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Design of Non-Standard Insulin Analogs for the Treatment of Diabetes Mellitus

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Abstract

Structure-based protein design has enabled the engineering of insulin analogs with improved pharmacokinetic and pharmacodynamic properties. Exploiting classical structures of zinc insulin hexamers, the first insulin analog products focused on destabilization of subunit interfaces to obtain rapid-acting (prandial) formulations. Complementary efforts sought to stabilize the insulin hexamer or promote higher-order self-assembly within the subcutaneous depot toward the goal of enhanced basal glycemic control with reduced risk of hypoglycemia. Current products either operate through isoelectric precipitation (insulin *glargine*, the active component of Lantus®; Sanofi-Aventis) or employ an albumin-binding acyl tether (insulin *detemir*, the active component of Levemir®; Novo-Nordisk). In the past year second-generation basal insulin analogs have entered clinical trials in an effort to obtain ideal flat 24-hour pharmacodynamic profiles. The strategies employ non-standard protein modifications. One candidate (insulin *degludec*; Novo-Nordisk a/s) undergoes extensive subcutaneous supramolecular assembly coupled to a large-scale allosteric reorganization of the insulin hexamer (the TR transition). Another candidate (LY2605541; Eli Lilly and Co.) utilizes coupling to polyethylene glycol to delay absorption and clearance. On the other end of the spectrum, advances in delivery technologies (such as microneedles and micropatches) and excipients (such as the citrate/zinc-ion chelator combination employed by Biodel, Inc.) suggest strategies to accelerate PK/PD toward ultra-rapid-acting insulin formulations. Next-generation insulin analogs may also address the feasibility of hepatoselective signaling. Although not in clinical trials, early-stage technologies provide a long-range vision of “smart insulins” and glucose-responsive polymers for regulated hormone release.

Keywords

glycemic control; protein engineering; polyethylene glycol; basal; prandial; insulin analog; diabetes mellitus

Introduction

The crystal structure of the zinc insulin hexamer, first elucidated by Hodgkin and colleagues in 1969 [1, 2], defines a landmark in the history of structural biology (Fig. 1A and 1B). Providing the first depiction of a protein homo-oligomer, this and related crystal structures

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Disclosure

Conflicts of Interest: The intellectual property pertaining to zinc-stapled human insulin analogs and its long-acting formulations are owned by Case Western Reserve University and licensed to Thermalin Diabetes, LLC. M.A. Weiss: holds shares in and is Chief Scientific Officer of Thermalin Diabetes, LLC.; he has also been a consultant to Merck, Inc. and the DEKA Research and Development Corp.; V. Pandyarajan: none. The authors otherwise declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

continue to provide a foundation for the design of insulin analogs and their stable pharmaceutical formulation [3, 4]. Analysis of the structure of an insulin hexamer at atomic-resolution [5] enabled design of rapid-acting (prandial) insulin analogs by targeted destabilization of self-association surfaces (Table 1A); their design and clinical use represent a triumph of rational protein design [6, 7]. Complementary efforts have led to the introduction of basal analog formulations superior to traditional NPH formulations and intended for once-a-day injection (Table 1B). The overarching therapeutic objective of insulin analog products, singly or in combination, is to recapitulate the physiologic pattern of insulin secretion by pancreatic β-cells in the course of metabolic homeostasis. Advances in the past year have focused on second-generation basal insulin analogs. In this mini-review we seek to relate therapeutic objectives to the utility of non-standard protein modifications.

Prandial Insulin Analogs

The design of rapid-acting insulin analogs provided an initial paradigm for the development and application of general principles of protein folding and assembly. Working toward the goal of accelerating hexamer disassembly [8, 9], these efforts posited that more rapid disassembly in the subcutaneous depot would facilitate capillary absorption of zinc-free insulin monomers and dimers [6, 10]. Mutations at subunit interfaces were found to weakened or prevent formation of dimers and hexamers with retention of native biological activity. Three such rapid-acting analogs are in current use (Table 1A): insulin *lispro* (the active component of Humalog®; Eli Lilly) [11], insulin *aspart* (Novolog®; Novo-Nordisk) [12, 13], and insulin *glulisine* (Apidra®; Sanofi-Aventis) [14, 15]. These products have been proven safe and effective in multi-injection regimens [7, 16] and for use in continuous subcutaneous infusion devices (insulin pumps) [17]. That distinct molecular strategies may equally well confer more rapid insulin absorption (e.g., introduction of a negative charge near the C-terminus of the B-chain in insulin *apart* (Asp^{B28}) or *glulisine* (Glu^{B29}) versus rearrangement of a positive charge adjoining a proline in insulin *lispro* (Lys^{B28}-Pro^{B29})) reflects the multiple ways that the wild-type insulin hexamer may be rendered less stable with maintenance of biological activity.

