Targeted delivery of antisense oligonucleotides to pancreatic β-cells

C. Ämmälä1*, W. J. Drury III1, L. Knerr1, I. Ahlstedt1, P. Stillemark-Bilton1, C. Wennberg-Huldt1, E.-M. Andersson1, E. Valeur1, R. Jansson-Löfmark1, D. Janzén1, L. Sundström2, J. Meuller2, J. Claesson2, P. Andersson3, C. Johansson3, R. G. Lee4, T. P. Prakash4, P. P. Seth4*, B. P. Monia4, S. Andersson1

Antisense oligonucleotide (ASO) silencing of the expression of disease-associated genes is an attractive novel therapeutic approach, but treatments are limited by the ability to deliver ASOs to cells and tissues. Following systemic administration, ASOs preferentially accumulate in liver and kidney. Among the cell types refractory to ASO uptake is the pancreatic insulin-secreting β-cell. Here, we show that conjugation of ASOs to a ligand of the glucagon-like peptide-1 receptor (GLP1R) can productively deliver ASO cargo to pancreatic β-cells both in vitro and in vivo. Ligand-conjugated ASOs silenced target genes in pancreatic islets at doses that did not affect target gene expression in liver or other tissues, indicating enhanced tissue and cell type specificity. This finding has potential to broaden the use of ASO technology, opening up novel therapeutic opportunities, and presents an innovative approach for targeted delivery of ASOs to additional cell types.

INTRODUCTION

A major advantage of using antisense oligonucleotides (ASOs) for therapeutic interventions is the ability to target genes coding for proteins linked to human diseases that are considered undruggable by classical small-molecule approaches or other traditional modalities (1–3). Therapeutic ASOs are single-stranded, chemically modified oligonucleotides, 14 to 20 nucleotides in length, with an overall negative charge that impedes diffusion across lipid bilayers. Progress has been made in improving the potency, efficacy, stability, and pharmacokinetic properties of ASOs (4), and several therapeutic ASOs are currently approved or in late-stage clinical development (3). However, uptake of ASO in tissues and cell types outside of the liver is still a major challenge (5, 6). Following systemic administration, liver and kidney show the highest ASO concentration and pharmacodynamic effect (“productive uptake”), whereas most other tissues show significantly lower uptake (7). Because of the low penetrance of ASOs in tissues such as brain, heart, and muscle, conjugation of thyroid hormone T3 to ASOs has been used as a strategy to minimize the side effects of thyroid hormone in these tissues while retaining the positive metabolic effects in liver (8). Furthermore, many cell types take up ASOs following systemic administration, but in most cases, this ASO uptake does not result in efficient inhibition of target gene expression (“nonproductive uptake”) (9).

Conjugation of oligonucleotides to triantennary N-acetylgalactosamine (GalNAc) greatly enhances productive uptake of oligonucleotides into hepatocytes through internalization of the ASO by the hepatocyte-specific cell surface lectin receptor asialoglycoprotein receptor 1 (ASGR1), improving potency 10– to 60-fold for reducing gene expression in this cell type (10). In this case, the uptake of the ASO via ASGR1 internalization further enhances the productive uptake in an already sensitive cell type (the hepatocyte). Ligand-induced internalization has also been exploited for delivery of microRNAs to pancreatic tumor cells targeting NRPI/integrin receptors (11), whereas G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors (GPCRs) have been explored to increase cellular uptake of ASO cargo conjugated to a peptide ligand (12) in vitro. Activation of GPCRs initiates receptor internalization, endocytosis, and trafficking through endosomes (13), with surface recycling controlling the rate and extent of receptor transported back to the cell surface. However, it has been postulated that the low abundance and limited ability to internalize a large amount of cargo makes GPCRs unsuitable for targeted drug delivery (14). Here, we have explored a novel approach: exploiting ligand-induced internalization of the glucagon-like peptide-1 receptor (GLP1R) to deliver ASOs to pancreatic β-cells, a cell type previously shown to be completely resistant to ASO uptake in rodents and nonhuman primates (9).

RESULTS

Conjugation of a GLP1R peptide agonist to an ASO

GLP1R is a GPCR belonging to the secretin receptor family, with limited human and rodent tissue distribution (15, 16). In human pancreatic islets of Langerhans, GLP1R expression is restricted to insulin-secreting β-cells and somatostatin-secreting δ-cells (17). Ligand activation of GLP1R leads to rapid internalization and recycling (18). Finan et al. (19) have shown that covalent conjugation of estrogen to an engineered GLP1R peptide ligand, generated by fusion of human GLP1 and exenatide fragments, increases uptake in pancreatic islets while eliminating estrogen-associated adverse pharmacology in non–GLP1R-expressing cells. This engineered peptide sequence, bearing a C-terminal cysteine (here referred to as eGLP1; for sequence information, see fig. S1), can be conjugated to ASOs via directed disulfide formation.

