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REVIEW

Enhancing the longevity and *in vivo* potency of therapeutic proteins: The power of CTP

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The relatively short serum half-life of polypeptides poses a challenge in the design of protein therapeutics. Extending the half-life and bioavailability while maintaining safety and efficacy has been attempted using various techniques such as sustained-release preparations and long-acting conjugations. A novel, alternative approach involves the fusion of a natural polypeptide, the C-terminal peptide (CTP) of human chorionic gonadotropin (hCG), to the target protein. The fertility drug FSH-CTP (Elonva®) was the first CTP-containing protein to receive marketing approval. This versatile platform has been utilized to improve the pharmacokinetic (PK) and pharmacodynamic (PD) properties of additional target proteins from different families. This includes CTP fused to erythropoietin (EPO), interferon beta (IFNβ) and coagulation factors VII and IX, as well as thyroid-stimulating hormone (TSH), oxyntomodulin, and growth hormone (GH). Two long-acting, CTP-containing chimeras are currently in clinical development by OPKO Biologics. Long-acting FVIIa-CTP (MOD-5014) is being developed for the treatment of hemophilia with an expected dosing regimen of 2-3 times a week. MOD-5014 exhibited improved *in vivo* PK/PD, coagulation, and safety parameters in comparison to rFVIIa. This supports the upcoming clinical evaluation of MOD-5014 in hemophilic patients. Long-acting CTP-modified hGH (MOD-4023), currently in advanced clinical development by OPKO, may replace current GH therapy in patients suffering from GH deficiency (GHD) by requiring fewer injections. Animal studies showed that once-weekly administration of MOD-4023 resulted in favorable weight gain response in comparison to daily hGH and established an excellent safety profile. A Phase 1 study in healthy volunteers and Phase 2 trial in GHD patients confirmed the safety and tolerability of MOD-4023 in adults; a pivotal Phase 3 study in GHD adults in ongoing. A recently completed Phase 2 study in GHD children demonstrated an improved PK/PD profile and efficacy for once-weekly MOD-4023 in comparison to daily hGH, supporting OPKO's upcoming Phase 3 trial in pediatric GHD patients. Based on recent pre-clinical and clinical experience, CTP technology was proven as a valid, flexible platform for the generation of safe and effective long-acting protein therapeutics, with the potential to offer an improved treatment for patients and better quality of life.

Keywords: drug design; growth hormone deficiency; hemophilia; obesity; recombinant fusion proteins

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Introduction

Polypeptides are susceptible to enzymatic degradation and therefore have a relatively short circulatory half-life of several hours. For this reason, peptide therapeutics are usually designed as sustained-delivery compounds, with the aim of maintaining an effective plasma concentration of the active peptide. This warrants the development of a suitable technology to prolong the serum half-life of therapeutic peptides, while also maintaining safety and high pharmacological efficacy. Several techniques have been developed to achieve this aim. One approach includes sustained-release preparations that utilize hydrogel or microsphere encapsulation [1, 2]. A second approach aims to directly increase the polypeptide's bioavailability and half-life, either by conjugation to moieties such as PEG [3], or by fusion to specific amino acid sequences such as the unstructured XTEN peptide [4] or hybrid Fc fragment [5]. An alternative to the latter approach is the extension of protein half-life by fusion to natural peptides such as human CTP.

CTP technology is based on a natural peptide, the C-terminal peptide (CTP) of the beta chain of human chorionic gonadotropin (hCG). This 31 amino acid-long hydrophilic terminus extension contains four O-glycosylation sites, and provides hCG with the required longevity to maintain pregnancy. In contrast, the beta chain of luteinizing hormone (LH), a gonadotropin that triggers ovulation, is nearly identical to hCGβ, with a 7 aa-long hydrophobic stretch instead of the 31 aa-long extension of hCG. As a result, hCG has a significantly longer serum half-life in comparison to LH (60-120 min for LH, several hours for hCG [6, 7]) as well as increased potency [8, 9]. Importantly, the CTP was shown to have no effect on the interaction between hCG and its receptor [10]. The CTP can be shuttled to different proteins to create chimeras with enhanced *in vivo* stability. Fusion of the CTP to the N-terminal region of hCGα significantly increased the *in vivo* activity of the protein but did not affect the ability of the chimera to bind the receptor. Moreover, it was shown that the signal for O-glycosylation is contained entirely within the CTP sequence and is independent of the surrounding protein [11, 12]. The O-linked oligosaccharides of the CTP add flexibility to the host protein and decrease its hydrophobicity, and thus lower renal and hepatic clearance and increase circulatory half-life [9, 13, 14]. The presence of terminal sialic acids in the CTP may also impede the hepatic clearance of CTP-modified proteins by reducing their affinity to asialoglycoprotein receptors in the liver [15, 16].

