Efficient mRNA display protocol yields potent bicyclic peptide inhibitors for FGFR3c: outperforming linear and monocyclic formats in affinity and stability

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Supporting Information
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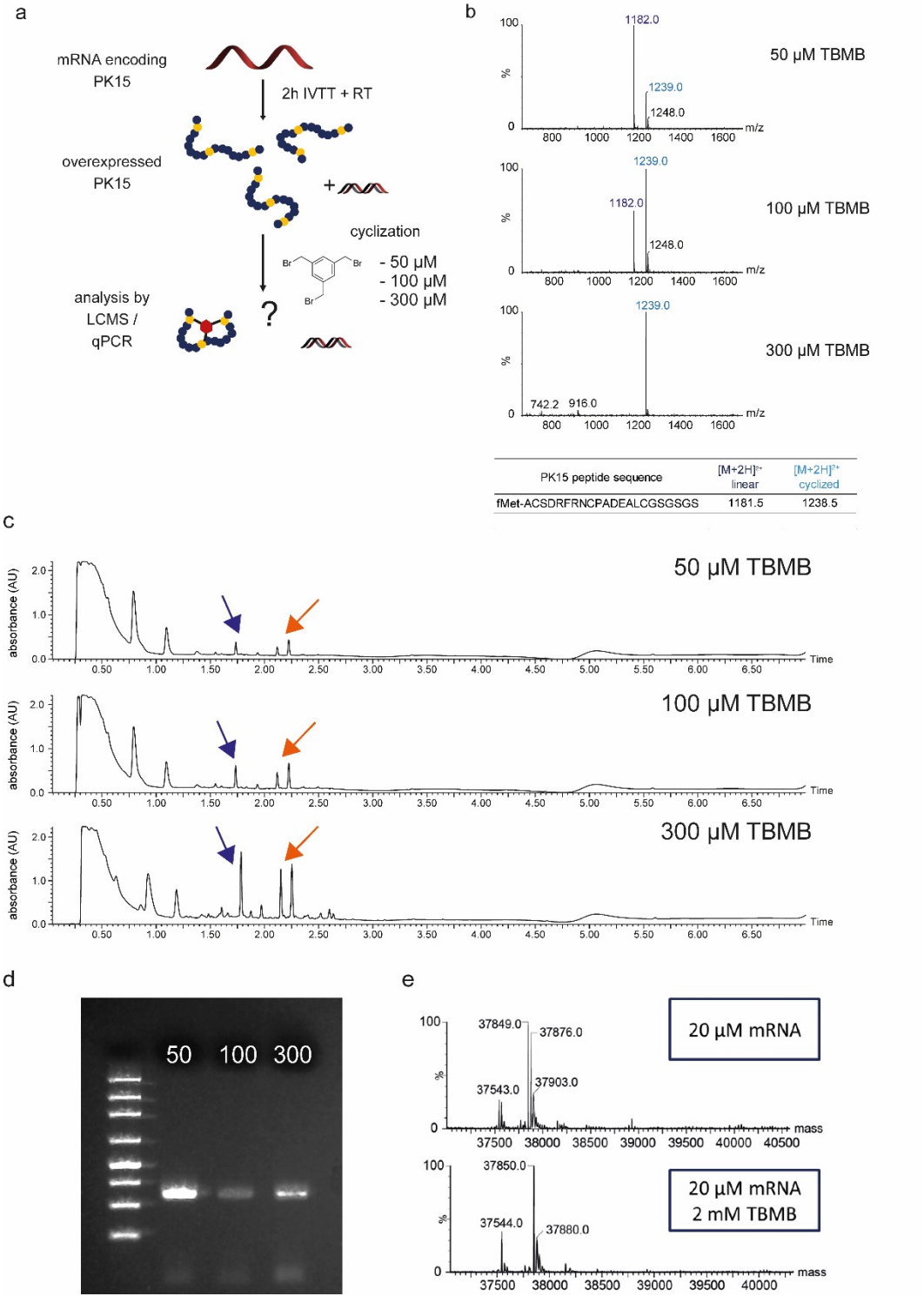
Supporting Figures and Tables

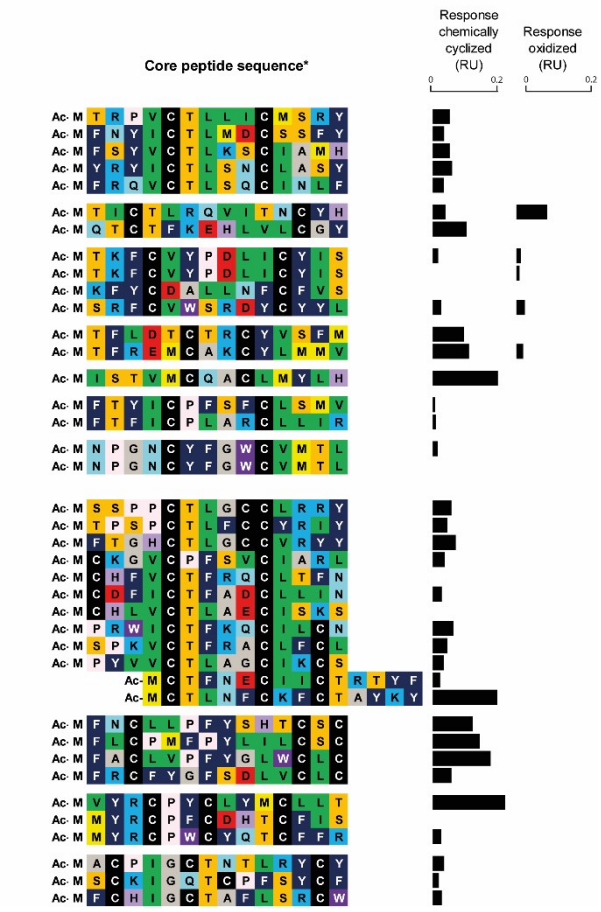
Reaction of *in vitro* translated PK15 peptides with TBMB



Supporting Figure S1: (a) a mRNA coding for PK15 was subjected to in vitro translation in the PURE system followed by reverse transcription to cDNA. The mixture of nucleic acid plus overexpressed peptides was incubated for 30 min with various concentrations of TBMB in ammonium bicarbonate buffer pH8. The cyclization efficiency is measured by LC-MS and the nucleic acid integrity is verified by qPCR followed by agarose gel (Supporting Figure S1.d) (b) MS signal detected after cyclization with TBMB, the signals corresponding to cyclized peptides appear in light blue whereas the linear

peptide masses are in dark blue. Calculated monoisotopic masses for the linear and cyclized peptides are shown in the table (only [M+2H]2+ was observed). (c) Analysis of the reaction of the *in vitro* translated PK15 peptides with various concentrations of TBMB. The peak indicated with the blue arrow corresponds to the PK15 peptide, for which the m/z signals are visible in Supporting Figure S1.b. The orange arrow indicates the absorbance signal of TBMB and its different oxidation products. (d) Agarose gel showing the amplicon obtained after PCR amplification of the IVTT mixtures submitted to different concentrations of TBMB. (e) Deconvoluted mass spectra comparing 20 µM PK15 mRNA with and without exposure to 2 mM TBMB.

BLI response of chemically and disulfide cyclized mono- and bicyclic peptides



Supporting Figure S2: BLI responses obtained for mono-/ bicyclic peptides cyclized with DBX/TBMB (response chemically cyclized) or oxidized in DMSO (response oxidized). Five of the tested monocyclic peptides showed binding both in their cyclized (via bridging reagent) and oxidized (cyclization via disulfide bond) form.

Overview of the purified peptides

Table S2: Overview of the purified peptides used in the study including their assigned names, structure, molecular weight and observed m/z signal.

