In Vivo Metabolite Profiles of an N-Acetylgalactosamine–Conjugated Antisense Oligonucleotide AZD8233 Using Liquid Chromatography High-Resolution Mass Spectrometry: A Cross-Species Comparison in Animals and Humans

Xue-Qing Li, Marie Elebring, Anders Dahlén, and Lars Weidolf

ABSTRACT

AZD8233, a liver-targeting antisense oligonucleotide (ASO), inhibits subtilisin/kexin type 9 protein synthesis. It is a phosphorothioated 3'-10-3 gapmer with a central DNA sequence flanked by constrained 2'-O-ethyl 2',4'-bridged nucleic acid (cEt-BNA) wings and conjugated to a triantennary N-acetylgalactosamine (GalNAc) ligand at the 5'-end. Herein we report the biotransformation of AZD8233, as given by liver, kidney, plasma and urine samples, after repeated subcutaneous administration to humans, mice, rats, rabbits, and monkeys. Metabolite profiles were characterized using liquid chromatography high-resolution mass spectrometry. Metabolite formation was consistent across species, mainly comprising hydrolysis of GalNAc sugars, phosphodiester-linker hydrolysis releasing the full-length ASO, and endonuclease-mediated hydrolysis within the central DNA gap followed by exonuclease-mediated 5'- or 3'-degradation. All metabolites contained the 5'- or 3'-cEt-BNA terminus. Most shorter metabolites had the free terminal alcohol at 5'- and 3'-positions of ribose, although six were found retaining the terminal 5'-phosphorothioate group. GalNAc conjugated shorter metabolites were also observed in urine. Synthesized metabolite standards were applied for (semi)quantitative metabolite assessment. Intact AZD8233 was the major component in plasma, whereas the unconjugated full-length ASO was predominant in tissues. In plasma, most metabolites were shortmers retaining the 3'-cEt-BNA terminus, whereas metabolites containing the 5'- or 3'-cEt-BNA terminus were detected in both tissues and urine. All metabolites in human plasma were also detected in all nonclinical species, and all human urine metabolites were detected in monkey urine. In general, metabolite profiles in animal species were qualitatively similar and quantitatively exceeded the exposures of the circulating metabolites in humans at the doses studied.

SIGNIFICANCE STATEMENT

This study presents metabolite identification and profiling of AZD8233, an N-acetylgalactosamine-conjugated antisense oligonucleotide (ASO), across species. A biotransformation strategy for ASOs was established by utilizing biologic samples collected from toxicology and/or clinical studies and liquid chromatography high-resolution mass spectrometry analysis without conducting bespoke radiolabeled absorption, distribution, metabolism, and excretion studies. The generated biotransformation package was considered adequate by health authorities to progress AZD8233 into a phase 3 program, proving its applicability to future metabolism studies of ASOs in drug development.
mainly metabolized via cytochrome P450 mediated phase I and conju-
gative phase II enzymes, ASOs are typically metabolized by exo- and
endonuclease-mediated hydrolysis to form truncated oligonucleotides
(Migliorati et al., 2022; Takakusa et al., 2023). Although it was reported
that pyrimidine-rich ASO sequences were more prone to endonuclease
hydrolysis than purine-rich oligonucleotides (Crooke et al., 2000), the
relationship of nuclease specificity versus sequence-based nucleotide bond
hydrolysis of therapeutic oligonucleotides remains largely unclear. Fur-
thermore, with the introduction of chemically modified nucleotide build-
ing blocks into ASO sequences, as well as the application of the ligand-
ASO conjugate concept to enhance druglike properties and achieve tar-
ged drug delivery (Andersson et al., 2018; Benizri et al., 2019; Bennett,
2019; Papargyri et al., 2020), it is important to understand the impact of
the chemical modifications on the metabolic fate of the newer generations
of ASOs, and consequently their implication on their efficacy and safety.
Therapeutic ASOs are so far classified as three generations: Gen 1.0,
replacing the phosphate (PO) backbone with a phosphorothioate (PS)
backbone; Gen 2.0, introducing 2′-O-methoxymethyl (2′MOE) on the ri-
boside, as well as the so-called Gen 2.5 by using 2′,4′-locked ribose moi-
eties (e.g., LNA and cEt-BNA) to increase target RNA affinity and nuclease resistance (Crooke, 2000; Benizri et al., 2019; Bennett, 2019;
Crooke et al., 2021; Shadid et al., 2021); and more recently Gen 3.0,
introducing mesylphosphoramidate internucleotide linkages on top of
previous generation modifications for enhanced activity and safety perfor-
mance (Zhang et al., 2022). Several ASO conjugates have been de-
veloped to achieve targeted drug delivery. The most successful example
is the triantennary N-acetylgalactosamine (GalNAc) cluster-conjugated
ASO that achieved target delivery to the liver with up to 20- to 30-fold in-
creases of potency (Wang et al., 2019). AZD8233, a GalNAc-conjugated
ASO, was developed to inhibit the hepatic enzyme proprotein conver-

