Emerging targets for diabetes

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The worldwide epidemic of type 2 diabetes (NIDDM) has been stimulating the search for new concepts and targets for the treatment of this incurable disease. Most current therapies were developed in the absence of defined molecular targets. Increasing knowledge on the biochemical and cellular alterations occurring in NIDDM has led to the development of novel and potentially more effective therapeutic approaches to treat the disease.

The role of peroxisome proliferator activated receptors (PPARs) in the regulation of lipid metabolism, insulin and triglycerides leads to the rational design of several PPAR agonists. However, many promising molecules, especially the dual-acting PPARγ/α, are yet to be approved due to safety issues. Meanwhile, two targets, protein tyrosine phosphatase 1B (PTP-1B) and glycogen synthase kinase-3 (GSK-3), have emerged as validated targets for treating this disease. The activity of various non-peptidic small molecules as well as small peptides like PTP-1B inhibitors has been studied. Likewise, GSK-3, which plays a key role in the insulin signalling pathway, has been intensely studied by various companies as a potential target for the development of antidiabetic therapies. This review focuses on PTP-1B and GSK-3 inhibitors studied until now.

DIABETES mellitus is a metabolic disorder characterized by hyperglycaemia, glycosuria, negative nitrogen balance and sometimes ketonaemia. It causes a number of complications like retinopathy, neuropathy and peripheral vascular insufficiencies[1]. The dramatic increase in the prevalence of diabetes can be attributed to several factors. Globally, diabetes has shadowed the spread of 'modern lifestyle' and can be linked to an increasingly overweight and sedentary population[2]. The chronic metabolic disorder that afflicts 150 million people is set to rise[3] to 300 million by 2025. There are two major forms of diabetes. Type 1 or insulin-dependent diabetes mellitus is an autoimmune genetic disease resulting from an absolute deficiency of insulin due to destruction of insulin-producing pancreatic β cells. Type 2 or non-insulin-dependent diabetes mellitus is a multi-factorial disease[4] which is characterized by insulin resistance associated not only with hyperinsulinaemia and hyperglycaemia but also with atherosclerosis, hypertension and abnormal lipid profile, collectively called syndrome X. Type 2 insulin-resistant diabetes accounts for 90–95% of

the diagnosed cases of the disease[5]. There is no single approach to treat this disease and usually a combination therapy is adopted from different approaches.

Different approaches for the treatment of diabetes[6,7] are given in Table 1.

Emerging biological targets

The worldwide epidemic of type 2 diabetes (NIDDM) has been stimulating the quest for new concepts and targets for the treatment of this incurable disease. Most current therapies were developed in the absence of defined molecular targets. The discovery of nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ) as the molecular target for glitazones-related drugs, heralded a new era in understanding the pathophysiology of insulin resistance and its related complications. PPARs belong to the superfamily of nuclear hormone receptors and are ligand-dependent transcription factors, whose mechanism of action is fairly well-understood today. PPARα is known to be receptor for the fibrate class of hypolipidemic agents, while PPARγ agonists reduce hyperglycaemia without increasing the amount of insulin secretion by increasing cellular glucose uptake, reducing cellular glucose production and increasing insulin sensitivity in resistant tissues[8]. Neither the fibrates nor the glitazones lower triglycerides and increase high density lipoprotein cholesterol (HDLc) simultaneously and thus improve insulin sensitivity. These observations helped in developing dual PPARα and PPARγ activators, which will be useful for the treatment of dyslipidaemia associated with atherosclerosis and type 2 diabetes. Recently, several groups have also reported the design and synthesis of many dual acting agonists (targeting both PPARγ and PPARα)[9,10]. Though some have shown great promise, there are concerns about safety issues and further clinical trial data are awaited.

The increasing knowledge on the biochemical and cellular alterations occurring in NIDDM has led to the development of other novel and potentially effective therapeutic approaches to treat this disease. Alongside, intensive studies of the mechanisms of action of the known drugs have provided further validation for several new molecular drug targets. In this review, we focus on two emerging targets which are insulin sensitizers: protein tyrosine phosphatase-1B (PTP-1B) and glycogen synthase kinase-3 (GSK-3).

