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

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BACKGROUND AND AIMS

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 would 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*.

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].

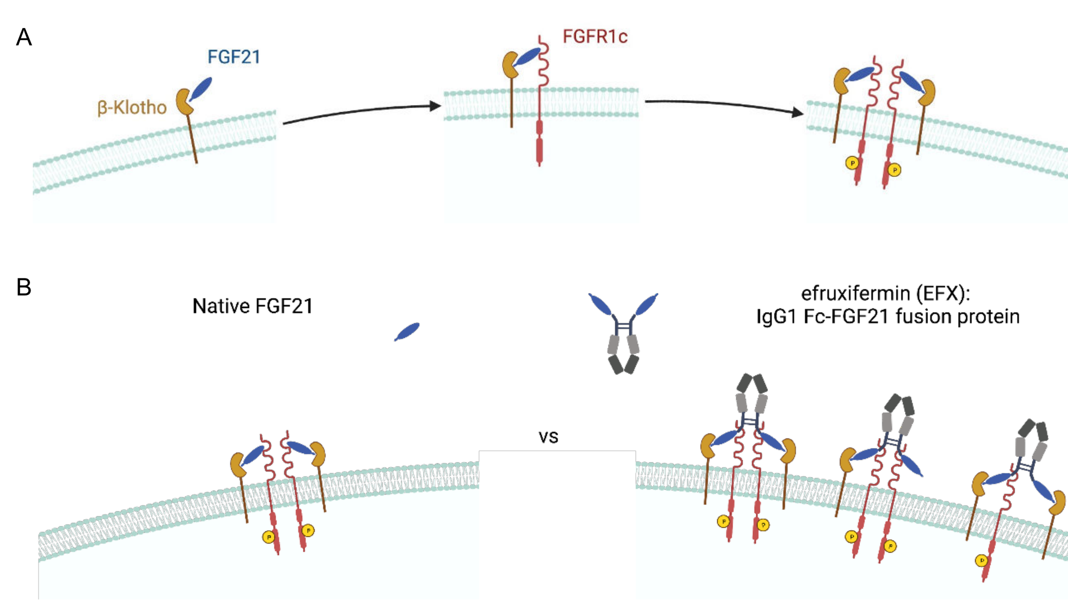


Figure 1. Schema of FGF21 or EFX binding to cognate FGF21 receptors in target tissues. Created with BioRender.com

MATERIALS AND METHODS

Monovalent analogs included RGE without an Fc domain (Figure 2A), an Fc dimer-fused RGE (Figure 2B), an FGF21 analog with a 30 kDa PEG attached to residue 108 (108-PEG30, Figure 2D), or an FGF21 analog with a 20 kDa PEG attached to residue 173 (173-PEG20, Figure 2E). Efruxifermin (EFX) comprises a homodimer of Fc-RGE (Figure 2C).

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 (Figure 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]

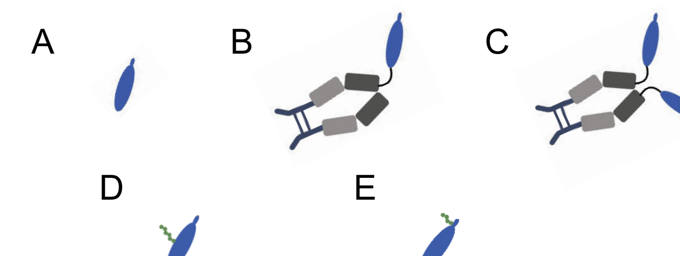


Figure 2. (A) monovalent RGE, (B) monovalent Fc-RGE, (C) bivalent Fc-RGE [EFX], (D) monovalent 108-PEG30, and (E) monovalent 173-PEG20

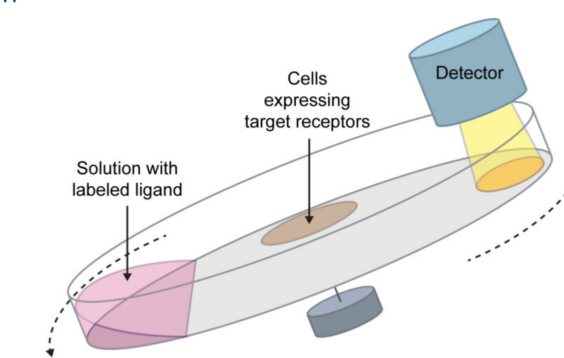
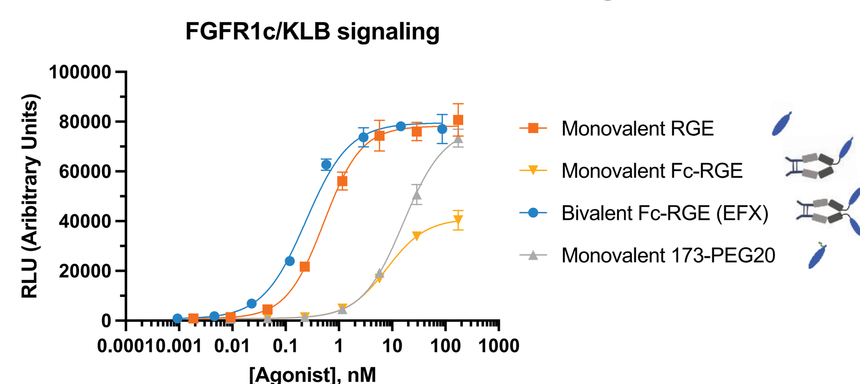


