Protein Engineering

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Multifunctional Antibody Agonists Targeting Glucagon-like Peptide-1, Glucagon, and Glucose-Dependent Insulinotropic Polypeptide Receptors

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Abstract: *Glucagon-like peptide-1 (GLP-1) receptor (GLP-1R), glucagon (GCG) receptor (GCGR), and glucose-dependent insulinotropic polypeptide (GIP, also known as gastric inhibitory polypeptide) receptor (GIPR), are three metabolically related peptide hormone receptors. A novel approach to the generation of multifunctional antibody agonists that activate these receptors has been developed. Native or engineered peptide agonists for GLP-1R, GCGR, and GIPR were fused to the N-terminus of the heavy chain or light chain of an antibody, either alone or in pairwise combinations. The fusion proteins have similar in vitro biological activities on the cognate receptors as the corresponding peptides, but circa 100-fold longer plasma half-lives. The GLP-1R mono agonist and GLP-1R/GCGR dual agonist antibodies both exhibit potent effects on glucose control and body weight reduction in mice, with the dual agonist antibody showing enhanced activity in the latter.*

GLP-1R, GCGR, and GIPR are three key metabolically-related peptide hormone receptors. GLP-1 receptor peptidic agonists, such as Exenatide,[1] Liraglutide,[2] Lixisenatide,[3] and Semaglutide,[4] have been proven effective therapeutic agents for glucose control and also lead to body weight loss. Recently, single peptides with agonist activities on GLP-1R and GCGR,[5] GLP-1R and GIPR,[6] or GLP-1R, GCGR and GIPR[7] have shown additional benefits in glucose control, weight loss, and fat mass reduction compared to mono GLP-1R agonists. Among them, Oxyntomodulin (OXM) is the only native human peptide that activates both GLP-1R and GCGR.[8] However, its activity on GLP-1R and GCGR is more than 10-fold lower compared to GLP-1 and GCG, respectively.[9] Other engineered synthetic peptides (for example, ZP2929 developed by Zealand Pharma) have higher activities on both receptors.[10] However, the engineered sequences, like GLP-1R mono-agonists, all suffer short half-lives in vivo.

Chemical conjugation[2,4,11] or genetic fusion of half-life extending agents (for example, PEGs, fatty acids, Fc fragment)[12] is necessary to generate long-acting versions of many peptide therapeutics to reduce injection frequency and increase compliance in clinical use. Chemical methods require highly selective reactions and often result in more complex and costly manufacturing processes, as well as reduced potency of the conjugated product. Albumin (monomer) or Fc (homodimer) fusions are limited to a single N-terminal fusion partner. As an alternative to short-lived peptides, functional antibodies that activate cell surface receptors are being developed as powerful research tools and therapeutics. However, selection of such antibodies from combinatorial antibody libraries or hybridomas has proven challenging, especially for antibodies with dual activities.[13] We have previously generated a bi-functional antibody by the simultaneous fusion of two distinct cytokines into CDR3H and CDR3L of the variable region, which retains the activities of the native proteins.[14] Herein, we extend this approach to generate multi-functional metabolically active antibodies by fusion of the same or distinct peptides to the N-terminus of the heavy or/and light chains of antibodies.

Previously, we genetically fused EX-4 into CDR3H of Herceptin directly adjacent to a Factor Xa cleavage site to release the free N-terminus of the fused EX-4 that is required for GLP-1R activation.[15] We reasoned that direct fusion of an agonist peptide to the N-terminus of an antibody heavy chain or light chain could further simplify the generation of agonist antibodies for many therapeutic peptides. More importantly, this strategy should also simplify the generation of antibodies with dual activities, given the two chain nature of the antibody molecule. To test this notion, a gene fragment encoding GLP-1, GCG, GIP, EX-4, and ZP[10] flanked with a C-terminal flexible linker or rigid helical linker were fused at the N-terminus of the heavy chain or light chain of Synagis (Syn), an FDA approved RSV neutralizing antibody (Figure 1) that has been used to treat pediatric patients since 1998. Syn does not bind with high affinity to any human proteins and has low immunogenicity, and therefore is ideal as a carrier scaffold to generate antibody fusions. Specifically, to create the antibody fusions we used an IgG1 heavy-chain constant region with seven mutations (SI) which have been shown to reduce complement dependent and antibody-dependent cell-mediated cytotoxicities.[16] We also mutated