tase subtilisin/kexin type 9 (PCSK9) and thereby decrease low-density
lipoprotein cholesterol levels and ultimately the risk of cardiovascular
termini and 10 DNA nucleotides in the central part. Additionally, the
5′-end of the ASO is conjugated to a GalNAc triantennary cluster via a
phosphodiester bond.
In this study, our goal was to investigate the biotransformation of
AZD8233 in animal species and in humans. Because the GalNAc trianten-
nary linker moiety in AZD8233 structure was previously applied in an-
other ASO conjugate, ION-681257, and the biotransformation of this
GalNAc-linker moiety was well described by Shemesh et al. (2016), the
focus of this study was therefore to compare the ASO-related metabolite
profiles of AZD8233 across species. Our internal strategy for biotransfor-
mation studies of ASO drug candidates described in this paper was ap-
plied. Liver, kidney, plasma, and urine samples obtained from the main
study animals in toxicology studies of AZD8233 after repeated dosing
were used to characterize metabolite profiles and compared with those in
human plasma and urine samples collected from a clinical multiple as-
cending dose (MAD) study. Metabolite characterization and quantification
in various sample matrices were achieved by applying liquid chromatogra-
phy high-resolution mass spectrometry (LC-HRMS) methodology without
conducting radiolabeled absorption, distribution, metabolism, and excretion
(ADME) studies. A full package of biotransformation data was generated
and supported clinical trial applications in drug development.

Materials and Methods

Test Compound

AZD8233 (Fig. 1) is a 16 nucleotide, 3-10-3 cEt-DNA-cEt gapmer, with a
full phosphorothioate backbone (i.e., three cEt-BNA-modified ribosyl nu-
clotides are flanking the 5′- and 3′-ends of a 10 DNA stretch). The 5′-end is conjugated
to a GalNAc triantennary cluster via a phosphodiester bond. The conjugate was
synthesized at AstraZeneca R&D Gothenburg, Sweden.

Chemicals and Reagents

Reference oligonucleotide shorter 5′ 3mer (5′-AA3′U) was obtained from
Ionis Pharmaceuticals (CA). Optima LC-MS grade methanol and 1,1,1,3,3,3-hex-
fluoro-2-propanol (HFIP) were obtained from Fisher Scientific (Loughborough,
UK). Triethylamine (TEA), ammonium hydroxide solution (28%–30%), phenol-
chloroform-isomylalcohol mixture (25:24:1, v/v), EDTA, and bovine serum

![Fig. 1. Sequence and structure of AZD8233 (GN-5′-AAMeUAATMeCTMeCATGTMeCAG-3′), a GalNAc (GN)-conjugated 16-nucleotide 3-10-3 gapmer with a full phosphorothioate backbone and constrained 2′-O-ethyl-2′,4′-bridged nucleic acid modification (cEt-BNA) at positions 1–3 and 14–16 (underlined). A, adenine; G, gua-
nine; GNs, a GalNAc triantennary cluster with three N-acetylgalactosamine sugars attached; Mc, 5-methyl cytosine; MeU, 5-methyl uracil; T, thymine.](image-url)
albinum (BSA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). 1,2-dichloroethane was obtained from Merck (Darmstadt, Germany). Ultra-pure water was obtained from an in-house Milli-Q water purification system (Merck KGaA, Darmstadt, Germany). All other chemicals and solvents were of the highest quality commercially available.

Nomenclature of Oligonucleotide Metabolites

The nomenclature of the oligonucleotide metabolites in this study was based on their length and the retention of the 3'-5' cEt-BNA terminal. For example, a metabolite sequence of 5'-wing AA-5'-BNA is described as the 5'-3'mer (i.e., defining a trimethylene metabolite with the retention of the 5'-cEt-BNA terminal). Furthermore, if not stated otherwise, the metabolite is a cleavage product with free hydroxyl groups at the 5'-3' terminal. A terminal phosphorothioate retained on the cleavage product is designated by the prefix "ps".

Synthesis of Oligonucleotide Shortmer Metabolites and Unconjugated ASO

A summary of dosing regimens of the samples analyzed from mice, rats, rabbits, monkeys, and humans is given in Table 2. All animal studies were performed with humane care, and all protocols and procedures were approved by the respective country and institutional animal care and use committees. The human study was conducted in accordance with the principles of the Declaration of Helsinki, the International Council for Harmonization Good Clinical Practice, and all applicable regulatory requirements. The clinical study was conducted at Parexel Early Phase Clinical Unit (Los Angeles, CA) and registered at clinicaltrials.gov with the identifier NCT04155645.

In nonclinical toxicology studies, 0.2–0.4 ml blood was collected into EDTA tubes at selected time points after multiple subcutaneous drug administration to mice, rats, rabbits, and monkeys. Plasma samples were separated for analysis. At the sacrifice time points after the last dose, animal liver and kidney cortex samples were collected and weighed (0.1–0.3 g) for analysis. Monkey urine samples were collected at the selected time intervals. All samples were stored at −80°C until analysis.

In brief, the clinical study included male and female subjects, 18–65 years of age, with documented dyslipidemia, with or without type 2 diabetes. Subjects were assigned to 8 weeks of multiple dosing of AZD8233 saline solution for subcutaneous injection. Plasma and urine samples were collected on day 57 in all subjects.

