New Horizons: Next-Generation Insulin Analogs: Structural Principles and Clinical Goals

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Abstract

Design of “first-generation” insulin analogs over the past three decades has provided pharmaceutical formulations with tailored pharmacokinetic (PK) and pharmacodynamic (PD) properties. Application of a molecular tool-kit—integrating protein sequence, chemical modification and formulation—has thus led to improved prandial and basal formulations for the treatment of diabetes mellitus. Although PK/PD changes were modest in relation to prior formulations of human and animal insulins, significant clinical advantages in efficacy (mean glycemia) and safety (rates of hypoglycemia) were obtained. Continuing innovation is providing further improvements to achieve ultra-rapid and ultra-basal analog formulations in an effort to reduce glycemic variability and optimize time in range. Beyond such PK/PD metrics, next-generation insulin analogs seek to exploit therapeutic mechanisms: glucose-responsive (“smart”) analogs, pathway-specific (“biased”) analogs, and organ-targeted analogs. Smart insulin analogs and delivery systems promise to mitigate hypoglycemic risk, a critical barrier to glycemic control, whereas biased and organ-targeted insulin analogs may better recapitulate physiologic hormonal regulation. In each therapeutic class considerations of cost and stability will impact utilization and global distribution. This review highlights structural principles underlying next-generation design efforts, their respective biological rationale and potential clinical applications.

Keywords: protein engineering/protein design/insulin action/insulin signaling/molecular pharmacology
**Abbreviations.** FDA, United States Food & Drug Administration; FRC, fixed-ratio combination; GLP-1, glucagon-like peptide 1; GLP-1RA, glucagon-like peptide 1 receptor agonist; GLUT1, glucose transporter 1; GRI, glucose-responsive insulin; HbA1c, hemoglobin A1c; HSA, human serum albumin; IDeg, insulin degludec; IDet, insulin detemir; IGLar, insulin glargine; IgG, immunoglobulin G; IR, insulin receptor; IRS, insulin receptor substrate; PBA, phenylboronic acid; PD, pharmacodynamic; PEG, polyethylene-glycol; PK, pharmacokinetic; PPG, postprandial glucose; SCI, single-chain insulin; SQ, subcutaneous; T1D, type 1 diabetes mellitus; T2D type 2 diabetes mellitus; and WT, wild type. Amino acids are designated by standard three-letter code; residue numbers in insulin are shown by chain in superscript. Names of insulin analogs are italicized in lower case.
Introduction

The present year marks both the centennial of insulin’s discovery (1921) (1) and the golden anniversary of its high-resolution structure (2). Whereas the former’s therapeutic impact was immediate, clinical translation of structural advances has occurred only gradually (3). Indeed, unmet clinical needs in the treatment of Type 1 and Type 2 diabetes (T1D and T2D) represent continuing sources of therapeutic innovation. In this review we highlight a creative interplay between basic science and molecular pharmacology. Together, a series of advances in diverse technologies—from recombinant expression and protein design to cell biology, “omics” data and transgenic model organisms—enabled and continue to inspire a search for the “perfect insulin” (4). This review highlights structural principles pertinent to clinical goals, including optimizing time in range and minimizing risk of hypoglycemia. Such goals reflect concurrent advances in insulin delivery (5,6) and glucose monitoring (7-9).

Insulin remains essential for the treatment of T1D (10), even as its role in T2D is changing due to an expanding armamentarium of unrelated therapeutic approaches (11-13). In either clinical setting novel analogs and reformulations seek to enhance glycemic control while minimizing hypoglycemic risk. Beyond hemoglobin A1c (HbA1c) as an indicator of mean glycemic control, the advent of continuous glucose monitoring (CGM) has enabled assessment of time in range (TiR) (14) and glycemic variability (9,15-17). In T2D broader clinical considerations led to insulin’s deemphasis in current treatment algorithms (12,18); preferred agents (whether oral or injected) mitigate hypoglycemic risk, avoid weight gain (or induce weight loss), and may be cardioprotective (19,20). These include metformin as a first-line agent with subsequent addition of one of several classes (DPP4 inhibitors, sulfonylureas, GLP-1 receptor agonists, or SGLT2 inhibitors) (21-24). For those T2D patients requiring insulin therapy, investigational approaches envision further optimization of pharmacokinetic (PK) and pharmacodynamic (PD) properties (such as ultra-rapid or ultra-basal), providing glucose-responsivity as a “smart” insulin analog (25,26), possible organ-selective insulin analogs, and bias insulin agonists. Complementary investigations exploit co-administration of insulin with another hormone to obtain additive or synergistic benefits. This review describes in turn these past and future frontiers of insulin technology in relation to structural principles and clinical needs.

Insulin Structure and Analog Design

The crystal structure of the zinc insulin hexamer was a landmark in molecular endocrinology (Fig. 1). Depicting a protein homo-oligomer for the first time, the structure provided a framework for investigating the biosynthesis and storage of insulin in pancreatic β-cells (27,28). Despite its small size (51 amino acids per monomer; Fig. 1A), the zinc hexamer exhibits key features of globular proteins in general: well-defined elements of secondary structure, tertiary organization with hydrophobic core, specific interfaces for self-assembly (Fig. 1B), and capacity for long-range conformational change (the TR transition; Fig. 1C) (29). The latter anticipated the recent elucidation of structural mechanisms by which conformational change in insulin on binding to the insulin receptor (IR) may be amplified and transmitted to effect transmembrane signaling (30). Crystallographic analyses of the TR transition rationalized the stability of classical zinc-based pharmaceutical formulations of insulin.
Insulin’s crystal structure underlay efforts to optimize its molecular properties for clinical use. Modification of the insulin molecule to modulate its pharmacokinetic (PK) and pharmacodynamic (PD) properties in “first-generation” insulin analogs provided a pioneering triumph of rational protein design. Products in current use are summarized in Table 1; these fall into two classes, rapid acting as intended for bolus injection before meals or use in insulin pumps (1A) and long acting as intended for once-a-day injection (Table 1B). This combination of products seeks to recapitulate the homeostatic pattern of insulin secretion by pancreatic β-cells. Respective time-action profiles of insulin products are shown in Figure 2A. The essential idea, first realized in the 1930s through studies of microcrystalline suspensions, posits that the physical chemistry of insulin (including its self-association equilibria) directly modulates the stability of a subcutaneous (SQ) depot and its rate of absorption (31). We describe in turn first-generation prandial and basal analogs with emphasis on structure-function relationships.

