Base Excision Repair Intermediates as Topoisomerase II Poisons*

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Abasic sites are the most commonly formed DNA lesions in the cell and are produced by numerous endogenous and environmental insults. In addition, they are generated by the initial step of base excision repair (BER). When located within a topoisomerase II DNA cleavage site, “intact” abasic sites act as topoisomerase II poisons and dramatically stimulate enzyme-mediated DNA scission. However, most abasic sites in cells are not intact. They exist as processed BER intermediates that contain DNA strand breaks proximal to the damaged residue. When strand breaks are located within a topoisomerase II DNA cleavage site, they create suicide substrates that are not religated readily by the enzyme and can generate permanent double-stranded DNA breaks. Consequently, the effects of processed abasic sites on DNA cleavage by human topoisomerase IIα were examined. Unlike substrates with intact abasic sites, model BER intermediates containing 5′- or 3′-nicked abasic sites or deoxyribosephosphate flaps were suicide substrates. Furthermore, abasic sites flanked by 5′- or 3′-nicks were potent topoisomerase II poisons, enhancing DNA scission ~10-fold compared with corresponding nicked oligonucleotides that lacked abasic sites. These findings suggest that topoisomerase II is able to convert processed BER intermediates to permanent double-stranded DNA breaks.

Topoisomerase II is an indispensable enzyme that regulates nucleic acid topology in vivo by passing an intact double-stranded DNA helix through a second segment of DNA. To create the DNA gate that allows this essential strand passage reaction to occur, the enzyme generates a transient double-stranded break in the second DNA segment (1–4).

To maintain the integrity of the genome during the strand passage event, topoisomerase II forms covalent bonds with the 5′-termini of the DNA gate (3, 5–9). This covalent topoisomerase II-cleaved DNA complex is referred to as the cleavage complex (10). Under normal circumstances, cleavage complexes are fleeting intermediates in the catalytic cycle of topoisomerase II and are tolerated by the cell (1, 8). However, conditions that increase the physiological concentration of cleavage complexes often lead to the formation of permanent double-stranded DNA breaks in the genome. When these breaks accumulate beyond threshold levels, they induce recombination events that lead to chromosomal translocations and in some cases trigger cell death pathways (8, 11–14). Thus, although the ability to cleave and religate the genetic material is critical for the physiological functions of topoisomerase II, the generation of double-stranded DNA breaks by the enzyme has potentially deleterious consequences.

As a result of its intrinsically lethal nature, topoisomerase II has been utilized as a target for a number of highly successful anticancer agents (8, 14–18). These drugs, which include etoposide, doxorubicin, and mitoxantrone, all work by dramatically increasing the cellular concentration of topoisomerase II-DNA cleavage complexes (8, 14–18). Because they convert an essential enzyme to a potent cellular toxin that generates breaks in the genome, these drugs are referred to as topoisomerase II poisons (19). This nomenclature is used to distinguish cleavage-enhancing agents from topoisomerase II catalytic inhibitors, which kill cells by robbing them of the critical catalytic functions of the enzyme (14, 15, 20).

It has been hypothesized that anticancer drugs are able to exploit the DNA cleavage activity of topoisomerase II because they mimic the actions of endogenous topoisomerase II poisons (14, 15, 21–23). In support of this theory, several DNA lesions have been shown to be position-specific poisons of the type II enzyme (21–26). For example, when located within the 4-base stagger that separates the two phosphodiester bonds cleaved by the enzyme, abasic sites often stimulate topoisomerase II-mediated cleavage 10–20-fold (22). Some DNA adducts, such as 1,N6-ethenoadenine, enhance scission to a similar extent. In contrast, other adducts such as 8-oxoguanine and N7-methyladenine are weak topoisomerase II poisons and have comparatively small effects on enzyme-mediated DNA cleavage (26).