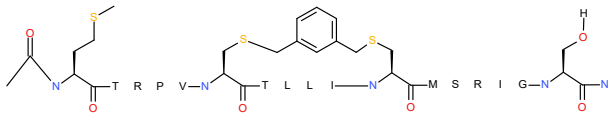
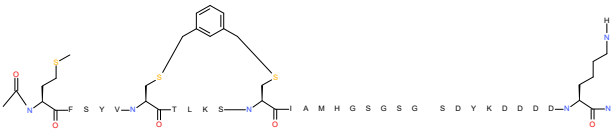
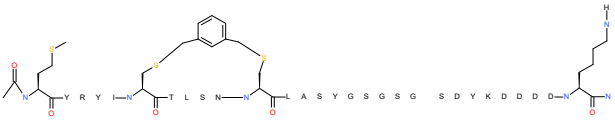
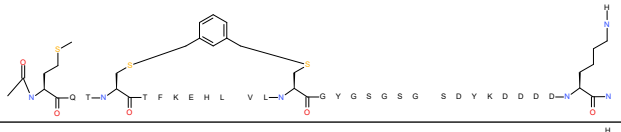
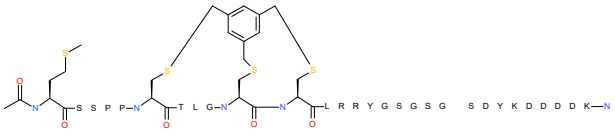
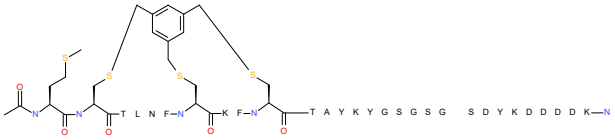
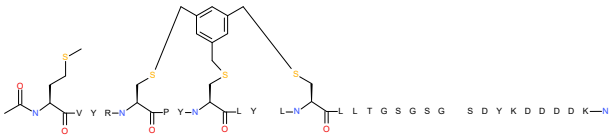
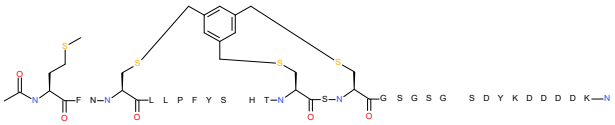
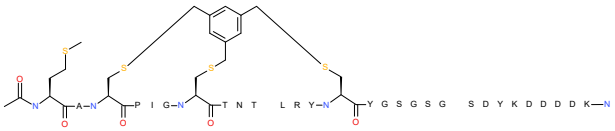
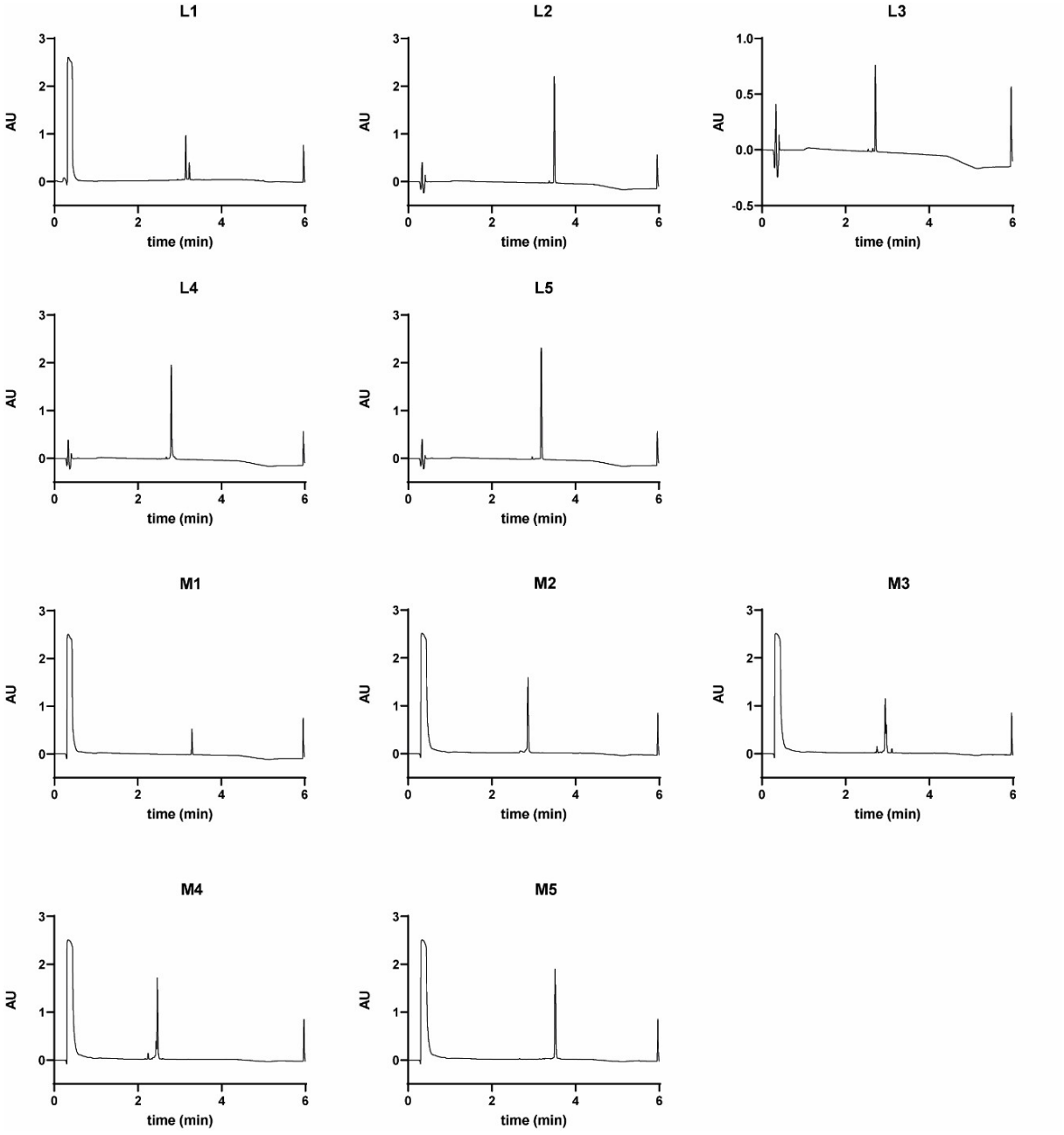
Peptide	Geometry	Exact amino acid sequence	Exact mass (Da)	Observed m/z
L1	Linear		2283.08	1143.04 $[M+2H]^{2+}$
L2			2135.09	1069.09 $[M+2H]^{2+}$
L3			2121.07	708.35 $[M+3H]^{3+}$
L5			2117.96	1060.47 $[M+2H]^{2+}$
L4			3644.65	1216.54 $[M+3H]^{3+}$
M1	Monocyclic		2024.54	1013.10 $[M+2H]^{2+}$
M2			3302.40	1102.33 $[M+3H]^{3+}$
M3			3369.42	1124.68 $[M+3H]^{3+}$
M4			3341.43	1115.37 $[M+3H]^{3+}$
M5			3386.39	1130.33 $[M+3H]^{3+}$

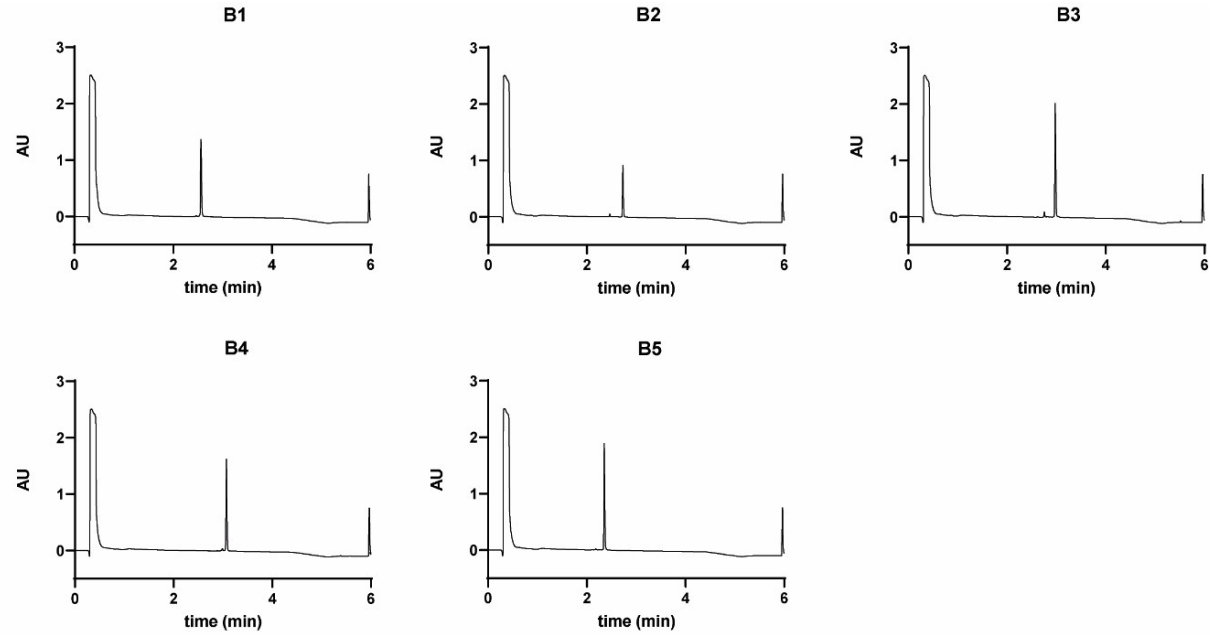
Table S2: Overview of the purified peptides used in the study including their referred names, structure, molecular weight and observed m/z signal. (cont.)

