PROTEIN THERAPEUTICS

Lasso-grafted designer cytokines

Cytokine receptor agonists can be designed with longer half-lives in circulation and with enhanced penetration of the blood–brain barrier by genetically grafting macrocyclic peptides into the structural loops of fragment crystallizable regions.

Aphrodite Kapurniotu and Jürgen Bernhagen

Cytokines and growth factors — which are small proteins that orchestrate cell communication and signalling — play central roles in immunity and in inflammatory conditions, in cancer and neurological diseases. Many cytokine-blocking therapeutics (such as soluble conjugates of receptors and fragment crystallizable (Fc) regions of antibodies targeting the tumour necrosis factor, and antibodies against interleukins (ILs) such as IL-6 or IL-1) have been developed, and some are clinically available, most notably to treat rheumatoid arthritis and cancer[1]. Yet, in addition to being targets for inhibition, cytokines can also act as therapeutics. In fact, several cytokines are clinically used as protein therapeutics against cancer, autoimmune diseases and viral infections[2]. However, as with many multifunctional proteins, therapeutic cytokines typically also cause adverse effects, and may have limited stability and poor pharmacokinetics. These can be alleviated via poly(ethylene glycol) conjugation, lipidation or fusion with immunoglobulin moieties, but these methods require extensive optimization and cannot be applied to all proteins[3,4].

Alternatively, cytokines can be designed as protein agonists with improved properties such as multiple functionalities and reduced immunogenicity[5]. In particular, macrocyclic peptides have emerged as an attractive alternative to protein therapeutics because they combine advantages of both small molecules and antibodies, such as small size, high affinity and high specificity, and can be efficiently generated by combined high-throughput screening and rational-design strategies. However, predictably designing protein or macrocyclic-peptide therapeutics with desired properties is difficult. Reporting in *Nature Biomedical Engineering*, Katsuya Sakai, Junichi Takagi, Kunio Matsumoto and colleagues now show that a surrogate agonist of Met (a human receptor tyrosine kinase that is dimerized and activated by the hepatocyte growth factor; HGF) can be generated by grafting macrocyclic peptide pharmacophores into suitable loops of a fragment crystallizable (Fc) region (or of a complete antibody) to yield potent HGF-mimicking cytokine receptor agonists with favourable pharmacokinetics and penetration of the blood–brain barrier (BBB).

Sakai and authors' approach capitalizes on prior work showing that macrocyclic peptides with target protein-binding affinity can be selected from a large pool of random sequences by a combination of mRNA-display and genetic code reprogramming (a method known as 'random non-standard peptides integrated discovery' or RaPID), and then inserted into surface-exposed loop domains of proteins serving as a scaffold and endowing the macrocycle with improved properties[7]. The authors termed this approach 'lasso grafting' because a lasso-like moiety (the macrocyclic peptide) is inserted into a loop of the harbouring protein; it involves the replacement of the thioether ring-closure bond of the selected macrocycle by the loop domain of the host protein.

By controlling cell growth, survival, and migration, the HGF–Met ligand–receptor axis plays central roles in development, organ homeostasis, and tissue repair. Recombinant HGF is therefore an attractive therapeutic candidate, but its translational potential is limited by its low bioavailability. Sakai and co-authors selected surrogate HGF-mimicking macrocylic peptide agonists by RaPID, and tested their incorporation by lasso grafting into various loops of human Fc. They identified two 15-residue peptide pharmacophores (aMD4 and aMD5) that bind to the ectodomain of Met and that dimerize the receptor[6]. To optimize the grafting site on the Fc protein, lasso grafting was tested in all eight possible Fc loops. Essentially, all Fc(MD) conjugates expressed well in eukaryotic cells, and two loops turned out to be compatible host scaffolds that preserved the agonistic properties of the parental macrocyclic peptide (Fig. 1). One of them, the conjugate Fc(aMD4)B3, was studied in detail via a variety of receptor-signalling assays and functional profiling. As Fc forms a homodimer, the lasso graft harbours two identical macrocyclic guest peptides in good proximity to each other, conveying a divalent nature to the HGF-like agonist predicted to enable the surrogate cytokine to initiate Met dimerization. In fact, the authors found that the potency of Fc(aMD4)B3 as a Met-receptor activator was similar to that of the non-grafted aMD4 dimer, as indicated by a comparably low nanomolar half-maximal effective concentration (EC_{50}) derived from agonist-induced Met-phosphorylation responses in a Met-expressing cell line. The agonistic potency of Fc(aMD4)B3 was 10-to-100-fold lower than that of natural HGF, but analysis of the downstream kinase-signalling pathways in a cell line and in primary hepatocytes showed that the lasso–Fc conjugate elicited cellular responses that were qualitatively comparable to those triggered by the natural ligand. Importantly, a survey of phosphorylation patterns of numerous receptors showed that the surrogate agonist was selective for its target receptor Met. Moreover, transcriptomic analysis of human hepatocyte spheroid cultures provided evidence that Fc(aMD4)B3 mirrored the alteration of gene-expression patterns by the natural cytokine to equally control key pathways of wound repair and liver functions[6]. Most experiments in the authors' study were performed with Fc(aMD4)B3, yet its potency was increased by the introduction of cysteine residues to the peptide pharmacophore (which led to a disulfide-mediated closed macrocyclic conformation; Fc(aMD4ds)B3).

