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Radiosensitization by a Modified Nucleotide that Produces DNA Interstrand Cross-Links under Hypoxic Conditions

In Seok Hong, Hui Ding, and Marc M. Greenberg*

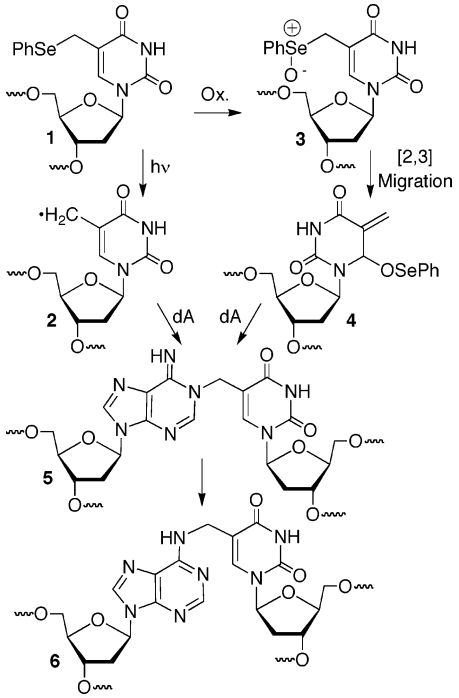
Department of Chemistry, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218

Received December 8, 2005; E-mail: mgreenberg@jhu.edu

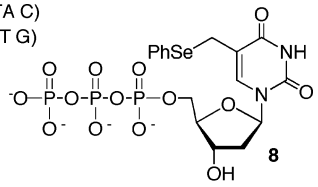
A number of anti-cancer agents induce cell death by oxidizing or alkylating DNA. Molecules, such as mitomycin, that produce interstrand cross-links (ISCs) by alkylating opposing DNA strands are particularly deleterious to the cell.[1−3] γ-Radiolysis is also a very common treatment for a variety of cancers in which DNA is the biologically relevant target.[4] Radiation damage is enhanced by O_2, which "fixes" damage by trapping DNA radicals and helps to explain why hypoxic solid tumors resist ionizing radiation. Exogenous molecules have been developed that sensitize DNA to γ-radiolysis under anoxic conditions, in some cases possibly by acting as a surrogate for O_2.[5,6] The non-native nucleosides, 5-bromo- and 5-iodo-2′-deoxyuridine, are also used as radiosensitizers. Random substitution of these molecules for thymidine enhances DNA damage by γ-radiolysis under anoxic conditions.[7] We wish to report on the first modified nucleotide whose nucleotide triphosphate is accepted as a substrate by DNA polymerases and that sensitizes DNA to γ-radiolysis by producing interstrand cross-links.

Recently, we showed that phenyl selenide **1** produces an ISC with the opposing dA upon UV photolysis or oxidation (Scheme 1).[8−10] Cross-linking proceeds through 5-(2′-deoxyuridinyl)methyl radical (**2**) upon photolysis or methide **4** under oxidative conditions. Both reaction conditions produce a kinetic ISC product believed to be **5** that isomerizes in solution to the isolated N6 adduct (**6**). The modest carbon−selenium bond dissociation energy, utility of aryl selenides in radical ion fragmentation chemistry, and their facile oxidation led us to investigate whether γ-radiolysis initiates ISC formation from **1**.[11,12]

Scheme 1. Interstrand Cross-Link (ISC) Formation via **1**



5′-d(AGA TGG AC**1** CAG GTA C)
3′-d(TCT ACC TGA GTC CAT G)
7



^{137}Cs irradiation of **7** produced ISC in a dose-dependent manner, as determined by denaturing polyacrylamide gel electrophoresis (Figure 1A). The determination that ISC yield is dependent upon O_2 concentration (Figure 1B) eliminated the radical as an intermediate upon radiolysis of **1** because cross-link formation via **2** is independent of O_2.[10] We investigated the possibility that ^{137}Cs irradiation produced ISC via the oxidation−rearrangement pathway (Scheme 1) by examining the effects of various additives that react with reactive oxygen species (Table 1). Addition of mannitol, which traps hydroxyl radical, prior to irradiation of **7**, has almost no effect on ISC yield. In addition, ISC formation increased slightly when superoxide dismutase was added to the mixture prior to irradiation, suggesting superoxide was not involved in oxidation of **1** but perhaps H_2O_2 was. The involvement of H_2O_2 produced during radiolysis was confirmed by the large decrease in ISC formation upon catalase addition. As expected based upon the effect of catalase on cross-link formation, reaction of **7** with added H_2O_2 also produces ISCs (data not shown).

The indicated involvement of H_2O_2 suggested that γ-radiolysis-induced cross-links result from oxidation of **1** to the selenoxide (**3**) and rearrangement to electrophilic **4**, as shown to occur upon

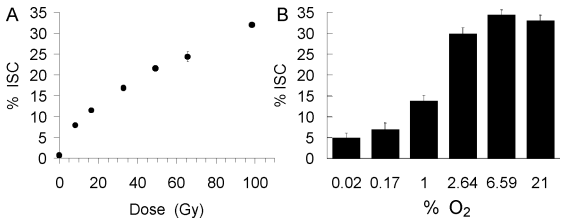


Figure 1. ISC formation from **7** upon γ-radiolysis: (A) as a function of dose under aerobic conditions; (B) as a function of O_2 concentration (98.4 Gy).

