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Investigating the Biochemical Impact of DNA Damage with Structure-Based Probes: Abasic Sites, Photodimers, Alkylation Adducts, and Oxidative Lesions†

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ABSTRACT: DNA sustains a wide variety of damage, such as the formation of abasic sites, pyrimidine dimers, alkylation adducts, or oxidative lesions, upon exposure to UV radiation, alkylating agents, or oxidative conditions. Since these forms of damage may be acutely toxic or mutagenic and potentially carcinogenic, it is of interest to gain insight into how their structures impact biochemical processing of DNA, such as synthesis, transcription, and repair. Lesion-specific molecular probes have been used to study polymerase-mediated translesion DNA synthesis of abasic sites and TT dimers, while other probes have been developed for specifically investigating the alkylation adduct O^6-Bn-G and the oxidative lesion 8-oxo-G. In this review, recent examples of lesion-specific molecular probes are surveyed; their specificities of incorporation opposite target lesions compared to unmodified nucleotides are discussed, and limitations of their applications under physiologically relevant conditions are assessed.

Within years of the discovery that DNA was responsible for transmitting hereditary information (*1*), and before publication of the double-helical structure of DNA (*2*), researchers began to probe its susceptibility to various types of damage (*3*). Exposure of DNA to UV[1] radiation, alkylating agents, and reactive oxidative species leads to the formation of abasic sites, pyrimidine dimers, alkylation adducts, and oxidative damage products. Each of these types of lesions can be mutagenic and sometimes carcinogenic, and this chemical process, therefore, is highly relevant to cellular biology and disease. For example, UV radiation damage-induced skin cancer was estimated to result in more than 1 million new cases of the disease in the United States in 2008 (*4*). Further, DNA alkylation by tobacco-derived carcinogens contributes to lung cancer, which is estimated will result in ~160000 deaths in the United States in 2009 (*5*, *6*). These examples of the biological consequences of DNA damage underscore the continued need for understanding the underlying molecular mechanisms.

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[1]Abbreviations: A, adenine; 2-Ap, 2-aminopurine; AP, apurinic; ARP, aldehyde-reactive probe; BER, base excision repair; Bn, benzyl; BzNU, *N*-nitroso-*N*-benzylurea; C, cytosine; ddATP, dideoxyadenosine triphosphate; dNTP, deoxyribonucleoside triphosphate; dPTP, deoxyribopyrene triphosphate; ELISA, enzyme-linked immunosorbent assay; exo$^-$, exonuclease-deficient; exo$^+$, exonuclease-proficient; G, guanine; GC, gas chromatography; gp43, T4 DNA polymerase; HPLC, high-performance liquid chromatography; Kf, Klenow fragment; MS, mass spectrometry; NBzMA, *N*-nitrosobenzylmethylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 8-oxo-G, 8-oxoguanosine; PCR, polymerase chain reaction; POB, pyridyloxobutyl; RIP, ribosome-inactivating protein; T, thymine; T4 endo V, T4 endonuclease V; THF, tetrahydrofuran; UV, ultraviolet; XP, xeroderma pigmentosa.

Cellular factors such as base excision repair (*7*, *8*) mitigate some damage to DNA; however, certain lesions evade repair, and through a variety of biochemical pathways, these DNA adducts may wreak havoc on normal cellular replication and its regulation. For example, misincorporation of nucleotides across from a lesion during polymerase-mediated DNA synthesis, in which the damaged DNA strand may act as a template, could lead to frame shift or substitution mutations (*9*). The ability of various polymerases to perform translesion synthesis has been studied in great detail over the past decade, yielding important mechanistic information regarding the origins of mutagenicity (*10*, *11*). Another mode of disruptive lesion formation involves DNA alkylation followed by hydrolytic base excision, i.e., depurination or depyrimidination. This reactivity mode has been demonstrated for various genotoxins such as the natural product leinamycin (*12*, *13*), the natural product derivatives acylfulvene (*14*, *15*) and azinomycin epoxide (*16*), the tobacco-derived nitrosamine NNK (*17*), and endogenous estrogen metabolites (*18*). In many cases, it is unclear whether cytotoxicity arises from initially formed DNA adducts or subsequently generated abasic sites. Finally, DNA lesions such as inter- and intrastrand cross-links formed by chemotherapeutic alkylating agents (i.e., nitrogen mustards and cisplatin) can be biochemically disruptive by stalling DNA replication and triggering apoptosis (*19*).

To elucidate biochemical mechanisms underlying the important role DNA lesions play in mutagenesis and carcinogenesis, their biological prevalence and chemical structure need to be determined. It is therefore of interest (1) to identify and quantify DNA lesions in model experimental systems and in vivo, (2) to determine influences of lesions on physical properties of DNA, such as helical structure and thermal stability, and (3) to characterize the impact of the lesion on DNA function, i.e., enzyme-mediated processes such as replication. With regard to

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identification and quantification, several approaches for detecting DNA lesions exist and have been reviewed; examples include ^{32}P postlabeling, electrochemical or fluorescence detection, immunoassay, mass spectrometry, accelerator mass spectrometry, and quantitative PCR (*20*−*23*). Described herein are emerging chemical and biochemical approaches for probing how specific adduct types affect molecular structure and function. Lesion-specific nucleoside probes have been reported for abasic sites, TT photodimers, an alkylation adduct (O^6-Bn-G), and an oxidative lesion (8-oxo-G). These have been used to probe abasic sites in DNA, as well as those formed in RNA by ribosome-inactivating proteins, to elucidate mechanistic aspects of polymerase-mediated translesion synthesis, including the impact of stereoelectronic properties of the incoming nucleotide on polymerase activity and exo/endonuclease activity opposite TT photodimers. Finally, lesion-specific probes under development for the study of the DNA adducts 8-oxo-G and O^6-Bn-G have been reported and will be discussed here.

ABASIC SITES

DNA abasic site formation resulting from nucleotide depurination or depyrimidination (i.e., AP site formation) is estimated to occur at a background physiological rate of ~10000 times per human genome per day (*24*). The depurination rate at physiological pH, ionic strength, and temperature has been estimated to be 3×10^{-11} s^{-1}, or one purine per *Escherichia coli* genome per generation (*25*, *26*), and depyrimidination is ~20 times slower than depurination (*27*). Abasic sites impact the local and global structure of DNA, and depending on the context, single-base loss destabilizes the duplex on the order of 3−11 kcal/mol (*28*). Base loss can occur spontaneously or be promoted by DNA alkylation or oxidation. Furthermore, abasic sites are intermediates during excision repair of damaged nucleotides(*29*), and healthy cells typically have a robust capacity for repair (*30*). Deficiencies in this process can alter normal biochemical processing of DNA, leading to mutation or acute toxicities (*27*). Because of the extensive role of AP sites in normal and disease-related processes, it is important to be able to detect their presence and improve our understanding of how they influence biochemical processing of DNA.