Figure 3. LigandTracer Assay Measures fluorescent ligand in cell area over time.

RESULTS

Monovalent and Bivalent FGF21 Analogs: Cell-based 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
Monovalent 173-PEG20	16.2	16.2	93	1.1
Monovalent 108-PEG30	*Not Determined*			

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 reduced both potency (~15-fold right-shift in EC_{50}) and agonist efficacy (~2-fold down-shift in E_{max}), likely due to steric hindrance of the N-terminal interaction with FGFR1c. Addition of a second RGE moiety to monovalent Fc-RGE, yielding bivalent Fc-RGE (EFX), more than overcame steric hindrance by the Fc domain, as potency increased 1-2-fold relative to monovalent RGE unconjugated to an Fc domain, and full agonism was restored. Addition of a 20 kDa PEG at position 173 (173-PEG20), amid residues important for β-Klotho-binding, significantly reduced potency relative to an unconjugated monovalent analog like RGE, consistent with the importance of unhindered FGF21 C-terminal binding to β-Klotho.

Investigation of Binding Mode for Monovalent and Bivalent FGF21 Analogs

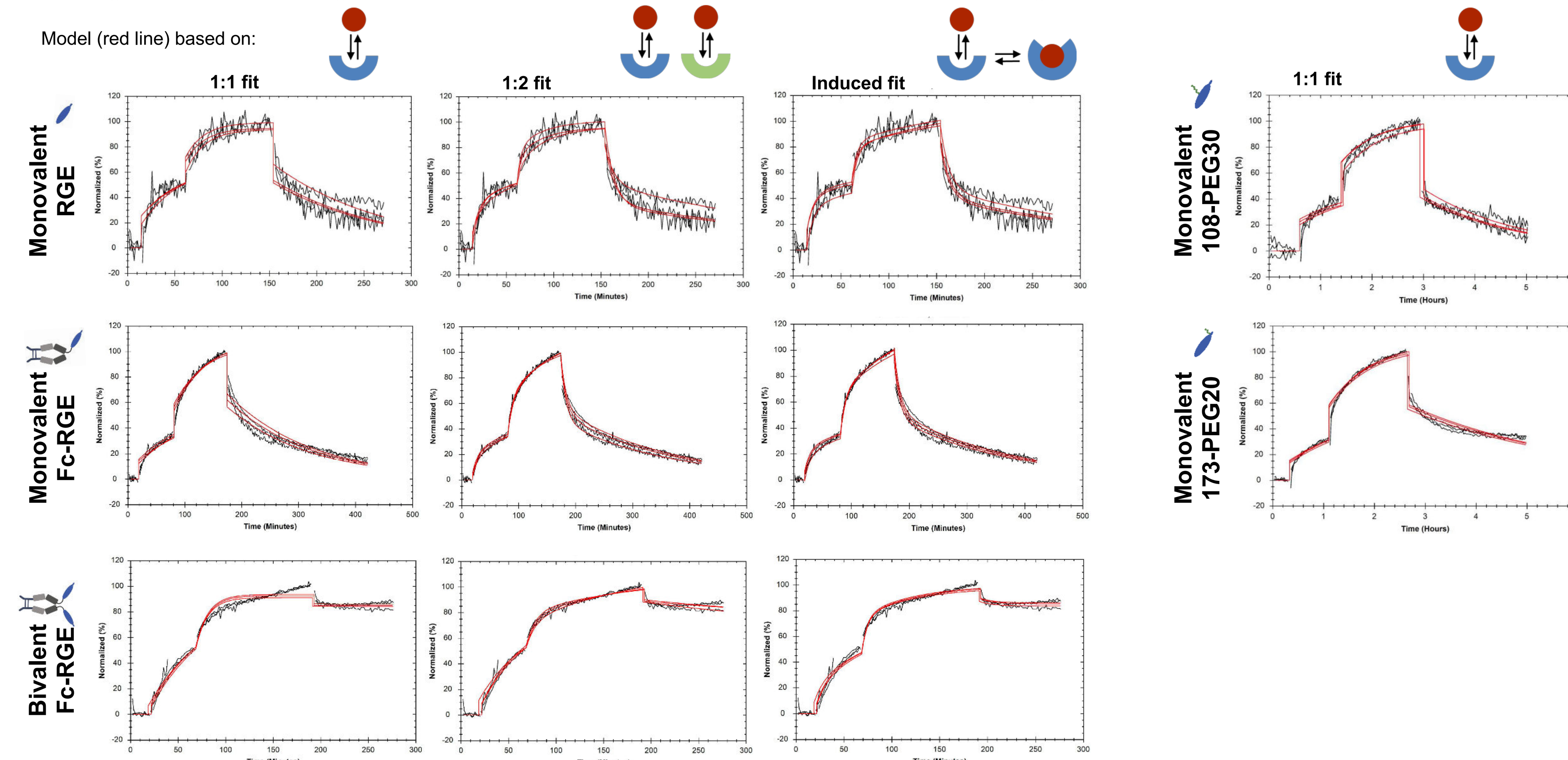


Figure 5. Binding mode modeling. To measure kinetics of association with the same cell line as employed in Figure 4, FITC-labeled analogs were incubated consecutively at two concentrations, the second one 3-5-fold higher than the initial concentration. Dissociation kinetics were then followed after replacement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

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}