Because the lasso-grafted peptides were generated de novo and do not share sequence homology with the Met-binding region of natural HGF, it is important to characterize the receptor binding epitope of the surrogate agonist. By characterizing the Met-receptor ectodomain of a chimera (Met_{ECD}) containing moieties of human and mouse Met_{ECD}, by performing ligand–receptor crosslinking, and by carrying out single-molecule examination of the crosslinked Met_{ECD}–Fc(aMD4)B3 complex by high speed-atomic force microscopy, Sakai and co-authors discovered that the Met-binding site of Fc(aMD4)B3 is distinct from that of the natural ligand, and show that Fc(aMD4)B3 induced Met-dimer formation in a

1:2 complex stoichiometry. This confirmed that although the surrogate agonist binds to a different site, it activates Met to the same extent as HGF.

Immunoglobulin half-life in blood is enhanced by the neonatal Fc receptor FcRn. Encouraged by results from surface plasmon resonance spectroscopy confirming that the affinity of Fc(aMD4)B3 to FcRn was preserved in the conjugate, Sakai and co-authors examined the stability and activity of the designer cytokine in a mouse model. In contrast to recombinant HGF, whose concentration decreased to less than 0.01 nM within 1 hour, the half-life of Fc(aMD4)B3 was approximately 50 hours and comparable to control Fc, with bioactive concentrations larger than 1 nM up to 7–8 days after a single intravenous injection. Importantly, the administered Fc(aMD4)B3 displayed potent hepatoproliferative bioactivity in a chimeric mouse model transplanted with human hepatocytes, and induced a transcriptomic signature featuring mitotic, cell-cycle and metabolic processes. Hence, the surrogate cytokine combines the potent agonistic activity of the guest macrocycle and the favourable bioavailability characteristics of the host scaffold[6].

Because HGF-induced Met activation is known to exert neuroprotective effects in preclinical models of cerebral ischaemia and neurodegeneration, Sakai and colleagues tested whether it is possible to generate lasso-grafted Met agonists that can cross the BBB (Fig. 1). Because it is well-established that antibodies against the transferrin receptor (TfR) can enhance the delivery of small-molecule drugs across the BBB, the authors applied lasso grafting of aMD4 on the Fc of an anti-mouse TfR (mTfR) antibody. They confirmed that lasso grafting on the full antibody preserved the binding affinity of the antibody for TfR, and that it penetrated the BBB and displayed Met-agonistic activity (although with a 10-fold lower EC_{50} compared to that of isolated Fc). They also show that a single intravenous injection at a reasonable dose (about 20 mg/kg) of the TfR(aMD4) construct resulted in antibody concentrations in brain parenchyma of 10 nM, a concentration that is sufficient for Met activation and that indicates substantial BBB penetration. Moreover, immunofluorescence microscopy showed the colocalization of the antibody with neurons, astrocytes and microglia, which for many brain functions are under control of the HGF–Met axis. Although the actual agonistic activity of TfR(aMD4) in the brain would need to be experimentally tested, BBB-penetrating Met agonists lasso-grafted on an anti-TfR antibody may have promising translational uses.

