A NOVEL PROTEOLYTICALLY STABLE ALKYLATED FGF21 ANALOGUE (0499) WITH EXTENDED HALF-LIFE LOWERS BODYWEIGHT, HEPATIC STEATOSIS AND FIBROSIS IN A MURINE MODEL OF NASH

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Aim

- This work aimed to develop a metabolically stabilised FGF21 analogue with long circulating half-life for the treatment of NASH.

Introduction

- Fibroblast growth factor 21 (FGF21) is a metabolic regulator with pleiotropic effects on food preferences, energy, and lipid metabolism. Non-clinical and short term clinical data have shown pronounced effects of FGF21 analogues on NASH resolution and fibrosis (1,2).
- FGF21 binds and activates the short isoform of FGFR1 and 3 but only in the presence of the beta-klotho co-receptor (KLB). FGF21 has a short half-life (T½) in circulation and is proteolytically cleaved in the C-terminal region (3) leading to inactive degradation products. The C-terminal region is critical for biological activity due to its binding to KLB.
- A novel C-terminally stabilised and protracted FGF21 analogue (0499) is presented which shows significant beneficial effects in a murine model of NASH.

Methods

- Engineered FGF21 analogues with cysteine residues in the C-terminal region were expressed and purified from *E. Coli*. The FGF21 analogues were alkylated using a repertoire of structurally diverse non-covalent albumin binders containing fatty acid motifs. The protracted FGF21 compounds were tested for in vitro potency (pErk signalling in HEK/hKLB cells). Efficacy (body weight lowering) and PK properties were evaluated in vivo. Degradation by fibroblast activation protein (FAP) in vitro, as well as pharmacokinetics (half-life) in multiple species was determined by LC/MS or immuno assay.
- Male C57BL/6JRj mice were fed either a standard chow diet or AMLN diet (DIO NASH mice) for 26 weeks (3). A liver biopsy was taken, and the mice were randomized into three groups (n=12) based on hepatic Collagen 1 expression. NASH DIO mice were treated with 0499 sc once daily) 0.2-0.05 mg/kg) and compared to diet restricted DIO NASH mice (WM control). At the end of the study liver TG and cholesterol content were determined biochemically and lipid also by morphometric analysis. Effect on liver inflammation and fibrosis were visualized by CD11b, alpha-smooth muscle actin (aSMA) and Picro-Sirius Red (PSR) IHC-staining.

Results

Identification of protraction site and type

- Mutations in the C-terminal of FGF21 can lead to a significant loss of activity as seen in table 1. C-terminal region 178C, 179C, and 181C mutations all caused >10 loss of potency, however, 180C mutation remarkably retained Met-FGF21 potency in vitro.

Analogue	Mutations	Potency (HEK293/hKLB EC50 (nM))
Met-FGF21	-1M	2.4
A	-1A, 121Q, 168L	2.2
B	-1A, 121Q, 168L, **181C**	26.7
C	-1A, 121Q, 168L, **180C**	2.5
D	-1A, 121Q, 168L, **179C**	69
E	-1A, 121Q, 168L, **178C**	104

Met-FGF21 is a reference compound carrying an N-terminal methionine as result of expression in *E. coli*. The potency of Met-FGF21 closely resembles that of wild-type FGF21 (data not shown). The mutations (-1A, 121Q, 168L) ensure stability and prevent oxidation and deamidation and have no effect on FGF21 potency. Introduced Cys residues were protected with cysteamine.

Table 1: In vitro potency of C-terminal Cys analogues.

- Therefore 180 C (Compound C) was selected for attachment of albumin binding protractors. Resulting compounds carrying C12 diacid through C18 diacid containing sidechains (table 2) showed a ~2-fold decreased potency in absence of albumin.
- Markedly increased circulating T½ and lowering of body weight in mice was seen with the C18 (0499) protracted compound but not with Met-FGF21 (table 2).

Analogue	Mutations	Protractor	HEK293/hKLB No albumin EC50/nM	PK in mice T½ (hrs)	Body weight loss mice, 6 days (g)
Met-FGF21	-1M	N/A	2.4	1.2	-0.5*
F	-1A, 121Q, 168L, 180C	C12 diacid	6.5	1.1	-0.6*
G	-1A, 121Q, 168L, 180C	C14 diacid	6.1	0.9	-0.9*
H	-1A, 121Q, 168L, 180C	C16 diacid	4.5	3.2	-1.1**
0499	**-1A, 121Q, 168L, 180C**	**C18 diacid**	**4.7**	**12.3**	**-2.6****

* 1 mg/kg BID dosing ** 1 mg/kg QD dosing
The protractors were linked to the introduced Cys residue via gGlu-OEG-OEG-C2DA-Ac

Table 2: In vitro potency and mouse PK/PD read-outs of 180C analogues

- The FGF21 analogue 0499 which carries the C-terminal C18 diacid protractor and three stabilising mutations (figure 1) was chosen for further development.

