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Efruxifermin, a bivalent Fc-FGF21 analog, demonstrates improved biophysical and pharmacological engagement with live cells compared to monovalent FGF21 analogs

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BACKGROUND

Efruxifermin (EFX) is a long-acting Fc-FGF21 fusion protein currently in Ph2b clinical trials for treatment of advanced (F2/F3) liver fibrosis and compensated cirrhosis (F4) due to non-alcoholic steatohepatitis (NASH). Unlike monovalent analogs of FGF21, one molecule of EFX comprises two molecules of an FGF21 variant, FGF21[L98R,P171G,A180E] (RGE).

One of FGF21's two receptors, β-Klotho, forms a high-affinity interaction with the C-terminus of FGF21. Subsequently, the N-terminus binds to one of FGFR1c, 2c, or 3c forming a multimeric complex required to mediate intracellular signaling (Figure 1A). A greater number of theoretical binding interactions for bivalent EFX than for monovalent FGF21 analogs could result in different pharmacological properties (Figure 1B).

We hypothesized this bivalent structure could result in greater affinity for FGF21's receptors on the cell surface compared to monovalent FGF21 analogs, potentially leading to more potent, durable, and effective agonism of FGF21's target receptors *in vivo*.

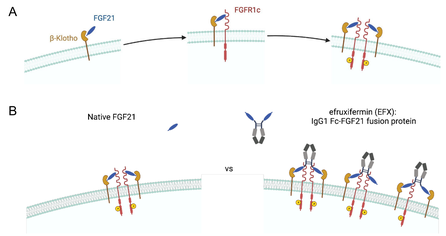


Figure 1. Schema of FGF21 or EFX binding to cognate FGF21 receptors in target tissues. Created with BioRender.com

AIMS

We aimed to elucidate the differences between monovalent and bivalent FGF21 analogs using a combination of biophysical and cell-based assays.

We sought to understand whether these *in vitro* differences could underlie observed clinical differences between monovalent and bivalent FGF21 analogs in patients with metabolic diseases, including obesity[1,2], type 2 diabetes[3,4], and NASH[5,6].

METHODS

Monovalent analogs included RGE without an Fc domain (Figure 2A), or an Fc dimer-fused RGE (Figure 2B). Efruxifermin (EFX) comprises a homodimer of Fc-RGE (Figure 2C).

Figure 2. Schema of (A) monovalent RGE, (B) monovalent Fc-RGE, and (C) bivalent Fc-RGE [EFX].

HEK293 cells overexpressing human β-Klotho and FGFR1c, as well as an Elk-1 promoter-driven luciferase vector, were cultured using standard methods. Luciferase expression is proportional to extent of FGF21 agonism of its receptors, enabling use of this cell line to measure potency of FGF21 analogs.

Association and dissociation kinetics of mono- or bivalent FGF21 analogs with the surface of live cells was measured using a fluorescence-based, real-time method called LigandTracer (Fig 3). The contribution of the interaction with β-Klotho to the overall association of mono- vs. bivalent analogs was assessed using a peptide which selectively inhibits binding to β-Klotho[7].

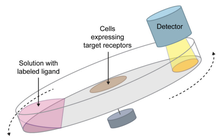
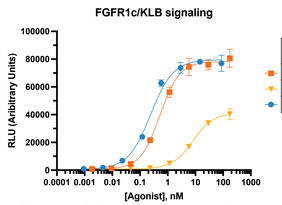


Figure 3. LigandTracer Assay
Measures accumulated fluorescent ligand in cell area over time.

RESULTS

Monovalent and Bivalent FGF21 Analog Potency as Agonists of FGF21's Receptors



Analog	EC_{50} (nM analog)	EC_{50} (nM FGF21)	Fold Induction	Hill Slope
Monovalent RGE	0.52	0.52	95	1.2
Monovalent Fc-RGE	7.93	7.93	49	1.2
Bivalent Fc-RGE (EFX)	0.24	0.48	114	1.1

Figure 4. Cell-based potency of monovalent and bivalent FGF21 analogs. Analogs were incubated with the HEK293-based bioassay for ~20 hours, and agonism was measured by FGF21 signaling-dependent luciferase activity.

Addition of an Fc domain to a monovalent FGF21 analog, RGE, significantly reduces both potency (~15-fold right-shift in EC_{50}) and agonist efficacy (~2-fold down-shift in E_{max}).

Addition of a second RGE moiety to monovalent Fc-RGE, yielding bivalent Fc-RGE (EFX), more than overcomes steric hindrance by the Fc domain, as potency is increased 1-2 fold relative to monovalent RGE unconjugated to an Fc domain.

