Mutagenesis by an Antisense Oligonucleotide and Its Degradation Product

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The European Medicines Agency has expressed concern regarding (1) the potential for antisense oligonucleotide (ASO) therapeutics to induce sequence-specific mutation at genomic DNA and (2) the capability of ASO degradation products (nucleotide analogues) to incorporate into newly synthesized genomic DNA via DNA polymerase and cause mutation if base pairing occurs with reduced fidelity. Treating human lymphoblastoid cells with a biologically active antisense molecule induced sequence-specific mutation within genomic DNA over fourfold, in a system where RAD51 protein expression was induced. This finding has implications for ASO therapeutics with individuals with an induced DNA damage response, such as cancer patients. Furthermore, a phosphorothioate nucleotide analogue potently induced mutation at genomic DNA two orders of magnitude above control. This study shows that a biologically active ASO molecule can induce heritable sequence alterations, and if degraded, its respective analogue may incorporate into genomic DNA with mutagenic consequences.

Key Words: antisense oligonucleotides; targeted nucleotide exchange; mutation; nucleotide analogues; genomic DNA.

The potential for antisense oligonucleotide (ASO) to modulate protein expression by translational repression or RNase H-mediated degradation has application if a powerful therapeutic tool (Denli and Hannon, 2003; Zamore, 2001). However, recent studies demonstrating the potential for oligonucleotides to induce site-directed mutation within reporter genes has caused concern regarding the potential for oligonucleotide-based pharmaceuticals to induce heritable sequence alterations (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC50003149.pdf). In a process known as targeted nucleotide exchange (TNE), oligonucleotides have been reported to induce mutation mainly within engineered reporter constructs (Bonner and Kmiec, 2009; Dekker et al., 2003; Olsen et al., 2009). Oligonucleotides, typically over 45 nt in length, are designed to bind complementarily to the nontranscribed (sense) strand within duplex DNA. Oligonucleotides with a single-mismatched base, relative to its homologous sequence, are capable of directing mutation to the site of the mismatch. For example, a mutant green fluorescent protein construct carrying a single inactivating point mutation was reported to be corrected, to the wild-type sequence restoring fluorescence, using a 74-mer oligonucleotide with correction frequencies in the order of 2% (Bonner and Kmiec, 2009).

Furthermore, the potential for nucleotide analogues, released as oligonucleotide degradation products, to enter intracellular nucleotide pools has been questioned by the European Medicines Agency but genotoxicity was deemed unlikely (Bonner and Kmiec, 2009). Oligonucleotide analogues have been well established (Wutzler and Thust, 2001), but these analogues are not used in ASO construction. Significantly, perturbation of endogenous nucleotide pools even by excess canonical nucleosides or nucleotides can also have a mutagenic consequence (Mattano et al., 1990; Phear et al., 1987).

This study has addressed these concerns by examining the genotoxicity of an ASO entity and its putative degradation products.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides were DNA based and obtained from Sigma Genosys. Oligonucleotides were chemically modified to contain four terminal phosphorothioate linkages and reverse phase purified. Oligonucleotide sequences are as follows: AD3-hprtPM 5′-A*C*A*G*TCATAGGAATGGATA*T*A*T*C-3′ and control 5′-A*C*C*T*TGATGGCAAATAGGT*A*A*T*A-3′. The “*” indicates the position of the phosphorothioate linkage.

Cell Culture

Human lymphoblastoid TK6 cells were obtained from American type culture collection. TK6 cells were cultured in RPMI 1640 media supplemented with 10% vol/vol heat inactivated horse serum, t-glutamine (2mM), penicillin...
Induction of RAD51 Protein Expression in TK6 Cells Using Methyl Methanesulfonate

Exponentially growing TK6 cells were treated with methyl methanesulfonate (MMS; 0, 0.1, 0.2, or 0.5 µg/ml) for 24 h to induce RAD51 protein expression. Following treatment, TK6 cells were washed in culture media and maintained in exponential growth for up to 72 h. Aliquots of TK6 cells were removed at 2, 4, 8, 24, 48, and 72 h following treatment and washed in ice cold PBS for protein extraction and subsequent immunoblot analysis to determine RAD51 protein expression. The optimum concentration of MMS was used to pretreat TK6 cells the day before treatment with oligonucleotide.