Here, we used ribonuclease (RNase) H1–dependent ASO gapmers (synthesis scheme 1 illustrated in fig. S2), as cargo conjugated to eGLP1, to validate an approach for productive delivery of ASO to pancreatic β-cells by exploiting internalization of ligand-activated
GLP1R. ASOs against two RNA transcripts were used to determine whether this conjugation strategy can render a completely refractory cell type (the β-cell) sensitive to ASO. ASOs used in this study targets metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), which is an evolutionary conserved noncoding RNA transcript highly expressed in many tissues (4, 9), and the forkhead box protein O1 (FOXO1) transcript, a protein-coding mRNA encoding a transcription factor important for both hepatic (20) and pancreatic β-cell function (21) (for ASO structures and sequence information, see fig. S3). With high expression in all cell types, knockdown of MALAT1 can be used as a quantitative marker for productive uptake of ASOs in GLP1R-expressing and non–GLP1R-expressing cells in vitro and in vivo. FOXO1 was used to investigate whether gene silencing reduces FOXO1 protein in pancreatic β-cells. The eGLP1 peptide was also conjugated to an ASO with a scrambled nucleotide sequence (eGLP1-Ctrl-ASO, sequence information in fig. S3) to serve as control. The eGLP1-ASO conjugates include two sites of metabolic vulnerability: the disulfide link between the peptide and the ASO and a trinucleotide phosphodiester sequence at the 5′ end of the ASO (Fig. 1A). These motifs are rapidly cleaved inside cells (22, 23), thus liberating the free ASO and maximizing RNAse H1 activity, but are stable enough to survive in vivo distribution from the site of administration to the pancreas.

The eGLP1-ASO conjugates maintain GLP1R signaling and internalization

The interaction of eGLP1 and MALAT1-ASO conjugated to the eGLP1 peptide (eGLP1-MALAT1-ASO) with GLP1R was investigated using human embryonic kidney (HEK) 293 cells stably expressing the human receptor (GLP1R-HEK293). Internalization was visualized by treating cells with fluorescein-labeled eGLP1 peptide (BODIPY) or eGLP1-MALAT1-ASO (Cy3) (Fig. 1A), monitoring the increase in cellular fluorescence. Under these conditions, both fluorescein-labeled eGLP1 peptide (Fig. 1B) and eGLP1-MALAT1-ASO (Fig. 1C) were rapidly internalized by GLP1R-HEK293 cells (Fig. 1C, b and c) but not by wild-type HEK293 cells (Fig. 1, B and C, a), demonstrating that the GLP1R can internalize the ligand also when conjugated to MALAT1-ASO.

Ligand-activated GLP1R signals through Gαs, leading to an increase in cytosolic adenosine 3′,5′-monophosphate (cAMP). Binding of agonists to GLP1R also promotes β-arrestin recruitment and GLP1R receptor internalization (18). In native pancreatic β-cells, GLP1R activation ultimately results in augmentation of glucose-stimulated insulin secretion (GSIS) and improvement of β-cell function (24), a property that has led to the development of GLP1R ligands as peptide therapeutics for the treatment of type 2 diabetes (T2D) (25). To explore the pharmacology in more detail, we compared the potency of eGLP1-MALAT1-ASO, eGLP1-FOXO1-ASO,
and eGLP1-Ctrl-ASO conjugates to that of the eGLP1 peptide and exenatide (Byetta, used in the treatment of T2D) in several assays: binding (Fig. 2A), dynamic mass redistribution (DMR; Fig. 2B), cAMP accumulation (Fig. 2C), β-arrestin recruitment (Fig. 2D), receptor internalization assays (Fig. 2E) in cell lines overexpressing GLP1R, and effects on insulin secretion in primary human islet microtissues (Fig. 2F). All ASO conjugates activated GLP1R in all assays with potencies similar to eGLP1 and exenatide (table S1) and potentiated GSIS equivalent to exenatide. However, the efficacy of eGLP1-conjugated ASOs in β-arrestin recruitment and receptor internalization was reduced compared to eGLP1 and exenatide (Fig. 2, D and E), indicating an opportunity to optimize the peptide-ASO conjugates to further increase ASO uptake.

**Conjugation to eGLP1 enhances the productive uptake of ASOs**

Productive uptake of ASO was measured by quantifying MALAT1 and FOXO1 transcripts using quantitative real-time polymerase chain reaction (qPCR). Overnight treatment of GLP1R-HEK293 cells with increasing concentrations of MALAT1-ASO or eGLP1-MALAT1-ASO resulted in a dose-dependent reduction of MALAT1 expression (Fig. 3A). There was a more than 40-fold increase in potency in cells treated with the eGLP1-MALAT1-ASO [half maximal inhibitory dose (IC₅₀) = 19 nM] compared to MALAT1-ASO (IC₅₀ = 874 nM) (table S2). Similar potency enhancements have been observed for ligand-conjugated ASOs in HEK293 cells expressing Stabilin-2 (27) and ASGR1 (27, 28). There was no difference in potency between MALAT1-ASO and eGLP-MALAT1-ASO in wild-type or GPR40-overexpressing HEK293 cells (fig. S4 and table S2), suggesting that the enhancement of productive uptake is specific to GLP1R and not due to a general effect from GPCR overexpression. eGLP1 conjugation also increased productive uptake in isolated mouse islets (Fig. 3B), and transcript levels of both MALAT1 and FOXO1 were reduced after overnight treatment with 1 μM of either eGLP1-MALAT1-ASO or eGLP1-FOXO1-ASO compared to unconjugated MALAT1 or FOXO1 ASO, respectively. In human islets, there was a

![Fig. 2. Characterizing GLP1R signaling pathways and pharmacology activated by eGLP1-MALAT1-ASO (•) and eGLP1-FOXO1-ASO (○) compared to eGLP1 peptide (○) and exenatide (△).](image)

