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ARTICLE

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Surfactant-modified parathyroid hormone fragments with high potency and prolonged action: Structure-informed design using glycolipid surfactant conjugation

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Abstract

The pharmaceutical properties of peptides (high potency and specificity with low toxicity) make them an attractive therapeutic class but typically short duration of action and low bioavailability can limit their usefulness. Here, we begin evaluation of a new class of peptide modification, glycolipid surfactant conjugation, designed to extend the half-life of peptide therapeutics. This work illustrates ease of synthesis and conjugation, range of modulation of pharmacokinetic/pharmacodynamic behavior and acceptability *in vivo* of this approach. Proof of concept used parathyroid hormone and showed that an N-terminal fragment can be modified, informed by the parathyroid hormone receptor x-ray structure, to produce high potency, enhanced intrinsic efficacy and prolonged action *in vivo*. This suggests daily (hypoparathyroidism) or weekly (osteoporosis) administration with biological sequelae (stem cell maturation to osteoblasts) throughout the week. Compound **7** was chosen for advanced study. Exploration of the physical properties and development potential of glycolipid surfactant-modified peptides are underway with additional peptide therapeutics.

KEYWORDS
glycolipid surfactant, peptide prolongation, peptide-surfactant conjugate, PTH

1 | INTRODUCTION

Peptide analogs have been recognized as having attractive features as therapeutics (high specificity, high potency, and low inherent toxicity), but with substantial limitations (short duration of action, susceptibility to protease action, poor oral or transmucosal bioavailability), for many decades.[1] A wide array of approaches were undertaken to avoid these limitations, mainly focused on avoiding proteolysis (PEGylation,[2] D-amino acid substitution,[3] foldamer incorporation,[4] etc.).[5]

However, it was realized early on that a major mechanism for short duration of action of short peptides was filtration into the dilute urine in the glomerulus. We therefore focused on a different approach to analog design, a hypothesized "whole-body depot effect." Thus incorporation of very hydrophobic unnatural amino acids was hypothesized to prevent rapid glomerular filtration and proteolysis, due to hydrophobic depot formation,[6,7] with slowed release from the site of injection, and by binding to hydrophobic carrier proteins such as serum albumin (SA). The proof-of-concept research[8] resulted in very hydrophobic analogs of GnRH ([D-Nal(2)^6]GnRH, Nal(2) is 2-naphthylalanine; nafarelin, Synarel) with exceptionally high potency and prolonged duration of action relative to the native hormone and to comparators at that time. Subsequent studies demonstrated high protein binding, confirmed that this binding was due predominantly to SA affinity,[9] and showed significant intranasal bioavailability (2-5%).[10] Extension of this concept to hydrophobic peptides containing hypothesized membrane binding motifs[7,11] generated a very long-acting GnRH antagonist ([N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^3, D-hArg(Et$_2$)^6,hArg(Et$_2$)^8,D-Ala^{10}]GnRH; Pal(3) is 3-pyridyl-alanine, (hArg(Et$_2$) is Ng,Ng'-diethyl-homoarginine; ganirelix, Antagon).

Others have used SA binding very successfully to prolong the duration of action of peptides and proteins using fatty acid acylation. For example, myristoylation (insulin detemir[12,13]), palmitoylation

(liraglutide[14]) and acylation through spacers with octadecanedioic acid (insulin degludec,[15] semaglutide[16]) generated analogs with prolonged duration of action, allowing daily (QD) to weekly (QW) administration. Accordingly, both hydrophobic amino acid substitution and acylation with fatty acids can result in depot formation and substantial SA binding, to result in remarkable improvements in duration of action.[1] Modification of peptides by the addition of unnatural amino acids with long alkyl side chains, unrelated to the peptide sequence, in some cases associated with separate addition of carbohydrate, has been extensively investigated under the rubric of "lipidation"[17–19] with variable results.

While the "whole-body depot effect" paradigm was used successfully to prolong duration of action and diminish the issue of proteolysis, more recently we considered the question of improvement of transmucosal bioavailability. Although formulation enhancer excipients such as bile salts and other surfactants had shown large increases in nasal bioavailability during our studies for the commercialization of nafarelin (to F > 20%),[10] they were too irritating to the nasal mucosa for continuous use. More recently, metabolizable nonionic surfactants of the glycolipid class (e.g., 1-dodecyl-β-D-maltoside, Intravail) were reported to be less irritating and to impart important increases in intranasal bioavailability,[20] presumably by disrupting intercellular barriers to allow paracellular absorption. We hypothesized that while effects of formulation excipients might be lost quickly by dilution, conjugation of this structure class to peptides might bring continued, explicit surfactant character and have multiple additional favorable features—transient hydrophobic self-association to form micelles for slowed release from the injection site, high affinity to SA for duration, steric hindrance to the approach of proteases, disruption of the tight junctions between cells in the nose or gut due to their surfactant character, increased stability in formulations and favorable receptor interactions. Thus, we visualized the potential to improve the pharmaceutical character of peptides by multiple mechanisms, employing this previously unused class of modification. The question then became what would be the effect of directly conjugating such surfactants to peptides to prolong duration of action once in the circulation and as an approach to the disruption of tight intracellular junctions.