Monovalent 108-PEG30	1.7×10^{4}	1.5×10^{-4}	9.0×10^{-9}
Monovalent 173-PEG20	1.7×10^{4}	8.3×10^{-5}	4.8×10^{-9}

Addition of an Fc domain to monovalent RGE resulted in an almost 2-fold weaker binding affinity. Similarly, PEGylated FGF21 analogs had 50% to 3-fold weaker binding affinity than monovalent RGE to the cell surface.

Addition of a second RGE moiety to monovalent Fc-RGE, yielding bivalent Fc-RGE (EFX), increased association rate approximately 10-fold (or 4-fold relative to monovalent RGE), and markedly stabilized the interaction, with dissociation rate about 30 or 40-times slower than that of monovalent analogs Fc-RGE or RGE. PEGylation of FGF21 near the C-terminus (residue 173) leads to an approximately 3-fold slower association rate, and 2-fold slower dissociation rate than an un-PEGylated analog like monovalent RGE.

Bivalent Fc-RGE (EFX) has a **>100-fold higher binding strength** (K_D) than any of the monovalent analog: **18 pM** vs. **3.0 – 9.0 nM**.

Investigation of Binding Stability for Monovalent and Bivalent FGF21 Analogs

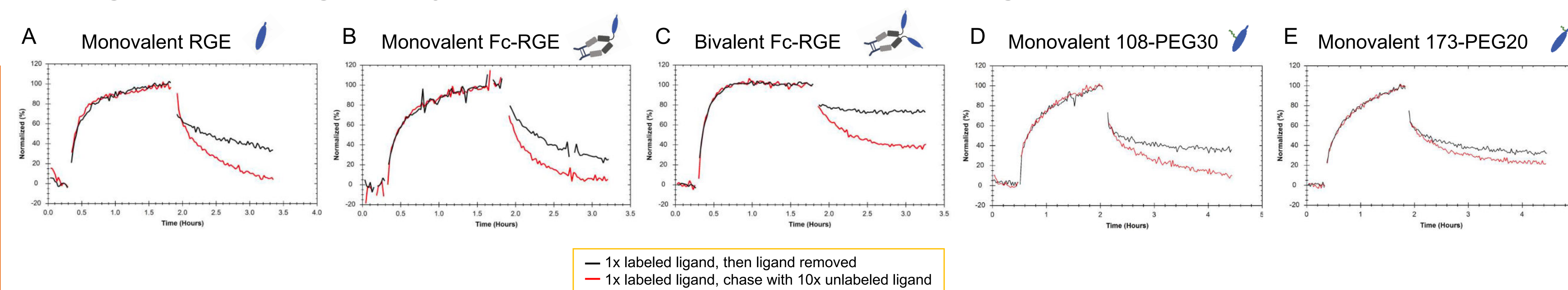


Figure 6. Displacement efficiency of monovalent vs bivalent FGF21 analogs. Cells were incubated with FITC-labeled (A) monovalent RGE, (B) monovalent Fc-RGE, (C) bivalent Fc-RGE (EFX), (D) monovalent FGF21-108-PEG30, or (E) monovalent FGF21-173-PEG20. Following equilibration of labeled ligand to the cell surface, dissociation was monitored after either ligand withdrawal (black line) or replacement with a 10-fold molar excess of unlabeled ligand (red line).

