ESI for

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

Materials and Instruments

Reagents and solvents were obtained commercially and used without further purification. All measurements were carried out at ambient temperature. Samples were protected from light when not being irradiated. Reduced glutathione (GSH, ≥ 99.8%), Tris (Tromethamine) (USP 99.0-101.0%), hydrogen peroxide (30%), ferric chloride (anhydrous), superoxide dismutase (SOD) and nitro blue tetrazolium chloride (NBT) were purchased from Fisher Scientific. 6-thioguanine (6-TG) was purchased from Alfa Aesar. Sodium azide (NaN_3 ≥ 99.5%), plasmid pBR322 DNA, agarose, TEX 1X buffer and ethidium bromide were purchased from Sigma-Aldrich.

Instruments used in this work include UVA lamp (Spectroline ® model EN 180L (115V, 60Hz, 0.20 AMPS), UV/Vis spectrophotometers (Biomate 3® UV-VIS Spectrophotometry from Thermo Electron Corp and Thermo Scientific Evolution 220 UV-Visible spectrophotometer), AccuTherm Microtube Shaking Incubator (Labnet International, Inc.), Acquity Ultra Performance LC/MS (Waters), and GelDoc-It2 UVP Gel Imaging System with VisionWorksLS image processing software.

Stock solution Preparations

- 50 mM pH 7.4 Tris-HCl buffer preparation: 6.0 g tromethamine was dissolved in ~ 700 mL deionized water. The pH of this solution was adjusted to 7.4 by adding appropriate amount of HCl solution. The mixture was then diluted to 1000 mL.
- NBT solution: 0.2071 g NBT was added into 20 mL Tris-HCL buffer solution. The mixture was kept under stirring until NBT was dissolved. This solution should be prepared freshly and kept in refrigerator.
- 6-TG solution: The laboratory lighting was dimmed. 0.003 g 6-TG was added into 5.00 mL Tris-HCl buffer or deionized water. The mixture was kept under stirring until 6-TG was dissolved. This solution should be prepared freshly and kept in refrigerator.
- GSH solution: 0.0038 g GSH was added into 1.00 mL Tris-HCl buffer or deionized waster. The mixture was kept under stirring until GSH was dissolved. This solution should be prepared freshly and kept in refrigerator.
- NaN_3: 0.0050 grams of NaN_3 was added into 2.00 mL Tris-HCl buffer or deionized water. The mixture was kept under stirring until GSH was dissolved. This solution should be prepared freshly and kept in refrigerator.
- SOD: 0.0020 g SOD was added into 2.00 mL Tris-HCl buffer or deionized water. The mixture was kept under stirring until GSH was dissolved. This solution should be prepared freshly and kept in refrigerator.

Sample preparation and measurements

- Production and determination of superoxide radicals ($O_2^{\cdot -}$) as a function of irradiation time: The amount of $O_2^{\cdot -}$ produced was determined by the formation of monoformazan (MF^+) via two electron

addition using a molar extinction coefficient of 1.7×10^4 M^{-1} s^{-1} derived from literature.[1] This method is widely used to quantitatively determine $O_2^{\cdot -}$.[2, 3] Under our experimental conditions, the concentration of NBT was controlled high enough to react with all of the $O_2^{\cdot -}$ produced upon UVA irradiation of 6-TG. Briefly, freshly prepared 2.0×10^{-5} M 6-TG and 3.2×10^{-4} M NBT in 50 mM pH 7.5 Tris-HCl buffer solutions were exposed to UVA light at 365 nm for up to 160 minutes under stirring in the presence and absence of 1.1×10^{-4} M GSH (freshly prepared), 2×10^{-4} M NaN_3 and 1000 U SOD (freshly prepared), respectively. The reduction of NBT by $O_2^{\cdot -}$ was monitored at its maximum absorption of 545 nm against water.

- LC/MS: The protocol described above for sample preparation was used except for the slightly different amounts of 6-TG (3.0×10^{-4} M) and GSH (3.0×10^3 M) in deionized water, rather than pH 7.5 Tris-HCl buffer. 6-TG samples were irradiated for 0, 20, 40, and 80 minutes in the absence of GSH, and 0 and 80 minutes in the presence of GSH. The irradiated samples were kept under darkness prior to LC/MS measurements. The mobile phase was composed of water and acetonitrile containing approximately 0.05% trifluoroacetic acid (TFA) at a flow rate of 0.1 mL/min.

