Investigations from the Belly of the Beast: N-Terminally Labeled Incretin Peptides That Are Both Potent Receptor Agonists and Stable to Protease Digestion

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Type 2 diabetes is an ever more common human disease, which has as its hallmark an inability of the body to properly maintain glucose homeostasis. If left unchecked, it can lead to heart disease, vision loss, and kidney disease; at this particular moment in history, it is also important to note that this condition is correlated with a greater risk for severe illness from COVID-19. Typically, when a healthy person digests a meal, peptide hormone glucagon-like peptide-1 (GLP1) is introduced into the bloodstream and goes on to activate, in several tissues, its cognate receptor GLP1R, initiating numerous events that work to ensure a constant blood sugar level. Thus, when this carefully orchestrated system breaks down, peptides related to GLP1 serve in the clinic as injectable therapeutics to manage the disease. The Achilles' heel of this class of active compounds, however, is susceptibility to cleavage by proteases and thereby reduced half-lives in patients. Protease sensitivity is frequently a stumbling block for the application of peptide-based drugs. The way this looks on the ground is that the top three clinically applied GLP1R agonists, exenatide, liraglutide, and semaglutide, require twice daily, once daily, and weekly injection schedules, respectively, representing an inconvenience for patients in the best case. Thus, the search for the holy grail of peptide therapeutics must continue, namely, a general strategy for modifying peptides to impart resistance to proteolysis while enabling retention of excellent receptor agonist potency. Or, perhaps, the search is over. In this issue of *ACS Central Science*, Kumar and coauthors[1] report how an N-terminal trifluoroethyl modification fits the bill, not only in the case of GLP1/GLP1R/DPP4, but also for other related peptides, receptors, and proteases.

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From the perspective of design, Kumar and coauthors began with the seemingly straightforward task of imparting protease stability to GLP1, a problem that other groups had tackled with success in the past, albeit almost universally at the expense of receptor agonist potency. DPP4, the greatest stumbling block to the therapeutic success of GLP1, is a serine protease that utilizes two active site glutamate residues to bind the positively charged N-terminus of active GLP1(7-37) and then hydrolytically remove the His7Ala8 dipeptide to yield inactive GLP1(9-37). The Kumar paper cites work from 20 years ago by Flatt et al.[2] and Wheeler et al.,[3] as well as a more recent patent[4] and paper from 2016 by Sexton et al.,[5] all of which explored various types of N-acylation as a successful strategy to confer resistance to DPP4 and also observed disappointing concomitant hits to agonist activity. Operating under the hypothesis that the acylated N-terminus may be refused by GLP1R because it appears to the receptor to be an improperly processed agonist, Kumar and coauthors instead opted for an N-alkylation strategy. The authors then further explored considerations about how N-terminal agonist modifications may impact meaningful binding of the agonist deep within the receptor, the belly of the beast, as the authors figuratively describe. Here the jumping off point was the impressive cryo-EM structure of a GLP1-GLP1R complex (PDB: 5VAI) solved in 2017 by Skiniotis and co-workers.[6] Unfortunately, though this structure clearly reveals the manner in which the receptor makes contact with the N-terminal His7 side chain (namely, a cation-π interaction with R299^{ECL} and hydrogen bonding to W306$^{5.36}$ and I309$^{5.39}$), no obvious direct contact to the N-terminus is observed to help specifically
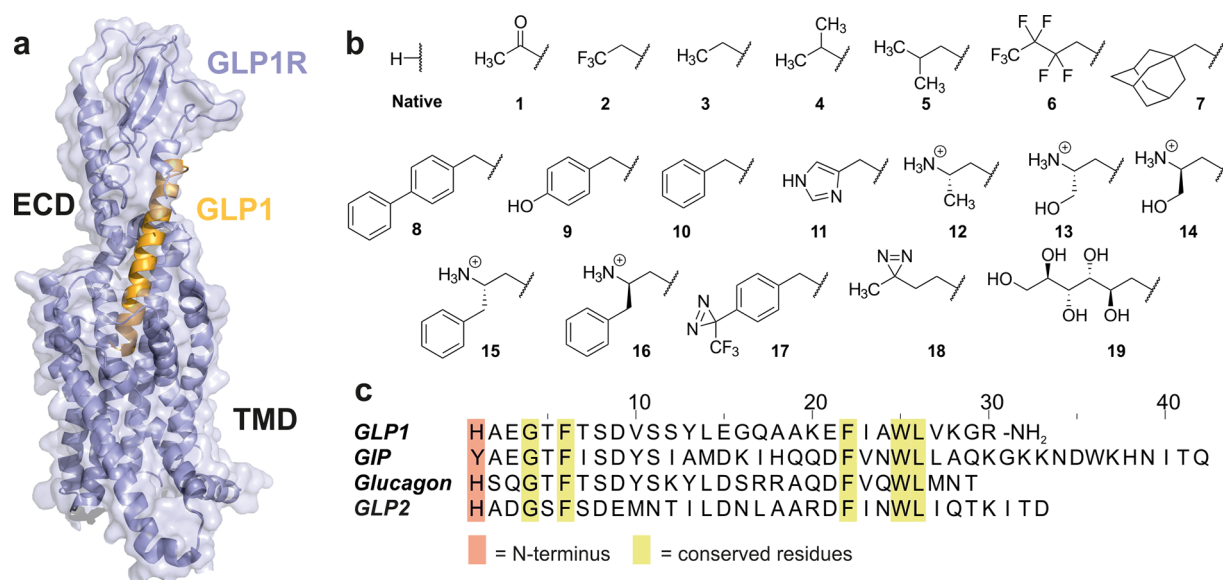
Figure 1. The Kumar study at a glance. (a) Prior cryo-EM structure of GLP1-GLP1R complex (PDB: 5VAI).[6] (b) N-Terminal modifications investigated. (c) Alignment of therapeutic peptides. Adapted with permission from ref 1. Copyright 2020 The Authors. Published by American Chemical Society.