To select an appropriate framework on which to initiate testing of these hypotheses we considered parathyroid hormone (PTH). The PTH N-terminal active fragment (rhPTH(1-34)) was accepted as the sole bone anabolic agent available for the treatment of osteoporosis for a number of years,[21] although a related parathyroid hormone related-protein (PTHrP) analog[22] and other anabolic agents were added recently.[23] Additionally, a full length PTH protein (rhPTH (1-84)) was approved for treatment of hypoparathyroidism, favored for its somewhat longer duration of action.[24,25] Nonetheless there is a continued desire for improved PTH analogs with greater convenience and decreased incidence of side effects.[26]

We studied the binding interaction of PTH and PTHrP with their common receptor (PTH1R) which, as a Class B GPCR, has both a seven-transmembrane region responsible for G-protein activation and a long extracellular region that adds additional binding energy to the interaction (Figure 1, PDB code 3H3G).[27,28] For both hormones the amino terminal 1 to 14 residues bind within the juxtamembrane region while the 15 to 34 region forms a predominantly helical conformation that binds to a hydrophobic channel in the extracellular portion of this receptor. Critically important interactions between the hormones and the hydrophobic channel of PTH1R are those between ligand residues at positions 23' (Trp in PTH, Phe in PTHrP) and 24' (Leu in both).[27] Similarly, the residue at 28' (Leu in PTH, Ile in PTHrP) also projects into an extended gray hydrophobic channel of the receptor (Figure 1). We hypothesized that a truncated ligand (representing the amino terminal portion of PTH or PTHrP) conjugated to a glycolipid surfactant moiety (replacing the C-terminal 15-34 region of the hormone), might present the hydrophobic tail of the surfactant to the hydrophobic channel of the extracellular region of the PTH1R, providing additional binding energy and generating a potent agonistic ligand. We undertook a proof-of-concept program in order to initiate a preliminary validation of these several hypotheses. As a new, fully virtual startup company we used contract research organizations (CROs) for all research.

The varied glycolipid surfactant reagents[29] (Scheme 1) were generated from the corresponding non-ionic glycolipid surfactants, some of which are widely used in the industry and considered to be generally recognized as safe (e.g., 1-octyl β-d-glucoside; 1-dodecyl β-d-

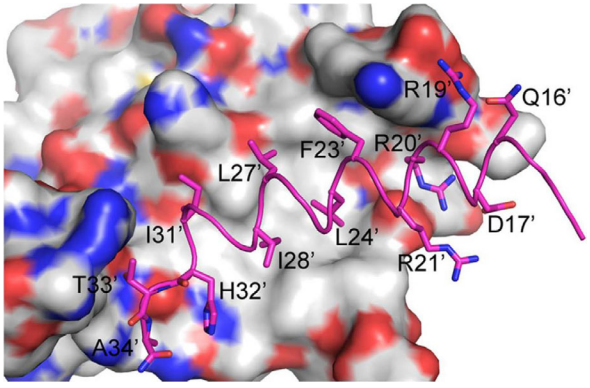
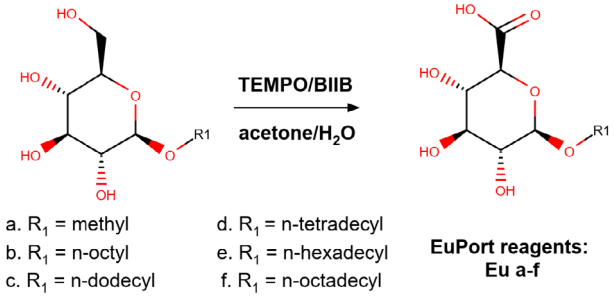


FIGURE 1 Structure of the parathyroid hormone R1 receptor with bound PTHrP-(12-34) (PDB code 3H3G). The molecular surface of the extracellular domain shows the hydrophobic groove contacted by Phe-23', Leu-24', Leu-27', and Ile-28' of PTHrP. The surface is colored according to atom type: gray for carbon, blue for nitrogen, and red for oxygen. Note the gray hydrophobic receptor pocket which extends from the binding region of Phe-23' to that of Ile-31' of PTHrP. The analogs studied are based on the hypothesis that the hydrophobic tail of the conjugated surfactant can bind to this region of the receptor and generate additional binding energy thereby, in order to increase the potency and intrinsic efficacy of the novel ligands. This Figure 1 is reprinted with permission and was originally published in the Journal of Biological Chemistry, Pioszak, AA; Parker, NR; Gardella, TJ; Xu, HE. "Structural Basis for Parathyroid Hormone-related Protein Binding to the Parathyroid Hormone Receptor and Design of Conformation-selective Peptides" J. Biol. Chem. 2009; 284:28382-28 391. ©The American Society for Biochemistry and Molecular Biology. Permission conveyed through Copyright Clearance Center, Inc

maltoside). The desired carboxylic acid reagents for conjugation are readily available through chemoselective 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO)-mediated oxidation, in the presence of water,[30] of the primary OH group(s) on such surfactants. The typical glycolipid structure therefore comprises 1-alkyl β-D-glycopyranosiduronic acids (alternatively, 1-alkyl β-D-glucuronic acids), a type of structure used extensively by the liver for solubilization and detoxification of hydrophobic molecules (phase II metabolism). Additional surfactants, including those with a disaccharide head group (maltoside, melibioside), were prepared similarly and evaluated (data not shown).