- DNA sample preparation and cleavage by 6-TG: The pBR322 DNA was prepared by mixing it at a 1:3 ratio with Tris-HCl buffer. Fe^{3+}, GSH and 6-TG solutions were freshly prepared in 50 mM pH 7.4 Tris-HCl buffer. 6-TG solution was stirred and heated for around one hour at 40°C. The effects of 6-TG on DNA strand breakage in the presence and absence of GSH were determined by converting supercoiled pBR322 plasmid DNA to the open circular and linear forms. Reactions were carried out in a total volume of 100 μL 50 mM pH 7.4 Tris-HCl buffer containing 10 μg pBR322 DNA and 50 mmol 6-TG in the presence and absence of 400 mmol GSH. The effects of SOD and Fenton reagents were tested. Samples were prepared in 50 mM pH 7.4 Tris-HCl under darkness. The UVA (365 nm) irradiation of DNA samples was carried out at 37°C for 100 minutes. Gel electrophoresis was then performed on irradiated DNA samples. Briefly, the agarose gel was prepared at a concentration of 0.8% using 0.400 g agarose and 50 mL of TBE 1X buffer. This solution was microwaved for 2 minutes, allowed to cool prior to adding 0.5 μl of ethidium bromide, poured into a gel casting tray with a well comb and was left for approximately 20 minutes to solidify. 5.0 μL of DNA sample was treated with 5.0 μL of Promega® 6190A blue orange 6x loading dye. The Promega® loading dye is a convenient marker dye containing 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA at pH 8.0), provided in a premixed, ready-to-use form. The dye is used for loading DNA samples into gel electrophoresis wells and tracking migration during electrophoresis. The well comb was removed and 10 μL of each DNA samples/loading dye mixture was then loaded onto the solidified gel apparatus. Horizontal gel electrophoresis was carried out in 50 mM TBE buffer for 60 minutes at 130 V, followed by photography with GelDoc-It^2 UVP Gel Imaging System and VisionWorks® image processing software.

  Both therapeutic and deleterious medication of 6-TG are believed to be associated with its combination in patients' DNA.[4, 5] 6-TG/UVA-induced DNA-cleavage was monitored by observing the conversion of supercoiled (form I) plasmid DNA to the circular nicked form (form II) occurred upon single-strand scission. All samples in Figure S1 contain same amount of DNA but show different ratios of form I over form II under photochemical reactions. The dark controls by DNA alone (sample 1) or in the presence of chemical additives (6-TG, GSH, Fe^{3+} and H_2O_2, sample 2) do not significantly cleave DNA. An enhancement of DNA cleavage by GSH is evident by the higher transformation of form I to form II in the presence of (sample 5) but not in the absence of (sample 4) GSH. The DNA cleavage upon UVA irradiation of Fe^{3+} and H_2O_2 in the absence of 6-TG is also observed (control sample 3) and appears quite different from other samples, in which linearized plasmid migration appears above form I (unwinding of form I). An addition of 6-TG (sample 6) and 6-TG + GSH (sample 7) into sample

3 results in significant DNA damage. The most efficient single-strand cleavage of plasmid DNA is observed in sample 7 under Fenton like conditions. The photooxidation enhancement by Fenton-like conditions can also be seen by comparing sample 5 (DNA, 6-TG, GSH) to sample 7 (DNA, 6-TG, GSH, Fe^{3+} and H_2O_2). It is worth noting that DNA cleavage assay could be oversensitive. Those results should be interpreted qualitatively.

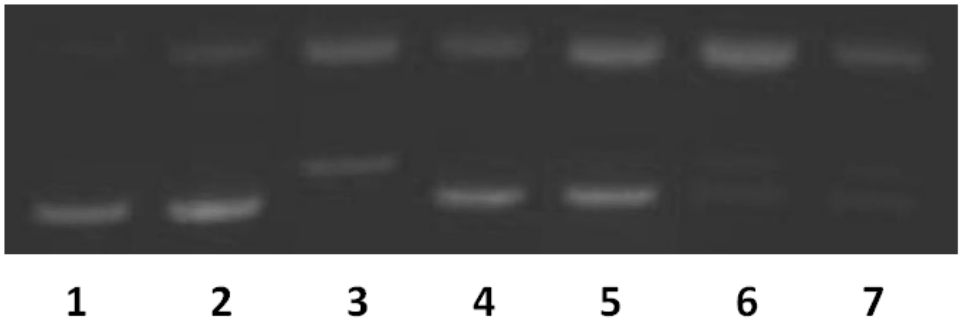

Figure S1. Cleavage of supercoil pBR322 DNA (10 μg) by UVA/6-TG (50 μmol) in the presence and absence of GSH (400 μmol), Fe^{3+} (5 pmol) and H_2O_2 (90 pmol). Controls 1-3: DNA alone under darkness (1), DNA, 6-TG, GSH, Fe^{3+} and H_2O_2 under darkness (2), DNA, Fe^{3+} and H_2O_2 under UVA (3). Effect of 6-TG in the absence of (4) and presence of (5) GSH under UVA. Effect of Fe^{3+} and H_2O_2 in the presence of 6-TG (6), and 6-TG + GSH (7) under UVA.

References

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