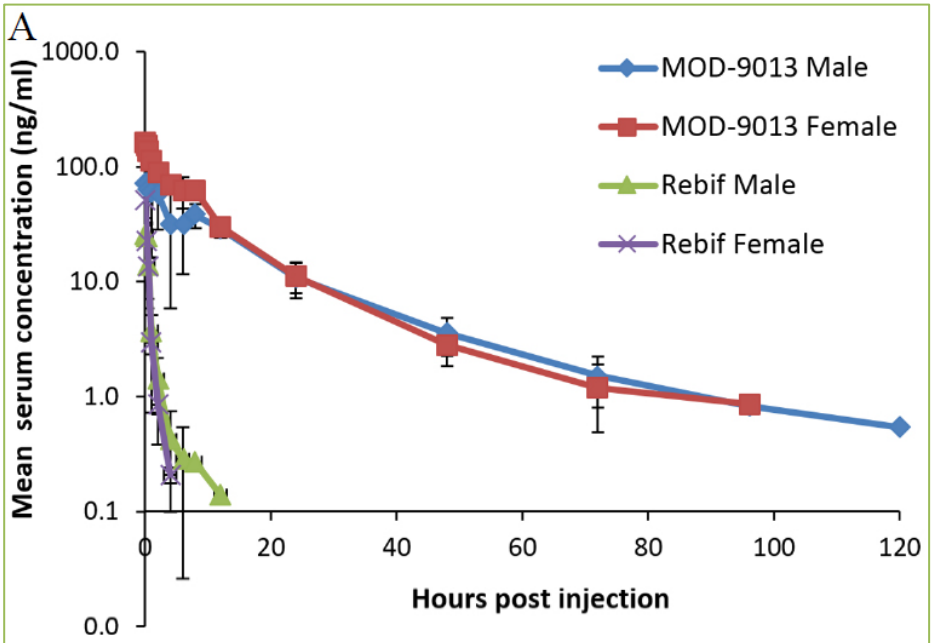
This review outlines the current development status of several CTP-containing compounds, utilizing different proteins from different sub-families. This allows the assessment of CTP's potential to serve as a platform technology for the production of long-acting protein pharmaceuticals. In addition to FSH-CTP (Elonva®), which is the first CTP-modified drug to receive marketing approval to date, this article briefly describes other CTP-modified proteins in several stages of development. Finally, an overview is provided of two CTP chimeras currently in clinical development at OPKO Biologics, namely FVIIa-CTP (MOD-5014) and hGH-CTP (MOD-4023).

FSH-CTP

Follicle-stimulating hormone (FSH), a glycoprotein secreted by the pituitary gland, plays a vital role in the development of ovarian follicles and testicular tubules. Fares *et al*. fused the CTP to the beta subunit of FSH using site-directed mutagenesis and gene transfer techniques, in order to prolong the half-life of the protein and increase its *in vivo* activity. The fusion of one or two copies of CTP resulted in enhanced FSH potency, while not interfering with the protein's assembly, secretion, or receptor binding [17]. FSH-CTP (corifollitropin alfa [Elonva®], Schering-Plough), currently the only CTP-containing product approved for clinical use, is a hormone fertility drug that contains one copy of CTP fused to the C terminus of the beta chain of FSH. Fusion of the CTP moiety nearly doubles the elimination half-life of the drug in comparison to recombinant FSH [18]. The *in vivo* biopotency of FSH-CTP is 10-fold higher, and its elimination half-life is 2-3 times longer, than recombinant FSH. Clinical studies have shown that a single dose of corifollitropin could replace the first seven daily injections of recombinant FSH required prior to *in vitro* fertilization [19]. Elonva® received marketing approval from the European Commission in 2010.