SCHEME 1 Preparation and structures of the glycolipid surfactant conjugation reagents used for modification of the peptides evaluated herein

We noted a minimized, optimized N-terminal PTH fragment[31] that appeared to have full intrinsic activity. In order to test the effects of our new modifications on pharmacokinetic (PK) and pharmacodynamic (PD) behavior we chose to modify peptides similar to this sequence with 1-alkyl β-D-glucuronic acid reagents of varying 1-alkyl substitution length (Scheme 1 and Table 1), acylated onto the ε-amino function of a Lys residue in the peptide sequence. Thus, we report shortened PTH fragments with high human PTH1R-activating potency (pEC_{50}), apparent super-agonistic efficacy (maximal cAMP accumulation >100%) and prolonged duration of action in rats.

2 | MATERIALS AND METHODS

Peptide synthesis and purification was carried out at CS Bio Co (Menlo Park, CA). Human cell line experiments were carried out at Cerep (Le Bois l'Evêque, France; now Eurofins Cerep).

2.1 | ETHICS STATEMENT

In vivo characterization in rats was carried out at AAALAC-accredited PharmaLegacy Laboratories (Shanghai, China) with a bioassay protocol modeled on a Lilly study.[34] All animal procedures were in compliance

TABLE 1 Structures and *in vitro* biological screening data for analogs on SaOS2 cells

Cmpd. Number	Analog structure[a]	PTH1R potency pEC_{50}[b] (SE)	Maximum response[c] (SE) %
	PTHrP (1-34)—assay internal standard	Circa 9.09-9.00	100
	hPTH (1-34)—submitted as sample	8.48 (0.06)	90 (3)
	[AC_5C^1,Aib^3,M]PTH (1-14)[d]	{8.92}[d]	{82 (12)}[d]
1	[AC_5C^1,Aib^3,Nle^8,I^{15},Z***c***^{16},M]PTH	7.17 (0.21)	115 (9)
2	[AC_5C^1,Aib^3,Nle^8,F^{14},I^{15},Q^{16},Z***a***^{17},M]PTH	7.23 (0.04)	114 (2)
3	[AC_5C^1,Aib^3,Nle^8,F^{14},I^{15},Q^{16},Z***b***^{17},M]PTH	7.50 (0.13)	124 (7)
4	[AC_5C^1,Aib^3, Nle^8,F^{14},I^{15},Q^{16},Z***c***^{17},M]PTH	7.4 (0.10)	131 (6)
5	[AC_5C^1,Aib^3, Nle^8,I^{15},Q^{16},Z***c***^{17},M]PTH	7.66 (0.18)	132 (8)
6	[AC_5C^1,$Aib^{3,17}$, Nle^8,I^{15},Q^{16},Z***c***^{18},M]PTH	7.30 (0.29)	>110
7	[AC_5C^1,$Aib^{3,17,19}$, Nle^8,I^{15},Q^{16},Z***c***^{18},M]PTH	8.75 (0.33)	130 (7)
8	[AC_5C^1,$Aib^{3,17,19}$, Nle^8,I^{15},Q^{16},Z***e***^{18},M]PTH	8.9 (0.50)	127 (6)
9	[AC_5C^1,$Aib^{3,17,19}$, Nle^8,I^{15},Q^{16},Z***f***^{18},M]PTH	>9	129 (5)
10	[AC_5C^1,$Aib^{3,17,21}$, Nle^8,I^{15},Q^{16},L^{18},Z***c***^{19}R^{20},M]PTH	8.48 (0.15)	130 (3)

Note: Z(***y***) indicates a surfactant-acylated Lys residue where ***y*** indicates length of alkyl substitution on 1-alkyl β-D-glucuronic acid. Z***a*** = Me; Z***b*** = C8; Z***c*** = C12; Z***d*** = C14; Z***e*** = C16; Z***f*** = C18.

Abbreviations: Aib, α-aminoisobutyric acid; AC_5C, 1-aminocyclopentane-1-carboxylic acid; hR, homo-arginine.

[a]Structures are analogs of [AC_5C^1,Aib^3,M]PTH (1-14),[31] which is an analog of the minimized [M]PTH (1-14),[32] [$A^{1,3,12}$,Q^{10},hR^{11},W^{14}]ratPTH (1-14). See detailed structures in the Supporting Information.

[b]Potency (pEC_{50}, -logEC_{50}) measured as percent of maximal cAMP accumulation in hSaOS2 cells achieved by internal standard (PTHrP) *in vitro* following stimulation with increasing concentrations of test article in single assay, in duplicate, at Cerep. This osteosarcoma cell line natively expresses the human PTH1 receptor.[33] Nonlinear regression analysis was performed on raw data in Prism 5 using standard equations (three parameter fit or variable Hill slope, to best fit). See experimental and Supporting Information for additional discussion and all fitted curve plots.

[c]Maximum response gives mean and SE of the top of curve range as given by Prism for production of cAMP as a percentage of the maximum response of internal standard PTHrP as 100%.