Several cytokines have been designed with intriguing properties. Examples are hyper-IL-6, a fusion protein between IL-6 and its soluble receptor, which enables trans-signalling, potently promoting liver regeneration on liver damage or toxicity-induced hepatic injury[8,9]; IC7Fc, an engineered gp130 ligand and cytokine with ciliary-neurotrophic-factor-like yet IL-6-receptor-dependent signalling properties that improves hyperglycaemia, prevents liver steatosis and modulates the loss of skeletal muscle mass and thus could have a utility in type-2 diabetes and muscle atrophy[5]; and surrogate bispecific Wnt agonists that induce receptor dimerization and canonical Wnt signalling which, owing to their improved solubility that spares the necessity for lipidation, can facilitate applications in regenerative medicine[10]. Yet Sakai and co-authors' work goes beyond such surrogate cytokines with novel functionalities and such fusion protein conjugates with antibody moieties. Notably, the combination of the RaPID macrocyclic-peptide-selection technology and a lasso-type grafting procedure applied to suitable loop structures of the Fc chain of antibodies[7] generates potent Met agonists with in vivo activity[6]. In fact, macrocyclic-peptide selection allows for non-natural agonist molecules to be selected from a myriad of molecule combinations, and the authors have shown that such surrogate agonists, which typically do not share sequence homology with the mimicked natural cytokine and may bind to different receptor sites, can have potent cytokine-like agonistic activity. In addition, the authors' work highlights the potential of macrocyclic peptides as a therapeutic modality that combines the advantages of peptides and antibodies[11–14]. To this end, the work is consistent with an intrinsic property of 15-residue macrocycles selected via lasso grafting to adopt a functional conformation in the context of an unrelated recipient scaffold protein. Furthermore, the functionality of the scaffold protein, at least in the case of Fc or full-size immunoglobulins, remains intact.

To harness the therapeutic utility of lasso-grafted peptides as cytokine mimics, some challenges would need to be addressed. First, for the surrogate Met agonists, and although predictable intra-brain Met-activation remains to be shown, it will be important to test whether this coincides with a neuroprotective effect in corresponding disease models. Second, although peptides usually exhibit higher specificity than small molecules, potential off-target effects of the selected random peptide segments remain to be excluded. Third, the observed partial steric interference of the Fab arm with Met-agonistic activity of the inserted macrocycle calls for the optimization of the topology and size of the anti-TfR antibody construct. It will also be important to clarify whether such effects are generally seen or whether they are specific to the conformational context. The observed enhancement of agonistic activity of grafted aMD4 by disulfide bridging highlights the importance of its conformation and that the function of aMD4 can be tailored by conformational control. For further guidance, obtaining the structures of Fc(aMD4)B3 and of Fc(aMD4ds)B3 via nuclear magnetic resonance may be able to confirm the macrocycle-like fold of the graft. Overall, Sakai and colleagues' study is suggestive of the translational potential of guest peptides grafted onto suitable loops of antibodies to generate cytokine mimics that combine the potent agonistic activities of macrocyclic peptides with the favourable pharmacokinetic properties of antibodies.

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Competing interests

A.K. and J.B. are co-inventors on patent applications related to the anti-macrophage migration inhibitory factor and to anti-chemokine strategies in inflammatory and cardiovascular diseases, and on patent applications related to the generation of amyloid-inhibitory peptides.