Structure of the FGF21 analogue 0499

```
A HPIPDSSPLL QFGGQVRQRY LYTDDAQQTE AHLEIREDGT VGGAADQSPE   50
  SLLQLKALKP GVIQILGVKT SRFLCQRPDG ALYGSLHFDP EACSFRELLL  100
  EDGYNVYQSE AHGLPLHLPG QKSPHRDPAP RGPARFLPLP GLPPALPEPP  150
  GILAPQPPDV GSSDPLSMVG PSQGRSPSYC S                      181
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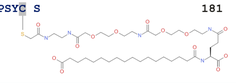

Figure 1: 0499 structure

0499 and Met-FGF21 display similar receptor selectivity

- As seen in table 3 Met-FGF21 and 0499 have similar receptor selectivity. Neither Met-FGF21 nor 0499 activated FGFR2c or FGFR4 in agreement with literature (4).

Analogue	FGFR1c/KLB EC50 (nM)	FGFR2c/KLB EC50 (nM)	FGFR3c/KLB EC50 (nM)	FGFR4/KLB EC50 (nM)
Met-FGF21	6.0 ± 0.3	No activation	10.6 ± 0.9	No activation
0499	**4.6 ± 1.1**	**No activation**	**12.2 ± 3.4**	**No activation**

Table 3: In vitro FGF receptor selectivity of 0499 vs Met-FGF21

0499 is proteolytically stabilised and shows extended half-life

- 0499 had increased stability towards degradation by fibroblast activation protein (FAP) (table 4).
- Both Met-FGF21 and 0499 were cleaved between 171P and 172S by FAP, but the presence of albumin stabilised 0499.
- Increased FAP stability was also observed in vivo.

Analogue	Stability towards FAP No albumin T½ (min)	Stability towards FAP 0.1% albumin T½ (min)
Met-FGF21	27	28
0499	**57**	**448**

Table 4: In vitro stability of 0499 and Met-FGF21 towards fibroblast activation protein (FAP) in the presence or absence of albumin.

- Extended half-life of 0499 was confirmed in mice, cynomolgus monkey, minipig, and LYD pig (Table 5).

Analogue	Mouse T½ (hrs)	Cynomolgus T½ (hrs)	Minipig T½ (hrs)	LYD pig T½ (hrs)
Met-FGF21	≤1	N/A	2	N/A
0499	**12**	**52**	**72**	**48**

Table 5: Circulating T½ of 0499 and Met-FGF21 across species

0499 lowered body weight and steatosis in DIO NASH mice

- A rapid loss of body weight was observed with 0.2 mg/kg 0499 and the dose was subsequently reduced to 0.1 and subsequently 0.05 mg/kg to keep body weight at approximately 90% of starting weight. At the end of the study mice treated with 0499 had lost 7% while the weight-matched (WM) group lost 11% compared to baseline as seen in figure 2A.
- 0499 treatment lowered ALT significantly more than the WM control group (figure 2B), while plasma AST was decreased to a similar degree in the two groups (data not shown).

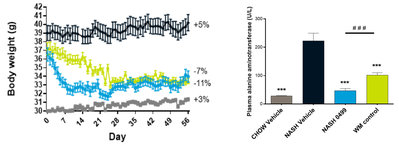

Figure 2: Effect of 0499 on BW (A) and plasma ALT (B).
DIO mice: black line, 0499: blue line, WM control: lime line and chow fed mice:: grey line

- As seen in figure 3A 0499 was more efficacious in reducing lipid content compared to the WM vehicle group based on quantitative morphology. Liver TG was lowered by a similar degree by 0499 and the WM control while 0499 also lowered hepatic cholesterol content (figure 3B) despite a significant increase in food intake compared to the NASH vehicle group (data not shown).

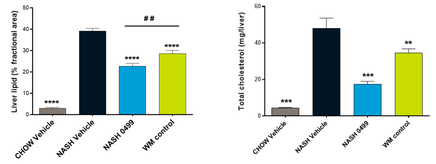

Figure 3: Effect of 0499 on liver lipids (A) and liver cholesterol (B)

- Both the 0499 treated group and the WM vehicle group decreased levels the infiltrating neutrophils and monocytes measured as CD11b staining at the end of the study, but this did not meet statistical significance (data not shown).

0499 lowered fibrogenesis in DIO NASH mice

- Mice treated with 0499 had lower % area of aSMA (figure 4A), a marker of hepatic stellate cell activation compared to the NASH vehicle group, while no significant effect was observed in the WM vehicle group. The effect of 0499 on fibrosis measured as PSR (figure 4B) did not reach statistical significance.

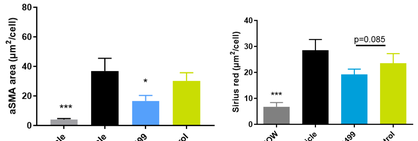

Figure 4: Effect of 0499 aSMA (A) and PSR (B) expression
*Statistical significance was determined by 2-tailed unpaired Student's t-test (chow vs NASH vehicle groups, and 0499 vs. WM control), or 1-way ANOVA with Tukey post-hoc test (excluding chow group). Results are shown as AVG. ± SEM Statistical significant shown as * when compared to NASH vehicle and as # when comparing 0499 to WM control. * or #: P<0.05, ** or ##: P<0.01, *** or ###: P<0.001.*

Summary

- Careful selection of position 180 as protraction site and of a C18 diacid as protractor enabled development of the novel FGF21 analogue 0499.
- In diet-induced NASH mice 8 weeks of treatment with 0499 (doses 0.05 to 0.2 mg/kg once daily) reduced hepatic steatosis (measured as % fraction area) and HSC activation more than the WM control, indicating an effect of 0499 on these parameters independent of BW loss.

Conclusion

- 0499 is a novel, proteolytically stable and protracted FGF21 analogue with significant beneficial effects on the liver in a murine model of NASH. Therefore, 0499 represents a new and competitive FGF21 analogue for the future treatment of NASH.

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