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2′-*O*-(2-methoxyethyl) nucleosides are not phosphorylated or incorporated into the genome of Human Lymphoblastoid TK6 Cells

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Running title: 2′-*O*-MOE phosphorylation and incorporation into cellular DNA and RNA

Abstract

Nucleoside analogues with 2′-modified sugar moieties are often used to improve the RNA target affinity and nuclease resistance of therapeutic oligonucleotides in preclinical and clinical development. Despite their enhanced nuclease resistance, oligonucleotides could slowly degrade releasing nucleoside analogues that have the potential to become phosphorylated and incorporated into cellular DNA and RNA. For the first time, the phosphorylation and DNA and RNA incorporation of 2′-*O*-(2-methoxyethyl) (2′-*O*-MOE) nucleoside analogues have been investigated. Using LC/MS/MS, we showed that enzymes in the nucleotide salvage pathway including deoxycytidine kinase (dCK) and thymidine kinase (TK1) displayed poor reactivity toward 2′-*O*-MOE nucleoside analogues. On the other hand, 2′-fluoro (F) nucleosides, regardless of the nucleobase, were efficiently phosphorylated to their monophosphate forms by dCK and TK1. Consistent with their efficient phosphorylation by dCK and TK1, 2′-F nucleosides analogues were incorporated into cellular DNA and RNA while no incorporation was detected with 2′-*O*-MOE nucleoside analogues. In conclusion, these data suggest that the inability of dCK and TK1 to create the monophosphates of 2′-*O*-MOE nucleoside analogues reduces the risk of their incorporation into cellular DNA and RNA.

Keywords: modified nucleosides, DNA incorporation, nucleoside phosphorylation, nucleoside kinase

Introduction

Oligonucleotide-based therapeutics have the potential to modulate gene expression in mammalian cells to treat a wide range of diseases including cancer, inflammatory, dyslipidemia and infectious diseases (Kole and Leppert, 2012; Shahbazi *et al.*, 2016). Various chemical modifications to the base, sugar, and backbone have been developed and applied to therapeutic oligonucleotides to improve their pharmacokinetics and pharmacodynamics properties (Deleavey and Damha, 2012). One of the most widely used modifications for antisense oligonucleotides is phosphorothioate internucleotide linkages whereby one of the non-bridging oxygen atoms in the phosphodiester linkage is replaced by sulphur (Eckstein, 2000). These first-generation phosphorothioate oligonucleotides (PS ODN) have sufficient nuclease resistance, support RNase H activity, and bind plasma proteins which is critical to maintain favourable pharmacokinetics and tissue distribution properties (Eckstein, 2000). Subsequently, the so-called second generation of chemical modifications were developed by modifying the 2′-position of the sugar moiety of the ODN with either an alkyl group (e.g., 2′-O-methyl, or 2′-O-methoxyethyl) or a 2′-fluoro group (Monia, 1997). These modifications increased the hybridization affinity by holding the ribose ring in a configuration that is optimal for hydrogen bonding with the target RNA strand (Kawasaki *et al.*, 1993). The 2'-F modification has been used extensively in siRNA constructs, whereas the single-stranded phosphorothioate ODNs more commonly use the 2′-alkyl modifications. The second generation chemistry that is most well studied in development is the 2′-*O*-MOE modified PS ODN (Geary *et al.*, 2001a).

The 2′-alkyl modifications have an added benefit in that they further enhance nuclease resistance and modulate the pharmacokinetic properties of antisense

ODNs (Prakash, 2011). As fully 2′-ribose modified oligonucleotides do not support RNase H to cleave the target RNA, so-called gapmers were developed whereby the ribose sugar modifications are confined to both ends of ODN leaving a central ‘gap’ of unmodified 2′-deoxyphosphorothioate nucleotides (Monia *et al.*, 1993). For example, the FDA-approved antisense drug “Mipomersen”, used to treat familial hypercholesterolemia, is a 20-mer fully phosphorothioated oligo with 2′-*O*-MOE modified ribose at both ends (Crooke and Geary, 2013). This makes the 3′- and 5′-end of the antisense oligonucleotide highly resistant to exonuclease-mediated metabolism.

The genotoxic potential for single-stranded PS ODN has been thoroughly examined, with and without 2′-alkyl modifications, and have been found to be uniformly absent of any genotoxicity potential, but the implications of the liberated mononucleotides have never been directly assessed (Berman *et al.*, 2016; Henry *et al.*, 2002). The released nucleotide analogues could have a genotoxic potential either via perturbation of the endogenous nucleotide pool or by incorporation into newly synthesised genomic DNA potentially leading to mutation or chromosome breakage (Mattano *et al.*, 1990; Phear *et al.*, 1987). The likelihood of unbalancing nucleotide pools through slow nuclease mediated metabolism seems an unlikely concern because this would be addressed by the standard genotoxicity assays where uptake and metabolism at extraordinarily high concentrations have been documented without genotoxic impact (Henry *et al*., 2002). However, the European Medicines Agency (EMA) raised a concern on the genotoxic potential of the liberated nucleotide analogues as they could serve as substrates for various intracellular kinases which would represent the first crucial step for their incorporation into newly synthesised DNA

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003149.pdf. Indeed, using *in vitro* polymerase extension assays, previous studies showed that 2′-fluorodeoxynucleotides were substrates for human DNA polymerases including polymerase alpha (pol α) and polymerase gamma (pol γ), and could be incorporated into primed DNA (Richardson *et al.*, 2000). Subsequently, the same group demonstrated that 2′-fluoropyrimidines (2′-F-C and 2′-F-U) could be incorporated into DNA and RNA of various tissues of rats and woodchucks following long-term treatment (90 days) (Richardson *et al.*, 2002). These studies raise a question as to whether other commonly employed 2′-ribose modifications such as 2′-*O*-MOE could also permit DNA incorporation with the potential for mutagenic consequences. Therefore, the purpose of this study was to evaluate if 2′-*O*-MOE nucleoside analogues are phosphorylated by nucleotide salvage enzymes and whether they are incorporated into cellular DNA and RNA. Here we confirm phosphorylation and DNA/RNA incorporation of 2′-F nucleosides but demonstrate that these properties are not shared by 2′-*O*-MOE nucleosides.