Sample Preparation

Biotransformation of AZD8233 was studied in samples from selected dose groups in animals including the dose group at No-Observed-Adverse-Effect Level (NOAEL), and in humans after multiple s.c. administration of AZD8233 at 90 mg once monthly. Pooled tissue homogenate, urine, and plasma samples were prepared across individuals in each dose group for metabolite identification and profiling.

Liver and Kidney Tissue Homogenization. Preweighed liver and kidney tissue samples (0.1–0.3 g) were placed in Precellys 2- or 7-ml reinforced tubes. Six 3-mm diameter ceramic balls were added to each tube. Ice-cold water was added to sample tubes at a ratio of 1:5 tissue weight to water volume. The samples were homogenized using a Precellys 24 homogenizer (Bertin Corp.), 2 × 20 seconds, 5000 rpm, with a 20-second pause between the intervals, and then placed on ice. When needed, the process was repeated until the samples were homogenized. Liver and kidney homogenates were then pooled equimetrically across individuals to obtain one pool of each type from each dose group.

Plasma. Plasma 0- to 24-hour pools were prepared using the trapezoidal area under the curve (AUC) pooling method (Hamilton et al., 1981; Hop et al., 1998) in each dose group and species. Plasma samples were first pooled across individuals at each time point between 0 and 24 hours by equivolumetric mixing. Subsequently, one single pool was prepared by mixing volumes in proportion to the sampling timespan to give one AUC0-24 h pool. A predose plasma pool was prepared by equivolumetric mixing across individuals.

Urine. Pooled urine samples were prepared across individuals and collection intervals in proportion to the total weight of the samples collected. A predose pool of human urine was prepared by equivolumetric mixing across individuals.

TABLE 1

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Sequence (5' to 3')</th>
<th>Length</th>
<th>MW</th>
<th>Purity</th>
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<tbody>
<tr>
<td>AZD8233</td>
<td>GN- AA\textsubscript{4}MeUAAT\textsubscript{5}M\textsubscript{5}T\textsubscript{4}C\textsubscript{5}M\textsubscript{4}CATG\textsubscript{5}M\textsubscript{4}CAG</td>
<td>16</td>
<td>6919.2</td>
<td>92%</td>
</tr>
<tr>
<td>Unconjugated ASO</td>
<td>AA\textsubscript{4}MeUAAT\textsubscript{5}M\textsubscript{5}T\textsubscript{4}C\textsubscript{5}M\textsubscript{4}CATG\textsubscript{5}M\textsubscript{4}CAG</td>
<td>16</td>
<td>5399.6</td>
<td>97%</td>
</tr>
<tr>
<td>5' 3mer</td>
<td>AA\textsubscript{4}MeU</td>
<td>3</td>
<td>1026.2</td>
<td>96%</td>
</tr>
<tr>
<td>5' 8mer</td>
<td>AA\textsubscript{4}MeUAAT\textsubscript{5}M\textsubscript{5}C</td>
<td>8</td>
<td>2645.2</td>
<td>95%</td>
</tr>
<tr>
<td>5' 9mer</td>
<td>AA\textsubscript{4}MeUAAT\textsubscript{5}M\textsubscript{5}C</td>
<td>9</td>
<td>2964.5</td>
<td>95%</td>
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<tr>
<td>5' 15mer</td>
<td>AA\textsubscript{4}MeUAAT\textsubscript{5}M\textsubscript{5}C\textsubscript{5}M\textsubscript{4}CATG\textsubscript{5}M\textsubscript{4}CA</td>
<td>15</td>
<td>5012.2</td>
<td>95%</td>
</tr>
<tr>
<td>3' 3mer</td>
<td>T\textsubscript{5}M\textsubscript{4}CAG</td>
<td>3</td>
<td>1042.0</td>
<td>99%</td>
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<tr>
<td>3' 4mer</td>
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<td>4</td>
<td>1362.2</td>
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<tr>
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<td>2357.0</td>
<td>96%</td>
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<td>8</td>
<td>2676.3</td>
<td>96%</td>
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</table>

GNs, a GalNAc triantennary cluster with three N-acetylgalactosamine sugars attached. M, 5-methylated cytosine; U, 5-methylated uracil; underline letter, phosphorothioate oligonucleotides with constrained 2'-O-ethyl 2', 4'-bridged nucleic acid; MW, molecular weight.
Metabolite Identification and Profiling by LC-HRMS

Preparation of Calibration Standards. A series of standard solutions containing a mixture of AZD8233 and its 11 synthesized oligonucleotide metabolite standards were prepared at concentrations ranging from 0.25 to 40 μM in 5% bovine serum albumin (BSA) aqueous solution. Pooled plasma, urine, liver, and kidney homogenates prepared from naive animals (control group) and pooled human plasma and urine samples from placebo subjects were used as blank biofluids.