Enzymes involved in the repair of abasic sites exist in prokaryotes and eukaryotes; these utilize polymerases that recognize abasic sites as intermediates in base excision repair (BER) (*8*, *31*−*33*). Briefly, AP endonucleases cleave at the 5′-position of the damaged DNA strand, forming a 5′-phosphodeoxyribose (dRP) and liberating a 3′-hydroxyl group. The deoxyribose phosphate group is excised by 2-deoxyribose-5-phosphate lyase, resulting in a single-nucleotide gap. Then, a DNA polymerase, such as pol β, inserts the missing nucleotide, which is finally ligated into the DNA strand by a DNA ligase (*32*).

Abasic sites are manifested in several chemical structures, beyond the hemiacetal moiety directly resulting from nucleobase hydrolysis [AP (Figure 1)]. For example, one-electron nucleotide oxidation results in the formation of 2′-deoxyribonolactone [L (Figure 1)] (*34*, *35*). Despite the structural similarity between L and AP, it was found that T is primarily incorporated opposite the L lesion by *E. coli* (*36*, *37*), while A is typically inserted opposite AP sites (a phenomenon often termed the "A-rule") (*36*−*41*). Another abasic site variant [C4-AP (Figure 1)] is formed after treatment of DNA with antitumor antibiotics such as bleomycin; similar to AP, it was found to block primer extension by Klenow fragment polymerases (*42*). Because of their

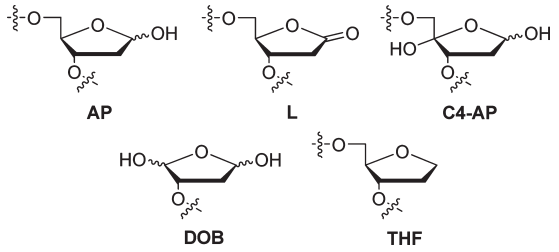


FIGURE 1: Structures of different forms of abasic DNA lesions.

lack of stability and difficulty in synthesis for each of these types of abasic sites, the lesions are often modeled by the stabilized tetrahydrofuran analogue [THF (Figure 1)] to investigate their biochemical impacts. Such studies have been conducted using lesion-specific molecular probes, including chemically reactive probes, non-natural nucleotide probes, and other non-nucleotide molecular probes.

Chemically Reactive Abasic Site Probes. The classic approach for detecting abasic sites involves aldehyde-reactive probes (ARPs). AP sites exist as an equilibrium mixture between the closed-ring hemiacetal and the corresponding open-chain aldehyde. ARPs consisting of a nucleophilic O-substituted hydroxylamine linked to a reporter substrate react with the aldehyde to covalently bind the abasic site (*43*, *44*). Methoxyamine was originally utilized as an AP site probe in BER studies. It was discovered that when methoxyamine binds the aldehyde, it blocks AP endonuclease and inhibits DNA cleavage 5′ of the AP site (*43*). Studies with colon cancer xenografts showed that when methoxyamine was administered together with a DNA methylating drug such as Temozolomide, the alkylation-induced AP sites reacted with methoxyamine, blocking BER and thus potentiating the therapeutic potential of the drug (*45*).

Various ARPs have been designed for quantitative analysis of abasic sites. For example, following incubation with DNA, the biotin-tagged reagent **1** (Figure 2) was treated with an avidin−biotinylated horseradish peroxidase complex, and any unbound DNA was removed by washing. In this ELISA-like assay, conjugated peroxidase activity colorimetrically quantified correlates with bound open-chain abasic sites in DNA (*46*). ARP **1**, now commercially available, was recently used to measure abasic sites formed from depurination of dibenzo[*a*,*l*]pyrene adducts in calf thymus DNA (*47*). In other studies, fluorescence intensities of the solutions of DNA incubated with luminescent ARPs, such as those containing dansyl or lissamine-rhodamine fluorophores [**2** and **3** (Figure 2)] were spectroscopically measured to determine the level of ARP adduct formation (*48*, *49*). The accuracy of the fluorescent ARP assay may be improved by utilizing capillary electrophoresis to separate any unreacted probe from the DNA sample, increasing the signal-to-noise ratio of fluorescence detection (*50*).

Chemically reactive probes have been designed for detecting oxidized abasic site products as well. The biotin-tagged cysteine probe **4** (Figure 2) selectively reacts with the butenolide formed from treating 2′-deoxyribonolactone L with *N*,*N*′-dimethylethylenediamine, creating an adduct that subsequently could be quantified by ELISA-like assays similar to those discussed above (*51*). Similarly, probe **5** (Figure 2) was designed to react with C4-AP and DOB abasic sites and be detected by an ELISA-like assay (*52*). These chemically reactive probes are useful for quantifying abasic sites in bulk DNA.

Abasic Site Nucleotide Probes. In seminal studies involving synthetic DNA, Matray and Kool introduced the pyrene

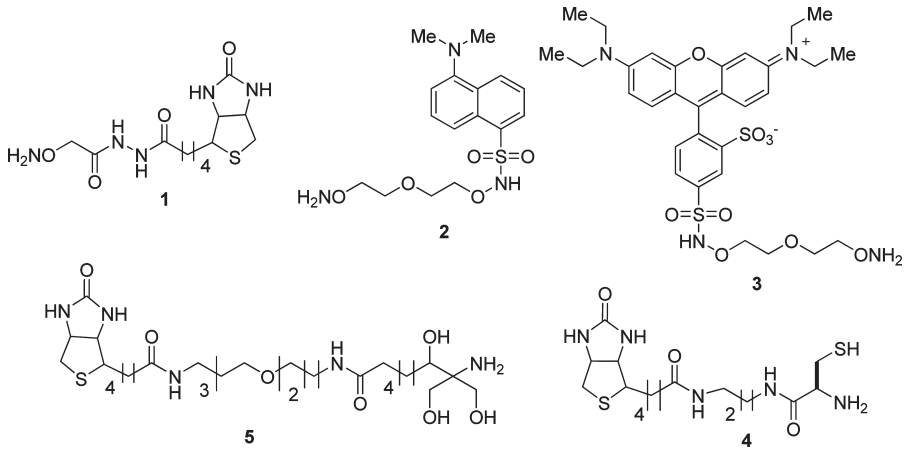


FIGURE 2: Chemically reactive probes for abasic lesions.