Total Protein Extraction

Total cell protein content was extracted by suspending cell pellets in freshly made lysis buffer (1 µl Halt protease inhibitor + 99 µl of 150 mM NaCl, 0.1% vol/vol igepal, 1 mM Tris [pH 7.4], and 1 mM EDTA) on ice for 20 min. Samples were then centrifuged at 10,000 × g for 10 min at 4°C. Supernatants were collected into fresh eppendorf tubes and stored at −20°C. Protein content in samples was determined using the bicinchoninic acid assay according to manufacturer's protocol (ThermoScientific).

Immunoblot

Protein expression was determined by immunoblot. In brief, for ADAMTS3 and RAD51 protein expression, total protein (20 µg) was loaded per well. Samples were electrophoresed through 10% SDS–polyacrylamide gel and transferred on to a PVDF membrane. Membranes were stained with primary antibody overnight; for RAD51, 1:2000 mouse antihuman RAD51 antibody and for ADAMTS3, 1:200 rabbit antihuman ADAMTS3 antibody. Following primary antibody staining and several wash steps, blots were stained with horseradish peroxidase-conjugated secondary antibody and visualized. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression was employed as a loading control using primary rabbit antihuman GAPDH antibody (1:1000). Protein expression was determined using densitometry and normalized to GAPDH-loading control.

Hypoxanthine-Guanine Phosphoribosyltransferase and Thymidine Kinase Forward Mutation Assay

Treating TK6 cells with oligonucleotide. For treatment of TK6 cells with oligonucleotide, the desired concentration of oligonucleotide was mixed with siPORT NeoFX (Ambion) and allowed to stand to allow complex formation. Meanwhile, TK6 cells were counted and 3 × 10⁶ cells were aliquoted per treatment and mixed with the oligonucleotide/siPORT complex. Treatments were in three independent cultures. Transfection was allowed to occur for 4 h at 37°C, 5% CO₂. TK6 cells were then washed and suspended for culturing overnight. As a positive genotoxic control, TK6 cells were treated with ethyl methanesulfonate (EMS; 5 µg/ml). To measure the spontaneous mutant frequency at each locus, oligonucleotide transfection mixtures were replaced with culture media (background). Following treatment of TK6 cells with oligonucleotide, cells were sampled (24, 48, and 72 h) for protein extraction, to determine ADAMTS3 protein expression, by immunoblot, as a marker of AD3-hprtPM antisense activity.

Treating TK6 cells with nucleotides. For treatment of TK6 cells with nucleotide, 4 × 10⁶ TK6 cells were aliquoted per 75-cm² flask in a 5 ml volume. Serial dilutions of nucleotides were dissolved in cell culture media to a final 2x concentration. Cells (5 ml) were mixed with 2x nucleotide (5 ml). As a negative control, culture media (5 ml) replaced the nucleotide. EMS (5 µg/ml) was used as a positive control. Cells were exposed to test compounds for 24 h. Following treatment, cells were pelleted at 200 × g for 5 min and washed with culture media.

Determining cytotoxicity and the TK and HPRT mutant frequency. Following treatment of TK6 cells with oligonucleotide or nucleotide, cells were counted daily for 3 days to determine the relative suspension growth (RSG; a measure of cell death and proliferative ability following treatment (Clive et al., 1995; Clements, 2000). On the third day, an aliquot of TK6 cells was plated at 1.6 cells per well in 96-well plates to determine cloning efficiency and 20,000 cells per well in trithorouthidinium to determine the thymidine kinase (TK) mutant frequency. This cloning efficiency was used to correct the RSG to give the relative total growth (RTG) as a measure of cytotoxicity following treatment. The remaining cells were maintained for a further 4 days phenotype expression. Following this, the hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutant frequency was then determined by seeding 20,000 cells per well in 96-well plates in 6-thioguanine (TG), and cloning efficiency was determined by seeding 1.6 cells per well in the absence of 6-TG. Plates were incubated for 14 days minimum at 37°C, 5% CO₂ and then colonies were scored. Mutant clones were individually isolated and expanded in 6-well plates for 7–10 days for genomic DNA extraction and PCR amplification.