Materials and Methods

Materials and Reagents

TK6 human lymphoblastoid cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated horse serum, 2 mM l-glutamine, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2. All cell culture reagents were purchased from Invitrogen (Paisley, United Kingdom). Nucleosides: natural nucleosides 2′-deoxyguanosine, 2′-deoxyadenosine, 2′-deoxycytidine ,2′-deoxythymidine and their corresponding 5′-O-monophosphate derivatives were purchased Sigma-Aldrich (Poole, United Kingdom).). 2′-*O*-MOE-modified

nucleosides including 2′-*O*-(2-Methoxyethyl)-5-methylcytidine, 2′-*O*-(2-methoxyethyl)-5-methylcytidine-5′-*O*-monophosphate,2′-*O*-(2-methoxyethyl)-5-methyluridine, 2′-*O*-(2-methoxyethyl)-5-methyluridine-5′-O-monophosphate,2′-*O*-(2-methoxyethyl) adenosine, 2′-*O*-(2-methoxyethyl)-adenosine-5′-O-monophosphate, 2′-*O*-(2-methoxyethyl)-guanosine, 2′-*O*-(2-methoxyethyl)-guanosine-5′-O-monophosphate. were obtained from Jena Bioscience. 2′-F nucleosides were obtained from Ionis (CA, USA). Enzymes: Human deoxycytidine kinase (DCK), human UMP-CMPK Kinases were obtained from Novocib (France). Thymidine Kinase 1 (TK1), human adenylate kinase 1 (AK1), human guanylate kinase 1 (GK1), human deoxythymidylate kinase (thymidylate kinase) (DTYMK) were obtained from ProSpec (Israel).

Enzymatic reaction

The phosphorylation reactivity of modified nucleosides by dCK was analysed by incubating 200 µM of the indicated nucleoside in 100 mM Tris, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP and 1 µg of enzyme in a 50 µl reaction volume at 37 °C for 2 h. Phosphorylation by TK1 was analysed in 50 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, 10 mM dithiotreitol (DTT) and 3 mM NaF. AK1 and GK1 were assayed with 1 µg of the respective enzyme, 1 mM ATP and 200 µM of substrate in 100 mM Tris-HCl (pH 7.8), 100 mM KCl and 10 mM $MgCl_2$. CMPK was assayed as above but in 100mM Tris-HCl (pH 9), 250mM KCl, 15mM Mg-acetate and 5mM DTT. DTYMK was assayed as above but in 50 mM Tris-HCl pH 7.5, 2.5 mM MgCl2, 10 mM dithiotreitol (DTT) and 3 mM NaF.

Nucleotide pool, RNA and genomic DNA extraction

TK6 cells (4 x 10^5 cell/ml) seeded in a 24-well plate were treated with the indicated concentration of the nucleotide for 24 h. Cells were treated with 100 µM of isotope-labelled cytidine (15N3) for 4 h before nucleic acid extraction. To remove

extracellular nucleosides for the analysis of intracellular exposure of modified nucleosides, cells were first pelleted by centrifugation for 5 min at 300 x *g* in a 1.5 ml microcentrifuge tube. The supernatant was completely removed and the cell pellet was suspended in 1 ml of ice-cold PBS by gentle vortexing. Cell were then centrifuged for 5 min at 300 x *g* before removing the PBS solution, and the washing step was repeated twice. The intracellular nucleotide pool was extracted by adding 1 ml of ice-cold 80% methanol to the cell pellet. The samples were then vortexed, centrifuged for 10 min at 20000 x *g* and the supernatant was transferred to a new microfuge tube and dried by SpeedVac at 37 °C for 1.5 h. The samples were then diluted in 50 µl of H_2O. For DNA and RNA extraction, the cells were washed 3 times with PBS. DNA was then extracted using the DNaesy Kit (Qiagen) according to the manufacturer protocol. In brief, the cell pellet was suspended in 200 µl of PBS before the addition of 20 µl of Proteinase K, 4 µl of RNase A (100 mg/ml) and 200 µl of lysis buffer provided with the kit. The samples were then mixed by vortexing and incubated at 56°C for 10 minutes and 200 µl ethanol (>99.6%) added to the sample and mixed by vortexing. The mixture was then pipetted into DNeasy Mini spin column (provided with the kit) and centrifuged at 6000 x *g* for 1 minute during which the DNA is bound to the DNeasy membrane. This is followed by two wash steps to remove contaminants. The DNA in the spin column was washed with 500 µl of ethanol-based wash buffer (provided with the kit) and centrifuged for 1 minute at 6000 x *g*. The spin column was placed in a new collection tube and washed with 500 µl of ethanol-based wash buffer (provided with the kit) and centrifuged for 3 minutes at 20000 x *g*. DNA was then eluted by pipetting 200 µl buffer EB onto the DNeasy membrane and incubation at room temperature for 1 minute before centrifugation for 1 minute at 6000 x *g*. Total RNA (longer than 200 nucleotides) was extracted and

