Definition of Endocrine FGF Structure as a Means to Super-Agonism

Agrawal, A[1,2]., Parlee, SD.[3], Perez-Tilve, D.[4], Li, P.[3], Pan, J.[3], Mroz, P.A.[1], Kruse Hansen[5], A.M., Andersen, B.[5], Finan, B.[3], Kharitonenkov, A.[3], DiMarchi, R.D.[1,2,3]

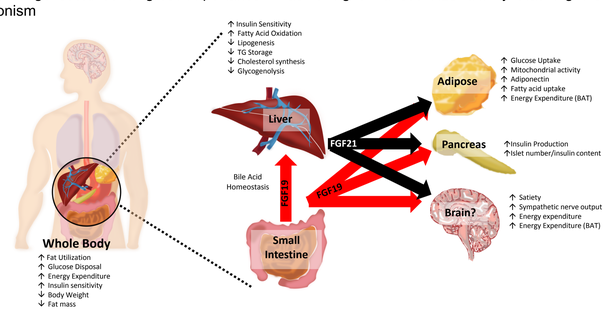
[1]Department of Chemistry, Indiana University, Bloomington, IN, 47405, USA, [2]Interdisciplinary Biochemistry Graduate Program, Indiana University, Bloomington, IN, 47405, USA, [3]Novo Nordisk Research Center Indianapolis, Indianapolis, IN, 46241, USA, [4]Metabolic Diseases Institute, Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, OH, USA, [5]Global Research, Novo Nordisk A/S, Novo Nordisk Park, Måløv, DK-2760, Denmark

Abstract

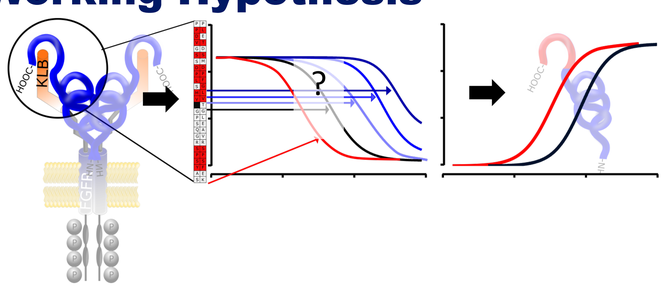
FGF21 has demonstrated curative pharmacology in murine models of obesity and diabetes that include ameliorating elevated lipids, glucose and insulin while simultaneously reducing body weight. In subchronic studies in humans, FGF21-therapy has demonstrated clinically meaningful reductions in triglycerides and other markers of lipid metabolism, without any significant effect on hyperglycemia. Assessment of FGF21 in non-human obese and diabetic primates suggests this glycemic outcome requires more intensive dosing. Accordingly, we explored the molecular elements within FGF21 responsible for receptor activity as a potential means for identifying a super-agonist. The endocrine FGFs (19 and 21) biochemically signal through a bipartite FGFR/KLB co-receptor complex, with the C-terminus believed to be of seminal importance to this interaction, and thus biological function. The 25-terminal amino acids of either FGF21 or 19 were determined to be necessary and sufficient for binding KLB and capable of antagonizing the action of each endocrine FGF. Amino acid scanning identified a common structural basis for this binding despite only partial sequence identity among FGF19 and 21. While the majority of alanine-mutations in the C-terminus peptide resulted in detrimental or neutral effects on KLB binding, a similar substitution for the terminal lysine of native FGF19 significantly improved its potency. When incorporated to full-length FGF21, the structure-activity relationship of these peptide-based antagonists translated to full length agonists, of correlative potency. A specific FGF21 analog with a C-terminal FGF19-based peptide proved pharmacologically superior to either hormone when studied *in vitro*, and in dietary obese mice. Collectively, these results define the structural signature for KLB-mediated signaling by endocrine FGFs and provide a directed approach to design of more efficacious FGF-based therapy for treatment of metabolic diseases.

