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Antibody-oligonucleotide conjugate achieves central nervous system delivery in animal models for spinal muscular atrophy

Brain delivery of ASO with antibody-ASO conjugates

Authors

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Conflict-of-interest:
M.J.G. and M.J.A.W. are co-founders and equity holders in PepGen, a company developing peptide-conjugated oligonucleotides. C.W., M.B., G.T., and I.G., are employed by AstraZeneca. All other authors declare no competing interests.
Abstract

Antisense oligonucleotides (ASOs) have emerged as one of the most innovative new genetic drug modalities. However, their high molecular weight limits their bioavailability for otherwise treatable neurological disorders. We investigated conjugation of ASOs to an antibody against the murine transferrin receptor (TfR), 8D3_{130}, and evaluated it via systemic administration in mouse models of the neurodegenerative disease, spinal muscular atrophy (SMA). SMA, like several other neurological and neuromuscular diseases, is treatable with single-stranded ASOs that modulate splicing of the survival motor neuron 2 (SMN2) gene. Administration of 8D3_{130}-ASO conjugate resulted in elevated levels of bioavailability to the brain. Additionally, 8D3_{130}-ASO yielded therapeutic levels of SMN2 splicing in the central nervous system of adult hSMN2 transgenic mice which resulted in extended survival of a severely affected SMA mouse model. Systemic delivery of nucleic acid therapies with brain targeting antibodies offers powerful translational potential for future treatments of neuromuscular and neurodegenerative diseases.
Introduction

RNA-based therapeutics are an emerging form of therapy amendable to treating multiple neurodegenerative and neuromuscular diseases, many of which are currently without a therapeutic intervention. Antisense oligonucleotides (ASOs) are synthetic single-stranded oligonucleotides or oligonucleotide analogues designed to bind to RNAs, either messenger (mRNA) or non-coding RNA (ncRNA), using Watson-Crick base pairing. ASOs modulate the function of RNA either through steric blockage of cis regulatory elements on mRNAs or by inducing RNase H1 mediated degradation of the targeted RNA (1). There are currently 8 FDA approved single-stranded ASO drugs and many more under pre-clinical investigation (2). One of these, nusinersen, has been approved for the treatment of spinal muscular atrophy (SMA). SMA is a neurodegenerative disease characterized by the degeneration of lower motor neurons found within the spinal cord and by subsequent skeletal muscle atrophy. SMA is caused by a reduced level of survival motor neuron (SMN) protein due to mutations and/or deletions in the *SMN1* gene. Humans carry a redundant parologue of *SMN1*, called *SMN2*, which is unaffected in the majority of SMA patients. However, *SMN2* has two main splice variants; full-length *SMN2* (*FLSMN2*) mRNA (~10%) yields functional SMN protein, while Δ7SMN (~90%) generates an unstable truncated SMN protein that is typically degraded (3–6). ASOs designed to bind to the intron splice suppressor N1 (ISS-N1) of *SMN2* pre-mRNA, can block splicing factors from binding and thus increasing the probability of exon 7 incorporation. This, in turn, increases the level of mature *FLSMN2* mRNA and functional SMN protein. The clinically successful ASO therapy for SMA (nusinersen, marketed as Spinraza®) has received worldwide regulatory approval (7).

Systemic ASO therapy of neurodegenerative diseases is made challenging by the need to cross the neurovascular unit in the brain (blood-brain barrier, BBB) and spinal cord (blood-
spinal cord barrier, BSCB). On their own, systemically administered ASOs are readily accumulated in the liver but are only very moderately distributed to peripheral tissues such as skeletal muscle. Advanced chemical modifications to the backbone and sugar moieties of ASOs have improved tissue uptake but have yet to achieve significant BBB/BSCB penetration for distribution in brain and spinal cord. As such, ASO compounds, either approved (including nusinersen) or in development for neurodegenerative disease, typically circumvent the BBB/BSCB via local administration directly into the circulating cerebrospinal fluid compartment, using intrathecal delivery (8). However, intrathecal administration typically results in lower levels of ASO exposure in higher (cervical) spinal cord and cerebral regions. It is also precluded in certain SMA patients such as those with spinal column abnormalities (9). Additionally, following intrathecal administration, only low levels of ASO have been reported outside the CNS, for example in important target tissues such as skeletal muscle and liver, both known to be affected in SMA (10). Therefore, a systemically administered ASO delivery system with biological activity in brain and spinal cord as well as in peripheral tissues is required to facilitate optimal treatment in all SMA patients.

The transferrin receptor (TfR) is the most widely studied pathway for transport of antibody-based drugs across the BBB, BSCB, and choroid plexus (11–14). TfR is expressed on the luminal side of brain capillary endothelial cells where it binds transferrin and traffics iron into the parenchyma. Anti-TfR antibodies and antibody fragments have successfully used this pathway to deliver drug cargoes into the brain parenchyma (11, 13, 15, 16). However, to achieve sufficient therapeutic levels of drug exposure within the brain, precision engineering of the binding affinity to TfR is required. Antibodies with a high affinity for TfR preferentially accumulate in brain capillaries, but do not release efficiently to the abluminal side (17–21). Elevating the levels of antibody accumulation in the brain parenchyma required engineering to optimise affinity, introducing pH-dependent binding, and/or creating
monovalent binding (21–23). The anti-mouse Tfr monoclonal antibody, 8D3₁₃₀, was
designed to bind specifically to mouse Tfr (mTfr) at sufficiently low affinity to permit BBB
transcytosis and release it from the mTfr once exposed to the brain parenchyma (16, 24).
Our previous work has demonstrated the ability of cell-penetrating peptides to deliver
morpholino phosphorodiamidate oligonucleotides (PMOs) across the BBB and BSCB at
therapeutically relevant doses (25). Here we report that systemic delivery of PMOs directly
conjugated to the anti-mouse Tfr monoclonal antibody 8D3₁₃₀ yields even greater
bioavailability in the CNS. This induces high expression of full-length SMN2 mRNA and
SMN protein in the CNS and in peripheral tissues of an adult hSMN2 transgenic mouse, and
rescues survival of severely affected neonatal SMA mice. This work provides a way forward
for systemic ASO treatment in SMA and other neurodegenerative diseases treatable with
ASOs including Huntington’s Disease (HD), amyotrophic lateral sclerosis (ALS),
spinocerebellar ataxia 2 (SCA2) and tauopathies such as Alzheimer’s disease (26).
Results

Synthesis and purification of anti-TfR antibody-PMO conjugates

The ISS-N1 regulatory element found in intron 7 of the hSMN2 gene is the most promising target for modulation of SMN2 splicing. Steric masking of ISS-N1 with a fully modified ASO favouring inclusion of exon 7 (27–29). We synthesized a 25-mer PMO targeting ISS-N1 and directly conjugated it to a short maleimide functionalized peptide linker (Mal-C3-FB[RB]6, B = beta-alanine; Figure 1A). The maleimide-functionalized PMO was then conjugated to the heavy chain of either a low affinity anti-mouse TfR (8D3130) or an isotype-control antibody against nitrophenol (NIP228) (16) (Figure 1A). The PMO-antibody conjugates were purified via size exclusion chromatography to remove any unreacted maleimide-functionalized PMO and antibody. Analysis of purified antibody-conjugates by MALDI-TOF mass spectrometry revealed a fully modified product with a drug to antibody ratio of 2, i.e., the incorporation of 2 PMOs per antibody, which correspond to an overall mass shift of 20.4 kD (Figure 1, B and C, Supplemental Figures S1 and S2).