Table 1. Effect of Reactive Oxygen Species Scavengers on ISC Formation

additive	% ISC (normalized)	additive	% ISC (normalized)
mannitol (1 mM)	93.9 ± 2.1	catalase (600 U)	8.5 ± 0.3
S.O.D. (0.6 U)	112.6 ± 6.6	S.O.D. (0.6 U)/ catalase (600 U)	8.6 ± 0.5

10.1021/ja058290c CCC: $33.50

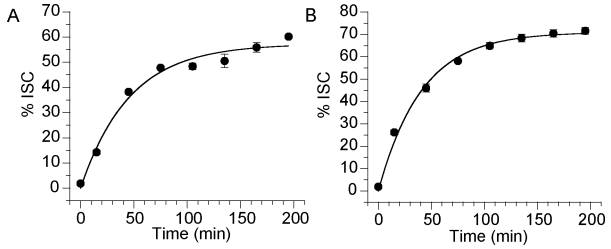


Figure 2. Rate of ISC growth from **7** upon (A) γ-radiolysis (235 Gy) or (B) $NaIO_4$ (5 mM) treatment.

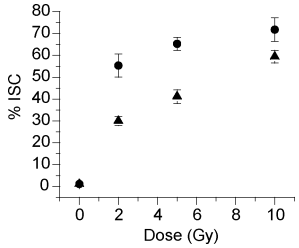


Figure 3. ISC formation upon γ-radiolysis: as a function of dose under aerobic (●, 21% O_2) and hypoxic (▲, 0.17% O_2) conditions.

reaction with $^1O_2$ or $NaIO_4$.[9] Further support for this mechanism was obtained by determining the rate constants for ISC formation in **7** treated with $NaIO_4$ (5 mM) or exposed to ^{137}Cs (235 Gy) (Figure 2). ISC growth followed first-order kinetics in each instance. Moreover, the observed rate constants for ISC formation when **7** was exposed to ^{137}Cs ($k_{obs} = 3.6 \pm 0.5 \times 10^{-4}$ s^{-1}, $t_{1/2} = 32.1$ min) or $NaIO_4$ ($k_{obs} = 4.1 \pm 0.3 \times 10^{-4}$ s^{-1}, $t_{1/2} = 28.2$ min) were within experimental error of one another.

Although radiolytically induced ISC formation from **7** is O_2 dependent, cross-links are observed even under severely hypoxic conditions (Figure 1B). A more realistic illustration of how **1** might perform in cellular DNA would be to examine cross-linking in a longer duplex containing randomly incorporated phenyl selenide. This requires demonstrating that **1** can be incorporated enzymatically into DNA. The viability of this process was established by determining the steady-state parameters for incorporation of **1** by a model DNA polymerase, the Klenow exo$^-$ fragment of *E. coli* DNA polymerase I.[13] The corresponding nucleotide triphosphate (**8**) was accepted as a substrate for incorporation opposite dA, albeit ~150 times less efficiently than was dTTP (Table 2). In addition, a template could be fully copied by Klenow exo$^-$ in the presence of dATP, dCTP, dGTP, and **8**, indicating that the modified nucleotide does not block extension and **1** can be incorporated at multiple sites within a duplex.[14]

A 7,200 nucleotide duplex containing **1** randomly incorporated in one strand was prepared by using Sequenase to copy linearized single-stranded M13mp7 plasmid.[14] Transformation of linearized plasmid to duplex form was carried out in the presence of equal concentrations of **8** and dTTP (0.5 mM), along with the three other native dNTPs (1.0 mM). Using the relative specificity constants for dT and **1** incorporation by Klenow exo$^-$ as a guide, on average, ~12 molecules of **1** are believed to be incorporated per duplex. Very high yields of ISC were obtained when the duplex was exposed to low doses of radiation (2−10 Gy) in aerated solution (Figure 3). No cross-linking was detected in a control duplex prepared without **8** (data not shown). Moreover, ~60% of the DNA molecules were cross-linked when exposed to 10 Gy in an O_2 environment (0.17%) found in severely hypoxic cells (Figure 3).

Table 2. Comparison of Incorporation of **1** and dT Opposite dA by Klenow exo$^-$

5′-d-ACC ATG GGA CGT ACG AC
3′-d-TGG TAC CCT GCA TGC TGA TGC TAG GTC GTG CAC GTC

N = T, **8** ↓ Klenow exo$^-$, dTTP or **8**

5′-d-ACC ATG GGA CGT ACG AC**N**
3′-d-TGG TAC CCT GCA TGC TGA TGC TAG GTC GTG CAC GTC

dNTP	V_{max} (%·min^{-1})	K_m (nM)	V_{max}/K_m (%·min^{-1}·nM^{-1})
dTTP	23.7 ± 1.4	8.5 ± 1.4	2.8 ± 0.3
8	10.7 ± 0.8	598.1 ± 58.7	$1.8 \pm 0.1 \times 10^{-2}$

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Supporting Information Available: Experimental procedures, sample velocity versus [dNTP] plots, and autoradiograms describing full-length primer extension for incorporating dTTP or **8**, and formation of 7,200 nt duplex from linearized M13mp7. This material is available free of charge via the Internet at http://pubs.acs.org.

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