**Fig. 2.** Characterizing GLP1R signaling pathways and pharmacology activated by eGLP1-MALAT1-ASO (•) and eGLP1-FOXO1-ASO (○) compared to eGLP1 peptide (○) and exenatide (△). (A) Displacement of 125I-GLP1 in membranes from GLP1R overexpressing HEK293. eGLP1-MALAT1-ASO (○) and eGLP1-FOXO1-ASO (■) displaced 125I-GLP1 equally well as the eGLP1 peptide (○) and exenatide (△). Functional assays measuring G protein signaling by (B) DMR and (C) cAMP accumulation in GLP1R-HEK293 cells showed that eGLP1-MALAT1-ASO (○) and eGLP1-FOXO1-ASO (■) were equally potent as the eGLP1 peptide (○) and exenatide (△), with only minor impact from conjugation of MALAT1-ASO and FOXO1-ASO to the eGLP1 peptide. (D) β-Arrestin2 recruitment in GLP1R-CHO-K1 and (E) receptor internalization in GLP1R-U2OS using PathHunter assays showed similar potencies for eGLP1, exenatide, and ASO-conjugated eGLP1, but with reduced maximal effect for both eGLP1-MALAT1-ASO (○) and eGLP1-FOXO1-ASO (■). Data are presented as mean ± SEM using exenatide as reference for 100% effect. (F) Effect of 10 nM exenatide (△), eGLP1-Ctrl-ASO (○), FOXO1-ASO (□), eGLP1-FOXO1-ASO (■), MALAT1-ASO (○), or eGLP1-MALAT1-ASO (●) on glucose GSIS in human reconstituted islet microtissues. Islets were treated with high glucose, and the compounds and insulin content were measured in the culture medium. Data were normalized to secretion at 11 mM glucose and presented as geometric mean ± 95% confidence interval (CI). Statistical analysis by one-way analysis of variance (ANOVA) adjusted for multiple comparisons.
trend toward an increase in productive uptake, with eGLP1-MALAT1-ASO compared to MALAT1-ASO (Fig. 3C), but the effect did not reach statistical significance. Together, this demonstrates that eGLP1 conjugation enhances productive uptake of ASO in mouse and possibly also human islet cells.

To explore whether silencing of gene expression also leads to reduction in protein levels, mouse islets were treated with 1 μM eGLP1-MALAT1-ASO for 96 hours, which reduced FOXO1 mRNA expression compared to FOXO1-ASO treatment (Fig. 3D). To control for any effects arising from activation of GLP1R signaling, one group of islets was treated with the eGLP1 peptide conjugated to the scrambled ASO (eGLP1-Ctrl-ASO), which showed no effect on FOXO1 mRNA levels (Fig. 3D) but retained GLP1R signaling and internalization properties (table S1). Inhibition of FOXO1 mRNA also caused a drastic reduction of FOXO1 protein, as shown by the Western blot (Fig. 3D, image of a representative gel) with the quantification of the FOXO1 protein intensity stain relative to α-tubulin (Fig. 3F).
Pancreatic islets internalize eGLP1-conjugated ASO in vivo, silencing gene expression

The ability of eGLP1-conjugated ASOs to target insulin-secreting β-cells within the pancreas in vivo was first investigated in normal lean mice treated twice a week with subcutaneous injections of saline, MALAT1-ASO, or eGLP1-MALAT1-ASO and intravenous injections of saline or eGLP1-MALAT1-ASO. At the end of the study, animals were euthanized 72 hours after the last dose, and pancreas and liver were collected and stained for MALAT1-ASO uptake by immunohistochemistry (IHC), with an antibody against the ASO phosphorothioate backbone and for MALAT1 expression by in situ hybridization (ISH). Figure 4A shows that in animals treated subcutaneously with saline (Fig. 4A, a) or MALAT1-ASO (Fig. 4A, b), there was no evidence of ASO uptake (top) or reduction of MALAT1 expression (bottom) within the islets (circled in blue), while in mice treated with eGLP1-MALAT1-ASO (Fig. 4A, c), there was ASO uptake and reduced MALAT1 gene expression in the islets.

The intravenous route administration was also evaluated, comparing eGLP1-MALAT1-ASO to saline treatment. Figure 4B shows that this route was equally effective targeting delivery of ASOs to pancreatic islets, causing similar uptake of ASO and reduction in MALAT1 RNA within the islets as subcutaneous dosing (Fig. 4A). In liver tissue collected from subcutaneously injected animals, there was no difference in ASO uptake (by IHC) or reduction in MALAT1 expression (by ISH) between animals treated with MALAT1-ASO or eGLP1-MALAT1-ASO (fig. S5A), suggesting that eGLP1 conjugation does not enhance uptake in liver, a tissue that does not express GLP1R (29). To determine whether the productive uptake conferred by eGLP1 conjugation was dependent of GLP1R expression in vivo, GLP1R knockout mice were treated with saline or eGLP1-MALAT1-ASO (Fig. 4C), and MALAT1 RNA levels were evaluated by ISH. Treatment with eGLP1-MALAT1-ASO reduced islet MALAT1 expression in wild-type mice (Fig. 4C, c) but not in GLP1R knockout mice (Fig. 4C, d). For comparison, MALAT1 RNA