PMO conjugation alters the pharmacokinetic properties of the 8D3130 antibody

To determine the pharmacokinetic (PK) properties of the antibody-PMO conjugates 8D3130–PMO and NIP228-PMO, and whether these altered the plasma, brain and spinal cord exposure over that of the antibody alone, we conducted a plasma PK, brain and spinal cord exposure study in vivo. Adult wild type C57BL/6J mice were administered a single intravenous (IV) dose of 20 mg/kg of 8D3130 (± PMO) and NIP228 (± PMO). Blood, brain and spinal cord homogenate samples were studied at regular time intervals over a one-week period. A MesoScale Discovery (MSD) Universal hIgG capture and detection assay for 8D3130 and NIP228 antibodies was used to determine exposure parameters.
Blood plasma was sampled at 17 min, 4-, 6-, 24-, 48-, 96-, 120- and 168-hours post-administration. At their highest, the maximum concentration (Cmax) of 8D3\textsubscript{130} was 8-fold higher than its PMO-conjugated antibodies, whilst that of NIP228 was 4-fold higher than its control. The addition of PMO correlated negatively with clearance data (Cl (ml/h/kg)), revealing a 4- and 2.5-fold higher clearance with PMO, compared to 8D3\textsubscript{130} and NIP228 without PMO (Table 1). During the first 4 days post-injection, unconjugated 8D3\textsubscript{130} had a significantly higher total plasma exposure (AUC\textsubscript{last}, the area under the plasma concentration-time curve from time zero to the last measured concentration) than 8D3\textsubscript{130}-PMO (Figure 2A and Table 1). One-week post-injection, the total plasma exposure (AUC\textsubscript{last}) was 7- and 3-fold higher for 8D3\textsubscript{130} and NIP228 compared with their respective PMO-conjugated antibodies (Table 1).

Brain and spinal cord samples were collected at 4-, 24-, 96- and 168-hours post-administration and homogenates processed for analysis via MSD assay (Figure 2, B and C). The MSD plate-based sandwich immunoassay was formatted whereby the anti-human IgG capture antibody bound to sample hIgG (± PMO), and a specific detection antibody labelled with SULFO-TAG emitted light on electrochemical stimulation. The levels of hIgG (± PMO) in plasma, brain and spinal cord were quantified by reference to standard curves generated using calibrator samples with a four-parameter nonlinear regression model. At 24 hours post-administration, the C\textsubscript{max} of 8D3\textsubscript{130} (± PMO) in the brain (Figure 2B), was significantly 6-fold higher than NIP228 and 10-fold higher than NIP228-PMO (Table 2). At one week, the total exposure to the drug (AUC\textsubscript{last}) of 8D3\textsubscript{130} and 8D3\textsubscript{130}-PMO was 8- and 5-fold higher, respectively, than NIP228-PMO (Table 2). Brain and spinal cord measurements for NIP228-PMO were below the lower limit of quantification (LLoQ) for the assay for all time points and are not presented here.
In the spinal cord, $C_{\text{max}}$ at 24 hours of $8D3\sub{130}$ ($\pm$ PMO) was 6-fold higher than NIP228 and 7-fold higher than NIP228-PMO (Figure 2C and Table 1). AUC$_{\text{last}}$ of $8D3\sub{130}$ and $8D3\sub{130}$-PMO was an impressive 51- and 22-fold higher, respectively, than for NIP228 (Table 2). Lower AUC for both $8D3\sub{130}$-PMO and NIP228-PMO are likely explained by the increased clearance of the conjugated forms.

**Dose-dependent SMN2 upregulation in brain and spinal cord by $8D3\sub{130}$-PMO**

The hSMN2 transgenic mouse strain (FVB.Cg-Smn1$^{tm1Hung}$ Tg(SMN2)2Hung/J) carries the entire human SMN2 gene, lives to adulthood, and has an unaltered BBB and BSCB (30, 31). This strain is therefore an ideal adult mouse model in which to evaluate the biodistribution and biochemical efficacy of nucleic acid drug compounds that regulate SMN2 gene expression and splicing. SMN2 transgenic mice were given a single IV administration of $8D3\sub{130}$-PMO or control NIP228-PMO with a 10, 20, 50, 80 or 100 mg/kg dose. A matched volume of 5 $\mu$l/g saline administration was used for untreated controls. Each dosage group had $n = 5$ mice per group with exception of 20 mg/kg dose where $n = 4$ mice. Tissues were harvested 7 days post-administration and analysed for SMN mRNA splicing by qRT-PCR (10, 20, 50, 80, and 100 mg/kg dosage) and SMN protein (by western blot; 10, 50, 100 mg/kg dosage) (Figures 3 and 4).

$FLSMN2$ mRNA (qRT-PCR expression of exon 7) was normalised to total SMN (qRT-PCR expression of exons 1-2a) and plotted as a fold change over saline treated expression (Figure 3, A and D). At the lowest dose of 10 mg/kg, $FLSMN2$ expression in the brain was statistically greater than saline treated mice (1.52 ± 0.08 vs 1.0 ± 0.098 fold change). A maximum average expression of 2.213 ± 0.145 fold expression over saline treatment was observed at 50 mg/kg dose. This activity was reflected in western blots of SMN protein (Figure 3B). A 50 mg/kg and 100 mg/kg administration of $8D3\sub{130}$-PMO yielded 1.6 ± 0.089
and 1.8 ± 0.128 fold change in SMN protein expression over saline treatment, respectively. The high activity was also notable in the spinal cord. FLSMN2 mRNA analysis of the thoracic region of the spinal cord yielded the highest fold change at 80 mg/kg (2.0 ± 0.2) and 100 mg/kg (2.4 ± 0.086) compared to saline-treated controls (Figure 3D). Protein was extracted from the cervical region of the spinal cord. At the 100 mg/kg dosing, SMN protein expression was significantly increased over saline treatment (1.52 ± 0.2 vs 1.0 ± 0.07), p = 0.009 (Figure 3E). In contrast, NIP228-PMO treated tissues were not found to be significantly changed from saline treatment. Discrepancies between RNA and protein results has been classically observed in both pre-clinical and clinical contexts of SMA in adults (9). However, the large difference between RNA and protein results in the spinal cord may also be due to morphological differences between cervical and thoracic regions of the spinal cord (32).

To determine the tissue concentration of PMO, a single IV dose of 50 mg/kg (n = 6 per group) was chosen given its elevated level of activity. Seven days post-administration, tissues were collected and the concentration of PMO was determined via ELISA (33) (Figure 3, C and F). A scrambled PMO conjugated to 8D3130 was used to control for aberrant probe binding. The tissue concentration of PMO delivered by 8D3130 within the brain (1796 ± 475 ng/g) and spinal cord (870 ± 610 ng/g) was significantly greater than PMO delivered by NIP228 (105 ± 88 ng/g and 340 ± 244 ng/g, respectively). While not directly measured, it can be assumed that active PMO accumulated in the tissue would no longer be attached to the antibody as the antibody would prevent the PMO from access the nucleus.