It is notable that most of these DNA adducts are repaired in the cell by base excision repair (BER) (27–30). The first step of all BER pathways is the removal of the damaged base by a DNA glycosylase, which converts the lesion to an abasic site (27, 28, 31). Thus, if a DNA adduct (even one with relatively little effect on topoisomerase II DNA cleavage) is located within the 4-base cleavage stagger, it has the potential to be converted to a strong topoisomerase II poison by the initiation of BER.

After the creation of abasic sites by the action of the glycosylase, a series of BER intermediates are generated that contain DNA nicks or flaps proximal to the site of the original lesion (32–34). Because these intermediates contain strand breaks, they may create substrates that are more lethal for topoisomerase II than the intact abasic site. Indeed, when DNA strand breaks are located between the scissile bonds of a topoisomerase II cleavage site, they often lead to the production of cleaved molecules that disassociate from the active site of the enzyme (35). Because these suicide substrates yield products that cannot be religated readily by topoisomerase II, cleaved

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1 The abbreviations used are: BER, base excision repair; dRP, deoxyribosephosphate; AP, apurinic/apyrimidinic.
molecules accumulate over time. Therefore, BER has the potential to create DNA substrates that can be converted directly to permanent double-stranded DNA breaks by topoisomerase II. If so, processed BER intermediates could be more dangerous to cells than the original damage.

To explore the effects of processed abasic sites on topoisomerase II activity, the ability of human topoisomerase IIα to cleave a series of oligonucleotide substrates that model processed short-patch (i.e. simple) BER intermediates was examined. Results indicate that when the original lesion was located within the 4-base stagger of a topoisomerase II DNA cleavage site, BER intermediates that contained nicks or DNA flaps acted as suicide substrates for topoisomerase IIα. Furthermore, 5'- and 3'-nicked intermediates generated by BER were potent topoisomerase II poisons. These findings support a model in which processed DNA lesions may adversely affect cells through their interactions with topoisomerase II.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human topoisomerase IIα was expressed and purified from *Saccharomyces cerevisiae* as described previously (36, 37). T4 pyrimidine dimer glycosylase/AP lyase was a generous gift from Dr. R. Stephen Lloyd (University of Texas, Galveston, TX); T4 polynucleotide kinase was obtained from New England Biolabs Inc.; [γ-32P]ATP (≈7000 Ci/mmol) was from ICN; *Escherichia coli* uracil-DNA glycosylase was from U. S. Biochemical Corp.; dSpacer (tetrahydrofuran abasic site analog) and 2'-deoxyuridine phosphorimidases were from Glen Research Corp. All other chemicals were analytical reagent grade.

**Preparation of Oligonucleotides**—A 40-base oligonucleotide corresponding to residues 87–126 of pBR322 (5,38) and its complementary oligonucleotide were synthesized. The sequences of the top and bottom strands were 5'-ACGTTGGCGGAGTGAAGCTGGTGGCATTTGAGGTG-3' and 5'-TGAAAACTCAAACTGGTGGCTC-3', respectively. A second 40-base single-stranded oligonucleotide corresponding to residues 1072–1111 of the *MLL* oncogene (36,39) and its complementary oligonucleotide also were synthesized. The sequences of the top and bottom strands were 5'-GGCTGGGTCGACCAAGC-3' and 5'-AATTTCCTTTTGCAGACATGG-3', respectively. Points of topoisomerase II-mediated cleavage in both sequences were denoted by down arrows. Single-stranded oligonucleotides containing an abasic site analogue or a deoxyuridine residue were prepared in a similar manner utilizing corresponding phosphorimidases. Single-stranded oligonucleotides were labeled with [γ-32P]phosphate on their 5'-termini with T4 polynucleotide kinase, purified by electrophoresis in a 7 M urea, 14% polyacrylamide gel, visualized by UV shadowing, and isolated using the Qiagen extraction protocol.