Introduction

- FGF21 and 19 are secreted endocrine fibroblast growth factors (FGF) factors that lack a classical heparin-binding domain, thus they signal through a compulsory bipartite receptor-complex of klotho beta (KLβ) and FGF receptor (FGFR1c, 2, 3 & 4)[1-4].
- Produced largely by the liver and upregulated under states of metabolic stress, including fasting [5,6], FGF21 is of great interest for its curative pharmacology in murine models of obesity and diabetes ameliorating elevated lipids, glucose and insulin while simultaneous reducing blood glucose in murine models [7-9].
- In subchronic studies in humans, FGF21-therapy demonstrated clinically meaningful reductions in triglycerides and other markers of lipid metabolism, without any significant effect on hyperglycemia[10,11].
- Indeed dose proportional glucose lowering was observed in diabetic, non-human primates in an acute manner only at supra-pharmacologic levels. Thus lack of efficacy in human studies may result from under-dosing and a need for a higher-potency FGF21 analogues.[12,13]
- Work by Kharitionenkov and colleagues identified that the N- and C-termini of FGF21 dictate its signaling; Cleavage of the N-terminus decreases FGF21 potency and efficacy via loss of FGFR activation but does not impede KLβ binding. By contrast cleavage of the C-terminus of FGF21 diminishes interaction with KLβ[14].
- Cleavage of 17 N-terminal amino acids of FGF21 (FGF21$^{18\text{-}181}$/ΔN17) results in a protein antagonist for FGF21 signaling by competing for KLβ binding [14].
- The basis of receptor signaling by FGF19 is largely thought to parallel FGF21.
- We sought to determine if there is a common basis to KLB binding for the C-terminal segments of FGF19 and FGF21, since these proteins are as different in amino acid sequence as they are alike. Such knowledge could be of great importance in achieving enhanced KLB affinity that might enable super-agonism



Working Hypothesis



We hypothesize that by exploring how individual amino acids within the C-terminus of FGF21 and 19 contribute to KLB binding we can optimize its potency thereby enhancing signaling via FGF receptors.

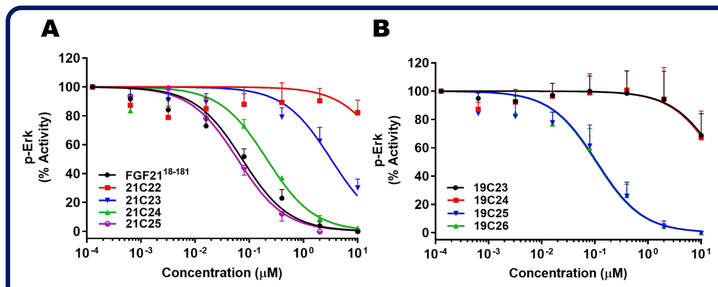


Figure #1: Short C-terminal peptides are sufficient to compete for FGF19 and FGF21 signaling. Removal of the 17-N terminal amino acids of FGF21 (FGF21$^{18\text{-}181}$) results in a protein which can compete with FGF21 for binding to KLβ, thus antagonizing FGF21 signaling. To identify the key amino acids that are necessary and sufficient within FGF21$^{18\text{-}181}$ to bind KLβ; N-terminal amino acids were sequentially removed from FGF21$^{18\text{-}181}$ and the resulting proteins and peptides evaluated for the ability to compete with FGF21 in a HEK293 that overexpress human-KLβ (293/KLβ). The 25-terminal amino acids of FGF21 (21C25) were found to be capable of competing for human-KLβ at a similar potency as FGF21$^{18\text{-}181}$ (**A**), further removal of amino acids results in decreases in potency. Similar results occur with the 26-C-Terminal amino acids of FGF19 (**B**).

Fold	IC_{50} (μM)	Position	Residue	Position	Residue	IC_{50} (μM)	Fold
1.00	0.17 ± 0.10	21C25		19C26		0.20 ± 0.10	1.00
2.47	0.42 ± 0.10	157	P	P	169	0.42 ± 0.38	2.13
4.88	0.83 ± 0.30	158	P	L	170	2.94 ± 2.26	14.93
6.76	1.15 ± 0.30	159	D	E	171	0.08 ± 0.07	0.39
63.00	10.71 ± 7.60	160	V	T	172	1.16 ± 0.76	5.90
0.71	0.12 ± 0.00	161	G	D	173	0.53 ± 0.28	2.69
14.35	2.44 ± 1.10	162	S	S	174	1.25 ± 0.38	6.36
1.71	0.29 ± 0.10	163	S	M	175	0.17 ± 0.14	0.87
-	NC	164	D	D	176	NC	-
-	NC	165	P	P	177	NC	-
-	NC	166	L	F	178	NC	-
0.94	0.16 ± 0.00	167	S	G	179	2.33 ± 1.17	11.81
-	NC	168	M	L	180	NC	-
25.12	4.27 ± 2.00	169	V	V	181	2.34 ± 1.02	11.90
-	-	-	-	T	182	0.59 ± 0.37	2.98
1.12	0.19 ± 0.10	170	G	G	183	0.39 ± 0.09	1.97
0.71	0.12 ± 0.00	171	P	L	184	0.18 ± 0.15	0.89
0.65	0.11 ± 0.10	172	S	E	185	0.20 ± 0.16	1.04
0.47	0.08 ± 0.00	173	Q	A	186	Native	-
0.82	0.14 ± 0.10	174	G	V	187	0.43 ± 0.28	2.17
0.59	0.10 ± 0.10	175	R	R	188	0.33 ± 0.23	1.69
22.41	3.81 ± 4.80	176	S	S	189	8.12 ± 4.98	41.20
49.12	8.35 ± 8.30	177	P	P	190	1.52 ± 0.37	7.69
100.24	17.04 ± 6.60	178	S	S	191	2.48 ± 1.38	12.59
23.76	4.04 ± 0.40	179	Y	F	192	2.22 ± 1.68	11.25
-	Native	180	A	E	193	0.47 ± 0.24	2.39
1.24	0.21 ± 0.10	181	S	K	194	0.007 ± 0.001	0.04