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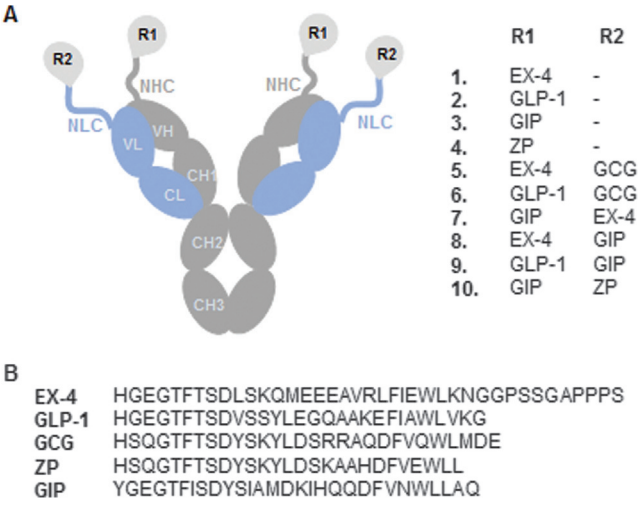

Figure 1. Design of mono-, dual-, and tri-agonist antibodies in Synagis (Syn). A) Mono- or dual- fusion of peptides at the N-termini of antibody heavy chain and light chains. All of the N-terminal antibody fusions were generated as full-length IgGs. B) Sequences of agonist peptides fused at the N-termini of heavy chain and light chain. NHC = N-terminal heavy chain, NLC = N-terminal light chain, HC = heavy chain, LC = light chain.

Table 1: Properties of the Synagis fusion antibodies and peptides.[a]

	Protein	EC_{50} GLP-1R	EC_{50} GCGR	EC_{50} GIPR	Yield
1	Syn-EX-4	5.0 ± 0.8	–	–	13
2	Syn-GLP-1	7.0 ± 0.6	–	–	44
3	Syn-GIP	–	–	6.0 ± 0.5	67
4	Syn-ZP	5.0 ± 0.4	17.0 ± 9.4	–	51
5	Syn-EX-4-GCG	6.0 ± 0.3	25.0 ± 1.6	–	15
6	Syn-GLP-1-GCG	14.0 ± 0.7	42.0 ± 3.3	-	24
7	Syn-GIP-EX-4	8.0 ± 0.8	–	6.0 ± 4.7	56
8	Syn-EX-4-GIP	6.0 ± 0.6	–	8.0 ± 1.0	10
9	Syn-GLP-1-GIP	10.8 ± 0.6	–	8.1 ± 0.3	15
10	Syn-GIP-ZP	5.0 ± 0.7	11.0 ± 0.9	9.0 ± 0.5	58
	GCG	–	68[18]	–	–
	EX-4	13.0 ± 0.8	–	–	–
	GLP-1	8[19]	–	–	–
	ZP2929	110[20]	160[20]	–	–
	GIP	–	–	20[6]	–

[a] EC_{50} (pM) for activation of GLP-1R, GCGR, and GIPR determined using HEK 293 cells overexpressing GLP-1R, GCGR, or GIPR and carrying a cAMP response element (CRE) luciferase (Luc) reporter. The yields [mg L^{-1}] of fusion antibodies were based on purified material after expression from FreeStyle 293-F cells by transient transfection.

Ala2 of GLP-1 and GIP to Gly to improve stability against proteolysis. Furthermore, we introduced two mutations, Asn28Asp and Thr29Glu, into GCG to increase stability, based on published results.[17] Finally, the GLP-1R/GCGR dual agonist peptide ZP was derived from Zealand peptide.[10] The heavy or light chain peptide fusions were next paired with the wild-type (WT) light or heavy chains of Synagis, respectively, to generate the mono-functional Synagis fusion antibodies. Alternatively, the heavy and light chain peptide fusions were paired with a distinct light or heavy chain peptide fusion to generate the multi-functional Synagis fusions (Figure 1).