Enhanced uptake and activity were also notable in the peripheral tissues. Neuromuscular diseases typically require therapies to target skeletal muscles. SMA in particular has also been shown to affect the liver and kidney tissues (10, 34, 35). Therefore, a potential advantage of a systemically administered drug treatment would be its activity within these...
tissues. We analysed the activity of 8D3_{130}-PMO and NIP228-PMO in tissues from the adult SMA mice treated with a single IV administration of 10, 20, 50, 80 or 100 mg/kg dose of antibody-PMO. Two skeletal muscles of the hindlimb, tibialis anterior (TA) and quadriceps (Quad) were selected for evaluation of FLSMN2 mRNA and protein expression over saline treated tissues (Figure 4, A and B). Elevated levels of expression and eventual plateau starting from 50 mg/kg was notable in the TA for both mRNA and protein (Figure 4, A and B). In the Quad, a 20 mg/kg dose of 8D3_{130}-PMO yielded a 2-fold enhanced expression over saline treated Quad (1.9 ± 0.6 vs 1.0 ± 0.17 fold change, respectively). The highest level of mRNA expression was achieved at the 50 mg/kg and 80 mg/kg doses (3.2 ± 0.7 and 3.7 ± 0.3 fold change, respectively), indicating a similar plateau in efficacy at higher doses. The control NIP228-PMO also yielded elevated levels of activity in Quad but not in the TA for both mRNA (Quad: 3.2 ± 0.5 fold change and TA: 1.7 ± 0.17 fold change) and protein (Quad: 1.9 ± 0.2 fold change and TA: 1.43 ± 0.17 fold change) (Figure 4, A and B). The observed molecular effects are correlated to the PMO concentration in the skeletal muscle: both 8D3_{130} and NIP228 delivered elevated levels of PMO into tibialis anterior and quadriceps (Figure 4, E and F). The difference in activity could be due to variations in muscle fibre composition between skeletal muscles.

Both 8D3_{130}-PMO and NIP228-PMO were active in the liver and kidney (Figure 4, C and D). FLSMN2 mRNA expression in liver was highest at 50 mg/kg for both 8D3_{130}-PMO (3.17 ± 3.6 fold change) and NIP228-PMO (3.6 ± 0.2 fold change). There was no significant difference between the two antibody-PMOs in mRNA expression, however, the protein expression was greater in 8D3_{130}-PMO treatment at 100mg/kg (3.2 ± 0.6 fold change) than NIP228-PMO (2.1 ± 0.5 fold change), p < 0.0001 (Figure 4B). Similarly, there is a significant difference between the concentration of 8D3_{130}-PMO and NIP228-PMO (p = 0.02) in the liver (Figure 4G). In the kidney, there appeared to be a plateau in activity from 50 mg/kg both
at the mRNA and protein levels (Figure 4, C and D). Kidney expression of *FLSMN* mRNA and protein was lower than the liver for all doses (Figure 4 C and D). While the activity of the PMO was lowest in the kidney, the PMO concentration was the highest of all tissues evaluated, indicating a non-functional accumulation of PMO or antibody-PMO (Figure 4H). However, the high PMO accumulation did not result in enhanced levels of kidney injury molecule-1 (KIM-1), a marker of kidney injury previously observed in animals treated with high concentrations of oligonucleotides (Supplemental Figure S3) (36).

**8D3\textsubscript{130}-PMO localisation throughout the brain**

Previous studies have shown that anti-TfR antibodies typically require 24 hours to translocate through the endothelia of the BBB or BSCB to enter the parenchyma of the brain and spinal cord (21). To assess the topography of distribution in the brain, we dosed *hSMN2* transgenic mice with 50 mg/kg 8D3\textsubscript{130}-PMO and NIP228-PMO (n = 3 per group) and perfused animals 24 hours later. Cryosections of the brain were immunohistochemically stained for 8D3\textsubscript{130}-PMO and NIP228-PMO using an Alexa Fluor 488 goat anti-human IgG secondary antibody (Figure 5, A and B). Whole brain images revealed a fluorescent signal for 8D3\textsubscript{130}-PMO throughout the brain. The strongest staining of 8D3\textsubscript{130}-PMO was observed in the thalamus, pons and cerebellum regions of the brain. In contrast, NIP228-PMO was not observable in the brain.

To validate that observed fluorescence correlates with drug activity within the brain parenchyma, we performed an endothelial brain tissue fractionation experiment on adult SMA mice dosed with 50 mg/kg (n = 6 per group) to separate the endothelium of the blood-brain barrier from the brain parenchyma. Tissues were collected 7 days post-administration, and for this experiment, we included the 8D3\textsubscript{130}-scrambled PMO (8D3\textsubscript{130}-ScrPMO) as a control. Enrichment of the endothelial cell (EC) and parenchyma fractions was validated with
qRT-PCR analysis of EC markers (Pecam1 and Vcam1), neuronal makers (b-tub III and Map2) as well as a glial marker (Gfap) (Figure S3). FLSMN2 mRNA expression was measured in both fractions (Figure 5, C and D). Both saline and 8D3\textsubscript{130}-ScrPMO controls yielded comparable results, indicating no activity from the 8D3\textsubscript{130} antibody itself. 8D3\textsubscript{130}-PMO was active in both the brain parenchyma (1.89 ± 0.28 fold change) and endothelial cell (1.93 ± 0.23 fold change) fractions. NIP228-PMO activity was not significantly different from saline or 8D3\textsubscript{130}-ScrPMO activity.

8D3\textsubscript{130}-PMO co-localizes to astrocytes of the spinal cord.

To evaluate which spinal cord cell populations were targeted by 8D3\textsubscript{130}-PMO, we dosed adult SMN2 transgenic mice with 50 mg/kg 8D3\textsubscript{130}-PMO or NIP228-PMO and perfused animals 24 hours later. Cryosections of spinal cord were then analysed for co-localization of the 8D3\textsubscript{130}-PMO and NIP228-PMO to motor neurons (using ChAT immunolabelling) and astrocytes (using GFAP immunolabelling). As TfR is found on the surface of neurons in the spinal cord, we expected a widespread cellular distribution of 8D3\textsubscript{130}-PMO (12). Instead, we observed a more cell-specific uptake of 8D3\textsubscript{130}-PMO in the astrocytes of the spinal cord, with minimal co-localization within motor neurons (Figure 6). The tight association of astrocytes with endothelial cells in the BBB and BSCB regulates passage of compounds into the nervous parenchyma. It is therefore likely that 8D3\textsubscript{130}-PMO remains sequestered in astrocytes, which are the first glial population encountered in the spinal cord parenchyma.

8D3\textsubscript{130}-PMO increases FLSMN2 expression and extends survival in severely affected SMA mice

The hSMN2 mouse strain (FVB.Cg-Smn1\textsuperscript{tm1Hung} Tg(SMN2)2Hung/J) can be bred to produce a severe SMA phenotype in neonatal pups. SMA pups exhibit lower body weight than their
unaffected littermates by day 5 and reduced movement from day 6-8, and subsequently die between 7-10 days of age (30). To assess the potential of 8D3\textsubscript{130}-PMO to prevent the onset of an SMA phenotype, pups were treated with single subcutaneous administration of 20 mg/kg or 50 mg/kg dose at post-natal day 0 (PND0). Survival and tissue expression of SMN2 mRNA was evaluated (Figure 7). Median survival following single subcutaneous administration of 20 mg/kg 8D3\textsubscript{130}-PMO (n = 7), NIP228-PMO (n = 15), 8D3\textsubscript{130}-scrPMO (n = 11) or 0.9% Saline (n = 17) was 24, 12, 11 and 7 days, respectively. Survival of 8D3\textsubscript{130}-PMO treatment was statistically greater than NIP228-PMO or 8D3\textsubscript{130}-scrPMO, p < 0.0001 (Figure 7A). Pups treated with the higher dose of 50mg/kg 8D3\textsubscript{130}-PMO (n = 7) or NIP228-PMO (n=8) survived a median of 22 and 21 days respectively (p = n.s.) (Figure 7B). Only at the lower treatment dose was the difference in activity between 8D3\textsubscript{130}-PMO and NIP228-PMO evident. \textit{FLSMN2} mRNA expression was measured in a separate group of pups treated at PND0 with 50mg/kg 8D3\textsubscript{130}-PMO, NIP228-PMO, 8D3\textsubscript{130}-Scrambled PMO or 0.9% saline. Brain, spinal cord, skeletal muscles from the hindlimbs, heart, kidney and liver tissues were collected PND7. In all tissues evaluated, both 8D3\textsubscript{130}-PMO and NIP228-PMO significantly enhanced \textit{FLSMN2} levels over saline and 8D3\textsubscript{130}-scrambled PMO treated tissues (Figure 7, C-H). 8D3\textsubscript{130}-PMO produced greater \textit{FLSMN2} expression over NIP228 within brain (3.5 ± 0.84 vs 2.3 ± 0.6 fold change), spinal cord (5.1 ± 1.24 vs 3.4 ± 1.2 fold change) and heart (3.8 ± 0.24 vs 2.96 ± 0.24 fold change) (Figure 7, C, D and F).
Discussion

There are currently over 80 antibody drugs approved by the FDA. The majority of them treat immune-mediated diseases and various cancers, including hematologic malignancies and solid tumours. Only nine of the 80 approved antibody drugs are antibody-drug conjugates (ADCs), all of which are approved for cancer therapies (37). These ADCs act by increasing the internalization of cytotoxic small molecules into cells expressing cancer cell membrane proteins such as CD30 and CD33. Antibody-ASO conjugates are a newer class of drug only recently developed for therapeutic application (38).