[d]Data calculated from table 2 in the study by Shimuzu *et al*[31] (EC_{50} = 1.2 ± 0.8 nM) for transfected HKrk-B28 cells, so analog likely would be less potent on natively-expressed PTH1R on SaOS2 cells.

with applicable animal welfare acts and were approved by the internal Institutional Animal Care and Use Committee (IACUC) of PharmaLegacy.

2.2 | Glycolipid reagent synthesis

The starting material for the modified glycolipid surfactant reagents (termed EuPort reagents) were commercial nonionic surfactants obtained from Anatrace, Inc. (Maumee, OH). Thus 1-alkyl β-D-glucosides with 1-methyl, 1-octyl, 1-dodecyl, 1-hexadecyl and 1-octadecyl chains were submitted to selective oxidation on the 6-position primary OH function at CS Bio Co. (Menlo Park, CA), to yield the desired 1-alkyl β-D-glucuronic acid reagents. This mild and very clean, chemoselective oxidation used TEMPO as the electron transfer agent and near stoichiometric amounts of the organic oxidant [bis(acetoxy)-iodo]benzene (BAIB), in the presence of water, to yield the corresponding glucuronic acids, with only volatile acetic acid and iodobenzene as reagent byproducts.[30] Following stripping to dryness, the crude glucuronic acid reagents were used directly for coupling to the desired lysine residue ε-amino function without protection of the glucuronic acid secondary OH groups. Sodium hypochlorite can be used alternatively as the stoichiometric primary oxidant.

2.2.1 | 1-Alkyl β-D-glucuronic acids. General oxidation method

To a solution of 1-dodecyl β-D-glucopyranoside (2.0 g, 5.74 mmol) in 20 mL of acetonitrile and 20 mL of deionized water is added BAIB (4.4 g, 13.7 mmol) and TEMPO (0.18 g, 1.15 mmol). The resulting mixture was stirred at room temperature until reaction completion (up to 20 hours) by tlc. The reaction mixture was diluted with water at pH ca. 3 and lyophilized to dryness to give crude product as a white powder (1.52 g, 73%) of sufficient purity for direct use in coupling to the peptide Lys side chain. In a like manner were prepared the other 1-alkyl β-D-glucuronic acids used to acylate the other peptide products described herein. For more hydrophobic molecules, additional organic solvent may be used.

2.3 | Peptide synthesis

Peptides were prepared as C-terminal amides at CS Bio Co., using standard Fmoc peptide synthesis protocols and employing t-butyl based side chain protection on Rink-Amide resin. In each case an Fmoc-Lys(ε-ivDde)-OH residue was incorporated at the desired conjugation position. The Lys side chain amine of this residue was deprotected by use of 4% hydrazine hydrate in DMF for 4 hours as the penultimate synthesis step. The free Lys side chain was modified by acylation with the appropriate EuPort reagent (6' carboxylic acid) using diisopropylcarbodiimide/1-hydroxybenzotriazole or similar catalyst for ca. 12 hours, or until completion by Kaiser test (ninhydrin). Final deprotection and resin cleavage was carried out using the standard trifluoracetic acid (TFA) cleavage cocktail (TFA/H_2O/triisopropylsilane/1,2-ethanedithol; 94:2:2:2) for 240 minutes at room temp. The mixture was filtered and the filtrate treated with Et_2O to precipitate the product, which was washed extensively with Et_2O and dried in vacuo to yield the crude product. Purification was carried out on preparative reversed-phase liquid chromatography (C18) columns using a buffer gradient (CH_3CN in 0.1%TFA; pH circa 2) from 5% to 70% CH_3CN. Product containing fractions were combined, neutralized, concentrated and lyophilized. Analytical characterization for purity was carried out on an Agilent hplc system using Phenomenex Luna C18, 5 μ 100 Å pore, 250 × 4.6 mm columns at 20 °C and employing various gradients appropriate for the structures (see footnote to Table S3; Figures S1-S10). Retention characteristics are reported as capacity factor values, k' [$k' = (t_R\text{-}t_0)/t_0$)]. All compounds were > 95% pure by hplc area integration. Peptides were characterized for identity and incorporation of the glycolipid modifier by LC-MS/MS (see Table S3).

2.4 | *In vitro* characterization

Human cell line experiments were carried out at Cerep (Le Bois l'Evêque, France; now Eurofins Cerep). This routine assay is listed in their *In Vitro* Pharmacology: Cellular Functional Assays as PTH1, utilizing human osteosarcoma cell line SaOS2 cells (i.e., native PTH1R expression) and is modeled on work by Orloff *et al.*[33] In each experiment, the reference compound (PTHrP (1-34)) was tested on duplicate cell populations concurrently with the test articles and the data were compared with historical values determined at Cerep. If the experiment was acceptable in accordance with Cerep's validation Standard Operating Procedure, the data were reportable. The results were reported as a percent of control specific agonist maximal response ((measured specific response/control specific agonist response) × 100) and, for antagonists, as a % inhibition of control specific agonist response (100 - ((measured specific response/control specific agonist response) × 100)) obtained in the presence of test compound.