purified using RNeasy Mini kit (Qiagen) according to the manufacturer protocol. In brief, the cell pellet was suspended in 350 µl of lysis buffer (provided with the kit) followed by vortexing. The Lysate was pipetted into QIAshredder spin column and centrifuged for 2 min at 20800 x *g*. One volume of 70% ethanol was added to the homogenised lysate and mixed well by pipetting. The mixture was then transferred to an RNeasy spin column and centrifuged for 15 seconds at 8000 x *g*. The column was washed with 350 µl wash buffer (provided with the kit) and centrifuged for 15 seconds at 8000 x *g*. DNase (10 µl) was added to the spin column and incubated at toom temperature for 15 minutes followed by the addition of 350 µl wash buffer (provided with the kit). Columns were then centrifuged for 15 seconds at 8000 x g and washed twice with 500 µl ethanol based wash buffer (provided with the kit). RNA was eluted by addition of 50 µl RNase-free water followed by centrifugation for 1 minute at 8000 x *g*. DNA and RNA and nucleotide pool extract were hydrolysed as described before (Bachman *et al.*, 2014). In brief, DNA or RNA (1-2 µg) was incubated with 5 U of DNA Degradase Plus (Zymo Research) in a total volume of 50 µl for 3 h at 37°C.

LC/MS/MS analysis

Analysis was performed on Acquity UPLC I-class system. The mobile phase solvent A consisted of HPLC graded water containing 0.1% formic acid, and solvent B consisted of acetonitrile containing 0.1% formic acid. Analysis was performed using a mobile phase flow rate of 0.6 ml/min using a linear gradient starting from 2% solvent B to 98% solvent B in 3.5 min and equilibrated as 2% solvent B for 1 min and a Waters XSelect HSS T3, 3.5 µm HPLC column (2.1 x 50 mm) (Waters). Samples were analysed on atmospheric pressure ionization (API) 4000 triple quadrupole mass spectrometer. Nucleosides were monitored in the selected reaction monitoring

(SRM) mode using ion atmospheric pressure ionization at a source temperature of 450 °C and an IonSpray voltage of 4200 V and a collision energy of 30 eV. The dwell time for each monitored transition was 30 ms. The SRM transitions were: dA 252→136, dG 268→152, dC 228→112, T 243→127, A 268→136, G 284→152, C 244→112.1, U 245→127, MOE-A 326→136, MOE-G 342→152, MOE-5mC 316→126, MOE-T 317→127, 2'-FA 270→136, 2'-FG 286→152, 2'-FC 246→112, 2'-FU 247→113. The SRM transitions of nucleoside monophosphates and diphosphates in the in the negative ion mode were: dAMP 330→79, dADP 330→79, dGMP 346→79, dGDP 330→79, dCMP 306→79, dCDP 330→79, TMP 321→79, TDP 330→79, MOE-AMP 404→79, MOE-ADP 330→79, MOE-GMP 420→79, MOE-GDP 330→79, MOE-5mCMP 394→79, MOE-5mCDP 330→79, MOE-TMP 395→79, MOE-TDP 330→79, 2'-FAMP 350→136, 2'-FGMP 366→152, 2'-FCMP 326→112, 2'-FUMP 327→113Data analysis and peak areas were acquired using Analyst Software, v3.3 (Applied BioSystems). The area under curve of the nucleoside analogue and the corresponding native or isotopically labelled nucleosides was obtained from the extracted ion chromatograms and expressed as a ratio.

Statistical analysis

The data were tested for statistical significant differences with one-way ANOVA.

Results

Phosphorylation of 2′-*O*-MOE and 2′-F nucleosides

To be utilised by cellular DNA polymerases for DNA incorporation, nucleosides analogues must be converted to their active triphosphate form via sequential phosphorylation by various kinases in the nucleotide salvage pathway. Therefore, we first examined the ability of salvage enzyme activity to convert of 2′-*O*-MOE nucleosides to their monophosphate forms by liquid chromatography/tandem mass

spectrometry (LC/MS/MS). Calibration curves, correlation coefficient (r^2), retention times and limit of detection (LOD) are shown in Supplementary Figure S1. We examined the substrate activity of the two main salvage enzymes, human deoxycytidine kinase (dCK) and thymidine kinase 1 (TK1), on 2′-*O*-MOE nucleoside analogues. We have chosen dCK and TK1 as both are located in the cytosol and can phosphorylate natural purine and pyrimidine deoxyribonucleosides (Johansson and Eriksson, 1996). In addition, comparisons were made with 2′-F nucleosides widely used in siRNA therapeutics (Watts *et al.*, 2008). To the best of our knowledge, there are no reports on the substrate activity of human dCK and TK1 towards 2′-F nucleosides except 2′-F-C (Kierdaszuk *et al.*, 1999). Structures of 2′-*O*-MOE and 2′-F nucleosides are shown in Figure 1A. The level of conversion was calculated based on relative peak area ratios of the reactant before and after the kinase reaction (supplementary Fig. S2 for extracted ion chromatograms). As shown in Figure 1B, dCK displayed poor reactivity towards 2′-*O*-MOE purine nucleosides and was only able to convert about 5% of 2′-*O*-MOE-G to monophosphate and was not able to convert 2′-*O*-MOE-A at all. Similarly, 2′-*O*-MOE-5meC and 2′-*O*-MOE-T were poor substrates for dCK and TK1 phosphorylating 5% of 2′-*O*-MOE-5mC and 4% of 2′-*O*-MOE-T respectively (Fig. 2, B). In contrast, 2′-F nucleosides were remarkably good substrates for both dCK and TK1, regardless of the nucleobase, exhibiting similar substrate activities (> 95 % conversion) to their respective natural 2′-deoxy nucleosides (Fig. 1, B).