Sample Preparation Using Liquid-Liquid Extraction. A Bravo automated liquid handling platform (Agilent Technologies, CA) was used for sample extraction in 96-well plates. Due to the high capacity of the automated sample preparation, all samples, including urine, were subjected to robot sample handling prior to analysis for metabolite profiling and identification. The liquid-liquid extraction (LLE) preparation also avoided adsorption of ASOs to plastic surfaces frequently observed with direct analysis of urine samples. An aliquot of 100 μl tissue homogenate, 100- to 400-μl urine sample, or 50- to 200-μl plasma sample was first diluted by water to a final volume of 400 μl. Then 150 μl ammonia solution was added and vortex mixed for 5 minutes, followed by the addition of 390 μl phenol/chloroform/isoamyl alcohol (25:24:1) to each sample well and pipette mixing for 50 cycles. After centrifugation of the sample plate at 4000 rpm, 4°C, for 10 minutes, an aliquot of 450 μl of the upper aqueous layer was transferred to a new plate and further purified by adding 300 μl dichloromethane, pipette mixing for 50 cycles, and again centrifuged at 4000 rpm, 4°C, for 10 minutes. The resulting aqueous layer was separated, and 350 μl was taken into a new plate and dried under gentle nitrogen flow at room temperature. The extract residue was reconstituted in 200 μl of 50 μM EDTA aqueous solution containing 10% methanol. Depending on the sample concentration and MS response, further 5- or 10-fold dilutions were made, when necessary, using the same EDTA solution before LC-HRMS analysis.

Preparation of Calibration Standards. A series of standard solutions containing a mixture of AZD8233 and its 11 synthesized oligonucleotide metabolite standards were prepared at concentrations ranging from 0.25 to 40 μM in 5% bovine serum albumin (BSA) aqueous solution. Pooled plasma, urine, liver, and kidney homogenates prepared from naive animals (control group) and pooled human plasma and urine samples from placebo subjects were used as blank biofluids. Blank matrices were spiked with the standard solutions to construct the calibration samples at a series of concentrations from 0.002 up to 500 nM depending on sample matrices. Calibration samples were subjected to their respective work-up procedure and treated identically to actual study samples.

Sample Preparation Recovery. AZD8233 and its 11 synthesized oligonucleotide metabolite standards were used to examine LLE sample preparation recovery. Stock solutions of the mixture of 12 standards were spiked into blank human plasma or monkey liver homogenates to final concentrations of 80 and 400 nM. Samples were mixed and subjected to LLE extraction. For comparison, blank plasma or liver homogenates were treated using the LLE method directly. After sample evaporation to dryness, solutions of standard mixture were added in the blank matrix residue. All sample extracts were then reconstituted in aqueous solution containing 10% methanol and 50 μM EDTA and mixed thoroughly before analyzed by LC-HRMS. Peak areas of each analyte in samples with standard mixture added before and after the LLE preparation were integrated to calculate sample preparation recovery.

Sample Analysis by LC-HRMS

All extracted samples were analyzed using an ACQUITY UPLC system connected to a SYNAPT G2-Si Q-TOF mass spectrometer (Waters, Milford, MA). The software used for instrument control and data acquisition was MassLynx (version 4.1; Waters). Chromatographic separations were performed on an ACQUITY Premier Oligonucleotide C18 column (130 Å, 1.7 μm, 2.1 mm × 100 mm; Waters). The injection volume was 10 μl, and the column temperature was set at 60°C. Mobile phase A consisted of 200 mM HFIP/7.5 mM TEA in water, and mobile phase B consisted of 200 mM HFIP/7.5 mM TEA in methanol. A gradient elution profile was used, starting at 5% B for 1 minute, continuing with a linear increase to 15% B in 3 minutes, followed by a linear increase to 45% B during 6 minutes. Then, the fraction of mobile phase B was increased to 80% in 0.5 minutes and maintained for 2 minutes before returning to initial conditions and reequilibrating for 4 minutes before the injection of the next sample. The total run time was 17 minutes. Flow rate was set to 0.4 ml/min.

The urine pools in monkey (0- to 16-hour) and human (predose and 0- to 24-hour) on day 57 were analyzed for metabolite profiles.

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in continuum mode on selected samples to conduct data processing using ProMass for MassLynx (version 2.0; Novatia LLC, Newtown, PA).

To compare software-assisted data processing for oligonucleotide metabolite identification, selected samples from the monkey study were analyzed using an Orbitrap Exploris 480 mass spectrometer coupled to a Vanquish binary LC system and controlled by Xcalibur software version 4.5.445.18 (Thermo Fisher Scientific, Bremen, Germany). Details of the LC-MS conditions are described in the Supplemental Method.