ASO therapies have become one of the most promising forms of gene therapies for a wide range of diseases. In their naked form, ASOs are unable to pass through the BBB or BSCB and therefore require invasive modes of delivery through either direct intracerebroventricular or intrathecal administrations to treat neurodegenerative diseases. The highly successful nusinersen, an ASO targeting \( SMN2 \) in patients with SMA, has extended survival and welfare for many children (8). However, the repeated intrathecal administrations required for treating neurodegenerative diseases subject patients to a lifetime of this invasive procedure. Reaching the CNS via systemic administration would be a major step forward in ASO therapies. We have previously used peptides to deliver ASOs to the brain and spinal cord at therapeutically relevant levels (25). Here we made use of the natural mechanisms for translocation across the BBB/BSCB by targeting the TfR with an anti-mouse TfR antibody 8D3\(_{130}\). Systemic administration of 8D3\(_{130}\)-PMO conjugates resulted in elevated levels of CNS exposure in an adult \( hSMN2 \) mouse model while NIP228-PMO control was found to have similar effects to 8D3\(_{130}\)-PMO in peripheral tissues.

TfR1 is a 97-kDa type II membrane protein expressed as a homodimer (39, 40). The TfR1 binds to iron-laden transferrin and translocates it across brain endothelial cells. Using an anti-TfR1 antibody to translocate a cargo across the BBB has been previously successfully carried
out with various cargos (11). Additionally, delivery of ASOs conjugated to anti-transferrin antibody have been used to image gene expression in rat models of brain ischemia and brain glial tumors (41–43). However, studies into antibody-delivery of ASOs to the brain are limited. These early studies did not investigate the cellular biodistribution of the anti-TfR conjugates, nor the activity of the ASOs. The lack of in-depth imaging leaves doubts into the mechanisms of transport of the anti-TfR-conjugates across the brain endothelium and of cell-specific uptake.

The anti-TfR antibody 8D3\textsubscript{130} was developed from the parent antibody 8D3, which has a stronger binding affinity to the mTfR, with respective $k_D$ of 130nM and 1.2nM (16). 8D3 is capable of transducing across the BBB into the brain parenchyma and has been used as a fusion protein with the neuroprotective glycoprotein cytokine erythropoietin (EPO) (44). The 8D3-EPO fusion antibody garnered a modest effect in the AD mouse model (45).

8D3\textsubscript{130}-PMO conjugate modified the PK and activity of both the 8D3\textsubscript{130} and PMO alone (Figure 2). PMO reduced the amount of 8D3\textsubscript{130} assayed in the brain from the first sampling but only reduced 8D3\textsubscript{130} in spinal cord 96 hours post-administration. Plasma levels of 8D3\textsubscript{130} were also reduced when conjugated to PMO. This suggests that the modified antibodies are interacting with cell surfaces or extracellular matrix, thus reducing their bioavailability.

However, despite the reduced plasma levels, 8D3\textsubscript{130}-PMO greatly improved uptake and activity of the PMO into the brain and spinal cord. To observe the effect of 8D3\textsubscript{130} delivery of PMO to the brain and spinal cord, we chose an adult mouse model with four copies of the human $SMN2$ transgene (31). This animal has no phenotype or observed disruption of the BBB or BSCB and should therefore recapitulate the biodistribution required for treating neurodegenerative and neuromuscular diseases. A two-fold change in $FLSMN2$ expression is clinically relevant for alleviating SMA disease pathology (46–48). Here, we have shown the 8D3\textsubscript{130}-PMO conjugates reaches above a 2-fold change to $FLSMN2$ expression in the brain.
and spinal cord following single dose administrations of 50 mg/kg and above. Although fold changes in protein levels are not as high, similar levels of FLSMN2 expression in the same mouse model have only been achievable by direct brain administrations of 100-200 µg PMO (49). Therapeutically relevant levels of FLSMN2 expression were also observable in skeletal muscles, tibialis anterior and quadriceps, as well as the liver while lower levels of expression was achieved in the kidneys. In addition, doses as high as 100 mg/kg had no observable negative effects on the mice.

Despite this accumulation of compound within the kidneys, preliminary toxicity data using the above mouse model shows no significant toxicity when analysing levels of kidney injury marker 1 (KIM-1) (Supplemental Figure S3), a urinary biomarker of acute kidney injury (36). We hypothesise that the lack of kidney toxicity previously seen in ASO treated mice (50) could be due to the natural path of kidney circulation for monoclonal antibodies (mAbs). The majority of mAbs are reabsorbed in proximal tubules and re-enter the systemic circulation. However, the highest tissue concentration of our compounds was observed in the kidneys (Figure 4 H). Whilst the molecular mass of 8D3_{130}-PMO (ca. 170 kD) is far greater than the 60 kD glomerular filtration cut-off, free PMO moieties resulting from metabolic cleavage (in the plasma or elsewhere) of the antibody from the PMO would be reabsorbed by the proximal tubule epithelia – which has previously been identified as the structure in the kidney with the highest localised concentrations of ASOs (51). Thus, while low levels of renal re-uptake were anticipated, the antibody-PMO conjugates face the common ASO renal accumulation phenomenon described in the field (52).

The regional pattern of PMO activity and uptake into the brain and spinal cord was region and cell specific. The BBB is not homogenous throughout the CNS and differences in permeability may allow for the targeted passage into specific regions of the brain (53, 54) . Immunohistochemistry of the 8D3_{130}-PMO compounds indicate a particularly elevated level
of uptake into pons and thalamus regions of the brain (Figure 5). A similar observation was made with another anti-TfR drug conjugate, JR-141. JR-141 is an anti-human TfR-human iduronate-2-sulfatase (IDS) protein conjugate generated to treat the lysosomal storage disease mucopolysaccharidosis II (MPSII). Preclinical studies in mice and monkeys given IV administration observed JR-141 within Purkinje cells of the cerebellum and pyramidal cells in the hippocampus (13). The authors also observed widespread biodistribution of JR-141 in the heart, kidney, liver, lung, and spleen. However, they did not use an antibody isotype control, so it is unclear if this is specific for TfR binding or due to a more general antibody uptake. These results led to a Phase I/II clinical trial which demonstrated reduction in heparan sulfate (a lysosomal glycosaminoglycan inadequately catabolised in MPSII) in the cerebral spinal fluid of treated patients (55).