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Table 1. Various approaches for treatment of diabetes

Insulin or insulin mimetics[74]	
Insulin/modified insulin	Provides better glycaemic control
Improved delivery vehicle	Shows more favourable pharmacokinetics
Insulin mimetics	Selectively activates the human insulin receptor
Enhancers of insulin release[75,76]	
Sulfonyl ureas	Act only in the presence of elevated glucose level
Glucagon like peptide	Stimulates β-cell growth and differentiation
Imidazoline	Potent effect on glucose tolerance
Inhibitors of hepatic glucose production (hgp)	
Glucagon receptor antagonists	Non-competitive action with glucagon receptor
Glycogen phosphorylase inhibitor	Decreases glucose-1-phosphate formation from glycogen
Pyruvate dehydrogenase kinase inhibitor	Increases oxidative glucose metabolism and decreases gluconeogenesis
Fructose-1,6-biphosphatase inhibitor	Decreases pyruvate conversion to glucose
Glucose-6-phosphatase inhibitor	Affects final step in both gluconeogenesis and glycolysis
Inhibitors of glucose uptake	
Glycosidase inhibitor	Inhibits α-glycosidase and decreases conversion of fructose to glucose
Inhibition of gastric emptying	Moderate postprandial glucose spikes
Inhibition of Na^+ glucose co-transporter (SGLT)	Blocks renal glucose reabsorption from urine, used to induce glycosuria
Enhancer of insulin action	
PPARα agonist	Decreases obesity
PPARγ agonist	Lipid and cholesterol homeostasis
Retinoid X receptor	Controls lipid and carbohydrate metabolism
β₃ Adrenergic receptor agonist	Decreases food consumption and leptin
Protein tyrosine phosphatase-1B inhibitor	Prevents dephosphorylation of activated insulin receptor
Glycogen synthase kinase-3 inhibitor	Activates glycogen synthase

Protein tyrosine phosphatase-1B

Tyrosine phosphorylation of proteins is a fundamental mechanism for the control of cell growth and differentiation. It is reversible and governed by the opposing activities of protein tyrosine kinases (PTKs), which catalyse phosphorylation and protein tyrosine phosphatases (PTPs), which are responsible for dephosphorylation[11]. Defective or inappropriate operation of these network leads to aberrant tyrosine phosphorylation, contributing to the development of many diseases like cancer and diabetes. PTPs can be divided into three major subfamilies – tyrosine-specific, dual-specific and low molecular weight phosphatases. The dual-specific phosphatase utilizes the protein substrate that contains pTyr as well as pSer and pThr[12].

Several PTPs have been implicated as negative regulators of the insulin signalling pathway; these include PTPα, PTP-LAR, and PTP-1B. PTP-1B is a cytosolic phosphatase consisting of a single catalytic domain. *In vitro*, it is a non-specific PTP and dephosphorylates a wide variety of substrates. *In vivo*, it is involved in down regulation of insulin signalling by dephosphorylation of specific phosphotyrosine residues on the insulin receptor. Administration of PTP-1B antisense oligonucleotides to diabetic obese mice reduces plasma glucose and brings insulin level to normal. PTP-1B knockout mice have shown increased insulin sensitivity and decreased weight gain after a high-fat diet. All these evidences help to validate PTP-1B as a key negative regulator of insulin signal transduction and a poten-

tial therapeutic target in the treatment of NIDDM and obesity[13].

On binding to its receptor, insulin induces activation of insulin receptor tyrosine kinase (IRTK) through autophosphorylation. This leads to recruitment of insulin receptor substrate (IRS) proteins, followed by activation of phosphatidyl inositol 3 kinase (PI3K) and subsequent translocation of glucose transporter-4 (GLUT-4) and glucose uptake in muscle (Figure 1). This process is negatively regulated by PTP-1B and in general mechanism for down regulation of IRTK activity[14].

The active site of PTP-1B contains a common structural motif His–Cys–Ser–Ala–Gly–Ile–Gly–Arg, forming a rigid, cradle-like structure that coordinates to the aryl phosphate moiety of the substrate. It contains an active site nucleophile, Cys 215. The dephosphorylation of tyrosine takes place via two steps. In the first step there is a nucleophilic attack on the substrate phosphate by the sulphur atom of Cys, coupled with protonation of tyrosyl leaving group by Asp181 acting as a general acid. This leads to the formation of cysteinyl-phosphate intermediate. The second step mediated by Glu262 and Asp181, leads to the hydrolysis of catalytic intermediate and release of phosphate[15].