The EC_{50} value (concentration producing a half-maximal specific response) was determined by interpolation from nonlinear regression analysis of the concentration-response curves generated with mean duplicate values using Hill equation curve fitting ($Y = D + [(A - D)/(1 + (C/C_{50})^{nH})]$, where Y is the specific response, D is the minimum specific response, A is the maximum specific response, C is the compound concentration, and $C_{50} = EC_{50}$ or IC_{50}, and nH = slope factor). This analysis was performed using a software developed at Cerep (Hill software) and validated by comparison with data generated by the commercial software SigmaPlot 4.0 for Windows (© 1997 by SPSS Inc.).

The nonlinear regression analysis of the cAMP responses also was carried out in Prism 5 and the logEC_{50} with SE values were by determined using the standard Prism log agonist vs response (three

parameters, Hill slope 1) or log agonist vs response variable slope (constrained to Hill slope < 2) methods, depending on best data fit. The goodness of fit was evaluated as R^2; in almost all cases >>0.90 (see Supporting Information for curve plots and summarized data). Test compounds were evaluated once, on cells in duplicate, and data are reported as pEC_{50} (the -log EC_{50}). For super-agonist compounds this is 50% of curve top, so slightly underestimates potency as measured by concentration showing 50% of maximal response of internal standard.

Dissolution of the more hydrophobic compounds, such as ≥C12 containing chains, required dissolution in water first (reaching pH near 3, due to TFA salt), then dilution with assay buffer containing 0.1% BSA in order to achieve dissolution without loss of peptide.

2.5 | *In vivo* characterization

Male Sprague Dawley rats (Sino-British SIPPR/BK Lab) of 8 to 10 week age (220-250 g) were purchased by PharmaLegacy Laboratories (Shanghai, China) and allowed to adapt for 7 days in the vivarium with room temperature 19 to 26 °C, relative humidity 40 to 70%, 12 hours. fluorescent light (8:00-12:00) and dark cycle, at two rat per cage housing density and with free access to rodent food (irradiated) and filtered municipal tap water. Groups of five animals were injected once sc with saline, compound **7** at 80 or 320 μg/kg, or hPTH (1-34) at 80 μg/kg, the latter from Bachem (H-4835.0001). Injection was done at 16 or 64 μg/mL concentration as appropriate to dose, actually five times more dilute than per protocol. Blood samples (circa 400 μg/sample) were taken via retro-orbital vein from rats after anesthesia by isoflurane at predose, 0.5, 1, 2, 4, and 5 hours post-dose, and placed in tubes on ice until centrifuged (10 minutes at 5000 rpm at 4 °C), within 1 hour of collection. Serum was transferred into labeled polypropylene tubes, protected from light and stored in a −80 °C freezer. Animals were euthanized after the final blood sample by CO_2 inhalation followed by cervical dislocation and discarded without necropsy. No treatment-related signs were noted and all animals survived to scheduled study termination. Blood phosphate and calcium levels were determined by Hitachi 7020 Automatic Analyzer using protocols received with the corresponding reagents. GraphPad Prism software (version 5) was used to conduct the statistical data analysis, performing Analysis of Variance (ANOVA) followed by Bonferroni test with $P < .05$ as the minimal level of significance. The means ± SEM are given in the Supporting Information.

3 | RESULTS AND DISCUSSION

3.1 | PTH analog design

All compounds were analogs of a "minimized" parent PTH sequence, [M]PTH(1-14).[32] Note that M here means minimized, not the amino acid Met. An analog containing an AC_5C residue in position 1 was substantially more potent[31] than the parent [M]PTH(1-14) and that became the actual parent of this series (Table 1), but further modification was made by replacement of Met^8 by Nle^8, in order to prevent facile oxidation. The glycolipid surfactant reagents used (Scheme 1 and Table 1) are 1-*n*-alkyl β-D-glucuronic acid moieties with varying 1-alkyl substitution length and, when acylated onto a Lys residue, they are designated as Z***y***, signifying a Lys(ε-(1-alkyl β-D-glucuron-6-yl)) residue, where ***y*** designates the alkyl chain length (Z***a*** = Me; Z***b*** = octyl; Z***c*** = dodecyl; Z***d*** = tetradecyl; Z***e*** = hexadecyl; Z***f*** = octadecyl). For the parent surfactants, it is well known that the length and hydrophobicity of the alkyl chains vary the general physical and surfactant properties[35,36] such as solubility in water, liquid crystal behavior, critical micelle concentration (CMC), micelle size, affinity for SA, and so on.[37] By extension, these properties are expected to the extend to the peptide conjugates (to a greater or lesser extent, depending on peptide size and placement) with clear potential to substantially affect, in a tunable way, solubility in presence of SA, PK/PD behavior *in vivo*, receptor affinity and kinetics, protease resistance, permeability across mucosal membranes, and so on. It was not feasible to investigate all of these aspects at this stage of preliminary proof-of-concept research, but such studies remain of substantial scientific interest.