We next examined the capacity of human nucleoside monophosphate kinases including cytidylate kinase 1 (CMPK1), thymidylate kinase (dTMPK), adenylate kinase 1 (AK1) and guanylate kinase 1 (GK1) to phosphorylate 2′-*O*-MOE-5mC, 2′-*O*-MOE-T, 2′-*O*-MOE-A and 2′-*O*-MOE-G monophosphates respectively. As shown

in Fig.1C, AK1, GK1, CMPK1 and dTMPK were not able to create diphosphates of 2′-*O*-MOE modified nucleoside monophosphates regardless of the nitrogenous base. In contrast, AK1 and GK1 were able to phosphorylate (about 95% conversion) dAMP and dGMP controls respectively. Similarly, CMPK1 and DTMK were able to phosphorylate >70% of CMP and TMP, respectively (Fig. 1, C). Extracted ion chromatograms are shown in supplementary figure S3.

Cellular DNA and RNA incorporation of 2′-O-MOE nucleosides

The final substrate required by DNA and RNA polymerases for nucleoside incorporation into DNA and RNA is the nucleoside triphosphate. Therefore, the minimal activity of nucleoside kinases and nucleoside monophosphate kinases on 2′-*O*-MOE nucleoside analogues likely to present a major barrier for their incorporation into cellular DNA and RNA. To confirm this, we asked whether the treatment with 2′-*O*-MOE- or 2′-F-modified nucleosides leads to their DNA and RNA incorporation in TK6 human lymphoblastoid cells. We used TK6 cells as they are human-derived and widely used for genotoxicity testing including the in vitro micronucleus assay and gene mutation assays (Lorge *et al*., 2016). It is important to add that TK6 cells are p53-competent and therefore mount a robust DNA damage response. In addition, the growth characteristics of TK6 cells and their identity (karyotypes and genetic status) are well-characterised. Accordingly, we incubated TK6 cells with different concentrations (10, 250 and 1000 μM) of 2′-*O*-MOE nucleosides or their monophosphate forms for 24 hr. Comparisons were made to 2'-F nucleosides as previous results showed that 2'-F pyrimidines can be incorporated into cellular DNA and RNA (Richardson *et al*., 2002). Following treatment, DNA and RNA were extracted and digested to nucleosides and analysed by LC/MS/MS as described under the method section.

- **Effect of 2′-*O*-MOE and 2′-F nucleosides on DNA and RNA synthesis**

Using the DNA and RNA incorporation of isotopically labelled cytidine C [+3], we first assessed the effect 2′-*O*-MOE and 2′-F nucleoside treatment on DNA synthesis as a measure of cell proliferation and overall DNA transcription (RNA synthesis) by LC/MS/MS. Results were expressed as % of labelled C [+3] in total dC or C. As shown in Fig. 2 B and C, treatment with 2′-*O*-MOE nucleosides or their monophosphate forms had minimal effect on the overall DNA and RNA synthesis even at the highest concentration (1000 μM) with the level of synthesis remaining similar to control untreated cells (*p*-value > 0.05). However, there was a concentration dependent decrease in DNA synthesis following treatment with all 2′-F nucleosides except 2′-F-U (Fig 2, B). For example, treatment with 250 μM and 1000 μM of 2′-F-A resulted in 50% and 92% reduction DNA synthesis compared to control respectively. When cells were treated with 1000 μM of 2′-F-G and 2′-F-C, a sharp decrease (> 90%) in DNA synthesis compared to control was observed (Fig 2, B). Likewise, treatment with all 2′-F nucleoside analogous resulted in a concentration dependent decrease in RNA synthesis (Fig 2, C).

- **Incorporation of 2′-*O*-MOE and 2′-F nucleosides into cellular DNA and RNA**

Using the same LC/MS/MS conditions described above, we next evaluated the genomic DNA and RNA incorporation of 2′-*O*-MOE and 2′-F nucleosides expressed as a ratio of the AUC of modified nucleosides compared to their respective natural equivalents. A schematic of the LC/MS/MS method to evaluate the DNA/RNA incorporation of modified nucleosides is shown in Figure 3, A. Figure 3 (B) shows examples of extracted ion chromatograms derived from hydrolysed DNA and RNA from cells treated with 250 μM of 2′-*O*-MOE-G and 2′-F-G nucleosides. Treating TK6

cells for 24 hours with 2′-*O*-MOE nucleosides or their monophosphates, regardless of the nucleobase, did not result in DNA or RNA incorporation using various concentrations up to 1000 μM. On the other hand, when cells were exposed to 2′-F nucleosides, we were able to detect incorporation of all nucleosides into DNA except 2′-F-U. For example, after treatment with 2′-F-G (250 μM), 12% of total dG contained 2'-F-G modifications (Figure 3, C).

As might be expected even higher levels of incorporation of 2'-F nucleosides were seen for RNA. For example, 2'-F-C nucleosides accounted for as much as 46% of total cytidine content after treatment with 1000 μM of 2'-F-C nucleosides (Fig. 3, C). It should be noted that when we analysed undigested DNA or RNA samples, we did not observe a detectable signal for any of the natural or modified nucleosides suggesting efficient removal of extracellular non-incorporated nucleosides from the DNA or RNA samples before hydrolysis.

- **Intracellular exposure of 2′-*O*-MOE nucleosides and their monophosphates**

To become incorporated into cellular DNA and RNA, nucleoside analogues must display efficient uptake across the plasma membrane. It is possible that the lack of 2′-*O*-MOE nucleosides incorporation into cellular DNA and RNA was due to poor cellular uptake. Therefore, we determined the intracellular exposure of modified nucleotides by measuring the ratio of the signal from the indicated nucleoside to endogenous nucleosides in the nucleotide pool extracts. Incubation of TK6 cells with various concentrations (10, 250 and 1000 μM) of 2′-*O*-MOE for 24 hr resulted in a significant intracellular uptake that was concentration-dependent (Fig. 4). However, 2′-*O*-MOE-A and 2′-*O*-MOE-G showed significantly lower cellular uptake than 2′-*O*-MOE-5mC or 2′-*O*-MOE-T (Fig. 4), the later of which accumulated to higher

concentrations than their native nucleoside counterparts. In general, the cellular uptake of 2′-*O*-MOE nucleoside monophosphates was lower than their respective nucleosides. Nonetheless, we were able to demonstrate intracellular uptake of 2′-*O*-MOE nucleoside monophosphates that was concentration dependent (Fig. 4). Similarly, treating TK6 cells with 2'-F nucleosides resulted in a concentration dependent increases in the cellular uptake up to 1000 μM with lower accumulation of 2'-F-A, -G and –U than 2'-F-C.