Genomic DNA Extraction

Genomic DNA was extracted from TK6 cells using the QIAamp DNA Blood mini kit (Qiagen, Crawley) according to manufacturer’s instructions. In brief, pelleted TK6 cells were suspended in PBS containing proteinase K and lysis buffer AL. Samples were mixed by pulse vortexing and incubated at 56°C for 10 min. Lysates were mixed with ethanol and added to a spin column followed by centrifugation. Columns were washed with wash buffer AWI and then AW2. DNA was eluted out of the spin column using DNase/RNase-free water. DNA was quantified using the NanoDrop 1000 spectrophotometer.

PCR and DNA Sequencing

PCR reactions were performed to amplify a 1-kb region of exon 3 in the HPRT locus that enclosed the AD3-hprtPM target sequence. A typical reaction was in a 50-μl reaction volume containing genomic DNA (300 ng–1 μg) from isolated HPRT mutant clones, 200nM of each forward (5'-AGGGCAAAAGGATGTGTTACG-3') and reverse (5'-AGTGGTCTTCGTGCGACTT-3') primer, dNTPs (200 μM), Tfi polymerase (5 units), and 1× Tfi PCR buffer supplemented with MgCl₂ (1.5 mM). Amplification was performed using a Peltier thermal cycler as follows: initial denaturation step at 94°C for 4 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s, and polymerization at 72°C for 1 min. The final polymerization step was extended to 7 min. Samples were then stored at 4°C until time of analysis. Aliquots of PCR products were resolved by electrophoresis through a 1.2% agarose gel containing ethidium bromide (0.5 µg/ml) and visualized under ultraviolet (UV). PCR products were extracted and purified for DNA sequencing using the PureLink Quick gel extraction kit (Invitrogen, Paisley, U.K.), according to manufacturers protocol. Purified PCR products were diluted to 10 ng/μl and sent for sequencing by Gene Service Ltd. (Source Biosciences).

Mbo I restriction enzyme digestion of PCR products. PCR products from HPRT mutant clones were subject to Mbo I restriction enzyme digestion to inform the integrity of the 5'-GATC-3' recognition sequence. PCR products containing wild-type sequence result in cleavage into two fragments: 474 and 544 bp. Mutation within the 5'-GATC-3' sequence results in resistance to Mbo I restriction enzyme digestion. Typically, PCR products were restriction enzyme digested with Mbo I restriction enzyme (4 units) at 1 h at 37°C. Digested PCR products were resolved on 1.2% wt/vol agarose gels containing ethidium bromide (0.5 µg/ml) and visualized under UV.

 Primer Extension Assay

This method was adapted from Lacenere et al., 2006. Primed template, with an overhang of five thymidine nucleotides, was made by annealing template strand (5'-TTTTTTCCCCAAACACAAAAAGGGAGACACA-3') with the complementary primer strand (5'-6-carboxyfluorescein-TGTGCTGGGCGGTGGTTGTGGGG-3'), which is labelled with a 5' fluorophore in annealing buffer (75mM Tris pH 7.2 and 75mM NaCl). A typical reaction mixture contained

(100 units/ml), and streptomycin (100 µg/ml). All cell culture reagents were obtained from Invitrogen, unless otherwise stated.
48 h and was further reduced by 45–70% by 72 h. Protein expression by 20–30% with 5 and 10 μM AD3-hprtPM induced a dose- and time-dependent knockdown of ADAMTS3 protein expression, whereas the HPRT locus served as a control oligonucleotide and ~4.4-fold with 10 μM AD3-hprtPM would act as an antisense molecule in modulating ADAMTS3 protein expression, whereas the HPRT locus served as a genomic reporter of sequence-independent genotoxicity following oligonucleotide treatments.