Investigation of Binding Mode for Monovalent and Bivalent FGF21 Analogs

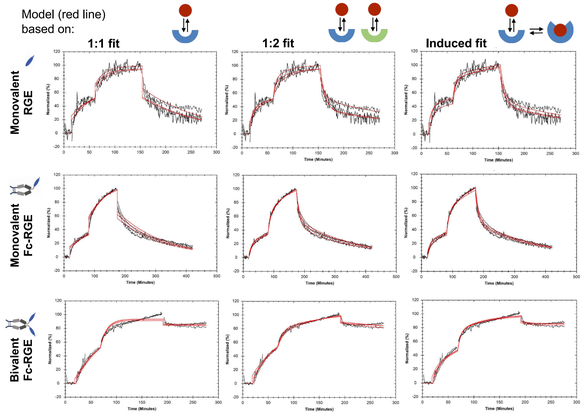


Figure 5. Binding mode modeling. To measure kinetics of association with the cell line employed in Figure 4, FITC-labelled analogs were incubated consecutively at two concentrations, the second one 3-5-fold higher than the initial concentration. Dissociation kinetics were then followed after replacement with incubation media containing no ligand.

While the 1:1 model adequately fit the data generated with monovalent RGE and monovalent Fc-RGE, a more complex model was needed to describe the interaction between bivalent Fc-RGE and the surface of target cells, particularly the long linear phase during association.

Table 1. Global fitting of kinetic parameters and affinity to a standard binding model. Triplicate measurements presented in Figure 5 were fit (red lines) using TraceDrawer software, and kinetic and affinity parameters were estimated.

Analog	k_a (1/[M*s])	k_d (1/s)	K_D (M)
Monovalent RGE	4.7×10^4	1.4×10^{-4}	3.0×10^{-9}
Monovalent Fc-RGE	2.1×10^4	1.1×10^{-4}	5.4×10^{-9}
Bivalent Fc-RGE (EFX)	1.8×10^5	3.3×10^{-6}	1.8×10^{-11}

Addition of an Fc domain to monovalent RGE decreases the association rate of ligand to the cell surface by approximately 2-fold, with minimal (<25%) impact on dissociation rate, resulting in a binding affinity that is almost 2-fold weaker.

Addition of a second RGE moiety to monovalent Fc-RGE, yielding bivalent Fc-RGE (EFX), increased association rate approximately 10-fold (or 5-fold relative to monovalent RGE), and markedly stabilized the interaction, with a dissociation rate about 30 or 40-times slower than that of monovalent Fc-RGE or monovalent RGE, respectively.

Together, these contribute to bivalent Fc-RGE (EFX) having a 100-fold higher binding strength (K_D) than either monovalent analog: **18 pM** vs. **3.0 – 5.4 nM**.

Investigation of Binding Stability for Monovalent and Bivalent FGF21 Analogs

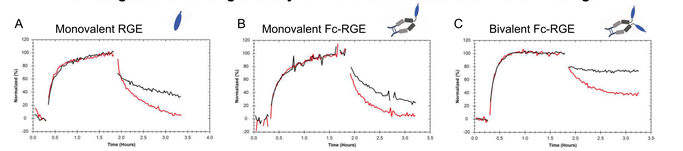


Figure 6. Displacement efficiency of monovalent and bivalent FGF21 analogs. Cells were incubated with FITC-labeled monovalent RGE (A), monovalent Fc-RGE (B), or bivalent Fc-RGE (EFX) (C). Following equilibration of labeled ligand to the cell surface, dissociation was followed after replacement with a 10-fold molar excess of unlabeled ligand.

A 10-fold excess of unlabeled analog maximally displaces each corresponding labeled monovalent analog (Figure 6A,B). In contrast, a significant proportion (~40%) of labeled bivalent Fc-RGE remains stably associated in the presence of 10-fold excess of unlabeled bivalent Fc-RGE (Figure 6C). This appears consistent with the much slower off-rate of the bivalent Fc-RGE (EFX) analog quantified in Table 1 and observed in Figure 5.

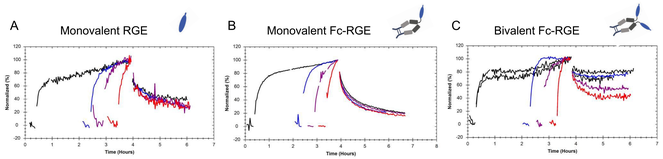


Figure 7. Stabilization of binding over time for monovalent and bivalent analogs. To investigate stabilization of the interaction, cells were incubated with labelled analogs for increasing durations.

With increasing incubation time during the association phase, monovalent analogs did not demonstrate stabilization of binding to the cell surface (Figure 7A,B).

In contrast, bivalent Fc-RGE (EFX) demonstrated stabilization: as association time increased from 30 minutes to 3.5 hours, the proportion of bivalent Fc-RGE (EFX) that remained bound during the dissociation phase increased (Figure 7F). One possible explanation for this stabilization over time is engagement of the cell surface by the second RGE moiety of the bivalent Fc-RGE analog, which is consistent with improved binding affinity (Figure 5, Table 1) and cell-based potency (Figure 4) relative to monovalent FGF21 analogs.