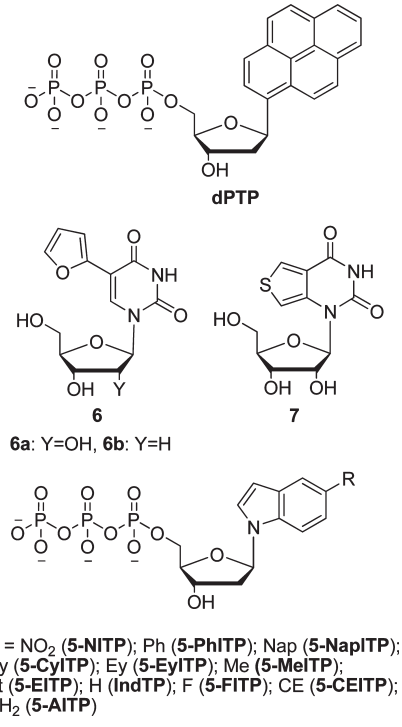


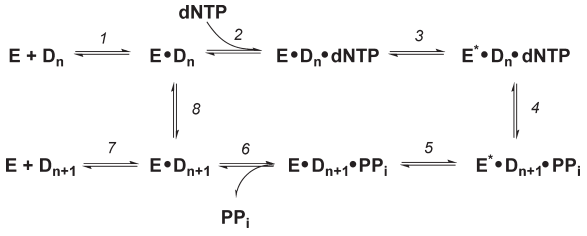
FIGURE 3: Nucleoside probes for abasic lesions and TT dimers: Ph, phenyl; Nap, naphthyl; Cy, cyclohexyl; Ey, ethylene; Me, methyl; Et, ethyl; CE, cyclohexenyl.

nucleotide [dPTP (Figure 3)] as a base surrogate that pairs opposite AP sites and the model THF (*53*). Across from an abasic deoxyribose in a template DNA strand, the pyrene nucleotide was preferentially incorporated over the four natural nucleotides by Klenow fragment (Kf) of *E. coli* DNA polymerase I (exo mutant). This phenomenon underscored the role of shape complementarity in molecular recognition in duplex DNA and also demonstrated that hydrogen bonding is not essential for stable pairing. Additionally, the pyrene probe could be used like a dideoxynucleotide terminator for sequencing abasic DNA because elongation stalled after dPTP incorporation. Synthetic 43-mer oligonucleotides containing one or multiple THFs were sequenced using a combination of canonical dideoxynucleotides and dPTP. Incorporation of dPTP was observed for DNA samples diluted to contain as little as 1% damaged strands among undamaged oligonucleotides. Further studies with the pyrene nucleotide have probed the abasic-like site generated when adenine-specific DNA methyltransferase M·*Taq*I binds to DNA (*54*), affirmed the significance of nucleobase shape complementarity in closed polymerase−DNA complexes (*55*), demonstrated the importance of π-stacking during template-independent nucleotide additions onto blunt-end DNA (*56*) and in DNA duplexes in aqueous solution (*57*), and revealed details regarding the base flipping mechanisms of uracil DNA glycosylase (*58*) and UV-induced TT dimer translesion DNA synthesis (vide supra).

Emissive nucleosides have recently emerged as an attractive strategy for spectroscopic detection and evaluation of abasic sites in DNA and RNA. For example, Greco and Tor reported a fluorescent thymidine analogue [**6a** (Figure 3)], which contained an appended conjugated furan ring at the 5-position of the nucleotide. Synthetic 12-mers in which analogue **6a** was paired with either A or THF opposite the probe exhibited identical thermal stabilities, but the duplex containing **6a** opposite the THF exhibited a 7-fold increase in emission intensity compared to that with **6a** opposite A (*59*). As a further example, a thiophene-based analogue [**7** (Figure 3)] was used to spectroscopically detect RNA abasic sites produced by ribosome-inactivating proteins (RIPs). Toxic RIPs catalyze N-deglycosylation of the conserved α-sarcin/ricin stem−loop domain region of rRNA, and treating this RNA sequence with the toxin saporin generates RNA abasic sites. When synthetic oligonucleotide hybridization probes containing **7** were paired opposite an abasic site, up to 6.5-fold enhancements in emission intensities were observed compared to those of **7**:A. These results demonstrate the effectiveness of these fluorescent nucleotides in signaling the occurrence of abasic sites in RNA, and probes such as **7** are expected to be useful in detecting ribosome-inactivating proteins that act on other specific RNA substrates, or for the discovery of RIP inhibitors or antidotes (*60*).

Insight regarding detailed molecular mechanisms of the polymerase-mediated synthesis template by damaged DNA has been gained from various recent studies (*10*), including experiments involving nucleotide probes. Before we discuss some significant examples in this area, a very brief overview of polymerase-mediated DNA synthesis is warranted (*10*, *61*, *62*). As shown in Scheme 1, the polymerase binds DNA (step 1), followed by reversible binding of an incoming dNTP (step 2). Following a proposed conformational change from the open to the catalytically active closed form of the polymerase (step 3), formation of a phosphodiester bond between the incoming dNTP and 3′-end of the oligonucleotide primer occurs (step 4). The polymerase

Scheme 1: Polymerase-Mediated DNA Synthesis[a]



[a]Abbreviations: E, "open" polymerase; D_n, deoxyribonucleotide; dNTP, deoxyribonucleotide triphosphate; E*, "closed" polymerase; D_{n+1}, deoxyribonucleotide extended by one nucleotide; PP_i, pyrophosphate.

relaxes to the open form (step 5) and releases the inorganic pyrophosphate byproduct of phosphodiester bond formation (step 6). Finally, the polymerase−oligonucleotide complex may dissociate (step 7) or continue directly to another round of elongation (step 8) in a processive fashion. Some polymerase−lesion combinations allow non-natural nucleotide incorporation via this DNA synthesis pathway, thus facilitating a means of testing molecular aspects of polymerase-mediated translesion DNA synthesis.

Exonuclease-deficient T4 DNA polymerase (gp43 exo$^-$)-mediated synthesis past abasic sites has been examined in studies conducted by Berdis and co-workers using a series of hydrophobic base analogues as polymerase substrates (*63*). Nucleoside triphosphates with varying π-surface areas, shapes, dipole moments, and energies of solvation were used as substrates in T4-mediated DNA synthesis templated by THF-containing DNA. The non-natural substituted indole nucleotides included 5-NITP (*64*), 5-PhITP (*65*), 5-NapITP (*66*), 5-CyITP, 5-EyITP, 5-MeITP, and 5-EITP (Figure 3) (*63*). Analogues with larger π-electron surface areas, such as 5-NITP, 5-PhITP, and 5-NapITP, possessed high binding affinities and were quickly incorportated opposite an abasic site but were poorly incorporated opposite templating nucleobases (*64*−*66*). Selectivity was diminished for non-natural nucleotides with π-electron surface areas >200 Å^2, which was attributed to the larger nucleobases nonselectively intercalating into the DNA. However, non-natural nucleotides with π-electron surface areas of <180 Å^2, such as 5-CyITP, 5-EyITP, 5-EtITP, and 5-MeITP, also demonstrated high catalytic efficiency opposite abasic sites. For these nucleotides, overall catalytic efficiencies for incorporation opposite templating nucleobases were 100-fold lower than opposite an abasic template (*63*).