Inhibitors of PTP-1B: Phosphatase LAR, CD45, SHP-2, cdc25c and T-cell PTP (TCPTP) share 50–80% homology in the catalytic domain with PTP-1B, which presents a challenging task of achieving selectivity, especially over TCPTP. Thus it was necessary for the inhibitors to inter-

act with the regions outside the catalytic site in order to be selective. A non-catalytic phosphotyrosine-binding site was identified, which seems to be ideal since it is close to the catalytic site and is less homologous between the PTP-1B and TCPTP when the amino acid sequences were compared[16]. Hence targeting both the sites simultaneously may show good activity and selectivity against PTP-1B. Several companies are pursuing the development of PTP-1B inhibitors as drugs[17]; Abbott, Wyeth, Merck-Frosst, Takeda and Nova Nordisk, to name a few.

Vanadium has a potential therapeutic value in human diabetes. It inhibits phosphotyrosine phosphatase and activates autophosphorylation of tyrosine residue. But vanadium compounds lack specificity and augment tyrosine phosphorylation of a wide variety of cellular proteins. They pose toxicity problems as they also target some ATPase, adenylate cyclase and Ca^{2+} channels[17].

High throughput screening identified 1,2-naphthoquinones as the lead molecule having IC_{50} values in micro-molar range. The derivatives of 1,2-naphthoquinone (1) (Figure 2) were evaluated for their *in vitro* inhibitory activity against recombinant human PTP-1B using fluorescein diphosphate. In this series, 4-cyclohexyl-1,2-naphthoquinone exhibited 10 to 60-fold selectivity over other PTPs[18].

Pyridazine analogues are reversible, non-competitive PTP-1B inhibitors. This indicates that they do not bind within the active site cleft of PTP-1B. High throughput screening showed compound (2) derivatives as being novel reversible inhibitors with an IC_{50} value in micro-molar range. Unlike many tyrosine phosphatase inhibitors, this compound class lacks negative charge and thus showed high permeability across the cell membrane[19].
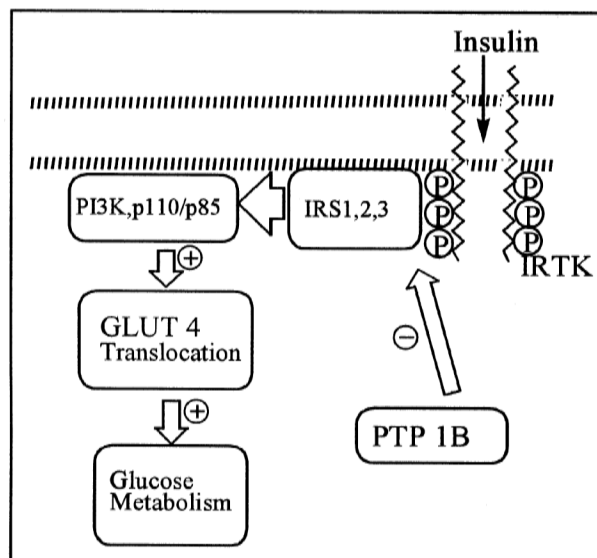

Figure 1. Negative regulation of insulin receptor by PTP-1B. (–) denotes inhibition whereas (+) denotes activation.

The non-peptidyl compounds having difluoromethylene phosphonic acid (DFMP) group were shown to be potent inhibitors of PTP-1B. The phenyl or ethynyl group at *meta* position increases potency by 15 to 17-fold. The increase is due to the pi–cation interaction of the phenyl ring with Lys-116 and Lys-120. The α,β-unsaturated allyl ester (3) moiety was the most potent reversible, competitive inhibitor[20].

3-Formylchromone is a neutral molecule and inhibits PTP-1B with potency of 73 µM. 6-Biphenyl-3-formylchromone (4) was found to be the most potent inhibitor in this series. It is suggested to have extended interaction of the extra phenyl ring with the surface near the active site of the enzyme[21].