TSH-CTP

Thyroid-stimulating hormone (TSH) is a heterodimeric glycoprotein secreted by the pituitary gland. Joshi *et al*. produced a human TSH-CTP chimera that demonstrated prolonged half-life as a result of decreased metabolic clearance as well as enhanced *in vivo* potency. Recombinant TSH is commonly used for the diagnosis and treatment of thyroid cancer, and a potent, long-acting variant would obviate the need to repress thyroid function in patients undergoing radioiodine uptake and imaging tests [20]. Conversion of the TSH heterodimer into a single-chain form was conceived as a way to enhance the protein's half-life, since the assembly of the α and β subunits is a rate-limiting step. Using the CTP as a linker between the two chains allowed the successful construction of a functional, single-chain TSH polypeptide. The presence of the CTP



B

MOD-9013					
	$T_{1/2}\alpha$ (h)	$T_{1/2}\beta$ (h)	AUC (ng-h/ml)	CL/F (ml/h/kg)	Vz/F (ml/kg)
Males	9.08±3.7	17.55±4.73	996.8±368.1	1.78±0.64	36.7±10.92
Females	5.5±0.44	13.32±2.54	1328.6±175.4	1.39±0.35	20.1±2.98
Rebif®					
Male	0.34±0.13	1.44±1.0	20.6±8.02	56.67±20.91	98.0±23.71
Female	0.22±0.05	0.91±0.35	22.0±9.33	55.62±20.91	24.17±15.92

Figure 1. Pharmacokinetics of IFNβ1A-CTP (MOD-9013) and Rebif® following a single IV dose in cynomolgus monkeys (n = 3/group). A. PK profiles of MOD-9013 (3.7 µg/kg) and Rebif® (6 µg/kg). Serum concentrations were determined using a commercial ELISA kit (Fujirebio). B. Summary of PK parameters.

sequence led to an increased expression and secretion of the chimera, but did not affect the interaction with the receptor *in vitro*. In addition, the single-chain variant was found to have a decreased rate of clearance, and increased thermostability and bioactivity in comparison to wild type [21, 22].

EPO-CTP

Erythropoietin (EPO) is a 34 kDa protein hormone that regulates the production of red blood cells in the bone marrow, with a relatively short half-life due to rapid renal clearance. A single copy of CTP fused to the N-terminal of EPO prolonged its half-life and increased its *in vivo* activity, while only minimally affecting secretion and receptor binding. Moreover, a single dose of EPO-CTP in mice was sufficient for maintaining sustained blood levels of EPO and for the stimulation of hematocrit production, while an equivalent dose of wild type EPO produced the same effect only when administered as three injections over a period of one week [23]. In order to further increase the longevity of EPO, a second chimera was generated that contains three copies of CTP (CTP-EPO-CTP-CTP). Once-weekly injections of this long-acting variant in mice dramatically increased hematocrit levels (~8-fold) and reticulocyte counts when compared to wild type and to recombinant human EPO [24].

IFNβ1a-CTP

Interferon beta (IFNβ), a member of the type I interferon family, is a 166 amino acid glycoprotein produced by fibroblasts and other cells, with anti-viral, cell proliferation and gene induction activities. Currently, type I interferons are used for the treatment of multiple sclerosis, although their efficacy is limited due to low molecular weight, which decreases vascular retention [25]. With the aim of developing a long-acting IFNβ1a, the native interferon protein was fused to three copies of CTP peptide (CTP- IFNβ1a-CTP-CTP; MOD-9013). A comparative *in vitro* and *in vivo* biopotency study (unpublished) was conducted by OPKO to evaluate the activity of MOD-9013 in comparison to commercial IFNβ1a (Rebif®). MOD-9013 exhibited a superior PK profile in

