

Structurally Modified Analogues of Glucagon-Like Peptide-1 (GLP-1) and Glucose-Dependent Insulinotropic Polypeptide (GIP) As Future Antidiabetic Agents

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Abstract: Glucagon-like peptide-1(7-36)amide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are gastrointestinal insulin-releasing hormones involved in the regulation of postprandial nutrient homeostasis. These two incretin hormones are glucose-dependent stimulators of pancreatic beta-cell function, exhibiting a spectrum of secondary extrapancreatic activities, which favour the efficient control of blood glucose homeostasis. Such actions of GLP-1 and GIP have generated considerable interest in their possible exploitation as novel agents for the treatment of type 2 diabetes. Despite the many attributes of GLP-1 and GIP as possible future antidiabetic agents, their rapid degradation in the circulation by dipeptidyl peptidase IV (DPP IV) to inactive truncated forms GLP-1(9-36)amide and GIP(3-42), severely limits their therapeutic usefulness. This review will consider recent developments in the design and effectiveness of synthetic DPP IV-resistant analogues of GLP-1 and GIP. Consideration will be given to the effects of N-terminal modification and amino acid substitution of GLP-1 and GIP either side of the DPP IV cleavage site on (i) susceptibility to enzymatic degradation, (ii) binding to native hormone receptor, (iii) ability to elevate intracellular cyclic AMP, (iv) potency as insulin secretagogues, and (v) antihyperglycaemic activity in type 2 diabetes. It will be shown that structural modification can produce a varied set of biological activities, ranging from more efficacious analogues to those which antagonise the activity of the native hormone. The antidiabetic properties of the best GLP-1 and GIP analogues indeed promise to provide the basis for novel, effective and long-acting drugs for type 2 diabetes therapy. This approach is currently being pursued actively by the pharmaceutical industry.

Key Words: Glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), GLP-1 analogues, GIP analogues, type 2 diabetes.

INTRODUCTION

Unger and Eisentraut coined the term 'enteroinsular axis' in 1969 to describe all of the gut factors, which contribute to enhanced insulin secretion following the ingestion of a meal [1]. Later in 1979, Creutzfeldt suggested that the axis encompassed nutrient, neural and hormonal signaling and he laid down the two main criteria required for the classification of incretin hormones [2]. Firstly, an incretin must be released in response to nutrients, particularly carbohydrates, and secondly, it must stimulate insulin secretion at physiological concentrations. At present two potent incretin hormones, glucagon-like peptide-1(7-36)amide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) have been discovered and characterised. These peptide hormones have exceeded expectations by not only possessing insulinotropic actions, but also by acting through a number of other glucose-lowering mechanisms. The scientific profile of incretin hormones has been further raised by mounting evidence which suggests that both GLP-1 and GIP enhance the growth, differentiation, proliferation and survival of pancreatic beta-cells [3-6].

GLUCAGON-LIKE PEPTIDE-1 (GLP-1)

GLP-1 was first described as glucagon-related peptide (GRP) by the cloning of the anglerfish proglucagon gene [7]. Following cloning of the bovine [8], hamster [9], rat [10] and human [11] proglucagon genes, it was realised that anglerfish GRP-1 was a homologue of GLP-1, encoded in mammalian genes. Although there was some evidence to suggest that GLP-1 was a potent insulinotropic hormone, great uncertainty existed as to which form of GLP-1 (GLP-1(1-37), GLP-1(7-37), GLP-1(1-36)amide and GLP-1(7-36)amide) was bioactive [12]. This was clarified in 1987 when the GLP-1(7-36)amide and GLP-1(7-37) were shown to be equipotent secretagogues of glucose-dependent insulin secretion [13-15], while GLP-1(1-37) and GLP-1(1-36) amide are completely inactive. For the purpose of this review, GLP-1 refers to the active GLP-1(7-36)amide. Years of subsequent research have widely confirmed the insulinotropic action of GLP-1 in islets [16, 17], normal and diabetic animals [18-22] and healthy and diabetic human volunteers [23].

GLP-1 is known to have physiological actions in pancreatic islets, liver, adipose tissue and skeletal muscle. In pancreatic islets, GLP-1 stimulates insulin and somatostatin secretion [13, 14, 24, 25] and inhibits glucagon secretion [25]. GLP-1 stimulates insulin gene transcription [26-28], pancreatic islet cell proliferation [3, 29] and beta-cell

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replication in mice [30]. GLP-1 causes the differentiation of pancreatic ductal AR42J cells into glucagon- and insulin-producing cells [4]. This differentiation to a beta-cell type occurs through a PDX-1 dependent pathway [31, 32]. Lesser-known actions of GLP-1 include promotion of glucose uptake and glycogen formation in liver and skeletal muscle [33-35]. GLP-1 also stimulates lactate production, glucose uptake and glycogen storage in diaphragm muscle from mice [35].

GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP)

GIP was originally isolated as an 'enterogastrone' [36] from a crude porcine CCK extract, where it was initially shown to inhibit histamine-induced gastric acid secretion from innervated canine Bickel-type pouches [37]. Due to this inhibitory action the hormone was initially dubbed 'gastric inhibitory polypeptide' (GIP) [38, 39]. However, subsequent studies designed to examine the wider physiological properties of GIP established that in the presence of glucose, physiological concentrations of GIP were capable of stimulating insulin secretion from pancreatic beta-cells [40, 41]. Based on this evidence, the hormone was subsequently renamed 'glucose-dependent insulinotropic polypeptide', thus retaining the original acronym GIP [42].

Following postprandial release from intestinal K-cells, the primary role of GIP is to modulate glucose-dependent insulin secretion [43]. Furthermore GIP stimulates proinsulin gene transcription and translation [44, 45]. In addition, GIP enhances the growth, differentiation, proliferation and survival of pancreatic beta-cells [5, 6]. It also appears to act as a beta-cell mitogenic and anti-apoptotic factor [46]. Extrapankreatic effects of GIP also enhance its glucose-lowering ability. Thus, GIP has been shown to inhibit hepatic glucose production [47] and to promote glucose uptake in isolated mouse diaphragm muscle [48]. Functional GIP receptors have also been identified on adipocytes [49], where GIP has been shown to stimulate glucose transport [50], fatty acid synthesis [51] and lipoprotein lipase activity [52]. As a consequence of these biological effects, GIP has recently been proposed as a potential therapeutic agent for the treatment of diabetes, obesity and related metabolic disorders [53, 54].