A 10-fold excess of unlabeled analog maximally displaced each corresponding labeled monovalent FGF21 analog (Figure 6A,B,D,E). In contrast, a significant proportion (~40%) of labeled bivalent Fc-RGE remained 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. The presence of a 20 kDa PEG molecule attached near the C-terminus may hinder the ability of unlabeled monovalent 173-PEG20 to displace pre-bound labeled ligand (Figure 6E).

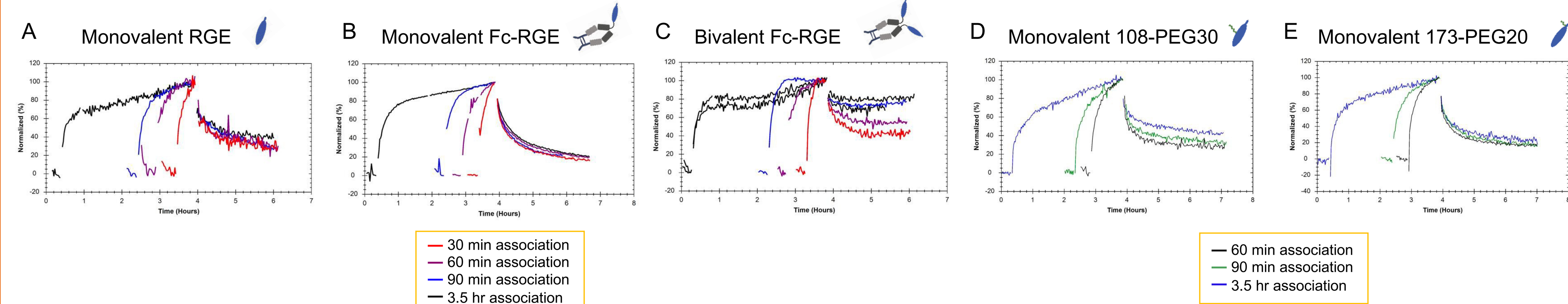


Figure 7. Stabilization of binding over time for monovalent and bivalent analogs. To investigate stabilization of the interaction (*i.e.*, slower dissociation rate), cells were incubated with labelled analogs for increasing durations, followed by removal in the dissociation phase.

With longer incubation duration during the association phase, binding of monovalent analogs to the cell surface was not noticeably stabilized (Figure 7A,B,D,E).

In contrast, bivalent Fc-RGE (EFX) demonstrated stabilization: as association time increased up to 3.5 hours, the proportion of bivalent EFX that remained bound during the dissociation phase increased (Figure 7F). One possible explanation for this stabilization is that more interactions are formed over time with target cell surface receptors by the second RGE moiety of the bivalent Fc-RGE analog, consistent with stronger binding affinity and cell-based potency relative to monovalent analogs driven by avidity effects.

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

	Monovalent RGE	Monovalent Fc-RGE	Bivalent Fc-RGE (EFX)	Monovalent 108-PEG30	Monovalent 173-PEG20
Half-life extension	minimal	Fc-fusion	Fc-fusion	30 kDa PEG at residue 108	20 kDa PEG at residue 173
FGF21 receptor hindrance	none	N-terminus linked to IgG1 Fc	N-terminus linked to IgG1 Fc	none	20 kDa PEG at residue 173
mol. FGF21 / mol. analog	1	1	2	1	1
Number of potential interaction points with cell surface	2	2	4	2	2
# of high-affinity interaction points (KLB-mediated)	1	1	2	1	1
# of low-affinity interaction points (FGFR-mediated)	1	1	2	1	1
K_D (affinity) on live cells	3 nM	5.4 nM	**0.018 nM**	9nM	4.8 nM
EC_{50} (potency),cell-based bioassay	0.52 nM	7.93 nM	0.24 nM	*Not determined*	16.2 nM

CONCLUSIONS

The **bivalent structure** of Fc-RGE (EFX), with two FGF21-variant moieties per molecule, resulted 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 translated 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).

While PEGylation may prolong pharmacokinetic half-life of an FGF21 analog, **PEGylation of the FGF core or C-terminal residues of FGF21 did not enhance, and may reduce, affinity for the surface of target cells.**

Bivalent FGF21 analogs like EFX therefore **may deliver differentiated pharmacology** due to **sustained engagement of receptors** on the surface of target cells, even **compared to molecules with apparently similar pharmacokinetic profiles** enabled by PEGylation-dependent C-terminal protection

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