Rapid-acting insulin analog formulations have proven to be safe and effective for use in insulin pumps [17]. Increasing interest in closed-loop systems (in which control of the pump is controlled by an algorithm based on feedback from a continuous glucose monitor [18, 19]) has highlighted the need for insulin analogs even faster than current products [20–23]. Although early-stage protein engineering efforts have been undertaken to address this need [24], in recent years progress has been obtained in small clinical trials and pre-clinical trials done in animals through ancillary technologies. These include use of heating pads at the site of injection to increase local blood flow [25, 26], co-injection of the enzyme hyaluronidase to break down connective tissue at the site of injection [27], trans-dermal micro-needle patches (with active infusion [28]), intradermal microneedles administration [29], needle-free jet injection [30], and excipients that accelerate insulin hexamer disassembly [31, 32]. Enthusiasm for approaches unrelated to molecular features of insulin itself in part reflects concern that any further pharmacokinetic advantage of accelerated protein disassembly in a subcutaneous depot (beyond that achieved by current products) may be incremental. Any further destabilization of the insulin hexamer may also incur a tradeoff between rapid action and the stability requirements of a pharmaceutical formulation. It is possible that extension of protein engineering to include use of unnatural amino acids (as can now be incorporated in microbial expression systems by expanded genetic-code technology [33]) may encourage efforts to combine novel molecular design with innovative delivery systems.

Basal Insulin Analogs

A cornerstone in the non-pump-based treatment of diabetes mellitus is provided by basal insulin analog formulations [34]. Whereas fast-acting analogs are essential for the management of Type 1 diabetes mellitus, basal insulin analogs enhance glycemic control in multi-injection regimens [35] and offer superior pharmacokinetic and pharmacodynamic profiles with fewer hypoglycemic events as compared to older basal insulin products which also benefits patients with Type 2 diabetes mellitus [36]. The current and predicted global need for such products exceeds that of rapid-acting formulations due to the increasing prevalence of the metabolic syndrome and Type 2 diabetes mellitus. In such patients controllable by either prandial or basal analogs alone, basal regimens are preferred to their simplicity and reduced risk of weight gain (as exclusive meal-time injections enhance insulin's anabolic effects) [37].

Targeted stabilization of the insulin hexamer has posed a more subtle challenge to the protein engineer than its targeted destabilization [4, 38]. Evolutionary optimization of the insulin hexamer and the molecular elegance of its conserved self-assembly surfaces [5] limit possible structural routes to further improvement. Although pioneering efforts in this direction were proposed by Dodson and colleagues [4, 5], current products (Table 1B) have employed non-peptidic modifications to circumvent the need for detailed molecular analysis. Insulin *glargine* (the active component of Lantus®; Sanofi-Aventis) thus exploits isoelectric precipitation, a reversible transition to insolubility that classically occurs between pH 5 and 6 (under which conditions wild-type insulin exhibits little or no net charge) [39, 40]. This strategy is robust to the details of molecular structure. Insulin *glargine* contains a two-residue basic extension of the B-chain (Arg^{B31} and Arg^{B32}) whose positive charges result in a shift in the isoelectric point to neutrality. Injection of an unbuffered pH 4 formulation results in precipitation within the subcutaneous depot with protracted absorption for 16–24 hours [41]. The di-arginyl extension is disordered and largely removed by endogenous exopeptidases. Lantus® is the most widely-used long-acting insulin currently on the market [42]. Related analogs containing additional basic residues N-terminal to Gly^{A1} have also been described but are not in clinical use [43]. Insulin *detemir* (the active component of Levemir®; Novo-Nordisk) by contrast contains a prosthetic fatty acyl group on Lys^{B29}, intended to mediate binding to serum albumin and hence provide a circulating depot [44]. Although this mechanism is active, the tethered moiety serendipitously also enhances the stability of hexamers of the modified insulin [45]. Levemir® is often administered twice a day as its duration of action is less prolonged than that of Lantus®. Thus, non-standard modification of the insulin molecule has enabled the exploitation of chemical tactics beyond those made possible by the 20 naturally occurring amino acids – and so complement classical structural relationships implicitly optimized by evolution.