**Prandial Analogs.** Design was based on the premise that more rapid disassembly in a SQ depot (or absence of self-assembly) would facilitate capillary absorption of zinc-free insulin monomers and dimers. Diverse amino-acid substitutions at subunit interfaces were characterized that weakened dimerization or hexamer assembly. Candidates were selected based on (a) compatibility with high-affinity IR binding with native activity in vivo and (b) amenability to stable pharmaceutical formulation. Detailed studies of immunogenicity were deferred until clinical trials. Three such rapid-acting analogs are in current use (Table 1A); in order of introduction, these are insulin lispro, insulin aspart and insulin glulisine. These products were proven safe and effective in multi-injection regimens and for continuous SQ infusion (insulin pumps) (32-34). Although the rapid-acting products meet regulatory criteria for chemical and physical stability, prandial analogs are generally more susceptible to degradation above room temperature than is human insulin (35). Current efforts focus on optimization of formulations (such as active excipients) to further accelerate SQ absorption (36-38). Ultra-rapid analog formulations promise to enhance feedback-based control of closed-loop systems (39). Because insulin assembly ordinarily protects the hormone from degradation, these efforts may encounter a tradeoff between more rapid action and formulation stability.

Crystallographic studies of insulin lispro and aspart informed related mechanisms of rapid action (40,41). Hexamer assembly in each case enhances formulation stability and requires specific binding of phenolic ligands, previously employed as antimicrobial agents. Ligands binding induces allosteric T→R reorganization of the zinc insulin hexamers (Fig. 1C) (41). Whereas the T-state protomer resembles the solution structure of an insulin monomer (42-44), in the R-state protomer the B-chain undergoes a change in secondary structure to form an elongated B1-B19 α-helix; a monomeric R-like conformation has not been observed. The crystal structure of insulin lispro was determined as a T3R3 zinc hexamer containing three bound phenolic ligands whereas the structure of insulin aspart was determined as an R6 hexamer containing six bound ligands. Respective dimer interfaces retain native-like anti-parallel β-sheets (residues B24-B28) with only subtle structural distortions near the sites of substitution. On SQ injection, the ligands rapidly diffuse from the depot (release of phenolic ligands from hexamers occurring on the millisecond time scale (45)), enabling the unliganded variant hexamers to rapidly disassemble for capillary absorption (2,46,47). In addition to their therapeutic utility, basic studies of these analogs (each with B28 substitutions) enriched the structural understanding of an otherwise conserved proline (ProB28) at the native dimer interface (40).
Unlike insulin *lispro* and *aspart*, insulin *glulisine* is formulated without zinc ions. The analog contains two substitutions (Asn$^{B3}$ → Lys and Lys$^{B29}$ → Glu) but retains Pro$^{B28}$ and therefore capacity for dimerization. Together, the two substitutions enhance the analog’s resistance to chemical and physical degradation with an increased net negative charge at neutral pH. The augmented intrinsic stability of the *glulisine* monomer renders hexameric assembly (and hence the need for zinc ions) dispensable in a formulation. The U-100-strength solution (0.6 mM as in U-100 WT formulations) contains both monomers and dimers in a dynamic equilibrium, each capable of rapid SQ absorption. Recent crystallographic studies of *glulisine* (48) may motivate further improvement in its formulation.

Advances in “smart” insulin pumps—closed-loop systems in which control of the pump is driven by an algorithm based on feedback from a continuous glucose monitor—have highlighted the need for ultra-rapid analog formulations (Table 1A). Ultra-rapidity would make more robust continuous glucose-monitor-based pump control algorithms to enhance time in range (6,39,49). A wide variety of approaches have been investigated, including heating pads at the site of injection to increase local blood flow, co-injection of the enzyme hyaluronidase to break down connective tissue at the site of injection, needle-free jet injection and fabrication of micro-needle patches (for review, see (50)). None achieved clinical adoption. Inhalable powdered form of insulin monomers with rapid onset (12 min) and short duration (3 h) of action have received regulatory approval (51). Marked progress has been made using additives to current formulations, such as “active excipients” that enhance rates of hexamer disassembly or that increase local blood flow (37). Two such reformulations of *aspart* and *lispro* have received regulatory approval as ultra-rapid insulin analog formulations (Table 2) (52-58).

Investigational strategies to combine ultra-rapidity with enhanced stability have focused on screening synthetic copolymers to minimize surface denaturation (53,54,56,57,59,60). A complementary frontier is defined by insulin concentration: of future interest toward the goal of extending pump therapy to T2D patients with marked insulin resistance would be development of novel ultra-fast, ultra-concentrated insulin analog formulations (i.e., at strengths in the range U300-U500/ml).