**RESULTS**

**Design of an ASO**

In the first instance, an ASO was designed to bind complementarily to the mRNA of the ADAMTS3 gene as well as contain sequence homology to the HPRT locus except a single-mismatched base (AD3-hprtPM; Fig. 1). It was proposed that AD3-hprtPM would act as an antisense molecule in modulating ADAMTS3 protein expression, whereas the HPRT locus served as a genomic reporter of off-target genotoxicity, which could be quantified using the established HPRT forward mutation assay. The integrity of the genomic TK locus served as a reporter of sequence-independent genotoxicity following oligonucleotide treatments.

As a negative control, an oligonucleotide was employed with the same base composition as AD3-hprtPM but in a random order (control; Fig. 1). Oligonucleotides were chemically modified to contain four terminal phosphorothioate linkages at either end to increase nuclease resistance (Kenner et al., 2002).

**Antisense Activity of AD3-hprtPM**

Human lymphoblastoid TK6 cells were treated with AD3-hprtPM and control oligonucleotides up to 10μM to inform antisense activity. Treated cells were sampled at 24, 48, and 72 h to determine ADAMTS3 protein expression (Figs. 2A–C). Gels were quantitated using densitometry, and ADAMTS3 protein expression was normalized to control oligonucleotide treatment at the respective dose and time (Fig. 2D); AD3-hprtPM induced a dose- and time-dependent knockdown of ADAMTS3 protein expression by 20–30% with 5 and 10μM treatment by 48 h and was further reduced by 45–70% by 72 h.

**Sequence-Specific Mutation at Genomic DNA by AD3-hprtPM**

To quantify off-target genotoxicity caused by the presence of AD3-hprtPM, the HPRT forward mutation assay was employed. Previous studies have proposed the involvement of homologous recombination (HR) repair in the process of TNE (Radecke et al., 2006; Morozov and Wawrousek, 2008). To investigate this, a dose range of the genotoxin MMS was used to induce RAD51 protein expression as a marker of HR repair activity (Gupta et al., 1997; Saleh-Gohari et al., 2005; Sung and Robberson, 1995). MMS treatment is likely to induce repair pathways other than HR repair, but it is HR repair that is most likely to facilitate oligonucleotide-mediated mutagenesis. Human TK6 cells were treated for 24 h with MMS up to 0.5 μg/ml and aliquots of cells were removed up to 72 h for determination of RAD51 protein expression (Supplementary figure 1). Treatment with 0.2 μg/ml MMS was found to induce RAD51 protein expression ~2.5-fold by 24 h and ~5-fold by 48 h. TK6 cells were then pretreated for 24 h with 0.2 μg/ml MMS to induce RAD51 protein expression followed by treatment with AD3-hprtPM or control oligonucleotide (Fig. 3). Treatment with oligonucleotide up to 10μM was not found to be excessively cytotoxic (Fig. 3A) and the pretreatment of TK6 cells with 0.2 μg/ml MMS was not found to be significantly genotoxic (comparing untreated vs. 0.2 μg/ml MMS–treated background). Interestingly, the biologically active ASO, AD3-hprtPM, was found to induce the HPRT mutant frequency in a dose-dependent manner (Fig. 3B). Treating TK6 cells with 5μM AD3-hprtPM induced the HPRT mutant frequency ~1.5-fold above 5μM control oligonucleotide and ~4.4-fold with 10μM treatment. Genotoxicity was not observed at the nontargeted TK locus following AD3-hprtPM treatment suggesting a sequence-specific mode of action (Fig. 3C).

Genotoxicity caused by AD3-hprtPM was proposed to be a result of an overactive HR repair pathway initiated by the MMS pretreatment. This conclusion was supported when AD3-hprtPM was found to induce mutation at the targeted HPRT locus following omission of the MMS pretreatment (Supplementary figure 2).