Next-Generation Insulin Analogs

Two candidate basal insulin analogs are under investigation (Table 1C). These employ different structural and biophysical principles as described in turn below.

Allosteric Assembly of a Subcutaneous Insulin Depot

A next-generation basal insulin analog developed by Novo-Nordisk, designated insulin *degludec*, is under clinical investigation by a network of academic collaborators [46–49]. Formulated at neutral pH, the protein solution contains a dimer of zinc hexamers linked by a novel acyl modification (Fig. 1C). Remarkably, the analog undergoes multi-hexamer assembly in the subcutaneous depot and thereby achieves protracted action. The designation "degludec" reflects three chemical features: *de*, the absence of Thr^{B30} (i.e., *des*-B30), *glu*, side-chain addition of a glutamic acid residue via a non-standard peptide bond between its δ-

carboxylate function and the ε-amino group of Lys^{B29}, and *dec*, referring to a dicarboxylic acid ($HOOC-(CH_2)_n-COOH$) in turn linked to the α-amino group of attached Glu. Optimization of this class of modifications by Novo-Nordisk led to the choice of n=14, corresponding to an ester of thapsic acid. Whereas insulin *glargine* contains two additional positive charges at neutral pH (Arg^{B31} and Arg^{B32}; see above) and is insoluble, the net formal charge of insulin *degludec* differs from wild-type insulin by -2 (loss of the positive charge of Lys^{B29} and gain of one negative charge from the peptide-linked thapsic acid), thereby permitting formulation as a clear solution at pH 7.4.

Results of two Phase 3 clinical trials of insulin *degludec* in the respective treatment of patients with type 1 and type 2 diabetes mellitus have been released [50, 51]. These open-label treat-to-target trials were designed to demonstrate non-inferiority to insulin *glargine* in a basal-bolus algorithm (the bolus component was in each case provided by insulin *aspart*). The primary endpoint was change in HbA1c over the course of 1 year. The trials showed that the efficacy of insulin *degludec* was similar to that of insulin *glargine*, confirming the findings of smaller previous studies [48, 49]. Notably, the Phase 3 studies documented lower rates of nocturnal hypoglycemia in both type 1 and type 2 patients; fewer overall hypoglycemic events were reported in the study involving type 2 patients. These differences were significant. Reduced risk of hypoglycemic events was hypothesized to be due to the flatter and more extended pharmacokinetic and pharmacodynamic profile of insulin *degludec*. These favorable features are presumably due to the prolonged duration of the subcutaneous insulin depot and subsequent binding of the circulating *degludec* monomer to albumin. Reduced risk of nocturnal and overall hypoglycemia may encourage more aggressive efforts by patients and providers to achieve individualized glycemic targets.

The mechanism of protracted action by insulin *degludec* exploits the classical structural reorganization of zinc insulin hexamers (designated the T → R transition) [52–54]. Higher-order assembly of insulin in the subcutaneous depot is associated with a change in hexamer conformation triggered by release of the phenolic preservative from its R-state binding sites. Thus, whereas the vial or pen formulations contain dimers of stable R-type hexamers (advantageous for stability and resistance to degradation), the depot contains supramolecular acyl-bridged stacks of T-type hexamers, yielding linear polymers that only slowly disassemble (Fig. 1E). Once in the bloodstream, the acyl modification also permits albumin binding as achieved by insulin *detemir*. Because the pharmacokinetic profile of insulin *degludec* exceeds 24 hours, once-a-day dosing promises to provide a flatter pharmacodynamic profile and reduced risk of hypoglycemia relative to first-generation products [47–49]. Acylated insulin analogs conferring protracted action have also been disclosed in patents awarded to the Lilly Research Laboratories [43, 55–58]. Although the physical state of proteins in a subcutaneous depot is not amenable to atomic-level characterization, many of these unanticipated structural features lead to the favorable pharmacokinetic properties of this next-generation analog and represent a combination of rational design and serendipity. The mechanism of action of insulin *degludec* highlights the structure and organization of a subcutaneous insulin depot as a biomaterial in its own right [59]. An independent approach to the supramolecular “depot engineering” in the absence of unnatural modifications exploits the introduction of novel zinc-ion binding sites on the surface of the insulin hexamer to enable its pH-dependent supramolecular assembly in the subcutaneous depot [60].

