

Integrating transcriptomics and proteomics to assess antisense oligonucleotide safety and efficacy: a time-resolved approach

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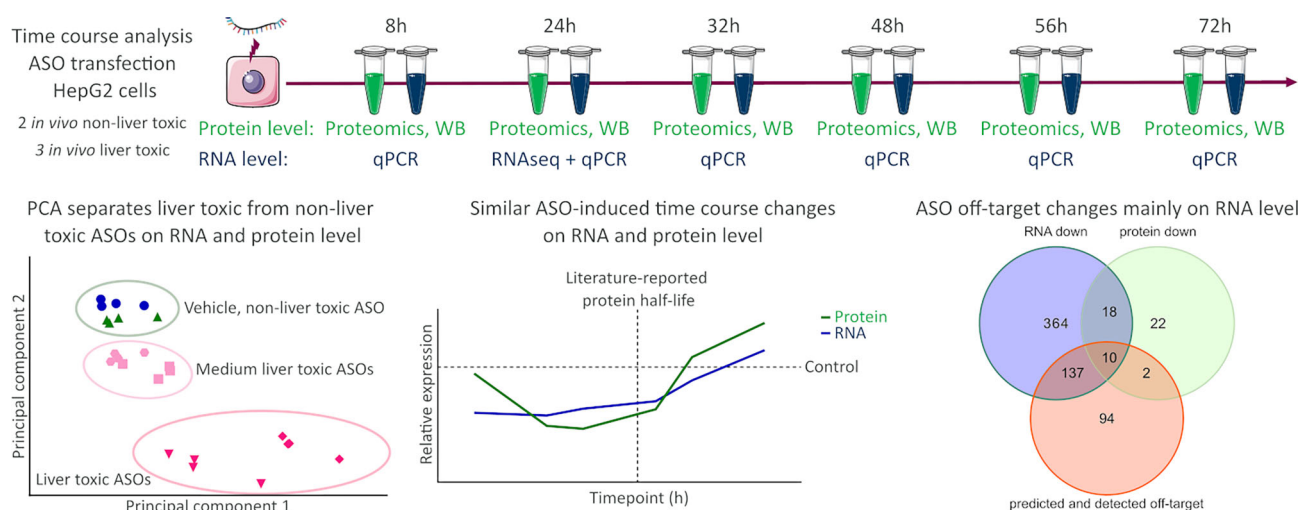
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Abstract

Antisense oligonucleotides (ASOs) are promising therapeutics, but safety concerns such as liver toxicity and off-target (OffT) effects necessitate thorough evaluation during the compound selection process. This study leverages time course global proteomics and transcriptomics to assess ASO-induced changes *in vitro*, comparing liver toxic *versus* non-liver toxic ASOs. The research confirms that ASOs perturb different cellular pathways at both RNA and protein levels, effectively discriminating between liver toxic and non-liver toxic ASOs. Contrary to expectations, protein level reduction isn't delayed relative to ASO-induced RNA reduction, highlighting the importance of understanding RNA and protein level relationships in specific model systems. Furthermore, many OffT effects observed at the RNA level do not directly translate to corresponding protein level changes. These findings suggest that current RNA-focused OffT assessment strategies capture predicted OffTs but could benefit from protein level studies that could potentially de-risk oligonucleotide drug (OND) candidates with seemingly problematic OffT profiles at the RNA level. The study underscores the value of global proteomics as a complement to RNAseq in ASO drug development, refining safety assessment and improving candidate selection.

Graphical abstract



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Introduction

Over the past three decades the field of nucleic acid therapeutics has made substantial progress, exemplified by regulatory approval of 20 oligonucleotide drugs (ONDs) [1–3]. While almost all approved ONDs act via binding to their target RNA in a sequence-specific manner, the resulting effect on RNA varies depending on the type of OND [4]. Reducing disease-associated target-transcript levels is one of the most common approaches, where RNase H-dependent antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) induce degradation of target RNA by recruiting endogenous enzymes. ASOs are 12–24 nucleotides long chemically modified single-stranded ONDs, which bind their cellular (m)RNA targets, forming a DNA:RNA hybrid which subsequently recruits RNase H1 to degrade target RNA [5]. RNase H1 is expressed in both cytoplasm and nucleus and can thus degrade target RNA in both compartments [6]. The unprotected end of the cleaved target RNA allows further degradation by the exosome complex and exonucleases, resulting in reduced expression of any encoded protein [7].

Identification of compounds that are safe to progress into clinical studies is a critical part of the drug discovery process. For ASOs, two important safety parameters are liver toxicity and hybridization-dependent off-target (OffT) effects. Early screening for liver toxicity often starts *in vitro* followed by *in vivo* tolerability studies in rodents [8–17]. Several published examples complemented acute tolerability studies with the assessment of genome-wide transcriptomic signatures using e.g. microarray and RNA sequencing (RNAseq). Although a correlation between liver toxicity and the number of differentially expressed genes (DEGs) was reported, no common, recurring pathways could be identified across different liver toxic ASOs [8, 9, 12, 14, 16].

All drug modalities exhibit potential for OffT effects. For ONDs, hybridization-dependent OffT effects are defined as OND activity on unintended transcripts [18, 19]. RNase H1 shows highest activity with full complementarity between ASO and RNA but can tolerate mismatches [20–22]. This results in a large number of potential OffT sites across the entire transcriptome that need to be assessed before entering clinical trials [20–22]. Recommendations from The Oligonucleotide Safety Working Group (OSWG) on OffT assessment strategies have focused on modulation of RNA level expression changes [23, 24].

Despite the most common goal of using RNase H1-dependent ASOs being reduction of the level of the encoded protein, evaluation of ASO activity is often carried out at RNA level, especially for studies of genome-wide effects [23, 24]. The capture range for transcriptomics studies (20–60k isoforms, spanning ~10k genes) is wider compared to proteomics (from hundreds to several thousand proteins) ones. Capturing protein level changes in such studies should ideally include a range of sampling timepoints to reflect the vastly different half-lives observed for proteins [25]. The historical scarcity of global proteomic ASO studies can partly be explained by low throughput and high costs preventing such time course analysis for multiple treatments. Recent advances in high-throughput, data-independent acquisition proteomics [26], both on acquisition [27] and data analysis level [28], has overcome some of these challenges [10], including increased throughput at reduced sample amounts required [29].

In this study, we leveraged access to internal transcriptomics and proteomics platforms, dubbed bulkSMARTseq [18, 19], and fast proteomics [27, 29], to do an initial exploration and characterization of ASO-induced genome-wide effects on RNA and protein levels and the co-variation between on- and OffT transcript reduction. We selected from the literature five RNase H1-dependent ASOs with the same chemistry and largely similar design that showed different degree of liver toxicity. We confirmed the published toxicity level in a head-to-head acute tolerability study in mouse, with results that correlated well with *in vitro* apoptosis induction in HepG2 cells after ASO transfection. This is in line with observations by others [11], supporting the use of the HepG2 model system for genome-wide comparison of ASO-induced RNA and protein level changes. To account for anticipated differences in protein half-lives, samples for proteomic analysis were taken across six timepoints. Our data show that (i) ASOs perturb different cellular pathways on RNA and protein level, discriminating between liver toxic versus non-liver toxic ASOs, (ii) similar time courses were observed for ASO-induced RNA and protein level changes, and (iii) the number and magnitude change of experimentally verified *in silico* predicted OffTs were higher on RNA level.

The results of this first study comparing genome-wide ASO induced changes on both RNA and protein level are largely in line with other studies using smaller datasets. Not being novel per se, it provides valuable confirmation to academic and industrial sponsors developing ASO therapeutics that many of the current assumptions underlying testing for safe ASO candidates are supported. However, some of our results on RNA:protein level relationships were surprising and warrant further studies that would be relevant not only for RNase H-dependent ASOs but also for siRNAs. Our assumption that reduction of protein levels would be delayed in relation to ASO-induced RNA reduction was not supported by our data and underscores the importance of understanding the relationship between RNA and protein level changes in a given model system to draw correct conclusions. Moreover, the observation that many off-target effects observed at RNA level did not result in corresponding changes at protein level suggests that the current RNA-focused OffT assessment strategies could be complemented by protein level studies, possibly de-risking OND candidates with seemingly problematic OffT profiles.

Materials and methods

Oligonucleotides

Five sequences with identical chemistry and design but different number of *in silico* predicted OffTs and safety profiles were selected based on previous literature findings [11, 14, 15, 30–32] (Table 1). Two additional ASOs, EZN-3042 and LNA41 targeting Survivin and MYD88, respectively, were used to study on-target knockdown in human cell lines. ASOs were synthesized on an ÄKTA Oligopilot 10 system by using commercially available DNA phosphoramidate building blocks and PS 5G UnyLinker support (GE Healthcare) (Supplementary Table S1). All ASOs were stored and provided as 1 mM stocks in water by AstraZeneca Compound Management.