**Preparation of Substrates Containing a 5'-Nick or a dRP-Flap**—Substrates that contained 5'-nicks or dRP-flaps were prepared synthetically by annealing three DNA segments, a complete 5'-nicked subsequence (with a nick or flap located at the desired position). The 5'-nick oligonucleotide was annealed as described above using a 1:1.25 ratio of full-length 40-mer to short oligonucleotides. The dRP-flap intermediates were annealed sequentially. The full-length bottom 40-mer and the short top oligonucleotide that did not contain the flap were annealed by incubating at 70 °C for 10 min and cooling below 55 °C. The third oligonucleotide, which contained the DNA flap, was added, and the annealing mixture was incubated at 55 °C for 10 min and cooled to 25 °C.

**Preparation of Substrates Containing a 3'-Nick**—The 3'-nicked substrate was prepared enzymatically. The top strand of the 40-mer oligonucleotide described above was synthesized with a uracil at the +2 position of the incorporated topoisomerase II cleavage site. This oligonucleotide was annealed to its complementary bottom strand as described under Preparation of Oligonucleotides. The uracil was replaced by treating 50 nm double-strand 0.2 units of uracil-DNA glycosylase in a reaction volume of 15 μl at 37 °C for 30 min. The resulting abasic oligonucleotide was treated with 1.5 ng/μl T4 pyrimidine dimer glycosylase for 30 min at 37 °C in 25 mM NaH₂PO₄, pH 6.8, 1 μl EDTA, 100 mM NaCl, and 100 μg/ml bovine serum albumin in a 20 μl reaction volume (40,41). The DNA was purified by passage through a 1 ml Sephadex G-25 column.

**RESULTS**

DNA lesions that cause relatively small perturbations in the structure of the genetic material such as base loss, oxidation, or alklylation generally are repaired by BER (30–34,42,43). Two primary pathways are used: short-patch (i.e. simple) BER (Fig. 1), in which only the damaged residue is excised and replaced, or long-patch BER, in which 2–13 residues are excised (32–34,44–47). Because short-patch BER is the primary pathway in mammalian cells (accounting for ~80% of BER) (31,32,44), and the initial intermediates are the same in both pathways, the present study focused on the potential of short-patch BER intermediates to act as topoisomerase II poisons.

As depicted in Fig. 1, short-patch BER is a bifurcated pathway. The fork that is chosen is determined by the specific DNA glycosylase that converts the damaged base to an abasic site.
The resulting gap is filled by DNA polymerase associated AP lyase activity, the glycosylase (step 2). This reaction is followed by DNA polymerase β, displacing the abasic site and leaving a dRP-flap (step 3). Finally, the flap is removed by the dRP lyase activity of DNA polymerase β (step 4), and the nick is sealed by DNA ligase I or the ligase III/XRCC1 complex (step 5) (33, 49).

If the glycosylase is a “bifunctional glycosylase” and has an associated AP lyase activity, the right fork predominates. In case of base loss, the enzyme cleaves the bond that bridges the scissile bonds. Residues numbered 1 to 4 are located between the scissile bonds.

Cleavage of an Oligonucleotide Containing an Abasic Site by Human Topoisomerase IIα—Previous studies demonstrated that topoisomerase II-mediated DNA scission is enhanced when an abasic site is included within the 4-base stagger of a cleavage site. Conversely, when an abasic site is located immediately outside the points of cleavage, scission often is depressed (22, 25, 36).

The initial model BER intermediate examined in the present study contained an abasic (apurinic) lesion at the +2 position of a well characterized topoisomerase II cleavage site (which is derived from pBR322 and shown in Fig. 2). This lesion was chosen as a control for two reasons. First, consistent with earlier work that examined this sequence (22), the +2 abasic site is recognized as a strong topoisomerase II poison. Inclusion of this lesion increased levels of cleavage nearly 9-fold (from 0.2 to 1.7%) compared with wild-type and stimulated enzyme-mediated DNA cleavage to a greater extent than abasic sites located at any other position (Fig. 2, right panel). Second, by situating the original lesion at the +2 position, the nicks and flaps in model BER substrates were located between the scissile bonds.