Fig. 4. GLP1R-dependent uptake of ASO and knockdown of gene expression in mice treated with eGLP1-ASO conjugates in vivo. (A) Representative pancreatic sections stained for ASO by IHC and MALAT1 RNA by ISH from mice treated for 2 weeks with three subcutaneous injections of (a) saline, (b) MALAT1-ASO (1 μmol/kg), or (c) eGLP1-MALAT1-ASO (1 μmol/kg). (b) ASO uptake by IHC and MALAT1 RNA levels by ISH in pancreatic sections from mice treated for 2 weeks with three subcutaneous injections of (a) saline or (b) eGLP1-MALAT1-ASO (1 μmol/kg). (C) MALAT1 gene expression by ISH in (a) wild-type (WT) and (b) GLP1R knockout (KO) mice 72 hours after a single subcutaneous dose of saline or eGLP1-MALAT1-ASO (1 μmol/kg). (D) Pancreatic section from wild-type mice stained using fluorescence in situ probes for MALAT1 (purple, arrows), insulin (green), and glucagon (red). Pancreatic sections were collected 72 hours after one subcutaneous administration of (a) saline or (b) MALAT1-ASO and (c) eGLP1-control-ASO or (d) eGLP1-MALAT1-ASO, and all compounds were dosed at 1 μmol/kg. Scale bars, 200 μm. Islets are circled in blue in (A) to (C).
was also visualized by ISH in pancreas from saline-treated wild-type (Fig. 4C, a) and GLP1R knockout mice (Fig. 4C, b).

Mouse islets have a distinct architecture, with a large central core of β-cells surrounded by a mantle of glucagon-secreting α-cells, somatostatin-secreting δ-cells, and a few pancreatic polypeptide-secreting cells (30). Of these cell types, the α-cell is the second most abundant cell type after the β-cell. In human islets, β-cells and δ-cells (30) express GLP1R with no or minimal expression in other islet cell types, and the same has been shown for mouse (16).

Figure 4D shows pancreatic sections from mice treated with saline (Fig. 4D, a), MALAT1-ASO (Fig. 4D, b), eGLP1 peptide (Fig. 4D, c), and eGLP1-MALAT1-ASO (Fig. 4D, d) stained for MALAT1, insulin, and glucagon by fluorescence ISH. MALAT1 RNA (purple) was reduced in the insulin-expressing central cells (green) but not in the peripheral glucagon-expressing cells (red) in the islet, only in mice treated with eGLP-MALAT1-ASO (Fig. 4D, d). In mice treated with saline, MALAT1-ASO, or eGLP1, MALAT1 was expressed throughout the islets (Fig. 4D, a to c). This demonstrates that GLP1R expression is also needed for productive uptake within the islet.

**eGLP1 conjugation targets ASO delivery selectively to pancreatic islets in vivo**

To determine the potency of eGLP1-ASO conjugates, MALAT1 and FOXO1 transcripts were measured by qPCR in islets isolated from mice 72 hours after single subcutaneous injections of increasing doses of either parent ASOs or eGLP1-ASO conjugates. At the doses tested, there was no significant reduction in either MALAT1 or FOXO1 expression in islets from mice treated with MALAT1-ASO (Fig. 5A) or FOXO1-ASO (Fig. 5B) compared to vehicle-treated mice. However, animals treated with either eGLP1-MALAT1-ASO (Fig. 5A) or eGLP1-FOXO1-ASO (Fig. 5B) showed a dose-dependent reduction in target gene expression, with a calculated IC$_{50}$ of 0.007 μmol/kg for MALAT1 and 0.04 μmol/kg for FOXO1 (table S3), equivalent to 0.07 mg/kg and 0.4 mg/kg, respectively. As the liver is one of the main organs for ASO accumulation and activity, the effect of ASO treatment on MALAT1 and FOXO1 transcripts in liver was also measured by qPCR in all animals. At all dose levels tested, there was little or no suppression of gene expression in liver in any of the treatment groups [MALAT1 (fig. S5B) and FOXO1 (fig. S5C)].

There was no difference in GLP1R mRNA expression in the islets isolated from animals in any of the treatment groups (fig. S6A). In liver, there were only marginal levels of GLP1R mRNA transcript detected (fig. S6B). Although it would be of interest to compare the abundance of GLP1R protein in islets to that of ASGR1 in liver, as it also has a high capacity for internalizing ASOs, comparing receptor abundance between the two tissues using conventional methods is challenging, particularly due to the low yield of receptor-bearing membranes for islets. To get at least an indication of how the

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**Fig. 5. Dose-dependent silencing of gene expression and reduction in protein levels in mice treated with eGLP1-ASO conjugates in vivo.** (A) Target gene expression in islets isolated from mice 72 hours after single subcutaneous doses of MALAT1-ASO (○) or eGLP1-MALAT1-ASO (•). (B) Target gene expression in islets isolated from mice 72 hours after single subcutaneous doses of FOXO1-ASO (○) or eGLP1-FOXO1-ASO (●). Data in (A) and (B) were normalized to vehicle control and presented as mean ± SE (n = 6 per group), assuming log-normal distribution. Solid line corresponds to a Hill equation fitted to the experimental data. For visual assessment, the vehicle group is included in the graph at the point where the y axis intercepts the x axis. (C) Representative Western blot of islet homogenates from animals treated for 6 weeks with weekly subcutaneous injections of saline, FOXO1-ASO (0.1 μmol/kg), eGLP1-FOXO1-ASO (0.1 or 0.03 μmol/kg), or eGLP1-Ctrl-ASO (0.1 μmol/kg), showing FOXO1 protein levels. (D) FOXO1 protein levels in individual animals quantified relative to α-tubulin and normalized to the geometrical mean of untreated mice. Data were represented as scatter dot plots for individual mice and the geometrical mean for each treatment group.