These fusion constructs were expressed in FreeStyle 293-F cells by transient transfection. Secreted fusion proteins were purified using protein A chromatography and analyzed by SDS-PAGE (Supporting Information, Figure S1) and LC-MS (Supporting Information, Figures S2–S5). The yields of the fusion proteins ranged from 10 to 67 mg L^{-1} (Table 1); all proteins can be concentrated to over 10 mg mL^{-1} in PBS (pH 7.4) without aggregation (analyzed by size-exclusion chromatography); we also measured the thermal stability of selected mono- and dual- fusion proteins and observed similar T_m values to that of Synagis (Supporting Information, Table S1). The similar thermal stabilities, yields, and solubility of the fusion proteins and Synagis indicate that N-terminal fusion does not negatively impact correct folding of the antibody scaffold as long as the fusion sites and linkers are properly selected.

The in vitro activities of the mono- and dual-Synagis fusions were next examined using HEK 293 cells overexpressing GLP-1R, GCGR, or GIPR and carrying a cAMP response element (CRE) luciferase (Luc) reporter. The EC_{50} values of some of the N-terminal antibody fusions on their cognate receptors are similar to or better than those of the parent peptides (Table 1). Indeed, Syn-GIP-ZP showed balanced and potent activities in the activation of all three receptors.[7] The ratio of the potencies on GLP-1R, GCGR, and GIPR for optimal efficacy depends on in vivo studies in higher species. To this end, the combinatorial strategy described here allows one to easily modulate the potencies of the antibody fusions on different receptors by simply engineering the linker (length or/and composition), switching the fusion sites between the heavy and light chains of the antibody scaffold, or altering the individual peptide sequences. The ability to independently adjust the activity of the antibody agonist on each receptor makes this attractive for cases where one needs to precisely control the relative agonist activities to obtain the maximal therapeutic benefit.

To determine whether fusion of peptides at the N-termini of the heavy and light chains of Synagis increases their plasma half-life, we carried out pharmacokinetic studies of the dual-agonist fusion protein Syn-EX-4-GCG in rats. The estimated terminal $t_{1/2}$ of Syn-EX-4-GCG after s.c. injection is 46 hours based on EX-4 detection and 11 h based on GCG detection (Figure 2). Compared to EX-4 ($t_{1/2} < 0.5$ h) and GCG ($t_{1/2}$ of 3–6 min),[21] which have rapid plasma clearance after s.c. administration, Syn-EX-4-GCG has a slower absorption phase, greater C_{max} (maximum concentration), longer residence time, and much slower plasma clearance. The serum from the dosed animals was also analyzed using the in vitro GLP-1R and GCGR activation assays, and showed a similar PK profile (Supporting Information, Figures S6, S7). The distinct half-lives of the EX-4 and GCG peptide fusions suggest that the degradation rate of the EX-4 and GCG moieties are most likely different. This could lead to different durations of action on GLP-1R and GCGR in vivo after dosing of the dual-fusion molecule, a property that might have to be optimized to achieve optimal efficacy.

We next evaluated the efficacy of representative antibody agonists in blood glucose control in mice by an oral glucose tolerance test (OGTT). Mice treated with a single dose of