A time course for the cleavage of a +2 abasic substrate by human topoisomerase IIα is shown in Fig. 2 (left panel). As observed previously (60), scission of both the abasic and wild-type substrates reached a stable equilibrium within 5 min. Formation of this equilibrium confirms that the enzyme does not release cleaved substrates. Rather, it remains covalently attached to the oligonucleotide throughout the scission event and is able to religate the cleaved DNA molecule.

Cleavage of an Abasic Substrate Containing a 5'-Nick—It has been estimated that some human tissues contain as many as 200,000 abasic sites per cell even in the absence of environmental insults (50, 61). After base loss (either by nonenzymatic hydrolysis or by the actions of a monofunctional glycosylase), the predominant BER pathway that is triggered enlists an AP endonuclease for initial processing. The endonuclease generates a nick immediately 5’ to the abasic site (33, 49). These 5'-nicked intermediates represent the most prevalent form of abasic sites in human tissues (61). Because of the physiological abundance of abasic sites with 5'-nicks, the ability of human topoisomerase IIα to cleave these processed BER intermediates was determined (Fig. 3).

A time course for cleavage of a substrate that contained a 5'-abasic site at the +2 position as well as the corresponding nicked substrate that lacked an abasic site is shown. In contrast to results obtained with intact abasic oligonucleotides, levels of cleavage for both nicked substrates increased with time and did not reach equilibrium within the 20-min assay (left panel). This finding strongly suggests that these oligonucleotides act as suicide substrates that are not readily religated readily by topoisomerase IIα.

Cleavage products generated in reactions that contained the 5'-abasic substrate accumulated at a much higher rate than products from reactions that used nicked oligonucleotides lacking an abasic site (Fig. 3, left panel). Thus, even when flanked by a 5'-strand break, abasic sites are recognized as topoisomerase II poisons. To further address this recognition, the ability of human topoisomerase IIα to cleave a series of substrates that contained 5'-abasic sites at different sequence positions was examined (Fig. 3, right panel). The pattern of DNA cleavage enhancement for the nicked abasic sites was similar to that observed for the corresponding non-nicked abasic substrates (compare with Fig. 2, right panel). However, when nicked substrates were employed, cleavage products accumulated with time (data not shown), and levels of cleavage significantly exceeded those observed with non-nicked oligonucleotides.

To ensure that results obtained with 5'-abasic abasic sites were not unique to the DNA sequence utilized for the above experiments, two additional oligonucleotide substrates containing well-characterized cleavage sites for human topoisomerase IIα were examined. Both were derived from the breakpoint cluster region of the MLL oncogene at chromosome.

FIG. 2. Cleavage of non-nicked DNA substrates by human topoisomerase IIα. Abasic sites were incorporated into the top strand of a 40-base pair sequence at the indicated positions. Arrows represent the points of topoisomerase II-mediated cleavage, with open arrows and asterisks denoting the 32P-labeled DNA strand. Time courses for the appearance of DNA breaks are shown for the wild-type (WT) (C); also see the inset and +2 abasic (+2 AP; (O)) substrates (left panel). The inset shows an enlargement of the wild-type data for detail. Cleavage of abasic oligonucleotides by topoisomerase IIα at 20 min are shown in the right panel. Levels of cleavage were calculated relative to that of a sequence with no abasic lesions (None, set to 1). Data represent the averages of ≥3 independent experiments, and S.E. are indicated by error bars.
band 11q23, with the points of topoisomerase II-mediated DNA cleavage on the individual substrates located at position 1067 or 1087 (36, 39). Similar results were obtained for the two substrates; data for the 1087 site are shown in Fig. 4. Overall levels of DNA cleavage with the MLL substrates were lower than observed with the pBR322-derived sequence. However, the presence of a 5'-nick abasic site at the +2 position of the 1087 MLL oncogene site dramatically stimulated DNA scission mediated by topoisomerase IIα. Furthermore, levels of cleavage increased with time, indicating that the nicked abasic substrate was a suicide substrate for the human enzyme. Thus, 5'-nick abasic sites appear to have a similar effect on topoisomerase II-mediated DNA cleavage even in different sequence contexts.