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**Table 1.** Summary of qPCR analysis of MALAT1 and FOXO1 expression in islets and liver.

| Treatment          | MALAT1 mRNA | FOXO1 mRNA |
|--------------------|--------------|------------|
| Vehicle            | 100          | 100        |
| eGLP1-MALAT1-ASO   | 75 ± 5       | 72 ± 4     |
| eGLP1-FOXO1-ASO    | 80 ± 6       | 78 ± 5     |
| eGLP1-FOXO1-ASO (0.1 μmol/kg) | 90 ± 7     | 88 ± 6     |

**Note:** All values are expressed as mean ± SE, n = 6 per group.
expression of GLP1R in the islet compared to that of ASGR1 in liver, we also measured the relative expression of ASGR1 in the liver samples. Figure S6C shows that ASGR1 levels in the liver samples were comparable to the GLP1R levels in the islet, suggesting that, at least by relative expression, the levels of internalizing receptors may not be vastly different between liver and islets.

**ASOs targeted to islets silence gene expression and reduce protein levels**

To investigate whether reduced mRNA expression leads to reduced protein levels in vivo, ob/ob mice were treated once weekly for 6 weeks with subcutaneous administrations of either saline, FOXO1-ASO (0.1 μmol/kg), eGLP1-FOXO1-ASO (0.03 and 0.1 μmol/kg), or eGLP1-CTRL-ASO (0.1 μmol/kg) to control for effects arising from GLP1R activation. Repeated administration of eGLP1-FOXO1-ASO resulted in potent reduction of FOXO1 mRNA in islets (fig. S7A) without affecting expression in liver (fig. S7B). The control-conjugated eGLP1-CTRL-ASO also had a small effect in reducing FOXO1 mRNA. FOXO1 protein levels in the islets from eGLP1-FOXO1-ASO–treated mice were reduced compared to untreated animals (Fig. 5C, lanes C and G), whereas the control eGLP1-CTRL-ASO increased FOXO1 protein levels (Fig. 5C, lane E) despite the reduction in mRNA, presumably through some feedback mechanism from GLP1R activation. Figure 5D summarizes the quantification of FOXO1 protein levels normalized to α-tubulin, showing a significant reduction of FOXO1 in islets from animals treated with eGLP1-FOXO1-ASO compared to eGLP1-CTRL-ASO, suggesting that the ASO treatment is silencing FOXO1 mRNA and overcoming the eGLP1-stimulated increase in protein levels.

**DISCUSSION**

Here, we describe a novel strategy for targeted delivery of ASOS to pancreatic β-cells by conjugating ASOs to a GLP1R peptide agonist. We show that peptide conjugation improves the potency of the MALAT1- and FOXO1-ASO in vitro and in vivo in a GLP1R-dependent manner and that silencing of FOXO1 mRNA leads to a reduction in protein levels. eGLP1 conjugation not only is able to deliver ASO to the pancreatic β-cells, a cell type otherwise resistant to ASO uptake (9), but also enhances the productive uptake in pancreatic islet relative to liver. Thus, systemic administration of ASOs conjugated to GLP1R agonists will significantly reduce the doses required for productive uptake in GLP1R-expressing cells, thereby limiting ASO exposure in other cell types and tissues. However, the experiments presented here cannot rule out that treatments with higher doses, or longer duration of repeated administrations with GLP1-conjugated ASOs, over time will lead to accumulation also in non–GLP1R-expressing cell types.

Conjugation of ASO to GLP1R peptide ligands represents a novel concept for targeted delivery of therapeutic oligonucleotides through internalization of GLP1R. As GLP1R belongs to a class of receptors previously considered unsuitable for in vivo cellular delivery of cargo, the data presented here add GLP1R to the receptor-ligand systems that can be exploited for targeted delivery of ASOs to a cell type previously inaccessible to ASO pharmacology. Our work opens the possibility to develop treatments for diseases caused by or associated with aberrant gene expression in the pancreatic β-cells—with broad opportunities for regenerative treatments to restore functional islet mass in T2D while reducing the risk for off-target effects in cells and tissues not expressing the GLP1R.