Oxalyl-arylamino benzoic acid derivatives are catalytic site-directed, competitive and reversible PTP-1B inhibitors. The dicarboxylic acid portion of the molecule binds in the catalytic site; compound (5) was found to be the most potent in this series. The S-isomer is 20-fold more active than the R-form[22]. Like (5), the oxamyl propionic acid analogue (6) also has selectivity over TCPTP. As the number of acid groups increases, the chance for the inhibitor to penetrate the cell membrane via passive diffusion is dramatically reduced. The monoacid analogue (7) has maintained most of the potency of the corresponding diacid and has selectivity greater than 23-fold over TCPTP.

The phosphopeptide Ac–Asp–Ala–Asp–Glu–*Xxx*–Leu–NH_2 (8a), derived from epidermal growth factor receptor is an excellent substrate for PTP-1B, when *Xxx* represents pTyr. But the major limitation of pTyr-containing peptides is their susceptibility to phosphorolysis and inactivation by PTPs. Since the phosphate group is crucial for PTP-substrate binding, an effective, non-hydrolysable phosphate mimetic is an important aspect of PTP-1B inhibitor design[23]. The most effective phosphate mimetic reported is the DFMP group. Peptide-bearing phosphono(di-fluoromethyl) phenylalanine (F_2Pmp) binds better than the analogue peptide substrates and can be up to three orders of magnitude more effective than the non-fluorinated analogues. But the dianionic nature of the DFMP group compromises cell permeability[24].

As the efficacy of the phosphonates is hampered by their inability to penetrate into cells, there is considerable interest in the development of non-phosphorus containing pTyr mimetics. The analogues that utilize the dicarboxylic acid-containing malonate structure as phosphate isosteres are the most successful non-phosphorus containing pTyr mimetics. These include *O*-malonyltyrosine (OMT) and fluoro-*O*-malonyltyrosine (FOMT), which in the context of peptides are among the most potent PTP-1B inhibitors. They are designed to potentially afford pro-drug protection strategies. It was envisioned that the charged malonyl carboxyl groups could be masked in their ester form, and then liberated once inside the cells to the free carboxyls via the action of cytoplasmic esterase. The limitation of OMT-containing peptide is the removal of only one ester

Figure 2. PTP-1B inhibitors.
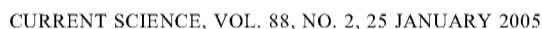
after esterase treatment[25]. Peptides containing dicarboxylic acid-based pTyr mimetics were prepared and evaluated for their PTP-1B inhibitory potency[26].

O-Malonyl tyrosine (**8b**) and *O*-carboxymethyl salicylic acid (**9**) containing peptides are found to be potent inhibitors of PTP-1B. Compound (**9**) has 94% inhibition at 100 μm concentration. Both were effective at enhancing the insulin stimulated uptake of 2-deoxyglucose by L6 myocytes[27].

Aryl α-ketocarboxylic acids comprise a new class of inhibitors for PTP-1B. The peptide containing phenyl gly-

oxalic acid (**10**) has shown some promise against PTP-1B. But these peptides are not as potent as other peptides containing FOMT and F_2Pmp. However, they have better activity than peptides with pTyr analogue having a single carboxylic acid[28].

Alpha-bromoacetophenone derivatives act as potent PTP inhibitors by covalently alkylating the conserved catalytic cysteine in the PTP active site. Derivatization of the phenyl ring with a tripeptide Gly–Glu–Glu[29] resulted in potent, selective inhibitors against PTP-1B.
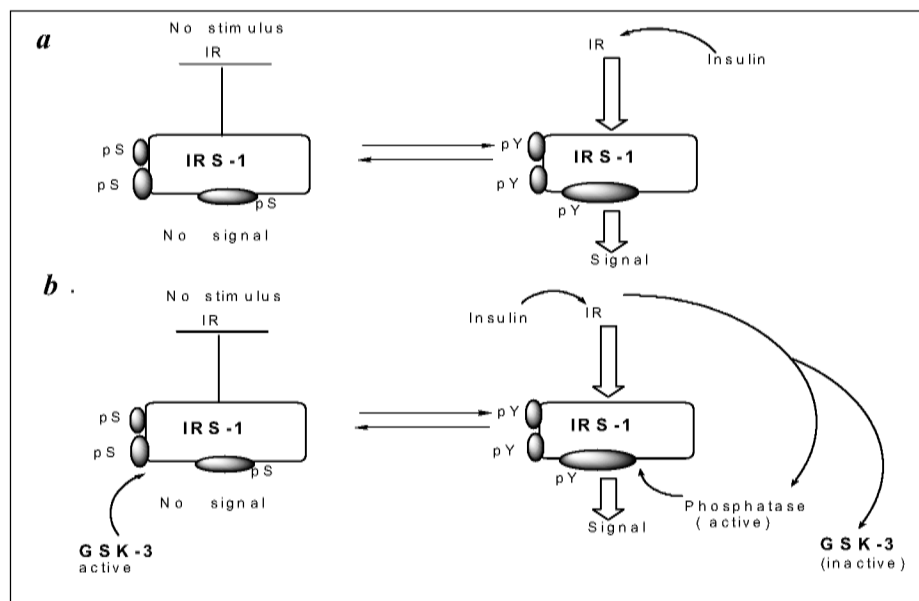
Figure 3. Role of GSK-3 in insulin receptor signalling.