B1	Bicyclic		3269.57	1090.70 $[M+3H]^{3+}$
B2			3418.76	1140.31 $[M+3H]^{3+}$
B3			3436.86	1146.50 $[M+3H]^{3+}$
B4			3348.62	1117.04 $[M+3H]^{3+}$
B5			3291.57	1098.01 $[M+3H]^{3+}$

Analytical UPLC profiles of purified peptides

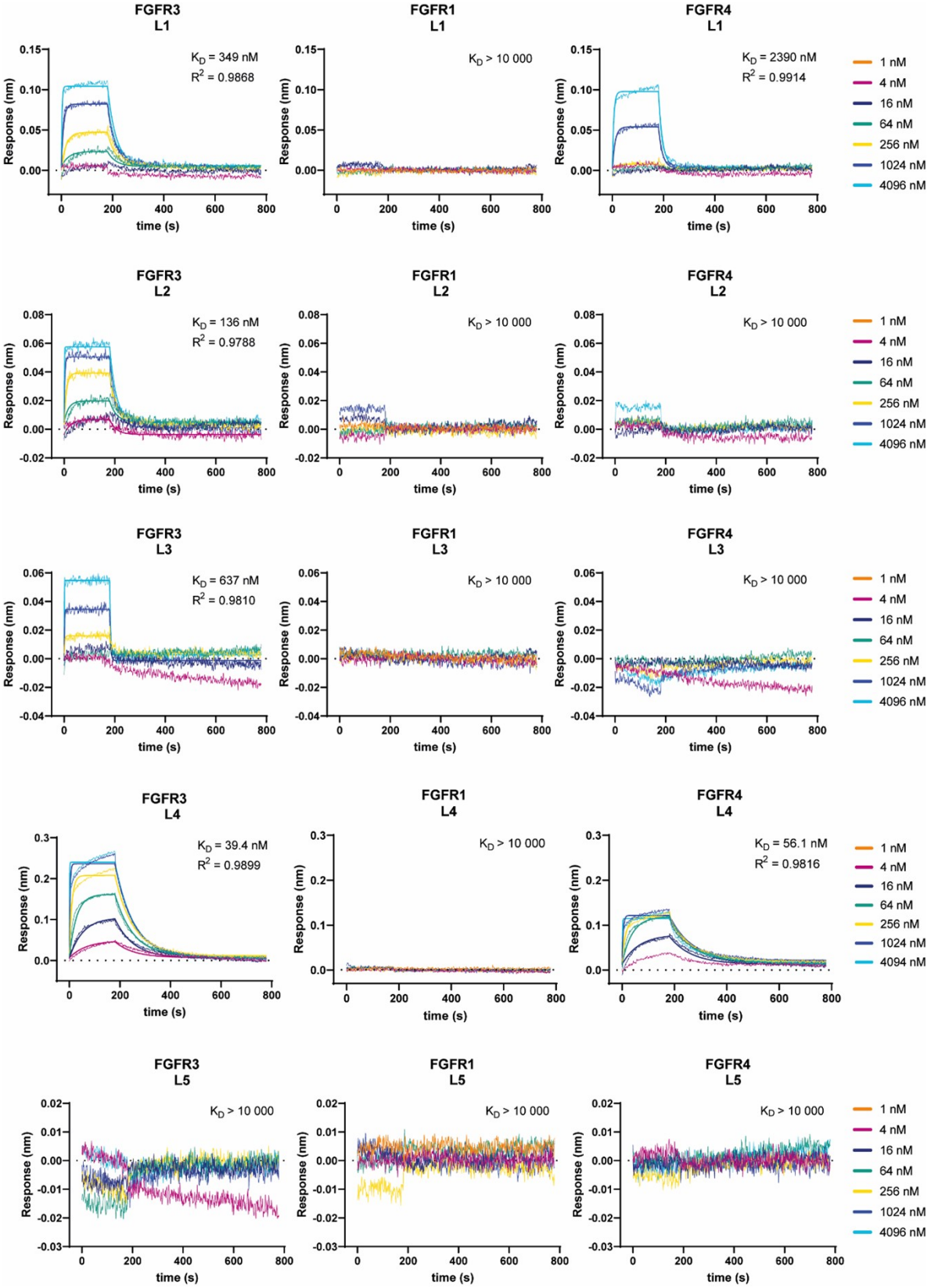


Supporting Figure S3: Analytical UPLC profiles of the purified peptides in this study. Peptide M6 is missing as it was not purified successfully.

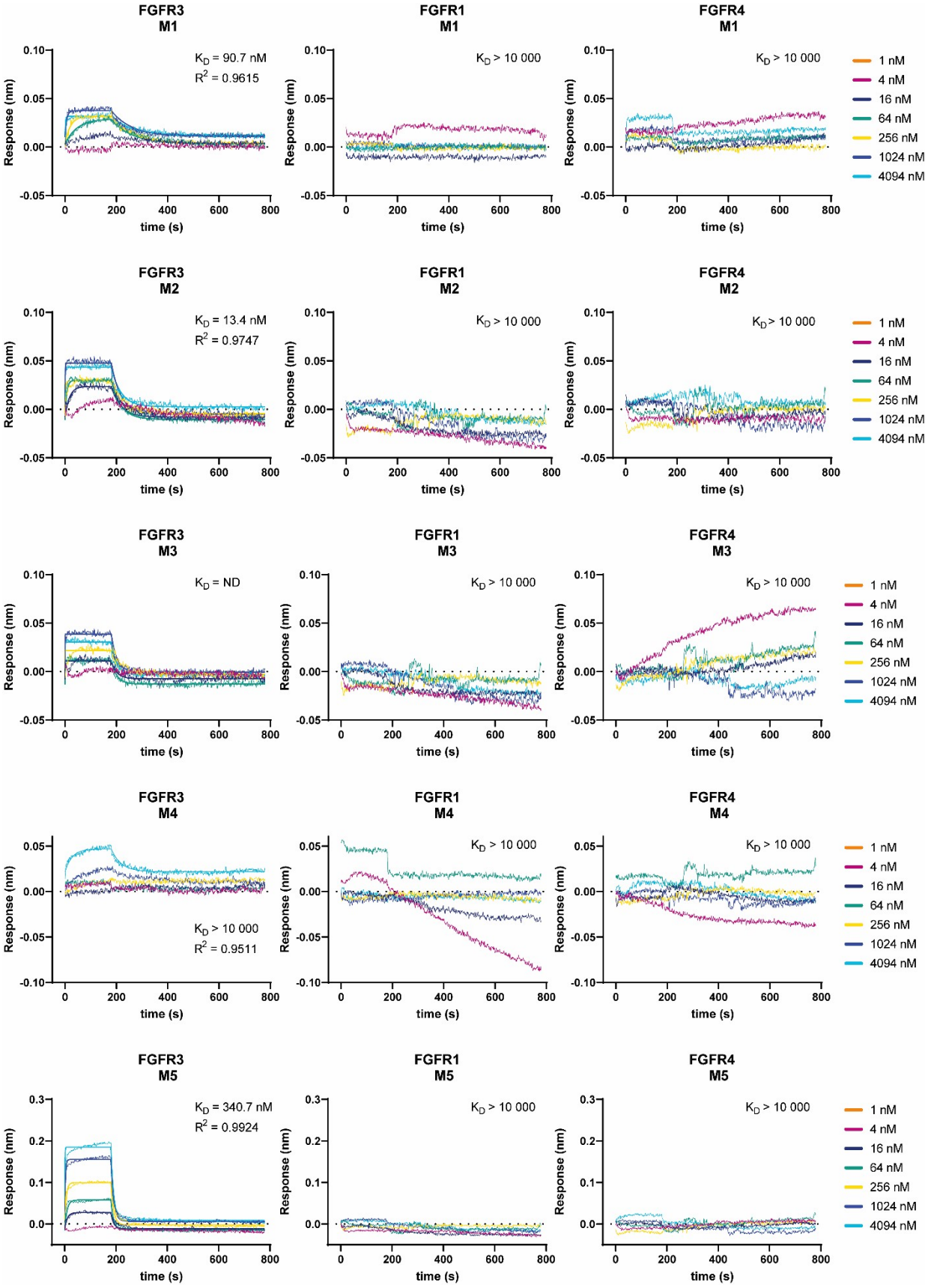


Supporting Figure S3: Analytical UPLC profiles of the purified peptides in this study. Peptide M6 is missing as it was not purified successfully. (cont.)