3.2 | *In vitro* characterization

The analogs are based on [AC_5C^1,Aib^3,Nle^8,M]PTH (1-14) (see Table S1 for detailed diagram of structures) and were evaluated for potency (pEC_{50}) and efficacy (maximal cellular accumulation of cAMP as a % of maximal effect for internal standard, PTHrP(1-34)) in screening assays on a human osteosarcoma cell line (SaOS2), natively expressing PTH1R (Table 1).[33] Extension of the parent structure into the desired binding region by a Leu^{15}-Lys^{16} provided a site for acylation of the Lys at position 16 with a hexadecyl glycolipid reagent (Z***c***) resulting in compound **1** ([AC_5C^1,Aib^3,Nle^8,I^{15},Z***c***^{16},M]PTH) which showed weak potency (pEC_{50} = 7.17), but already is indicating slightly higher maximal efficacy for cAMP accumulation (115%) compared to PTH itself. Testing a slighter longer peptide chain and the replacement of the Trp^{14} with Phe^{14} resulted in moderately potent analogs and the efficacy increased with surfactant chain length (methyl, octyl, dodecyl; compounds **2**-**4**), with a maximum efficacy of 131% with the dodecyl moiety (**4**; Table 1). The Trp^{14} residue (compound **5;** pEC_{50} 7.66) gave a more potent analog than the Phe^{14} (compound **4**). Stepping out an additional residue and incorporating an Aib at position 17 yielded a weaker analog (compound **6**), but addition of an Aib at both residues 17 and 19 gave a sharp increase in potency and efficacy (compound **7**). Compound **7** had a pEC_{50} = 8.75, near equivalency to full length hPTH(1-34) and with apparent super-agonist efficacy, 130% of the internal standard (Figure S15). We interpret this as likely a combination of helix stabilization by the Aib residues and slowed receptor off-rate, resulting in super-agonistic activity for cAMP formation. Hydrophobic analogs in the GnRH area similarly have shown super-agonistic activation of second messenger systems, attributed to slowed receptor kinetics.[38] It is very interesting to note that when analogs **2**, **3**, **4** were tested for antagonist character, in the presence of 10 nM

PTHrP (1-34) internal standard, they gave substantially increased maximal efficacy values compared to those achievable by PTHrP alone (124%, 121%, 147%, of the PTHrP response, respectively), supporting the super-agonist concept discussed here (Figure S13).

Further lengthening the alkyl chain and the glycolipid surfactant character, resulted in the hexadecyl and octadecyl-substituted analogs (compound **8** and **9**, respectively) which exhibited increased potency (pEC_{50} of 8.9 and >9, respectively) and continue the super-agonist activity trend (circa 127-129%). Further lengthening the peptide sequence by 4 residues (**10**) did not increase potency (Table 1) but maintained super-agonist efficacy (130%). Homologous compounds **7**, **8**, **9** (surfactant *n*-alkyl chain length C12, C16, C18, respectively) appear to offer a spectrum of molecules that present very high potency, equal or superior to that of hPTH(1-34) and PTHrP(1-34), and with super-agonistic efficacy on PTH1R in a native expression format (Figure 2). Although only **7** was tested *in vivo*, compounds **8** and **9** are expected to have significantly longer PK profiles, based on historical precedent,[39] and due to their increased side chain length and hydrophobicity, which will likely result in tighter SA binding.

The objective with these modifications was to demonstrate the effect of varying the chain length of the surfactant as this is known to affect the CMC, solubility and micellar behavior of such surfactants.[36,37] The duration of action *in vivo* is anticipated to vary with chain length, with longer chains expected to show slower uptake and tighter binding to SA, resulting in lower Cmax and longer $T_{1/2}$, although this has not yet been evaluated in detail for this series due to funding limits. Further variation in physical properties and PK profile are possible by variation of the surfactant head group (e.g., mono vs disaccharide; α or β configuration) and alkyl tail group functionalization.[40] Compound **7** was considered a highly interesting molecule with reduced size, higher hydrophobicity and surfactant character while maintaining high potency, near that of hPTH(1-34), coupled with super-agonist activity. Historically such physical properties also have led to increased transmucosal bioavailability. Accordingly, its PD behavior was examined *in vivo*.