**Analysis of AD3-hprtPM–Induced HPRT Mutant Clones**

According to the proposed TNE model, mutation within duplex DNA is directed to the site of the mismatched base (Aarts et al., 2006; Bonner and Kmiec, 2009; Dekker et al., 2003; Morozov and Wawrousek, 2008; Olsen et al., 2009). Thus, to confirm sequence-specific mutation by AD3-hprtPM, a restriction fragment length polymorphism assay was designed. The target region of AD3-hprtPM, within exon 3 of the HPRT locus, was PCR amplified to yield a 1-kb fragment. AD3-hprtPM–mediated mutation at the mismatched base would be expected to result in a G > T transversion causing the loss of a 5’-GATC-3’ Mbo I restriction enzyme recognition sequence. PCR fragments containing the mutant sequence
would be rendered resistant to Mbo I digestion. Those that retained the wild-type sequence would yield two cleavage products of ~550 and ~450 bp.

Mbo I digestion of 14 control and 29 AD3-hprtPM–induced HPRT mutant clones resulted in cleavage of all PCR products into two fragments, contradicting the expected mechanism of mutagenesis (Supplementary figure 3). DNA sequencing of PCR-amplified HPRT mutant clones (41 AD3-hprtPM–induced HPRT mutant clones and 29 control) supported the retention of the wild-type 5’-GATC-3’ sequence at position 138 (Fig. 4). However, DNA sequencing also revealed a single-base deletion (position 171; 34%) adjacent to a single G > A transition mutation (position 172; 29%) downstream of the AD3-hprtPM target sequence (underlined; position 134–156). These mutations were primarily found in AD3-hprtPM–induced HPRT mutant clones and not control. The frequency

FIG. 2. Antisense activity of AD3-hprtPM oligonucleotide. TK6 cells were treated with 2, 5, or 10 μM AD3-hprtPM or control oligonucleotide. ADAMTS3 protein expression at (A) 24, (B) 48, and (C) 72 h was determined by immunoblot. ADAMTS3 expression was quantified by densitometry and corrected for GAPDH-loading control. ADAMTS3 protein expression following AD3-hprtPM treatment was normalized to control oligonucleotide treatment at the respective time and dose (D). Data represent mean ± SD of three independent treatments. ADAMTS3 protein expression from AD3-hprtPM treatment is compared with control oligonucleotide at the same dose and time using a two-way Student’s t-test. *p < 0.05.

FIG. 3. Genotoxicity of AD3-hprtPM in a HR repair–induced system. Human TK6 cells were pretreated with 0.2 μg/ml MMS for 24 h to induce the HR repair pathway. The untreated group measures the cytotoxic/genotoxic effect of the MMS pretreatment. The spontaneous mutant frequency following MMS pretreatment was determined as background. EMS (5 μg/ml) was used as a positive genotoxin. Cytotoxicity below the minimum accepted 20% RTG is not evident following treatment of TK6 cells with 2, 5, or 10 μM oligonucleotide (A). AD3-hprtPM–induced a dose-dependent increase in HPRT mutants relative to control oligonucleotide (B). AD3-hprtPM oligonucleotide failed to induce mutation at the nontargeted TK locus (C). Data represent mean ± SD of three independent treatments. *p < 0.05 using two-way Student’s t-test.
Incorporation of ASO-Derived Nucleotide Analogues
Into a Primed DNA Template

Following reports of serum nucleases capable of degrading phosphorothioate oligonucleotides after 1h (Hoke et al., 1991; Morvan et al., 1993), this study has also addressed the capability of nucleotide analogues to be incorporated into newly synthesized DNA. In the first approach, an in vitro primer extension assay, adapted from Lacenere et al. (2006), was engineered to inform the potential of Tfi DNA polymerase to incorporate nucleotide analogues commonly used in ASO design. Incorporation of nucleotide analogue was informed by extension of primed template by five nucleotides. Using this model, DNA polymerase was able to extend the primed template to full length using 0.5 μM deoxyadenosine triphosphate (dATP), as a control, in a 10-min reaction (Fig. 5A).