Development of a PEGylated Basal Insulin Analog

A derivative of insulin *lispro* containing a single polyethylene-glycol (PEG) moiety is under clinical investigation as a basal insulin analog formulation. Developed at the Lilly Research Laboratories and designated LY2605541, the analog contains a 20-kDa PEG unit attached to Lys^{B28} via its ε-amino group by means of a urethane bond (Fig. 1D). The modified insulin

analog exhibits a duration of action that is more protracted than that of Lantus and offers the potential promise of reduced intra-patient variability [61]. Although no formal reports have been published, presentations and posters at the recent 2012 American Diabetes Association's 72nd Scientific Sessions have provided evidence that LY2605541 may be associated with reduced risk of nocturnal hypoglycemia relative to Lantus [62]. The Lilly investigators hypothesize that the increased hydrodynamic radius of the analog (a fourfold increase afforded by the attached PEG moiety; see below) retards both absorption from the subcutaneous depot and renal clearance [63]. Preliminary data suggest that treatment with LY2605541 is associated with modest weight loss rather than the weight gain often seen on treatment with Lantus. Studies of the analog in dogs further suggest that the PEGylated analog may have hepatoselective action to blunt hepatic glucose output [64]. Although no molecular mechanism has been established for the partial hepatoselective effect exhibited by this analog, we speculate that it may be a consequence of reduced receptor-mediated clearance and reduced uptake by hepatic Kupffer cells due to the bulk of the PEG moiety, leading in turn to longer residence times in the microcirculation of the liver [65].

PEGylation, although novel in the context of insulin, provides a well-established strategy to enhance the therapeutic properties of proteins [66]. Pioneered by F. Davis (Rutgers University) more than 40 years ago, PEGylation leads to increased solubility (due to its hydrophilicity), decreased degradation (due to decreased access of proteases to the protein itself), and augmented hydrodynamic radius, markedly decreasing renal clearance [67]. Molecular diversity can be explored as PEG polymers can vary by size (5–40 kDa), degree of branching, and site (or sites) of attachment to a protein. To date, several PEGylated proteins have been approved for US Food & Drug Administration for human use, including PEGylated adenosine deaminase (for treatment for severe combined immunodeficiency syndrome) and PEGylated interferon alpha (for treatment of treatment for Hepatitis C).

Conclusions

The discovery of insulin in 1922 was a transformational event in molecular medicine and elicited broad public support for the development of innovative therapeutic products [68]. Characterization of the atomic-level structure of insulin and its conformational repertoire has extended over eight decades and involved international networks of laboratories, with current focus on the relationship between classical structures [5] and the mechanism of binding to (and triggering) the insulin receptor [69]. It is remarkable that this small globular protein continues to inspire molecular innovation, motivated by unmet clinical needs. Short-term risks of insulin replacement therapy reflect daily steering between treatment-related hypoglycemia (with its neurocognitive and adrenergic symptoms), on the one hand, and hyperglycemic excursions on the other. In addition to acute metabolic decompensation, hyperglycemic excursions are associated with omnipresent long-term risks of microvascular, macrovascular, and neurologic complications.