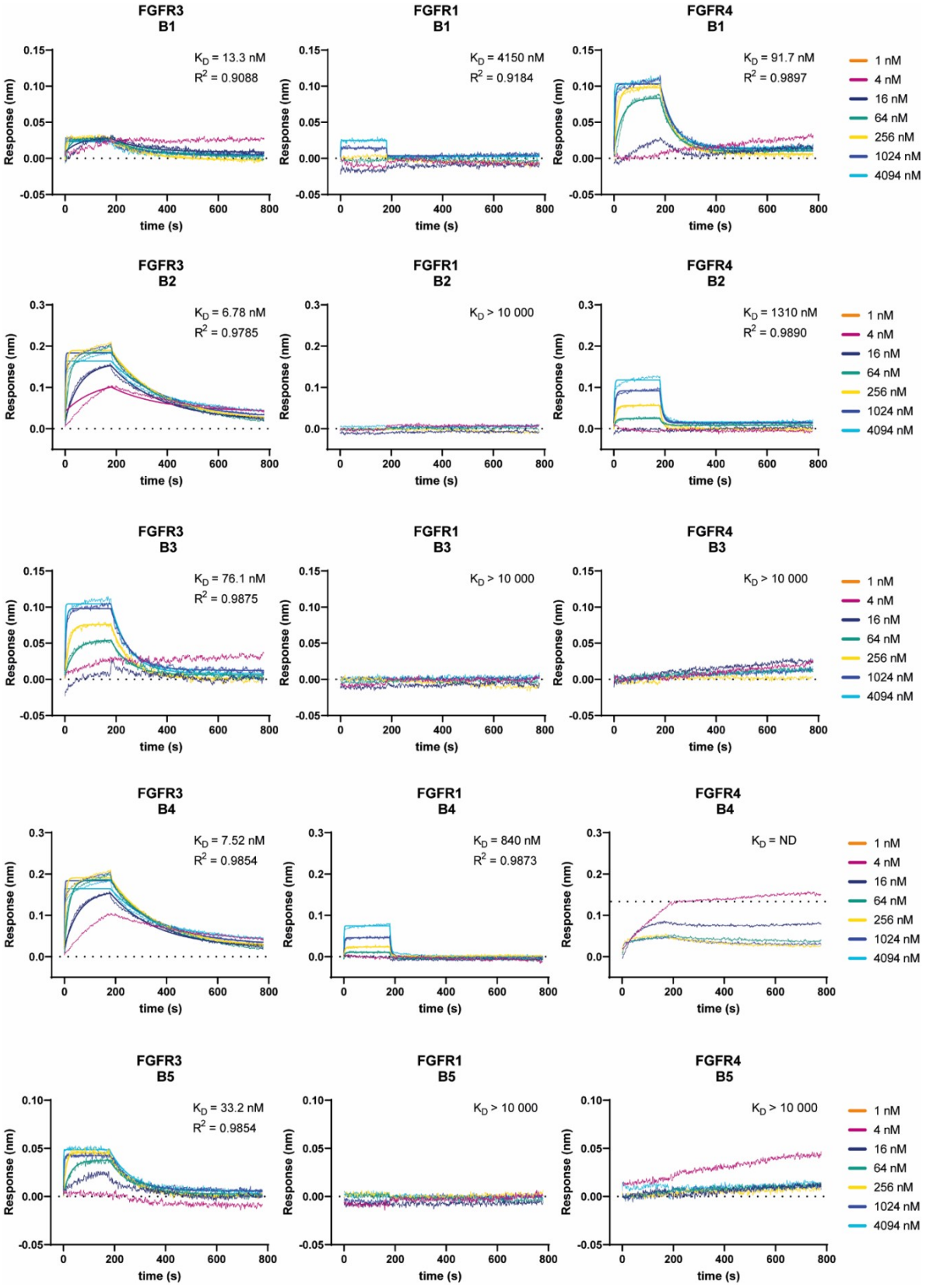
Multiple concentration BLI binding curves



Supporting Figure S4: Fitted BLI dose-response curves for the purified peptides. For each peptide the dose-response curves for the target FGFR3 as well as two additional FGF receptors are shown.



Supporting Figure S4: Fitted BLI dose-response curves for the purified peptides. For each peptide the dose-response curves for the target FGFR3 as well as two additional FGF receptors are shown. (cont.)



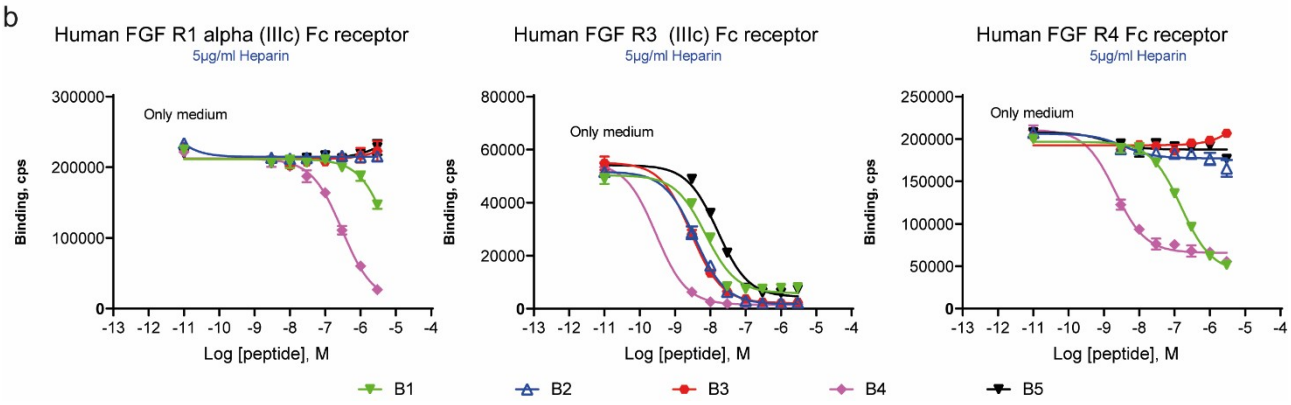
Supporting Figure S4: Fitted BLI dose-response curves for the purified peptides. For each peptide the dose-response curves for the target FGFR3 as well as two additional FGF receptors are shown. (cont.)