Cleavage of a SubstrateContaining a dRP-Flap—After the creation of a 5'-nick abasic site by an AP endonuclease, the next BER intermediate that is generated contains a dRP-flap (formed by the displacement of the abasic site) (33, 49). As seen in Fig. 5, topoisomerase IIα cleaved the dRP-flap oligonucleotide, and cleavage products increased over time. However, levels of scission observed for the flap-containing oligonucleotide were no greater than those obtained with nicked substrates that lacked abasic sites (see Figs. 3 and 7). These results suggest that the DNA strand break that accompanies the flap converts the oligonucleotide to a suicide substrate but that the dRP-flap itself does not enhance topoisomerase II-mediated DNA scission (i.e. the flap does not act as a topoisomerase II poison).

To further explore the effects of flaps on topoisomerase II-mediated DNA cleavage, the ability of the human enzyme to cut substrates with flaps comprised of intact nucleotides (i.e. the base as well as the deoxyribose moiety) was characterized. Two substrates were employed that incorporated flaps immediately downstream from the +2 position. The first contained a guanidine flap (guanidine is the residue located at the +2 position of the wild-type oligonucleotide). One caveat regarding this substrate is that the guanidine has the potential to reanneal to the duplex portion of the oligonucleotide, displace the +2 residue, and transpose the flap to a location immediately upstream of the +2 position. To overcome this problem, a second substrate that contained a mismatched uridine flap also was examined.
A basic site, and a nick was generated (UDG). Uracil DNA glycosylase was used to create an abasic site (Fig. 1A) and an oligonucleotide containing the corresponding nick but no abasic site (Fig. 1B). Data represent the averages of ≤2 independent experiments, and S.E. are indicated by error bars.

FIG. 6 . Preparation of 3′-nicked abasic oligonucleotide substrates. A 3′-nicked substrate was generated in the top strand of a 40-base pair sequence that contained a uracil at the +2 position (−UDG/−T4 pdg). Uracil DNA glycosylase was used to create an abasic site, and a nick was generated 3′ to the abasic site via a β-elimination reaction using T4 pyrimidine dimer glycosylase/AP lyase (1.5 ng/μl T4 pdg) (40, 41). Excess catalyst (7.5 ng/μl T4 pdg), base/heat treatment (NaOH/heat), or piperidine treatment (piperidine) generated primarily a δ-elimination product (62). 21-, 22-, and 23-mer DNA markers also are shown.

Time courses for cleavage of the two nucleotide-flap substrates are shown in Fig. 5. DNA scission increased over time, and levels of cleavage were somewhat higher than those obtained with the dRP-flap oligonucleotide. These results provide further evidence that dRP-flaps located within the 4-base cleavage overhang create suicide substrates but do not themselves act as topoisomerase II poisons.

Cleavage of an Abasic Substrate Containing a 3′-Nick—If damaged bases are removed by the actions of a bifunctional glycosylase, the associated AP lyase activity generates a nick immediately 3′ to the resulting abasic site (see Fig. 1) (32–34). Generally, lyases cleave DNA chains in cells via a β-elimination reaction. This action fragments the deoxyribose moiety and generates a DNA species that contains an abasic site (see Fig. 1A) and an oligonucleotide containing the corresponding nick but no abasic site (Fig. 1B). Data represent the averages of ≤2 independent experiments, and S.E. are indicated by error bars.

To generate a model BER substrate that contained the physiologically relevant β-elimination product, a three-step protocol was used. First, an oligonucleotide was synthesized that contained a uracil in place of the guanine at the +2 position of the top strand of the topoisomerase II-DNA cleavage site. Second, the uracil residue was converted to a natural abasic site by treatment with uracil-DNA glycosylase. Third, a nick 3′ to the abasic site was generated by the lyase activity of T4 pyrimidine dimer glycosylase/AP lyase (40, 41). Reaction products are shown in Fig. 6. The resulting substrate contained >85% of the desired β-elimination product.