**Basal Insulin Analogs**

Basal formulations (including neutral protamine Hagedorn; NPH) can enhance glycemic control in multi-injection regimens and are of continuing importance in a subset of T2D patients. In the latter, basal regimens are preferred (relative to prandial) due to simplicity and reduced weight gain (61). Although the global need for basal products exceeds that of rapid-acting formulations (given the emerging pandemic “diabesity” (62)), targeted stabilization of the insulin hexamer poses a subtler challenge than its mutational destabilization. Evolutionary optimization of the hexamer’s self-assembly surfaces obscures structural strategies to achieve further improvement, a challenge circumvented by current products. For example, insulin *glargine* (IGlar) (63) exploits isoelectric precipitation (64), a phenomenon robust to structural details. Reversible loss of solubility ordinarily occurs in wild-type (WT) insulin between pH 5 and 6 wherein the protein exhibits negligible net charge. To shift such precipitation to physiological pH, IGlar contains a two-residue basic extension of the B-chain (Arg$^{B31}$-Arg$^{B32}$). Injection of an unbuffered pH 4 formulation containing zinc ions hence results in SQ precipitation leading to protracted absorption (the acidic conditions necessitates a substitution [Asn$^{A21}$→Gly] to avoid chemical degradation). The precipitate, which may be
microcrystalline, is presumed to contain zinc hexamers within which zinc ions both stabilize self-assembly and fine-tune the isoelectric point. The di-Arg extension, otherwise mitogenic, is removed by endogenous SQ exopeptidases (65,66); principal metabolites M1 and M2 (GlyA21-insulin and its des-B30 derivative, respectively) are no more mitogenic than is WT insulin. Increasing the IGLar concentration (from 0.6 mM to 1.8 mM; U-300 strength) leads to further PK/PD prolongation as a near-peakless once-a-day formulation (Table 1B) (67,68); bioavailability decreases with increasing formulation strength (67,69). The U-300 formulation is of particular utility in T2D patients with marked insulin resistance who would otherwise require a large injection volume of a U-100 formulation (70).

Insulin detemir contains a prosthetic fatty-acyl group modifying LysB29, which stabilizes the SQ depot and mediates binding to human serum albumin (HSA) to create a circulating depot (71,72) (Fig. 3). ThrB30 is absent to simplify recombinant manufacture. The modification impairs activity, however, and so a U-100 formulation contains fourfold more protein molecules per ml than in other U-100 products. Unlike IGLar or degludec (IDeg), detemir (IDet) is often administered twice a day as its duration of action is less than 18 hours (73). Fortuitously, treatment with IDet is uniquely associated with less weight gain than treatment with other insulins. Although the mechanism is not well understood, it is possible that IDet enhances hypothalamic signaling to control appetite (Begg, 2015 #233), a feature that foreshadows objectives of next-generation analog design (below). Some clinical data suggest that glycemic variability is lower in patients treated with IDet relative to NPH and glargine (74-76).

Foreshadowing next-generation basal analogs, insulin IDeg was developed as a true 24-hour peakless formulation (Table 1B). This des-B30 analog is formulated at neutral pH as a novel dimer of T3R3 zinc hexamers linked by an acyl modification of LysB29 (hexadecanedioic acid with γ-L-glutamyl spacer; Fig. 2B, 3A). Remarkably, the analog undergoes multi-hexamer SQ assembly to achieve protracted action (Fig. 2C). Such higher-order assembly is triggered by a T3R3→T6 change in hexamer conformation on release of the bound phenolic ligand (77). The B29 modification also mediates albumin binding more effectively than the shorter acyl chain of IDet (78-80). Both IDeg and IGLar are known for their near-peakless PD profiles. Open-label studies have provided evidence of reduced day-to-day variability and risks of severe hypoglycemia among patients using IDeg than among those who received IGLar (81-84). This may be due to its flatter PK/PD profile (85,86).

Next-generation once-a-week insulins. The efficacy and convenience of once-a-week GLP-1 agonists (87-89) motivated design of analogous insulin products. Insulin Icodec exhibits time to maximum concentration of 16 h with half-life 196 h (90,91). Its ultra-long PK profile is enabled by (i) strengthening HSA binding and (ii) weakening its binding to the IR, each delaying clearance. HSA binding was strengthened by further lengthening the dicarboxylic adduct at LysB29 (C-20 diacid linked through a 2xOEG-γ-Glu linker; Fig. 3) (92). IR binding was decreased by paired aromatic substitutions in the B chain (TyrB16→His and PheB25→His (93-96), which also augments stability toward proteolysis (97)); these side chains pack at or near the hormone-receptor interface (Fig. 3A-C). Thermodynamic stability is augmented by a “reverse hydrophobic” substitution at the A-chain surface (TyrA14→Glu). Because intercurrent illnesses can unexpectedly interrupt food consumption, the safety of once-a-week insulin products (if not glucose responsive) will require careful assessment with respect to hypoglycemia.
Ultra-basal formulations can in principle be engineered by fusion of insulin to other moieties (98). For example, polyethylene-glycol (PEG) can enhance overall hydrodynamic radii, thus delaying renal clearance to prolong duration of action. This principle was exemplified by insulin peglispro (LY2606641) (99-101), discontinued after phase-3 trials due to risk of hepatotoxicity (102). An alternative approach involves fusion of proteins or peptides to the immunoglobulin Fc domain of s provides a general method to prolong plasma half-life (103,104), due in part to Fc-receptor-mediated recycling (105); such binding is pH-dependent. Whereas in the bloodstream (at neutral pH) such binding is weak, within an acidic endocytic vesicle binding is strong, protecting the tethered fusion peptide from degradation and enabling its recycling. Investigational application to single-chain insulin (SCI) analogs has been described (Fig. 2D, E): basal insulin Fc (LY3209590) is such an SCI-IgG Fc-fusion protein. To weaken receptor binding, the SCI contains paired B-chain substitutions at the same sites as in icodec (Tyr816->Glu and Phe825->His) with an unstructured peptide linker (Table 3, Fig. 2E and Fig. 3A-C). A half-life of 17 days was observed in patients with T2DM with near-peakless PK/PD profile with a once-week dosing (106). Alternative embodiments of this strategy include (LAPS®Insulin115; Fig. 2F) that employed a PEG linker (107), and a novel investigational heterodimeric two-chain insulin-Fc fusion protein(108).