Table #1: Defining the structure-activity relationship in FGF21 and 19 C-terminal peptides. To better understand how each of the amino acids within 21C25 and 19C26 contribute to KLβ binding, an alanine scan was performed and subsequently tested for the resulting peptides ability to compete with FGF21 in 293/KLβ cells. IC_{50} values (presented in μM, mean ± SD, n=3) and fold-changes in antagonistic activity *versus* respective native peptides are listed above. Results indicate 2-key corresponding regions in each native peptide, FGF21$^{158\text{-}169}$ & FGF21$^{176\text{-}179}$ as well as , FGF19$^{170\text{-}181}$ & FGF19$^{189\text{-}192}$ which significantly decreased the potency of peptide compared to its native counterpart. These regions are therefore thought to be optimized. One peptide 1926 K194A displayed a ~10-20x increase in potency over 21C25 and 19C26.

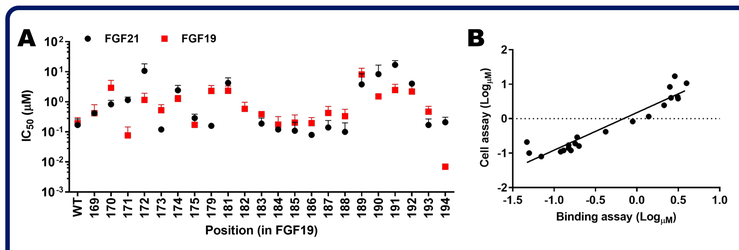


Figure #2: Alanine-substitutions in FGF21 and 19-based peptides result in equipotent antagonists, and for FGF21-based peptides correlate well with receptor binding. Comparison of calculated IC_{50}s in the 293/KLB assay from corresponding alanine-mutations in 21C25 and 19C26 peptides display similar potencies (**A**)**.** Further comparison of log-transformed IC_{50} values of 21C25 ala-scan peptides from the 293/KLβ signaling assay with their competitive displacement of FGF21 from a soluble human FGFR1c/KLβ binding assay likewise display a high level of correlation (r^2 = 0.90).

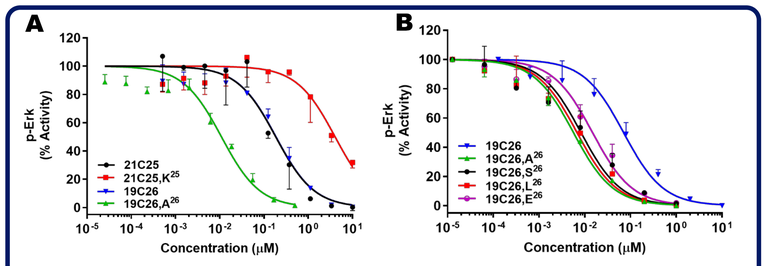


Figure #3: An alanine substitution in FGF19-based peptide but not FGF21 improves antagonistic potency as a result of the loss of lysine and not the gain of alanine. Given the increase in potency resulting from the K194A modification in 19C26, the question as to whether the loss of lysine or gain of alanine was driving its effects was investigated. Replacement of 21C25 with a terminal lysine (21C25,K^{25}) resulted in a significant loss of potency, while a replacement of native 19C26 with alanine (19C26,A^{26}) displayed the expected increase in potency (**A**)**.** To explore whether alanine alone bestowed this increase in potency, the terminal lysine of 19C26 was replaced with, serine, leucine and glutamic acid all of which displayed a similar increase in potency as the alanine substitution (**B**)**.**

