Running title: Mutation by an antisense oligonucleotide

Mutagenesis by an antisense oligonucleotide and its degradation product

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Abstract

The European Medicines Agency (EMA) has expressed concern regarding (i) the potential for antisense oligonucleotide (ASO) therapeutics to induce sequence specific mutation at genomic DNA and (ii) the capability of ASO degradation products (nucleotide analogues) to incorporate into newly synthesised 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 four fold, 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.

Introduction

The potential for ASO to modulate protein expression by translational repression or RNase H mediated degradation has application as 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 (European Medicines Agency, 2004).

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 nucleotides in length, are designed to bind complementary to the non-transcribed (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 GFP 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 EMA but genotoxicity was deemed unlikely (European Medicines Agency, 2004); if base pairing with the nucleotide analogue occurred with reduced fidelity, mutation might be expected. The genotoxicity of antiviral nucleoside analogues has 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*TGAT GGCAAATAGGT*A*A*T*A-3'. The * indicates the position of the phosphorothioate linkage.

Cell culture. Human lymphoblastoid TK6 cells were obtained from ATCC. TK6 cells were cultured in RPMI 1640 media supplemented with 10%$_{v/v}$ heat inactivated horse serum, L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). All cell culture reagents were obtained from Invitrogen, unless otherwise stated.

Induction of RAD51 protein expression in TK6 cells using methyl methanesulfonate (MMS). Exponentially growing TK6 cells were treated with MMS (0 μg/ml, 0.1 μg/ml, 0.2 μg/ml 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 h, 4 h, 8 h, 24 h, 48 h 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 pre-treat 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%$_{v/v}$ igepal, 1 mM Tris (pH 7.4) and 1 mM EDTA) on ice for 20 mins. Samples were then

centrifuged at 10,000x g for 10 mins 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 (BCA) assay according to manufacturer's protocol (Thermo Scientific).

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 anti-human RAD51 antibody; for ADAMTS3, 1:2000 rabbit anti-human ADAMTS3 antibody. Following primary antibody staining and several wash steps, blots were stained with HRP-conjugated secondary antibody and visualised. GAPDH protein expression was employed as a loading control using primary rabbit anti-human GAPDH antibody (1:1000). Protein expression was determined using densitometry and normalised to GAPDH loading control.

***HPRT* and *TK* forward mutation assay.**

a. 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 $3x10^6$ 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% CO_2. TK6 cells were then washed and suspended for culturing overnight. As a positive genotoxic control, TK6 cells were treated with ethyl methanesulphonate (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 h, 48 h and 72 h) for protein extraction, to determine ADAMTS3 protein expression, by immunoblot, as a marker of AD3-hprtPM antisense activity.

b. Treating TK6 cells with nucleotides. For treatment of TK6 cells with nucleotide, $4x10^6$ TK6 cells were aliquoted per 75 cm^2 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 200x g for 5 mins and washed with culture media.

c. Determining cytotoxicity and the *TK* and *HPRT* mutant frequency. Following treatment of TK6 cells with oligonucleotide or nucleotide, cells were counted daily for three 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, TK6 cells were plated at 1.6 cells per well in 96 well plates to determine cloning efficiency and 20,000 cells per well in trifluorothymidine to determine the *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 four days phenotypic expression. Following this, the *HPRT* mutant frequency was then determined by seeding 20,000 cells per well in 96 well plates in 6-thioguanine and cloning efficiency 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%CO_2 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 with proteinase K and lysis buffer AL. Samples were mixed by pulse vortexing and incubated at 56 ºC for 10 mins. Lysates were mixed with ethanol and added to a spin column followed by centrifugation. Columns were washed with wash buffer AW1 and then AW2. DNA was eluted out of the spin column using DNase/RNase free water. DNA was quantified using the NanoDrop ND1000 spectrophotometer.