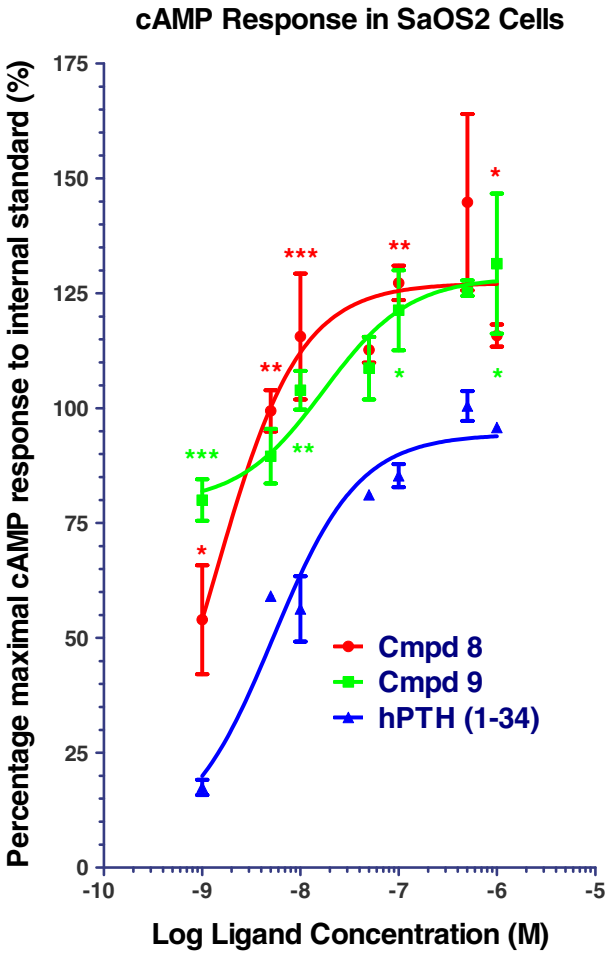


FIGURE 2 Cellular assay results showing potency and efficacy of selected analogs on cAMP response (in duplicate) in SaOS2 cells (human osteosarcoma cell line), natively expressing PTH1R. Here data for analogs, and for hPTH(1-34) submitted as blinded sample, are plotted from a single experiment, in duplicate. Compound **7** pEC_{50} (8.75) was essentially equipotent to hPTH(1-34) when submitted as a sample in parallel (pEC_{50} = 8.48). Here the homologous compound series had the surfactant modifier n-alkyl chain extended from C12 in **7**, to C16 and C18 for **8** and **9**, respectively, with pEC_{50} of 8.9 and > 9, respectively. Maximal response >100% is referred to herein as "super-agonist" activity and is consistently seen for surfactant alkyl chains >C12 in this study (see Table 1, Figure 2, and curves Figures S11-S15 presented in the Supporting Information). GraphPad Prism software (version 5) was used to conduct the statistical data analysis, performing ANOVA followed by Bonferroni tests with $P < .05$ as the minimal level of significance. Compared to hPTH: $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

3.3 | *In vivo* characterization

A convenient *in vivo* characterization assay makes use of the hypophosphatemia response to PTH(1-34) in rats.[34] In that assay, an effective dose of hPTH(1-34) causes a slow drop of serum phosphate to a nadir at 60 minutes, with return to normal levels by 240 minutes (Figure S16). For our study (Figure 3 and Table S2), there is an initial response to the dilution by a large injection bolus that blocks the effect normally seen at 80 μg/kg, presumably due to the ×5 lower than protocol-specified formulation concentration at the CRO. However, the ×4 higher dose of compound **7** gives a strong and very long-lasting hypophosphatemia effect (through end of assay at 5 hours; see Figure S17 for plot of individual animal data), supporting the analog design hypothesis. The PK profile concept desired here was for a single dose to provide a ca. 24 hours infusion-like effect in humans, and this PD profile in the rat suggests achievement of that target. Such a profile might be useful for treatment of hypoparathyroidism, where infusion gives superior treatment and quality of life compared to daily or multiple daily doses.[41] Interestingly, a 24 hours infusion of PTH (1-34) once weekly in rat models of osteoporosis provides an anti-osteoporotic effect similar to that of daily injection of PTH(1-34).[42] Such a treatment course (24 hours infusion-like effect; 6 days off during stem cell maturation into osteoblasts) might offer advantages for convenience and efficacy in humans, and might also result in

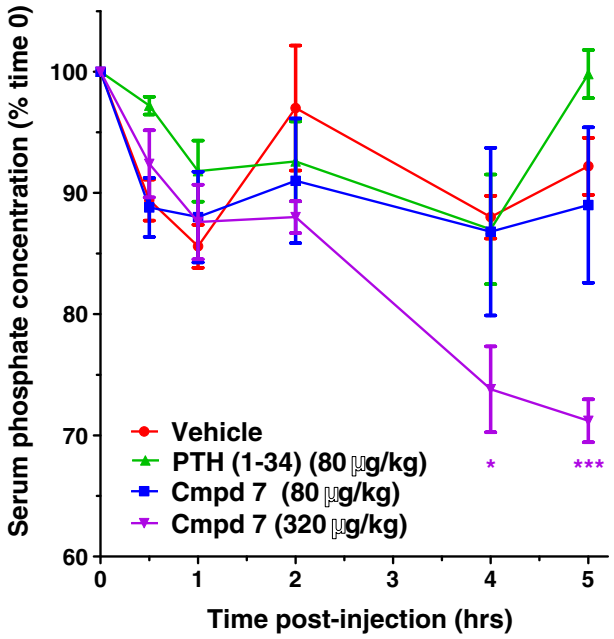


FIGURE 3 Serum phosphate response to hPTH (1-34) in male Sprague Dawley rats (n = 5). While neither PTH (1-34) nor compound **7** gave a statistically significant response at 80 µg/kg., a fourfold higher dose gave a strong and long-lasting decrease in serum phosphate lasting through the final assay time point at 5 hours. GraphPad Prism software (version 5) was used to conduct the statistical data analysis, performing ANOVA followed by Bonferroni test with $P < .05$ as the minimal level of significance. Compared to vehicle, $^{*}P < .05$; $^{***}P < .001$

decreased potential for the osteosarcoma seen with PTH in rat chronic toxicology.[43] There is speculation that differing receptor kinetics for different PTH1R conformations and G protein status may offer particular advantages for selected applications,[44] but this will require more detailed *in vivo* studies.