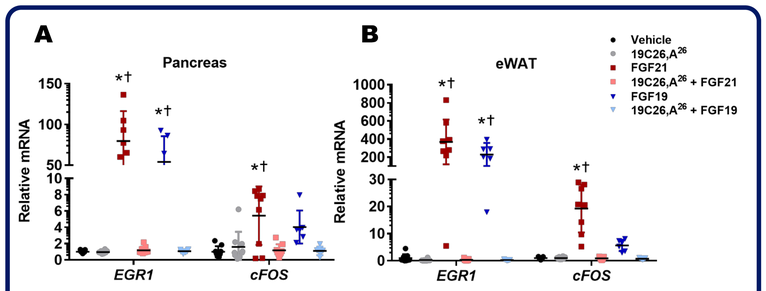


Figure #4: 19C26,A^{26} peptide antagonizes FGF21 and 19 signaling *in vivo* marked by decreased *EGR1* and *cFOS* expression in pancreas and eWAT. To explore whether the 19C26,A^{26} peptide displayed antagonism *in vivo*, lean (C57BL/6J) mice were pre-treated with 19C26,A^{26} (30 mg/kg) (or vehicle) prior to treatment with either FGF19 or FGF21 (1 mg/kg). The gene induction of *EGR1* and *cFos* expression was determined one hour post-treatment in (**A**) pancreas and (**B**) epididymal adipose tissue (eWAT) tissue. FGF21 and 19 significantly increased *EGR1* expression in both pancreas and eWAT that was inhibited by 19C26,A^{26} . Likewise FGF21 increased the expression of *cFOS* at one hour that was inhibited by 19C26,A^{26} . Mean ± SEM, n = 5–8, 1-way ANOVA with Tukey post-hoc analysis *P < 0.05 versus vehicle and †P < 0.05 versus 19C26,A^{26}

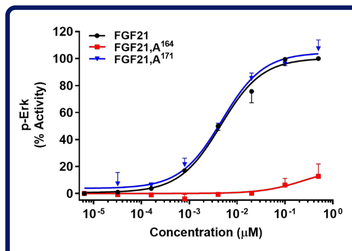


Figure #5: Null and detrimental mutations in FGF21-based peptides are paralleled when incorporated into full length protein. To explore whether null and detrimental substitutions identified via antagonistic alanine-scan translated when incorporated into full length protein; FGF21 analogues based on 21C25,A^{164} and 21C25,A^{171} were expressed, purified and tested for *in vitro* potency in 293/KLβ cells. In line with their antagonistic potencies FGF21,A^{164} displayed decreased potency and efficacy while FGF21,A^{171} displayed similar activity when compared to native FGF21.

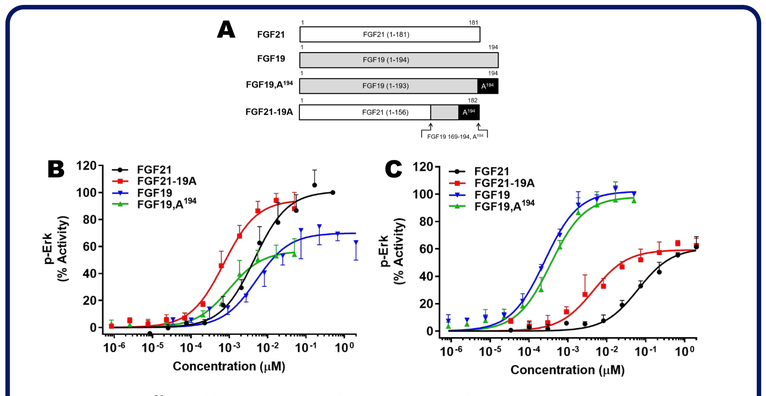


Figure #6: 19C26,A^{26} substitution into the C-terminus of FGF21 or 19 improves agonistic potency *in vitro*. Given the increase in antagonistic potency of 19C26,A^{26}, *in vitro* the question as to whether a parallel increase in potency could be achieved in FGF21 and 19 by replacing the c-terminus of the protein with the aforementioned peptide was explored. A schema displaying native FGF21 and FGF19 sequences along with the modified analogs FGF19,A^{194} and FGF21-19A which used in the subsequent studies (**A**). For both analogues incorporation of 19C26,A^{26} into the protein significantly increased the potency (~2-3x FGF21-19A & ~10x FGF19,A^{194}) compared to their respective control in 293/KLβ. In human Hep3B hepatocytes, which comparatively express higher levels of FGFR4, only the FGF21-19A maintains this greater increase in potency.