AlphaScreen binding assay

a

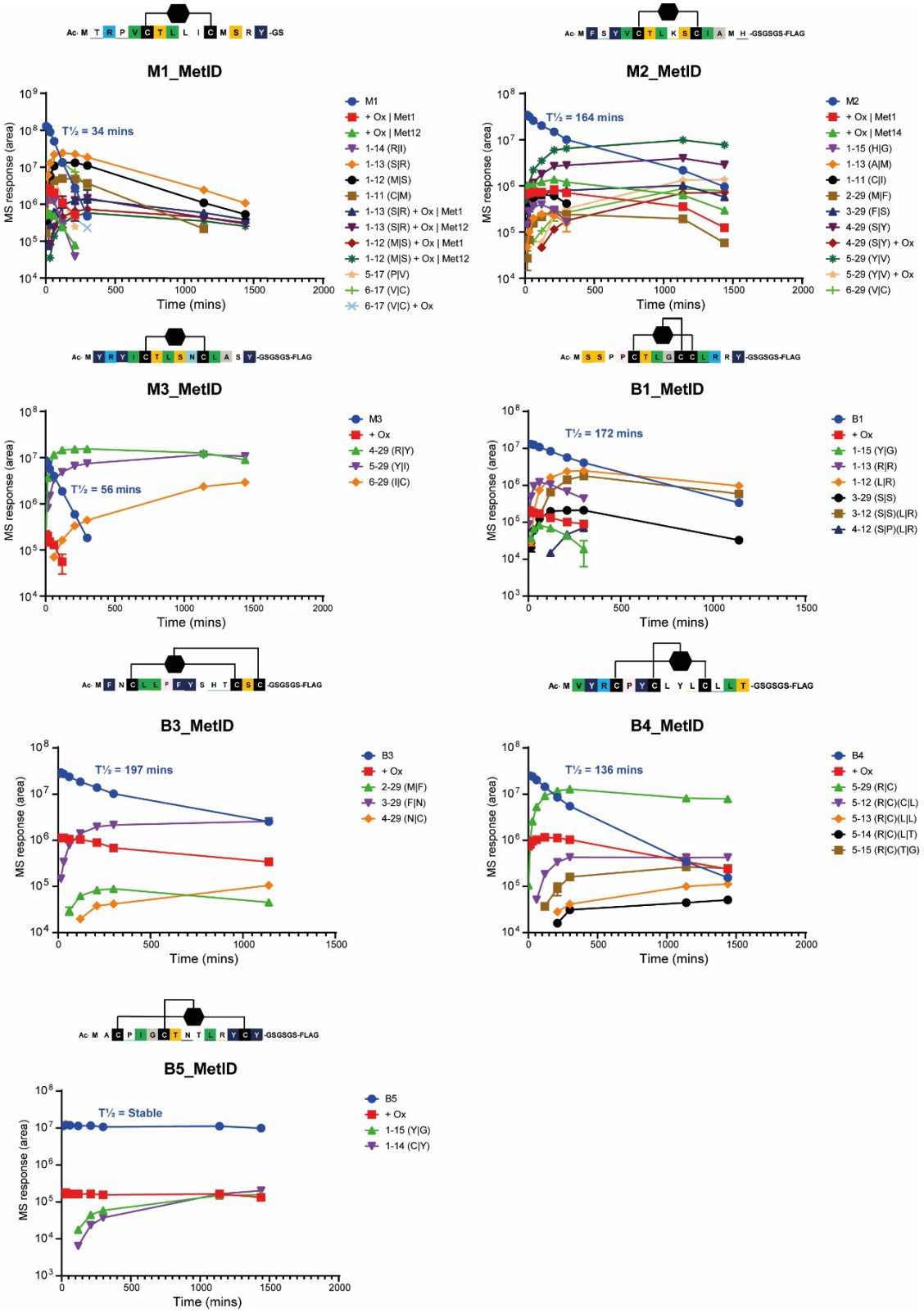
	IC_{50}		
Peptide	FGFR1c	FGFR3c	FGFR4c
B1	>3000 nM	7.3 nM	154 nM
B2	>3000 nM	3.7 nM	>3000 nM
B3	>3000 nM	2.8 nM	>3000 nM
B4	301 nM	<1.0 nM*	2.1 nM
B5	>3000 nM	16 nM	>3000 nM

b



Supporting Figure S5: (a) IC_{50} obtained from the AlphaScreen assay showing displacement of the FGF21/FGF19 from soluble FGFR(1,3,4)/Beta klotho complex by the bicyclic peptides. (b) Assay curves used for calculation of IC_{50}. * Not enough points in the curve for precise IC_{50} determination

Plasma stability assay



Supporting Figure S6: Metabolites identification for selected peptides. The cleavage products are shown as fragments with first – last amino acid in the metabolite (for example 1-14), as well as the cleaved amino acid bond in brackets. The cleavage products are listed in hypothetical order of appearance.

Materials and Methods

List of the primers and nucleic acid used

Oligonucleotide name	Sequence (5'-3')
Primer 1	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG
Primer 4	TTTCCCGCCCCCGACCTATGAACCAGAACCGCTGCC
Primer 6	CTATGAACCAGAACCGCTGCC
Puromycin-oligo	/5Phos/CT CCC CGC CCC CGACC/iSp18/ /iSp18//iSp18//iSp18//iSp18/CC /3Puro/
PK15 sequence	CTTTAAGAAGGAGATATACATATGGCGTGCAGCGATCGTTTTCGCAACTG TCCGGCGGATGAAGCCCTGTGCGGCAGCGGTTCTGGTTCATAG
NNK library for cyclic peptides - 1	CTTTAAGAAGGAGATATACATATGNNKNNKNNKNNKNNKTGCNNKNNK TGCNNKNNKNNKNNKNNKGGCAGCGGTTCTGGTTCATAG
NNK library for cyclic peptides - 2	CTTTAAGAAGGAGATATACATATGNNKNNKNNKNNKNNKTGCNNKNNKNNKTGC NNKNNKNNKNNKGGCAGCGGTTCTGGTTCATAG
NNK library for cyclic peptides - 3	CTTTAAGAAGGAGATATACATATGNNKNNKNNKNNKTGCNNKNNKNNK NNKTGCNNKNNKNNKNNKGGCAGCGGTTCTGGTTCATAG
NNK library for cyclic peptides - 4	CTTTAAGAAGGAGATATACATATGNNKNNKNNKNNKTGCNNKNNKNNK NNKNNKTGCNNKNNKNNKGGCAGCGGTTCTGGTTCATAG
NNK library for cyclic peptides - 5	CTTTAAGAAGGAGATATACATATGNNKNNKNNKTGCNNKNNKNNKNNKNN KNNKTGCNNKNNKNNKGGCAGCGGTTCTGGTTCATAG
NNK library for cyclic peptides - 6	CTTTAAGAAGGAGATATACATATGNNKNNKTGCNNKNNKNNK NNKNNKNNKNNKNNKTGCNNKNNKGGCAGCGGTTCTGGTTCATAG
NNK library for cyclic peptides - 7	CTTTAAGAAGGAGATATACATATGNNKTGCNNKNNKNNKNNKNNKNNKNNK NNKNNKNNKTGCNNKGGCAGCGGTTCTGGTTCATAG
NNK library for cyclic peptides - 8	CTTTAAGAAGGAGATATACATATGTGCNNKNNKNNKNNKNNKNNKNN NKNNKNNKNNKNNKTGCGGCAGCGGTTCTGGTTCATAG
Trim library for linear peptides	CTTTAAGAAGGAGATATACATATG T18 T18 T18 T18 T18 T18 T18 T18 T18 T18 T18 T18 T18 T18 GGCAGCGGTTCTGGTTCATAG (T18 = all AA encoded by a single codon but Met and Cys)