GLP-1 AND GIP AS POTENTIAL DRUGS FOR TYPE 2 DIABETES

Type 2 diabetes represents about 90% of all cases of diabetes and is characterised by defective beta-cell function, insulin resistance and a relative rather than absolute insulin deficiency [55-58].

Incretin hormones exhibit many of the desirable attributes of a drug for treatment of type 2 diabetes. Their insulinotropic effects on pancreatic islets and glucose-lowering actions in peripheral tissues promise effective blood glucose control in type 2 diabetic patients [59, 60]. The glucose-dependent action of incretin hormones means that they are unlikely to cause hypoglycaemic episodes such as encountered with sulphonylureas currently used for type 2 diabetes therapy [53, 61, 62]. Furthermore, the ability of both GLP-1 and GIP to stimulate insulin gene transcription

and increase pancreatic beta-cell mass [3, 5, 6, 26, 29], may compensate for the dysregulation of insulin secretion synonymous with type 2 diabetes.

DPP IV DEGRADATION: DIFFICULTIES AND STRATEGIES FOR PREVENTION

One of the major hurdles in progressing GLP-1 and GIP to the clinic has been their rapid degradation in the circulation, which chiefly occurs as a result of the enzyme, dipeptidyl peptidase IV (DPP IV; EC 3. 4. 14. 5) [63]. DPP IV displays a strict substrate specificity, hydrolysing peptides from the NH₂-terminus following a penultimate proline, alanine or hydroxyproline residue. In addition to other regulatory peptides [63], DPP IV cleaves N-terminal dipeptides from both GLP-1 (His⁷-Ala⁸) and GIP (Tyr¹-Ala²) removing their insulinotropic activity (Fig. 1). Presently, inhibition of GLP-1 and GIP degradation by DPP IV is being tackled by a two-pronged approach. These include the use of inhibitors of DPP IV and the development of degradation-resistant analogues of GLP-1 and GIP.

DPP IV INHIBITORS

Marked improvements of metabolism and glycaemic control in diabetes have been reported following treatment with a number of DPP IV inhibitors [64-69]. Administering NVP-LAF237 with pioglitazone, appears to normalise glucose concentrations in adult obese Zucker rats [70]. Another DPP IV inhibitor, FE 999011, delayed the onset of type 2 diabetes in Zucker diabetic rats [71]. Oral administration of the DPP IV inhibitor, P32/98, to VDF (*fa/fa*) Zucker rats caused sustained improvements in glucose tolerance, insulin sensitivity and beta-cell glucose responsiveness [72]. Treating these animals for a prolonged period with P32/98 also improved hepatic and peripheral insulin sensitivity [73]. In streptozotocin-induced diabetic rats, beta-cell survival and islet neogenesis was enhanced by P32/98 [6]. Additionally, type 2 diabetic subjects showed improved metabolic control following 2-3 times daily administration of NVP DPP728 over a 4-week study period [74].

Despite beneficial actions of DPP IV inhibitors on glycaemic control, the physiological actions of DPP IV inhibition on many other regulatory peptide substrates (at least 35) could be adversely affected [75]. Until a fully comprehensive evaluation of the consequences of widespread DPP IV inhibition has been carried out in clinical trials, the potential of DPP IV inhibitors in diabetes therapy will remain uncertain. A major advantage of using DPP IV inhibitors is the possibility of oral administration, but some uncertainty exists regarding their efficacy [76]. However, an attractive alternative to enzyme inhibition involves the development of specific GLP-1 and GIP analogues modified at the N-terminus around the enzyme cleavage site which confers DPP IV resistance [53].

ENZYME-RESISTANT ANALOGUES OF GLP-1 AND GIP

Due to relatively close structural similarity, GLP-1 and GIP possess similar N-terminal regions, i.e His⁷-Ala⁸-Glu⁹- and Tyr¹-Ala²-Glu³-, respectively (Fig. 1). This explains their susceptibility to DPP IV degradation, as this enzyme prefers

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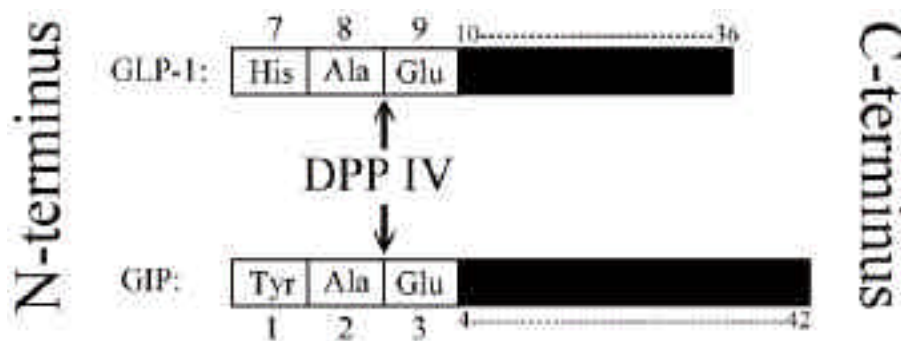


Fig. (1). The similarities of the N-terminal regions of GLP-1 and GIP. Amino acid residues can be modified at positions 7, 8 and 9 in GLP-1, corresponding to positions 1, 2 or 3 in GIP, to create synthetic analogues which may have increased resistance to DPP IV degradation.

an alanine, proline or hydroxyproline residue in the penultimate N-terminal position. A number of structural modifications of GLP-1 and GIP have been used to safeguard against DPP IV degradation (see Tables 1-3). Firstly, the N-terminal His⁷ and Tyr¹ positions of GLP-1 and GIP, respectively, have been extended by various chemical modifications [77, 78]. Secondly, Ala⁸ [79] and Glu⁹ [80, 81] positions of GLP-1, and Ala² [82, 83] and Glu³ [84, 85] positions of GIP, respectively, have been substituted with other amino acids. Thirdly and more recently, GLP-1 has been acylated with long- or short-chain fatty acids to enhance its half-life and bioavailability (see section on drug delivery).