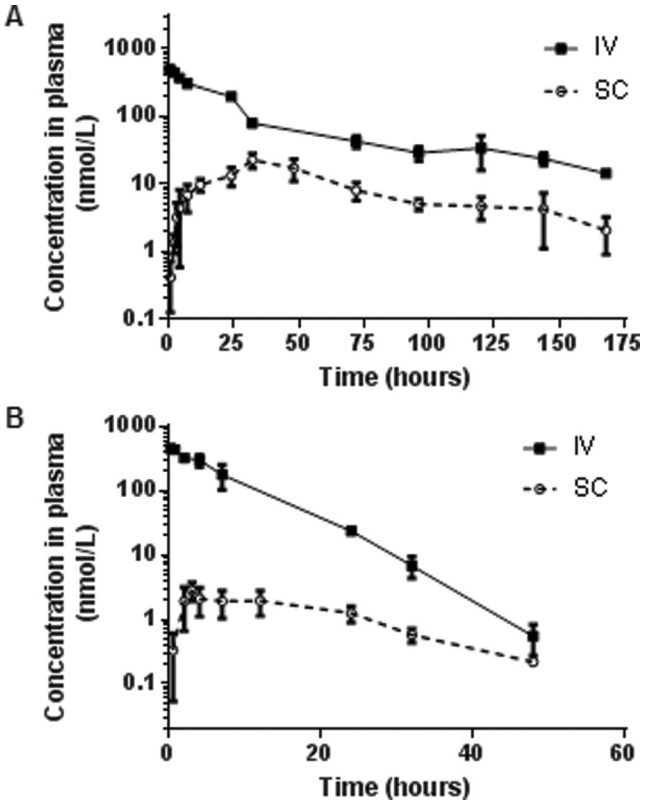

Figure 2. Pharmacokinetics of the dual agonist Syn-EX-4-GCG in rats. Dual agonist (2 mg kg^{-1}) in PBS (pH 7.4) was administered by i.v. or s.c. injection into SD female rats ($N=3$). Blood samples were collected at the indicated time points and analyzed by a sandwich ELISA using an *anti*-human Fc antibody and an EX-4 or GCG-specific antibody. A) Concentration of Syn-EX-4-GCG based on detection of *anti*-human Fc and *anti*-EX-4. B) Concentration of Syn-EX-4-GCG based on detection of *anti*-human Fc and *anti*-GCG. Data were analyzed by WinNonlin using a two-compartment model.

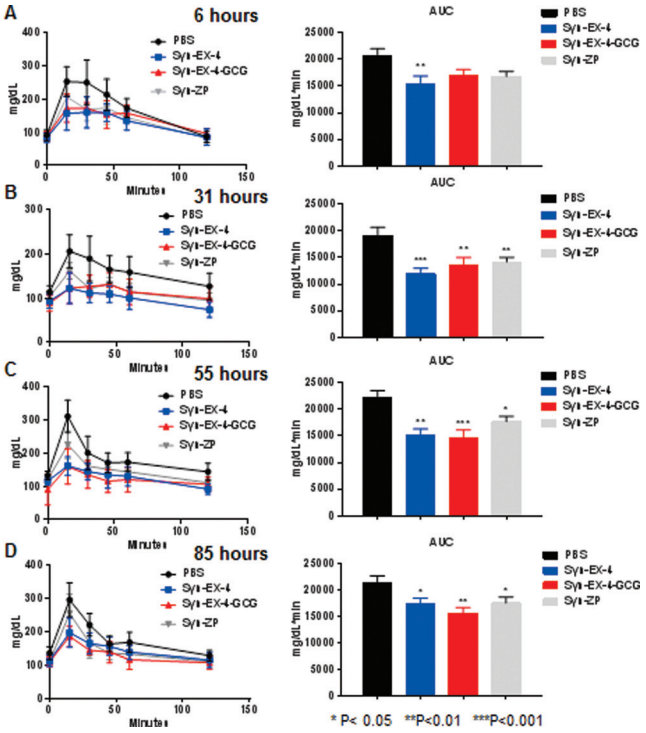

Figure 3. Pharmacodynamics of the Syn-EX-4 (**1**), Syn-EX-4-GCG (**5**), and Syn-ZP (**4**) in mice. Single doses of vehicle (PBS, pH 7.4), Syn-EX-4, Syn-EX-4-GCG, and Syn-ZP (8 mg kg^{-1}) were subcutaneously administered into CD1 mice ($N=5$). OGTTs (3 g kg^{-1} d-glucose; p.o.) were performed at 6 h (A), 31 h (B), 55 h (C), and 85 h (D) after single-dose treatment. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ (one-way ANOVA test).

Syn-EX-4 (**1**), Syn-EX-4-GCG (**5**), and Syn-ZP (**4**; 8 mg (50 nmol) kg^{-}, subcutaneous injection) showed sustained control of blood glucose levels for up to 85 hours (Figure 3). This result confirms that the extended half life translates to a long lasting effect in vivo. It is interesting to note that the effects of the dual agonist Syn-EX-4-GCG relative to the mono GLP-1 agonist Syn-EX-4 become more significant at later time points, which is probably due to the counter but shorter activity of the GCG moiety relative to the EX-4 moiety in the dual fusion protein.