DNA Sequence of FGFRs cloned for expression

FGFR1c-ECD	AGGCCCTCCCCCACACTGCCCGAACAAGCTCAACCTTGGGGAGCTCCCGTGGAGGTGGAAAGCTTTCTGGTGCACCCCGGCGATTTACTG CAGCTGAGATGTCGTCTGAGAGACGACGTCCAGAGCATCAACTGGCTCAGAGACGGCGTGCAGCTGGCCGAATCCAATCGTACCAGAAT CACCGGCGAGGAGGTCGAGGTCCAAGATAGCGTGCCCGCTGATTCCGGTTTATATGCTTGCGTGACCAGCTCCCCTAGCGGCAGCGATAC CACCTACTTCAGCGTGAATGTCTCCGACGCTTTACCCAGCAGCGAGGATGACGACGACGATGACGACTCCTCCTCCGAGGAGAAGGAGAC AGATAACACCAAGCCCAATCGTATGCCCGTGGCCCCCTACTGGACATCCCCCGAGAAGATGGAGAAGAAACTCCATGCCGTGCCCGCTGC TAAGACCGTGAAATTCAAGTGTCCCAGCTCCGGCACCCCCAATCCTACTTTAAGGTGGCTCAAGAATGGCAAGGAGTTCAAGCCCGACCAT CGTATCGGCGGCTATAAGGTGAGGTACGCCACTTGGAGCATCATCATGGACTCCGTCGTGCCCAGCGACAAGGGCAATTACACTTGTATC GTGGAGAACGAGTACGGAAGCATCAACCACACCTACCAGCTGGATGTGGTGGAGAGGAGCCCCCATCGTCCTATTCTGCAAGCTGGACT GCCCGCTAACAAGACCGTCGCTTTAGGCAGCAACGTGGAGTTCATGTGCAAGGTCTACTCCGATCCCCAGCCCCACATTCAGTGGCTGAA GCACATTGAGGTGAATGGCAGCAAGATTGGCCCCGACAATTTACCCTACGTGCAGATTTTAAAGACCGCCGGCGTCAACACCACCGACAA GGAGATGGAGGTGCTGCACCTCAGAAACGTGAGCTTCGAGGACGCCGGCGAATATACTTGTCTGGCCGGCAACAGCATCGGACTGAGCC ACCACAGCGCTTGGCTGACAGTTTTAGAGGCTCTGGAAGAGAGGCCCGCCGTGATGACCTCCCCTCTGTATTTAGAG
FGFR3c-ECD	GAGTCTTTAGGCACCGAACAAAGGGTCGTGGGCAGAGCTGCCGAAGTGCCCGGTCCCGAACCCGGACAGCAAGAACAGCTGGTGTTCGG AAGCGGCGACGCTGTCGAGCTCAGCTGTCCTCCTCCCGGTGGCGGACCTATGGGCCCCACAGTGTGGGTGAAGGACGGCACCGGTTTAG TCCCTAGCGAGAGAGTGCTGGTCGGCCCTCAGAGGCTGCAAGTTCTGAACGCCTCCCACGAGGATTCCGGCGCCTACTCTTGTAGACAGA GACTCACCCAGAGGGTGCTGTGCCACTTCAGCGTGAGGGTGACAGATGCCCCCAGCTCCGGCGATGACGAGGATGGAGAGGATGAGGC CGAGGACACTGGTGTGGATACCGGCGCTCCCTACTGGACAAGGCCCGAGAGGATGGATAAGAAATTATTAGCCGTGCCCGCCGCTAACA CCGTGAGATTTCGTTGTCCCGCTGCCGGAAACCCCACCCCTAGCATTAGCTGGCTGAAGAACGGTCGTGAGTTTAGAGGCGAGCACAGAA TCGGCGGCATCAAGCTGAGGCACCAGCAGTGGTCTTTAGTGATGGAGAGCGTGGTGCCCAGCGACAGAGGAAACTACACTTGTGTCGTG GAGAACAAGTTCGGCAGCATTCGTCAGACCTACACTTTAGACGTTTTAGAGAGATCCCCCCACAGACCCATTTTACAAGCTGGACTGCCCG CTAATCAGACAGCCGTGCTGGGCTCCGATGTCGAGTTCCACTGCAAGGTGTACTCCGACGCCCAGCCCCACATCCAGTGGCTGAAGCACG TGGAGGTGAATGGCAGCAAAGTGGGACCCGATGGCACCCCTTACGTGACCGTGCTGAAGACCGCCGGCGCTAACACCACCGATAAGGAG CTGGAGGTGCTGTCTTTACACAACGTGACCTTCGAGGATGCCGGCGAGTACACTTGTCTCGCCGGCAATTCCATCGGCTTTTCCCACCACA GCGCTTGGCTGGTGGTGCTGCCCGCTGAGGAAGAACTGGTGGAAGCTGACGAGGCCGGAAGCGTGTATGCCGGT
FGFR4c-ECD	CTGGAGGCCAGCGAGGAGGTGGAACTGGAGCCTTGTTTAGCTCCTTCTTTAGAGCAGCAAGAACAAGAACTGACAGTGGCCCTCGGACA GCCCGTGAGACTGTGCTGTGGTCGTGCTGAGAGGGGAGGCCACTGGTACAAGGAGGGCTCCAGACTGGCCCCCGCTGGAAGAGTGAGA GGCTGGAGAGGAAGGCTGGAGATCGCCAGCTTCCTCCCCGAAGACGCCGGTCGTTATTTATGTCTGGCTCGTGGCTCCATGATCGTGCTG CAGAATTTAACACTGATCACCGGCGACTCTTTAACCAGCAGCAACGACGATGAAGATCCTAAGTCCCATAGAGACCCCAGCAATCGTCACA GCTATCCTCAGCAAGCTCCTTACTGGACCCACCCCCAGAGGATGGAGAAGAAGCTGCATGCCGTGCCCGCCGGCAACACAGTGAAGTTTC GTTGTCCCGCTGCCGGAAACCCCACCCCCACCATTCGTTGGCTGAAGGACGGCCAAGCTTTCCACGGCGAGAATCGTATCGGCGGCATTC GTCTGAGGCACCAGCACTGGTCTTTAGTGATGGAGAGCGTCGTGCCCTCCGATCGTGGCACCTATACATGTTTAGTGGAGAACGCCGTGG GAAGCATTCGTTACAATTATTTACTCGACGTGCTGGAAAGGTCCCCCCACAGACCTATTTTACAAGCTGGACTGCCCGCTAACACAACCGC CGTGGTGGGCTCCGACGTGGAGCTGCTGTGTAAGGTGTACAGCGATGCCCAGCCTCACATCCAGTGGCTGAAGCACATCGTGATCAACG GCTCCAGCTTTGGCGCCGACGGCTTCCCCTATGTCCAAGTTCTGAAAACCGCCGACATTAACAGCAGCGAGGTGGAGGTGCTGTATCTGA GAAACGTGTCCGCCGAGGATGCTGGCGAGTACACTTGTCTCGCCGGAAACAGCATCGGCCTCTCCTACCAGTCCGCTTGGCTGACCGTTTT ACCCGAAGAAGATCCTACATGGACAGCCGCCGCTCCCGAGGCTAGATACACCGAT

Protein expression and purification of hFGFRs

Materials: EndoFree Plasmid Maxi Kit was purchased from QIAGEN. Optimum Growth™ Flasks were purchased from THOMSO. FreeStyle™ 293 Expression Medium, Geneticin™ Selective Antibiotic, Pluronic™ F-68 Non-ionic Surfactant (100 X), 293fectin™ Transfection Reagent, Opti-MEM™ Reduced Serum Medium were purchased from Invitrogen. Stericup Quick Release-HV Sterile Vacuum Filtration System was purchased from Merck. NuPAGE™ 4 to 12%, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels was purchased from Invitrogen. Superdex® 75 was purchased from GE Healthcare. SEC-HPLC was performed on TSKgel G2000 SWxl column coupled with Agilent HPLC. LC-MS was performed on Agilent PLRP-S column coupled with Waters UPLC Xevo G2 Q-TOF. Kinetic Turbidimetric LAL Test kit was purchased from Charles River Laboratories.

Preparation of plasmids: Three different DNA constructs (FGFR1_ECD, FGFR3_ECD, FGFR4_ECD) with C-terminal Avi and HPC4 sequences were generated and cloned into the pJSV002 expression vector using seamless assembly cloning technology (Taihe Biotechnology Co.,LTD). The expression vectors were transformed separately into Escherichia coli (E. coli) DH5a, plated onto a LB plate containing 100 ug/mL ampicillin, and incubated overnight at 37 °C. Plasmid DNA was obtained from the culture medium of transformed cells and sequenced to confirm the proper reading frame of constructs. For transfection in mammalian cells, endotoxin-free plasmids were purified from large-volume E. coli cultures using plasmid preparation kits (EndoFree Plasmid Maxi Kit).

Expression of recombinant FGFRs: Plasmids of FGFRs were transiently transfected in suspension-growing mammalian cells using a FreeStyle 293 Expression Medium (Gibco). Briefly, HEK 293-6E cells were maintained as a suspension culture in FreeStyle 293 expression medium with 1% F-68 and 25 ug/mL Geneticin (Gibco). The culture was produced in a shake flask with a vented cap at 37 °C, in a humidified atmosphere containing 5% CO2 with 50 mm orbital shaking (135 rpm/min). For transient transfection of recombinant protein, the cells were split on the day before transfection at density of 0.7~1 × 10^6 cells/ml and allowed to grow overnight. Cells were diluted to a final volume of 560 mL with the density of 1.07×10^6 viable cells/mL. Transfection was done by: 1) gently mixing 600 ug expression plasmid(s) and Opti-MEM in a total volume of 20 mL; 2) adding diluted DNA to the diluted 293fectin (600 uL 293fectin in 20 mL Opti-MEM, incubated for 5 min at room temperature) followed by mixing and incubation at room temperature for 20 minutes; 3) adding the DNA/293fectin mixture drop wise to 560 mL cells in a 1.6-L Optimum Growth™ flask and incubating on an orbital shaker in a 37°C incubator with a humidified atmosphere of 5% CO_2. Cell culture medium was harvested 5 days later by centrifugation at 6000rpm for 30 minutes and filtered with a 0.45 μm membrane, and protein expression level was determined with SDS-PAGE.

Purification of recombinant FGFRs: All the purification steps were performed at 4 °C. The cell culture was loaded onto an in house prepared anti-HPC4 column and washed with washing buffer (20 mM Tris-HCl, 100 mM NaCl, 1mM $CaCl_2$, pH 7.5), and then eluted with elution buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5). The eluted fractions were biotinylated using in house produced BirA biotin ligase. Finally, the biotinylated protein was purified using gel filtration with Superdex® 75 (1x PBS, pH 7.4). The eluted fraction was concentrated using Amicon Ultra concentrator (Millipore) with a 10 kDa cut-off filter. Protein purity and integrity was analyzed by SDS-PAGE and SEC-HPLC (TSKgel

G2000 SWxl column on Agilent HPLC). The protein mass was confirmed with LC-MS (Agilent PLRP-S column on Waters UPLC Xevo G2 Q-TOF), and the endotoxin was determined using Kinetic Turbidimetric LAL Test kit.