Considerable attention has been paid in the past year to potential public-health concerns regarding a possible link between basal insulin analogs and cancer. These concerns were stimulated by a study in 2008 suggesting that use of Lantus at high doses lead to an increased risk of several common cancers. Such concerns were exacerbated by reports that the analog exhibits a sixfold-increased affinity relative to human insulin for the mitogenic IGF-1R and by *in vitro* studies suggesting an almost eightfold increase in mitogenic potential in cancer cell lines [70–72]. The claimed association between insulin *glargine* and risk of cancer is controversial and is not supported by recent studies [73–75]. Additional population-based studies of cancer prevalence in long-term users of insulin products (ideally prospective and randomized) will nevertheless be required to address the issue of carcinogenicity and its potential relationship to *in vitro* properties of insulin analogs.

Next-generation insulin analogs in affluent societies seek to enhance the convenience and safety by which patients can achieve metabolic control. Ultra-rapid delivery technologies and ultra-flat basal insulin analog formulations promise to enable patients to recapitulate with greater precision endogenous mechanisms of hormonal regulation. The cost-effectiveness of such technologies, an of increasing societal concern in relation to aggregate health-care expenditures [76–78], will require population-based analysis of relative impact on rates of complications leading to expensive interventions and long-term disability [79]. The majority of patients in the coming decades will be living in underprivileged regions of the developing world. In such regions intertwined scientific, technical, and societal challenges are posed by the cold chain of insulin delivery in the absence of refrigeration [80–82]. Given this humanitarian need and its growing scale, we anticipate that third-generation insulin analogs must combine ultra-stability with optimized pharmacokinetic properties. Such efforts define a new frontier of translational research with application to global health.

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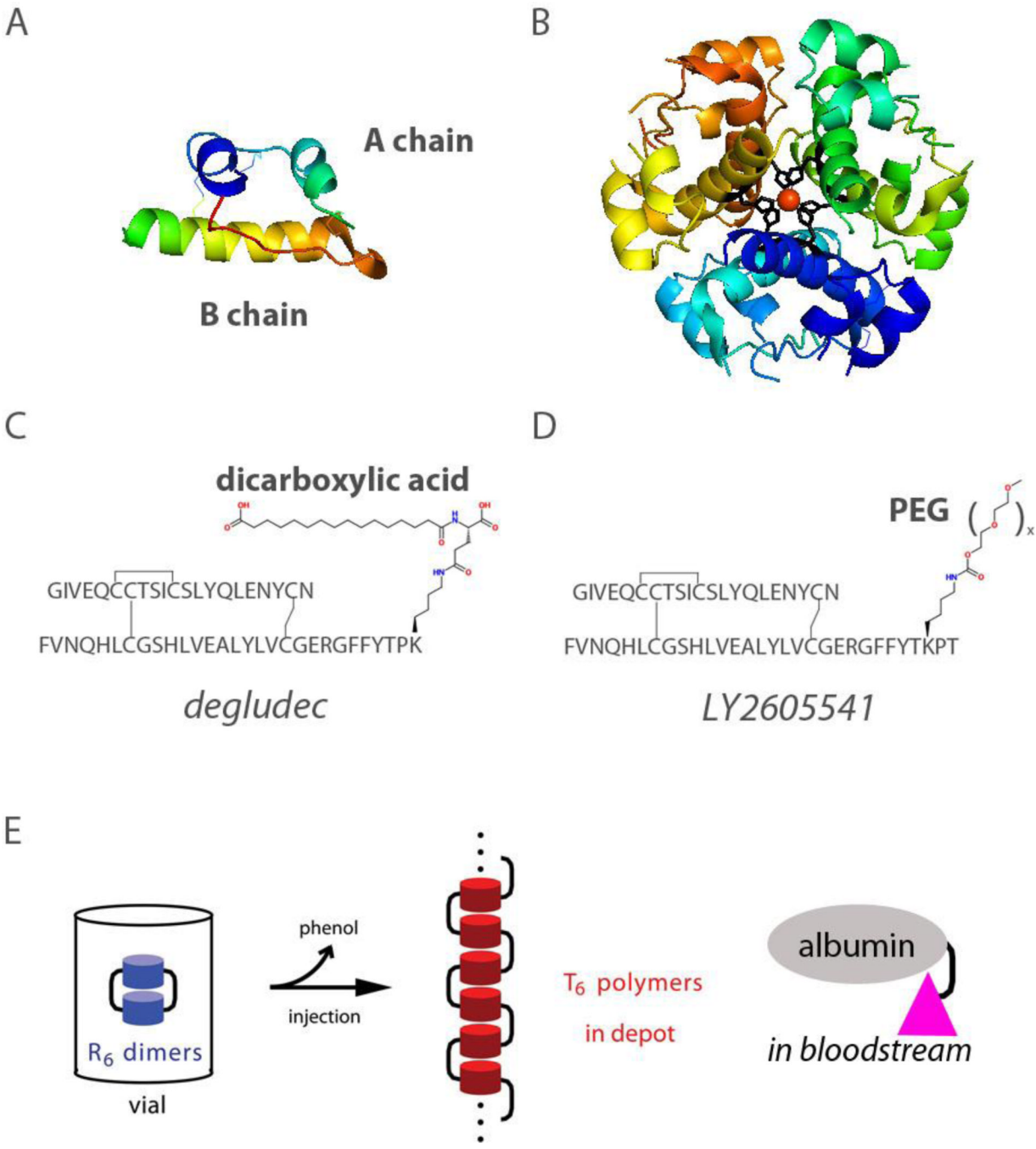