Next we measured the ability of the GLP-1R mono-agonist and GLP-1R/GCGR dual agonist to reduce body weight in the high-fat diet induced obese (DIO) mouse model. We first performed a dose-titration study of Syn-EX-4 in DIO mice. In this study, Syn-EX-4 reduced body weight in DIO mice in a dose-response manner (Supporting Information, Figure S8); at 2 mg kg^{-1} the fusion antibody reduced body weight $15.2\pm2.4\%$ in only 6 days. Next we measured the efficacy of Syn-EX-4 and Syn-EX-4-GCG in the same model using a sub-optimal dose of 0.5 mg kg^{-1} to better differentiate the activity of mono- and dual agonists. Syn-EX-4-GCG (**5**) at 0.5 mg kg^{-1} significantly reduced body weight $12.6\pm1.4\%$ after every other day s.c. dosing for 9 days (Figure 4 A), while the vehicle (PBS, pH 7.4) group showed a slight increase in body weight. Syn-EX-4 (0.5 mg kg^{-1}) also reduced body weight but to a lesser degree ($8.1\pm1.8\%$ reduction after 9 days) than the dual Syn-EX-4-GCG fusion protein at the same dose level. The higher efficacy of the dual agonist antibody fusion Syn-EX-4-GCG suggests that there is a benefit for co-agonism of both GLP-1R and GCGR, which is consistent with the previous findings using receptor knock-out mice.[22] The dual agonist Syn-ZP fusion also induced weight loss in the DIO model ($4.8\pm1.3\%$), but again not to the same degree as Syn-EX-4-GCG after 6 day treatment (Figure 4 B). This result may stem from distinct receptor activity or half-lives and reinforces the utility of being able to independently modulate these properties in dual chain antibody fusion format.

In summary, we demonstrate that mono-, dual-, and tri-agonist antibody fusion proteins can be generated by fusing peptides at the N-terminus of the heavy chain or/and light chain of Synagis. The fusion antibodies potently activate GLP-1R, GCGR, and GIPR in vitro and have significantly extended half-lives in rodents. The long lasting efficacy to control glucose level and body weight reduction in mice will likely translate into greater than 1X/week dosing in humans. Although we observed improved efficacy for the GLP-1R/GCGR dual agonist relative to the mono GLP-1R agonist in body weight reduction, it will be important to evaluate dual

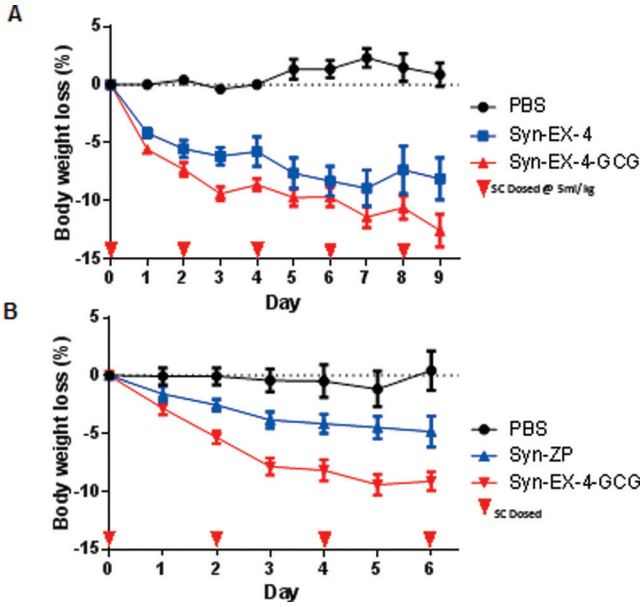

Figure 4. The dual agonist Syn-EX-4-GCG significantly reduced body weight in DIO mice. Vehicle (PBS, pH 7.4), Syn-EX-4, Syn-EX-4-GCG, and Syn-ZP (0.5 mg kg^{-1}) were subcutaneously administered to DIO mice ($N = 8$) every other day. Mouse body weight was measured daily. Percent weight loss was according to body weight change from Day 0.

agonists in non-human primate models to select the optimal clinical candidates.

Keywords: antibodies · dual agonists · obesity · peptides · protein engineering

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