Data Analysis

For metabolite identification, a list of theoretical masses of AZD8233, its unconjugated ASO and expected shortimer ASO metabolites formed via the hydrolysis of the phosphodiester bonds, was calculated at various charge-states with the aid of an Excel-based calculation tool for ASOs, ProMass, or MassLynx (v. 4.1; Waters) wherever applicable. The extracted ion chromatogram (XIC) of full-scan MS (Function 1, F1) were then generated using the theoretical masses of expected metabolites. The XIC of the diagnostic fragment ions of [O2PS]− (m/z 94.936) or cEt-BNA fragments (cEt-A, m/z 370.038, C12H14N5O6PS; cEt-MeU, m/z 361.028, C12H14N5O6PS; cEt-McC, m/z 360.042, C12H14N5O6PS; and cEt-G, m/z 386.033, C12H14N5O6PS) were extracted from the high energy MS² function (F2 and/or F3) with a mass tolerance of 5 mDa. Chromatographic XIC peaks of the fragment ions m/z 94.936 aligning with the retention time of peaks in the XICs of precursor ion scan function F1 indicated the presence of AZD8233 and its oligonucleotide metabolites in the analyzed samples. When a significant chromatographic peak from the m/z 94.936 trace did not correspond to an expected ASO metabolite peak in the F1 XICs (i.e., indicating the presence of an unexpected metabolite), the MS spectra in F1 at the indicated retention time was extracted to obtain precursor ions for metabolite identification.

For quantification and semiquantification of ASO and metabolites, TargetLynx (v. 4.1; Waters) was used for data processing. Mass chromatographic peaks of AZD8233 and its metabolites were integrated for quantitative analyses with a mass tolerance of 50 mDa. Concentrations of AZD8233 and metabolites in tissue homogenates, plasma, and urine were determined using 12 calibration standards [i.e., intact AZD8233, unconjugated ASO, six synthesized nucleotide shortimer standards retaining the 3′ cEt-BNA terminal (3′- to 8mer), and four synthesized shortimer standards retaining the 5′ cEt-BNA terminal (5′- to 15mer)]. For metabolite of which there was no synthesized standard available, a calibration curve of the parent or a shorter standard of a similar length at the same ion-charge state and bearing the same 3′- or 5′-cEt-BNA terminus when possible was used as a surrogate for its semiquantitative estimation. When ASO diastereoisomers appeared as split chromatographic peaks, their total peak areas were integrated for quantification. The total drug-related material (DRM) in each sample was therefore calculated as the sum of molar concentrations of AZD8233 and its (semi)quantified metabolites; their relative abundance (% DRM) was calculated using the molar concentration of each metabolite divided by the summed total DRM concentration.

Software-assisted data processing was performed in selected samples using ProMass software for Waters Q-TOF MS data and BioPharma Finder (BPF) V5.1 (Thermo Fisher Scientific) for Orbitrap Exploris 480 MS data. Deconvoluted MS spectra were generated to give accurate mass of the metabolites and compared with their corresponding theoretical masses. Using BioPharma Finder intact oligonucleotide analysis, the mass tolerance for sequence matching was set to 20 ppm; and for the sequencing analysis, the mass accuracy was set at 6 ppm and minimum confidence at 0.8.

Results

Analytical Strategies for AZD8233 Metabolite Identification and Profiling Using LC-HRMS

To establish analytical strategies for AZD8233 ASO metabolite identification and profiling, LC-HRMS characteristics of AZD8233 and its 11 synthetic ASO metabolite standards of three to 16 oligonucleotides in length were studied (Supplemental Fig. 1). Under the applied LC-HRMS conditions, observed molecular charge distributions (i.e., charge envelope) of parent and ASO metabolites were between one and eight, with the most abundant charge states being one to three depending on the length of the ASO sequences. LC retention times of these unconjugated ASO metabolites depended on the length of the oligonucleotide, where the retention time decreased with decreasing length. Because of the chirality of the phosphorothioate ASO backbone, the chromatographic peaks of the short ASO metabolites appeared as split, indicating their diastereoisomeric features. MS/MS or high-energy MS² spectra of the ASO standards showed complicated fragment patterns. However, a few distinct product ions were observed (Fig. 2). Consistent with the reported phosphorothioate backbone-specific [O_PS]− fragment detected in PS-ASOs (Husser et al., 2017; Romero-Palomo et al., 2021), the corresponding product ion at m/z 94.936 was detected in the high energy MS² spectra of all synthesized standards, particularly in MS function F3 with target enhanced fragmentation, leading to increased signal-to-noise ratio. The resulting XIC chromatograms of the m/z 94.936 fragment ion were therefore used to guide ASO metabolite identification. In addition, AZD8233 contains modified cEt-BNA nucleotides at both 5′ and 3′ wings. These cEt-BNA nucleotides are unique and not present in endogenous nucleotides. Therefore, the observed cEt-BNA specific fragment ions generated using high energy MS² function F2 (i.e., cEt-A in both wings, cEt-McC from the 5′ end, cEt-McC, and cEt-G from the 3′ end) were also unique diagnostic ions assisting the identification of metabolites retaining the 3′ or 5′ cEt-BNA wings. As a result, identification of AZD8233 ASO metabolites was established with the following criteria: 1) the agreement of observed and expected metabolite spectra of singly and multiply charged precursor ions (charge envelope) and their isootope distribution (isootope envelope) in full-scan HRMS spectra, 2) the concurrence of metabolite XIC peaks with the corresponding XIC peaks of diagnostic fragment ions of m/z 94.936 and/or cEt-BNA product ions, and 3) the nucleotide length-dependent LC retention times. The assignment of MS/MS fragments were performed for synthetic ASO references and a few identified metabolites in biologic samples using BioPharma Finder sequence analysis. ASO sequence-specific backbone cleavages (McLuckey et al., 1992) were observed, leading to predominant fragment ions as a-, b-, c-, w-, x- and y-series ions (Supplemental Fig. 2).