Testing the reactivity of *in vitro* translated peptides with tris-(bromomethyl)benzene (TBMB)

For each concentration of TBMB (1,3,5-Tris(bromomethyl)benzene 657336 from Sigma-aldrich) tested, a ssDNA encoding for the peptide PK15 was amplified in 7 cycles of PCR with primer 1 and 4 using Roche KAPA HiFi HotStart Library Amp Kit. Around 1 µg of PCR product was then subjected to mRNA transcription using T7 RiboMAX™ Express Large Scale RNA Production System. Around 60 pmoles of mRNA was added to 25 µL of a PURE *in vitro* translation system (NEB PURExpress®) containing 4% v/v RNASIN plus RNase inhibitor (Promega). The mix was incubated 2h at 37°C before denaturation of the ribosomes and other translation proteins 15 min at 60°C. After spinning down the precipitated material, the clear mixture containing the translated peptide, nucleic acids etc. is used for a reverse transcription 45 min at 50°C using SuperScript™ III First-Strand Synthesis System and primer 6, in a total volume of 50 µL. Once the samples have gone through the major molecular steps of the mRNA display protocol, the mixture is topped up with 40 µL of 80 mM NH_4HCO_3 pH 8and 10 µL of TBMB dissolved in DMSO is added to reach a concentration of either 50, 100 or 300 µM (10% v/v DMSO in the final volume). The reaction is incubated 1 hour at 30°C. 1 µL is saved for PCR with primer 1 and 4 with subsequent agarose gel analysis and 10 µL were measured on LC-MS Waters Xevo G2-XS Q-TOF.

mRNA display

Preparation of the mRNA display library

For the cyclic libraries, we used ssDNA encoding for NNK random sequences as well as two fixed cysteines in various positions (see list of oligonucleotides). The linear library was designed to encode for 14 random amino acids generated with 18 trinucleotide phosphoramidites (all amino acids but Cys and Met, see list of oligonucleotides).

In the first step of library preparation, ssDNA libraries were amplified in 7 cycles of PCR with primer 1 and 4 using Roche KAPA HiFi HotStart Library Amp Kit. The PCR also appended flanking regions coding for sequences enabling transcription, translation and puromycin ligation. Subsequently, the different PCR-amplified cyclic libraries were pooled at equimolar concentration. Amplicons were then subjected to *in vitro* transcription using T7 RiboMAX™ Express Large Scale RNA Production System. 250 pmoles of the mRNA of each of the libraries were ligated with 300 pmoles of puromycin oligonucleotide (see list of oligonucleotides) at RT for 1-2h using T4 RNA ligase (Promega). As a result, we typically observed 70 -90 % ligation efficiency on gel.

After mRNA ligation to puromycin, the libraries were translated using NEB PURExpress® Δ RF123 Kit at a scale of 25 µL for the first round and 10 µL for all subsequent rounds. As the kit consists of a purified and reconstituted *E.coli* translation system, all peptides sequences started with a N-terminal formyl-Methionine. All translation steps were done in presence of 4% v/v RNASIN plus RNase inhibitor (Promega). *In vitro* translation reactions were incubated 45 min at 37°C before denaturation of the ribosomes and other translation proteins 15 min at 60°C. The libraries were then

reverse transcribed 45 min at 50°C using SuperScript™ III First-Strand Synthesis System and primer 6. Right after reverse transcription, the cyclic libraries were reacted with 40, 200, 1000 µM cyclization reagents (m-dibromoxylene 125911 from Sigma-aldrich only used at 200 uM or 1,3,5-Tris(bromomethyl)benzene 657336 from Sigma-aldrich) at 30°C in 80 mM NH_4HCO_3 pH 8 / 10% DMSO. Finally, the libraries were desalted using spintrap G25 columns pre-equilibrated with selection buffer (PBS 1x, 0.05% Tween-20 pH 7.4).

Selection against biotinylated FGFR3

The above-prepared libraries were then subjected to 3 steps of negative selection against 20 µL streptavidin or neutravidin-coated magnetic beads and in absence of protein (either Dynabeads™ M-280 Streptavidin or SpeedBeads™ Magnetic Neutravidin Coated particles). This was done in order to avoid background bead-related peptide binders. After completion of the negative selections, 1 µL of the library was sparred in order to quantify the amount of cDNA in the input library by qPCR. The rest of the libraries was then used for positive selection against about 20 pmoles of FGFR3 protein immobilized on 25 µL of either streptavidin or neutravidin coated beads. Libraries and the protein were incubated 45 min and then washed 3 times with 100 µL of selection buffer (PBS 1x, 0.05% Tween-20 pH 7.4). The volume of the washes were increased to 200 & 300 µL in Round 2 & 3, respectively. The washed beads were then resuspended in 25 µL of qPCR mix (minus polymerase and SYBR green) and incubated 5 min at 95°C in order to elute the cDNA from the beads. The first set of beads used for negative selection was subjected to a similar treatment. The amount of cDNA in the input and in the output of negative and positive selections was then quantified by qPCR using Roche KAPA HiFi HotStart Library Amp Kit and primer 1 & 4. The PCR-amplified cDNA of the positive selection was used for preparing the libraries in the next round and for next-generation sequencing.

Next-generation sequencing and bioinformatic analysis

The output of each selection round was amplified in two PCR steps to append barcodes and constant regions required for sequencing with Illumina MiSeq. The PCR amplification was performed using Roche KAPA HiFi HotStart Library Amp Kit following the manufacturer's protocol. PCR products were gel purified, quantified using Qubit Broad range kit and pooled into 20 pM libraries containing 10 % PhiX DNA in order to achieve good read quality. Finally, the NGS library was sequenced using MiSeq® Reagent Kit v3 (150 cycles). See already published procedures for further details (Bhushan, B. et al, Chem Sci 2022).

Synthesis of peptide hits for primary screens

Selected peptides were synthesized in a 96-well plate format using Intavis Multipep RSI instruments. The synthesis scale was 2.5 µmole using 0.3 mM resin pre-loaded with the sequence: GSGSGSDYKDDDDK-azido-Lys-$CONH_2$, which corresponds to the spacer sequence present in our mRNA display libraries as well as a Flag-tag added for solubility and potential detection in bioassays. The amino acids were dissolved in 0.1 M oxyma DMF and coupled using standard Fmoc Chemistry with 3 M DIC/collidin as coupling reagents (double couplings 60 + 120 min). After each addition, we added a capping step with 0.3 M AcOH in DMF/ 0.1 M oxyma. Fmoc deprotection was done using 20% piperidine in DMF/ 0.1 M oxyma. All peptides were synthesized with an Nter acetylated Met, to mimic the formyl-Methionine present during the mRNA display selections.

After completion of the synthesis, peptides were cleaved from the resin using the following mixture: 94% TFA/ 2% Water / 2% TIS/ 2% DTT. About 1.2 mL of cleavage mixture was added stepwise to the wells over the course of two hours. After incubation, the peptides were precipitated with diethylether and the remaining cleavage solution/diethylether was separated from the precipitated peptide using gravity filtration. The peptides were then further washed 3 times with 500 µL of diethylether and dried using vacuum filtration. Finally, the dried peptides were re-dissolved in 1 mL DMSO and transferred to a deep-well master plate. Concentration in the master plate was usually around 1 – 2 mM.

For cyclization, 50 µL (about 50 nmoles) of the master plate were transferred to a folding plate containing 750 µL of 60 mM NH_4HCO_3-buffer pH 8, 100 µL of DMSO and 5 Eq of TCEP. The plate was incubated 15 min at RT. In the meantime, stock solutions of chemical linkers were prepared at 6 mM concentration in DMSO. Finally, 50 µL of linker (6 eq) were added to the folding plate and reacted with the peptide for an hour. The reaction was then quenched by addition of 10 eq of L-Cys to the peptides.

The identity and purity of peptides was determined by a Waters Acquity system, equipped with a TUV detector (F08UPT321M) and a QDA detector (KDA3351) and an Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm X 50 mm). The different runs included a linear gradient off 10 % to 90 % acetonitrile 0.1 % TFA and 3 min gradient run-time. Peptides were quantified using UHPLC-CAD on a Thermo Fisher Vanquish system equipped with an ACQUITY UPLC CSH C18 Column, (130Å, 1.7 µm, 2.1 mm X 50 mm) and a Charged Aerosol Detector H. (Solvent A: water with 0.1% TFA, solvent B: acetonitrile with 0.1% TFA, gradient 0-80% B 0.0 – 4.0 min flow rate 0.45 mL/min).