Table 1. Properties of the tool ASOs used in this study including literature-reported and confirmed toxicity classification

ASO	2' Ribose LNA modification pattern	Literature reported safety		References	Confirmed liver toxicity (average ALT [IU/l])	Intended on-target transcript
		<i>in vitro</i>	<i>in vivo</i>			
PBS	NA	NA	NA	NA	31 ± 2	NA
569713	<u>3-10-3</u>	NA	Non-toxic	[14]	31 ± 5	None
569720	<u>3-10-3</u>	NA	Toxic	[14]	>10 000	mFXI
LNA32	<u>3-10-3</u>	Non-toxic	Non-toxic	[11, 15]	29 ± 1	mMYD88
LNA43	<u>3-8-3</u>	Toxic	Toxic	[11, 15]	>10 000	mMYD88
SPC5001	<u>3-8-3</u>	Toxic	Toxic	[30-32]	159 ± 39	hPCSK9

Safety characterization is based on *in vitro*, *in vivo*, and clinical data from literature and an in-house study where ALT levels were measured in an acute tolerability study in male BALB/c mice administered with 150 mg/kg ASO by subcutaneous injection. Three animals were used per treatment group. ALT is reported as average ± standard deviation. ALT is a biomarker of liver toxicity correlating well with histopathology changes. All ASOs are fully phosphorothioate (PS)-modified. ALT = alanine transaminase, h = *Homo sapiens*, IU/l = International Units per Liter, LNA is underlined, m = *Mus musculus*, NA = not applicable.

Mouse acute tolerability studies

Acute tolerability studies were performed with male BALB/c mice, approximately 6 weeks old, at the Korean Institute of Technology (KIT) in Daejeon, South Korea. The animal study protocol was reviewed and assessed by the Institutional Animal Care and Use Committee of KIT and all procedures were performed in compliance with Animal Welfare Act and Guide for the Care and Use of Laboratory Animals (by Institute for Laboratory Animal Research publication). Animals were dosed once subcutaneously with 150 mg/kg ASO and compared against PBS control. Blood samples for clinical pathology were taken, 72 h after injection, prior to the terminal sacrifice from all surviving animals (one mouse dosed with LNA43 died before the study end point). In short, approximately 0.7 ml of blood was collected and transferred into tubes without anticoagulant for serum separation. The tubes were kept at room temperature for a minimum of 90 min and subsequently centrifuged at 3000 rpm for 10 min to obtain serum. Finally, alanine aminotransferase (ALT) levels were measured using a Toshiba 200FR NEO Chemistry Analyzer.

Cell culture

A549 cells (ATCC, CCL-185) were cultured in Ham's F12 Kaighn's Modification Medium with 10% fetal bovine serum (FBS) (Gibco). HepG2 cells (ATCC, HB-8065) were cultured in Eagle's Minimum Essential Medium (MEM) with 10% FBS (Gibco). PC9 cells (ECACC, 90 071 810) were cultured in Roswell Park Memorial Institute 1640 Medium with 10% FBS (Gibco). All cell lines were maintained at 37°C with 5% CO₂. The Vi-CELL XR Cell Viability Analyzer (Beckman Coulter) was used to count cells when seeding for experiments. The cells tested negative for mycoplasma contamination and were authenticated by the AstraZeneca CellBank through STR profiling.

ASO treatment

Six-well plates

Western blot: A549, HepG2, and PC9 cells were seeded in Six-well plates (Falcon), 300 000 cells per well, and allowed to attach overnight. ASOs were transfected using Lipofectamine 2000 as detailed in the manufacturer's instructions. Final ASO and Lipofectamine 2000 concentrations were 50 nM and 0.25%, respectively.

24-Well plates

RT-qPCR: A549, HepG2, and PC9 cells were seeded in 24-well plates (Falcon), 100 000 cells per well, and allowed to attach overnight. ASOs were transfected using Lipofectamine 2000 as detailed in the manufacturer's instructions. Final ASO and Lipofectamine 2000 concentrations were 50 nM and 0.25%, respectively.

96-Well plates

Cytotoxicity time course, imaging, proteomics, and RNAseq: HepG2 cells were seeded in 96-well plates (Greiner 655946, Revvity PhenoPlate for imaging) using the Multidrop Combi Reagent Dispenser with a small tube plastic tip dispensing cassette (Thermo Scientific), 100 000 cells per well in 50 µl of medium, and allowed to attach overnight. To prepare ASO plates, 1 µl of ASO stock solution (5 µM ASO stock concentration for 50 nM final) was added per well in 96-well plates (Greiner 651201) using the Multidrop. Then, the Multidrop was used to add 49 µl of Lipofectamine2000 master mix in opti-MEM (Thermo Scientific) to each well, and the ASO plate was incubated for 30 min at room temperature. Finally, the contents of the ASO plate were transferred to the HepG2 cells using the Bravo Automated Liquid Handling Platform with 96-well head (Agilent) for a final Lipofectamine 2000 concentration of 0.25%.

384-Well plates

Caspase 3/7 dose-response time course: HepG2 cells were seeded in 384-well plates (Greiner 781091) using the Multidrop Combi Reagent Dispenser with a small tube plastic tip dispensing cassette (Thermo Scientific), 2000 cells per well in 10 µl of medium, and allowed to attach overnight. ASO plates (Greiner 784201) with 10 point half-log (1:3.162) dose-response, top concentration 1.25 µM, were prepared on the Echo Acoustic Liquid Handler (Beckman Coulter) using intermediate 0.1, 10, and 100 µM ASO stock solutions. Then the Multidrop was used to add 10 µl of Lipofectamine 2000 (Thermo Scientific) master mix to each well, and the ASO plate was incubated for 30 min at room temperature. Finally, the contents of the ASO plate were transferred to the HepG2 cells using the Bravo Automated Liquid Handling Platform with 384-well head (Agilent) for a final Lipofectamine 2000 concentration of 0.25%.

Confocal imaging

Imaging was performed 32- and 72 h post transfection. First, cells were washed with 100 μ l of PBS (Gibco). Cells were fixed in 50 μ l of 4% paraformaldehyde (PFA) solution (Merck) in PBS for 20 min at room temperature. Afterwards PFA solution was washed away with three PBS washes. Second, cells were permeabilized in 100 μ l of 0.1% Triton-X solution (Merck) in PBS for 15 min at room temperature. Triton-X solution was washed away with three PBS washes. Then, cells were blocked in 100 μ l of 1% Bovine Serum Albumine (BSA) solution (Merck) in PBS for one hour at room temperature. BSA solution was washed away with three PBS washes. Finally, cells were incubated overnight at 4°C with 50 μ l of primary antibody, 1:1000 diluted monoclonal rabbit anti-PS-ASO antibody (developed in-house) in 1% BSA. Primary antibody was washed away with three PBS washes. Cells were subsequently incubated with secondary antibody for 1 h in the dark at room temperature, 1:1000 diluted goat anti-rabbit Alexa Fluor 647 antibody (Thermo Scientific) in 1% BSA. Secondary antibody was washed away with three PBS washes. Hoechst was used to stain nuclei: cells were treated for 5 min at room temperature with 1:5000 diluted Hoechst33342 (Thermo Scientific) in PBS. Hoechst was washed away with three PBS washes. Finally, cells were stained with HCS CellMask Green (Thermo Scientific) for 30 min in the dark at room temperature, diluted 1:5000 in PBS. HCS CellMask Green was washed away with three PBS washes. All wells were acquired on the CellVoyager 8000 (Yokogawa) using an 20 $\times$ air objective. Nine tiles were acquired per well and only maximum-intensity projections were exported to Signals Image Artist (Revvity) for data analysis. Nuclei were detected using option C and cytoplasm was detected based on nuclei selection using option B. A mean intensity cut-off was set for transfected cells, 30 and 40 for 32- and 72-h post transfection respectively, and subsequently cellular, cytoplasmic, and nuclear PS-ASO intensity was quantified (transfected cells only).

Cytotoxicity assays

Cytotoxicity assays were run on ASO-treated cells 8-, 24-, 32-, 48-, 56- and 72-h post transfection. The CellTiter-Fluor Kit (Promega) was used to measure cell viability. Reagents were thawed at room temperature. 5 μ l of GF-AFC substrate was added per 5 ml assay buffer and 50 μ l of reconstituted buffer was added per well using the Multidrop. Cells were put on an orbital shaker for 30 s at 700 rpm and incubated at 37°C for 30 min. Fluorescence was measured on the Pherastar FSX Microplate Reader (BMG Labtech) using an optical filter (excitation 410 nm and emission 480 nm). Data were normalized to mock (cells incubated in medium with Lipofectamine 2000 transfection reagent only). The Caspase 3/7-Glo kit (Promega) was used to measure apoptosis. Reagents were thawed at room temperature. 10 μ l or 50 μ l reconstituted Caspase 3/7-Glo reagent (for 384- and 96-well plates, respectively) was added to each well (96-well plates were previously treated with CellTiter-Fluor). Cells were put on an orbital shaker for 30 min at 700 rpm before read-out on the Envision Multilabel Plate Reader (PerkinElmer) using a standard luminescence protocol. Data were normalized to mock (cells incubated in medium with Lipofectamine 2000 transfection reagent).