Investigation of spinal cord delivery showed a heterogenous patterning of 8D3_{130}-PMO activity seen in FLSMN2 and SMN protein levels across the cervical, thoracic and lumbar regions of the spinal cord (Figure 3, D and E). This could be accounted for by the different levels of permeability between the sections of the BSCB (32). A high rate of uptake was observed in the astrocyte cell population but could not be seen in the motor neurons of the spinal cord (Figure 6). Astrocytes are a critical component of the BBB and BSCB and they are the first point of contact for compounds translocating across the endothelium. Their expression of the Fc gamma receptor (FcγRI), which binds to the Fc component of the immunoglobulin IgG (56), may facilitate cell uptake of the antibody-PMO conjugate. Some experimental evidence also suggests TfR expression on astrocytic cell membrane (57–59). Astrocytes provide metabolic support to motor neurons and low levels of SMN in astrocytes exacerbate motor neuron death in SMA (60). Increasing astrocyte-directed SMN expression extended survival in severe SMA line of mice by rescuing defects in NMJs and proprioceptive synapses, and can extend lifespan and gross motor function in SMA mice (60,
Similarly, neurons co-cultured with astrocytes cultured from SMA mice had reduced synaptic formation and transmission indicating a cell-autonomous effect in SMA derived astrocytes (62). SMN deficiency alters intracellular calcic signalling in astrocytes which in turn may affect communication with motor neurons (62, 63). SMN deficiency in astrocytes also results in lower levels of Ephrin2 expression – an astrocytic membrane protein involved in axon guidance (62). Additionally, SMA motor neurons co-cultured with SMA or wild type astrocytes resulted in similar numbers of synapses and excitatory postsynaptic current, highlighting the importance of the astrocyte-motor neuron interaction in SMA (62).

Recent work has also suggested that a dysfunction of neuronal circuits is a primary causal event for SMA. In a severe SMA mouse model, the loss or reduction of proprioceptive synapses on motor neurons precedes motor neuron loss (64). Pharmacological rescue of the synapses resulted in improved motor behaviour (65). In our study, it is possible the rescue of survival of severe SMA mice following treatment with antibody-PMOs was due to a rescue of SMN in peripheral neurons rather than the astrocytes. Further study into the 8D3130-PMO bio-distribution of the peripheral nervous system is an exciting next step for this work.

Delivery of oligonucleotides and siRNA with antibodies has been mostly studied using the transferrin receptor as the antibody target. Early work by Penichet et al. used a system whereby an antibody is fused to avidin to act as a carrier for biotinylated pharmaceutically active drug – in their case, as a carrier for a biotinylated peptide nucleic acid (PNA) oligo (66). However, only 0.12% injection dose/gram was observed in brains of treated rats, far less than the 13 ug per g tissue which equates to 2.7% injection dose/gram observed in our study. A similar compound, anti-TfR avidin linked to biotinylated luciferase targeting siRNA, was tested in rat brain tumour expressing luciferase and reduced luciferase levels were observed 48 hrs after IV administration (67). Similar to our antibody-ASO design, Sigo et al., utilized a linker to covalently conjugate the anti-TfR (antiCD71) to an siRNA (68). AntiCD71-siRNA
compounds were only shown to be active in peripheral tissues, liver, heart, and skeletal muscle. Unlike our study, delivery into the CNS was not reported and their IgG control-siRNA was not active in the skeletal muscle.

In addition to improved uptake into the CNS and peripheral tissues, 8D3\textsubscript{130}-PMO also rescued survival in a severe mouse model of SMA. A severe SMA pups carries two copies of SMN2 and deletion of exon 7 from the endogenous mSmn. These pups are born indistinguishable from littermates but begin to show reduction in weight and movement within a few days with early lethality at an average of 7 days. Due to the early onset of a disease phenotype, treatment of PMO is required within a day or two after birth. The BBB and BSCB is immature at this early stage of development, leaving the brain and spinal cord exposed high molecular weight molecule like PMOs (25). It was unclear if the conjugation of a PMO to 8D3\textsubscript{130} would improve uptake over NIP228-PMO control given the observed activity of NIP228-PMO in peripheral tissues (Figure 4). Indeed, using a high dose of 50 mg/kg, both 8D3\textsubscript{130}-PMO and NIP228-PMO rescued survival and tissue expression of full length SMN2 mRNA. However, a significant improvement in survival of 8D3\textsubscript{130}-PMO over NIP228-PMO was observable using a lower dose of 20 mg/kg, indicating the TfR is active in BBB transport at these early developmental stages and the BBB is acting as a barrier, albeit a weak one (69). Alternatively, conjugation to the anti-TfR antibody may facilitate improved cellular uptake of the PMO via TfR on the target cells compared to the control antibody.

The BBB and BSCB represent a significant barrier to the delivery of biologic drugs, both protein and nucleic acid based. Improving CNS exposure using anti-TfR antibodies, which allow transcytosis across the BBB, can be exploited for the delivery of drugs to the brain. We have found that systemic dosing of an anti-TfR antibody-PMO conjugate can access the central compartment and affect the splicing of the SMN2 mRNA to levels only previously observed with direct cerebral or intrathecal drug administration, rescuing survival in severe
SMA mice. Whilst this study provides a proof of concept for therapeutic, systemic dosing of antibody-ASO conjugates, there remains much to do in translating these results to the clinic, including understanding any toxicological liabilities. Notwithstanding, this our study offer the hope of improved therapy discovery and delivery for many debilitating neurological diseases.
Methods

**Preparation of antibodies**

The rat anti-mouse transferrin receptor (TfR) antibody 8D3 was mutated to reduce its affinity for mouse TfR to generate 8D3_{130} as described (16, 24). 8D3_{130} and an isotype-control antibody, used as a negative control, against the hapten nitrophenol (NIP228) were expressed as chimeric human IgG1 molecules with a cysteine residue inserted in the CH2 domain of the heavy chain to allow site specific conjugation of the PMOs (70). Antibodies were expressed in transiently transfected Chinese hamster ovary (CHO) cells in serum-free medium as described previously (71). Cultures were maintained in a humidified incubator at 37°C, 5% CO2 for 14 days after which the media was harvested. Antibodies were purified from the medium using protein affinity chromatography followed by size exclusion chromatography. The concentration of IgG was determined by A280 using an extinction coefficient based on the amino acid sequence of the IgG (72).

**Conjugation of thiol-derivatized antibodies with Mal-C3-FB[RB]₆-PMO**

A 25-mer maleimide-functionalized phosphorodiamidate morpholino oligo (PMO) conjugate targeting the ISS-N1 site of the SMN2 gene was synthesized by conjugation of the 3’-end of the PMO to the C-terminal carboxylic acid moiety of the linker through amide coupling (Supplemental Figure S1) (73). Sequence for ISS-N1 targeted PMO (5’-3’) was GTAAGATTCACTTTTCATAATGCTGG and sequence for scrambled PMO (5’-3’) was CCTCTTACCCTCAGTTACAATTTATA. Both were fully modified phosphorodiamidate morpholino oligos.

IgG (15 ml at 10 mg/ml, ~1 µmol in PBS) was reduced with 40 eq. of TCEP (0.5M in water, 80 µl, 40 µmol) for about 3 hours at room temperature under mild agitation. Samples were taken at different time points and analysed by MALDI-TOF mass spectrometry to
follow the reduction reaction. Afterwards, the buffer was exchanged to DPBS containing 1 mM EDTA by using a HiPrep 26/10 desalting column at a flow rate of 10 ml/ml to remove unreacted TCEP. The reduced antibody-containing fractions (~20 ml) were combined and re-oxidized with 400 µl of a 50 mM (20 µmol, 20 eq.) solution of dehydroascorbic acid in DMSO for 4 hours at room temperature. Subsequently, the reaction mixture was desalted three times and concentrated by ultrafiltration (Ultracel 100 kD Ultrafiltration disc with 100 mM phosphate buffer, pH 6.9, Merck Millipore, Burlington, MA, USA) to a final volume of 12 ml.