**MATERIALS AND METHODS**

**eGLP1 peptide-ASO conjugate synthesis**

The synthesis of eGLP1-conjugated ASOs MALAT1 (9), FOXO1 (10), and scrambled control (11) was accomplished, as described in scheme 1 (fig. S1). 5′-Hexylamino ASOs 1 to 3 were synthesized using standard solid-phase oligonucleotide synthesis procedure (31). eGLP1 (8) was generated on Rink Amide-ChemiMatrix by microwave-assisted 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis using a Biotage Initiator+ Altra synthesizer. All peptide couplings were mediated by ethyl cyanohydroxyiminoacetate (Oxyma Pure) and N,N′-diisopropycarbodimide. Compound 4 [3-pyridinylthio]-2,5-dioxo-1-pyrrolidinyl ester was obtained from Chem-Impex International Inc. (935 Dillon Drive Wood Dale, IL 60191, USA). A solution of compound 4 (3 μmol) in N,N′-dimethylformamide (DMF; 22 μl/μmol) was added dropwise to a solution of ASOs 1 to 3 (1 μmol) in aqueous sodium tetraborate [80 μl/μmol, 0.1 M (pH 8.5)], and the resulting mixture was stirred at room temperature for 3 hours. The reaction mixture was diluted with water [fivefold of reaction solution volume (v/v)] and purified by high-performance liquid chromatography (HPLC) (Waters Source 30Q resin; buffer A: 100 mM ammonium acetate in 30% acetonitrile in water; buffer B: 1.5 M sodium bromide in A; 10 to 60% B in 28 column volumes, flow 14 ml min⁻¹, λ 260 nm). Fractions containing full-length ASOs 5 to 7 [assessed by liquid chromatography–mass spectrometry (LC-MS) analysis] were pooled together, diluted to obtain an acetonitrile concentration of 10%, and desalted by HPLC on a reverse-phase column. Product fractions were concentrated to yield 5 to 7 (80 to 83%). Compounds 5 to 7 (1.2 μmol) were dissolved in mixture of degassed water (192 μl/μmol) and 0.1 M sodium bicarbonate solution (192 μl/μmol). eGLP1 peptide 8 (1 μmol) was dissolved in 50% DMF in 0.1 M aqueous sodium bicarbonate solution (215 μl/μmol). eGLP1 peptide solution was added to the above solution of compounds 5 to 7 in small portions (30% of total volume each time) in 5 min intervals. After stirring for 1 hour at room temperature, the reaction mixture was diluted with water [10-fold of reaction solution volume (v/v)], and the product was purified by HPLC (conditions same as above). Product fractions (assessed by LC-MS analysis) were pooled together and desalted by HPLC on a reverse-phase column to yield eGLP1 ASO conjugates 9 to 11 (55 to 65%). LC-MS analysis characterized compounds 9 to 11. 9: ultraviolet (UV) purity, 98.3%; calculated mass, 10766.1; found mass, 10765.1; 10: UV purity, 98.1%; calculated mass, 10838.2; found mass, 10837.8; 11: UV purity, 88.7%; calculated mass, 107780.5; found mass, 107778.5.

**Cell culture**

Human GLP1R or GPR40 was stably overexpressed in HEK293 cells (GLP1R-HEK293 and GPR40-HEK293). Wild-type HEK293 cells (American Type Culture Collection) were routinely cultured in Dulbecco’s modified eagle medium (DMEM; Gibco, #31966) with 10% fetal bovine serum (FBS; Gibco, #10270) and GLP1R-HEK293 and GPR40-HEK293 in the same medium supplemented with hygromycin B (100 and 200 μg/ml; Invitrogen, #10687). For gene expression analysis, cells were cultured in poly-d-lysine
(PDL)–coated 96-well plates (Corning) at 37°C, 95% humidity, and 5% CO2 and then incubated with increasing concentrations of ASO or ASO conjugates for 24 hours, followed by RNA extraction and expression analysis. Cells for β-arrestin and receptor internalization assays were cultured according to the manufacturer’s protocol (DiscoverX Corporation, Fremont, CA).

Fluorescence imaging

Wild-type HEK293 and GLP1R-HEK293 cells were seeded to 16,000 cells/cm² in DMEM with 10% FBS to 96-well PDL plates (Greiner, #655946) and cultured at 37°C and 5% CO2. The following day, the medium was changed to DMEM with 0.1% bovine serum albumin (BSA; Sigma-Aldrich, #A1595), and cells were incubated in 100 nM BODIPY-eGLP1 peptide at 37°C for the indicated time. Alternatively, 33 nM Cy3-eGLP1-MALAT1-ASO was added to the cells, in the presence of single-stranded DNA (10 μg/ml; Sigma-Aldrich, #D7656), to prevent nonspecific adherence of the conjugate to the PDL-coated plate and incubated at 37°C and 0°C, respectively, for the indicated time.

A nuclear stain, Hoechst 33342 (Thermo Fisher Scientific, #62249), was added to the cells during the last 15 min of the incubation. At the end of the staining protocol, cells were washed in phosphate-buffered saline (PBS), fixed in 4% buffered paraformaldehyde for 10 min at room temperature, and washed in PBS. Images were acquired on an ImageXpress Micro fluorescence microscopy (Molecular Devices) instrument at 20× to 40× resolution and analyzed using ImageJ (Fiji platform).

Receptor-binding assay

Binding was measured by a competition binding assay using isolated membranes from GLP1R-HEK293 cells. Membranes were incubated for 2.5 hours at room temperature in Hanks’ balanced salt solution (HBSS), 20 mM Hepes, 0.1% BSA (pH 7.4), 0.1% BSA with 0.075 nM 125I-GLP1 (7–36) (PerkinElmer #NEX308) as a tracer in the absence or presence of compounds over a range of concentrations. Following incubation, membranes were filtered through a filter plate (Millipore, #MSFCNXB50) and washed with ice-cold 25 mM Hepes, 1.5 mM CaCl2, 1 mM MgCl2, 100 mM NaCl, and 0.1% BSA (pH 7.4) to separate bound from unbound radioligand and subsequently quantitated by scintillation counting (MicroBeta TriLux, PerkinElmer).

DMR assay

DMR was measured using an Epic biosensor system (Corning). GLP1R-HEK293 and wild-type HEK293 cells were cultured in DMEM with 10% FBS in fibronectin-coated 384-well Epic plates (Corning, #5042) at 37°C, 95% humidity, and 5% CO2 for 24 hours. Following the incubation, culture medium was replaced with HBSS (Invitrogen), 20 mM Hepes (Invitrogen) (pH 7.4), 0.1% BSA (Sigma, #A6003), and 0.04% dimethyl sulfoxide (DMSO) and equilibrated to 26°C in the Epic system for 30 min before addition of compounds and detection of DMR.

cAMP accumulation assay

cAMP accumulation was measured in GLP1R-HEK293 cells using the cAMP Dynamic 2 Kit from Cisbio Bioassays (Colodet, France). Cells were incubated with compounds for 30 min at room temperature in HBSS supplemented with 20 mM Hepes (pH 7.4), 0.1% BSA, and 1 mM 3-isobutyl-1-methylxanthine, followed by detection with homogeneous time-resolved fluorescence according to the manufacturer’s assay protocol.