Co-administration of formulations or hormones

Biphasic insulin formulations. Premixed insulin formulations have long provided biphasic PD profiles as a simplified regimen (109,110) Such products, which provide both mealtime and basal glycemic control, are of widespread use in the developing world (111,112) and as a mode of T2D intensification in the developed world (113). Examples of existing premixed formulations are provided by 25% soluble insulin aspart and 75% aspart as NPH microcrystals; an analogous formulation contains 30% soluble insulin lispro and 70% lispro as NPH microcrystals (110). The development of IDeg as a peakless basal analog formulation led in turn to its use in a fully soluble premixture with 25% insulin aspart (114-116). It is possible that the latter product may be further modified to contain active excipients (as in Fiasp®) to promote ultra-rapid absorption of the aspart component (117,118). A further frontier may exploit ultra-stable biphasic single-chain insulin (SCI) analogs (119) to simplify distribution, storage and use (120).

Insulin/GLP-1RA co-administration. Current guidelines recommend avoidance of both hypoglycemia and weight gain as important therapeutic considerations when individualizing regimens (121). Combination therapy with insulin and a glucagon-like peptide-1 receptor agonist (GLP-1RA) shows promise in T2D by significantly reducing HbA1c, glycemic variability and body mass in patients otherwise not well controlled (89,122-126). Relative to short-acting GLP-1Ras, these agents exhibit increased effectiveness on overnight and fasting plasma-glucose concentration associated with lower HbA1c. In a recent meta-analysis use of long-acting GLP-1RAs (rather than short-acting agonists) in combination therapy with basal insulin therapy demonstrated therapeutic advantages, including lower HbA1c, fasting plasma-glucose concentration and body mass (127). The pending introduction of once-a-week basal insulin analog formulations (91,128) is likely to further encourage studies of its synchronous co-administration or FRC combination with once-a-week GLP-1RAs.
Insulin/amylin co-administration. Combination of insulin analogs with other hormones may also provide clinical benefit. In T1D a favored approach is co-administration of insulin analogs and pramlintide (an amylin analog), which resembles their endogenous co-secretion from β-cells. Like co-administered GLP-1, amylin can enhance glycemic control, mitigate weight gain and reduce the frequency of hypoglycemia (129,130). Physiological mechanisms include reduced food intake (increased satiety), slower gastric emptying, and inhibition of glucagon secretion (Fig. 4A). Such use of pramlintide use has been hampered by its formulation incompatibility with insulin, leading to the need for separate injections. Circumventing this barrier in an investigational dual-hormone pump led to increased time in range with reduced hypoglycemic events (131). Novel polymer-stabilized co-formulation of insulin and pramlintide is under investigation (60).

Glucose-responsive insulins

A long-standing goal is design of a glucose-responsive insulin (GRI) or deliver system to protect patients from hypoglycaemia (for reviews, see (25,132,133). Intrinsic (or unimolecular) GRIs define a novel investigational class of analogs wherein the modified hormone itself confers glucose-dependent activity or bioavailability (26). Initial efforts focused on sequestration of the active insulin hormone within the SQ space or bloodstream (as inactive complexes) with release (or activation) enhanced by hyperglycaemia. Recent bio-inspired advances exploit specific endogenous features of the SQ space, potential hormone-carrier proteins or cellular clearance systems (98). The following investigational schemes exploit chemical or protein-based glucose sensing (Fig. 3-D-G).

(i) Phenylboronic acid (PBA). This diol-binding element senses carbohydrates (134,135) and was first exploited in studies of PBA-modified insulin(136). The essential idea envisioned a glucose-responsive polymer-based release system. (25,132). GRI activity was subsequently described in a murine peritoneal glucose-infusion assay using PBA-modified IDet analog (137) (Fig. 3A). Although glucose-dependent HSA binding was hypothesized, this mechanism could not be demonstrated.

(ii) Diboronate sensors. Novo Nordisk prepared diboronate insulin derivatives exhibiting glucose-sensitive HSA binding (138) in the physiologic range (K_d 0.2-5 mM) and with good selectivity relative to lactate. A substantial literature pertains to mechanisms of monosaccharide binding and selectivity among such sensors (139,140).

(iii) GLUT1 as GRI carrier. Proteins other than HSA can sequester properly modified insulins (98,141). Gu and colleagues exploited glucose transporter GLUT1 (as found on the surface of erythrocytes) to sequester insulin molecules linked to a GLUT1-specific antagonist (142,143) (Fig. 3). This technology circumvents the need for a chemical glucose sensor (144,145). Ambient glucose at high concentration displaces the antagonist to liberate the tethered insulin moiety.