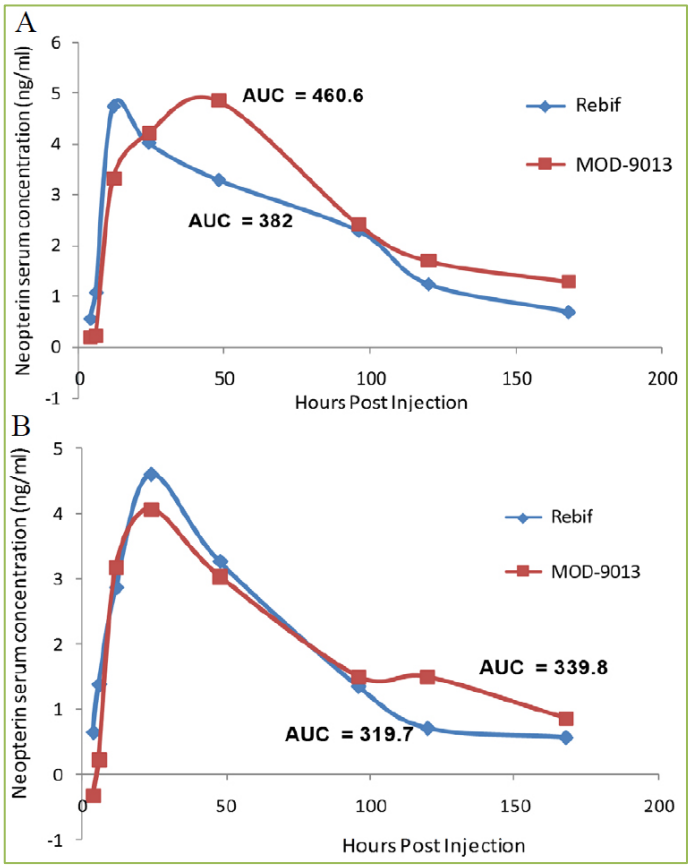


Figure 2. Average PD profile of MOD-9013 vs. Rebif® in cynomolgus monkeys following a single IV administration. MOD-9013 (6 µg/kg) or Rebif® (3.7 µg/kg) were injected in male (A) and female (B) monkeys, and neopterin levels were determined using a radioimmunoassay (ICN Biomedical).

comparison to Rebif® in a single-dose IV study in cynomolgus monkeys, with 50-fold higher AUC and 12-fold longer half-life (Figure 1). The *in vitro* anti-viral activity of MOD-9013 was found to be ~8-fold lower than Rebif®, and anti-proliferation and MHC-I induction assays also demonstrated reduced *in vitro* activity for MOD-9013 (not shown). The *in vivo* pharmacodynamics profile of MOD-9013 was compared to Rebif® by evaluating the serum levels of neopterin (a marker of cellular immune response) in cynomolgus monkeys. As shown in Figure 2, the serum profile of neopterin following a single IV administration of MOD-9013 demonstrated a slower return to baseline compared to Rebif®. In order to evaluate the *in vivo* biological efficacy of MOD-9013, the ability of the compound to inhibit the formation of a tumor following intradermal injection of melanoma cells was tested in nude mice. A single injection of MOD-9013 resulted in a significant inhibition of tumor progression, while Rebif® had only a minor effect in comparison to control (Figure 3). Taken together, the decrease in potency of IFNβ1A-CTP caused by reduced binding affinity is compensated by an increase in the overall systemic exposure caused by the