The interaction of human topoisomerase IIα with a substrate that contained a 3′-nicked abasic site at the +2 position is shown in Fig. 7. The enzyme cleaved the model BER intermediate in a time-dependent fashion. Levels of scission were not as high as those observed with substrates that contained 5′-nicked abasic sites (compare with Fig. 3, left panel). However, they were considerably higher than those obtained for an oligonucleotide that contained a nick but lacked an abasic site. These results indicate that the presence of a 3′-nicked processed abasic site converts the oligonucleotide into both a suicide substrate and a topoisomerase II poison.

DISCUSSION

Abasic sites represent the most commonly formed DNA lesions in the cell (31, 42, 63, 64). They are produced by a variety of endogenous and environmental insults including spontaneous cleavage of the nucleotide glycosidic bond, oxidation, exposure to ionizing radiation, and chemical modification (31–34, 42, 43). In addition, abasic sites are generated by the initial step of BER pathways that are used to repair a broad spectrum of base lesions (27, 28, 30–32). Regardless of how they are formed, abasic residues ultimately are converted to their original nucleotides by BER.

Because base loss creates non-coding DNA lesions, abasic sites represent formidable obstacles to the preservation of genetic information (31, 64–66). Beyond the documented mutagenic properties of abasic sites, they also appear to be position-specific topoisomerase II poisons (see Fig. 8) (22, 25).

DNA strand breaks generated by topoisomerase II, even those formed in the presence of topoisomerase II poisons, are reversible (see Fig. 8) (8, 9, 67). However, when tracking systems (such as those associated with the replication or transcription machinery) attempt to pass through enzyme-DNA cleavage complexes, the transient topoisomerase II-bound nucleic acid gates are converted to permanent double-stranded breaks (68–70). These breaks induce recombination events, which can generate chromosomal aberrations and, in extreme cases, initiate cell death pathways (8, 11–13).

The effects of intact abasic sites on topoisomerase II-mediated DNA scission have been characterized in previous studies (21, 22, 25, 36). However, the majority of abasic sites exist in the cell in processed forms that contain DNA strand breaks proximal to the site of damage (61). Therefore, the ability of human topoisomerase IIα to cleave processed abasic sites that modeled BER intermediates was determined. Results indicate
Based on the results of the present study and other recent work (26), the positional poison model has been extended to include DNA adducts and repair processes as depicted in Fig. 8. Although not all forms of base damage enhance enzyme-mediated DNA cleavage, a variety of lesions that are generated by endogenous or environmental insults act as topoisomerase II poisons (21, 22, 24–26). Irrespective of their initial effects on the enzyme, many of these lesions are repaired by BER pathways (27–30). The first step of all of these pathways is the removal of the damaged base by a DNA glycosylase, which leads to the generation of an abasic site (27, 28, 30, 31). Thus, BER converts many divergent forms of base damage to a common topoisomerase II poison.

In the next step of BER, abasic sites are processed further by an AP endonuclease or a lyase. Either action generates an intermediate that contains a DNA strand break proximal to the site of the original damage (32–34, 48). Because DNA strand breaks located in the vicinity of topoisomerase II cleavage sites can generate suicide substrates for the enzyme (35), processed abasic sites could potentially be converted to permanent double-stranded breaks by topoisomerase II even in the absence of DNA tracking systems. Clearly, most DNA lesions that are processed by BER pathways are ultimately restored to their original bases. Furthermore, it has not yet been demonstrated that topoisomerase II can compete successfully with the BER machinery for the processed lesions. However, under conditions of duress, it is conceivable that interactions between processed abasic sites and topoisomerase II could contribute to an adverse cellular outcome. Further studies will be necessary to determine the extent to which topoisomerase II impacts the physiological processing of these BER intermediates.

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