For quantification purposes, the mass responses of the full-scan MS and the diagnostic fragment ions of the oligonucleotide standards with various lengths were compared. Up to 50% deviation from the average mass response of all standard references was observed either by comparing the XIC peak areas of the most abundant ions of the ASO standards or their MS intensities in deconvoluted mass spectra processed by ProMass and/or BioPharma Finder software (Fig. 3). The summed XIC profiles of the ASO standards using their respective most intense precursor ions in full-scan mode were similar to the profiles using the diagnostic [O_PS]− fragment ion m/z 94.936 or cEt-BNA fragment ions when applicable (Fig. 2). Therefore, for metabolite profiling, quantitative and semiquantitative analyses were conducted with the use of calibration curves of the oligonucleotide standards ranging from three to 16 in length to achieve optimum (semi)quantitative estimations. The precursor ion(s) of the oligonucleotide of interest at the most intense charge state(s) and of the most abundant isotope ion(s) was used to extract ion chromatograms for peak integration.

In this study, LLE sample preparation was applied to extract AZD8233 and metabolites from biologic samples for quantitative metabolite profiling. The extraction recovery was examined using AZD8233 and its 11 synthetic oligonucleotide shorter standards in plasma and liver homogenates. A mean extraction recovery of 96% ± 8% in liver homogenates and 94% ± 5% in plasma was obtained for all reference standards independent of their oligonucleotide lengths and sequences (Supplemental Table 1).
AZD8233: GN₃-5’-AA^MeUAAT^MeCT^MeCATGT^MeCAG-3’

Fig. 2. Extracted ion chromatograms (XICs) of synthesized standards of AZD8233 and its ASO shorthmers. (A) and (B) total XICs of 3’- (A) and 5’-cEt-BNA (B) containing ASOs; (C–E) XICs of diagnostic fragment ions: (C) [O₂PS]⁻, (D) cEt-MeU, and (E) cEt-MeC.
Metabolite Identification and Profiling of AZD8233 in Mouse, Rat, Rabbit, Monkey, and Human

Applying the established analytical strategy, metabolite identification and profiling of AZD8233 were conducted in liver and kidney homogenates from animals, in plasma from animals and humans, and in urine from monkey and human after repeated subcutaneous administration. A summary of ASO metabolites found in animals and humans is given in Supplemental Tables 2–6. Representative metabolite profiles in monkey samples are shown in Fig. 4. In total, 53 parent AZD8233 related oligonucleotide components were observed. The structures of 11 metabolites were confirmed by comparison of MS, MS², MS/MS, and retention time data to the synthesized standards as the full-length unconjugated ASO and its truncated ASO metabolites (i.e., the shortmers retaining the modified 3’ cEt-BNA terminal as 3’ 3mer to 3’ 8mer, and the shortmers retaining the 5’ cEt-BNA terminal as 5’ 3-, 8-, 9- and 15mers). A further 42 shortmer metabolites were tentatively identified on account of the three

Fig. 3. Comparison of mass responses of AZD8233 and its synthesized ASO shortmers at a concentration of 2 μM. Relative abundances of the components were calculated using peak areas of the most abundant ions in extracted ion chromatograms and mass intensities of the deconvoluted mass spectra.

Fig. 4. Representative extracted ion chromatograms (XICs) of AZD8233 and its identified metabolites (left panel) and XICs of diagnostic fragment ion [O₂PS]⁻ m/z 94.936 (right panel) after repeated subcutaneous administration of AZD8233 in monkeys (8 mg/kg, once monthly). Details of dosing schedule are given in Table 2.
criteria for ASO metabolite identification as described in our analytical strategy above (i.e., 28’ short’mers including 22 GalNAC0-3 conjugated 5’ short’mers, 11 3’ short’mers including six retaining the terminal 5’-phosphorothioate group, and three metabolites as products of loss of one to three GalNAc sugars from parent AZD8233 molecule).