(iv) Glucose-dependent clearance. A novel GRI scheme exploited glucose-dependent clearance of a saccharide-modified insulin derivative by the ubiquitous mannose receptor (146). Such clearance was more rapid under conditions of hypoglycaemia than under conditions of hyperglycaemia (Fig. 3A). This mechanism differs from the above albumin-bound circulating depots as mannose receptor clearance is irreversible. Clinical trials exhibited only limited efficacy (147).
A novel class of unimolecular GRLs operates independently of a carrier protein, transporter or repurposed clearance receptor. Proof of principle was recently provided by a fructose-responsive switch inserted between the C terminus of the B chain and N terminus of the A chain in an HAs-KP framework (Fig. 3A)(148). This analog is “closed” at low monosaccharide concentration, but openable and active at high monosaccharide concentration (Fig. 4B,C) (148). This conformational cycle is in principle reversible (depending on metabolic state). The monosaccharide-opened state is compatible with native insulin-IR binding (Fig. 4C) (149). NMR studies documented changes in protein conformation regulated by fructose but not glucose, consistent with the monosaccharide-binding selectivity of the sensor. Beyond translational guidance, the cellular studies validated a structural coupling between hinge opening in the insulin B chain and reorganization of the IR ectodomain leading to transmembrane signalling (30).

Seminal studies in dogs (150) demonstrated that, whereas insulin is a potent inhibitor of glucagon activity under hyperglycemic conditions (151), hypoglycemia sensitizes the liver to glucagon signaling, thus overcoming insulin’s otherwise inhibitory effect on hepatic glucose production (150,152). These findings motivated simultaneous administration of both hormones as an investigational strategy for glycemic control with reduced risk of hypoglycemia (153). Investigations at different insulin/glucagon ratios suggested that a fixed mixture can reduce risk of hypoglycemia, mitigate insulin-induced weight gain (and even cause weight loss) when using a long-acting glucagon analog (154,155). Because such co-administration exploits a physiologic switch in the liver (Fig. 4D), the need for chemical glucose sensors or glucose-dependent carrier (or clearance system) is circumvented (152). Further analysis of integrative hormonal signaling in the liver on co-administration of insulin/glucagon analogs promises to be of marked interest.

**Organ-specific insulins.** In the past two decades organ-specific knock-out of the IR or IRS genes have generated valuable mouse models to probe organ-specific insulin signaling (156-158). Such insights complement long-standing concerns regarding peripheral administration of insulin versus endogenous portal secretion (159). Next-generation insulin analog design thus envisions organ-specific targeting.

(i) **Liver.** First-pass hepatic metabolism does not occur with SQ insulin, resulting in relative under-insulinization of the liver with suboptimal suppression of hepatic gluconeogenesis and relative over-insulinization of peripheral tissues. This can increase the risk of insulin resistance, hypoglycemia and weight gain (159). Peglispro, a PEG-linked analog, exhibited partial hepatic selectivity(160,161), but its clinical development was abandoned due to signs of hepatic toxicity in clinical trials (102).

(ii) **Brain.** Insulin signaling in the brain regulates food intake (162,163), leading to insulin’s classification as a neuroendocrine hormone (164,165) acting in the amygdala and hypothalamus (166). Interestingly, peripheral administration of Idet increases brain signaling relative to WT insulin (167), presumably due to enhanced transport through the blood-brain barrier. Idet may thus reduce food intake and mitigate weight gain relative to non-acetylated analogs. Mechanisms related to HSA binding are under investigation (168-170). The IR glycoform mass in the brain differs from glycoform masses elsewhere in the body (171), suggesting that specific targeting may be achievable (172). Such targeting may also be relevant in Alzheimer’s disease (169).
(iii) Adipocyte. Insulin signaling in adipose tissue regulates lipid storage and in turn whole-body glucose homeostasis (158,173). In mice palmitoleic acid inhibits lipogenesis and increase insulin sensitivity in liver and skeletal muscle (174). Novel branched fatty acid esters of hydroxyl fatty acids exert anti-diabetic and anti-inflammatory effects (175); their deficiency can lead to glucose intolerance. Although insulin-induced adiposity is undesirable, adipocyte-targeted insulins may, as a seeming paradox, reduce insulin resistance (176,177).

**Biased signaling.** Biased signaling (or “biased agonism”) is defined as ligand-dependent functional selectivity leading to different signaling outputs by the same receptor. Such bias can reflect subtle conformational differences among receptor-ligand complexes as exemplified by G-protein-coupled receptors (178-182). Extension of this paradigm to the IR could be transformational in T2D: Brown and Goldstein highlighted selective insulin resistance in the liver as a key unsolved problem related to hepatic steatosis (183-185). In T2D insulin becomes ineffective at glycemic control and yet can continue to drive excess lipid biosynthesis (186) and mitogenic outputs (187).

How might biased agonists of the IR be designed? Hints are provided by advanced phosphoproteomics approaches (188) (Fig. 5A, B), including bioinformatic analysis of regulatory networks (189). Unlike first-generation insulin analogs designed to improve PK/PD, a search for biased agonists seeks to exploit subtle linkages between IR structure and dynamics. Proof of principle has been obtained using IR-targeted phage-display peptides (190)(Fig. 5C), whose binding sites define three non-overlapping sites (sites 1, 2, and 3; (191)). Lawrence and colleagues have determined the structure of a Site-1 peptide bound to the IR L1 domain, overlapping with its native αCT-binding surface (192). The distinct phosphoproteomic signatures of phage-display-derived IR agonists (relative to insulin) suggest that, as in G-protein coupled receptors, subtle conformational features of the IR-ligand complex can indeed bias signaling outputs (Fig. 5D) (191). Such efforts may exploit yeast-display technology (193-195) as demonstrated by Chou and colleagues (196). The epidemiological relationship between T2D and increased risk of several common cancers (including breast- and colon cancer) (197-199) has focused particular attention on attenuation of mitogenic signaling by insulin analogs (200-202). Comparison of mitogenic signaling outputs triggered by binding insulin versus insulin-like growth factors I and II to the type 1 IGF receptor may be regarded as an experiment of nature demonstrating biased agonism in a receptor tyrosine kinase (203). Insulin analogs are routinely characterized for extent of cross-binding to type 1 IGF receptor and ratio of affinities for the IR isoforms (IR-A and IR-B).