Discussion

In the current study, we demonstrated that 2′-*O*-MOE nucleosides are neither effectively phosphorylated by cytosolic nucleoside kinases, nor are they incorporated into cellular DNA or RNA. In contrast, 2′-F modified nucleosides are readily phosphorylated and under our experimental conditions are incorporated at relatively high levels into cellular DNA and RNA, which in turn is accompanied by reductions in DNA and RNA synthesis. To the best of our knowledge, this is the first report evaluating the phosphorylation and DNA/RNA incorporation of 2′-*O*-MOE nucleoside analogues and provide confidence in the safe application, with regard to liberated nucleosides, of oligonucleotide therapeutics containing this modification.

Much of the understanding of nucleoside salvage pathways comes from the area of nucleoside analogues used as antiviral and oncology drugs aimed at inhibiting DNA synthesis and RNA transcription (Sofia, 2011; Van Rompay *et al.*, 2000). These drugs are often given as prodrugs that require phosphorylation by endogenous kinases to their triphosphate forms to become incorporated into viral or cellular DNA. In general, the formation of the monophosphate is the rate-limiting step in the activation of nucleosides drugs.

Our data suggests that the limited activity of nucleoside salvage kinases towards 2′-*O*-MOE nucleosides represents a major barrier for their cellular DNA and RNA incorporation. The absence of DNA and RNA incorporation of 2′-*O*-MOE nucleosides is unlikely to be due to the lack of cell exposure or inhibition of DNA and RNA synthesis as we were able to detect 2′-*O*-MOE nucleosides in the intracellular nucleotide pool and 2′-*O*-MOE treatments even at the highest concentrations used (1000 μM) had minimal effect on DNA and RNA synthesis. In addition, the LC/MS/MS assay described in this paper to detect 2′-*O*-MOE and their respective native nucleosides has a limit of detection in the femtomole range which is in line with previous reports (Bachman *et al*., 2014).

With the exception of 2′-F-U, all 2′-F nucleoside analogues were incorporated into cellular DNA and RNA indirectly suggesting that 2′-F nucleosides were phosphorylated to their respective 5′- triphosphates and incorporated by cellular DNA and RNA polymerases. This is consistent with previous reports showing that human cellular polymerases (pol α and pol γ) could incorporate 2′-F nucleosides into primed DNA in vitro (Richardson *et al*., 2000). Later work from the same lab demonstrated incorporation of 2′-F-C and 2′-F-U into DNA and RNA of various tissues of rats and woodchucks following long-term in vivo treatment (Richardson *et al*., 2002). Although showing almost complete phosphorylation by TK1, in our hands, 2′-F-U nucleosides were not incorporated into cellular DNA and RNA. One potential reason for this lack of incorporation could lie in the limited intracellular exposure observed with the 2'-F-U nucleosides (Figure 4). Also, the calibrated sensitivity of our LC/MS/MS methodology for detection of 2'-F-U was lower than for the other modified nucleosides (Fig. S1), therefore incorporation might have been below our detectable limits. Indeed, the LC/MS/MS assay used by Richardson *et al*. (2002) was able to

detect 2′-F-U as low as 400 pM while the limit of 2′-F-U detection described herein is 10 nM. In addition, it is possible that the difference in 2′-F-U incorporation into DNA/RNA *in vivo* and the lack of incorporation *in vitro* as described in this paper was due to variations in the cellular uptake or the phosphorylation capacity of salvage enzymes. Variations in the phosphorylation and anti-influenza activity of 2′-F nucleosides in different cell lines was previously reported (Tisdale *et al*., 1993), thus it is possible that certain phosphorylating enzymes of the nucleotide salvage pathway are absent or expressed differently in TK6 cells, potentially inhibiting the incorporation of 2'-F-U into cellular DNA/RNA.

In contrast to 2′-*O*-MOE nucleosides, 2′-F nucleosides treatment resulted in concentration dependent inhibition of DNA synthesis which was evident with 2′-F-A, 2′-F-G and 2′-F-C. Several previous studies showed that 2′-F-C inhibits cell growth and induces cytostasis due to an S-phase arrest (Brox *et al.*, 1974; Stuyver *et al.*, 2004). Similarly, data presented here showed that 2′-F-C reduced DNA synthesis by about 30% at 250 μM (Fig. 3, B). This inhibitory effect on cell growth could be promoted by genomic DNA incorporation potentially leading to double strand DNA breaks (Shen *et al.*, 2015). Yet, we cannot exclude other mechanisms of toxicity including disruption of the endogenous nucleotide pool balance or disruption of mitochondrial DNA synthesis (Kunz *et al.*, 1994; Pan-Zhou *et al.*, 2000). Further studies are needed to understand the underlying mechanisms responsible for this activity of the 2′-F nucleosides. However, taken together, these data suggest that caution should be applied when considering the use and application of 2′-F modifications in therapeutic oligonucleotides.