N-TERMINAL MODIFICATION OF GLP-1 (His⁷) AND GIP (Tyr¹)

Analogues of (His⁷)GLP-1

Hareter *et al.* [86] replaced His⁷ of GLP-1 with several other amino acids, and concluded that the imidazole ring of His⁷ was crucial for GLP-1 action. This group also demonstrated that N-terminal extension of GLP-1, with insertion of an acetyl group at the α -amino group of His⁷, did not significantly affect GLP-1 action. However, DPP IV stability was not determined. Another study characterised N-terminally extended peptides with imidazole-lactic acid, N-

Table 1. Characteristics of Position 1 (His⁷/Tyr¹) Analogues of GLP-1 and GIP

Position Modified	Analogue	DPP IV resistance	In vitro testing			In vivo testing			Reference	
			cAMP	Receptor binding	Insulin	Glucose	Insulin	Animal used		
His ⁷ : GLP-1	DesaminoGLP-1			15-fold				Minipig	87, 93, 94	
	(D-His ⁷)GLP-1			9.2-fold	N	N	N	N	94	
	N-Glucitol GLP-1		N	N				Wistar rats, (ob/ob) mice	20, 21, 22	
	N-Imi-GLP-1			2-fold	N	N	N	N	87	
	N- Me-GLP-1			3-fold	N	N	N	N	87	
	N-Me-GLP-1			4-fold	N	N	N	N	87	
	N-Acetyl-GLP-1							-	(ob/ob) mice	77, 97
	N-Pyroglutamyl-GLP-1							(ob/ob) mice	77	
Tyr ¹ : GIP	N-Acetyl-GIP			N				(ob/ob) mice	89	
	N-Pyroglutamyl-GIP			N				(ob/ob) mice	89	
	N-Glucitol-GIP		N	N				(ob/ob) mice	48, 78	
	N-Palmitate-GIP			N				(ob/ob) mice	88	
	N-Fmoc-GIP			N				(ob/ob) mice	88	

Analogues are modifications of GLP-1(7-36)amide, GLP-1(7-37) or GIP(1-42). Abbreviations used: N, parameter not measured; \uparrow , Exceptional improvement; \uparrow , improvement; \sim , similar; \downarrow , deterioration compared with native peptide; Imi, imidazole-lactic acid group; Me, methyl group; Fmoc, N-(9-fluorenyl)methoxycarbonyl group.

Table 2. Characteristics of Position 2 (Ala⁸/Ala²) Analogues of GLP-1 and GIP

Position modified	Analogue	DPP IV resistance	In vitro testing			In vivo testing			Reference
			cAMP	Receptor binding	Insulin	Glucose	Insulin	Animal used	
Ala ⁸ : GLP-1	(D-Ala ⁸)GLP-1			3.4-fold				Minipig	93, 94
	(Gly ⁸)GLP-1							Zucker rats C57BL/6J mice Minipig	90, 92, 94, 95
	(Ser ⁸)GLP-1		N					Wistar rats Minipig	91, 92, 94
	(Aha ⁸)GLP-1							Zucker rats Wistar rats	95
	(Thr ⁸)GLP-1		N			N	N	N	92
	(Aib ⁸)GLP-1		N			N	N	N	92
	(Abu ⁸)GLP-1							(ob/ob) mice	79
(Val ⁸)GLP-1							(ob/ob) mice	79	
Ala ² : GIP	(Gly ²)GIP			N				(ob/ob) mice	82
	(Ser ²)GIP			N				(ob/ob) mice	82
	(D-Ala ²)GIP				N			VDF Zucker rats	96
	(Abu ²)GIP			N				(ob/ob) mice	83
	(Sar ²)GIP			N				(ob/ob) mice	83

Analogues are modifications of GLP-1(7-36)amide, GLP-1(7-37) or GIP(1-42). Abbreviations used: N, parameter not measured; \uparrow , Exceptional improvement; \uparrow , improvement; \sim , similar/unchanged; \downarrow , deterioration compared with native peptide. Amino acids: Alanine, Ala; Glycine, Gly; Serine, Ser; Aminohexanoic acid, Aha; Threonine, Thr; Aminoisobutyric acid, Aib; 2-Aminobutyric acid, Abu; Valine, Val; Sarcosine, Sar.

Table 3. Characteristics of Position 3 (Glu⁹/Glu³) Analogues of GLP-1 and GIP

Position modified	Analogue	DPP IV resistance	In vitro testing			In vivo testing			Reference
			cAMP	Receptor binding	Insulin	Glucose	Insulin	Animal used	
Glu ⁹ : GLP-1	(Asp ⁹)GLP-1	N			N		N	(db/db)mice	97
	(Ala ⁹)GLP-1	N			N		N	(db/db)mice	97
	(Pro ⁹)GLP-1							(ob/ob) mice	80
	(Phe ⁹)GLP-1							(ob/ob) mice	80
	(Lys ⁹)GLP-1		Antag.		Antag.	Antag.	Antag.	(ob/ob) mice	81
	(Tyr ⁹)GLP-1							(ob/ob) mice	80
Glu ³ : GIP	(Pro ³)GIP		Antag.	N	Antag.	Antag.	Antag.	(ob/ob) mice	84, 85

Analogues are modifications of GLP-1(7-36)amide or GIP(1-42). Abbreviations used: N, parameter not measured; \uparrow , Exceptional improvement; \uparrow , improvement; \sim , similar/unchanged; \downarrow , deterioration; \downarrow , detrimental deterioration, compared with native peptide; Antag.= receptor antagonist. Amino acids: Aspartic acid, Asp; Alanine, Ala; Proline, Pro; Phenylalanine, Phe; Lysine, Lys; Tyrosine, Tyr.