Glycogen synthase kinase-3

GSK-3, initially described as a key enzyme involved in glycogen metabolism, is now known to regulate a wide range of cell functions[30]. This enzyme is centrally involved in regulating cellular structure, function and survival. It is also linked to several diseases like cancer, type 2 diabetes, chronic inflammatory processes, stroke and neurological diseases such as bipolar disorder or Alzheimer's disease[31].

GSK-3 refers to two isoforms – GSK-3α and GSK-3β. The two isoforms are encoded by different genes and share almost 98% homology in their catalytic domains. Both isoforms are ubiquitously expressed in cells and tissues, and have similar (though not identical) biochemical properties[32]. Few enzymes exert as broad a regulatory influence on cellular function as GSK-3. More than 40 proteins have been reported to be phosphorylated by GSK-3, including over a dozen transcriptional factors. Hence it seems that the activity of GSK-3 must be carefully regulated by mechanisms individually tailored for each substrate, to avoid indiscriminate phosphorylation by GSK-3. Though the regulatory mechanisms of GSK-3 are not fully understood, precise control appears to be achieved by a combination of phosphorylation, localization and interactions with GSK-3-binding proteins[33].

GSK-3 in diabetes and insulin resistance: Normally insulin-induced inactivation of GSK-3 contributes to glucose uptake and glycogen synthesis. The mechanisms contributory to insulin resistance and type 2 diabetes are multifactorial, but one factor[34] is certainly due to inadequate inhibitory control of GSK-3. Interesting reports have shown that ele-

vated levels of GSK-3 have been observed in diabetic and obese mouse stain and in skeletal muscles from patients with type 2 diabetes[35]. To understand this, let us take a look at the molecular mechanism of insulin signalling via insulin receptor substrate (IRS; Figure 3).

IRS-1 is the immediate substrate of insulin receptor tyrosine kinase, which phosphorylates the protein on multiple tyrosine residues in response to insulin[36]. In addition, IRS-1 is predominantly phosphorylated on serine/threonine residues in the absence of stimuli. It appears that this type of phosphorylation of IRS-1 converts the protein from a positive to a negative regulator of insulin receptor signalling. Tyrosine phosphorylation (pY) of IRS-1 is a positive trigger of insulin action, which initiates numerous signalling components. Yet its serine/threonine phosphorylation (pS) results in the opposite effect, presumably by direct interaction of IRS-1 with the insulin receptor[37]. This was demonstrated by two studies. In the first study, treatment with okadaic acid, a protein phosphatase inhibitor, followed by hyperphosphorylation of IRS-1 on serine/threonine residues, led to inhibition of the insulin-signalling pathway. In the second study, the cytokine tumour necrosis factor-α (TNF-α), which mediates insulin resistance, was shown to activate serine phosphorylation of IRS-1 and inhibit insulin-induced tyrosine phosphorylation of the insulin receptor.

These studies implicated serine/threonine protein kinases as important regulators in insulin resistance. Phosphorylation of IRS-1 on multiple serine residues by GSK-3 impaired insulin receptor tyrosine kinase activity and insulin action in intact cells. This notion fits well with the fact that GSK-3 is constitutively active and phosphorylates IRS-1 in the absence of stimulus. Thus, GSK-3 serves as a

'gatekeeper' to limit activation of insulin receptor signalling. In the absence of insulin, GSK-3 maintains the phosphorylation state of the multiple serine residues on IRS-1, thereby limiting insulin receptor signalling. In the presence of insulin, GSK-3 is inhibited, and tyrosine phosphorylation of IRS-1 mediates the downstream insulin signalling pathway[38]. Thus it is clear that GSK-3 inhibits insulin receptor coupled protein IRS-1, which in turn inhibits glycogen synthesis and glucose uptake.