Polymerase chain reaction and DNA sequencing. PCR reactions were performed to amplify a one Kb region of exon 3 in the *HPRT* locus which enclosed the AD3-hprtPM target sequence. A typical reaction was in a 50 µl reaction volume containing genomic DNA (300 ng to 1 µg) from isolated *HPRT* mutant clones, 200 nM of each forward (5'-AGGGCAAAGGATGTGTTACG-3') and reverse (5'-AGTGGTTTCTGGTGCGACTT-3') primer, dNTPs (200 µM), Tfi polymerase (5 units) and 1x Tfi PCR buffer supplemented with $MgCl_2$ (1.5 mM). Amplification was performed using a Peltier thermal cycler as follows: initial denaturation step at 94 °C for 4 mins, followed by 35 cycles of denaturing at 94 °C for 30 secs, annealing at 58 °C for 30 secs and polymerisation at 72 °C for 1 min. The final polymerisation step was extended to 7 mins. 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 visualised under UV. PCR products were extracted and purified for DNA sequencing using the PureLink Quick gel extraction kit

(Invitrogen, Paisley), according to manufacturer's protocol. Purified PCR products were diluted to 10 ng/µl and sent for sequencing by Gene Service ltd (Source Bioscience).

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 results in cleavage into two fragments; 474 bp 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) for 1 h at 37 °C. Digested PCR products were resolved on 1.2%$_{w/v}$ agarose gels containing ethidium bromide (0.5 µg/ml) and visualised under UV.

Primer extension assay. This method was adapted from Lacenere and co-workers (Lacenere *et al.*, 2006). Primed template, with an overhang of 5 thymidine nucleotides, was made by annealing template strand (5'-TTTTTCCCACAAACCAAAAGCCCAGACACA-3') with the complementary primer strand (5'-6FAM-TGTGTCTGGGCTTTTGGTTTGTGGG-3') which is labelled with a 5' fluorophore in annealing buffer (75 mM Tris pH 7.2 and 75 mM NaCl). A typical reaction mixture contained primed template (25 nM), Tfi DNA polymerase (0.6 units), $MgCl_2$ (3.5 mM), 1x Tfi PCR buffer and nucleotide in a 20 µl volume. As a negative control in experiments, nucleotide was omitted. Reactions were allowed to incubate for the required length of time at 72 °C to permit extension of the primed duplex. Following this, EDTA (5 µl of 1M) was added to stop the reaction. Primed template in the reaction mixture was then denatured by adding 25 µl of UREA gel loading buffer (1.5 M sucrose, 7 M UREA, 10 mM EDTA, 0.1% bromophenol blue) and heated at 95 °C for 5 mins with immediate transfer to ice slurry. Samples were then subject to electrophoresis through a 14% 8 M

UREA-polyacrylamide gel at 120 V for 3 h. 6FAM labelled primer strand was visualised at 490 nm/530 nm.

Results

Design of an ASO.

In the first instance, an ASO was designed to bind complimentary to the mRNA of the *ADAMTS3* gene as well as contain sequence homology to the Hypoxanthine-guanine phosphoribosyltransferase (*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 whilst 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 Thymidine kinase (*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 4 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 24h, 48h and 72h to determine ADAMTS3 protein expression (**Fig. 2, A-C**). Gels were quantitated using densitometry and ADAMTS3 protein expression was normalised 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 5μM and 10μM treatment by 48h and was further reduced by 45-70% by 72h.

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 methyl methanesulfonate (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 mutation. Human TK6 cells were treated for 24h with MMS up to 0.5μg/ml and aliquots of cells were removed up to 72h for determination of RAD51 protein expression (**Suppl. Fig. 1**). Treatment with 0.2μg/ml MMS was found to induce RAD51 protein expression ~2.5 fold by 24h and ~5 fold by 48h. TK6 cells were then pre-treated for 24h with 0.2μg/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 pre-treatment of TK6 cells with 0.2μg/ml MMS was not found to be significantly genotoxic (comparing Untreated vs.0.2μg/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 non-targeted *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 pre-treatment. This conclusion was supported when

AD3-hprtPM failed to induce mutation at the targeted *HPRT* locus following omission of the MMS pre-treatment (**Suppl. Fig 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 (RFLP) assay was designed. The target region of AD3-hprtPM, within exon 3 of the *HPRT* locus, was PCR amplified to yield a 1Kb 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 which retained the wild-type sequence would yield 2 cleavage products of ~550bp and ~450bp.