Fast proteomics

Cells were washed three times with 50 μ l of PBS (Gibco) on the Bravo Automated Liquid Handling Platform after 8, 24, 32, 48, 56, or 72 h of ASO transfection. Last remaining PBS was removed by hand using a 10 μ l multichannel pipet and the plates were immediately snap frozen on dry ice. Cells were lysed in 50 μ l of PreOmics iST lysis buffer and subsequently incubated at 95°C for 10 min for reduction and alkylation of protein thiols. Proteins were subjected to enzymatic cleavage overnight by adding equal amounts of endoproteinase Lys-C and trypsin in a 1:50 (w/w) enzyme:protein ratio. Desalting of peptides was performed according to the PreOmics iST protocol on a styrene divinylbenzene reversed-phase sulfonate sorbent. Purified peptides were vacuum centrifuged to dryness and resuspended in 50 μ l of 2% acetonitrile and 0.1% formic acid in water. Peptide concentrations were quantified and normalized using Quantitative Colorimetric Peptide Assay (Thermo Scientific) prior to LC-MS analysis. Peptides were loaded onto EvoTips (EvoSep Biosystems) and were analyzed using an EvoSep One liquid chromatography system coupled to a timsTOF Flex mass spectrometer through an 8 cm $\times$ 150 μ m, 1.5 μ m C18-based column (EvoSep Biosystems, EV1109). The LC was operated using the 60 samples per day factory method. Mass spectra for samples were acquired using data independent acquisition parallel accumulation-serial fragmentation using a window schema defined and optimized in part by py_diAID [33]. Data processing was conducted through the DIA-NN module using standard settings [29] utilizing deep learning-based spectra, retention times (RTs), and ion mobilities prediction enabled. Neural network classifier was set to double-pass mode, with cross-run normalization set to RT-dependent. All other analysis settings were kept at factory settings and identifications were set to 1% false discovery rate for precursors. We ran four or more biological replicates (five replicates for mock and four replicates for ASO-treated cells).

bulkSMARTseq

After 24 h of ASO transfection cells were washed three times with 50 μ l of PBS (Gibco) on the Bravo Automated Liquid Handling Platform. Last remaining PBS was removed by hand using a 10 μ l multichannel pipet. Cells were lysed in 63 μ l of LBE lysis buffer (Beckman Coulter), including 3 μ l of proteinase K (Beckman Coulter), and incubated for 30 min at room temperature while shaking. After the incubation the plate was snap frozen on dry ice until total RNA extraction using RNAdvance Cell v2 kit on the Biomek i7 Automated Liquid Handler (Beckman Coulter). Total RNA quality was confirmed using the Standard Sensitivity RNA Analysis Kit DNF-471 (Agilent) on a Fragment Analyzer 5300 (Agilent). Library preparation was performed using an in-house protocol adapted from SMARTseq technology: the bulkSMARTseq method with template-switching oligo [34–36]. In short, RNA concentrations were first normalized using the Biomek i7 Automated Liquid Handler (Beckman Coulter) to achieve 1 ng/ μ l, and 4 ng of RNA was used as input for reverse transcription. All master mixes were dispensed using the Mantis Microfluidic Liquid Dispenser (Formulatrix). Reverse transcription was performed using Maxima H Minus Reverse Transcriptase (Thermo Scientific) in presence of Oligo-dT, and template switching oligonucleotide (TSO) reactions were assembled using the Mantis

Microfluidic Liquid Dispenser (Formulatrix) and run on a thermal cycler (Bio-Rad): 42°C for 90 min, 10 cycles of 50°C for 2 min, and 42°C for 2 min, final step of 85°C for 5 min. The whole reaction was pre-amplified using KAPA HiFi Hot-Start ReadyMix for 8 cycles. Complementary DNA (cDNA) was diluted 1:10 and 5 µl of this dilution was used for tagmentation. Tn5 tags (Illumina) were added: 10 min at 55°C on a thermal cycler and reactions were blocked by addition of 0.04% SDS followed by 5 min room temperature incubation. Indexes were added using Phusion (Thermo Scientific) PCR on a thermal cycler in the presence of 0.05% Tween-20: 72°C for 3 min, 98°C for 30 s, 12 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min, and final step of 72°C for 5 min. Finally, bead cleanup was performed (AMPure XP Beads, Beckman Coulter): 50 µl of beads were added to 50 µl of sample, incubated at room temperature for 8 min followed by 2 min on the magnet. Beads were washed twice with 80% ethanol (Merck), dried for 5 min, incubated, resuspended in 20 µl with water for 5 min, put on the magnet for 2 min, and finally resuspended in 14 µl recovered. Library quality was checked using the High Sensitivity Next-Generation Sequencing fragment kit (Agilent) on a Fragment Analyzer 5300 (Agilent). Libraries were multiplexed and sequenced using a S2 flow cell on the NovaSeq 6000 (Illumina) using 51 cycles paired end. We ran four or more biological replicates (five replicates for mock and four replicates for ASO-treated cells).

RT-qPCR

Cells were trypsinized, pelleted, and snap frozen after 8, 24, 32, 48, 56, or 72 h of ASO transfection. Total RNA was purified using the RNeasy Micro Kit (Qiagen) on a QIAcube instrument (Qiagen). cDNA was synthesized from 100 ng input total RNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Scientific) on a VeritiPro thermal cycler (Thermo Scientific). cDNA was diluted 3× in RNase-free water before proceeding with quantitative PCR (qPCR): 3 µl of diluted cDNA was combined with 7 µl of Taqman Fast Advanced Master Mix (Thermo Scientific) in each well, the qPCR plate spun down and run a QuantStudio 6 instrument (Thermo Scientific) using standard settings. The following Taqman Gene Expression assays (FAM) were used: ATXN2 (Hs00268077_m1), BIRC5/Survivin (Hs04194392_s1), FNDC3B (Hs00981553_m1), GAPDH (Hs99999905_m1), MPP6 (Hs00212785_m1), MYD88 (Hs01573837_g1), PCSK9 (Hs00545399_m1), and RRM2 (Hs00357247_g1).

Western blot

Cells were trypsinized, pelleted and snap frozen after 8, 24, 32, 48, 56, or 72 h of ASO transfection. Proteins were extracted using RIPA buffer (Thermo Scientific) containing phosphatase- and protease inhibitor (Roche) and benzonase nuclease (Merck, 1 µl per ml RIPA). In short, samples were incubated on ice for 30 min, while being agitated every 3 min, spun down at 14 000 × g for 10 min and aliquoted for protein quantification and western blot analysis. Pierce BCA kit (Thermo Scientific) was used to measure protein concentrations: absorbance (562 nm) measurements were taken on the Pherastar FSX Microplate Reader (BMG Labtech). 10–30 µg total protein was diluted in Pierce LDS Sample Buffer and run together with Novex Sharp Pre-stained Protein Standard on 4%–12% NuPAGE Bis-Tris Mini Protein Gels in MOPS

SDS buffer for 1 h at 200 V (all Thermo Scientific). Samples were transferred with Trans-Blot Turbo Mini PVDF kit in the Trans-Blot Turbo System using the standard 7 min protocol (all Bio-Rad). Membranes were washed three times with TBS-T between every step, blocked at least 30 min in 5% milk, and incubated overnight at 4°C with primary antibodies in 1% BSA (all Merck). The next day membranes were incubated with secondary antibodies in 5% milk and revealed on the ChemiDoc (Bio-Rad) using the SuperSignal West Pico Plus kit (Thermo Scientific). The following primary antibodies were used: mouse anti-GAPDH 1:10 000 (Abcam 6C5), rabbit anti-ATXN2 1:500 (Cell Signaling E3B3Z), rabbit anti-FNDC3B 1:1000 (Proteintech 22605-1-AP), rabbit anti-MPP6 1:1000 (Cell Signaling F3T9S), rabbit anti-MYD88 1:1000 (Cell Signaling D80F5), rabbit anti-PCSK9 1:1000 (Cell Signaling D7U6L), rabbit anti-RRM2 1:1000 (Cell Signaling E7Y9J), and rabbit anti-Survivin 1:1000 (Cell Signaling 71G4B7). Polyclonal Goat Anti-Mouse ImmunoGlobulins/HRP and Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP (both antibodies from Agilent) were used as secondary antibodies.

In silico off-target prediction

OffTs were predicted against the full human transcriptome based on basic mismatch criteria [19, 37] and reported in [Supplementary Table S2](#). For 16 nucleotides long ASOs, this is defined as one mismatch, insertion, or deletion (indel) or two mismatches/indels with a contiguous match of at least 14 nucleotides, using an in-house *in silico* prediction tool. The sequence of each ASO is aligned against the full human transcriptome, Human Genome Annotation database v31 [38], including and excluding introns as ASOs have shown to target both exonic and intronic sequences, using *Bowtie*. Gapped OffTs were predicted by first introducing insertions and deletions to the input oligonucleotide sequence before aligning against the full human transcriptome in the same manner.

Data analysis

Data from acute tolerability study, Caspase 3/7 activation, cell viability, image quantification, and qPCR assays are represented as mean ± standard deviation. Results from RNAseq and proteomics are depicted as mean ± standard error.

Data analysis and normalization of the cytotoxicity assays was performed in PRISM version 10 (GraphPad).

qPCR data were analyzed using the 2(-Delta Delta C(T)) method [39].