Although non-natural 2′-sugar modifications of therapeutic oligonucleotides can enhance target binding affinity and nuclease stability, the potential of 2′-nucleosides

analogues to become incorporated into genomic DNA potentially leading to DNA replication errors or mutations raises a safety concern for therapeutic oligonucleotides. In general, 2′-F nucleoside analogues are commonly used in siRNA-based therapeutics to increase binding affinity and reduce immune activation (Watts *et al*., 2008). However, only a limited number of nuclease resistant phosphorothioate linkages can be tolerated while still providing siRNA efficacy. Moreover, while 2′-F modification confers increased hybridisation affinity, it does not confer nuclease resistance (Manoharan, 1999). These two factors are likely to increase the potential of 2′-F catabolism and nucleoside release and therefore subsequent conversion by cellular kinases to their triphosphate forms for incorporation into newly synthesised DNA. This could have genotoxic consequences as 2′-F sugar modifications have been demonstrated to destabilise B-DNA duplex by inducing a more A-like conformation (Ikeda *et al.*, 1998). Furthermore, once incorporated into DNA, 2′-F nucleosides appear resistant to normal mechanism of DNA repair as a fluorine at C2′ blocks the activity of glycosylases, thus inhibiting base excision repair (BER) for these analogues (Schroder *et al.*, 2016; Su *et al.*, 2016). Notwithstanding these considerations, chronic safety studies in rats and woodchucks have shown that despite 2′-F-U and 2′-F-C incorporation into tissue DNA and RNA, there are minimal toxicological consequences of the incorporation (Richardson *et al.*, 1999; Richardson *et al*., 2002). Furthermore, to our knowledge the siRNA constructs that utilize the 2′-F modified nucleosides have been negative in genetic toxicity studies, although there are limited primary references from which to draw (Berman *et al*., 2016; Janas *et al.*, 2016).

In contrast to the more metabolically labile siRNA, single stranded antisense oligonucleotides are usually designed with phosphorothioate backbone linkages

throughout the molecule with the modified nucleotides at terminal ends (e.g. 2′-*O*-MOE) providing further nuclease resistance (Bennett and Swayze, 2010). Therefore, the nucleosides concentrations used in this in vitro study are unlikely to be achieved following *in vivo* administration due to the slow degradation of oligonucleotides (Watanabe *et al.*, 2006). Ultimately, the 2'-*O*-MOE modified wings are largely excreted in urine with limited degradation of the modified nucleotides (Geary *et al.*, 2001b). Although they are not incorporated into DNA and RNA, 2′-*O*-MOE nucleosides could exert mutagenic consequences via a mechanism other than direct DNA incorporation such as nucleotide pool balance disruption although this potential mechanism is unlikely given the lack of phosphorylation, the slow rate of metabolism and the consistent lack of genotoxicity that has been reported (Berman *et al*., 2016; Henry *et al*., 2002).

The utility of non-natural 2′-sugar modification in oligonucleotide-based gene silencing technologies has proven valuable for their clinical development. These 2′-sugar modifications confer an RNA-like C3′-endo conformation which enhances the binding affinity to the target RNA (Kawasaki *et al*., 1993). The chemical structure of 2′-*O*-MOE and 2′-F nucleosides analogous closely mimics the natural nucleosides where the nucleobase component is unmodified. It is possible that the reduced steric interactions of the less bulky fluorine atom combined with its higher hydrophobicity is likely responsible for the higher ability of kinase salvage enzymes and cellular polymerases to accept 2′-F nucleoside analogue as a substrate resulting in their incorporation into genomic DNA. Further understanding of structure-activity relationship will help in the design of novel chemical modifications at the 2′-postion of the sugar moiety with improved chemical and biological stability.

In conclusion, we found that 2′-*O*-MOE nucleoside analogues are poor substrates for salvage enzymes largely preventing the formation of their corresponding active 5′-triphosphates and subsequent incorporation into cellular DNA and RNA. To our knowledge, this is the first study exploring the phosphorylation and DNA incorporation of 2′-*O*-MOE modified nucleotides. It is clear from the current study that there is a link between the substrate specificity of nucleotide salvage enzymes towards 2′-sugar modified nucleoside analogues and cellular DNA incorporation. We believe the current work provides insight for the design of nucleoside analogues with higher activity and improved safety profile when incorporated into therapeutic oligonucleotides.

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Figure legends

Figure 1. Phosphorylation of 2′-modified nucleoside analogues. A) Molecular structures of deoxyribonucleosdies, 2′-O-MOE and 2′-F nucleoside analogues, B) The ability of dCK and TK1 to phosphorylate was analysed by incubating 200 μM of the indicated nucleoside with 1 mM ATP and 1 μg of dCK (for dA, dG, dC and their 2′-*O*-MOE analogues) or TK1 (for T and 2′-O-MOE-T) in a 50 μl reaction volume at 37 °C for 2 h. The samples were then then analysed by LC/M/SMS and the % conversion was calculated based on the peak area of the substrate before and after the addition of the enzyme. C) The ability of cytidylate kinase 1 (CMPK1), thymidylate kinase (dTMPK), adenylate kinase 1 (AK1) and guanylate kinase 1 (GK1) to phosphorylate natural and 2′-*O*-MOE-modified CMP, TMP, dAMP, dGMP

respectively was analysed by as indicated above. N.D, not detected. Means ± S.D. of three independent experiments are indicated.

Figure 2. Effect of 2′-*O*-MOE and 2′-F nucleosides on cellular DNA and RNA synthesis.. A) DNA synthesis: TK6 cells were incubated with various concentrations of 2′-*O*-MOE, 2′-*O*-MOE monophosphate and 2′-F nucleosides for 24 h. The extracted DNA from cells were hydrolysed to nucleoside and the base composition (dC, dC+3) was then analysed by LC/MS/MS. Data show the abundance of dC+3 relative to dC. B) RNA synthesis: TK6 cells were incubated with various concentrations of 2′-*O*-MOE, 2′-O-MOE monophosphate and 2′-F nucleosides for 24 h. The extracted RNA from cells were hydrolysed to nucleoside and the base composition (C and C+3) was then analysed by LC/MS/MS. Data show the abundance of C+3 relative to C. Means ± S.D. of three independent experiments are indicated. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ as compared with control untreated cells.

