.[40] Thiyl radicals have been rarely observed directly with few reports through EPR in frozen solutions for their derivatives,[44, 45] resonance Raman spectra,[46] and X-ray absorption spectroscopy.[47, 48] In the current work, the formation of 6-TG thiyl radical was confirmed by the specificity of GSH reduction that led to the formation of GSSG (mass spectrum F). Our results are in line with the literature reports in which the formation of thiyl radicals was acknowledged in cyclic voltammograms at less positive potentials for 6-TG,[49] 6-mercaptopurine[50] and 6-mercaptopurine riboside.[51] In contrast to the extensive investigations on persulfoxide (R_2SOO^*), a key intermediate in the reactions of $^1O_2$ with sulfides,[3] limited information is available regarding the adduct between $^3O_2$ and thiyl radicals. The thioperoxyl radicals or the adducts of 6-TG thiyl radicals with oxygen have not been observed directly. The proposed mechanism is derived from the formation of thioether at M/Z 300.3-301.2 and is supported by the literature reports of transient infrared spectra of CH_3SOO[52] and density functional and ab initio calculations,[53] in which the well depth for converting $CH_3S^{\cdot} + {}^3O_2$ to $CH_3SOO^{\cdot}$ adduct was shallow at 9.7 kcal/mol, while low reaction barriers existed for the possible exit channels to form $CH_2S + HO_2$, $CH_3 + SO_2$, $CH_3SO + O$ or back to $CH_3S^{\cdot} + O_2$.

In a mixture of 0.3 mM 6-TG and 3 mM GSH, GSH (MW 307.32) is presented as a strong peak at M/Z 306.8-309.1 due to its much higher concentration than that of 6-TG before (mass spectrum E) or after (mass spectrum F) UVA irradiation. A tiny but labeled peak at M/Z 612.8 indicates the formation of GSSG (MW 612.63 in the mass spectrum F). The restoration of 6-TG is demonstrated by its peak at M/Z 168.07 even after 80 minutes of UVA irradiation in the presence of sufficient GSH (comparing mass spectra F to C and D) while the peak of 6-TG thioether (MW 300.30) is not observed (comparing mass spectra D to F). The proposed Type I mechanism in Scheme 1 is further confirmed by 6-TG/UVA-mediated supercoil pBR322 DNA cleavage tests. DNA damage was more prominent in the presence than in the absence of GSH. In addition, the most efficient single-strand cleavage of plasmid DNA was observed under Fenton like conditions, which specified the restoration of 6-TG by GSH and conversion of $O_2^{\cdot -}$ to $^{\cdot}OH$ (Figure S1 in ESI).

Conclusions

This work for the first time provides quantitative information for GSH-enhanced $O_2^{\cdot -}$ production upon UVA irradiation of 6-TG. 6-TG acts as a continuing endogenous source of oxidants via type I photosensitization-$O_2^{\cdot -}$ oxidation-GSH reduction cycles. The production of $O_2^{\cdot -}$ and the restoration of 6-TG occur concurrently but are finally abolished over time with the accumulation of 6-TG thioether in the absence of GSH. As GSH is naturally produced in human body, these findings provide an overall picture of the molecular mechanism underlying thiol-regulation for chemotherapy.

There are still unsolved mysteries in this area of research. For instance, what are the properties of intermediates (thiyl radicals, disulfides and thiylperoxyl radicals) formed during thiobase photosensitization and how would the type I and type II pathways compete with each other? Those questions are worth further investigations theoretically and experimentally e.g., with the aids of time-resolved techniques in different media.

Conflicts of interest

The authors declare no competing financial interests.

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