Sequencing reads were filtered with *RiboDetector* to exclude ribosomal reads and adaptor on reads trimmed with *NGMerge*. Quality control of raw sequencing data was performed with *FASTQC* ([Supplementary Table S3](#)). For generating count data, sequencing reads were assigned to genes based on Human Genome Annotation database v31 [38] using *Salmon*. Transcript and gene level-abundance were transformed into txdb database using *tximport*, which were subsequently used as input for differential expression analysis with *DESeq2*. Principal component analysis (PCA) was based on centred and scaled log(TPM + 1) data. One sample was excluded for differential gene expression due to not passing QC (read count < 10 M): mock replicate 5. DEGs were based on significance (Benjamini–Hochberg multiple testing adjusted *P*-value < 0.05), absolute log-fold change of gene expression (abs(log2FoldChange_shrunken) > 0.5)

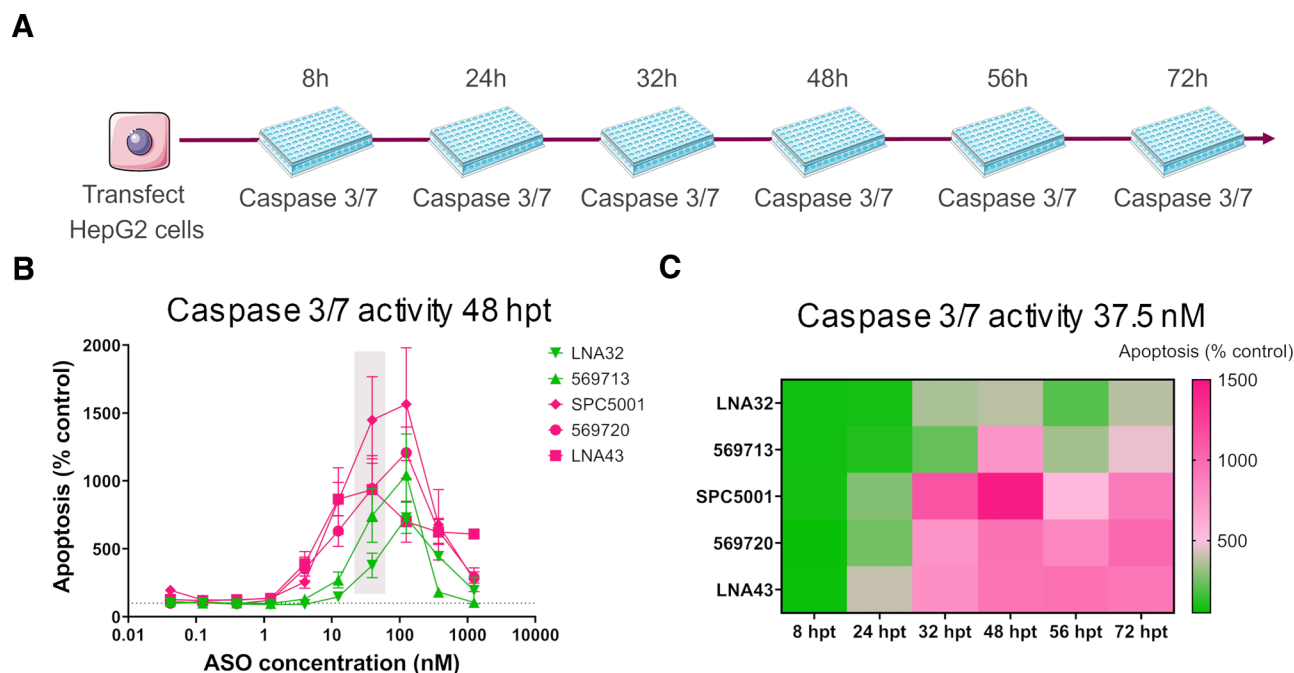


Figure 1. Tool ASO *in vitro* cytotoxicity classification. **(A)** Schematic overview of HepG2 *in vitro* cytotoxicity assay using Caspase 3/7 activation as a read-out for apoptosis. **(B)** Caspase 3/7 activation read-out upon tool ASO transfection for 48 h. Highlighted concentration (37.5 nM) was picked to study Caspase 3/7 activation time courses. **(C)** Caspase 3/7 activation read-out upon 37.5 nM transfection of tool ASOs at six different timepoints. For Caspase 3/7 assays, all ASOs were transfected at 0.042–1250 nM in a 10-point half-log dose curve, and samples were normalized to mock control (Lipofectamine 2000 transfection reagent only). $N = 3$ biological replicates. h = hour, hpt = hours post transfection.

and low variance in gene expression among samples ($\text{abs}(\text{salmon_log10_cv_mean}) < 1$) unless stated differently.

Peptide intensities were first aggregated at the protein level. These aggregated intensities were subsequently filtered to remove contaminants and normalized to the median intensity of each sample. Differential proteomics analysis was performed using the R package *limma*. Differentially expressed proteins (DEPs) were identified based on statistical significance (Benjamini–Hochberg multiple testing adjusted P -value < 0.05) and a threshold of absolute $\log_2$ -fold change > 0.5 for at least 2 out of 6 measured time points. PCA was conducted using centred and scaled normalized peptide intensities.

DEGs and proteins were further visualized in R v4.3.0 using cross-plots based on *stats*, *ggplot2*, and *ggExtra* packages.

Enrichment of off-targets among down-regulated gene and protein sets was assessed using one-sided Fisher's exact tests (alternative = “greater”) with all genes/proteins overlapping between transcriptomics and proteomics datasets used as the background universe. P -values were adjusted for multiple testing across compounds using the Benjamini–Hochberg procedure.

Results

Tool ASO safety profile characterization *in vitro* and *in vivo*

Having access to bulkSMARTseq and fast proteomics platforms allowed us to compare global changes at transcriptomic and proteomic levels. We focused on sequence-dependent effects and identified five RNase H-dependent ASOs from literature [11, 14, 15, 30–32] with identical chemistry and similar design that differed in safety profiles and number of *in silico* predicted OffTs [19] (Table 1 and Supplementary Table S2).

The safety profiles described in literature were confirmed in an acute tolerability study in mice. Increase in plasma levels of alanine transaminase (ALT) is a sensitive biomarker of liver toxicity that correlates well with histopathological changes observed with ASOs in liver [8]. Three days after a single high dose (150 mg/kg body weight), mice treated with LNA32 and 569713 showed no ALT change compared to vehicle control whereas SPC5001 treated mice showed slightly elevated ALT levels. ASOs 569720 and LNA43 resulted in significantly elevated ALT levels ($> 10\,000$ IU/l), confirming their previously reported severe liver toxicity (Table 1).

To study the *in vitro* safety of these ASOs, we adapted an assay described by Dieckmann *et al.* [11]. We transfected HepG2 cells with ASOs at 10 concentrations and measured Caspase 3/7 activation at different time points from 8 to 72 h post transfection as a proxy for apoptosis (Fig. 1A). Caspase activation peaked at 48 h post transfection, with results correlating with the mouse tolerability study; the *in vivo* liver toxic 569720, LNA43 and SPC5001 activated Caspase 3/7 from a low concentration of 3.9 nM while *in vivo* non-liver toxic 569713 and LNA32 showed Caspase 3/7 activation from a 10-fold higher concentration of 37.5 nM (Fig. 1B). The highest ASO-induced Caspase 3/7 activation was observed at 125 nM where activation peaked at 1500% for the *in vivo* liver toxic 569720, LNA43, and SPC5001. The *in vivo* non-liver toxic LNA32 reached a maximum of 500%, whereas the *in vivo* non-liver toxic 569713 reached an intermediate maximum at 1000%, indicating that the safety profile of this ASO may have been underestimated in the short duration (72 h) mouse *in vivo* tolerability study. Finally, we observed similar apoptosis time course patterns for all three liver toxic ASOs (Fig. 1C and Supplementary Fig. S1): activation started at 24–32 h post transfection, peaked at 48 h post transfection, and decreased at 56–72 h post transfection. In summary, our tool ASO

results from *in vitro* and *in vivo* studies are largely in line with their previously published safety profiles [11, 14, 15, 30–32].

RNAseq and proteomic profiles correlate with ASO liver toxicity

To ensure sufficient sample yield and minimal cell death caused by culture conditions, the cell culture protocol was further optimized to support genome-wide proteomics analysis. Initial tests showed that increasing HepG2 cell numbers to 100 000 per well in a 96-well plate maintained Caspase 3/7 activation by the liver toxic ASOs, with minimal cell death in the 10–50 nM ASO concentration range (data not shown). An initial time course experiment was run at 50 nM before proceeding with the final RNAseq and proteomics experiments (Supplementary Fig. S2A), confirming that the liver toxic ASOs induced apoptosis at multiple timepoints (Supplementary Fig. S2B), with cell viability of 80% or higher throughout the experiment (Supplementary Fig. S2C).

RNAseq samples were taken 24 h after transfection to ensure robust on-target knockdown while reducing time for secondary effects to occur [23]. This timepoint also captures cellular events preceding significant Caspase 3/7 activation. For the proteomics analysis, samples were taken at six timepoints ranging from 8 to 72 h post transfection (Fig. 2A).

In our RNAseq analysis, 95% of reads passed quality checks, 75%–77% aligned to the *H. sapiens* genome; 17 000–19 000 genes were quantified per library (Supplementary Table S3). In the proteomics analysis, peptides from approximately 5900 proteins were identified at every timepoint, except for 8 h post transfection for which the lower number of peptides could be explained by lower cell number and sample yield (Supplementary Fig. S3A). Out of the 6097 assessed proteins, 5903 had their corresponding mRNA transcripts captured by RNAseq (Supplementary Fig. S3B). For all subsequent differential analyses, we focused on this set of 5903 overlapping proteins and RNA transcripts identified by both methods. At the overlapping 24 h post transfection timepoint, RNAseq and proteomics log₂(fold changes) display similar dynamic ranges (RNAseq: –1.79 to 3.75; proteomics: –3.11 to 2.50; Supplementary Fig. S4A–E and Supplementary Table S4), with consistent magnitude of changes on both RNA and protein level. However, their linear co-variation (assessed using Pearson correlation, r^2), ranges from 0.33 to 0.49 for differentially expressed genes (DEGs) and 0.26 to 0.37 for differentially expressed proteins (DEPs) (Supplementary Fig. S4F), suggesting consistency in patterns, but not overall amplitude for the full dataset. At 8, 56, and 72 h post transfection proteomics timepoints the linear co-variation is lower than at 24 h post transfection. On the other hand, the linear co-variation is highest at 32 and 48 h post transfection timepoints (Supplementary Fig. S5). Interestingly, the density plots (Supplementary Fig. S4A–E) show that DEG are mostly downregulated, with median log₂(fold change) values < –0.5 for each ASO treatment (data not shown). In contrast, DEPs are preferentially upregulated.