β-Arrestin assay

β-Arrestin recruitment was measured in Chinese hamster ovary (CHO) K1 hGLP1R β-arrestin2 (DiscoverX, #93-0300C2) cells after 90 min incubation with compounds in Ham’s F-12, 1% FBS, and 0.3% DMSO at 37°C according to the manufacturer’s PathHunter assay protocol (DiscoverX Corporation, Fremont, CA).

Receptor internalization assay

GLP1R internalization was measured using the PathHunter eXpress GLP1R Activated GPCR Internalization Assay (DiscoverX, #93-0724E3CP0L) in GLP1R-overexpressing U2OS cells. Cells were incubated with compounds for 3 hours at 37°C, and internalization was quantified according to the manufacturer’s protocol (DiscoverX Corporation, Fremont, CA).

RNA preparation and qPCR

Wild-type, GLP1R-expressing, or GPR40-expressing HEK293 cells were seeded at a density of 30,000 cells per well in 96-well plates and treated with ASO or ASO conjugates as indicated for approximately 24 hours before harvest. Total RNA was extracted from cells or tissue samples using the RNeasy Micro Kit or Mini Kit (Qiagen). Complementary DNA (cDNA) was generated using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Reverse transcription qPCR analysis was performed with a QuantStudio 7 Flex using MALAT1 TaqMan assays (human HS01054576 and mouse Mm01227912, Applied Biosystems) following the manufacturer’s instructions. Mouse FOXO1 was measured using the following primer probe set: forward sequence, CAAAGTCACATACGGCCCAATCC; reverse sequence, CGTAACCTTGGTCTGTCCTGAA; and probe, TGAGCCCTTTGGCCCATGCTTAT. The data were normalized against acidic ribosomal phosphoprotein P0 (36B4) expression measured using the following primer probe sets: HEK293 cells, CCAATTCTATCATCAACCGGTACAA (forward sequence) and ACGAAGTGGAAAGGTGTAATCC (reverse sequence); mouse islets, GAGGAGTATGAGGATATGGGA (forward sequence), AAGCAGGGTACTTGTTG (reverse sequence), and TCAGTCTCTTCGACTAATCCGGCCA (probe sequence).

Mouse islet isolation

Mice were anesthetized with isoflurane and euthanized by cervical dislocation. Pancreatic islets were prepared by retrograde injection of a collagenase solution via the biliopancreatic duct and digestion at 37°C for 18 min. Liberated islets were handpicked under a stereo microscope at room temperature. Before use, the islets were cultured in RPMI1640 medium containing 11 mM glucose, 10% (v/v) FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml; Sigma, #P3333). Islets were dispersed using TrypLE Express (Thermo Fisher Scientific, #12605036) and plated in 96-well PDL-coated plates.

Human islets

Human primary islets were purchased from Prodo Laboratories Inc., providing islets isolated from donor pancreas obtained from deceased individuals with research consent from organ procurement organizations. The use and storage of human islets were performed in compliance with the Declaration of Helsinki, International Conference on Harmonisation/Good Clinical Practice, and AstraZeneca code.
of conduct. The human islets were maintained in tissue culture in PIM(S) Complete (Prodо Laboratories Inc.) before use.

**Western blot**

Protein extracts were generated by homogenization in an ice-cold M-PER protein extraction reagent (Thermo Fisher Scientific, Rockford, IL) containing a protease inhibitor cocktail (Complete Mini and phosphoSTOP, Roche Diagnostics, Basel, Switzerland). The protein content of the lysate was quantitated using Pierce BCA Assay Reagent (Thermo Fisher Scientific). Primary antibodies for Western blot analysis were FOXO1 (C29H4) (#2880, Cell Signaling, Boston, MA, USA) and α-tubulin (#16074, Sigma-Aldrich, St. Louis, MO, USA). A horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit (#P0448, DAKO, Glostrup, Denmark) was used as secondary antibody for FOXO1, and for the anti-α-tubulin antibody, an HRP-conjugated goat anti-mouse (#P0447, DAKO) was used. Enhanced chemiluminescence reagents (Pierce, Thermo Fisher Scientific) were used for detection. Gels were imaged and analyzed with Image Lab Software (Bio-Rad Laboratories Inc., CA, USA).

**GSIS in islet microtissue**

3D InSight Islet Microtissues were purchased from InSphero AG, Switzerland. GSIS was run by sequential incubation in 2.8 mM glucose followed by 11.1 mM glucose Krebs-Ringer buffer. Super-natants were analyzed using a human insulin immunoassay kit (#K151BZC, Meso Scale Discovery).

**Mice**

C57BL/B6Crl and B6.Cg-Lep ob/J mice were purchased from Charles River. Experiments in GLP1R knockout mice were performed at MedImmune, an AstraZeneca subsidiary. Animals were housed in microisolation cages on a constant 12-hour light/dark cycle with controlled temperature and humidity and with access to normal chow and water ad libitum. All in vivo experiments were performed in accordance with institutional and Institutional Animal Care and Use Committee guidelines.