Table 4. Characteristics of acylated analogues of GLP-1

Position modified	Analogue	DPP IV resistance	In vitro testing			In vivo testing			Reference
			cAMP	Receptor binding	Insulin	Glucose	Insulin	Animal used	
Lys ²⁶	[Lys(pal) ²⁶]GLP-1							(ob/ob) mice	106
Ala ⁸ , Lys ²⁶	[Abu ⁸ , Lys(pal) ²⁶]GLP-1							(ob/ob) mice	106
Ala ⁸ , Lys ²⁶	[Val ⁸ , Lys(pal) ²⁶]GLP-1							(ob/ob) mice	106
Lys ²⁶ , Lys ³⁴	NN2211				N			(ob/ob) mice Zucker rats Pigs	103, 117, 118, 119
Lys ³⁷	CJC-1131				N		N	db/db mice	105
Lys ³⁴	Ly315902	N	N	N	N	N		Sprague-Dawley rats	104, 120

Analogues are GLP-1(7-36)amide or GLP-1(7-37). Abbreviations used: N, parameter not measured; \uparrow , Exceptional improvement; \uparrow , improvement; \sim , similar/unchanged, deterioration; \downarrow , detrimental deterioration, compared with native peptide. A recent review provides a detailed overview of acylated GLP-1 modified analogues [102].

Table 5. Characterisation, Degradation, Maximal Insulin Secretion and cAMP Production of N-Terminal GLP-1 and GIP Analogues

	Molecular mass (Da)		Half-life in h (t _{1/2})		Maximal insulin secretion (ng/ml/20min)	CAMP EC ₅₀ (nmol/l)
	Measured	Theoretical	DPP IV	Plasma		
GLP-1	3297.3	3297.5	5.8	6.2	3.97 ± 0.40	6.6
N-acetyl-GLP-1	3338.9	3339.6	>12	>12	3.89 ± 0.22	10.9
N-pyroglutamyl-GLP-1	3408.4	3408.7	>12	>12	3.83 ± 0.30	26.4
GIP	4982.5	4980.5	2.3	6.2	3.95 ± 0.12	19.1
N-acetyl-GIP	5027.0	5026.0	>12	>12	4.62 ± 0.05	2.4
N-pyroglutamyl-GIP	5094.9	5094.2	>12	>12	5.01 ± 0.01	2.8

Half-life data were calculated from the percentage of major degradation fragments, GLP-1(9-36)amide and GIP(3-42) (following HPLC separation), relative to the intact peptide following incubation with purified DPP IV or human plasma. Further data on cAMP production and insulin secretion can be viewed in Figs. 2 and 3, respectively. Data and experimental details can be found in Green *et al.* [77] and O'Harte *et al.* [89].

methyl, and β -methyl groups at His⁷ (Table 1) [87]. These analogues were more resistant to DPP IV than native GLP-1 but showed reduced affinity for the GLP-1 receptor and compromised ability to stimulate cAMP production [87]. In our laboratory, His⁷-glucitol-GLP-1 was shown to exhibit resistance to DPP IV whilst maintaining antihyperglycaemic activity *in vivo* (Table 1) [20]. More recently, two additional analogues, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1, have been shown to be completely resistant to DPP IV and human plasma degradation, also exhibiting potent receptor binding, cAMP production and insulin secretory activity *in vitro* (Table 5, Fig. 2A and 3A). In obese diabetic (*ob/ob*) mice, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 display potent insulinotropic actions (Fig. 4), with N-pyroglutamyl-GLP-1 being particularly effective compared with the antihyperglycaemic effects of native GLP-1 (Fig. 4A) [77].

Analogues of (Tyr¹)GIP

During the past few years, several novel Tyr¹-modified analogues of GIP have been developed and characterised. These include N-acetyl-, N-Fmoc-, N-glucitol-, N-palmitate-, and N-pyroglutamyl-GIP [20, 21, 48, 78, 88, 89]. These analogues modified at the α -amino region of Tyr¹ exhibited complete resistance to purified DPP IV with *in vitro* half-lives greater than 12 h compared with 2.3 h for native GIP (Table 5). All Tyr¹-modified analogues stimulated 2 to 10-fold increases in cAMP production (Fig. 2B) and approximate 1.4-fold increases in insulin secretion *in vitro* (Fig. 3B). Subtle differences in individual analogues were noted. N-Fmoc- and N-palmitate-GIP (Table 1) appeared to be 14-20% less potent *in vitro* than N-acetyl-, N-glucitol- and N-pyroglutamyl-GIP (Fig. 3B). In obese diabetic *ob/ob*