PCA was applied on the RNAseq dataset showing the two most liver toxic ASOs separating from the remaining samples on PC1 axis, explaining 12.48% variance and PC2, explaining 8.61% variance (Fig. 2B). We also performed PCA on the proteomics dataset across all timepoints. For the 8 and 24 h time points, we did not observe any separation between the ASOs (Supplementary Fig. S6A and B). However, from

32 h post transfection (Supplementary Fig. S6C) and 48 h post transfection (Fig. 2C) onwards, the two most liver toxic ASOs clustered from the rest of the samples in PC1. In addition, the medium liver toxic ASO SPC5001 clustered together with 569713 from 32 h post transfection onwards (Fig. 2C and Supplementary Fig. S6C–E), based on PC1. The majority of variance is explained by the first four Principal Components, except for RNAseq and proteomics 8 h post transfection timepoint (Supplementary Fig. S6F). For 569713, this classification contrasts to the ALT response in the acute *in vivo* tolerability study (Table 1) but is in line with an elevated response in the HepG2 caspase assay (Fig. 1B and C, and Supplementary Fig. S1).

Using R packages *clusterProfiler* and *gene set enrichment analysis* (GSEA) mining, we investigated differences in signalling pathway responses between liver toxic and non-liver toxic ASOs in the RNAseq and proteomics datasets but no common toxic ASO classifiers could be identified (data not shown). This is in line with previous studies in mice which also could not identify common pathway changes for liver toxic LNA ASOs [8, 9, 14, 16].

To investigate if ASO liver toxicity correlated with the number of DEGs or DEPs, we used the following criteria for a relevant change: $|\log_2(\text{fold change})| \geq 0.5$, capturing both up-regulated and downregulated genes, representing >41% increase or 29% decrease, and Benjamini–Hochberg adjusted P -value ≤ 0.05 compared to mock treated samples. For the proteomics samples, at least two out of six timepoints had to meet these criteria. Treatment of HepG2 cells with the highly liver toxic 569720 and LNA43 ASOs resulted in the highest number of DEGs and DEPs, followed by medium *in vitro* toxic 569713 and SPC5001. The non-liver toxic LNA32 had the lowest number of DEGs and DEPs (Fig. 2D). PCA analysis on RNAseq and proteomics data and “simply” counting DEGs and proteins correlated well with the *in vitro* and *in vivo* toxicity classification.

Time course profiles of ASO on-target reduction are similar on RNA and protein level

Next, we studied time course profiles of ASO-induced on-target reduction on both RNA and protein level. Using the SPC5001 ASO, we examined the time course reduction for the intended target PCSK9 (Proprotein Convertase Subtilisin/Kexin Type 9). RNase H-dependent ASOs reduce protein levels by inducing degradation of mRNA and thereby inhibiting replenishment of proteins undergoing physiological turnover. With half-lives of proteins reported to be on average five times longer than RNA half-lives [25], we hypothesized that ASO-induced RNA reduction would be followed by a delay in PCSK9 protein level decrease. PCSK9 protein half-life was reported to be 44 h in HeLa cells [40]. However, the expected delay in protein reduction should be shorter since not all PCSK9 proteins are synthesized at the exact time of ASO-induced mRNA loss. To complement the RNAseq and proteomics studies discussed above, we also performed time course experiments in three human cell lines where RNA levels were assessed by qPCR and protein levels by western blot with a study design outlined in Fig. 3A.

To our surprise, maximum reduction of PCSK9 protein levels was already observed after 24 h, returning to mock levels after 56 h (Fig. 3B). Interestingly, running qPCR time courses in three human cell lines revealed that SPC5001-induced

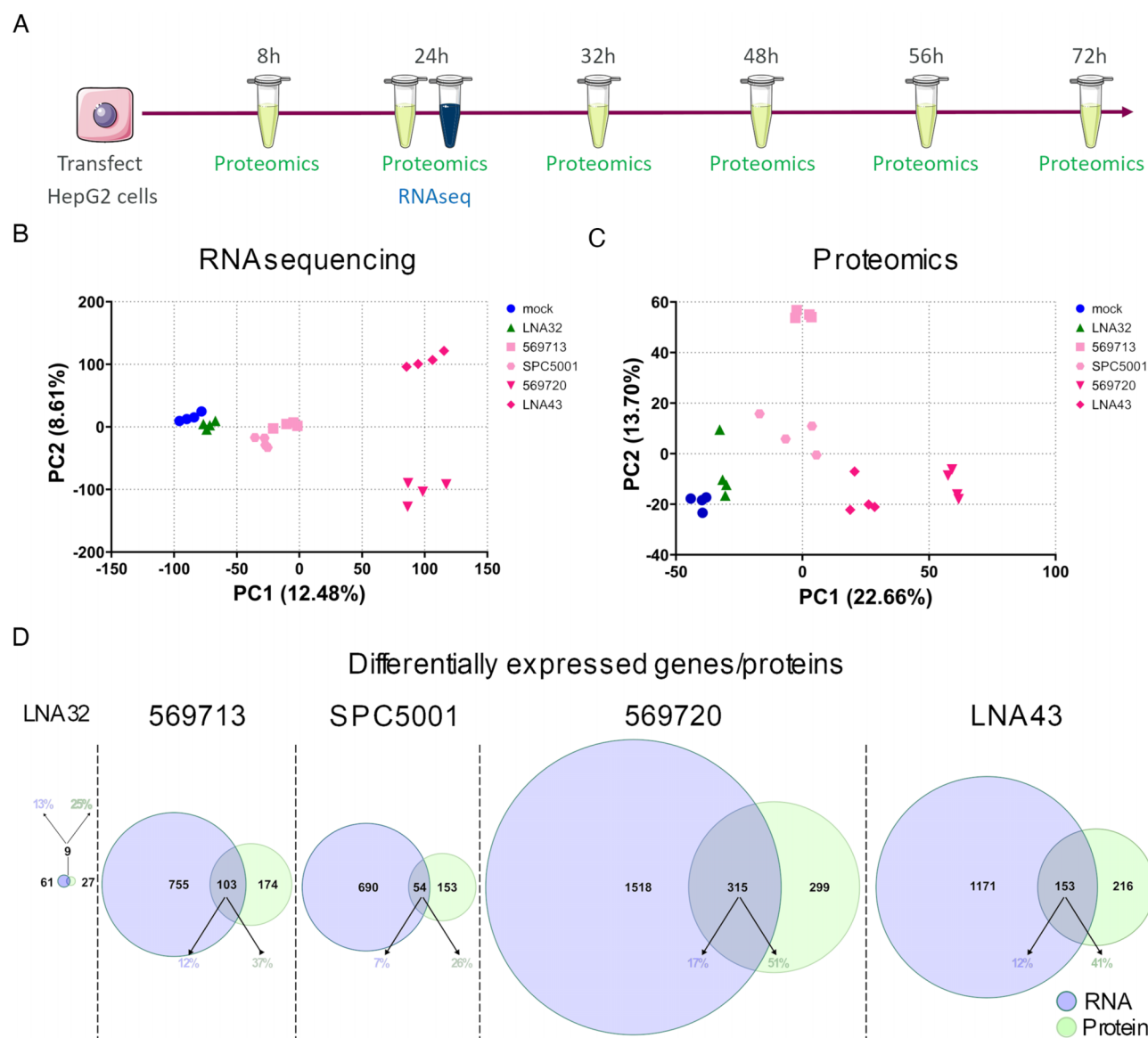


Figure 2. RNAseq and proteomics results in HepG2 cells correlates with tool ASO liver toxicity. **(A)** Schematic overview of HepG2 experiment: cells were transfected with 50 nM tool ASOs and samples for proteomics were taken at six different points, after 8, 24, 32, 48, 56, and 72 h, while RNAseq samples were only harvested after 24 h. **(B)** PCA of RNAseq samples after 24 h of tool ASO transfection. **(C)** PCA of proteomics samples after 48 h of tool ASO transfection. **(D)** Quantification of numbers of differentially expressed genes and proteins upon tool ASO transfection. Percentages of overlap are displayed for each ASO treatment. RNAseq parameters: absolute $\log_2(\text{fold change}) > 0.5$, Benjamini–Hochberg adjusted P -value < 0.05 . Proteomics parameters: absolute $\log_2(\text{fold change}) > 0.5$ at two or more timepoints, Benjamini–Hochberg adjusted P -value < 0.05 . $N = 4$ or more biological replicates. h = hour, PC1 = principal component 1, PC2 = principal component 2, PCA = principal component analysis, RNAseq = RNA sequencing.

reduction of PCSK9 mRNA (Fig. 3C) had a similar time profile as PCSK9 protein reduction, measured by both proteomics (Fig. 3B) and western blot (Fig. 3D–F). Interestingly, PCSK9 protein levels were not reduced after 8 h in HepG2 cells while RNA level was measured to be 0.60-fold relative to the mock control, indicating a 40% decrease (Fig. 3C). These results confirmed that, there is only a short delay in protein reduction.