**In vivo productive uptake of ASO**

For experiments evaluating ASO uptake (by IHC) and gene expression (by ISH or qPCR), C57BL/B6Crl mice were given a single subcutaneous or intravenous administration of vehicle, ASO (MALAT1-ASO or FOXO1-ASO), or eGLP1-ASO conjugates (eGLP1-MALAT1-ASO or eGLP1-FOXO1-ASO) (n = 6 mice per treatment group). Dose levels were as indicated in figures. Seventy-two hours after the last dose, animals were euthanized, pancreas was fixed in formalin and paraffin-embedded or islet-isolated, and liver samples were collected and fixed in formalin.

For effects of gene silencing on FOXO1 protein levels, B6.Cg-Lep ob/J (ob/ob) mice were treated with vehicle, FOXO1-ASO (0.1 μmol/kg), eGLP1-FOXO1-ASO (0.03 and 0.1 μmol/kg), or eGLP1-Ctrl-ASO (0.1 μmol/kg), once a week for 6 weeks (n = 8 mice per group) by subcutaneous injection. At the end of the study, 72 hours after the last dose, animals were euthanized, liver samples were collected, and pancreatic islets were isolated from all mice. All compounds were administered in PBS vehicle.

**ASO IHC**

IHC for detection of ASO was carried out in a Ventana Discovery XT immunostainer (Ventana Medical System Inc.) according to the manufacturer’s recommendation. All reagents were Ventana products (Roche Diagnostics, Basel, Switzerland). Antigen retrieval was done by protease 1 incubation for 8 min followed by 4 min incubation in antibody blocker and anti-ASO primary antibody incubation for 1 hour at 37°C (dilution 1:5000, Ionis Pharmaceutical). As detection systems, OmniMap anti-rabbit HRP incubated for 16 min and a chromogenic detection kit (DISCOVERY ChromoMap DAB, RUO) were used. Slides were counterstained with hematoxylin for 4 min followed by bluing for 4 min. Stained slides were analyzed under a standard bright-field microscope.

**MALAT1 RED and DAB ISH**

The in situ RNA amplification and labeling process were performed on the automated ISH platform Ventana Discovery ULTRA (Ventana Medical System Inc.) or the Bond RX (Leica Biosystems) for the probe detection RNAscope 2.5 VS Reagent Kit-RED and Bond Polymer Refine DAB Detection Kit, respectively. ISH probe for MALAT1 and RNAscope VS or LS Assay reagents were obtained from Advanced Cell Diagnostics (ACD) (Newark, CA) and used according to the manufacturer’s instructions. Stained slides were analyzed under a standard bright-field microscope.

**RNAscope 4-plex ISH**

The in situ RNA amplification and labeling process were performed at ACD (Newark, CA) by the Pharma Assay Services group using the RNAscope LS Multiplex Reagent Kit along with the 4-Plex Ancillary Kit according to the manufacturer’s instructions on a Leica Bond Rx autostainer. Probes were assigned to the following channel and fluorophore configuration: Mm-Ins2-C1 (Opal 520)/Mm-Gcg-C2 (Opal 570)/Mm-Sst-C3 (Opal 620)/Mm-Malat1-C4 (pooled probe, Opal 690). Using the positive and negative control probes, optimization was performed to establish the following pretreatment conditions: 15 min ER2 at 88°C and 15 min protease III at 40°C.

**Statistical analysis for concentration and dose-response curve estimates**

GLP1R signaling pathways and pharmacology in vitro potency data (Fig. 2 and table S1) were analyzed with a four-parameter logistic fit using the equation \( y = Bottom + (Top − Bottom)/(1 + 10^{(\log_{10}(ED_{50}−x) \times \text{HillSlope})}) \), where \( y \) is the response, \( x \) is the base 10 logarithm of the drug concentration, Bottom is no activation, and Top is full activation using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Potency estimates were reported as mean ± SEM derived from independent experiments, as indicated in table S1.

In vitro potency estimates of the effect on gene expression in cell lines (Fig. 3) were estimated with a three-parameter logistic fit using the equation \( y = Bottom + (Top − Bottom)/(1 + 10^{((\log_{10}(ED_{50})−x)/\text{HillSlope})}) \), where \( y \) is the response, Bottom corresponds to the maximum reduction achieved, Top is the least level achieved, and \( \log_{10}(ED_{50}) \) and Log Drug Concentration were stated in molar concentrations using GraphPad Prism. From each incubation concentration, the average and SD of three biological replicates were calculated and used as input variables in the regression analysis. Data were presented as mean and 95% asymptotic CIs.

In vivo potency estimations for RNA knockdown in islet cells (Fig. 5B and table S3) were based on fitting an \( I_{\text{max}} \) model (Hill equation), according to the equation RNA knockdown = Baseline \( x (1 – (I_{\text{max}} \times \text{Dose}^n)/(ED_{50}^n + \text{Dose}^n)) \), where the baseline is RNA.
Residual Error is the residual error estimate. To evaluate the model and model fit and normalized to vehicle levels. The distribution, and the model fit curve was derived from the bootstrap to vehicle levels and presented as mean ± SEM, using log-normalmates. For visual assessment, the experimental data were normalized variability was allowed for the baseline value, and an exponential effects approach, as implemented in Phoenix WinNonlin 7.0 and 4. W. B. Wan, P. P. Seth, The medicinal chemistry of therapeutic oligonucleotides.

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