**Concluding Remarks**

The discovery of insulin in 1921, a landmark in endocrinology, galvanized broad public support for biomedical research (1). The ensuring efforts of the late D. C. Hodgkin extended over six decades—inspiring an international network of laboratories—defined the atomic structure of insulin and its conformational repertoire. The 50th anniversary of the first high-resolution crystal structure of insulin comes at a time of transition from rapid-acting analogs to ultra-rapid and from basal to ultra-basal. The frontiers of insulin analog design extend beyond first-generation mutagenesis to highlight optimization of protein adducts and formulation excipients (204). Synergies among diverse technologies are of particular clinical promise, as ultra-rapid analog formulations may enhance the
performance of algorithm-regulated closed-loop systems (as captured by continuous glucose monitor -defined time-in-range metrics) and once-a-week ultra-basal analog formulations may enhance the efficacy of once-a-week GLP-1 agonists (88,89).

Optimization of the PK/PD properties of insulin analog formulations promise to enhance glycemic control in T1D beyond HbA1c (10,205). Ultra-rapid reformulations of prandial analogs may enhance TiR in closed-loop systems and thereby mitigate glycemic variability, including risk of hypoglycemia (39,118). Investigational ultra-basal insulin analog formulations (currently in clinical trials) may simplify basal-bolus regimens in T1D and basal-only (or basal-bolus) regimens in T2D. Therapeutic considerations in T2D are holistic, integrating glycemia control with management of cardiovascular risk, weight and the multiple components of the metabolic syndrome (12,13,206). For patients requiring insulin, glycemic targets may be moderated on an individual basis (15). Frontiers of innovation among insulin technologies focus on yet-unmet clinical needs (207,208), such as glucose-responsive strategies to avoid hypoglycemia (25,141,209). Fundamental features of metabolism in specific organs and cell types may be exploited to mitigate weight gain. For example, potential organo-specific insulin analogs may circumvent a current feature of SQ delivery, relative over-insulinization of the periphery and under-insulinization of the liver (156-158). Biased control of specific post-receptor signaling pathways in the liver may be of particular benefit to treat or avoid hepatosteatosis (183,185). Combination of insulin with other hormones, such as GLP-1R agonists or amylin analogs, promises to exploit physiological synergies (89,130) whereas co-administration of insulin and glucagon may buffer hepatic glucose output to reduce hypoglycemic risk (Pedersen, 2018 #175;Bode, 2020 #187).

The insulin analogs highlighted in this review, including those under investigation, were designed to meet standards of stability appropriate in the developed world. Whereas in affluent societies thermal degradation of insulin and insulin analogs is uncommon, the majority of patients in the coming decades will be living in underprivileged regions of the developing world (210). In such regions intertwined medical and societal challenges are posed by the cold chain of insulin delivery in the absence of refrigeration or a reliable electrical grid (120,211). Given this growing global-health need, we envisage a third-generation of insulin analogs: combining the present desiderata of properties with ultra-stability. Such efforts are likely to require further structural analysis of degradation mechanisms, including metastable partial folds and amyloid. The rugged landscape of protein folding and misfolding, for the present a foundational topic in biophysics, may thus emerge as a new translational frontier in molecular pharmacology.
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**Conflict of Interest Statement:** M.A.W. holds shares in and is Chief Innovation Officer of Thermalin Diabetes, LLC.; he has also been a consultant to Eli Lilly and Co., Merck, Inc. and the DEKA Research and Development Corp. The authors otherwise declare that the research was conducted in the absence of any commercial or financial relationships that could pose a potential conflict of interest.

**Data Availability Statement:** Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.
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<table>
<thead>
<tr>
<th>Analog</th>
<th>Modification</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A rapid-acting analogs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lispro</em> (Humalog®)</td>
<td>Pro⁸²⁸ → Lys</td>
<td>IGF-I-related transposition impairs dimerization</td>
</tr>
<tr>
<td>Eli Lilly and Co.</td>
<td>Lys⁸²⁹ → Pro</td>
<td></td>
</tr>
<tr>
<td>(Admelog®)</td>
<td>FDA approved Biosimilar</td>
<td>not currently approved as interchangeable with Humalog®</td>
</tr>
<tr>
<td>Sanofi-Aventis</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>aspart</em> (NovoLog®)</td>
<td>Pro⁸²⁸ → Asp</td>
<td>charge repulsion at dimer interface</td>
</tr>
<tr>
<td>Novo-Nordisk</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>glulisine</em> (Apidra®)</td>
<td>Asn⁸¹ → Lys</td>
<td>decreased zinc-free self-association</td>
</tr>
<tr>
<td>Sanofi-Aventis</td>
<td>Lys⁸²⁹ → Glu</td>
<td></td>
</tr>
<tr>
<td>Technosphere insulin (Afrezza®)</td>
<td>Approved (T2D, T1D)</td>
<td>monomeric human insulin adsorbed onto Technosphere microparticle</td>
</tr>
<tr>
<td>Mannkind Corp</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B basal analogs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>glargine</em></td>
<td>Arg⁸¹¹-Arg⁸¹² extension</td>
<td>shift in pI to pH 7 leads to isoelectric precipitation on injection</td>
</tr>
<tr>
<td>(Lantus® U100)</td>
<td>Asn⁸²¹ → Gly</td>
<td></td>
</tr>
<tr>
<td>(Toujeo® U300)</td>
<td>FDA approved Biosimilar</td>
<td>not currently approved as interchangeable with Lantus®</td>
</tr>
<tr>
<td>Sanofi-Aventis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Basaglar® U100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eli Lilly and Co.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>des-B30</td>
<td></td>
</tr>
<tr>
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<td>--------------------------------------</td>
</tr>
<tr>
<td><strong>detemir (Levemir&lt;sup&gt;®&lt;/sup&gt;)</strong></td>
<td>acylation of Lys&lt;sub&gt;B29&lt;/sub&gt; by a C14 fatty acid</td>
<td>stabilization of hexamer and binding to serum albumin</td>
</tr>
<tr>
<td>Novo-Nordisk</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>degludec (Tresiba&lt;sup&gt;®&lt;/sup&gt;)</strong></td>
<td>acylation of Lys&lt;sub&gt;B29&lt;/sub&gt; by gGlu-C16-diacid</td>
<td>unique self-assemblies of multihexamers and binding to serum albumin</td>
</tr>
<tr>
<td>Novo Nordisk</td>
<td></td>
<td></td>
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</tbody>
</table>

