Robust and Durable RNA Editing *In Vivo* with Axiomer™ Editing Oligonucleotides in Non-Human Primates

First proof of concept and target engagement (NTCP) leading to changes in biomarkers in NHPs using ADAR RNA editing technology

Nicole H. P. Cnubben[1], Marko Potman[1], Tsinatkeab Hailu[1], Francesco De Chiara[1], Tessa Schoon[1], Katarzyna E. Kolodziej[1], Seda Yilmaz-Elis[1], Sjef de Kimpe[1], Stan F. J. van de Graaf[2], Gerard Platenburg[1]

Introduction

One of the most promising breakthroughs in the genetic medicine field is Adenosine Deaminase Acting on RNA (ADAR) based RNA editing. Axiomer™, an RNA editing platform, uses chemically modified editing oligonucleotides (EONs) to recruit and guide endogenous ADAR to perform Adenosine (A) to Inosine (I) editing on double stranded RNA with high specificity. (**Fig. 1**) The Inosine (I) is then translated as a Guanosine (G), changing an amino acid in the protein and impacting its function. To date, Axiomer EONs have shown robust editing *in vitro* and *in vivo* and have the potential to become a new class of medicine offering new treatment possibilities for previously untreatable diseases.

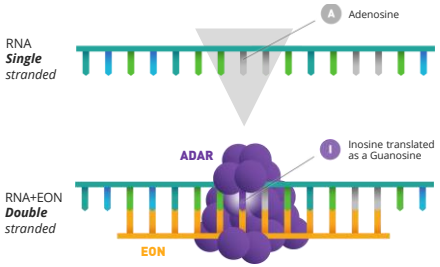

Figure 1. Editing Oligonucleotide (EON)-directed therapeutic editing *(A-to-I)*.

Objectives

To evaluate EON intrinsic editing capabilities of the liver to edit ACTB RNA in non-human primates (NHPs) and to assess the potential of Axiomer to modulate NTCP protein function in cholestatic diseases.

Results

Robust intrinsic editing capabilities reported with editing oligonucleotide in non-human primates

In a primary experiment assessing EON editing capabilities in the liver *in vivo*, cynomolgus monkeys were dosed with three intravenous (IV) infusions each at Day D1, D8, D15 with ACTB EON at a dose of 2mg/kg (n = 4) or control EON (n = 4). Editing efficiency of ACTB RNA reached up to 70% in the ACTB EON group in comparison to 0.1% in the control group at D4 (1 dose) and was durable with an average of 39% editing efficiency observed 24 days following last dose. (**Fig. 2**) No significant off-target editing was observed in NHP livers upon treatment with EON targeting ACTB RNA (data not shown).

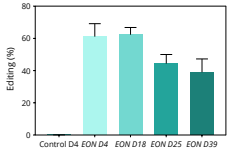

Figure 2. Editing of ACTB RNA in NHP *in vivo* (IV, 2mg/kg, 3 doses at D1, D8 and D15, LNP formulation, n=4, D4 to D39 data, dPCR, mean±SEM).

Editing oligonucleotides to target NTCP in cholestatic diseases as novel therapeutic strategy

Harmful accumulation of bile acids (BAs) contributes to cellular stress and significant tissue damage in cholestatic disorders. The consequences of these disorders can be devastating, highlighting the need for an on target therapeutic approach impacting the progression of the disease.

AX-0810, a program using Axiomer technology, is a targeted and transient approach that aims to reduce BAs load in the liver. By specifically affecting the main transporter for BAs reuptake from the portal vein circulation to the liver, called NTCP (Na-taurocholate transporting polypeptide, *SCL10A1* gene), the AX-0810 program represents a promising avenue to ameliorate the progression of cholestatic disorders. (Fig. 3) We assessed the potential of EONs to impact NTCP BAs reuptake function in preclinical models.

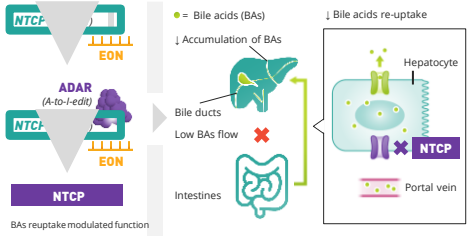

Figure 3. AX-0810 program aims to reduce bile acids reuptake into liver and limit toxic buildup.

NTCP variant affecting Na⁺ binding pocket leads to reuptake modulation

Evidences show that impacting NTCP BAs reuptake function can enhance liver function by reducing the levels of toxic BAs, improving liver damage markers and lowering inflammation biomarkers.[1] NTCP variants naturally occur in some people, changing the capacity to recycle BAs into the liver and increasing level of BAs in the serum, without causing a phenotype or being associated with cholestasis.[2-5]

NTCP Q68R is a variant located inside of a Na⁺ binding pocket and can indirectly affect NTCP function by altering the precise geometry required for BAs binding.[6] One hypothesis is that by potentially increasing affinity for negatively charged substrates, Q68R variant affects protein transport dynamics. (**Fig. 4** and **5**) Further assessment of Q68R variant in a BAs uptake assay showed a near complete inhibition of BAs (specifically Taurocholic Acid or TCA) uptake in transiently transfected U2OS cells, confirming findings in 3D model and corresponding to the mechanism of interest for AX-0810 program (**Fig. 6**).

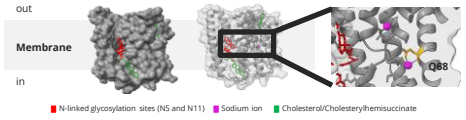

Figure 4. Structure of the human sodium/bile acid cotransporter (NTCP) and position of Q68 in the Na⁺ binding pocket.