Kinetic data obtained from the study of 5-CyITP, 5-EyITP, 5-EtITP, and 5-MeITP supported Berdis' proposed model for gp43 exo$^-$ polymerase-mediated synthesis, which posited that a templating base is oriented extrahelically within the polymerase, creating an abasic-like void in the DNA. Following association of an incoming nucleotide, the polymerase undergoes a conformational change that positions the templating base intrahelically for proper alignment during phosphoryl transfer; the rate constant for polymerization, k_{pol}, reflects the rate constant for this conformational change (*64*−*68*). It was observed that the presence of a templating nucleobase modestly influenced the binding affinities of incoming dNTPs of the smaller non-natural nucleotides; K_d values measured opposite T were ~3-fold higher compared to those measured opposite an abasic site. However, a templating thymine opposite incoming 5-CyITP, 5-EyITP, 5-MeITP, and 5-EtITP led to 30−60-fold lower k_{pol} values compared to the values of those opposite an abasic site. These data are consistent with discrimination between incoming nucleotides being largely controlled during the phosphoryl transfer step, instead of during the initial ground state binding (*63*). Thus, although the increased π-electron surface area of an incoming nucleotide leads to favorable stacking interactions within the abasic site that reduce K_d, the more important contributor to efficiency and selectivity of polymerase-mediated synthesis opposite templating or abasic sites is the overall size of the incoming nucleotide, which sterically interacts with templating bases, controlling k_{pol}.

Nucleobase-Derived Abasic Probes. With the goal of potentiating the activity of anticancer methylating agents, nucleobase-derived probes with the capacity to hinder BER of abasic sites induced by the drugs have been investigated. Demeunynck and co-workers designed a series of heterodimeric compounds consisting of purine nucleobases linked to an acridine moiety through polyamine or guanidine-containing tethers. NMR studies, in which the probes were added to duplex DNA containing abasic sites modeled by THF, showed that the purine nucleobases insert within the abasic site pocket and form a hydrogen bond to a partner T. The tethered acridine intercalates into the DNA duplex adjacent to the abasic site (*69*). The cytotoxicity and affinity of the probe for binding duplex DNA-containing abasic sites were modulated by varying the length and chemical identity of the tether (*70*). In cancer cell lines and in a P388 leukemia mouse model, co-administration of the heterodimeric probes with the anticancer DNA-alkylating drug bischloroethyl nitrosourea potentiated the anticancer activity of the drug (*69*−*71*).

Bulky Triphosphate Probes of Abasic DNA. With the aim of determining the minimal structural requirements of DNA polymerase substrates, Maga and co-workers investigated the suitability of nucleoside-free triphosphates for incorporation into DNA. A series of probes [**8a**−**c** (Figure 4)] consisting of bulky hydrophobic groups linked to a triphosphate, lacking any bases or sugar moieties, were investigated as DNA synthesis substrates for human polymerases α, β, and λ, *Saccharomyces cerevisiae* polymerase IV, *E. coli* polymerase I, and HIV-1 reverse transcriptase (*72*). These were screened for their ability to incorporate the analogues opposite either templating bases or THF. Generally, the non-nucleoside triphosphates competitively inhibited natural nucleotide binding and blocked template extension by human polymerases α, β, and λ and *S. cerevisiae* polymerase IV, while *E. coli* polymerase I and HIV-1 reverse transcriptase were not affected. Polymerase λ, which was known to perform translesion synthesis opposite abasic sites, was able to incorporate **8a** across from THF but not opposite templating nucleobases. Additionally, the mutant polymerase λ Y505A, which possesses a larger nucleotide binding site than the wild-type enzyme, incorporated both **8a** and **8b** opposite the abasic sites but not opposite normal substrates. It was rationalized that although the non-nucleoside triphosphates generally accessed polymerase binding pockets, causing polymerase inhibition, the polymerases did not undergo a transition from the catalytically inactive open position to the active closed position and thus were unable to align the non-nucleoside analogue with the primer strand and form a phosphodiester bond. However, polymerase λ and λ Y505A, which adopt a closed structure with or without nucleotide substrates, were able to catalyze the formation of a phosphodiester bond between the probes and primers. Since the probes contain no sugar moiety and thus no 3′-OH group, no

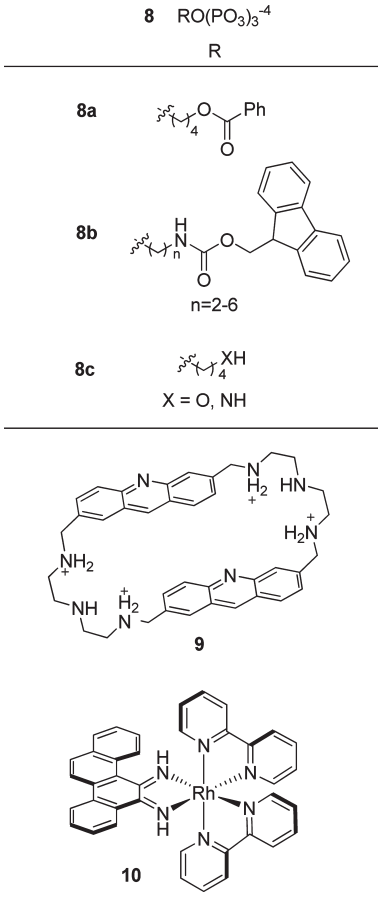


FIGURE 4: Non-nucleoside probes for abasic lesions.

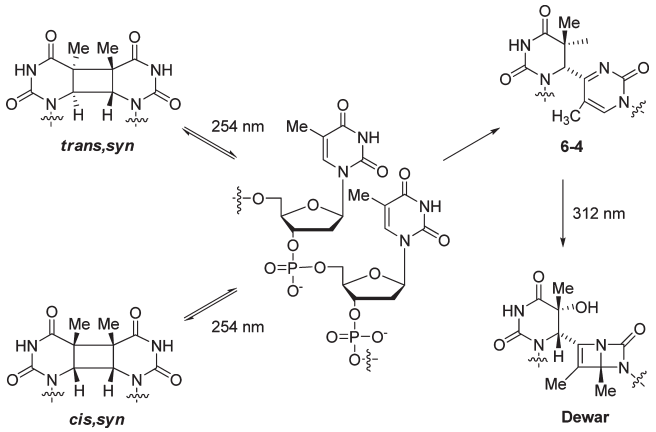
further extension is possible; therefore, these non-nucleoside analogues may have potential biotechnological applications as chain terminators for specifically detecting abasic sites.

Other Non-Nucleotide Probes. Various non-nucleotide probes that do not fit into the categories described above have been reported; some notable recent examples are illustrated in Figure 4. Cyclobisacridine [CBA, **9** (Figure 4)] selectively intercalates into duplex DNA at an abasic site and, upon irradiation, photocleaves both strands (*73*). Photoinduced cleavage at an abasic site was also demonstrated with $[\Delta\text{-Rh(bpy)}_2(\text{chrysi})]^{3+}$ [**10** (Figure 4)], a sterically bulky rhodium complex that inserts (not intercalates) at abasic sites or single-base bulges in DNA and ejects the mismatched base pair into the major groove before cleavage (*74*). Finally, several recent non-nucleobase abasic site probes include fluorescent intercalators (*75*), a hybridization probe detected using electrochemical methods (*76*), a naphthyridine-based hydrogen bonding fluorescent probe (*77*), and a ferrocenyl aminonaphthyridine-based hydrogen-bonding probe that can be detected both spectroscopically and electrochemically (*78*).