Panel A lists rapid-acting analogs employed in prandial regimens and in insulin pumps whereas B describes basal insulin analogs with protracted action. The first fixed-ratio basal insulin/GLP-1RA combination products (IGlarLixi, Xultophy™ 100/3.6) and (IDegLira, Soliqua™ 100/33) were approved by the US Food and Drug Administration (FDA) in 2016. The European Medicines Agency approved Xultophy™ in 2014 and Soliqua™ in 2017.
Table 2. Excipients, surfactants or chaperones used for ultra-rapid insulin analogs

<table>
<thead>
<tr>
<th>Analog</th>
<th>Modification</th>
<th>Mechanism</th>
</tr>
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<tbody>
<tr>
<td>Faster insulin aspart (Fiasp®)</td>
<td>niacinamide / L-Arg formulation</td>
<td>vasodilator promotes transport of hexamer</td>
</tr>
<tr>
<td>Novo Nordisk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultra-rapid lispro (URLi®) (Lyumjev®)</td>
<td>treprostinil / citrate formulation</td>
<td>vasodilator promotes vascular permeability</td>
</tr>
<tr>
<td>Eli Lilly and Co.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insuman® (U400)</td>
<td>poly(polypropylene) polymer</td>
<td>modify the monomer-air-liquid interface</td>
</tr>
<tr>
<td>Sanofi-Aventis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BioChaperone Lispro (BCLIS®)</td>
<td>BC222 excipient, citrate</td>
<td>promote hexamer dissociation monomer adsorption from SQ formulation stabilizer</td>
</tr>
<tr>
<td>Adocia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>investigational biocompatible and non-toxic acrylamide carrier/dopant copolymers (59,212)</td>
<td>investigational biocompatible and non-toxic acrylamide carrier/dopant copolymers (59,212)</td>
<td>modifying the monomer-air-liquid interface</td>
</tr>
</tbody>
</table>
Table 3. Insulin Analogs in Clinical Trials and Modes of Action