Figure 3. A) Schematic of the LC/MS/MS method to evaluate nucleoside analogue incorporation into cellular DNA and RNA. B) Extracted ion chromatograms of nucleosides from digested DNA and RNA of TK6 cells treated with 250 μM of 2′-*O*-MOE-G and 2′-F-G nucleosides for 24 hr analysed by LC/MS/MS. C) Incorporation of 2′-F nucleosides into cellular DNA and RNA. TK6 cells were treated with various concentrations of 2′-F for 24h. The DNA and RNA was then extracted and hydrolysed to nucleosides. Incorporation of 2′-F nucleosides was expressed as the ratio of AUC of 2′-F nucleosides relative to their natural counterpart. Means ± S.D. of three independent experiments are indicated. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. ns, not significant.

Figure 4. Cellular exposure to 2′-*O*-MOE and 2′-F nucleosides analogous. TK6 cells were treated for 24 hr with various concentrations of 2′-*O*-MOE, 2′-O-MOE monophosphate and 2′-F nucleosides. The intracellular nucleotide pool was then extracted and digested to nucleosides. The cellular uptake was expressed as the ratio of AUC of modified nucleosides relative to their natural counterpart. Means ± S.D. of three independent experiments are indicated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ as compared with control untreated cells.

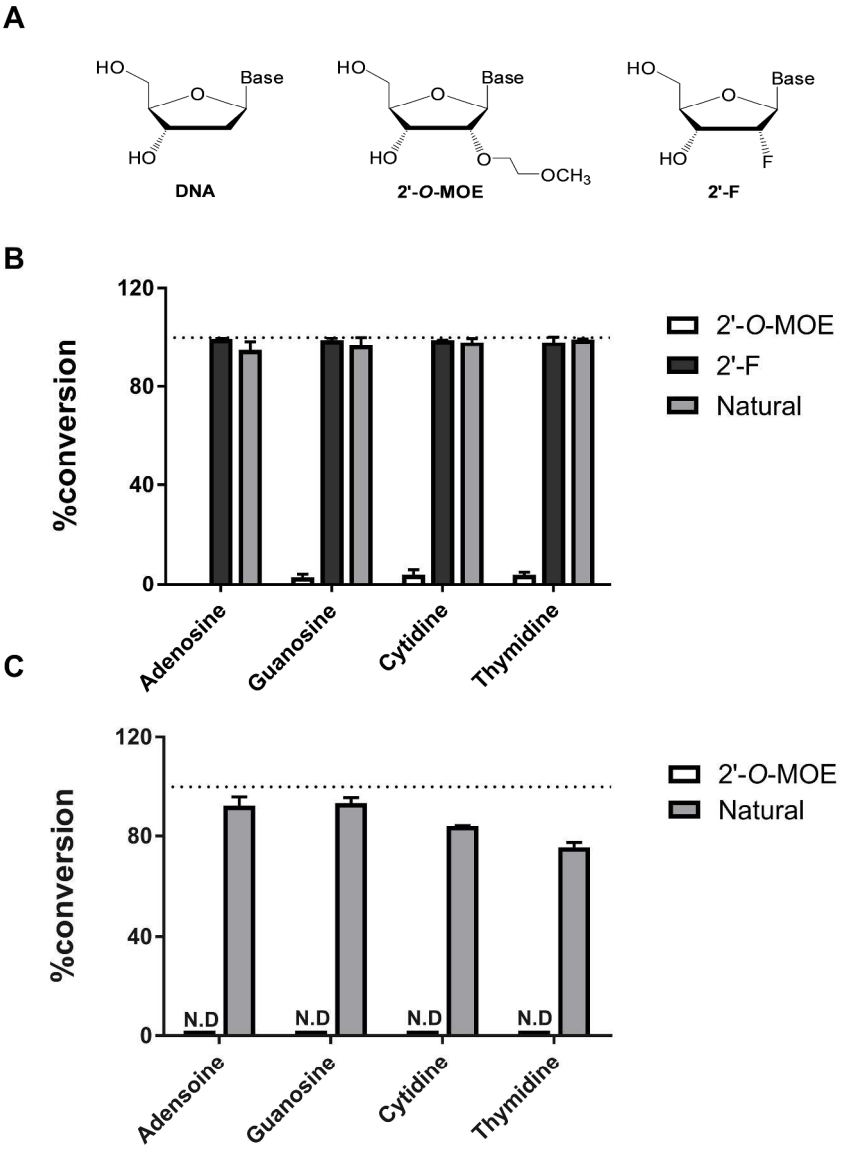

Figure 1.

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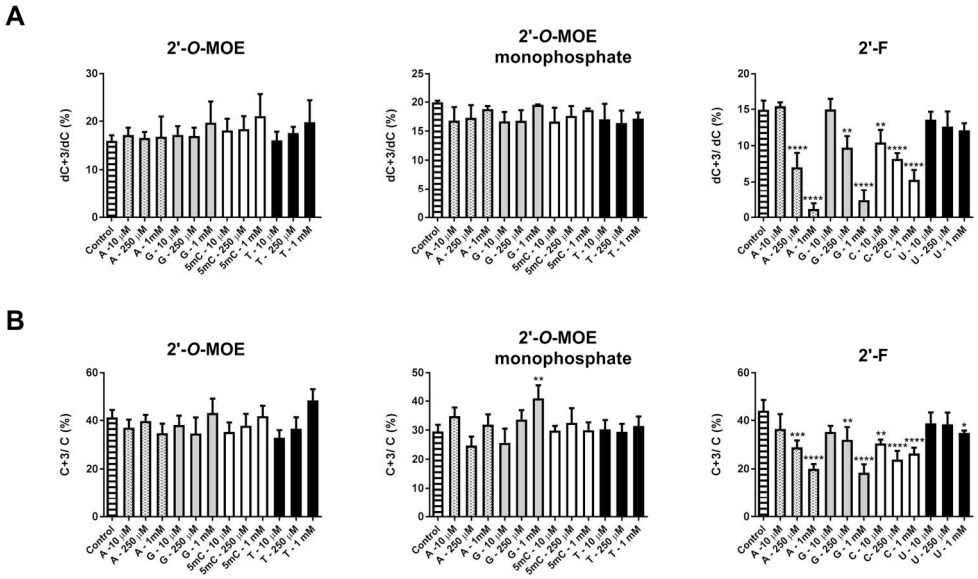