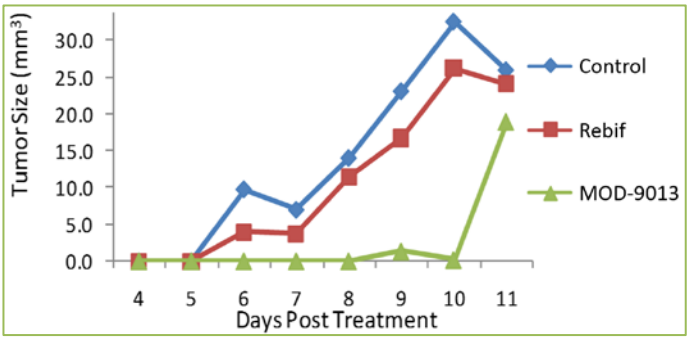
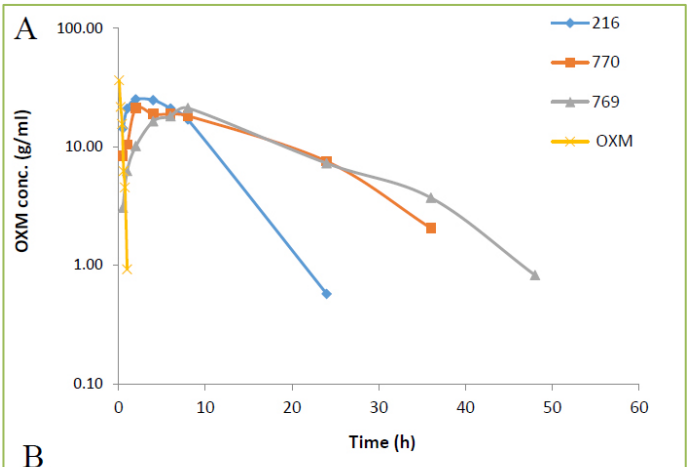


Figure 3. Efficacy study of MOD-9013 vs. Rebif® in nude mice. A single dose of MOD-9013, Rebif® (2*106 IU) or vehicle (control) were injected in eight nude mice carrying SK-MEL-1 melanoma cells, and tumor size was measured on a daily basis.



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	T1/2	C_{max} (ng/ml)	T_{max} (h)	AUC_{0-t} (ng-h/ml)	AUC_{∞} (ng-h/ml)	CL (ml/h/kg)	V_d (ml/kg)
OXM	0.18	36.4	0.08	13.1	13.3	16999	4375
216 (OXM-CTPx3)	3.78	25.1	2.0	308.9	312	512.8	2797
770 (OXM-CTPx4)	10.15	21.1	2.0	400.4	431.9	355.4	5379.6
769 (OXM-CTPx5)	9.63	21.2	8	431	442	346.2	4812

Figure 4. Pharmacokinetics of OXM-CTP variants (153 µg/kg) vs. native OXM (230 µg/kg) following a single SC injection in SD rats (n = 6/group/time point). A. PK profiles of the three variants and native OXM. Serum concentrations of OXM were quantified using a commercial ELISA kit (Bachem). B. Summary of non-compartmental PK parameters.

prolonged plasma circulating time.

OXM-CTP

Oxyntomodulin (OXM), a 37 amino acid peptide secreted by intestinal L cells, was shown to inhibit food intake and reduce body weight in both rodents and humans. In a study conducted in overweight human subjects, OXM was found to have pharmacological effect only after three daily subcutaneous injections [26]. However, OXM has a relatively short half-life. Using CTP technology, OPKO has created several long-acting variants of OXM and compared their serum pharmacokinetic profiles, as well as their *in vitro* and

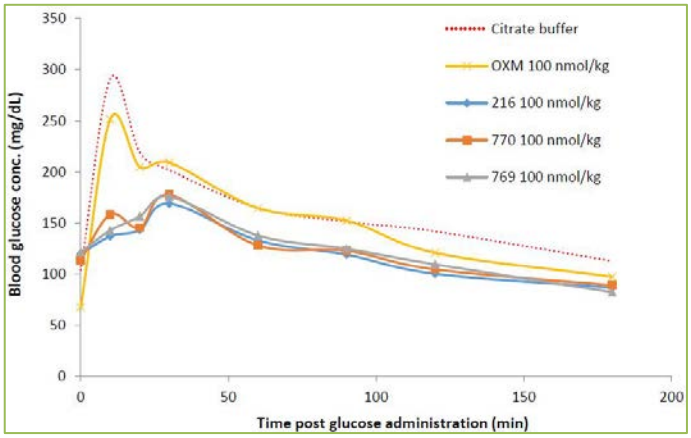

Figure 5. Glucose tolerance test (IPGTT) in mice following a single dose of OXM-CTP variants vs. native OXM. Overnight fasted C57BL/6 mice were injected IP with OXM peptide or OXM-CTPx3-5 (variants 216, 770 and 769, respectively) followed by IP injection of glucose (1.5 g/kg) 15 min later. Blood glucose levels were measured from tail vein samples using a glucometer.