In most cases, this protocol yielded 30 – 60 µM peptide solutions with a purity ranging from 30 to 70%.

High-throughput screening of peptide arrays

The binding of the peptides hits was assayed using Biolayer interferometry. The peptides in the folding plate were diluted 50x in assay buffer (PBS/ 0.05 % Tween-20/ 0.1 % BSA) to reach a final concentration approaching 1 µM. A running buffer was also prepared for dissociation steps by diluting 50x the peptide folding buffer 60 mM NH_4HCO_3-buffer pH 8/ 20% DMSO in assay buffer. Streptavidin biosensors (Fortebio) were used for coating biotinylated hFGFR3c .

Synthesis and purification of the selected peptides

All chemicals were of analytical grade or higher. Triisopropylsilane (TIPS), N,N´- diisopropylcarbodiimide (DIC) were from Sigma–Aldrich, Chemie GmBH (Steinheim, Germany). Acetonitrile (ACN) (LiChrosolve), trifluoroacetic acid S3 (TFA), and diethyl ether were purchased from Merck KGaA (Darmstadt, Germany). Water came from a MilliQ equipment (Advantage A10) from Millipore (Molsheim, France). Standard Fmocamino acids, resins and coupling reagents, Oxyma Pure were from Novabiochem (Darmstadt, Germany) or Protein Technologies (Tucson, USA). N-Methyl pyrrolidone (NMP) dimethylformamide (DMF) and piperidine were from Biosolve (Dieuze, France). PS resin, and Fmoc-Rink amide PS resin, (synthesis of peptide 20) were all purchased from Merck Millipore (Novabiochem).

Peptides were either synthesised by standard SPPS using PAL-AM or pre-loaded wang resins (loading ~0.3M) on Gyros Protein Technologies Prelude or Symphony X machines (125 µmol scale) or purchased from Apigenex, Prague, Czech

Republic. The resin was washed with dichloromethane (DCM), and peptides were cleaved from the resin by incubating with TFA/TIPS/DTT/water (94:2:2:2) for 3h at room temperature, followed by precipitation with diethyl ether. The precipitated peptides were washed three times with diethyl ether, and diluted to 50 µM in folding buffer (100 mM HEPES pH 7.4, 20% DMSO) to induce disulfide-mediated macrocyclisation. Purification was performed on Gilson LC systems, equipped with a 322 pump module, 155 UV/vis detector and GX-271 automated sample collector. A typical gradient of 20% - 60% solvent B over 30 min (flow rate of 25 mL/min, Axia Gemini 5uM NX-C18 110 Å columns, 250x30 mm) was employed (Solvent A: 0.1% TFA in water, Solvent B: 0.1 % TFA in Acetonitrile). The final peptides were isolated by lyophilisation.

Precise K_D measurements for FGFR1c, FGFR3c and FGFR4c using BLI

For accurate KD determination, dilution series of each peptide were performed, and association and dissociation kinetics curves were obtained for each concentration in order to fit a multi-point Kd. Data collection and analysis was performed using the Octet Instrument Control and Data Analysis software packages (ForteBio, Ver 9.0.0.10) and Prism (GraphPad Software, Ver. 7.05).

Alphascreen binding assay

We used the method described in Agrawal A, Parlee S, Perez-Tilve D, et al. Molecular elements in FGF19 and FGF21 defining KLB/FGFR activity and specificity. Mol Metab. 2018;13:45-55.

Measurements were done in triplicates with concentrations ranging from 0.01, 3, 10, 30, 100, 300 ,1000 and 3000 nM of peptide.

In vitro plasma stability and quantification by LC-MS

In vitro plasma stability of the peptides was assessed by incubation of 1 µM peptide in 80% pooled human Li-heparin plasma (mixed male and female from BioIVT, Westbury, NY, US) and 20% PBS buffer at pH 7.4 and 37°C with shaking. Samples were taken at selected timepoints: 2, 15, 30 mins, 1, 2, 3.5, 5, 19 and 24 hours by taking one volume of the samples and subjected to protein precipitation using three volumes of ice-cold ethanol, followed by centrifugation and dilution of one volume of the supernatant with one volume of water containing 1% formic acid (FA) before LC-MS analysis.

The LC-MS analysis were performed using a TurboFlow HPLC system from Thermo Fisher Scientific (Bremen, Germany) coupled to a Q Exactive HF Orbitrap Mass Spectrometer. The LC mobile phases consisted of A: MQ water with 5% organic solvent (50% methanol / 50% acetonitrile) and 1% FA and B: MQ water with 95% organic solvent (50% methanol / 50% acetonitrile) and 1% FA. The prepared samples were directly loaded onto the analytical column in Lx mode (Aeris Peptide XB-C18, 3.6µm, 2.1 x 50 mm column (controlled at 60°C) from Phenomenex (Torrence, CA)) using a flow rate of 400µl/min and eluted using a linear gradient of 10-90 %B over 5 mins. The Orbitrap mass spectrometer was operating in positive ionization mode with a spray voltage of 4 kV, with a resolution of 60K using a m/z 300-1500 full scan mode.

The LC-MS data was processed and quantified using the Quan Browser in the Xcalibur software from Thermo Fisher Scientific (Bremen, Germany) using the most abundant isotopes of the most abundant charge state of the peptides of interest. Plotting of data and calculation of T½ was done in Prism (version 8.02, GraphPad Software, Inc.)

Cell based Antagonism assays

Cell lines:

BaF3-(FGFR1c or FGFR3c or FGFR4)/KLB The BaF3 cell line is an immortalized murine bone marrow-derived pro-B-cell cell line, that grows in suspension. BaF3 cells have no endogenous FGF receptor expression. The BaF3 cells used in this protocol have been stably transfected with the human beta klotho receptor and the human FGFR1C/FGFR3c/FGFR4.

Culture medium:

RPMI1640 (Invitrogen, cat# 72400021)

+ 50 ml of heat inactivated FBS (GIBCO, cat# 16140-071)

+ 5 ml Penicillin/Streptomycin (GIBCO, cat# 15140)

+ 0.5 ng/ml IL-3 (250 µl of 1 µg/ml, cat# 14144 from Sigma)

+ 1 µg/ml puromycin (50 µl of 10 µg/µl cat# 8833 from Sigma)

+ 1 mg/ml G418 (10 ml of cat# 10131-027 from Gibco)

Assay medium:

RPMI 1640 + GlutaMAX-I + hepes

(cat#72400021 from Invitrogen)

+0,02% Tween 20 (final conc. is 0.01%). Freshly prepared from a 2% solution in water

+10 µg/ml Heparin, i.e. for 50 ml of assay medium add 10 µl from a stock of 50 mg/ml (final conc. is 5 µg/ml)

Starvation medium:

RPMI 1640 + GlutaMAX-I + hepes (cat# 72400021 from Invitrogen) without any additives (used for seeding of the cells)

Assay kit:

AlphaScreen, SureFire pERK 1/2 Assay Kits, PerkinElmer, cat# TGRES10K

contains:

Activation buffer

dilution buffer

Reaction buffer

5x Lysisbuffer

Reaction Mix (7.5 µl per well):

60 parts Reaction buffer + 10 parts Activation Buffer + 1 part Donor beads + 1 part Acceptor beads.

Beads:

Protein A general IgG detection kit, PerkinElmer, cat# 6760617M

AlphaScreen Protein A Acceptor Beads

AlphaScreen Streptavidin Donor Beads

Procedures:

pERK SureFire-assay: Alpha SureFire® p-ERK 1/2 (Thr202/Tyr204) sandwich immunoassays for quantitative detection of phospho-ERK 1/2 (phosphorylated on Thr202/Tyr204) in cellular lysates. Cells are seeded in 96-well (Nunclon Surface, cat# 167008, Nunc): density 3.0x105 cells/well/50 µl (i.e. 6.0x106 cells/ml) in starvation medium. On the nex day the SureFire/pERK assay is performed. Agonists: For FGR1C and FGFR3C: FGF21, for FGFR4: FGF19. Antagonists: dilution series, 1000 nM, 100 nM, 10 nM, 0 nM. Cells are pre incubated with antagonist 30 min, then 10 nM FGF19/21 is added. The assay is stopped by quickly adding 25 µl of undiluted Lysis Buffer per well (on top of compound, cells etc.) 4 µl of lysate is transferred to a 384 well Proxiplate and Acceptor and Donor Beads are added. To this 7.5 µl Reaction Mix per well are transferred, the plate is sealed and gently mixed on a plate shaker and incubated for 4 hours at room temperature. After reading of the plate, data is analysed using GraphPad Prism.