Metabolites in Liver and Kidney Homogenates. In liver and kidney samples, the unconjugated full-length ASO was the predominant component in all animal species, resulting from the hydrolysis of the phosphodiester bond that linked the full-length ASO with the GalNAc triantennary cluster. A number of ASO shortmer metabolites were identified with their oligonucleotide length ranging from three to 15 and all containing the modified cEt-BNA moieties at 3’ or 5’ ends (Figs. 4 and 5). The detection of shortmer metabolites retaining cEt-BNA moieties suggests that the metabolism of the full-length ASO was predominantly initiated via endonuclease-mediated hydrolysis of the internucleotide phosphodiester bonds followed by exonuclease catalyzed cleavage toward the modified 3’ and 5’ ends. Two N-1 metabolites (i.e., 3’ and 5’ 15mers) with the loss of the terminal cEt-BNA from full-length ASO were detected in rabbit liver homogenates 10 days postdose, each accounting for approximately 4% of total ASO exposure. However, these two metabolites were determined to be less than 1% of total ASO exposure in tissues 48 hours postdose in all other studied animal species. Intact AZD8233 was not detected in liver or kidney samples in any of the animal species. In all tissue samples collected 48 hours postdose, relative abundances of each of the shortmer ASO metabolites accounted for less than 8% of total DRM exposure. Metabolites 3’-4- and 5mer and 5’-8- and 9mer showed relatively higher exposure than the other shortmers. However, in rabbit liver samples 10 days postdose lower relative exposure of full-length ASO (49% of the total DRM) but higher relative exposures of shortmer metabolites were observed with the 5’-9mer being the most abundant metabolite accounting for 12% of total DRM, whereas other shortmer metabolites each accounted for less than 8% DRM. The observation of the apparent higher relative abundance of ASO metabolites, including the N-1 shortmers, in rabbit liver samples 10 days postdose than those in tissues 48 hours postdose in other animal species suggested cEt-BNA modified oligonucleotides with PS linkage were still subjected to endo- and exonuclease-mediated hydrolysis, although at a rather slow rate. In monkey liver samples collected from the recovery animals three months after the last dose, evidence of the very slow elimination rate was clear as unconjugated ASO and four ASO shortmer metabolites (3’-3- and 4mers, and 5’-8- and 9mers) were still detectable, albeit at 8- to 20-fold lower concentrations compared with those at 48 hours postdose (Supplemental Fig. 3), suggesting possible tissue accumulation after once monthly administration at the given doses.

In general, metabolite profiles were similar in liver tissues across all studied animal species (Fig. 5). Similar profiles were also observed in kidney tissues, in male and female animals (data on file), and in different dose groups, although the absolute exposures between the groups were different.

Metabolites in Urine. In monkey urine 0- to 16-hour pool at day 57, parent AZD8233, unconjugated ASO, a large number of 3’-shortmer metabolites with 4 to 12 oligonucleotides in length and 5’-shortmers (5’-3- to 11mer) with and without retaining the GalNAc0-3 triantennary cluster moiety were detected (Fig. 4; Supplemental Table 5). Among the shortmer metabolites, 16 contained free terminal alcohol groups at the 3’ and 5’ ends, six were 3’-shortmers retaining the terminal 5’-phosphorothioate group (i.e., ps-3’-shortmers) (Supplemental Fig. 4), and 19 were conjugated 5’-shortmers with GalNAc0,3 triantennary cluster still attached to the 5’ cEt-BNA terminal. Quantification and semi-quantification of parent compound and conjugated and unconjugated ASO metabolites were conducted using the synthesized standards. It is worth noting that the semi-quantification of GalNAc-conjugated 5’-shortmer metabolites was based on the unconjugated synthetic ASO standards with the assumption that their mass responses were similar. However, the LC retention times of the conjugated 5’-shortmers showed opposite eluting characteristics compared with the naked shortmers (i.e., conjugates with the truncated oligonucleotides attached eluted with longer column retention times) (Supplemental Table 2), indicating that their physical-chemical properties shifted to be more GalNAc triantennary cluster moiety-like than oligonucleotide-like. Therefore, the assumption of the similarity in mass responses of the conjugated and naked shortmers needs to be verified when representative reference standards of the GalNAc-conjugated 5’-shortmers are available. Nevertheless, among the quantified metabolites in monkey urine samples, the 3’-5- and 6mers were the most abundant 3’-shortmers accounting for approximately 11% and 7% DRM, respectively; the GalNAc3-conjugated 5’-3- and 4mers were the most abundant 5’-shortmer metabolites accounting for 16% and 28% DRM, respectively, in the 8 mg/kg dose group, and 7% and 20% DRM, respectively, in the 32 mg/kg dose group. Each of the remaining ASO metabolites accounted for less than 3% of DRM. Intact AZD8233 accounted for 4% and 19% of DRM, respectively, in the 8 and 32 mg/kg dose groups in monkey urine.

In human urine 0- to 24-hour pool, concentrations of all ASO-related components were low. AZD8233, unconjugated ASO, 3’-shortmer metabolites with 4 to 7 oligonucleotides in length (3’-4- to 7mer), and a cluster of GalNAc-conjugated 5’-shortmers were detected (Fig. 6). AZD8233 and unconjugated ASO accounted for 1.4% and 3.4% of DRM, respectively, in 0- to 24-hour urine. Similar to metabolites found in monkey urine samples, the 3’-5- and 6mers and the GalNAc3-conjugated 5’-4mers were the most abundant metabolite components accounting for 36, 18% and 7% DRM, respectively. Each of the remaining ASO components accounted for less than 5% of DRM. In the predose urine sample at day 57, chromatographic peak corresponding to the 3’-4mer was the only ASO related metabolite detected but at a concentration below the low limit of quantification.