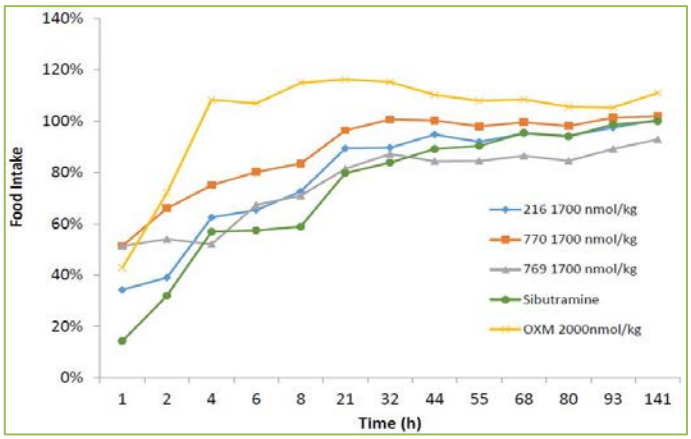

Figure 6. Food intake following a single dose of OXM-CTP variants vs. native OXM in mice. C57BL/6 mice (n = 4/group) were fasted for 17 h prior to treatment. Food intake were measured daily for 6 d, and the results are shown as % of citrate buffer control (=100%). Sibutramine, an anorexiant, was used as a positive control (20 mg/ml).

in vivo biological activity, to native OXM (unpublished results). Fusion of 3-5 copies of CTP to OXM led to significant extension of serum half-life up to ~10 h, and exposure (AUC) increased ~30-fold in comparison to native OXM (Figure 4). Moreover, injection of the OXM-CTP variants to mice, followed by sugar loading 15 min later, resulted in enhanced glucose clearance in comparison to native OXM, reflected by a reduction of ~20% of blood glucose AUC in comparison to control (Figure 5). When glucose was injected 2 h after OXM-CTPx4 administration, glucose tolerance activity was retained, while the activity of the native OXM was no longer apparent (not shown). Finally, a single SC dose of OXM-CTPx3, OXM-CTPx4 or OXM-CTPx5 in mice resulted in significant reduction in

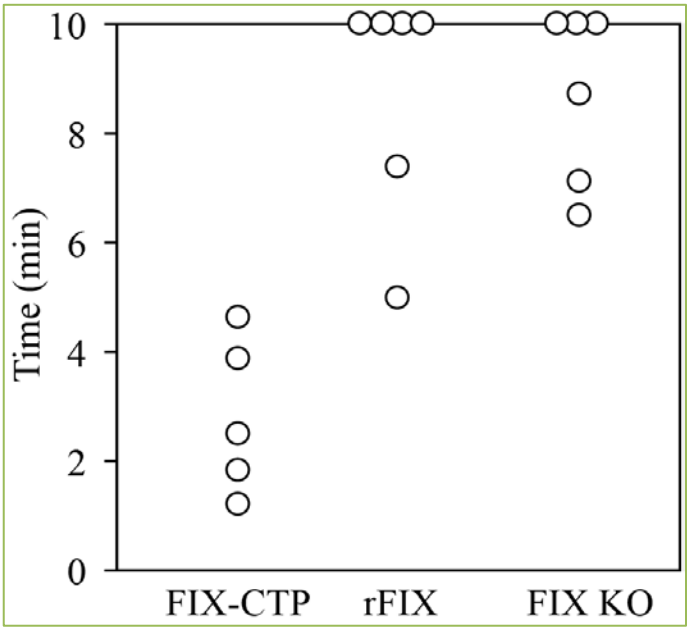

Figure 7. Bleeding time in FIX-deficient mice following a single IV dose (100 IU/kg) of FIX-CTP (n = 5) vs. rFIX (n = 6). The results show tail vein bleeding time after a second bleeding challenge 48 h post-dosing. FIX KO = untreated FIX-deficient mice as control (n = 6).

food intake in comparison to native OXM. The effect of the OXM-CTP variants lasted for up to 5 days, while the effect of native OXM peptide was abolished after 4 h (Figure 6).