UV-INDUCED DNA DAMAGE

An important class of DNA lesions consists of those induced by UV radiation, a critical contributor to skin cancer etiology. This correlation was initially made in studies of xeroderma pigmentosa (XP) patients, who exhibit high incidences of skin cancer, and whose somatic cells, unable to repair pyrimidine dimers, were hypersensitive to killing by UV radiation (*79*). Further, the action spectra (a plot correlating radiation frequency to relative biological response) for killing, mutating,

Scheme 2: Pathways to Thymine Dimers



and transforming cells were similar to the action spectra for pyrimidine dimerization, which falls within the DNA absorbance spectrum (*80*, *81*). Interestingly, it was also shown that reversal of pyrimidine dimers by photolyases reduced the incidence of tumors in experimental animals (*82*, *83*), which suggested that pyrimidine dimers were at least partially responsible for UV-induced biological effects (*84*).

The location and identity of UV-induced DNA lesions can be determined by HPLC analysis with UV spectroscopic detection (*85*), immunofluorescence (*86*), ^{32}P postlabeling (*87*), gas chromatography−mass spectrometry (GC−MS) (*88*), and HPLC−electrospray ionization MS/MS (*89*). Cyclobutane pyrimidine dimers, which may occur at TT, CC, CT, or TC dipyrimine sites within the same DNA strand, are the most common UV-induced photolesions (70−80% occurrence) (*90*). 6-4 photoproducts are less frequent (20−30% occurrence) (*90*), and it was discovered that sunlight causes them to photoisomerize to Dewar valence isomers (Scheme 2) (*91*). While the *cis-syn* thymine dimer is most prevalent and was implicated in early studies linking UV-induced DNA damage to skin cancer, it has been suggested that the less common 6-4 photoproducts play a more significant role in mutagenesis and lethality (*92*−*95*). DNA repair mechanisms, such as nucleotide excision repair or photoreactivation, undoubtedly also have a large impact on UV-induced mutagenesis; the biological effects of various lesions depend on which genes are damaged and what repair mechanisms are active in affected cells (*84*, *94*, *96*).

Photodimer Translesion DNA Synthesis. Mechanistic aspects of TT and CC *cis-syn* dimer-induced mutagenesis have been deduced through studies of translesion DNA synthesis by various polymerases, as reviewed in ref *97*. It has been observed that *E. coli* DNA polymerase I (pol I) bypasses the TT *cis-syn* dimer, and that in this process, the only nucleotide incorporated across from each T in the dimer is its natural pair, A (*97*). A putative mechanism accounting for this observation is that the specificity of incorporation of AA opposite the TT dimer results not from correct coding but from the inherent tendency for polymerases to incorporate A across from noninstructional (i.e., non-hydrogen bonding) DNA sites, as per the A-rule. Contradicting the noncoding hypothesis were NMR data for a DNA octamer containing a central TT dimer that appeared, in fact, to form a hydrogen bond to the complementary AA in the opposite strand (*98*). Further evidence that UV-induced lesions were templating, rather than noninstructional, was that *E. coli* under SOS conditions also incorporated AA opposite a TT dimer

at a much higher frequency than A was incorporated opposite a noninstructional abasic site (*99*−*101*). Additionally, GA was found to be selectively incorporated opposite 6-4 TT photoproducts (*101*). In DNA synthesis reactions mediated by *E. coli* polymerase V (pol V), similar selectivities were observed (*102*). However, other polymerases, including T7 exo$^-$ (*103*), *E. coli* pol III (*102*), and those found in yeast (*104*) and human cell extracts (*105*), incorporated AA opposite both *cis-syn* TT dimers and 6-4 TT photoproducts. Collectively, these data suggested that while lesion bypass mechanisms vary among different polymerases, generally lesion bypass involves enzyme-based molecular interactions that dictate the specificity of translesion nucleotide incorporation.

Probing TT Dimer Translesion Synthesis. Detailed information regarding the unique mechanisms for TT dimer bypass by specific enzymes has been gained through studies utilizing nucleotide probes. A crystal structure of the T7 exo$^-$ DNA polymerase bound to a DNA template in the presence of ddATP showed that the 5′-end of the template strand was bent out of the active site (*106*). On the basis of this structure, and in light of the fact that T7 exo$^-$ incorporates AA across a *cis-syn* TT dimer, Lloyd and co-workers proposed a mechanism for the bypass of the dimer in which the 3′-T, bent out of the active site due to its covalent bond to the downstream 5′-T, acts as an abasic-like noninstructive lesion, while the 5′-T, once moved back into the active site, acts as a templating base. Each insertion would be expected to template an A, as per the A-rule for the first insertion, and through normal nucleobase specificity for the second insertion (*103*). To test this hypothesis, Lloyd and co-workers took advantage of the pyrene nucleotide (dPTP in Figure 1), which was previously shown by Kool and Matray to be incorporated opposite abasic sites and analogues by T7 DNA polymerase, in preference to all four natural nucleotides (*53*). Assuming that the 3′-T of a TT dimer was indeed bent out of the active site of the polymerase, thus presenting an abasic site on the template strand, it was expected that dPTP would be selectively incorporated rather than adenine. Primer extension analyses confirmed that dPTP was in fact inserted by a >12:1 preference to dATP across the 3′-T of a template strand *cis-syn* TT dimer. However, dPTP was very inefficiently incorporated opposite the 5′-T of the dimer, while dATP was preferentially inserted, suggesting that the 5′-T was instructional during polymerase bypass of the lesion. Similar results with 6-4 TT photoproducts, their Dewar isomers, and *trans-syn* II TT dimers corroborated the transient abasic site model for T7 DNA polymerase UV lesion bypass (*103*).

To evaluate whether the transient abasic site model proposed for T7 DNA polymerase applies to other high-fidelity polymerases, Berdis and co-workers conducted primer extension assays with the bacteriophage T4 DNA polymerase (gp43) (*67*). In these studies, the rates of gp43-mediated incorporation of various natural and non-natural nucleotide probes across either a *cis-syn* TT dimer or a THF abasic site analogue in an oligonucleotide template were compared. It was expected that if gp43 employed the transient abasic site mechanism, then it would incorporate analogues such as 5-NITP, 5-PhITP, and 5-NapITP, which were previously demonstrated to have high binding affinities and incorporation rates across from abasic sites (*64*−*66*), opposite both the TT dimer and the abasic site with nearly equal kinetic parameters.