Using a phosphorothioate analogue of dATP (dATPαS), where a nonbridging oxygen in the α-phosphate moiety is replaced with sulfur (the most common type of chemical modification used in ASO design (Buchini and Leumann, 2003)), Tfi DNA polymerase was able to extend the primed template to full length using 0.5 μM deoxyadenosine triphosphate (dATP), as a control, in a 10-min reaction (Fig. 5A).

In comparison to the unmodified dATP, this correlates to a relative incorporation efficiency of ~12%. On the contrary, using a 2'-O-methyl-ATP–modified analogue (2’OMe-ATP), where the 2’ hydroxyl group on the ribose moiety of ATP is replaced with O-methyl to increase ASO target binding affinity (Yoo et al., 2004), Tfi polymerase failed to extend the primed template in a 60-min reaction (Fig. 5C).

Genotoxicity of Phosphorothioate Nucleotide Analogues

Having shown that DNA polymerase was capable of incorporating the noncanonical phosphorothioate analogue of particular point mutations upstream (G > A position 126; 58% control vs. 81% AD3-hprtPM) and downstream (G > A position 158; 19% control vs. 51% AD3-hprtPM) of the AD3-hprtPM target sequence were also found to be effected following AD3-hprtPM treatment.

Incorporation of nucleotide analogue was informed by extension of primed template by five nucleotides. Using this model, DNA polymerase was able to extend the primed template to full length using 0.5 μM deoxyadenosine triphosphate (dATP), as a control, in a 10-min reaction (Fig. 5A). Incorporation of the phosphorothioate nucleotide analogue, dATPαS, was in a 20-min reaction at 2 μM (Fig. 5B). Here, dATP (5 μM) was used as a positive control (lane C) to reference an extended template. DNA polymerase failed to incorporate the nucleotide analogue, 2’OMe-ATP, into the primed template in a 60-min reaction (Fig. 5C).
dATPαS into a primed template \textit{in vitro}, genotoxicity caused by incorporation of this analogue into genomic DNA \textit{in vivo} was assessed through the integrity of the HPRT and TK loci in human TK6 cells. Of important consideration was that degradation of oligonucleotides would not yield nucleotides as a triphosphate but rather as monophosphates. Thus, to reflect a true biologically relevant event, taking into account the prerequisite for phosphorylation of monophosphate nucleotides into triphosphates before utilization by DNA polymerase, TK6 cells were treated with monophosphate phosphorothioate nucleotide (dAMPαS).

Human TK6 cells were treated for 24h with dAMPαS up to 1mM. As a control, TK6 cells were also treated with 1mM of the unmodified dAMP nucleotide to allow comparison of genotoxicity caused by the single substitution of oxygen for sulfur in the 5’ phosphate group.

Cytotoxicity following dAMPαS treatment was acceptable up to 0.5mM (30% RTG) whereas treatment with the nucleotide control, dAMP, was much less cytotoxic (60% RTG; Fig. 6A). Treatment of TK6 cells with dAMP and dAMPαS failed to induce mutation at the HPRT locus (Fig. 6B). In contrast, treatment with dAMPαS resulted in a dose-dependent increase in TK mutants ~20-fold (0.5mM) and ~96-fold (1mM) above control (Fig. 6C). As 1mM dAMPαS failed to induce mutation at the HPRT locus (Fig. 6B), a cytotoxicity-based mechanism of mutation at the TK locus could be excluded. In contrast, 1mM dAMP treatment marginally induced the TK mutant frequency (Fig. 6C). Thus, the genotoxicity of 1mM dAMPαS was significantly (~56-fold) more potent than the unmodified counterpart. In fact, genotoxicity at either the HPRT or TK loci was no greater than ~3-fold for treatment of TK6 cells with canonical deoxyadenosine, dAMP or dATP (Supplementary figure 4).

**DISCUSSION**

Data presented in this study suggest that a biologically active ASO, AD3-hprtPM, and its phosphorothioate nucleotide analogue degradation product are capable of inducing mutation at genomic DNA in human lymphoblastoid TK6 cells.