Metabolites in Plasma. AZD8233 was the predominant component in all pooled AUC0-24h plasma in all species and a total of nine ASO metabolites were detected (Fig. 7; Supplemental Table 6), among which three shortmer metabolites, 3’-5-, 6-, and 7mers, were detected in humans (Figs. 6 and 7). Metabolite profiles in human plasma at individual time points are shown in Supplemental Fig. 5. All human metabolites were determined at lower exposure than their corresponding levels in any of the animal species at the doses studied including NOAEL.
doses. Unconjugated ASO and conjugated metabolites resulting from AZD8233 losing one to three GalNAc sugar moieties were also found in at least one of the animal species. In general, plasma exposures of ASO metabolites in AUC0–24h pools were low and estimated to be altogether less than 26% of intact AZD8233. The pooled pre-last-dose plasma samples were also analyzed in humans, monkeys, and mice. However, neither AZD8233 nor any of the ASO metabolites were detected.

Metabolite Profiling Using ProMass and BioPharma Finder. To evaluate the utility of commercially available oligonucleotide MS data processing software, ProMass and BPF, in metabolite identification and profiling, selected samples were analyzed and the qualitative and quantitative results were compared with those applying our analytical strategy and calibration standards. In general, ASO sequence derived metabolites with good MS intensities in clean sample matrices were found by both software products using mass deconvolution data process. However, metabolites of low abundance in complicated matrices (e.g., tissue samples) as well as conjugated ASO metabolites with GalNAc-sugar cleavages were not found using generic data processing methods. No additional significant ASO metabolites were identified by the software data processing than the expected ones. As a result, software-based data processing using deconvolution (both ProMass and BPF) and sequencing functions (BPF) were mainly used to confirm the identified major metabolites qualitatively when applicable in this study (Supplemental Fig. 2).

Discussion

In the public domain, the precedence of biotransformation studies of ASO therapeutics is limited. Dedicated regulatory recommendations for assessing safety of ASO metabolites are not clearly defined. In response to the challenges of biotransformation studies of ASOs, new strategies and analytical approaches are required to generate comprehensive ASO

Fig. 6. Extracted ion chromatograms (XICs) of AZD8233 and its identified metabolites (left panel) and XICs of diagnostic fragment ion [O2PS]− (right panel) in human plasma and urine at day 57 after repeated subcutaneous administration of AZD8233. Details of dosing schedule are given in Table 2.

Fig. 7. Exposure comparison of ASO metabolites in pooled AUC0–24h plasma after repeated subcutaneous administration of AZD8233 in human, mouse, rat, rabbit, and monkey. Details of dosing schedule are given in Table 2.
metabolism data to support safety assessment in humans. In drug development, radiolabeled drug substances are often used in ADME studies and metabolite profiles are generated by radioactivity measurement. However, there are obvious pitfalls when applying this approach to ASOs, as indicated in a few animal ADME studies using radiolabeled ASOs (Zhang et al., 1995; Bosgra et al., 2019; Post et al., 2019), such as poor excretion recoveries (less than 60% of dose recovered over a collection period of up to 10 weeks) and tissue distribution and metabolite profiles reflecting only a fraction of the metabolites after cleavage of the parent ASO into both radiolabeled and “radio-silent” products. We previously discussed the limitations of current analytical approaches in ASO biotransformation studies, including radiolabeling, UV, and MS detection (Weidolf et al., 2021). Considering the DMPK properties of ASOs, we conclude that utilizing in vivo samples and LC-HRMS are detection (Weidolf et al., 2021). Considering the DMPK properties of."
In conclusion, metabolite profiles of AZD8233 were qualitatively similar in the studied animal species (i.e., mouse, rat, rabbit, and monkey). All human metabolites were also found in all animal species. Plasma exposures of human metabolites in animals from NOAEL dose groups were higher than the exposures determined in humans (90 mg, once monthly). The metabolic pathways of AZD8233 after subcutaneous administration were via hydrolysis of GalNAc sugars of AZD8233, hydrolysis of the phosphodiester bond of the linker to release the unconjugated full-length ASO, and the endonuclease-mediated hydrolysis at the DNA central part, as well as the exonuclease-mediated 5’- or 3’-degradation, to form shorter metabolites. These metabolic pathways are consistent with the reports of other ASOs or their GalNAc conjugates (Geary, 2009; Husser et al., 2017; Post et al., 2019; Kim et al., 2020), suggesting common metabolic reactions of ASOs. Furthermore, we have established a biotransformation strategy for ASO drug candidates to use tissue, plasma, and urine samples collected from toxicology studies and/or clinical studies and analyzed by LC-HRMS for metabolite identification and profiling rather than conducting bespoke radiolabeled ADMET studies. The generated in vivo biotransformation package for safety assessment of ASO metabolites was considered adequate by health authorities to support progression of AZD8233 into the phase 3 program, proving its applicability to future metabolism studies of ASO candidates in drug development.

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Data Availability

Data underlying the findings described in this manuscript may be obtained in accordance with AstraZeneca’s data sharing policy described at https://astrazenecagrouptrials.pharmacm.com/ST/Submission/Disclosure.

Authorship Contributions

Participated in research design: Li, Elebring, Dahlén, Weidolf.
Conducted experiments: Li, Dahlén.
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References