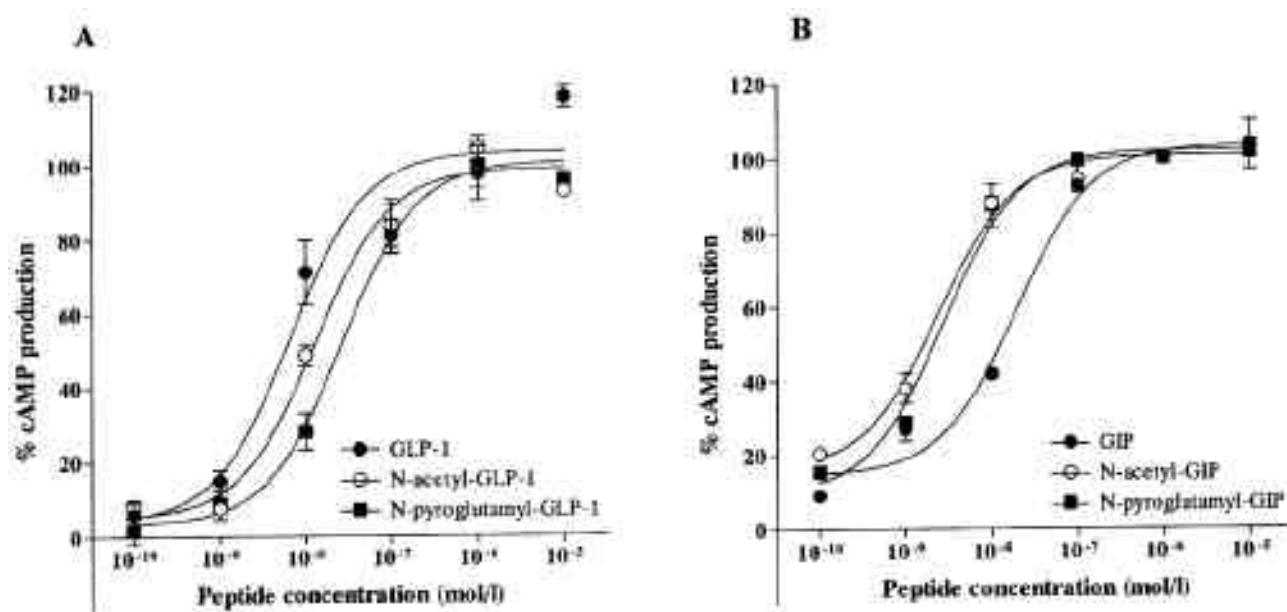


Fig. (2). Effects of His⁷/Tyr¹ analogues of GLP-1 and GIP on cAMP production in BRIN-BD11 cells. (A) Intracellular cAMP production by GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 (B) Intracellular cAMP production by GIP, N-acetyl-GIP and N-pyroglutamyl-GIP. BRIN-BD11 cells were exposed to peptides in the presence of 5.6mM glucose for 20 min. Experiments were performed in triplicate with data expressed (mean \pm SEM) as a percentage of the maximal response. Data taken from [77, 89].

mice, Tyr¹-modified analogues were noticeably superior at stimulating insulin release and lowering blood glucose compared with native GIP. Consistent with *in vitro* data, these results indicated that N-acetyl-, N-glucitol- and N-pyroglutamyl-GIP were slightly more potent than either N-Fmoc- or N-palmitate-GIP. Of the Tyr¹-modified analogues tested, N-acetyl-GIP [89] appeared to be the most impressive (Fig. 4). Importantly, the severe insulin resistance and beta cell defect of obese diabetic (*ob/ob*) mice (including poor response to native GIP) was largely overcome by N-acetyl-GIP, making the use of such an analogue for type 2 diabetes therapy a feasible objective. Thus any defect in the insulinotropic response to GIP in diabetes appears to be overcome by these stable and chemically modified GIP agonists.

POSITION 2 MODIFICATION OF GLP-1 (ALA⁸) AND GIP (ALA²)

Analogues of (Ala⁸)GLP-1

Burcelin *et al.* [90] substituted Ala⁸ for Glycine (Gly) in GLP-1 and found that this conferred resistance to DPP IV and corrected both the fasting hyperglycaemia and glucose intolerance of diabetic mice despite a reduced affinity for the GLP-1 receptor (Table 2). Similarly, Ritzel *et al.* [91] showed that substitution of Ala⁸ for Serine (Ser) significantly increased the plasma stability of GLP-1 without impairing its insulinotropic activity in Wistar rats (Table 2). Deacon *et al.* [92] generated four synthetic GLP-1 analogues by substituting Ala⁸ for threonine, glycine, serine and -aminoisobutyric acid (Thr⁸, Gly⁸, Ser⁸, Aib⁸) (Table 2). All of these analogues exhibited a significantly prolonged biological half-life *in vivo* and bound the GLP-1 receptor with high affinities. However, only native GLP-1 and (Aib⁸)GLP-1

significantly improved insulin output over basal. Similarly, Siegel *et al.* [93, 94] reported analogues with D-Alanine (D-Ala), Serine (Ser) and Glycine (Gly) substitutions at position 8 which were substantially more resistant to DPP IV than native GLP-1 and had similar or enhanced biological half-life and potency (Table 2). Doyle *et al.* [95] substituted Ala⁸ for Glycine (Gly) and aminohexanoic acid (Aha) (Table 2). These analogues stimulated insulin secretion and intracellular cAMP to a similar degree to native GLP-1. *In vivo* they lowered blood glucose and increased blood insulin levels in fasted Zucker rats. A recent study [79] characterised two novel Ala⁸-substituted analogues of GLP-1, namely (Abu⁸)GLP-1 and (Val⁸)GLP-1. These were shown to be completely resistant to degradation by DPP IV or human plasma (Table 2). Receptor binding studies demonstrated that (Abu⁸)GLP-1 and (Val⁸)GLP-1 bound the GLP-1 receptor with high affinity. However, the extent of binding was reduced compared with native GLP-1 (Table 2). Although active stimulators of intracellular cAMP, both (Abu⁸)GLP-1 and (Val⁸)GLP-1 were 1.5- and 3.5-fold less potent than native GLP-1. However, these losses in receptor affinity and cAMP production were not translated into compromised insulinotropic activity either *in vitro* or *in vivo* (Table 2). This may be explained by the diverse mechanisms of action of GLP-1 on the pancreatic beta-cell. Glucose tolerance testing in obese diabetic (*ob/ob*) mice showed that (Abu⁸)GLP-1 had similar *in vivo* glucose-lowering ability to native GLP-1, while (Val⁸)GLP-1 was up to 37% more potent. This glucose-lowering activity was coupled with enhanced insulin levels following peptide administration. (Abu⁸)GLP-1 and (Val⁸)GLP-1 appeared to be equipotent secretagogues *in vivo*. Amino acid substitution of GLP-1 at Ala⁸ appears to be a particularly viable strategy for