One technical explanation for the rapid recovery of both RNA and protein level reduction could be an ASO dilution effect resulting from HepG2 cells dividing throughout the experiment. HepG2 cells have an estimated doubling time of 48 h [41], where the fraction of transfected cells or ASO concentration could diminish over time. To rule this out, we per-

formed immunofluorescence experiments with an antibody recognizing the ASO phosphorothioate (PS) backbone. High-content confocal imaging followed by PS–ASO signal quantification confirmed that HepG2 cell division did not result in an ASO dilution effect; PS–ASO fluorescence intensity increased over time and 60%–90% of all identified cells were transfected at both tested timepoints, 32 and 72 h post transfection (Supplementary Fig. S7).

Interestingly, maximum achieved PCSK9 mRNA knock-down at 24 h post transfection was more efficient in lung-derived A549 and PC9 cells, 79% and 74% respectively, compared to 42% in hepatocyte-derived HepG2 cells. In line with observations in HepG2 cells (Fig. 3D), we found that the most significant SPC5001-induced reduction in PCSK9

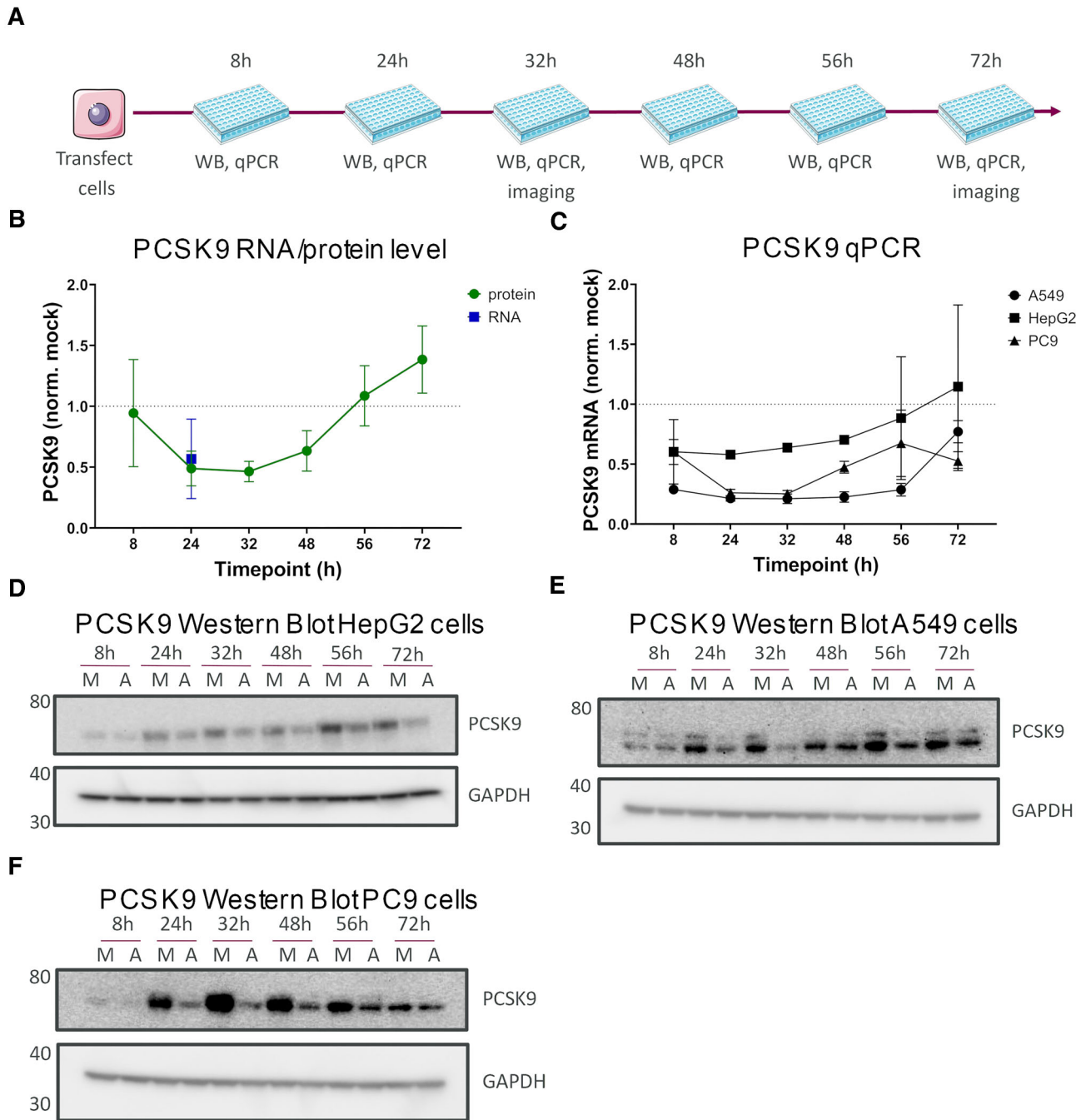


Figure 3. SPC5001 on-target RNA and protein reduction time course. **(A)** Schematic overview of on-target degradation experiment: cells were transfected with 50 nM SPC5001 and samples for western blot and qPCR were taken at six different points, after 8, 24, 32, 48, 56, and 72 h, while imaging plates were fixed after 32 and 72 h. **(B)** SPC5001 on-target (PCSK9) reduction as measured by RNAseq and proteomics. **(C)** SPC5001 on-target (PCSK9) mRNA reduction in three different human cell lines as measured by qPCR, SPC5001 on-target (PCSK9) protein reduction in **(D)** HepG2, **(E)** A549, and **(F)** PC9 cells as measured by western blot. $N = 1$ biological replicate for western blot, $N = 3$ biological replicates for qPCR, $N = 4$ or more biological replicates for multi-omics. Protein size in kilo Dalton. A = ASO treatment (50 nM SPC5001), h = hour, M = mock control (Lipofectamine 2000 only), RNAseq = RNA sequencing, WB = western blot.

protein in A549 (Fig. 3E) and PC9 cells (Fig. 3F) was observed at 24 and 32 h post transfection, respectively, with protein levels returning to mock level at the end of the experiment (56–72 h post transfection). Importantly, PCSK9 protein levels were not yet reduced at the earliest timepoint, 8 h post transfection, while RNA was down to 0.29- and 0.60-fold relative to the mock control respectively in A549 and PC9 cells (Fig. 3C). To recapitulate these results with other tar-

get genes, we used two other RNase H-dependent ASOs of LNA chemistry: EZN-3042 [14, 42, 43], targeting human baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) (Supplementary Fig. S8A), and LNA41 [11], targeting human Myeloid differentiation primary response 88 (MYD88) (Supplementary Fig. S8B). BIRC5 and MYD88 exhibited similar protein level time course profile as observed for PCSK9 using SPC5001, despite different protein half-lives. BIRC5

protein half-life was previously established to be 2.14, 3.35, and 5.93 h in HCT116, U2OS, and HEK293T cells, respectively [44] while MYD88 protein half-life was determined to be 45 h in THP1 cells (unpublished internal data). Analyzing time courses of PCSK9 RNA and protein reduction in two additional cell lines and using ASOs against two additional targets with large difference in reported protein half-lives, resulted in similar profiles of limited delay in protein reduction as the initial observation of the PCSK9 protein in HepG2 cells (Fig. 3C, E, and F).

ASO off-target RNA reduction correlates well with off-target protein reduction in HepG2 cells

Next, we sought to determine ASO-induced reduction of *in silico* predicted OffTs on both RNA and protein level. To our knowledge, no OND OffT study has yet been published comparing global changes at both RNA and protein levels. Based on the above described on-target silencing results we hypothesized that OffT RNA will be reduced for up to 48 h with the protein level following a similar time course pattern.

Hybridization-dependent OffTs were predicted *in silico* based on basic mismatch criteria [19], allowing for up to two nucleotide mismatches or a one nucleotide deletion or insertion anywhere in the ASO. Using these criteria, between 7 and 317 of the predicted OffTs for the different ASOs were detected in our overlapping set of 5903 transcripts and proteins from the RNAseq and proteomics experiments in HepG2 cells (Fig. 4A). As expected, predicted and detected OffTs were enriched in downregulated DEGs and DEPs (Supplementary Fig. S9). Between 6 and 147 of these detected OffTs were significantly downregulated on RNA level, illustrating the well-known high rate of false positive OffT predictions using currently available models [23]. It is worth noting that despite being designed to not have any OffTs in the human transcriptome according to the criteria above, dosing 596 713 resulted in 755 DEGs and 144 DEPs (Fig. 2D), illustrating that other causes than hybridization-dependent OffTs can result in differential expression.

At the protein level, only between 2 and 12 OffTs were significantly downregulated, i.e. clearly fewer OffTs predicted at RNA level showed corresponding reduction also at protein level. For two of the ASOs with predicted and detected OffTs, all downregulated proteins were also reduced at RNA level. For SPC5001 and LNA43, a few proteins encoded by RNA-level predicted OffTs did not show corresponding reduction at RNA level (4 out of total 7 DEPs and 2 out of total 12 DEPs for SPC500 and LNA43, respectively). With the high rate of false positive *in silico* OffT predictions and these two ASOs having many predicted and detected OffTs (240 and 317, respectively), we cannot rule out that the protein level reduction is the result of another mechanism than RNase H-dependent OffT activity.