Oligonucleotides have been previously reported to be capable of inducing mutation within a homologous sequence in duplex DNA, in a process known as TNE, where mutation is a result of a mismatched base within the oligonucleotide (Aarts et al., 2006; Bonner and Kmiec, 2009; Dekker et al., 2003; Morozov and Wawrousek, 2008; Olsen et al., 2009). An important point to consider is that the oligonucleotides employed in these studies tend to be greater than 45 nt in length. In this study, AD3-hprtPM (23 nt in length) was engineered to reflect the length of an ASO therapeutic and chemically modified to contain the commonly employed phosphorothioate linkages (Buchini and Leumann, 2003; Geary, 2009).

Although AD3-hprtPM was biologically active as an antisense molecule, mutation at the targeted HPRT locus in human
lymphoblastoid cells was not observed above the detection limit of the assay, unless RAD51 protein expression was stimulated prior to treatment. In that instance, AD3-hprtPM–induced locus and sequence-specific mutation ~4.4-fold above control. Sequencing HPRT mutant clones revealed mutation at the site of the mismatched base was absent in all clones. However, among other mutations, a single-base deletion and point mutation adjacent to but downstream of the AD3-hprtPM target sequence was predominant in mutant clones derived from AD3-hprtPM treatment and not control.

We propose that AD3-hprtPM binding to its target sequence on the sense strand (nontranscribed) is facilitated by the strand-pairing properties of RAD51 protein, perhaps during DNA replication (Gupta et al., 1997; Kow et al., 2007; Sung and Robberson, 1995). Indeed, the ability of RAD51 to pair single-stranded DNA with homologous double-stranded DNA has been previously reported (Gupta et al., 1997; Sung and Robberson, 1995). Upon strand invasion, a “displacement loop” structure is formed, which results in the displacement of the antisense strand. Following this, the model can be extrapolated from Hanawalt (1994) and Wang et al. (1996). The displacement loop structure may result in a physical blockade to a progressing replication/transcription fork causing it to revert back to a natural pause site generating a reiterative repair patch. Repeated attempts in replication/transcription may result in mutation introduced by the natural error frequency of the DNA repair polymerase. It may be the bound AD3-hprtPM that is removed by the helicase activity associated with a progressing fork but repeated cycles of binding, inhibition of replication/transcription, and reiterative repair increase the probability of a mutagenic event.

Alternatively, perhaps through HR repair during S-phase (Johnson and Jasin, 2001; Takata et al., 1998), AD3-hprtPM physically incorporates into the genome following RAD51-mediated strand invasion (Radecke et al., 2006). Once incorporated, AD3-hprtPM with the mismatched base and the noncanonical phosphorothioate linkages at either end may cause replication fork arrest. The sulfur within the phosphodiester backbone is likely to be recognized as a lesion by the nucleotide excision repair (NER) pathway ultimately resulting in the excision of an encompassing fragment of 27–29 nt in length (Huang et al., 1992; Svoboda et al., 1993). In support of this, the introduction of phosphorothioate bonds in a DNA double helix has been reported to cause structural alterations (Kanaori et al., 1999). This mechanism may also explain why the mismatched base from AD3-hprtPM treatment was not observed in HPRT mutant clones but was observed in previous studies using oligonucleotides that are far greater in length than the NER repair pathway is able to cleave.

In this study, human TK6 cells were transfected with oligonucleotide for 4h, which was then removed by washing. Exposure of cells to oligonucleotide for longer periods of time may increase the probability for ASO hybridization to genomic DNA and mutation. For example, in a Phase I/II clinical trial using a 2′OMe/phosphorothioate ASO (PRO051, Prosensa Therapeutics), plasma half-life of oligonucleotide was between 19 and 56 days (Goemans et al., 2011). Additionally, sc injection of a phosphorothioate ASO has been shown to rapidly distribute to the liver in mice and remain there with a half-life up to 19 days (Yu et al., 2001). In fact, ASO elimination from the liver was reduced with increasing ASO dose. Thus, accumulation and constant exposure of cells to ASO may increase the probability of ASO hybridization to genomic DNA and subsequent mutation; repeated cycles of ASO binding may further increase the likelihood of mutation. This hypothesis is supported from a study by Leonetti et al. (1991) and Chin et al. (1990), where microinjected oligonucleotides were shown to rapidly accumulate in the cell nucleus and not the cytoplasm. ASO binding to genomic DNA is even more likely when ASO accumulate in the cell nucleus.