Mbo I digestion of 14 control and 29 AD3-hprtPM induced *HPRT* mutant clones resulted in cleavage of all PCR products into 2 fragments, contradicting the expected mechanism of mutagenesis (**Suppl. Fig. 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 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 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 synthesised 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 0.5μM deoxyadenosine triphosphate (dATP), as a control, in a 10 minute reaction (**Fig. 5A**).

Using a phosphorothioate analogue of dATP (dATPαS); where a non-bridging oxygen in the α-phosphate moiety is replaced with sulphur (the most common type of chemical modification used in ASO design (Buchini and Leumann, 2003)), Tfi DNA polymerase was able to fully extend the template using 2μM dATPαS in a 20 minute reaction (**Fig. 5B**). 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 minute reaction (**Fig. 5C**).

Genotoxicity of phosphorothioate nucleotide analogues.

Having shown DNA polymerase was capable of incorporating the non-canonical phosphorothioate analogue dATPαS into a primed template *in vitro*, genotoxicity caused by incorporation of this analogue into genomic DNA *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 utilisation 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 sulphur in the 5' phosphate group.

Cytotoxicity following dAMPαS treatment was acceptable up to 0.5mM (30% RTG) whilst 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 (**Suppl. Fig. 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 oligonucleotides employed in these studies tend to be greater than 45 nucleotides in length. In this study, AD3-hprtPM (23 nucleotides 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, amongst 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 (non-transcribed) 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 is removed by the helicase activity associated with a progressing fork but repeated cycles of binding, inhibition of replication/transcription and re-iterative 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 non-canonical phosphorothioate linkages at either end may cause replication fork arrest. The sulphur within the phosphodiester backbone is likely to be recognised as a lesion by the nucleotide excision repair (NER) pathway ultimately resulting in the excision of an encompassing fragment of 27-29nt 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 hybridisation to genomic DNA and mutation. For

example, in a Phase I/II clinical trial using a 2'OMe/phosphorothioate ASO (PRO051, Prosensa Therapetucis), plasma half-life of oligonucleotide was between 19 and 56 days (Goemans *et al.*, 2011). Additionally, subcutaneous 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 hybridisation 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 micro-injected 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 pre-requisite 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). Since p53 negatively regulates RAD51 gene expression these patients may also present with elevated RAD51 protein expression (Arias-Lopez *et al.*, 2006, Hannay *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,

Raderschall *et al.*, 2002). However, how our *in vitro* genotoxicity data translates to an *in vivo* system is unknown and warrants further investigation.

ASO that are degraded can result in the release of non-canonical nucleotides, which may enter endogenous nucleotide pools and incorporate into newly synthesised DNA during replication. Data presented here suggest that a DNA polymerase can utilise a phosphorothioate nucleotide analogue (dATPαS), albeit with reduced efficiency (~8 fold relative to unmodified counterpart), but failed to incorporate a 2'Omethyl 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 dAMPαS resulted in significant mutation with an apparent thresholded effect. Remarkably, mutation at the genomic *TK* locus was up to 2 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 whilst the *TK* locus is a more robust reporter capable of detecting point mutations and even inter-gene 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 dAMPαS and so for incorporation into genomic DNA (i) the concentration of dATPαS must exceed a threshold for insertion of analogue rather than endogenous dATP and (ii) dAMPαS must first become a substrate for various kinases to convert the monophosphate into a triphosphate for utilisation 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 the mechanism of mutation by dAMPαS 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' to 3') is governed by the availability of the next (3')

nucleotide in sequence. If a misinserted nucleotide is followed 3' by a highly abundant nucleotide, polymerisation of this next nucleotide is favoured rather than the excision of the incorrect one by the 3' to 5' exonuclease activity associated to DNA polymerase (Fersht, 1979). However, in this case, the insertion of the phosphorothioate analogue, dATPαS, would render the bond nuclease resistant thereby locking the error into the sequence.

It is particularly important to note that although 0.5mM dAMPαS 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 is 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 degradation products can contribute to genomic instability.

Supplementary Data

Supplementary Figures 1-4 can be found online.

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Figure legends

Figure 1. Oligonucleotide sequences. The target sequence in exon 3 of the *HPRT* locus is shown as a duplex and underlined. AD3-hprtPM antisense oligonucleotide is designed to bind complementary to the non-transcribed strand, except a single mismatched base (bold lowercase). Control oligonucleotide has no sequence similarity to the *HPRT* locus.