Further, studies in various model systems[39] showed that inhibitors of GSK-3 enhanced response to insulin, e.g. GSK-3 inhibits lowered blood glucose levels and stimulated glucose transport and glycogen synthesis in skeletal muscle from insulin-resistant Zucker rats[40] and increased IRS-1 expression and glucose uptake in human skeletal

muscle[41]. These findings indicate that development of specific inhibitors for GSK-3 could have therapeutic implications for type 2 diabetes.

GSK-3 inhibitors: Given the various signalling pathways impacted by GSK-3, it is difficult at the present state of research to design inhibitors which may only alleviate a particular disease. However, efforts are on and many MNCs have successfully come up with potent GSK-3 inhibitors that lower blood glucose level (discussed later in the article). Presently, three distinct regions on the GSK-3 molecule are being targetted to suppress enzyme activity: (i) metal ion (Mg^{2+}) binding site; (ii) substrate interaction domain, and (iii) ATP-binding pocket[42].
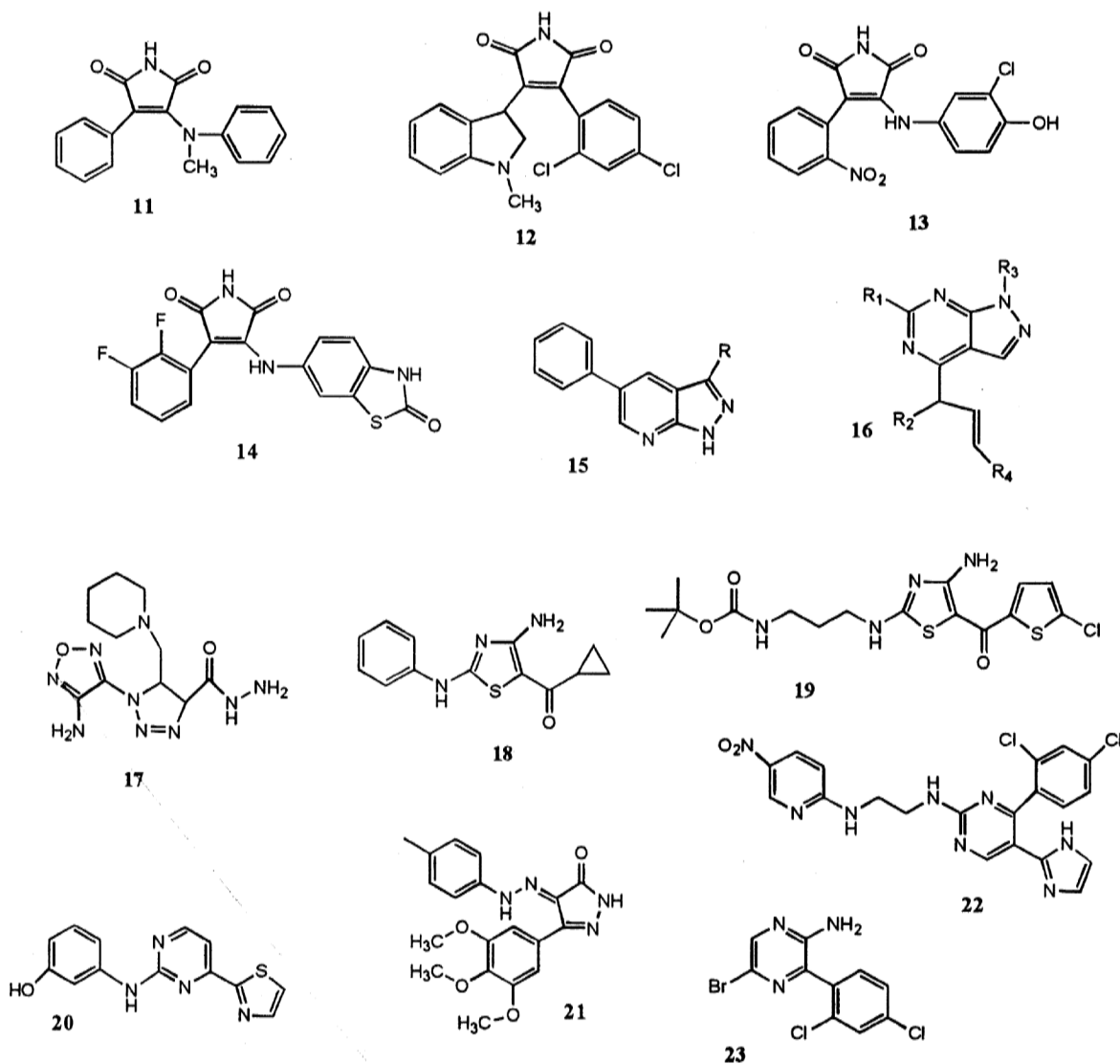