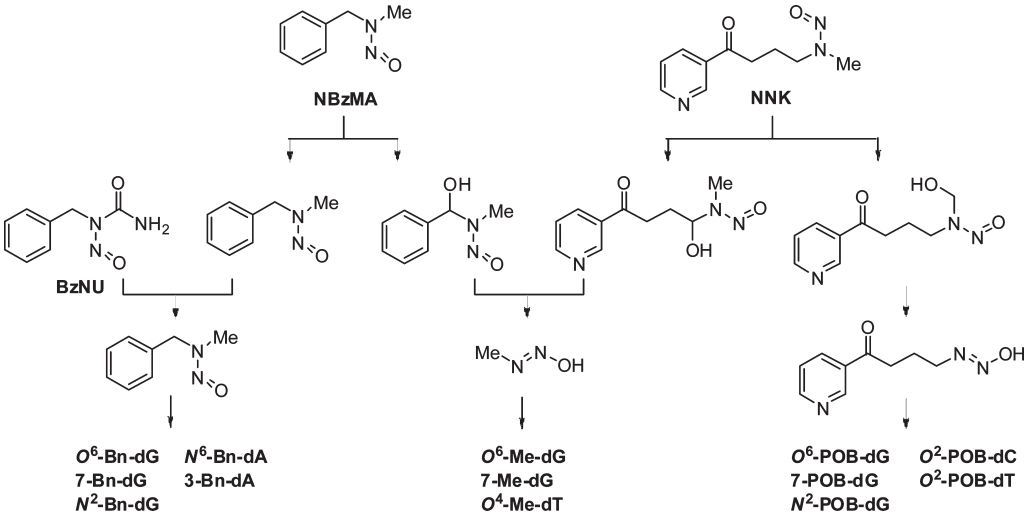
Different rates of incorporation of nucleotide probes across from TT dimers and abasic sites were observed in experiments involving exonuclease-deficient gp43 exo$^-$. In particular, nucleotides that were readily inserted opposite abasic sites, such as IndTP, 5-FITP, 5-AITP (Figure 3), 5-NITP, and dATP, were poorly inserted opposite the TT dimer. Additionally, although dATP was the only natural nucleotide to be incorporated across the TT dimer, the rate was ∼21-fold slower than the rate of incorportation opposite the abasic site, even at high concentrations such as 1 mM (*107*). These results implied that gp43 exo$^-$ does not entirely follow the A-rule or the transient abasic site model for TT dimer bypass. The significant differences between incorporation rates for each nucleotide probe opposite the TT dimers indicated that π-electron density and the overall size of the analogue both play a large role in the incorporation efficiency. On the basis of these studies, Berdis and co-workers concluded that the TT dimer was templating the incorporation of A via molecular interactions with T, not as an abasic-like site.

To test the templating TT dimer hypothesis, the rates of incorporation of the nucleotide probes across from an unmodified T were also evaluated. Comparison of the kinetic parameters showed that analogues with a large π-electron surface area [5-CEITP (Figure 3), 5-PhITP, and 5-NapITP] were incorporated opposite the TT dimer more efficiently than across T, while those with a smaller π-electron surface area (5-NITP, 5-FITP, and 5-CyITP) were more effectively incorporated opposite T. These observations led to the proposal that the TT dimer is replicated by gp43 exo$^-$ via a hybrid mechanism. For unmodified DNA, the templating nucleobases are oriented in an extrahelical position, creating an abasic-like void that can readily accept a bulky nucleotide probe like 5-PhITP. However, the bulkiness of the probe would hinder the repositioning of the extrahelical templating nucleobase back into its intrahelical position, diminishing the rate of incorporation of the probe. In contrast, the k_{pol} values for incorporation of the probes opposite abasic sites are much faster since there is no extrahelical base to reposition. The kinetics of incorporation of nucleotide probes opposite TT dimers support the model in which the 3′-T of the dimer is partially extrahelical, creating an abasic-like site into which the probe is incorporated at a lower k_{pol} than at an actual abasic site.

Probing TT Dimer-Induced Exo- and Endonuclease Activity. In addition to comparing the rates of gp43 exo$^-$-mediated incorporation of nucleotides opposite a TT dimer and abasic site, Berdis and co-workers also examined the ability of gp43 exo$^+$ to excise nucleotides opposite these lesions or T (*67*). It had been previously shown that gp43 removed A opposite an abasic site ∼30-fold faster than A opposite T (*108*). On this basis, altered hydrogen bonding and stacking interactions at the abasic site were thought to distort the primer−template geometry such that exonuclease activity increased. However, while gp43 exo$^+$ removed 5-PhITP opposite an abasic site, it did not remove the probe opposite a TT dimer, even though the exonuclease easily excised A opposite either lesion. It was hypothesized that the diminished reactivity was due to aberrant stacking interactions caused by the proximity of the phenyl moiety of 5-PhITP to the 5′-T of the TT dimer, which may have hindered the proofreading ability of gp43.

The mechanism of base excision by T4 endonuclease V (T4 endo V) has also been evaluated by a similar approach using the fluorescent nucleotide probe 2-aminopurine (*109*). T4 endo V is a DNA glycosylase that selectively binds to a *cis-syn* TT dimer and cleaves the glycosidic bond of the damaged base. The crystal structure of E23Q (a catalytically inactive T4 endo V mutant), cocrystallized with a substrate DNA, showed that the adenine opposite the 5′-T of a TT dimer was flipped extrahelically into an

Scheme 3: Representative Nitrosamine Alkylation Pathways



enzyme pocket, and that the DNA substrate had a 60° bend at the dimer (*110*). To study the role of base flipping in T4 endo V recognition of thymine dimer damage, Lloyd and co-workers utilized 2-Ap for monitoring the binding of the enzyme and the relative position of the 2-Ap in the T4 endo V−substrate DNA complex. The probe was selected because it had been previously shown to form a Watson−Crick base pair with thymine (*111*) and its fluorescence was significantly quenched in duplex DNA compared to that in singled-stranded DNA (*112*).

Lloyd and co-workers showed that placing 2-AP in either the 3′- or 5′-position opposite a TT dimer in oligonucleotides does not alter the binding or catalytic activity of wild-type T4 endo V or E23Q (*109*). The specificity of base displacement observed in the cocrystal structure was corroborated by titration assays, which showed no fluorescence enhancement (which would indicate extrahelical displacement of the probe) upon addition of E23Q to a duplex containing 2-Ap opposite the 3′-T of the TT dimer, while the converse was true for a duplex with 2-Ap opposite the 5′-T of the dimer. The specificity of E23Q for inducing base flipping solely in damaged DNA was established by a control experiment in which no fluorescence enhancements were observed for a duplex containing 2-Ap opposite an undamaged T (*109*). Thus, data regarding the TT dimer-induced endo- and exonuclease activity of T4 endo V and gp43 exo$^+$ obtained from experiments involving 2-AP and 5-PhITP, respectively, provided insight into enzyme−lesion interactions involved in these processes.