Figure 2. Antisense activity of AD3-hprtPM oligonucleotide. TK6 cells were treated with 2 μM, 5 μM or 10 μM AD3-hprtPM or control oligonucleotide. ADAMTS3 protein expression at 24 h (A), 48 h (B) and 72 h (C) was determined by immunoblot. ADAMTS3 expression was quantified by densitometry and corrected for GAPDH loading control. ADAMTS3 protein expression following AD3-hprtPM treatment was normalised 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 to control oligonucleotide at the same dose and time using a two-way student's t-test. * $p<0.05$.

Figure 3. Genotoxicity of AD3-hprtPM in a HR repair induced system. Human TK6 cells were pre-treated 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 pre-treatment. The spontaneous mutant frequency following MMS pre-treatment was determined as Background. EMS (5μg/ml) was used as a positive genotoxin. Cytotoxicity below the minimum accepted 20% RTG is not evident following treatment of TK6 cells with 2 μM, 5 μM 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 non-targeted *TK* locus (C). Data represent mean ± SD of three independent treatments. * $p<0.05$ using two-way students t-test.

Figure 4. Distribution of AD3-hprtPM mediated mutation in exon 3 of the *HPRT* locus. Sequences are numbered from the first base of exon 3 (1 to 164). The AD3-hprtPM target sequence is underlined from position 134-156. * above guanine (position 138) indicates the site of the mismatched base in AD3-hprtPM where G>T transversion mutation was predicted. Numbers and letters within a circle represent the frequency (%) and type of mutation from 41

AD3-hprtPM induced *HPRT* mutant clones whereas those in a square are from 29 control oligonucleotide induced *HPRT* mutant clones. + indicates an insertion and Δ indicates a deletion.

Figure 5. Efficiency of nucleotide analogue incorporation into a primed DNA template. Two complimentary oligonucleotide strands (25 nt and 30 nt) were annealed together to produce an overhang of five thymidine nucleotides. The ability to extend the short strand by five nucleotides is used as the marker for incorporation of nucleotide. The values above each lane are dose of nucleotide analogue (μM). Full extension of the primed template was found to occur in a 10 minute reaction with 0.5 μM dATP (A). Incorporation of the phosphorothioate nucleotide analogue, dATPαS, was in a 20 minute reaction at 2 μM (B). 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'Omethyl-ATP, into the primed template in a 60 minute reaction (C).

Figure 6. Genotoxicity of dAMPαS nucleotide analogue incorporation into genomic DNA. Human TK6 cells were treated with nucleotide analogue dAMPαS from 20 μM up to 1mM for 24 h. As a nucleotide control, TK6 cells were also treated with 1 mM dAMP. The zero dose is the negative control (solvent). EMS (5 μg/ml) was used as a positive genotoxin. For mutation assays, RTG above 20% are considered acceptable; only treatment with 1 mM dAMPαS (10% RTG) was below this threshold (A). Mutation at the *HPRT* locus was not observed (B). However, treatment with dAMPαS resulted in a dose dependent increase in *TK* mutants up to 96 fold above control (C). The unmodified nucleotide, dAMP (1mM), induced the *TK* mutant frequency ~1.7 fold (C). * $p<0.05$; ** $p< 0.01$; *** $p<0.001$ using one-way ANOVA with Dunnett's post-hoc test compared to control.

Figure 1

Figure 1
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Figure 2

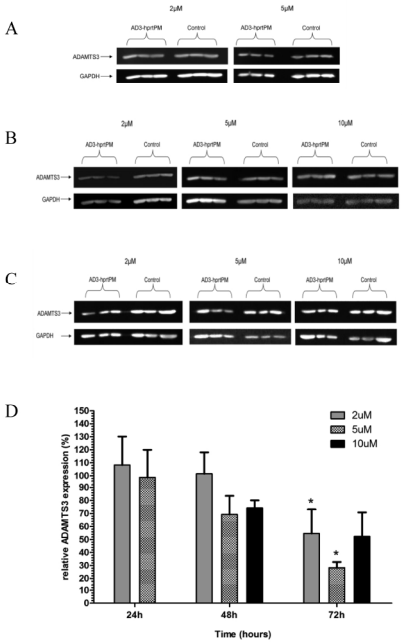

Figure 2
230x230mm (300 x 300 DPI)