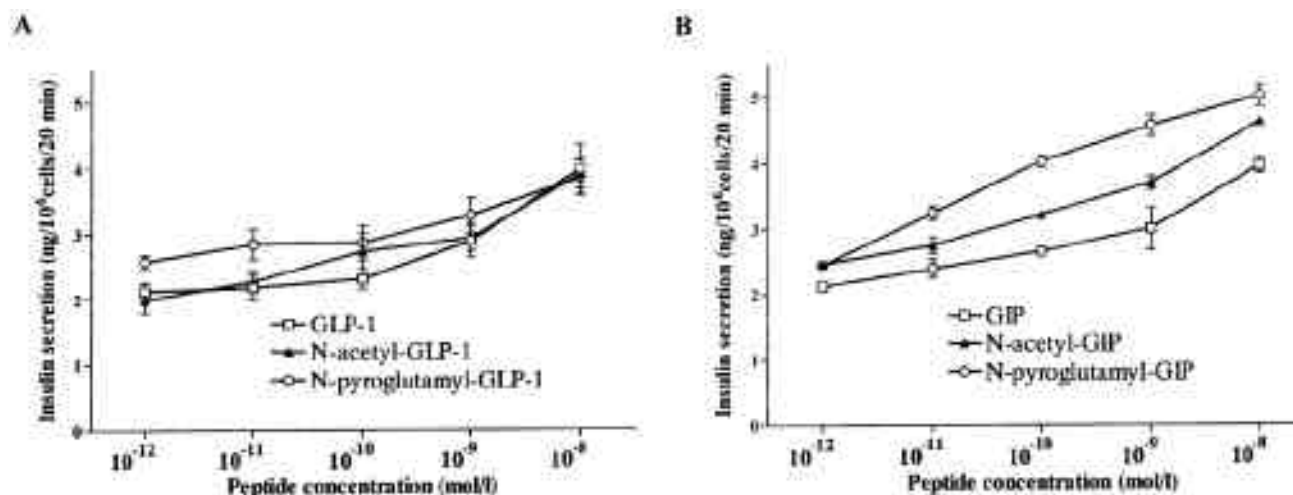


Fig. (3). Effects of His⁷/Tyr¹ analogues of GLP-1 and GIP on *in vitro* insulin secretion in BRIN-BD11 cells. (A) *In vitro* insulin secretion by GLP-1, N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1 (B) *In vitro* insulin secretion by GIP, N-acetyl-GIP and N-pyroglutamyl-GIP. BRIN-BD11 cells were exposed to peptide analogues in the presence of 5.6mM glucose for 20 min. Data are mean \pm SEM (n=8). Data taken from [77, 89].

generating worthwhile therapeutic candidate peptides for the treatment of type 2 diabetes.

Analogues of (Ala²)GIP

As shown in Table 2, several Ala²-substituted GIP analogues, including (Abu²)GIP, (Gly²)GIP, (Sar²)GIP, (Ser²)GIP and (D-Ala²)GIP have been synthesized and tested for DPP IV stability and biological activity [82, 83, 96]. (Abu²)GIP and (Sar²)GIP did not exhibit resistance to DPP IV and had disappointing *in vitro* biological activities. However, both analogues displayed antihyperglycaemic and insulinotropic activity comparable to native GIP when administered to obese diabetic *ob/ob* mice (Table 2) [83]. Contrastingly, (Gly²)GIP and (Ser²)GIP [82] were more resistant to DPP IV than native GIP, and displayed enhanced ability to elevate cAMP and stimulate insulin secretion *in vitro* (Table 2). These actions resulted in (Gly²)GIP and (Ser²)GIP having significantly improved insulinotropic and antihyperglycaemic activities in *ob/ob* mice compared with native GIP. Similarly, Pederson, McIntosh and colleagues investigated the substitution of L-alanine in position 2 of GIP with D-alanine (Table 2) [96]. This enzyme-resistant analogue exhibited moderately reduced biological activity *in vitro*, but significantly improved the glycaemic excursion in *fa/fa* VDF Zucker rats. (D-Ala²)GIP demonstrated similar activity to (Gly²)GIP or (Ser²)GIP (Table 2) [82]. Despite notable improvements in biological activity compared with native GIP, the efficacy of Ala²-substituted analogues was not as impressive as Tyr¹-modified GIP analogues (Table 2).

POSITION 3 MODIFICATIONS OF GLP-1 (GLU⁹) AND GIP (GLU³)

Analogues of (Glu⁹)GLP-1

Xiao *et al.* [97] demonstrated that substituting Glu⁹ for either Ala or Asp dramatically altered GLP-1 receptor affinity and biological activity. Until recently, no study had

investigated how modifications at Glu⁹ affected degradation of GLP-1 by DPP IV. Substituting Glu⁹ with Pro⁹ yielded a peptide, which was substantially more stable than native GLP-1, and which retained all of the usual biological actions (Table 3) [80]. Other analogues, (Phe⁹)GLP-1 and (Tyr⁹)GLP-1, were not as resistant to DPP IV as (Pro⁹)GLP-1 but were more resistant than native GLP-1 [80]. Of these novel analogues, only (Pro⁹)GLP-1 was highly potent in lowering plasma glucose and raising insulin levels *in vivo* in obese diabetic (*ob/ob*) mice [80]. Furthermore, replacing Glu⁹ with Lys⁹ (Table 3), produced a GLP-1 analogue which was profoundly resistant to DPP IV and which possessed cellular and metabolic actions similar to those of the established antagonists, GLP-1(9-36)amide and exendin (9-39) [81].

Analogues of (Glu³)GIP

In the case of GIP, substitution of Glu³ with Pro produced a novel GIP receptor antagonist, (Pro³)GIP, which was completely resistant to DPP IV mediated degradation (Table 3) [84]. (Pro³)GIP inhibited GIP-stimulated cAMP production and insulin secretion with high sensitivity and specificity *in vitro*. Furthermore, (Pro³)GIP effectively countered the insulin-releasing and antihyperglycaemic actions of the native GIP in obese diabetic *ob/ob* mice [84, 85]. These actions were similar to the GIP(3-42) antagonist but more specific than exendin (9-39) [85]. Recent studies employing (Pro³)GIP indicate that GIP is the major physiological incretin, perhaps accounting for up to 80% of nutrient-induced enteroinsular pancreatic beta-cell stimulation [85]. The potential therapeutic usefulness of this selective and potent GIP receptor antagonist is emphasised by studies which suggest that GIP plays a key role in lipid metabolism and the development of both genetically-inherited and diet-induced obesity [98]. These recent observations add to a growing body of evidence, which links GIP to fat deposition, obesity and glucose intolerance [54, 98-100].