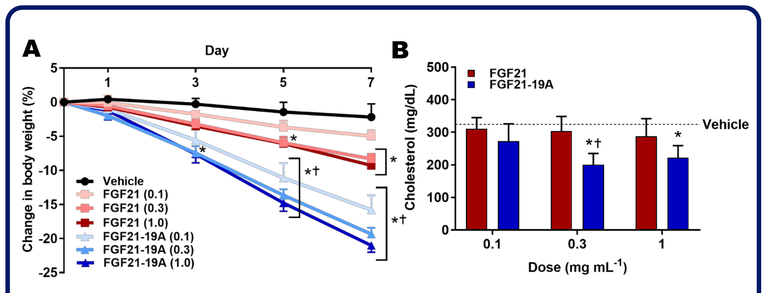
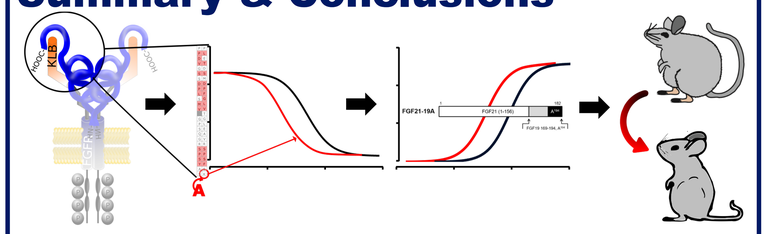


Figure #6: 19C26,A^{26} substitution into the c-terminus of FGF21 results in greater potency and efficacy on weight loss and reduction of circulating cholesterol compared to native FGF21. To explore the *in vitro- to in vivo* translation of the potent FGF21-19A analogue, DIO C57/BL6 mice were administered vehicle, FGF21 or FGF21-19A (0.1, 0.3 and 1 mg/kg) daily via *subcutaneous* injections. Body weight measurements were subsequently measured on alternative days. At the end of 7 day treatment, blood was collected from tail veins and serum cholesterol levels were analyzed using colorimetric assay. In line with the increase in potency *in vitro* , FGF21-19A displayed ~10x increase in potency *in vivo;* significantly reducing body weight and total cholesterol. N=8, 2-way ANOVA with Tukey post-hoc analysis †P < 0.05 was FGF21 versus FGF21-19A at equivalent doses and *P < 0.05 versus vehicle was calculated (**A**) **&** 1-way ANOVA with Tukey post-hoc analysis †P < 0.05 was FGF21 versus FGF21-19A and *P < 0.05 versus vehicle was calculated (**B**)**.**

Summary & Conclusions



- Short 25 Amino acid C-terminal of FGF21 and 19 are fully sufficient to support interaction with KLβ. Moreover, the limited sequence identity in the C-terminal region of FGF19 and FGF21 defines to a substantial degree the common functional elements of utmost importance to KLB binding
- The correlative manner in which Ala-substituted peptides block signaling by either hormone also suggests that FGF19 and FGF21 utilize common KLβ binding interfaces with 2-key corresponding regions in each native peptide; FGF21$^{158\text{-}169}$ & FGF21$^{176\text{-}179}$ as well as, FGF19$^{170\text{-}181}$ & FGF19$^{189\text{-}192}$ being of particular importance.
- The selective increase in peptide antagonism identified via Ala-Scan in 19C26,A^{26} was unexpected. Study of additional substitutions, nevertheless made it clear that alanine is not unique in its potency-enhancing properties, rather it is the loss of terminal lysine that appears to dictate the increase in potency.
- A single site mutation that selectively destroyed peptide-based antagonism demonstrated an analogous effect to eliminate agonism of full-length proteins suggesting the regions of peptide important for KLβ remain so in the full length agonist.
- In line with our hypothesis when the enhanced KLB binding potency of 19C26,A^{26} was incorporated into a full-length FGF21 sequence (FGF21-19A) the resulting FGF21 analog displayed a parallel increased *in vitro* activity that was also pharmacologically superior when studied in DIO mice This translation of peptide antagonism into super-agonism of the full-length protein is precedent setting.
- Overall our studies identified key regions of FGF21 and 19 that regulate KLβ binding and further provide proof of principal that by optimizing the C-terminus a more potent analogue of FGF21 can be identified. What remains to be seen is whether this increase in potency can overcome limitations of previous clinical FGF21 compounds.

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For complete list of references please see Agrawal, A. *et al.* (2018) *Molecular elements in FGF19 and 21 defining KLB/FGFR activity and specificity. Mol Metab. 2018 Jul; 13: 45-55*

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