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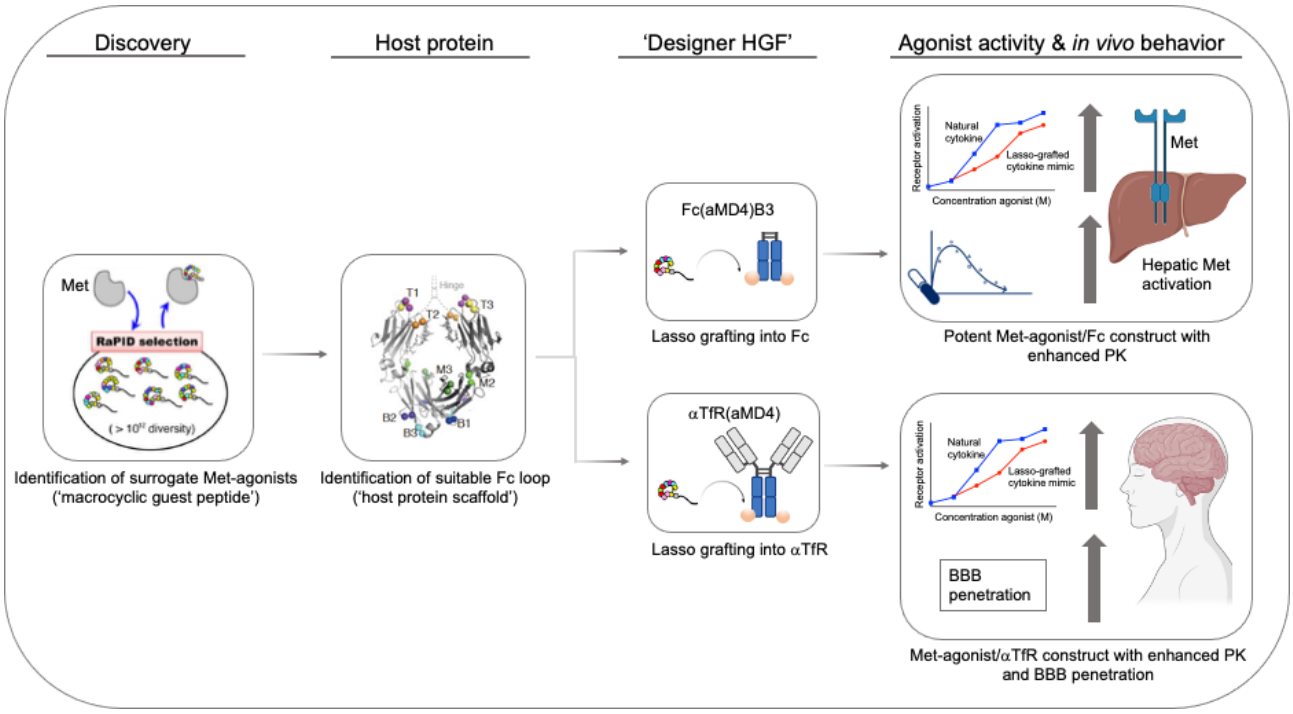
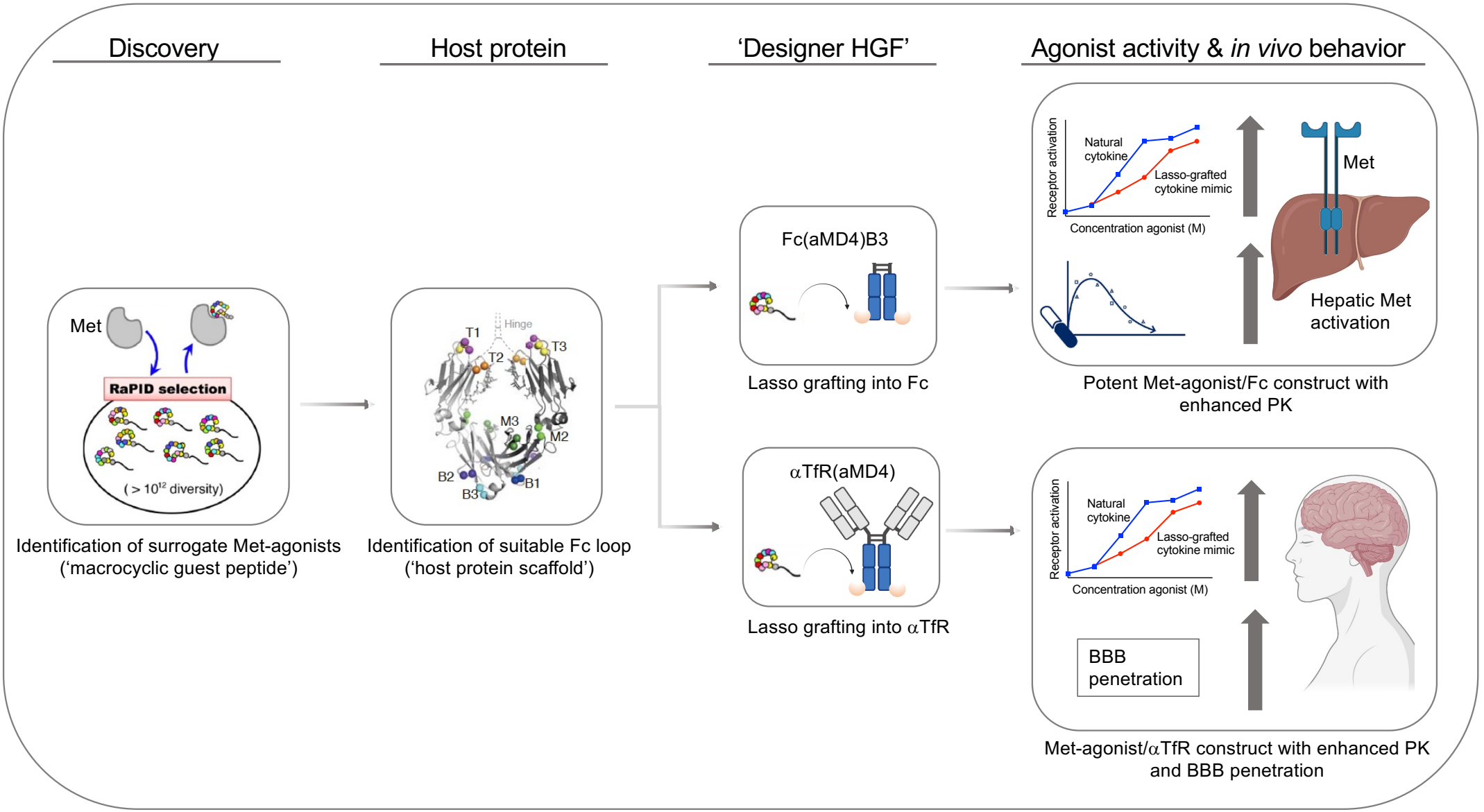