These data suggest that a mismatched base within an ASO may not be a prerequisite for mutation at its homologous sequence in genomic DNA providing RAD51 protein expression is sufficient to mediate strand invasion of duplex DNA. The dependence of AD3-hprtPM–mediated mutagenesis on RAD51 protein induction is clinically relevant to patients with p53 mutant tumors that are often found to have elevated RAD51 protein expression; it has been proposed that RAD51 overexpression may contribute to drug resistance and genomic instability (Klein, 2008). Furthermore, the majority of ASO currently in clinical trials are for cancer therapy and p53 is inactivated in half of human cancers (Soussi and Lozano, 2005). Because p53 negatively regulates RAD51 gene expression, these patients may also present with elevated RAD51 protein expression (Arias-Lopez et al., 2006; Hanay et al., 2007). For example, the extent of RAD51 protein expression in invasive ductal breast cancer correlated with the histological grading of tumors and RAD51 is reportedly induced 2–7-fold in several cancer cell lines similar to that reported here (Maacke et al., 2000; Radershall et al., 2002). However, how our in vitro genotoxicity data translate to an in vivo system is unknown and warrants further investigation.

ASO that are degraded can result in the release of noncanonical nucleotides, which may enter endogenous nucleotide pools and incorporate into newly synthesized DNA during replication. Data presented here suggest that a DNA polymerase can utilize a phosphorothioate nucleotide analogue (dATP[s]), albeit with reduced efficiency (~8-fold relative to unmodified counterpart) but failed to incorporate a 2′OMe RNA–based nucleotide (2′OMe-ATP) into a primed template. It would appear that incorporation of nucleotide analogues may entirely depend on the nature of the chemical modification.

Treating human lymphoblastoid cells with the phosphorothioate analogue dAMP[s] resulted in significant mutation with an apparent thresholded effect. Remarkably, mutation at the genomic TK locus was up to two orders of magnitude above control, yet mutation at the HPRT locus was not observed. Previous studies suggest that a large proportion of mutations at the HPRT locus can be deleterious to the cell, whereas the
TK locus is a more robust reporter capable of detecting point mutations and even intergene deletions (Doak et al., 2007; McGregor et al., 1996), and we suggest that this may be the case here.

Human TK6 cells were treated with the monophosphate analogue dAMPtS and so for incorporation into genomic DNA (1) the concentration of dATPαS must exceed a threshold for insertion of analogue rather than endogenous dATP and (2) dAMPtS must first become a substrate for various kinases to convert the monophosphate into a triphosphate for utilization by DNA polymerase. In essence, both these limitations would contribute to a genotoxic threshold as observed in the data presented here. Considering this thresholded effect at the TK locus, we suggest that the mechanism of mutation by dAMPtS may be in accordance with the next nucleotide effect model (Phear et al., 1987). The speed at which DNA polymerase extends an elongating strand (5′–3′) is governed by the availability of the next (3′) nucleotide in sequence. If a misinserted nucleotide is followed by a highly abundant nucleotide, polymerization of this next nucleotide is favored rather than the excision of the incorrect one by the 3′–5′ exonuclease activity associated to DNA polymerase (Fersht, 1979). However, in this case, the insertion of the phosphorothioate analogue, dATPαS, would render the bond nucleic resistant thereby locking the error into the sequence.

It is particularly important to note that although 0.5mM dAMPtS treatment was mutagenic in an in vitro system, the in vivo relevance is unknown; phosphorothioate oligonucleotides are thought to slowly degrade over time, but data are lacking to inform in vivo intracellular concentrations of ASO-derived nucleotide analogues. Nevertheless our findings raise the question as to whether ASO modalities and their respective degradations contribute to genomic instability.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