Figure 1.

(A) Ribbon model of wild-type insulin (B) Structure of insulin hexamer. Two axial zinc ions (*red*; overlaid at *center*) are coordinated by six histidine side chains (residue B10; *black*). The structure shown is the R_6 hexamer form characteristic of a pharmaceutical formulation [83]; coordinates were obtained from Protein Databank entry 1ZNJ. (C) Structure of insulin *degludec* showing the dicarboxylic acid attached to the ε-amino group of Lys^{B29}. (D) Structure of LY2605541 showing PEG attached to the ε-amino group of Lys^{B28}. (E) Exploiting the TR transition in supramolecular protein engineering: schematic representation of the mechanism of insulin *degludec*. *Left*, Insulin *degludec* is formulated at neutral pH as dimers of phenol- (or *meta*-cresol) stabilized R_6 zinc insulin hexamers (*blue*). The acyl

modification of Lys^{B29} is shown in schematic form as a *black bar* (in principle 6 per hexamer); for simplicity only two are shown. *Center*, On subcutaneous injection, diffusion of the phenolic ligand into cellular membranes triggers the R → T transition, leading in turn to linear polymerization of T_6 zinc hexamers (*red*). Classical hexamer reorganization is thus coupled to a change in mode of hexamer-hexamer assembly mediated in part by the B29-linked acyl group. *Right*, Upon entering the bloodstream as monomers (*pink*) insulin *degludec* binds to circulating albumin forming another depot that further protracts its action.

Table 1

Current Insulin Analogs and Modes of Action[a]

Analog	Modification	Mechanism
A		
Lispro (Humalog®) Eli Lilly and Co.	$Pro^{B28} \rightarrow Lys$ $Lys^{B29} \rightarrow Pro$	IGF-I-related motif impairs dimerization
Aspart (NovoLog®) Novo-Nordisk	ProB28 → Asp	Charge repulsion at dimer interface
Glulisine (Apidra®) Sanofi-Aventis	$Asn^{B3} \rightarrow Lys$ $Lys^{B29} \rightarrow Glu$	Decreased zinc-free self-association
B		
Glargine (Lantus®) Sanofi-Aventis	Arg^{B31}-Arg^{B32} tag $Asp^{A21} \rightarrow Gly$	Shift in pI to pH 7 leads to isoelectric precipitation on injection
Detemir (Levemir®) Novo-Nordisk	Modification of Lys^{B29} by a tethered fatty acid	Stabilization of hexamer and binding to serum albumin
C		
Insulin *degludec* Novo-Nordisk	Modification of Lys^{B29} by a dicarboxylic acid	Allosteric assembly of a linear T_6 polymer and albumin binding
LY2605541 Eli Lilly and Co.	PEGylation of Lys^{B28} in insulin *lispro*	Increased hydrodynamic radius

[a] *Panel A* lists rapid-acting analogs employed in prandial regimens and in insulin pumps. *Panel B* describes basal insulin analogs with protracted action. *Panel C* lists novel basal insulin analogs undergoing Phase 3 clinical trials; PEG, polyethylene glycol.