Figure 3

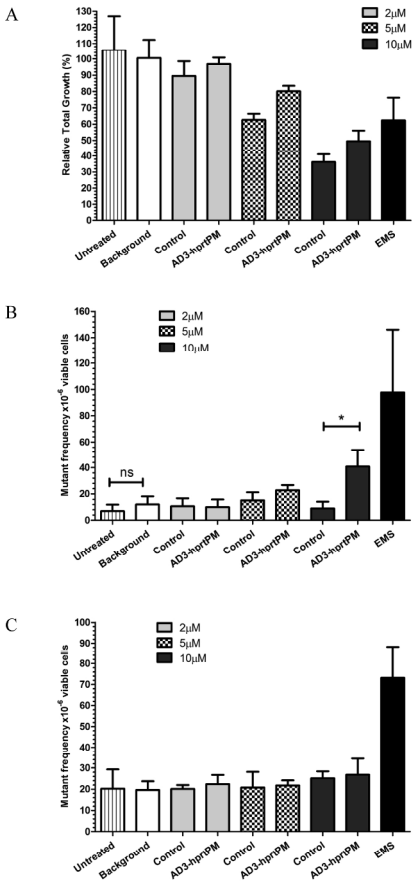

Figure 3
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Figure 4

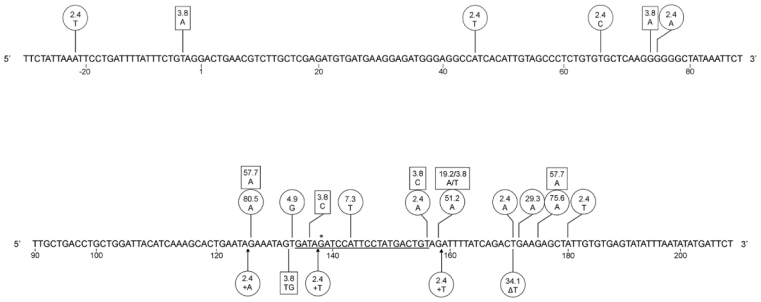

Figure 4
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Figure 5

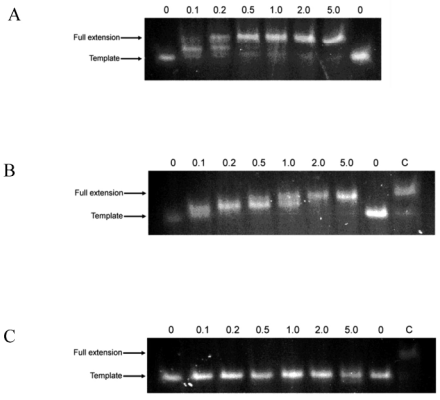

Figure 5
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Figure 6

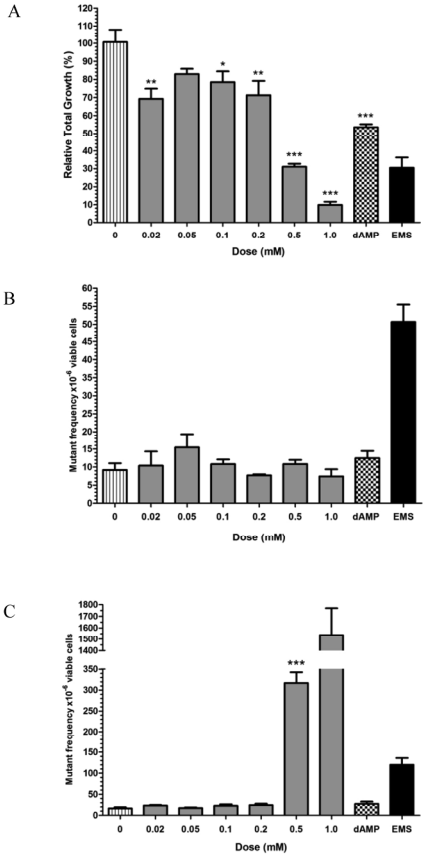

Figure 6
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