After determination of protein concentration, a 5-fold excess of PMO-maleimide conjugate in 100 mM PBS buffer (pH 6.9) was added. After overnight incubation, the conjugation reaction was purified by size exclusion chromatography (GE HiLoad 26/600 superdex 200pg column at a flow rate of 2 ml/min PBS). Product containing fractions were combined and concentrated by ultrafiltration (Ultracel 100 kD Ultrafiltration disc, Merck Millipore) to 10 mg/ml in DPBS.

**Antibody concentrations in mouse brain, spinal cord, and plasma via MesoScale Discovery (MSD) assay platform**

Male C57B/6 mice, age 10–12 weeks were intravenously injected with anti-TfR antibody (8D3130) or control IgG (NIP228) with or without PMO at 20 mg/kg (2 mg/ml on DPBS) or molar equivalent. Intravenous doses were administered into a tail vein at a constant dose volume of 10 ml/kg. Antibodies were supplied in D-PBS (Sigma). Following dosing, blood plasma samples were collected from the lateral tail vein (ca 200 µL) into a Li-Hep microvette (BD Diagnostic Systems, Franklin Lakes, NJ, USA) from each of six animals per time point per dose group. A second sample (ca. 600 µL) was collected by cardiac puncture under isoflurane anaesthesia into a Li-Hep microtainer (BD Diagnostic Systems). Following collection, blood samples were allowed to clot for 30 min and centrifuged at 10,000 rcf for 2
min at 4°C. The resultant plasma was collected and flash frozen on dry ice for subsequent measurement of antibody concentration.

After final blood collection, the mice were perfused with DPBS at a rate of 2 ml/min for 10 min until the extremities appeared white. The spinal cord and brain were removed, weighed, and homogenized in 5 volumes of ice-cold PBS containing 1% NP-40 and Complete® protease inhibitor cocktail tablets (Roche Diagnostics, Basel, Switzerland) using 2x10 clockwise strokes with 5 s rest time. Homogenates were rotated at 4°C for 1 h before centrifugation at 13,000 g, 4°C for 20 min. The supernatant was collected for measurement of antibody concentration. In life phase and sample preparation were performed by Pharmaron (Rushden, UK).

Antibody concentrations in mouse plasma and brain- and spinal cord homogenates were measured using the MesoScale Discovery (MSD) assay platform (Meso Scale Discovery, Rockville, MD, USA). This is a plate-based sandwich immunoassay format where the anti-human IgG capture antibody binds to sample hIgG (± PMO), and subsequently, a specific detection antibody labelled with SULFO-TAG emits light on electrochemical stimulation. Levels of anti-TfR and control antibody in plasma, brain, and spinal cord samples were quantified by reference to standard curves generated using calibrator samples with a four-parameter nonlinear regression model. Statistical analysis was performed using a 2-way analysis of variance, where appropriate, were made using Tukey test in GraphPad Prism. Data shown as the mean ± standard error of the mean, n = 3-4 per group.

In vivo PMO activity in SMA mouse model

The hSMN2 transgenic mouse (SMN2, FVB.Cg-Smn1^tm1Hung^Tg(SMN2)2Hung/J) was generated as previously described (30, 31) and maintained at the Biomedical Sciences Unit, University of Oxford. Handlings of Tg(SMN2)2Hung/Tg(SMN2)2Hung mice (SMN2 offspring that carry the wild-type mouse Smn1 gene) were conducted according to procedures
authorized by the UK Home Office under the Animal [Scientific Procedures] Act 1986. In vivo dosing studies were performed in mice at 8–9 weeks of age. 8D3130-PMO and NIP228-PMO were diluted in 0.9% saline and given at a volume of 10 ml/kg body weight. Seven days post administration animals were culled via rising CO₂ and tissues were collected, flash frozen in liquid nitrogen, and stored at -80°C until analysed. Each dosage group had n = 5 mice per group with exception of 20 mg/kg dose where n = 4 mice. A mix of males and females was used for each study.

**RNA extraction and qRT-PCR**

RNA extraction from harvested tissues was performed using a Maxwell® RSC simplyRNA Tissue Kit (Promega) and cDNA generated using ABI High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Waltham, MA) following manufacturer’s instructions. For skeletal muscle, a 10 min, 55°C incubation of homogenised tissue was added prior to addition of lysis buffer before the RNA extraction. qRT-PCR reaction using TaqMan® Fast Advanced Mastermix (Applied Biosystems) was performed and analysed on StepOnePlus™ real-time PCR system (Applied Biosystems). FLSMN2 and Total SMN2 transcripts were amplified using gene-specific primers (Supplemental Table S1) (74). Significance was determined via 2-way ANOVA with Dunnett multiple comparisons using GraphPad software (*p < 0.05, **p< 0.01, ***p < 0.001).

**Protein extraction and western blot**

Protein was harvested from approximately 300 mg of flash-frozen tissue homogenized in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5) with Complete mini proteinase inhibitors (Roche). A total of 20–30 μg of protein was separated on 10% Novex Tris-Glycine gels (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membranes. Total protein stain (30% methanol, 6.7% acetic acid, 0.0005% Fast Green FCF (Merck KGaA, Darmstadt, Germany)) stain was used as a
loading control and imaged prior to blocking. Post-blocking, human SMN protein was probed for using anti-SMN, clone SMN-KH monoclonal IgG1 (Merck Millipore, MABE230) and secondary antibody IRDye® 800CW goat anti-mouse IgG (LI-COR Biosciences, 926-32210). Membranes were imaged on Li-Cor Odyssey® FC imager and analysed with Image Studio™ software (LI-COR Biosciences).

**ELISA based measurements of oligonucleotide concentrations in tissues.**

To detect concentrations of PMOs in the tissues of treated mice, ELISAs were conducted as described in Burki et al. (33), using a phosphorothioate probe double-labelled with digoxigenin (DIG) and biotin (BIO), with the following sequence (5’-3’): [DIG]-\*C*A*G*C*A*T*T*A*T*G*A*A*A*G*T*G*A*A*T*C*T*T*A*C[BIO].

**Immunohistochemistry**

SMN2 mice (12–13 week old; Tg(SMN2)2Hung/Tg(SMN2)2Hung) were administered with a single intravenous dose of 50 mg/kg 8D3130-PMO or NIP228-PMO. Animals were perfused with 4% paraformaldehyde (PFA, in sterile PBS) 24 h after treatment. Brain and spinal cords were isolated and fixed in 4% PFA overnight. Tissues were then washed 4 times in 1× PBS and cryopreserved in 30% sucrose in 1× PBS for 3 days at 4°C. Tissues were frozen in O.C.T. compound and stored at −80°C. Brains were cut 20 μm thick along the sagittal axis while spinal cords were sectioned transverse. Slides were stored at −80°C before proceeding. Groups of n = 3 were used for each treatment group.

**Whole Brain Images**

Slides were thawed at RT, rehydrated in PBS for 40 min, permeabilized in 0.1% Triton X (in PBS) for 10 min, washed twice 5 min in PBS and blocked overnight at 4°C in 3% BSA (in PBS). The next day, slides were incubated with Alexa Fluor 488 goat anti-human IgG(H+L) (Invitrogen, A-11013) at 1:500 (in 3% BSA/PBS) for 2 h at room
temperature (RT). Slides were imaged at Manchester University, Bioimaging Facility on a 3D Histec pannoramic250 slide scanner at 20X.

**IgG/Nissl NeuroTrace co-staining**

Slides were thawed at RT, rehydrated in PBS for 40 min, permeabilized in 0.1% Triton X (in PBS) for 10 min, washed twice 5 min in PBS and blocked overnight at 4°C in 3% BSA (in PBS). The next day, slides were incubated with Alexa Fluor 488 goat anti-human IgG(H+L) (Invitrogen, A-11013) at 1:500 (in 3% BSA/PBS) for 2 h at room temperature (RT). Slides were then washed thrice 5 min in PBS before incubation with Nissl NeuroTrace 530/615 (Invitrogen, N21482) at 1:200 (in 3%BSA/PBS) for 20 min at RT.