Factor IX-CTP

Factor IX (FIX) is a 55 kDa glycoprotein, a member of the vitamin K-dependent protein factors associated with the coagulation system. Following proteolytic cleavage, the inactive zymogen is converted to active FIX (FIXa), which forms a complex with FVIII and in turn cleaves its natural substrate, FX. Intravenous administration of recombinant FIX (rFIX) is currently used for the treatment of patients suffering from hemophilia B, although the rapid clearance of FIX from the circulation limits its efficacy [27]. To produce a long-acting version of FIX, three tandem copies of CTP were fused to the C-terminus of FIX (MOD-3013). In an unpublished study, a comparative *in vitro* clotting assay (aPTT) showed that the coagulation activity of FIX-CTP is lower than commercial rFIX (BeneFIX®; data not shown). When the chimera's PK profile was compared to BeneFIX® in FIX-deficient mice following a single IV dose, a more favorable PK profile was demonstrated for MOD-3013 in comparison to BeneFIX® in terms of serum half-life and clearance (Table 1). A comparison of the PD profile of a single IV dose of MOD-3013 vs. BeneFIX® in FIX-deficient mice using a one-stage FIX activity assay revealed that MOD-3013-induced clotting activity was maintained for a significantly longer period of time than BeneFIX® (not

Table 1. Summary of PK parameters of FIX-CTP (MOD-3013; n = 12) vs. rFIX (BeneFIX®; n = 18) following a single IV dose (100 IU/kg) in C57BI FIX-deficient mice

	$T_{1/2}^{\alpha}$ (1/h)	$T_{1/2}\beta$ (1/h)	AUC (ng/ml*h)	CL (ml/kg/h)
FIX-CTP$_3$	5.2	28.7	39770	19
BeneFIX	3.4	12.7	22428	29

shown). MOD-3013-treated mice also exhibited a reduced bleeding time in a tail clip assay, demonstrated that MOD-3013 was potent at later time points than BeneFIX® (Figure 7).

Factor VIIa-CTP

Activated FVII (FVIIa) stimulates the extrinsic coagulation cascade and can thereby bypass deficiencies or inhibitors that abrogate the intrinsic coagulation pathway. The pro-hemostatic effect of FVIIa was also shown to be mediated by its direct interaction with the surface of activated platelets [28]. The short half-life of rFVIIa (~2.7 h) limits its use as a prophylactic agent to reduce the frequency of spontaneous bleeding episodes, as it necessitates frequent IV administration [29, 30]. A long-acting, CTP-modified FVIIa (MOD-5014) is currently in clinical development by OPKO for the treatment of patients with hemophilia A and B in cases of spontaneous hemorrhage, as well as for prophylactic use with an anticipated dosing regimen of 2-3 times a week. To increase the serum half-life of rFVIIa, CTP was fused to the C-terminus of FVIIa. MOD-5014 administered IV in mice, rats, monkeys and dogs exhibited an extended half-life, increased exposure, improved coagulopathy parameters, and its long-acting properties were confirmed (3-4-fold higher in comparison to rFVIIa in hemophilic dogs; unpublished results). Subcutaneous injections of MOD-5014 in rats and FVIII-deficient mice demonstrated an enhanced PK/PD profile, prolonged half-life and improved recovery in comparison to rFVIIa, as well as a confirmed safety profile in toxicological studies. The improved bioavailability of MOD-5014 resulted in significantly improved survival rates of normal animals and FVIII-deficient mice in a bleeding challenge and reduced the duration and intensity of bleeding in comparison to rFVIIa *in vivo*. MOD-5014 will be evaluated in an upcoming clinical study in hemophilic patients.