Contribution of High-affinity Interaction β-Klotho to Binding of Monovalent and Bivalent Analogs

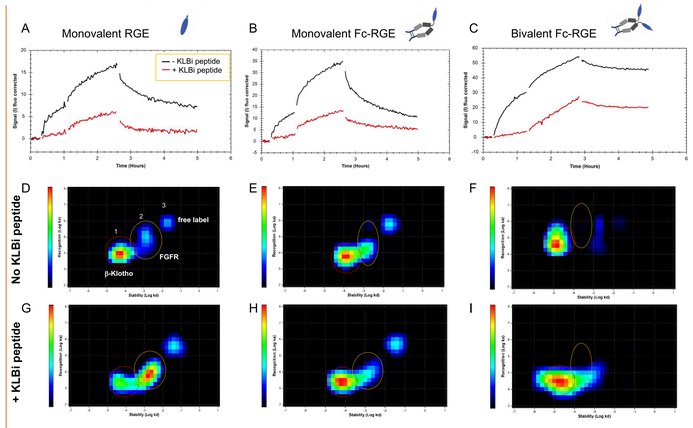


Figure 8. Impact of β-Klotho inhibition on kinetics of monovalent and bivalent FGF21 analog binding to the cell surface. Cells were incubated as per Figure 5, with or without 100 nM of a peptide which specifically inhibits binding to β-Klotho[7]. InteractionMap analysis (panels D-I) deconvolutes binding traces into their weighted, 1:1-like interaction components without *a priori* specifying the number of interactions present. Interactions with β-Klotho (red circle) and FGFR1c (orange circle) are indicated.

The presence of a β-Klotho inhibitor greatly decreased the association rate of monovalent RGE to the cell surface (Figure 8A), as indicated by a shift in the relative contribution of β-Klotho-driven interactions toward predominantly FGFR1c-mediated interactions (Figure 8D,G). Association of monovalent Fc-RGE (Figure 8B) was also greatly reduced, although the relative contribution of FGFR1c binding did not appear to be clearly altered, perhaps due to steric hindrance between the Fc-domain and FGFR1c (Figure 8E,H).

In contrast to the monovalent FGF21 analogs, the interaction peak representing binding to β-Klotho is more elongated along the Y-axis [$Log(k_a)$] for the bivalent Fc-RGE (EFX), suggesting heterogeneity in target recognition consistent with multivalent binding to β-Klotho (Fig 8F).

In the presence of the peptide inhibitor, the algorithm could not clearly separate the contributions of FGFR1c and β-Klotho to the overall interaction (one broad peak in Fig 8I). Nevertheless, a significant contribution of β-Klotho-binding appeared to be maintained by bivalent Fc-RGE (EFX) despite a >10-fold molar excess of the peptide inhibitor (Figure 8I). Moreover, while the association phase becomes more linear for all analogs in the presence of the inhibitor, the shift is more pronounced for bivalent Fc-RGE (Figure 8C vs A,B). Taken together, these data suggest that due to its bivalent structure, EFX may be better able to displace the β-Klotho inhibitor during the assay compared to monovalent FGF21 analogs.

Table 2. Summary data comparing monovalent and bivalent FGF21 analogs.

	Monovalent RGE	Monovalent Fc-RGE	Bivalent Fc-RGE (EFX)
FGF21 receptor hindrance	none	N-terminus linked to IgG1 Fc	N-terminus linked to IgG1 Fc
mol. FGF21 / mol. analog	1	1	2
Number of potential interaction points with cell surface	2	2	4
# of high-affinity interaction points (KLB-mediated)	1	1	2
# of low-affinity interaction points (FGFR-mediated)	1	1	2
K_D (affinity) on live cells	3 nM	5.4 nM	18 pM
EC_{50} (potency),cell-based bioassay	0.52 nM	7.93 nM	0.24 nM

CONCLUSIONS

- The **bivalent structure** of Fc-RGE (EFX), with two FGF21-variant moieties per molecule, results in much stronger affinity—predominantly because of **more stable binding**, *i.e.*, slower dissociation (Figure 5, Table 1)
- Much stronger affinity of Fc-RGE (EFX) for the target cell surface translates into **greater cell-based potency** compared to the monovalent RGE and monovalent Fc-RGE analogs (Figure 4).
- This may be due to **avidity effects** based on bivalent Fc-RGE (EFX) having **more simultaneous high- and low-affinity interactions** with its receptors on the surface of target cells than monovalent FGF21 analogs (Table 2).
- Any such avidity effects are consistent with the apparently greater efficiency of EFX in **overcoming competitive inhibition of the β-Klotho binding site** on the cell surface (Figure 8).
- EFX's much greater affinity for target cells and its ability to displace a (monovalent) competitive inhibitor of β-Klotho-binding suggests EFX **may retain pharmacological engagement** of its receptors for longer than monovalent analogs.
- Bivalent FGF21 analogs like EFX therefore **may deliver differentiated pharmacology** due to **sustained engagement of receptors** on the surface of target cells.

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