**IgG/IBA1 and IgG/GFAP co-staining**

Slides were thawed at RT, rehydrated in PBS for 40 min, permeabilized in 0.1% Triton X (in PBS) for 10 min, washed twice for 5 min in PBS and blocked overnight in 3% BSA (in PBS). The next day, slides were incubated with rabbit polyclonal antibody to GFAP (Abcam, ab33922) at 1:5000, or with rabbit anti-IBA1 antibody (Wako, Osaka, Japan, 019-19741) at 1:1000, for 24 h in 3% BSA (in PBS) at 4°C. The next day, slides were washed thrice for 5 min in PBS before incubation with Alexa 594 goat-anti rabbit secondary antibody (Invitrogen, A-11012) at 1:1,000 and Alexa Fluor 488 goat anti-human IgG(H+L) at 1:500 (Invitrogen, A-11013) in PBS for 2 h at RT. Samples were imaged on Olympus FV1000 confocal microscope using Fluoview software. Minimal post-imaging processing was done with FiJi (ImageJ).

**IgG/ChAT co-staining**

Slides were thawed at RT, rehydrated in PBS for 3 h and dried overnight at 4°C. The next day, slides were permeabilized and blocked by incubation for 4 h in 0.3% Triton X + 5% BSA (in PBS), then washed for 5 min in PBS. Slides were incubated with a rabbit antibody anti-ChAT (EPR 16590; Abcam) at 1:250 in the blocking solution for 48 h at 4°C. Slides
were then incubated with Alexa 594 goat-anti rabbit secondary antibody (Invitrogen, A-11012) at 1:750 and with Alexa Fluor 488 goat anti-human IgG(H+L) (Invitrogen, A-11013) at 1:500 in PBS for 2 h at RT.

All slides were washed three times for 5 min at RT and dried before mounting with the DAPI-containing VectaMount Permanent Mounting Medium (Vector Laboratories, Burlingame, CA, USA), and sealed with nail varnish. Slides were stored at 4°C in the dark before imaging on Olympus FV1000 confocal microscope. All images across conditions were taken on the same day for a given staining. Microscopy images were processed minimally on Fiji (ImageJ).

**Isolation of endothelial cells**

Cerebral endothelial cells (EC) from treated mice were extracted essentially as previously described (75). The brain was immediately extracted post euthanasia; cerebellum and olfactory bulbs removed; and the remaining brain tissue cut in half in ice cold DMEM. The brains were individually homogenized in fresh cold DMEM. After a brief spin at 1500 rcf and 4°C, the pelleted homogenate was resuspended in 18% dextran. The EC fraction was separated from the myelin/parenchyma layer with a 10 min centrifugation at 5000 rcf and 4°C. mRNA was extracted from EC and parenchyma using Maxwell RSC simplyRNA kit according to manufacturer’s instructions. Reverse transcription and qRT-PCR was performed as before. To ascertain the quality of the EC isolation, a series of qRT-PCRs were run on the cDNA with primers (Supplemental Figure S4 and Supplemental Table S2) towards targets enriched in EC, neurons, or glial cells.

**In Vivo PMO toxicity in the SMA mouse model**

Adult hSMN2 transgenic mice were bred regulations and treated following the method described above for the in vivo PMO activity in the SMA mouse model. Urine was collected 2- and 7-days post treatment and stored at -80 °C until analysed. KIM-1 levels
in urine were analysed using the Mouse TIM-1/KIM-1/HAVCR Quantikine ELISA Kit (R&D Systems™) and normalised to urinary creatinine levels during data analysis. Urinary creatinine levels were analysed using the clinical chemistry analyser at MRC Harwell, UK.

Statistics

Statistical analysis was performed using a 2-way ANOVA or 1-way ANOVA and corrected for multiple comparisons using Tukey Test in GraphPad Prism. Individual statistical information, including p value, is found within the figure legends.

Study Approval

The present studies in animals were reviewed and approved by the University of Oxford ethics committee (Oxford, UK). Studies were conducted according to procedures authorized by the UK Home Office under the Animal [Scientific Procedures] Act 1986.

Author contributions:

MG, MJAW and CW conceptualized the project. SMH, FA, MG, and CW were responsible for designing the research studies. SMH, FA, MB, GT, LG and JS conducted the experiments. The original draft was written by SMH and SMH, LG, JS, NA, MJAW were responsible for review and editing of the manuscript. SMH and FA are co-first authors in this manuscript. FA generated the antibody-ONs critical for this work. SMH was responsible for experimental design, conducting experiments and project management, as such SMH is ordered first in authorship.
Acknowledgments:

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Figures and Tables:

Figure 1: Synthesis of antibody-PMO conjugates. (A) Schematic of conjugation synthesis between 25 mer PMO to the free thiol group of a solvent exposed engineered cysteine residue in the CH2 domain of the heavy chain of either a low affinity mouse TfR (8D3130) or an isotype-control antibody (NIP228). (B) Chemistry of linker between PMO and antibodies. (C) Maldi-Tof spectra of the unmodified antibodies (black trace) and the purified antibody-PMO-conjugates (red traces) for the 8D3130 (left) and NIP228 antibodies (right).
Figure 2: Pharmacokinetics of antibody and antibody-PMO conjugates in mouse. Plasma, brain and spinal cord exposure following 20 mg/kg dose of 8D3_{130} (± PMO) and NIP228 (± PMO); unconjugated and conjugated to the PMO in a Universal PK assay. (A) Plasma PK of antibodies with or without PMO over a one-week period. High statistical significance (****) shown for 8D3_{130} 20 mg/kg vs 8D3_{130}-PMO 20 mg/kg for the first three time points and a lower significance (*) for 24 and 96 h time-points. No statistical significance shown for NIP228-PMO 20 mg/kg vs 8D3_{130}-PMO 20 mg/kg at any time point. (B) Brain exposure as a measure of µg compounds per gram of brain. Statistical significance (****) shown for 8D3_{130} 20 mg/kg vs 8D3_{130}-PMO 20 mg/kg for first three time points. Statistical significance (####) shown for NIP228-PMO 20 mg/kg vs 8D3_{130}-PMO 20 mg/kg at all time points. (C) Spinal cord exposure as a measure of µg compound per gram of spinal cord. Statistical significance (***P, <0.001) shown for 8D3_{130} 20 mg/kg vs 8D3_{130}-PMO 20 mg/kg at 96 h time point. Statistical significance (####P, <0.0001) shown for NIP228-PMO 20 mg/kg vs 8D3_{130}-PMO 20 mg/kg at first two time points and a lower statistical significance (#) at the last two time points. Statistical significance (representative P values) for exposure in brain, spinal cord and plasma between 8D3_{130} 20 mg/kg vs 8D3_{130}-PMO 20 mg/kg (*) and NIP228-PMO 20 mg/kg vs 8D3_{130}-PMO 20 mg/kg (#) at all time points evaluated. Statistical analysis was performed in GraphPad Prism. Data shown as the mean +/- standard error of the mean, n = 3-4 per group. Statistical significance shown using 2-way analysis of variance, where appropriate, were made using Tukey test. *P, <0.05; **P, <0.01; ***P, <0.001; ****P, <0.0001; #P, <0.05; ##P, <0.01; ###P, <0.001; ####P, <0.0001; ns, not significant.
Figure 3: In vivo activity and concentration of antibody-PMOs in the central nervous system of adult transgenic mice bearing the human SMN2 gene. Tail vein administration of 8D3<sub>130</sub>-PMO and NIP228-PMO were given at 8 weeks of age and tissues harvested 7 days post-administration. Splice switching activity of the compounds compared to saline treatment on the human SMN2 transgene was analysed via qPCR and western blots (mean ± S.E.M.). (A) qRT-PCR and (B) western blot analysis of brain shows distinct splicing activity between transferrin receptor targeted 8D3<sub>130</sub>-PMO and isotype control NIP228-PMO treatment groups. (C) PMO concentration as determined by ELISA. 8D3<sub>130</sub>-ScrPMO is an 8D3<sub>130</sub>-PMO conjugate (with scrambled PMO sequence) used as negative control. (D) qRT-PCR from the thoracic region of the spinal cord shows elevated levels of activity. (E) Western blot analysis from the cervical region of spinal cord indicates little activity in this region. (F) PMO concentration of 8D3<sub>130</sub>-PMO from the whole spinal cord. Statistical significance (representative P values) between 8D3<sub>130</sub>-PMO vs Saline (*) and 8D3<sub>130</sub>-PMO vs NIP228-PMO (#) was performed in GraphPad Prism. Data shown as the mean ± standard deviation, n = 5-6 per group. qRT-PCR and western blots were analysed with 2-way ANOVA corrected for multiple comparisons using Dunnett Test. ELISA for PMO concentration was analysed with 1 Way ANOVA corrected for multiple comparisons using Tukey Test. P values adjusted to account for each comparison, confidence level 0.95%. *p, <0.05; **p, <0.005; ***p, <0.0005; ****p, <0.0001; #p, <0.05; ##p, <0.005; ###p, <0.0005; ####p, <0.0001; ns, not significant.
Figure 4: In vivo activity and concentration of antibody-PMOs in peripheral tissues of adult SMN2 transgenic mice. Tail vein administration of 8D3130-PMO and NIP228-PMO were given at 8 weeks of age and tissues harvested 7 days post-administration. Splice switching activity of the compounds compared to saline treatment on the human SMN2 transgene was analysed via qPCR and western blots (mean ± S.D.). qRT-PCR (A) and western blot analysis (B) of skeletal muscles tibialis anterior and quadriceps. TA exhibited differences in activity between 8D3130-PMO and NIP228-PMO treatment groups. Quads show equal activity of both 8D3130-PMO and NIP228-PMO. qRT-PCR (C) and western blot analysis (D) of liver and kidney tissues. Both 8D3130-PMO and NIP228-PMO are highly active in the liver and less active in kidney. PMO concentration as determined by ELISA in (E) tibialis anterior, (F) quadriceps, (G) liver and (H) kidney following 50 mg/kg administration. 8D3130-ScrPMO, with scrambled PMO sequence, is used as negative control. Statistical significance (representative P values) between 8D3130-PMO vs Saline (*) and 8D3130-PMO vs NIP228-PMO (#) was performed in GraphPad Prism. Data shown as the mean ± standard deviation, n = 5-6 per group. qRT-PCR and western blots were analysed with 2 Way ANOVA corrected for multiple comparisons using Dunnett Test. ELISA for PMO concentration was analysed with 1 Way ANOVA corrected for multiple comparisons using Tukey Test. P values adjusted to account for each comparison, confidence level 0.95%. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001, #p, <0.05; ##p, <0.005; ###p, <0.0005; ####p, <0.0001; ns, not significant.
Figure 5: Whole brain biodistribution of antibody-ASO conjugates. Representative images of SMN2 transgenic mouse brain treated with single 50 mg/kg administration of (A) 8D3_{130}-PMO or (B) NIP228-PMO. The CNS was isolated from adult mice 24 hours post administration following perfusion fixation. 8D3_{130}-PMO and NIP228-PMO were identified by human secondary antibody (IgG(H+L)). Whole brain slides were imaged at 20x on 3D Histec pannoramic250 slide scanner. Images represent n = 3 mice. The greatest level of 8D3_{130}-PMO uptake into the brain was observed in the thalamus, pons and cerebellum regions of the brain. (C) FLSMN2 expression via QPCR was analysed in endothelium (BBB) and parenchyma of the brain fractionated by EC extraction. Mice were treated with 50 mg/kg 8D3_{130}-PMO, NIP228-PMO, 8D3_{130}-scrPMO or 0.9% saline. Statistical significance (representative P values) was performed in GraphPad Prism. Data shown as the mean ± s.d., n = 6 per group. Results analysed with one way ANOVA corrected for multiple comparisons using Tukey’s Test.
Figure 6: Cellular localisation of 8D3\textsubscript{130}-PMO to the astrocytes in the spinal cord.

Representative confocal images of spinal cord following single 50 mg/kg administration of 8D3\textsubscript{130}-PMO and NIP228-PMO. The spinal cord was isolated from adult mice 24 hours post administration following perfusion fixation. (A) Motor neurons (ChAT) in the anterior horn of the spinal cord, and 8D3\textsubscript{130}-PMO identified by human secondary antibody (IgG(H+L)) showed no overlap (merge). Fluorescence indicated a retention of the 8D3\textsubscript{130}-PMO (IgG(H+L)) in the vasculature. (B) Astrocytes of the anterior grey horn (GFAP) were colocalised with 8D3\textsubscript{130}-PMO (IgG(H+L)) (arrow heads). Scale bar represents 200 µm.
Figure 7: Survival and mRNA levels in severe SMA pups treated with antibody-PMOs. (A) Survival following single subcutaneous administration of 20 mg/kg 8D3\textsubscript{130}-PMO (n = 7), NIP228-PMO (n = 15), 8D3\textsubscript{130}-scrambled PMO (n = 11) or 0.9% Saline (n = 17). Median survival was 24, 12, 11 and 7 days, respectively. Mean survival after treatment with 8D3\textsubscript{130}-PMO was significantly greater than NIP228-PMO, p<0.0001 (Log-Rank (Mantel-Cox) test).

(B) Survival following single subcutaneous administration of 50 mg/kg 8D3\textsubscript{130}-PMO (n = 12), NIP228-PMO (n = 11), 8D3\textsubscript{130}-scrambled PMO (n = 11) or 0.9% Saline (n = 17). Median survival was 22, 21, 8 and 7 days, respectively. Both 8D3\textsubscript{130}-PMO and NIP228-PMO was statistically significant from 0.9% saline treated group, p < 0.0001 (Log-Rank (Mantel-Cox) test). However, there was no statistical difference between 8D3\textsubscript{130}-PMO and NIP228-PMO. Statistics determined by. (C-H) qRT-PCR measure of mRNA from tissues treated with 50 mg/kg antibody-PMO and collected 7 days post administration. Results were normalised to saline treatment controls. FLSMN2 mRNA represented at ratio to total SMN2 transcripts. One-Way ANOVA with Tukey’s multiple comparison test. All data represent mean values ± S.D. of two replicates. P-value representations: p**** < 0.001, p***<0.005, p** <0.005, p* <0.05.
### Table 1 – Summary of plasma exposure of 8D3<sub>130</sub> and NIP228 with and without PMO in C57BL/6J mice following intravenous administration.

<table>
<thead>
<tr>
<th>Construct</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ug/ml)</th>
<th>AUC&lt;sub&gt;last&lt;/sub&gt; (h*ug/ml)</th>
<th>Cl (ml/h/kg)</th>
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<td>0.054</td>
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<tr>
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<td>NIP228-PMO</td>
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<td>0.193</td>
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<td>Spinal Cord</td>
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<tr>
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<td>$T_{\text{max}}$ (h)</td>
<td>$C_{\text{max}}$ (µg/g)</td>
<td>$\text{AUC}_{\text{last}}$ (h*µg/g)</td>
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Table 2 – Summary of brain and spinal cord exposure of 8D3$_{130}$, 8D3$_{130}$-PMO, and NIP228 in C57BL/6J mice following intravenous administration.