hGH-CTP

Growth hormone (GH) is a 191-amino-acid pituitary protein that stimulates the hepatic production and release of insulin-like growth factor-1 (IGF-1), which is instrumental in the promotion of linear growth in children, and in the control of metabolism and body-mass composition in adults. Existing GH formulations have limited therapeutic utility since they require daily administration, which may cause problems such as concomitant side effects and non-compliance [31, 32]. A long-acting form of GH (MOD-4023; CTP-hGH-CTP-CTP), with the potential to reduce discomfort by requiring fewer injections, is currently in advanced clinical development by OPKO. In comparative long-term studies in animal models, MOD-4023 administered once weekly demonstrated a greater duration in weight gain response in comparison to daily hGH and an excellent safety profile. A Phase 1 clinical study in healthy adults has shown MOD-4023 to have a favorable safety and tolerability profile. In a Phase 2 trial in GHD adults, once weekly, repeated doses of MOD-4023 were found to be safe and well-tolerated. OPKO is currently investigating MOD-4023 in a pivotal Phase 3 study in GHD adults. In addition, a Phase 2 safety, tolerability, and dose-finding study in pediatric patients with GHD has been recently completed. 12-month data from this trial (unpublished results) suggest extended half-life, increased exposure, and reduced clearance for MOD-4023 in comparison to hGH with comparable annual height velocity as daily GH. This supports once weekly injections in pediatric GHD population that can potentially replace seven consecutive daily injections of currently marketed hGH, and provides the basis for the company's upcoming Phase 3 trial.

Conclusions and Future Perspective

This paper provided an overview of CTP technology, a promising, valid platform utilized to extend the half-life and provide superior *in vivo* activity for a wide array of different peptides and proteins, each with its own unique properties and mechanism of action. FSH-CTP (Elonva®) is currently the only marketed drug to utilize this technology. While several CTP-modified proteins such as IFNβ1a-CTP and FIX-CTP remain relatively unexplored beyond the early proof-of-concept stage, FVIIa-CTP and hGH-CTP have successfully moved forward in the development pipeline and are currently undergoing clinical trials.

We propose that CTP has a competitive advantage over other long-acting technologies such as PEG conjugation and Fc fusion. The relatively small increase in molecular weight following the fusion of CTP means that the recombinant protein contains a high percentage of the active moiety. Unlike PEG, addition of CTP does not increase viscosity, and thus enables formulation in high concentrations that could be administered using a small needle size. In addition, humans are exposed to the naturally-occurring CTP during embryonic development. This reduces the probability of immunogenic response in patients. This technological platform was proven to be highly modular, both in terms of the variety of protein families into which CTP was

successfully incorporated (e.g. hormones, cytokines, enzymes, and peptides), and in the ability to tailor the CTP to different target proteins by using different repeats in various positions in the protein chains. Based on recent experience with Elonva® and hGH-CTP, this innovative technology appears to be effective as well as safe, and has the potential to provide novel long-acting drugs that can improve treatment and the quality of life of patients.

Conflicting interests

PM is a consultant for OPKO Biologics.

List of abbreviations

AUC: area under curve; CL/F: apparent total clearance; C_{max}: maximum concentration; CTP: C-terminal peptide; EPO: erythropoietin; FSH: follicle-stimulating hormone; FVII: factor VII; FVIII: factor VIII; FIX: factor IX; GHD: growth hormone deficiency; hCG: human chorionic gonadotropin; IFNβ: interferon beta; IGF-1: insulin-like growth factor-1; IV: intravenous; LH: luteinizing hormone; OXM: oxyntomodulin; PD: pharmacodynamics; PEG: polyethylene glycol; PK: pharmacokinetics; SC: subcutaneous; $T_{1/2}$: half-life; T_{max}: time of maximum concentration; TSH: thyroid-stimulating hormone; V_d: volume of distribution; Vz/FL: apparent volume of distribution.

Authors' contributions

DC performed the literature research and drafted the manuscript. GH and OH coordinated the team and assisted with drafting of the manuscript. PM, LIY and YT performed FIX-CTP experiments. ABI performed the EPO-CTP and IFNβ-CTP experiments. OH and YT performed the OXM-CTP experiments. LB, MH, YT, MZ, RG, YF, LM, ABI, GH and OH were involved in the development of FVIIa-CTP. ABI, YT, MZ, RG, YF, LM, GH and OH were involved in the development of hGH-CTP.

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