Figure 2

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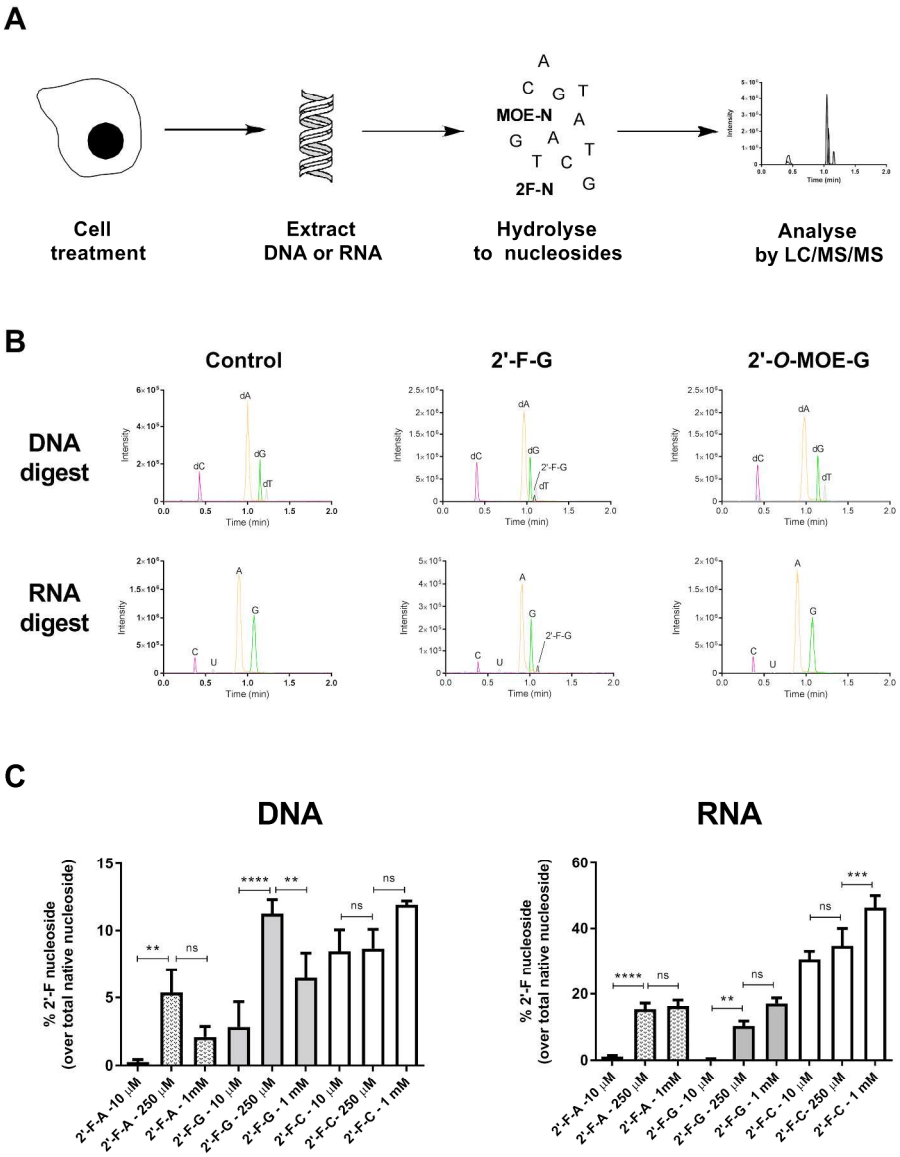

Figure 3

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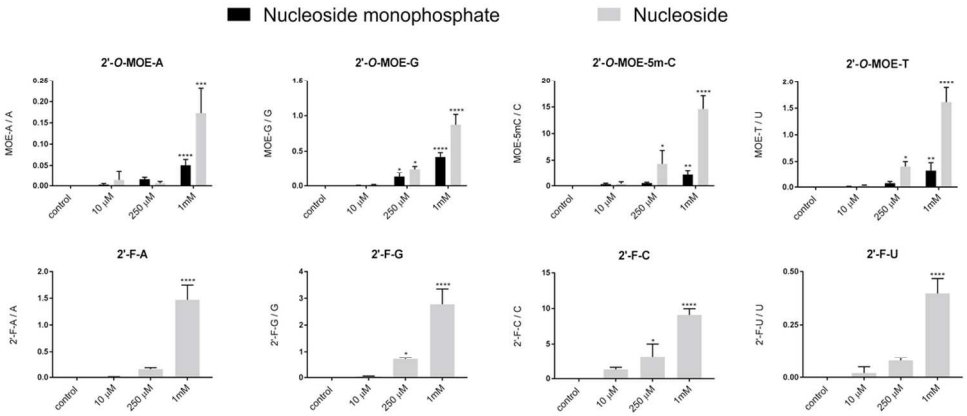

Figure 4

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