Comparing negative OffT $\log_2(\text{fold changes})$, i.e. reduction, between RNA and protein level, most predicted and detected OffTs exhibit larger effects at the RNA level than protein level (Fig. 4B). For example, SPC5001 shows median $\log_2(\text{fold changes})$ of -0.64 on RNA versus -0.33 on protein level, 569720 shows -0.83 on RNA versus -0.49 on protein level and LNA43 shows -0.38 on RNA versus -0.23 on protein level. In contrast, LNA32 displays comparable magni-

tudes, with median $\log_2(\text{fold changes})$ of -0.16 on RNA and -0.23 on protein level.

These observations corroborate the common approach of focusing experimental OND OffT assessment to changes on RNA level [23, 24]. To assess how the RNA and protein time course profiles observed for on-target reduction compared to OffT reduction, we selected one OffT per ASO for further validation. We could once again confirm the RNAseq and proteomics results (Supplementary Fig. S10) using alternative qPCR and western blot methods (Supplementary Fig. S11). Not unexpected, reduction of OffTs with at least one mismatch was less efficient (Supplementary Fig. S10) than on-target reduction based on perfect RNA sequence homology (Fig. 3).

In summary, we observed that more predicted OffTs were affected on the RNA level than on the protein level. These results support the common approach of studying OND activity on RNA level but highlights the importance of also studying protein level changes to get a comprehensive understanding of the activity of a specific OND.

Discussion

Despite RNase H-dependent ASOs triggering degradation of RNAs leading to reduction of encoded proteins [5], analysis of broad ASO activity is often focused on changes at the RNA level [8, 9, 14, 23, 24]. The scarcity of broad protein level studies may be explained by past limitations of cost-efficient technologies allowing sampling at multiple time points to allow for anticipated differences in time course of downstream protein level reductions.

Global ASO-induced changes on RNA level have been studied in experiments of ASO-induced liver toxicity and analysis of hybridization-dependent OffT effects [12–14, 45]. These are both important safety parameters to assess when identifying safe candidate compounds for clinical studies [46]. To date, beyond correlation to the sheer number of DEGs, no mechanistic (pathway) association has been identified between *in vivo* liver toxicity and transcriptomic analyses [8, 9, 11, 14, 16]. In this study, we leveraged recent improvements in proteomics technologies [27–29] to also study broad protein level changes when addressing two important questions: (i) can acutely liver toxic and non-liver toxic ASOs be discriminated by *in vitro* changes in transcriptome or proteome and (ii) how do ASO-induced changes compare on RNA and protein levels for hybridization-dependent on- and off-targets over time.

To address the first question, we selected from the literature five well-characterized tool ASOs of same chemistry and very similar design with different reported *in vitro* and *in vivo* liver safety profiles [11, 14, 15, 30–32] and performed a head-to-head comparison with safety focus *in vitro* and *in vivo*. With one notable exception, our *in vitro* results (Fig. 1B and C) showed overall good correlation with our *in vivo* data (Table 1) and previously reported findings. The 569713 ASO was reported to be non-liver toxic in acute *in vivo* studies [14] which was confirmed in our acute *in vivo* study (Table 1), whereas our *in vitro* results in the Caspase 3/7 assay indicate an intermediate toxicity profile (Fig. 1B and C, and Supplementary Fig. S1). It is possible that 569713 would show more prominent liver toxicity in longer duration *in vivo* tolerability studies.

A

	Predicted ^a	Detected ^b	Only RNA down ^c	Only protein down ^d	Both down
LNA32	393	76	14	0	4
569713	0	0	0	0	0
SPC5001	1131	243	137	2	10
569720	43	7	4	0	2
LNA43	2059	326	114	4	3

No predicted off-targets for 569713.

a. *in silico* predicted sequence homology.

b. Detected RNA + protein dataset.

c. $\log_2(\text{fold change}) < -0.5$, adjusted p-value < 0.05 .

d. $\log_2(\text{fold change}) < -0.5$ at ≥ 2 timepoints, adjusted p-value < 0.05 .

B

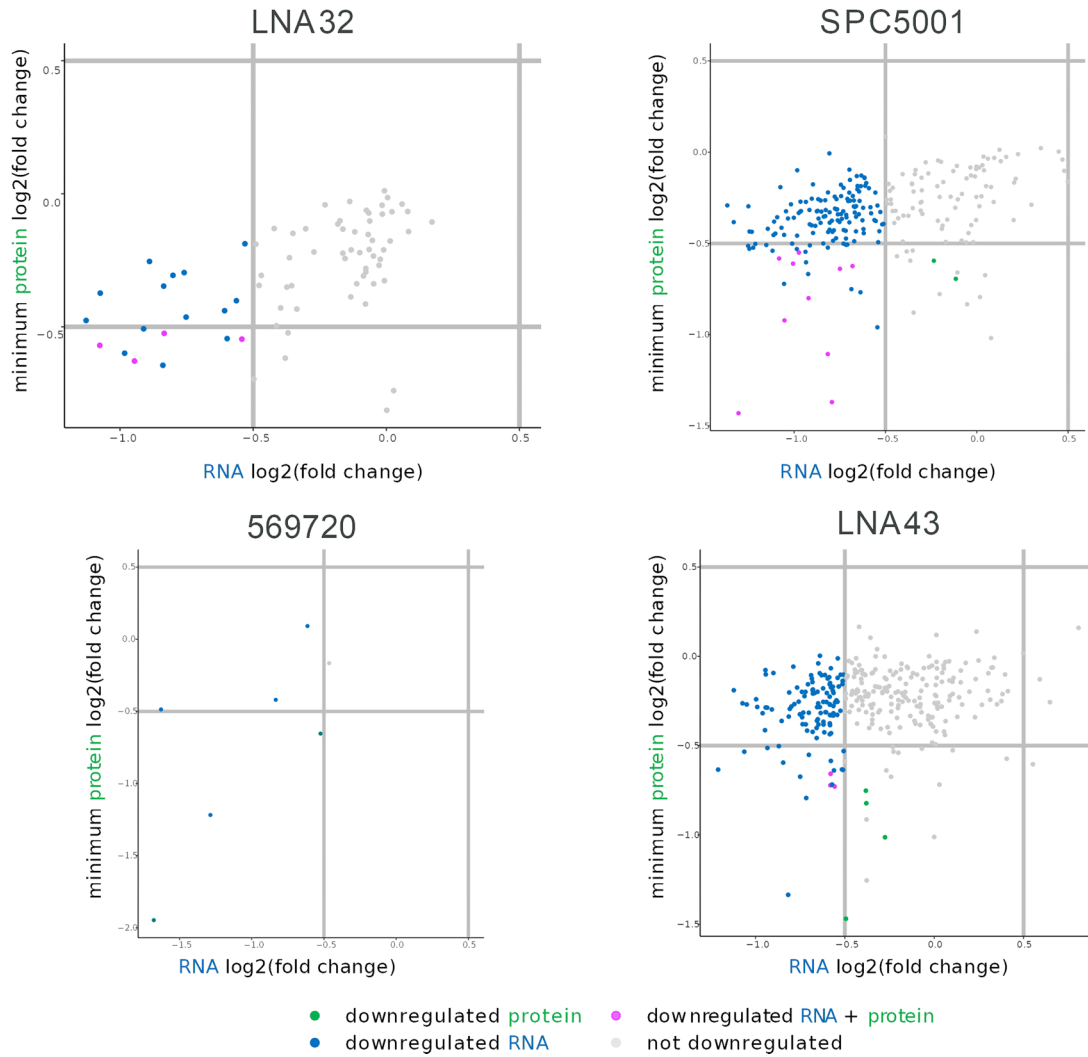


Figure 4. Overlap between off-target protein and RNA reduction. **(A)** Overview off-target downregulation by RNAseq and proteomics. **(B)** Cross-plots showing distribution of $\log_2(\text{fold change})$ for RNAseq quantification (x-axis) versus proteomics quantification (y-axis) for predicted and detected off-targets. Colors of points indicate downregulation status in each dataset; downregulation in the RNAseq dataset is based on $\log_2(\text{fold change}) < -0.5$ and Benjamini-Hochberg adjusted P -value < 0.05 and in the proteomics dataset downregulation is based on $\log_2(\text{fold change}) < -0.5$ and Benjamini-Hochberg adjusted P -value < 0.05 for at least two timepoints. The minimum (i.e. most negative) $\log_2(\text{fold change})$ across all timepoints is displayed for proteomics. Solid guide-lines show ± 0.5 $\log_2(\text{fold change})$ for each parameter. $N = 4$ or more biological replicates, RNAseq = RNA sequencing.