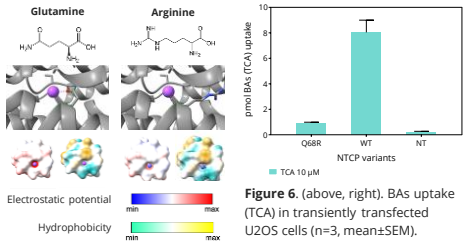

Figure 6. (above, right). BAs uptake (TCA) in transiently transfected U2OS cells (n=3, mean±SEM).

Figure 5. (above, left). 3D Model of Q68R variant impact on Na⁺ binding pocket of NTCP. Within the Na⁺ binding pocket, some hydrogen bonds and contacts are disrupted. The metal coordinating bond is disrupted, likely leading to the inability to bind one of the sodium ions. Clashes are inevitable since the Arg side chain is buried and likely to be found in one or another unfavorable rotamer state.

The Q68R variant solely affects NTCP bile acid reuptake function making it a variant of interest for Axiomer EON therapeutic application

Comprehensive verification of *SCL10A1*/NTCP expression with or without the Q68R variant was carried out. No significant difference in mRNA expression (**Fig. 7**) and protein levels (**Fig. 8**) were detected. The plasma membrane location of NTCP Q68R was also unaffected (**Fig. 9**) confirming that the variant only modulate NTCP function.

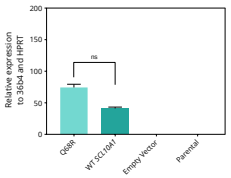

Figure 7. *SCL10A1* (NTCP) mRNA expression. (n=3, mean±SEM).

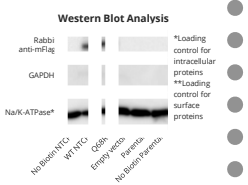

Figure 8. NTCP protein expression was detected on western blot using the anti-FLAG antibody for all constructs.

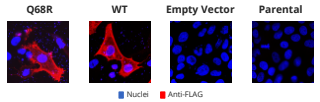

Figure 9. NTCP protein localization in transiently transfected U2OS cells.

Editing of NTCP leads to change in BAs *in vitro* and in NHPs *in vivo*

Following validation of the target, early generation of EONs were designed to lead to NTCP Q68R variant (EON1) and tested in a preliminary study *in vitro* (**Fig. 10**). EON1 induces a dose-response inhibition of BAs (specifically Tauro-nor-THCA-24-DBD) in HepaRG cells confirming that BAs in this model are mediated by NTCP.

Furthermore, linear regression analysis between EON1 concentration and BAs uptake showed a negative coefficient ($R2 = 0.56$) suggesting that increased EON1 concentrations lead to a decrease in BAs uptake (data not shown).

In cynomolgus monkeys (n=6), editing and serum BAs levels were analyzed after administration of EON1 (**Fig. 11**). High correlation between change in serum BAs and EON1 editing level in *SCL10A1* (NTCP) mRNA was observed, with linear regression reporting a $R^2 = 0.51$. With EON1, 29% editing in NHP led to a change in serum BAs of 8-fold 72 hours after treatment.

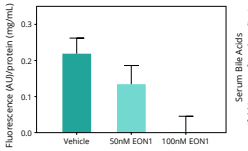

Figure 10. NTCP-mediated BAs uptake in HepaRG cells (50-100nM, n=3, 72 hours, mean±SEM).

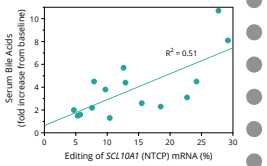

Figure 11. Correlation between change in serum BAs and editing of *SCL10A1* (NTCP) mRNA in NHPs *in vivo*.

Optimization of EONs' potency

In vitro and *in vivo* results in the previous sections have been generated with early generation of EONs (EON1). Leveraging expertise in EONs optimization, including adjustment of sequence and chemistry, lead to increased potency of EONs targeting *SCL10A1* (NTCP) mRNA. Further optimizations have enabled achievement of up to 60% editing, representing an increase in 3-fold versus EON1. (**Fig. 12**)

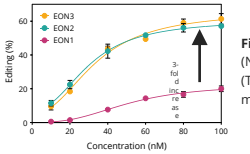

Figure 12. EONs targeting *SCL10A1* (NTCP) mRNA optimization in PHH, (Transfection, n=3, 72 hours, dPCR, mean±SEM).

Conclusions & next steps

- **For the first time in the ADAR RNA editing field, we reported *in vivo* proof of concept and target engagement (NTCP) correlated with desired changes in biomarkers in NHPs using Axiomer EONs**
- **Axiomer EONs have the potential to specifically modulate NTCP protein BAs reuptake function in cholestatic diseases**
- **Axiomer EONs reported robust intrinsic editing capabilities in non-human primates (NHPs)**
- **These findings support the new therapeutic application of Axiomer EONs targeting NTCP and the clinical candidate will be announced in 2024 for the AX-0810 program**

Literature

1. Slijepcevic D, et al. Hepatology. 2018 Sep;68(3):1057-1069
2. Ho RH, et al. J Biol Chem. 2004 Feb 20;279(8):7213-22;
3. Vaz FM, et al. Hepatology. 2015;61(1):260-267;
4. Vaz FM, et al. Dig Dis. 2017;35(3):259-260;
5. Mao F, et al. J Biol Chem. 2019;294(31):11853-11862
6. Yan H, et al. J Virol. 2014 Mar;88(6):3273-84