<table>
<thead>
<tr>
<th>Analog</th>
<th>Trial Phase (Reference)</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fast-Acting Insulins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BioChaperone Lispro (BCLIS®)</td>
<td>phase II (106)</td>
<td>ultra-rapid release</td>
</tr>
<tr>
<td>Adocia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal insulin Fc (BIF; LY3209590) is</td>
<td>phase II (106)</td>
<td>Fc-fusion protein prolongation SCI human IgG2 Fc fusion</td>
</tr>
<tr>
<td><strong>Oral and Inhalation Insulins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icodec (NNC0148-0287 C (Insulin 287) Novo Nordisk</td>
<td>Phase II (T2D) (91)</td>
<td>Glu&lt;sup&gt;A14&lt;/sup&gt;, His&lt;sup&gt;B16&lt;/sup&gt;, His&lt;sup&gt;B25&lt;/sup&gt; C-20 diacid group linked 2xOEG-gGlu</td>
</tr>
<tr>
<td>OI338 and OI320 Novo Nordisk</td>
<td>phase II (97)</td>
<td>Glu&lt;sup&gt;A14&lt;/sup&gt;, His&lt;sup&gt;B25&lt;/sup&gt;, des-B30, gGlu-2xOEG C18-diacid superior to C20-diacid</td>
</tr>
<tr>
<td>Tregopil (Biocon)</td>
<td>phase II / III (213)</td>
<td>single PEG unit linked to Lys&lt;sup&gt;B29&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Figure 1. Insulin structure and self-assembly. (A) Insulin monomer from 2-Zn (T₆) hexamer (PDB 4INS): A chain (residues A1-A21; light gray) and B chain (B1-B30; dark gray); and disulfide bridges A6-A11, A7-B7, and A20-B19 (yellow). (B) Insulin dimer in T₆ hexamer. (C) Structures of three types of zinc hexamers: T₆, T₃R³, and R₆ (PDB codes 1ZNJ, 1TRZ, and 1ZNJ). T and R (or R') protomers exhibit distinct B1-B8 conformations: extended (green) or α-helical or frayed α-helical (red). T- and R-state protomers are otherwise shown in dark and light gray, respectively; axial zinc ions as purple spheres.
Figure 2. Insulin activity profiles and representative structures. (A) PD profiles: (a) ultra-rapid-acting reformulations Fiasp and URLi (Table 1); (b) first-generation prandial formulations (Humalog, Novolog and Apidra); (c) “regular” WT formulation; (d) NPH insulin is an intermediate-duration microcrystalline formulation; (e, f) basal analog formulations Levemir and Lantus (Table 1; different depot-precipitation mechanisms); (g) Tresiba (Table 1) provides >24 h duration; (h) initial target profile for novel once-a-week analogs. *Inset shaded box* (top left): blue triangles represent successive insulin monomer, dimers and hexamers in a self-association equilibrium. (B) Structure of degludec as dimers of R6 zinc hexamer. As formulated, degludec forms T3R3 dimers, whereas the analog (C) reorganizes in the SQ depot as linear T6 polymers (77). In structure the A- and B chains are shown as light- and dark gray ribbons, respectively; zinc ions, as purple spheres; the B29 modification, as a green line; and the bound phenolic ligands, as space-filling spheres (CH groups, magenta; and hydroxyl group, red). (D) Prototypical single-chain insulin (SCI): B domain (B1-B30, dark gray), foreshortened C domain (6-30 residues, red) and A domain (A1-A21, light gray). Disulfide bridges (A6-A11, A7-B7, A20-B19) are shown as yellow sticks. (E, F) Ultra-basal insulin-Fc fusion proteins. (E) BIF; LY3209590) contains a (Gly4-Gln)2 linker (tan ovals) between C-terminal insulin residue A21 and the N-terminal Fc residue (106). The construct also contains modifications GluB16 and HisB25 at receptor interface (Fig. 3B-D); residues B27-B30 are substituted by Gly. (F) Alternative ultra-basal scheme conjugates SCI to an aglycosylated IgG4-derived Fc fragment via a PEG linker (3.4 kD, green oval); designated LAPSInsulin115, only preclinical data are available (107).
Figure 3. Chemical modifications of Lys in B chain. (A) Receptor binding tolerates diverse modifications at Lys\textsuperscript{B29} or Lys\textsuperscript{B28} (in lispro): (a) detemir, B29-linked C-14 acyl group; (b) degludec, B29-linked 16-carbon hexadecanoic diacid (50); (c) icodex, B29-linked C-20 diacid via 2xOE\textsuperscript{G}gGlu linker (blue); (d) GRI candidate, GLUT inhibitors (black; Glut-i2 (214)) linked at B29 via chemoselective bifunctional linkers (142) to mediate reversible binding to GLUT channels; (e) GRI candidate, B29-linked C-11 acyl-3-fluoro-PBA (red (137)); (f) GRI model, B28-A1-positioned fructose-responsive switch comprising Lys\textsuperscript{B28}-linked 4-dihydroxybenzoic acid (black) and Gly\textsuperscript{A1}-linked 3-fluoro-PBA (red) on a His\textsuperscript{A8}-modified lispro scaffold (148); (g) Investigational Merck GRI candidate NK-2640 (146), B29-saccharide-modified derivatives. (B-D) Hormone-receptor interface: once-a-week insulin analogs contain substitutions at positions B16 and B25 to attenuate IR affinity and hence delay IR-mediated clearance. (B) Structural overview of WT residues Phe\textsuperscript{B25} and Tyr\textsuperscript{B16} at the hormone-receptor interface (PDB entry 4OGA); the two side chains are shown as sticks in relation to the surfaces of the L1 domain (first leucine-rich repeat domain; cyan); the \(\alpha\text{CT}'\) element, (magenta). The bound insulin is shown as a ribbon, A chain in green and B chain in blue. (C) Model of His\textsuperscript{B25} and His\textsuperscript{B16} at variant interface. Phe\textsuperscript{B25} binds against an \(\alpha\text{CT}'\) cleft that accommodates Phe, Tyr and Trp residues. (D) WT insulin residues Phe\textsuperscript{B25} and Tyr\textsuperscript{B16} at receptor interface. Side chains are shown as sticks and methyl groups as spheres at one-third effective van der Waals radius). Images were generated using PYMOL.
Figure 4. Novel frontiers: bi-hormonal delivery and switchable insulin analogs. (A) Left, co-administration of basal insulins with GLP-1 agonists at fixed-ratio combinations (See Table 1 footnote): Right, co-administration of insulin and pramlintide. (B, C) Switchable “fructose-responsive insulin” in tethered (closed) conformation. Fructose-sensor (N-fPBAA1) is attached to GlyA1 whereas LysB28 is modified by aromatic diol, 3,4-dihydroxy benzoic acid (labelled K(DHBA)B28). (C) Model of a complex between open form and IR ectodomain (PDB entry 6HN5) on monosaccharide displacement of the switch (148). (D) Co-administration of insulin and glucagon is under investigation to reduce hypoglycemic risk via an endogenous “switch” in the liver’s relative hormone responsiveness (150,153,215).
Figure 5. IR signal transduction and biased agonism. (A) Insulin signaling via IR substrate (IRS) proteins that interact with downstream target proteins to activate phosphoinositide 3-kinase (PI3K) and AKT pathways. The Ras/Raf-mediating MAPK pathway is critical for proliferative responses. (B) Schematic phosphoproteomics workflow. Following exposure of cells to a hormone under two conditions (gold and blue), respective lysates are digested with protease(s). Phosphopeptides are identified by LC-MS/MS. (C, D) Biased signaling can be triggered by agonists via subtle differences in ligand-receptor conformations. (C) Left, IR-targeted phage-display-derived peptides (purple circle, pentagon, and diamond; 190) defined potential IR allosteric control sites (191,216). Bidirectional horizontal arrow between tyrosine kinase (TK) domains indicates their proximity on receptor activation. (D) IR signaling pathways mediate the divergent downstream outputs. The binding of a ligand can result in unbalanced activation of signaling by enhancing one output and reducing another. In hepatocytes (left) the goal is to minimize lipid synthesis relative to control of glucose output; in cancer cells (right) the goal is to inhibit mitogenicity (183).