DNA ALKYLATION ADDUCTS

Despite the typically low abundance and cellular repair of bulky DNA alkylation adducts, they can severely impact DNA structure and function, resulting in dramatic consequences such as mutation and apoptosis. Representative alkylation pathways are shown in Scheme 3. Nitrosamines are important DNA alkylators due to human exposures from diet, drinking water, and occupational hazards, and their link to carcinogenesis. Among the earliest characterizations of DNA lesion formation is methylation by *N*-nitrosodimethylamine at the 3- and 1-positions of A and the 3-, 7-, and O^6-positions of G (*113*, *114*). Other nitrosamine alkylating agents include metabolites of nicotine, such as α-hyroxylated NNK, which breaks down to generate the methanediazonium ion that subsequently methylates the 7- and O^6-positions of G and O^4-position of T (*115*). NNK may also be metabolized to produce pyridyloxobutyl (POB) adducts with the 7-, N^2-, and O^6-positions of G and the O^2-position of T and C (*116*). Benzylation of purine bases, at the 3-position of adenine and the 7-, N^2-, N^6-, and O^6-positions of G, by carcinogens such as hydroxylated *N*-nitrosobenzylmethylamine (NBzMA) (*117*) or *N*-nitroso-*N*-benzylurea (BzNU), also occurs (*118*). Of these, O^6-alkyl-G adducts have been paid particular attention because of their high mutagenicity (*119*−*121*).

Guengerich and co-workers have extensively evaluated O^6-alkyl-G lesion bypass by both replicative polymerases and Y-family polymerases, which are known to perform translesion bypass. They showed that human replicative polymerase δ, human Y-family polymerases η and κ, and model replicative polymerase HIV-RT all have similar efficiencies of incorporation of C and T opposite both O^6-Me- and O^6-Bn-G, while human Y-family polymerase 1 and model replicative *E. coli* polymerase T7$^-$ preferentially incorporate T opposite O^6-Me- and O^6-Bn-G (*122*, *123*). The model Y-family polymerase, *Sulfolobus solfataricus* DNA polymerase Dpo4, was shown to preferentially incorporate C opposite O^6-Me-G and O^6-Bn-G, at rates 14- and 62-fold slower, respectively, than the rate of incorporation of C opposite unmodified G (*124*, *125*). Crystal structures of ternary Dpo4 complexes revealed O^6-Me- and O^6-Bn-G in a "wobble pairs" opposite incoming dCTP, leading to the suggestion that the open, flexible active site of Dpo4 accommodates the wobble pair, favoring incorporation of C, while more rigid replicative polymerases enclose O^6-alkyl-G:T mispairs and force a greater fraction of T incorporation (*124*, *125*). While it is difficult to know which polymerases were responsible for translesion bypass in cells, it has been shown that in both human cells and *E. coli*, T is preferentially inserted opposite O^6-alkyl-G adducts during translesion DNA synthesis, leading to G to A transitions (*126*).

The mutagencity of O^6-alkyl-G adducts in cells also depends on their ability to be repaired by O^6-alkyl-G-DNA alkyltransferases (AGTs), as reviewed by Pegg et al. (*119*). The timing of repair by AGTs, which function by directly transferring the alkyl group to a reactive cysteine within the protein (*127*), may be crucial to whether the adduct persists, leading to mutation. In their excellent review of DNA alkylation damage, He et al. noted that while human AGT (hAGT) can repair O^6-alkyl-G paired opposite T, it was unclear whether *E. coli* AGT (C-Ada)

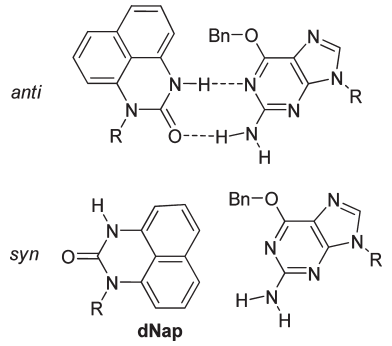


FIGURE 5: *anti*-dNap:O^6-Bn-G and *syn*-dNap:O^6-Bn-G base pairing. R denotes deoxyribose or the DNA backbone.

could perform the same function, though it was known to remove alkyl lesions when the G adduct was paired across from C (*128*− *130*).

O^6-Alkyl-G Adduct Probe. With the aims of understanding the occurrence, properties, and biological impacts of O^6-alkyl-G adducts in DNA and investigating the molecular requirements of a nucleotide partner for these important adducts, Gong and Sturla have conducted studies using O^6-Bn-G as a representative adduct (*131*). A diaminonaphthyl-derived adduct-specific nucleotide probe [dNap (Figure 5)] was designed to form a stable pair with O^6-Bn-G, and the specificity of this probe:adduct pair was evaluated by comparing the melting temperatures of 15-mer DNA duplex containing O^6-Bn-G or dNap paired opposite each other or natural bases. A 15-mer duplex with the O^6-Bn-G:dNap pair in the place of X:Y in the sequences 5′-TTGTCGGTA-TAXCGG-3′ and 5′-CCGYTATACCGACAA-3′ was more thermally stable than any pairing of O^6-Bn-G opposite a natural base by 5.6 °C on average, as well as more thermally stable than any dNap:natural nucleoside pair by an average of 5.5 °C.

It was hypothesized that the O^6-Bn-G:dNap pair is stabilized by a combination of $\pi-\pi$ stacking and hydrophobic interactions with the benzyl portion of the adduct, while the urea moiety provides points of contact for hydrogen bonding with the guanosine 1- and N^2-hydrogens. Although NOESY and X-ray analysis showed that the free nucleoside possesses a *syn* glycosidic torsion angle (in which the urea hydrogen bonding moieties are oriented over the ribose subunit), as opposed to the *anti* conformation that would allow for the hypothesized hydrogen bonding and $\pi-\pi$ stacking interactions, energy barriers between nucleoside conformations are usually low (*132*). Further, conformation depends on the chemical environment, be it free solution or solid state, in a mixture with other nucleosides, or in duplex DNA (*133*−*135*).

While the O^6-Bn-G:dNap pair is the first example of a stable adduct:probe pair, limitations in the stability and specificity of the pair underscore the need for further modifications to this probe. For example, a dNap:dNap self-pair also had a thermal stability similar to that of the G:C duplex, which is sometimes the case for hydrophobic nucleobase surrogates (*136*, *137*). Generally, the realization of synthetic nucleotides that pair with bulky adducts is at an early stage compared with studies involving the detection and evaluation of abasic sites and TT dimers. Thus, fundamental information regarding their physical interactions in the DNA duplex or compatibility with biochemically mediated processes, such as synthesis and repair, is lacking. This situation also holds true for probes under investigation for the key DNA oxidative lesion, 8-oxo-G, discussed in the next section.

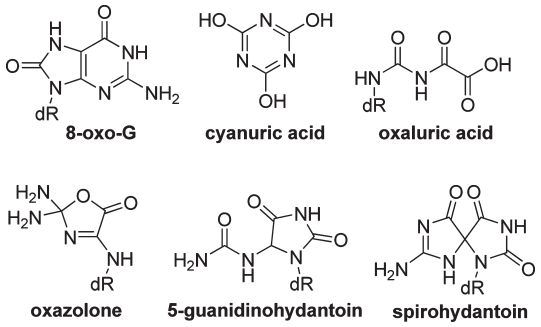


FIGURE 6: 8-Oxo-G and other oxidation products. dR is deoxyribose.