direct the design of the covalent modifications. Thus, an appreciable and diverse library of 18 alkyl modifications was designed and investigated in this work, numbered consistently with the peptide prefixes used throughout the text, which are shown in Figure 1.

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Kumar and coauthors convincingly show that N-alkylation, most thoroughly investigated in the case of the trifluoroethyl group, of not only GLP1, but also of glucagon-like peptide-2 (GLP2), glucagon, glucose-dependent insulinotropic polypeptide (GIP), and the clinically administered liraglutide, imparts protease resistance while enabling retention of agonist potency against their cognate receptors. For example, using HEK293 cells expressing receptors GLP1R, GIPR, GCGR, or GLP2R corresponding to peptides GLP1, GIP, glucagon, and GLP2, respectively, and a luciferase reporter system, it is shown that, whereas the potency of GLP1 toward GLP1R upon incubation with protease DPP4 drops from 3.5 to 2.8×10^3 pM, the potency of N-trifluoroethyl-GLP1 after similar incubation is found to remain at the very high level of 5.4 pM. Importantly, no trans or off target activation is observed. Excellent potencies and DPP4 resistance are shown almost

universally across the N-alkyl library, with all modified GLP1 species possessing single digit pM activation, except in the cases of the sterically bulky biphenyl (17.6 pM) and trifluoromethylphenyl diazirine (41.6 pM) groups, or due to stereochemical preference (L-serine, 25.3 pM) in one case; it is important to note that even though potency is somewhat compromised with the latter modifications, these variants still perform better than N-acetyl GLP1 for GLP1R activation. The study is nicely rounded out by demonstrations of protease stability of N-trifluoroethyl GLP1 against related proteases DPP9 and FAP, both members of the S9B prolyl oligopeptidase subfamily; no change in potency was observed subsequent to incubation in the presence of these enzymes. Finally, the results of in vivo glucose tolerance tests in mice and serum stability assays in rats strongly support the therapeutic utility of the N-trifluoroethyl group.

The results shown by Kumar and coauthors are very exciting indeed from the perspective of a simple and easy to introduce N-terminal modification, in particular N-alkylation, which is able to broadly confer protease stability and enable retention of agonist potency within the secretin family of receptors and their ligands. This approach is an impressive addition to the chemical toolbox that already houses strategies such as cyclization, unnatural building blocks, staples, depsipeptides, and lipidated side chains, among others.[7] Although N-alkylation may not prove to be a panacea in the field of receptor-based therapies, due to limitations that the authors themselves point out with respect to their results with the δ-opioid receptor, it certainly must from now on be

considered when imparting protease stability is called for, at least to exopeptidases.

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Beyond this particular field, however, we feel that N-alkylation must also be investigated with respect to myriad other peptide properties that are key for function: transport, trafficking, structure stabilization,[8] oligomerization state, and the likelihood of changing a peptide for injection into one that can be orally administered, an important and growing field of study.[9] Especially considering the observation here that receptor activation, long thought to be sacrosanct, can tolerate such a broad variety of N-alkyl groups, such covalent modification can be expected to impart benefits to these other properties as well. Furthermore, not only may peptide therapeutics be improved by N-terminal alkylation but also peptide diagnostic tools and peptide-based biomaterials. This must be explored, and we will wait expectantly to see into what directions the authors' results will take these areas of research.

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