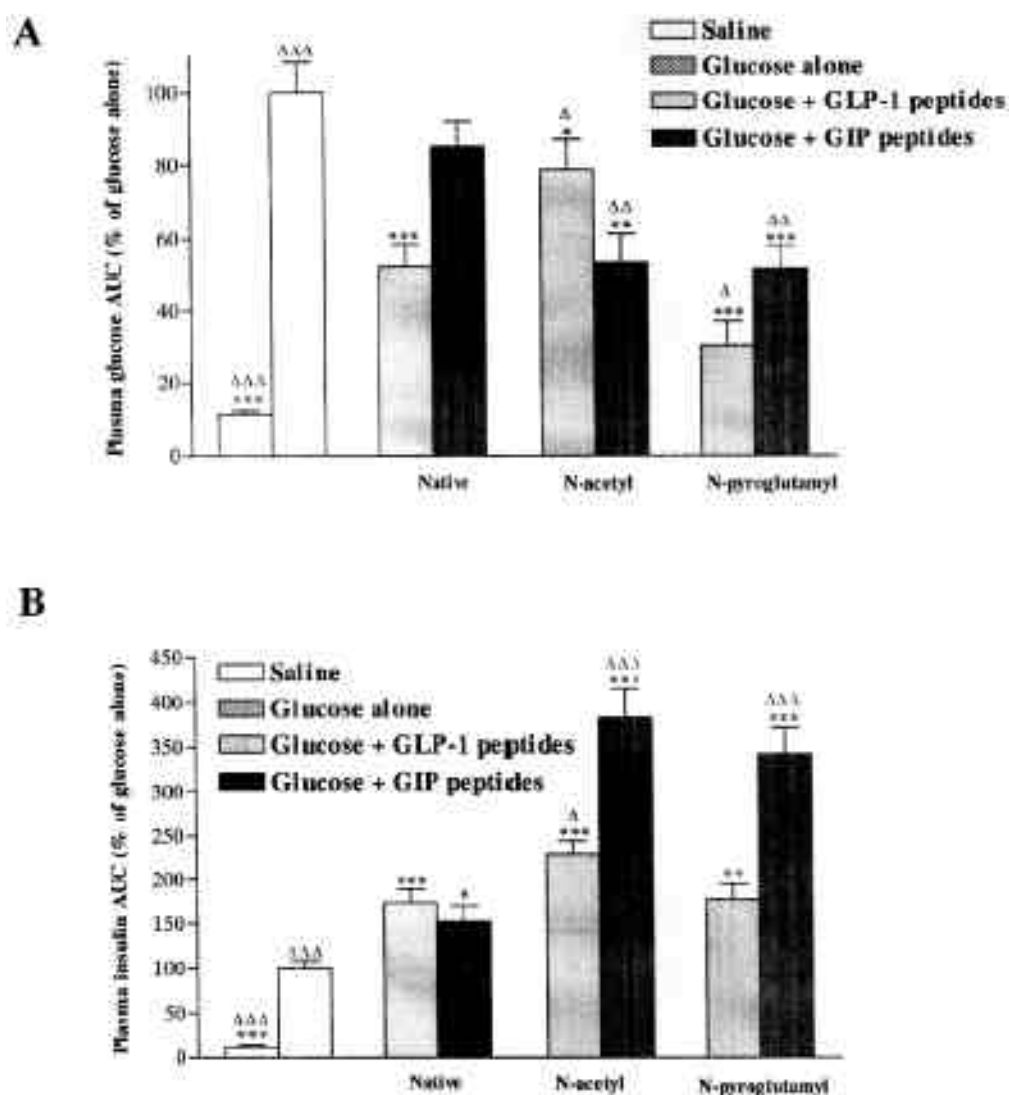


Fig. (4). *In vivo* actions of His⁷/Tyr¹ analogues of GLP-1 and GIP on (A) glucose-lowering and (B) plasma insulin in obese diabetic (*ob/ob*) mice. Glucose and insulin concentrations were measured prior to and after administration of saline (0.9% w/v), glucose alone (18mmol/kg), or in combination with peptide or peptide analogue. Data are mean \pm SEM for 8 mice with AUC values for 0-60 min post-injection calculated using the trapezoidal rule. *P<0.05, **P<0.01, ***P<0.001 compared with glucose alone. P<0.05, P<0.01, P<0.001 compared with native peptide. Data taken from [77, 89].

DRUG DELIVERY OF INCRETIN HORMONE ANALOGUES

Despite development of a number of potentially useful DPP IV resistant analogues of GLP-1 and GIP, the action of these peptides is likely to be lost within 4 hours of administration, probably due to renal clearance [88, 101]. One means of circumventing this problem is the attachment of acylated groups at selected residues, as covered in a recent review [102]. (Lys²⁶-(N-(-Glu(N-(-hexadecanoyl))-GLP-1, otherwise known as NN2211 has an extended half-life of 8 h in human subjects and a promising metabolic profile (Table 4) [103]. Other analogues modified in this way possess prolonged half-lives. Lys³⁴-(octanoyl)-GLP-1 (Ly315902) has a half-life of 3-6 h in dogs (Table 4) [104] and Lys³⁷-(2-(2-(2-maleimidopropionamido (ethoxy)ethoxy)-

acetamide-GLP-1 (CJC-1131) has a half-life of 18 h in rats (Table 4) [105]. Another study [106] indicates that acylated GLP-1 peptides can be problematic in terms of bioactivity and bioavailability in acute experiments (Table 4).