There has been speculation that PTH binds to PTH1R through capture of the C-terminal region of PTH by the extracellular receptor domain[45] (3D-2D search), followed by binding of the N-terminal region of PTH to the juxtamembrane region, causing activation of intracellular signaling through this latter interaction. However peptide structure is typically disordered in water and we hypothesize that the opposite sequence of events is more likely, at least for these truncated analogs. This visualizes adoption of the biologically active conformation (bent helix) on the cell membrane surface, as hypothesized for peptide ligands by Robert Schwyzer (Membrane Compartments Theory),[46] loading of the integral membrane region of PTH1R through 2D-2D search ("reduced dimensionality"),[47] followed by interaction of the peptide hormone's C-terminal region with the receptor extracellular region, giving additional binding energy and potentially altered receptor binding kinetics. The binding sequence for truncated analogs such as **7**, where virtually the entire C-terminal region of the ligand is removed, yet full potency is achieved, makes initial binding to the receptor extracellular region seem unlikely.

This work comes from a line of investigation[1] focusing on protection of peptides from clearance due to glomerular filtration by using various mechanisms— hydrophobic depot formation at site of injection by self-association, transient binding to serum albumin,[9] enhanced binding to cell membranes,[7] and blocking of proteolysis, which are now extended using surfactant conjugation to bring additional flexibility and, possibly, breadth of application (surfactant-assisted transmucosal bioavailability[20]). While alternative lipid acylation approaches may or may not bring surfactant-like character to peptides, dependent on design/placement, glycolipid surfactant conjugation does so explicitly, with a single moiety.

In view of the significant effect of native glycosylation on targeting, structure and stabilization of peptides[48] and proteins, a number of instances of glycosylation have been carried out on peptides.[49–52] However while glycosylation can have positive (blockade of proteolysis, increased solubility, altered selectivity, modest increases in duration of action) and negative effects (decreased potency), it is not conducive to substantial blockade of clearance by glomerular filtration since there is no provision of depot formation or protein binding to hinder that route. An instance of lipidic amino acid modification and glycosylation in a series of endorphin analogs was reported, but at separate sites on the peptide.[53] Other instances of lipidation of peptides accompanied by glycosylation similarly present separated units of lipid and hydrophobic character,[54] while explicit surfactant conjugation as shown here presents a variable hydrophobic tail rigidly mounted and presented directly to the solvent or binding partner. After the completion of this work a "neoglycolipid" modification[55] was invoked (essentially a short linear polyhydroxyl chain as part of a spacer, however not containing a directly bonded, lipid-sugar moiety, so not a glycolipid, per se) but there appears to be no particular function demonstrated for this unit regarding physical properties, presentation, rigidification, recognition or binding.

Similarly, after completion of this work, we became aware of reported favorable effects of incorporation of *N*-acetyl-D-glucosamine (GlcNAc) at certain sites in PTH(1-34) (positions 14, 18, 22, 26).[56] Although there apparently was greater than 100% efficacy shown on a cell line overexpressing hPTHR1 (GP-2.3), this increased efficacy appeared to be not significant on cells endogenously expressing the receptor (cell line SGS-72; SaOS2 derived). In contrast, the superagonism reported herein was on natively expressed hPTH1R (on human SaOS2 cells). One would expect GlcNAc modification to have some stabilizing effect due to potential blockage of some proteolysis (site and protease dependent), but only modest and nonflexible effects on prolongation of action of peptides *in vivo*.

4 | CONCLUSION

A primary goal of this research was the proof of concept and initial evaluation of the potential of a new class of peptide modification, glycolipid surfactant conjugation, for improving the pharmaceutical properties of peptides (potency, efficacy, duration of action, solubility, stability and bioavailability). As hypothesized, this resulted in analogs with a range of receptor activation, with EC_{50}s in the low nM range (Table 1), many with apparently greater intrinsic efficacy for activation

of the PTH1R on cells compared to PTHrP(1-34), the internal assay standard (> than 130% of the efficacy of the internal standard, as measured by cAMP production on the natively expressed hPTH1R on SaOS2 cells). Prolonged duration of action in a rat model also was observed. These data suggest an ability to give more prolonged coverage, akin to infusion, from daily administration at low dose (hypoparathyroidism) or perhaps once weekly administration (osteoporosis), from a larger, single dose, with biological sequelae (stem cell maturation to osteoblasts) lasting throughout the week.

These results with glycolipid surfactant conjugation, and their successful extension[40] to the GLP-1/glucagon receptor dual agonist space (see ALT-801, in clinical development), illustrate the ease of synthesis and incorporation, the retention of high or increased efficacy, a range of modulation of PK/PD behavior and its acceptability *in vivo*. Additional exploration of the physical properties and drug development potential of surfactant-modified peptides is underway and in planning.

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CONFLICT OF INTEREST

John J. Nestor owns shares in EuMederis Pharmaceuticals, Inc. and holds patents related to the peptide analogs and method of use of EuPort glycolipid surfactant-modified peptides.

AUTHOR CONTRIBUTIONS

John J. Nestor conceived and designed the EuPort glycolipid surfactant reagents and peptide analogs, synthetic routes, bioassays; interpreted data, wrote and approved manuscript. Wei Wang supervised peptide syntheses, adapted the published oxidation method, reviewed and approved manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supporting Information of this article

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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