Figure 4. GSK-3 inhibitors.

Lithium salts (Li$^+$) weakly inhibit GSK-3 through competition with the binding of Mg^{2+}, the essential metal ion cofactor of the enzyme[43,44]. Inhibition of GSK-3 by lithium salts causes enhanced glycogen synthase activity and reduced phosphorylation of various GSK-3 substrates.

Based on the mechanism phosphorylation at Ser-9/Ser-21 several phosphopeptides, derived from the amino-terminal end of GSK-3β, have been produced in an effort to compete with the binding of substrates to the phosphate interaction site of the enzyme. One such phosphopeptide, Thr–Thr–pSer–Phe–Ala–Glu–Ser–Cys, was found to inhibit the phosphorylation of glycogen synthase.

Using the screening programmes specifically aimed at finding GSK-3 inhibitory activity in compounds previously reported with other biological properties, hymenialdisine, paullones, indirubins and maleimides were picked up[45–48].

High throughput screening of the SmithKline Beecham compound collection has identified 3-anilino-4-arylmaleimide (11) (Figure 4) as potent GSK-3 inhibitors[49]. Pharmacological studies conducted on maleimide derivatives, SB-216763 (12) and SB-415286 (13), have shown that they stimulated glycogen synthesis in human liver cells. SB-517955 (14) had the capacity to lower glucose level in animal models.

Johnson and Johnson Pharmaceutical Research and Development developed a novel series of macrocyclic bisindolylmaleimides containing linkers with multiple heteroatoms having high selectivity for GSK-3β. Another series developed by Johnson and Johnson is polyoxygenated bis-7-azaindolyl maleimides[50–52].

Glaxo SmithKline Research and Development afforded a series of pyrazolo[3,4-b]pyridines (15) and pyrazolo[3,4-b]pyridazines (16) with IC$_{50}$ value in nM range. Several compounds synthesized in this series[53–61] may be useful in the treatment of diabetes mellitus.

Novo Nordisk reported the discovery of GSK-3 inhibitory activity within various chemical series, including substituted oxadiazepines[62]. 1-(4-amino-1,2,5-oxadiazolyl)-1,2,3-triazole derivatives (17)[63,64] and 2,4-diaminothiazoles (18, 19)[65,66]. Vertex Pharmaceuticals described the preparation of 4-arylpyrimidine-2-amines (20)[67,68] and 4,5 dihydro-1H-pyrazole-5-one (21)[69] as GSK-3 inhibitors. Their potential in treatment of type 2 diabetes is still to be evaluated.

Chiron Corporation claimed the discovery of several GSK-3 inhibitors comprising substituted 2-aminopyridazines (22)[70], 2-aminopyrimidines[71] WO 02/020495 and 2-aminopyridines[72] WO 99/065897. CT-98023 (23) developed by Chiron has shown good oral bioavailability, which reduced plasma glucose levels in fasted hyperglycaemic rats, improved hyperglycaemia and glucose disposal in diabetic mice[73].

Conclusion

Though we have come a long way from random *in vivo* drug screening, there are still many gaps in our understanding of the biological functions of the target. Yet, this does not prevent scientists from going ahead with the information available at hand to screen suitable molecules in the hope of coming up with new drugs. The diverse molecules designed as inhibitors for the above two targets bear testimony to this fact.

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