Fig. 1 | Lasso-grafted peptides and antibodies mimicking the properties of the human hepatocyte growth factor. Methodology and process for the generation of the lasso-grafted peptide-mimics and antibody-mimics, from the discovery of surrogate Met-receptor agonists by the RaPID (for 'random non-standard peptides integrated discovery') selection method, to the selection of suitable host-protein loops (here within the Fc moiety of a human immunoglobulin), to the grafting of the identified macrocyclic peptides into the host scaffold (into a fragment crystallizable (Fc) region, top; or into a full-size anti-transferrin receptor antibody (TfR), bottom) to yield the actual designer cytokine (here mimicking the human hepatocyte growth factor; HGF), to the characterization of the Met-agonistic activity (the graph icon shows an exemplary agonist comparison between natural cytokine (blue) and mimic (red)), and the in vivo pharmacokinetics (PK; icon shown symbolizes the in vivo PK of a drug) and blood–brain-barrier (BBB) penetration properties of the surrogate Met-receptor agonists. Met receptor expression in liver is indicated.

[Art Editor: The two schematics on the left are from here and from Fig. 1 in the Sakai Article. The rest of the schematics are original and were created with Biorender and Prism 9 / Graphpad (licenses of MedUni Muenchen, Institute for Stroke and Dementia Research). Please remove the axis ticks, label units and numbers in the two graphs with red blue and green curves.]


Discovery
Host protein
'Designer HGF'
Agonist activity & *in vivo* behavior
Met
RaPID selection
(> 10^12 diversity)
Identification of surrogate Met-agonists
('macrocyclic guest peptide')
Hinge
T1
T2
T3
M3
M2
B2
B3
B1
Identification of suitable Fc loop
('host protein scaffold')
Fc(aMD4)B3
Lasso grafting into Fc
αTfR(aMD4)
Lasso grafting into αTfR
Receptor activation
Natural cytokine
Lasso-grafted cytokine mimic
Concentration agonist (M)
Met
Hepatic Met activation
Potent Met-agonist/Fc construct with enhanced PK
Receptor activation
Natural cytokine
Lasso-grafted cytokine mimic
Concentration agonist (M)
BBB penetration
Met-agonist/αTfR construct with enhanced PK and BBB penetration