OXIDATIVE DAMAGE

Cellular reactive oxygen species, such as hydroxyl and superoxide radicals, react with DNA and induce single-strand breaks, DNA−protein cross-links, abasic sites, and base-oxidized lesions. In the case of oxidation-induced lesions, there are ∼50 known adducts (*138*) and 8-oxoguanosine [8-oxo-G (Figure 6)] is considered the most abundant (*139*). The oxidation potential of G in DNA is 1.3 V; upon oxidation to 8-oxo-G, the oxidation potential is lowered to 0.7 V, making this species more reactive toward oxidation compared to other bases and prone to oxidation under physiological conditions (*140*, *141*). Thus, further oxidation and other chemical transformations of 8-oxo-G yield secondary DNA adducts. For example, peroxynitrite, an endogeneous product of the reaction of nitric oxide and superoxide, oxidizes 8-oxo-dG to yield several mutagenic compounds such as cyanuric acid, oxaluric acid, and oxazolone (Figure 6) (*142*−*146*). Furthermore, 5-guanidinohydantoin and 2-imino-5,5′-spirohydantoin (Figure 6), generated from one-electron oxidation of 8-oxo-G or direct oxidation or photooxidation of G, are potentially mutagenic, leading to G → T and G → C transversion mutations in *E. coli*-based mutagenesis assays (*147*−*152*). If unrepaired before replication, 8-oxo-G itself can lead to G → T transversion mutations, a common somatic mutation in human carcinomas (*153*, *154*). Thus, 8-oxo-G is considered to be important in human carcinogenesis and has emerged as a biomarker for cellular oxidative damage (*138*, *155*, *156*).

8-Oxo-G Probes. There is extensive literature concerning methods for detecting 8-oxo-G and related oxidation adducts, including LC−MS, accelerator mass spectrometry, ^{32}P postlabeling, and immunochemical assays (*157*−*163*). These approaches can be extremely sensitive and specific; however, because they are generally sample-destructive and high background oxidation can be quite problematic during sample preparation, there is a continued need for in situ detection strategies involving 8-oxo-G-specific molecular probes.

With a goal of developing an 8-oxo-G-specific nucleotide probe, Sasaki and co-workers have reported cytosine analogues with the potential to preferentially bind 8-oxo-G over G (*164*). The structure of cbz-8-oxo-G-clamp **11** (Figure 7) was optimized through testing analogues with various terminal substituents, and its selectivity for binding 8-oxo-G was shown by ^{1}H NMR to result from multiple hydrogen bonds between the adduct and probe. Tricyclic probe **11** has an emission maximum at 450 nm upon excitation at 365 nm, which permits the binding characteristics of the probe to be detected by fluorescence changes. Binding studies were conducted by titrating silyl-protected nucleosides into buffered chloroform solutions of the probe. The emission intensity of **11** was drastically decreased when 8-oxo-G was

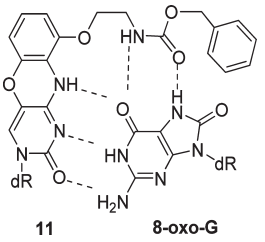


FIGURE 7: Proposed **11**:8-oxo-G complex. dR is deoxyribose.

added, while G caused no significant quenching. Under more physiologically relevant aqueous conditions, adding 8-oxo-G to a solution of **11** in water (solubilized by the detergent Triton X-100) resulted in fluorescence quenching patterns similar to those observed in chloroform.

Despite the favorable binding properties of free **11** with 8-oxo-G, mixed results were obtained when the system was evaluated in an oligonucleotide context (*165*). Thermal denaturation data indicated that the stability of a duplex containing G clamp **11** opposite 8-oxo-G was, on average, 2.5 °C lower than that of a duplex with **11** opposite G. This result led to the conclusion that the steric hindrance from the benzyloxycarbonyl group of the probe precluded it from forming the desired hydrogen bonding contacts in the context of duplex DNA. However, the fluorescence quenching of the probe in a duplex opposite 8-oxo-G was greater than that of the probe across from unaltered nucleotides, although some quenching was observed opposite G. Again, steric bulk of the probe was postulated to be the cause of the diminished ability of 8-oxo-G to quench the fluorescence of the probe relative to quenching by G. Therefore, derivatives of **11** with alternative terminal groups are being investigated to improve selectivity and efficacy while minimizing detrimental steric interactions in the oligonucleotide system.

Recently, Tor and co-workers reported the application of 5-furanyl deoxycytosine **6b** (Figure 3), the deoxyribonucleotide version of their previously reported abasic site probe **6a**, as an emissive probe that distinguishes among G, 8-oxo-G, and T in duplex DNA (*166*). Minimal disruptions to thermal stability (< 1 °C) occurred when the probe was placed opposite G, 8-oxo-G, or T in 13-mer oligonucleotide duplexes, relative to similar duplexes containing C instead of the probe. However, notable differences in the fluorescence intensities of duplexes containing the probe were observed around 440 nm. The emission intensity of the duplex with the probe:8-oxo-G pair was quenched 2-fold relative to the duplex with the probe:G pair, while the emission intensity of the duplex containing the probe:T pair was 4-fold higher than the intensity of that containing the probe:G pair. The quenching of the probe opposite 8-oxo-G relative to G was ascribed to 8-oxo-G's lower redox potential, which gives the probe higher excited-state quenching ability. The drastic increase in the emission intensity of the probe:T pair was attributed to the probe's likely extrahelical placement within the duplex, where it would be exposed to a more polar environment (thermal denaturation and emission studies were performed in aqueous buffer). There was speculation that the 5-furanyl deoxycytosine probe could be used in future nondestructive real-time fluorescence-based methods for monitoring 8-oxo-G formation in vitro.

CONCLUSION

DNA lesions, such as abasic sites, TT dimers, bulky alkylation adducts, and 8-oxo-G, play important biological roles due to disruptions in critical cellular processes. An emerging experimental strategy for elucidating mechanistic details regarding how these lesions physically interact with and affect biochemical processing of nucleic acids involves the use of chemical probes. Lesion-specific probes are reviewed here, organized on the basis of target lesions, and emphasizing chemical concepts behind the development of the probes as well as their application and pitfalls in biochemical studies. Much attention has been paid to non-natural nucleotide probes, especially for abasic sites, because of information that can be gained as these are incorporated opposite lesions in a polymerase-mediated manner. The mechanism of translesion DNA synthesis opposite TT dimers was discussed; in light of studies using non-natural nucleotide probes, it appears that TT dimers act either as transient abasic sites or as templates, depending on the polymerase. Finally, recent reports involve the design and evaluation of molecular probes for the oxidative lesion 8-oxo-G and the representative bulky alkylation adduct O^6-Bn-G; these examples represent new classes of molecular probes for nucleobase adducts but require further structure optimization and a better understanding of the physical basis of interactions with DNA and proteins before they can be effectively utilized for elucidating biochemical mechanisms or for novel damage detection strategies.

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