There is also a need to create more effective and user-friendly means of administering DPP IV-resistant analogues to avoid frequent injections or continuous infusion. A few investigations have explored alternative routes of administration with encouraging results. Gutniak *et al.* [107] administered GLP-1 in a buccal tablet formulation, which stimulated insulin secretion and lowered blood glucose in normal human volunteers. This study was extended to human subjects with type 2 diabetes, and effectively lowered blood glucose levels [108]. Another group experimented with the use of a biodegradable and biocompatible polymer to encap-

sulate a DPP IV-resistant analogue of GLP-1, ((D-Ala⁸)GLP-1) [109]. These microspheres of (D-Ala⁸)GLP-1 were administered orally to diabetic *db/db* mice and successfully lowered basal glucose levels. A somewhat more unconventional approach has been suggested, namely the development of encapsulated engineered cells which secrete GLP-1 [110]. Transgenic expression of exendin-4 in mice demonstrated that there is a preserved responsiveness to exendin-4, even after several months [111]. This novel approach may represent a potential future delivery system for GLP-1. Other drug delivery systems are open to investigation, such as the use of inhalers and nasal sprays/transdermal patches as adopted for insulin and vasopressin [112-114]. Since many type 2 diabetes patients develop progressive beta-cell failure on conventional oral agents, more research on delivery of incretin hormones is required before they can be explored as a safe mainstream diabetic therapy, to preserve or augment beta-cell function.

RELATIVE MERITS AND FUTURE OF GLP-1 OR GIP ANALOGUES

It has been broadly demonstrated that strategically modifying GLP-1 or GIP at the N-terminus causes an inhibition or even complete prevention of DPP IV-mediated degradation (Tables 1-3). Such modifications can have enhanced, neutral or even detrimental effects on the biological activities of GLP-1 and GIP (Tables 1-3). Furthermore, certain modifications can have opposite effects on GLP-1 and GIP. For example, N-terminal extension of GLP-1 with glucitol, acetyl or pyroglutamyl groups reduced receptor binding, cAMP production and insulin secretion [20, 77, 89]. The identical modification of GIP enhanced each of these bioactivities and provided strong antihyperglycaemic effects *in vivo* (Table 1; Figs 2-4) [48, 89]. Furthermore, when Glu⁹ of GLP-1 and Glu³ of GIP, are substituted for Pro there were stark differences in biological actions compared with native peptides. (Pro⁹)GLP-1 appears to be a novel and DPP IV stable GLP-1 agonist [80], whereas (Pro³)GIP is a specific and potent functional antagonist of the GIP receptor [84, 85]. Studies with this new GIP receptor antagonist have illustrated the dominance of GIP as the major physiological incretin hormone [85]. Interestingly, (Lys⁹)GLP-1 represents a novel functional GLP-1-receptor antagonist, (Table 3) [81]. Such structural modifications disclose parallels in the prevention of DPP IV-mediated degradation of GLP-1 and GIP, but reveal specific differences in their effects on receptor activation. Also apparent are the capabilities of relatively small changes in molecular structure to make major alterations to the properties of incretin hormones.

A large number of studies have created GLP-1 analogues which are more resistant to DPP IV than native GLP-1, making it a relatively straightforward attribute to engineer. Much more difficult, is the task of creating DPP IV resistant GLP-1 analogues with enhanced biological actions. Although many GLP-1 analogues can have similar *in vivo* activities, it is clear that they invariably exhibit reduced cAMP production and receptor binding affinity (Tables 1-3). This is a fundamental problem with present GLP-1 analogues and loss of post-receptor activity seems to occur regardless of whether the peptide is modified at position 7, 8

or 9. The challenge with GLP-1 is to synthesise DPP IV resistant analogues which do not suffer from impaired receptor activation. In the defence of GLP-1 analogues, some studies have shown that the observed losses in receptor binding and cAMP production do not necessarily lead to a reduced insulinotropic action *in vivo* [79, 80]. As pointed out, this may be due to other actions of GLP-1 on the beta-cell, such as inhibition of K_{ATP} channels leading to an influx of Ca²⁺ [115]. Thus, receptor binding and cAMP production may not be particularly good indicators of the insulin-releasing efficacy of GLP-1 peptides. Potent GLP-1 analogues could in future be generated by amino acid substitutions at either Ala⁸ and Glu⁹. In this regard, (Val⁸)GLP-1 and (Pro⁹)GLP-1 display excellent *in vivo* glucose-lowering and insulinotropic actions, in addition to profound DPP IV stability.

Bioactivity of GIP, is not as sensitive as GLP-1 to compromise when structural changes are introduced to impart DPP IV stability (Tables 1-3). With the exception of (Pro³)GIP, GIP analogues do not generally experience substantial losses in post-receptor or insulin secretory potency. Most notable is the modification of GIP at Tyr¹ which generates 'super GIP agonists'. Furthermore, specific GIP-receptor antagonists, such as (Pro³)GIP, have great potential as anti-obesity agents. Thus GIP receptor deficient (GIPR^{-/-}) mice exhibit glucose intolerance and diminished insulin secretion [116] and they also appear to be resistant to the development of obesity when placed on a high-fat diet [98]. Therefore, (Pro³)GIP is an important agent which could be employed for elucidation of the role of GIP in obesity and the treatment of insulin-resistant type 2 diabetes.

CONCLUSION

GLP-1 and GIP analogues discussed here may be classified into sub-types based on the site of structural modification. These include position 1 analogues (His⁷/Tyr¹), position 2 analogues (Ala⁸/Ala²) and position 3 analogues (Glu⁹/Glu³) which all have the potential resistance to degradation by DPP IV. Whilst Tyr¹-modified GIP analogues are especially potent, His⁷-modified GLP-1 analogues suffer losses in biological activity due to compromised post-receptor activity. Ala⁸ or Ala²- substitutions in GLP-1 and GIP, respectively, can produce competent analogues, equipotent to the native peptide and with resistance to DPP IV. Analogues of GLP-1 modified at Glu⁹ can be stable agonists ((Pro⁹)GLP-1) or stable antagonists ((Lys⁹)GLP-1). The Glu³-modified GIP analogue, (Pro³)GIP, is a stable and highly potent GIP receptor antagonist. It is clear that these different analogues of GLP-1 and GIP present with different properties and potential uses. Several of the analogues created so far represent important therapeutic and mechanistic tools, which may become instrumental in the crusade against the rising tide of type 2 diabetes.

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