RNAseq and proteomics methods are well established for the derisking of small molecule-induced liver toxicity, as they provide comprehensive insights into cellular responses, contributing to the identification of adverse response patterns [47, 48]. Inspired by this, we sought to discriminate between liver toxic and non-liver toxic ASOs *in vitro* based on transcriptomics and proteomics data. Both datasets were analyzed using differential expression, pathway enrichment analysis and PCA approaches. Applying PCA showed a separation between liver toxic and non-liver toxic ASOs for both the RNAseq and proteomics datasets (Fig. 2B and C), aligning with previously published [11, 14, 15, 30–32] and our experimental *in vivo* (Table 1) and *in vitro* (Fig. 1B and C) data. Similar to the Caspase 3/7 assay results, 569713 showed an intermediate response and clustered together with SPC5001 in the PCA analysis. The consequences of the different dynamic ranges are also projected on the PCAs (generally higher % of variance explained for the proteomics, compared to the transcriptomics); in both modalities the structure of the experimental design was recapitulated in the PCA groupings (Fig. 2B and C). In line with previous studies [8, 9, 11, 14, 16], we could not identify common pathway perturbations for liver toxic ASOs in either RNAseq or proteomic datasets using differential expression and pathway enrichment approaches. We hypothesize that several pathways with different triggers could be involved to converge on Caspase 3/7 activation. Nevertheless, our results show that liver toxic ASOs induce a higher number of DEGs on both RNA and protein level than non-liver toxic ASOs (Fig. 2D). The ability to distinguish between liver toxic and non-liver toxic ASOs indicates that early safety-focused animal studies, exemplified by the acute tolerability studies described here, could potentially be replaced [49] by *in vitro* RNAseq and proteomics experiments. To summarize, unbiased analyses, such as PCA, can be used on both proteomic and transcriptomic datasets to discriminate between acutely liver toxic and non-liver toxic ASOs, as exemplified by our HepG2 results.

To address the second question on how ASO-induced changes compare on RNA and protein level, we determined their linear co-variation using Pearson's correlation. The correlations between transcriptomic and proteomic quantifications and amplitude of the variation in expression can vary significantly, especially between different cell lines [50] or tissues [51]. Ponomarenko *et al.* [52] note that correlation coefficient between mRNA and proteins levels for different tissues is typically below 0.5. It has been hypothesized that such differences are due to factors including variable protein translational efficiency, degradation, half-life, number of free ribosomes, post-transcriptional modifications, secondary structure, subcellular localization, transport, and tRNA availability [53, 54]. None of this information is captured by RNAseq. Li *et al.* [55] show that utilizing Ribo-seq data and cellular context can improve predictive power of protein translation algorithms, while Srivastava *et al.* [56] improve their predictions by focusing protein–protein interactions. In line with the above, here, we also observe low correlation at the 24 h post transfection timepoint for which we have obtained both RNAseq and proteomics data (Supplementary Fig. S4). Highest correlation was observed at 32 or 48 h post transfection, depending on the ASO treatment (Supplementary Fig. S5).

Transient transfection of 30–100 nM potent ASOs into *in vitro* cancer cell models typically induces maximum on-target RNA knockdown already 4–24 h after transfection [7, 11,

57–59]. The extent of on-target silencing efficiency varies between cell lines due to differences in transfection efficiency and cell model properties, but it also depends on ASO chemistry and sequence, target expression levels and transient transfection methods used [60]. Here, we observed maximum on-target mRNA reduction between 8 and 32 h post transfection for PCSK9 mRNA in three different cell lines (Fig. 3C). The comparatively large differences in maximum on-target RNA knockdown at the 24 h timepoint between cell lines (range 42%–79%, Fig. 3C) are not uncommon to observe across *in vitro* models, even in cell lines of the same tissue origin [61].

Interestingly, PCSK9 protein reduction was observed already 8 and 24 h post transfection. This was an unexpected finding considering the 44 h reported protein half-life in HeLa cells [40]. Protein half-lives can vary from cell line to cell line, as observed previously for primary cells with different tissue origin [62]. In HepG2 cells, PCSK9 mRNA levels returned to mock control levels 56–72 h after ASO transfection. This aligns with our previous observations and other studies that efficient ASO-induced target RNA silencing typically lasts for up to 3–5 days in cancer cell models, depending on the ASO chemistry and sequence [63] (data not shown). Interestingly, PCSK9 protein levels also returned to mock control levels after 56 and 72 h in all three cell lines without time delay in relation to mRNA levels (Fig. 3). This is consistent with the calculated translation dynamics in HepG2 cells, where PCSK9 mRNA is estimated to be converted into protein in 2 min, assuming a translation rate of six amino acids per second [64].

De-risking potential off-target effects is an essential part of the process to select and characterize therapeutic oligonucleotide candidate compounds for clinical studies [23, 24, 46]. We hypothesized that most OffTs showing significant down-regulation on the RNA level would also show reduction on the protein level. To our surprise, only a fraction (between 2.5% and 33% depending on the ASO sequence) of OffTs downregulated on the RNA level were also reduced on the protein level (Fig. 4A). This largely correlated with bigger negative fold changes observed for OffTs on the RNA level (Fig. 4B). Using refined cut-off criteria for DEG and DEP could potentially explain the low number of observed OffT protein changes. We have evaluated different combinations of RNAseq and proteomics differential expression cut-off values, but this only slightly increases OffT protein down numbers (data not shown).

In line with our PCSK9 time course observations, representative OffTs showed only a short time delay between RNA and protein reduction (Supplementary Figs S10 and S11). Since both on- and hybridization-dependent OffTs act via the same RNase H-mediated degradation mechanism, the on-target reduction data in three different human cell lines confirm that we are studying genuine OffT reduction time courses in our proteomics dataset. Our results indicate the importance of having a robust understanding of baseline and OND-induced RNA and protein reduction kinetics in the experimental system used to avoid incorrect assumptions and interpretation of the data. The ideal experiment would avoid transfection and instead use free, “gymnotic” ASO uptake in a cell model for which basal protein turnover rates are understood. A recent study by Damle *et al.* [37] showed that RNAseq upon ASO gymnotic uptake with a range of concentrations can also be efficiently used for OffT screening, where they report higher sensitivity and selectivity compared to single dose level experiments.

Updated industry recommendations [23] and a recently published draft FDA guidance [20] focus recommendations for OND OffT assessment on RNA level analysis. With a much higher number of OffTs captured on RNA than protein level, our results support this approach to experimentally capture as many *in silico* predicted OffTs as possible. However, with OND induced changes at protein level being the main concern for protein encoding transcripts, it is possible that an RNA level only approach is too conservative. A combination of high-content analysis technologies at both RNA and protein level such as RNAseq and proteomics could lead to improved understanding of biologically relevant OffT effects, with more granular analyses using e.g. qPCR and targeted proteomics [65] applied later in the compound selection process for refined analysis of any remaining OffTs.

To summarise, our results illustrate that global proteomics methods can serve as a valuable addition to the commonly used RNAseq, when studying key properties of therapeutic oligonucleotides. We show for a small set of reference ASOs that both RNAseq and proteomics quantification approaches can be used to separate *in vivo* liver toxic from non-liver toxic ASOs using conventional data analysis methods such as PCA. If this correlation could be explored across wider, more diverse sets of ASOs, *in vitro* -omics analysis may have the potential to replace *in vivo* tolerability rodent studies that are commonly used for early drug candidate selection. Larger datasets would also facilitate the development of AI/ML-based prediction models for use already at the early stage of oligo design. In this study, we focused on ASO-induced *in vivo* liver toxicity. For other ASO-induced toxicities such as inflammatory responses and kidney toxicity [46, 66], it will be essential to identify other cell models and tool compounds for validation of the predictive value.

We showed that ASO-induced on- and off-target reduction has a short duration on both RNA and protein level after transfection in three human cell lines. Given the reported longer protein half-lives in other cell lines, this observation was a surprise to us and underscores the importance of basic research, including robust understanding of RNA and protein turnover in the model system used. We also demonstrate that the vast majority of OffTs are captured by the currently recommended RNA focused assessment strategy. Analysis of protein abundance by proteomics and more directed analyses of protein abundance have the potential to add more nuance to OffT assessment by possibly “rescuing” and being able to progress an otherwise promising oligo clinical candidate with only problematic RNA level OffT data. Therefore, protein level studies can serve as an important complementary approach to current RNA level-focused OffT assessment. Outcomes of future work using larger datasets and different model systems will determine if the current RNA level analysis should be complemented or even be replaced by assessment at the protein level.

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Author contributions: D.v.L. (Conceptualization, Formal analysis, Investigation, Visualization, Writing—review & editing, Writing—original draft), M.C. (Formal analysis, Software, Writing—review & editing), E.C.W. (Formal analysis, Software, Writing—review & editing), A.I. (Conceptualization, Investigation, Methodology, Data curation, Writing—review & editing), B.C. (Formal analysis, Software, Writing—review & editing), J.W. (Formal analysis, Software, Writing—review & editing), G.H. (Conceptualization, Methodology, Writing—review & editing), D.C. (Data curation, Formal analysis, Software, Writing—review & editing), N.G. (Methodology, Writing—review & editing), J.Y.T. (Formal analysis, Software, Supervision, Writing—review & editing), E.M. (Conceptualization, Methodology, Writing—review & editing), R.S. (Supervision, Writing—review & editing), I.M. (Supervision, Writing—review & editing), S.P. (Supervision, Writing—review & editing), and P.A. (Conceptualization, Supervision, Writing—review & editing, Writing—original draft).

Supplementary data

Supplementary data is available at NAR Molecular Medicine online.

Conflict of interest

D.v.L., M.C., A.I., B.C., J.W., G.H., D.C., N.G., J.Y.T., E.M., S.P., and P.A. were employed by and in most cases holding shares in AstraZeneca R&D when this study was conducted.

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Data availability

The RNAseq data underlying this article are available in the NCBI Sequence Read Archive (SRA) at <https://www.ncbi.nlm.nih.gov/sra>, under project accession PRJNA1302113. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository at <https://www.ebi.ac.uk/pride/> with the dataset identifier PXD065583.

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