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Research paper

# Glucose-derived spiro-isoxazolines are anti-hyperglycemic agents against type 2 diabetes through glycogen phosphorylase inhibition

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ABSTRACT

Glycogen phosphorylase (GP) is a target for the treatment of hyperglycaemia in the context of type 2 diabetes. This enzyme is responsible for the depolymerization of glycogen into glucose thereby affecting the levels of glucose in the blood stream. Twelve new D-glucopyranosylidene-spiro-isoxazolines have been prepared from *O*-peracylated *exo*-D-glucals by regio- and stereoselective 1,3-dipolar cycloaddition of nitrile oxides generated *in situ* by treatment of the corresponding oximes with bleach. This mild and direct procedure appeared to be applicable to a broad range of substrates. The corresponding *O*-unprotected spiro-isoxazolines were evaluated as glycogen phosphorylase (GP) inhibitors and exhibited $IC_{50}$ values ranging from 1 to 800 μM. Selected inhibitors were further evaluated *in vitro* using rat and human hepatocytes and exhibited significant inhibitory properties in the primary cell culture. Interestingly, when tested with human hepatocytes, the tetra-*O*-acetylated spiro-isoxazoline bearing a 2-naphthyl residue showed a much lower $IC_{50}$ value (2.5 μM), compared to that of the *O*-unprotected analog (19.95 μM). The most promising compounds were investigated in Zucker *fa*/*fa* rat model in acute and sub-chronic assays and decreased hepatic glucose production, which is known to be elevated in type 2 diabetes. This indicates that glucose-based spiro-isoxazolines can be considered as anti-hyperglycemic agents in the context of type 2 diabetes.



## 1. Introduction

Type 2 diabetes (T2DM) or non-insulin-dependent diabetes mellitus (NIDDM) is characterized by two defects: relative insulin deficiency and liver and peripheral insulin resistance. T2DM, which accounts for 90–95% of the diabetic cases, is a multi-factorial disease of largely unknown etiology involving both genetic and environmental factors and it is closely associated to the metabolic syndrome. The worldwide prevalence of obesity and diabetes has increased substantially in recent decades, also among young adults and children. The rising incidence of these pathologies has grown to alarming levels in developing countries. It is expected that the coming decades these countries will face severe health service burdens as chronic hyperglycaemia is associated with long-term damage, dysfunction and failure of various organs such as eyes, kidneys, nerves, heart and blood vessels. To minimize such health-threatening complications, patients with T2DM must control their

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glycaemia by intensive lifestyle intervention as a primary treatment, with adequate diet and exercise, along with pharmacological therapies [1,2].

Biguanides and α-glycosidase inhibitors have therapeutic value as they limit, respectively, hepatic glucose output, and intestinal absorption of carbohydrates. Sulfonylureas and incretin mimetic drugs act by directly or indirectly increasing insulin release from the β-cells in the pancreas, while thiazolidinediones (TZD) activate peroxisome proliferator-activated receptors (PPARs). Metformin is a biguanide now believed to be the most widely prescribed antidiabetic drug. As pharmacological treatments are inadequate for 30–40% of T2DM patients, combination therapy is frequently applied. With increasing severity of diabetes, insulin administration is prescribed and many patients progress to insulin therapy with time. In spite of substantial progress in the management of diabetic pathologies, the limitations or adverse effects of current treatments are incentive for improving diabetes management. Glycogen phosphorylase (GP) inhibition is one of the pharmacological approaches currently investigated [3].

GP isozymes [4] have been identified and characterized in a large number of organisms (bacteria, fungi, yeast, plants, insects, animals) and in mammalian tissues. GP is expressed mainly in the muscles, liver and brain where it permits the breakdown by phosphorolysis of glycogen to glucose-1-phosphate. GP*a* and GP*b* represent, respectively, the phosphorylated (active) and unphosphorylated (less active) isoforms [5]. This enzyme has been thoroughly studied by kinetic investigations and X-ray diffraction analysis of enzyme–ligand complexes. These studies provided evidence on the binding site and binding mode of ligands to the enzyme. The accumulated information forms a rational basis for the kinetic data, but more significantly, provides a detailed view of the GP structural features with identification of several binding sites such as the active site, the inhibitor site, the allosteric and the new allosteric sites, the glycogen storage site, the phosphorylation site and an understanding of their roles at molecular level [6]. As glycogenolysis is a key component to hepatic glucose production, generally observed excessive in T2DM, a large variety of synthetic molecules has been investigated as GP inhibitors, as a possible pharmacological control of glycaemia. The studied inhibitors mainly target the allosteric [7] and new allosteric site [8], and the active site which accommodates glucose and glucose-based and related analogs [9–14], as can be seen also from general reviews [3,15–20]. NMR spectroscopy has been shown recently through the fragment-based approach to offer additional techniques for probing, in solution, the binding pockets of GP, and investigating cooperativity between the various binding sites [21].

Even though a large set of data has been reported from *in vitro* enzymatic experiments, much need to be clarified through pharmacological studies to get a better understanding of the *in vivo* specific response of a drug under evaluation. Its effects, which depend on pharmacokinetic and pharmacodynamic properties, are unpredictable and sometimes difficult to rationalize. For example, the inhibitory effects of indole-site effectors has been reported to be modulated by endogenous small-molecular-weight effectors of GP*a* activity, although at higher concentrations, indole-site GP inhibitors almost completely inhibit phosphorylase activity and retain a glucose concentration dependence [22,23]. A series of benzamide derivatives, presumed to bind at the new allosteric site of GP (dimer interface) as suggested by molecular docking simulation, was found to simultaneously inhibit GP and activate glucokinase [24]. GP inhibitors can not only interfere with glycogenesis, as mentioned, but also with gluconeogenesis, and the *in vivo* effects of AMP and indole site inhibitors have been reviewed [18,25,26]. Recently, the effect of D-glucopyranosylidene-spiro-thiohydantoin (Fig. 1A) on glycogen metabolism in liver tissues of streptozotocin-induced and obese diabetic rats has been investigated. This showed the coordinated regulation of glycogen phosphorylase and synthase by 50 μM A in liver extracts of Wistar rats, resulting in the activation of synthase by a shortening of the latency compared to control animals. Compound A was also effective in lowering blood glucose levels and restoring hepatic glycogen content in streptozotocin-induced diabetic rats. Furthermore, intravenous administration of **A** to Zucker Diabetic Fatty (ZDF) rats significantly decreased hepatic GP*a* levels, and the activation of synthase was initiated without any delay [27].

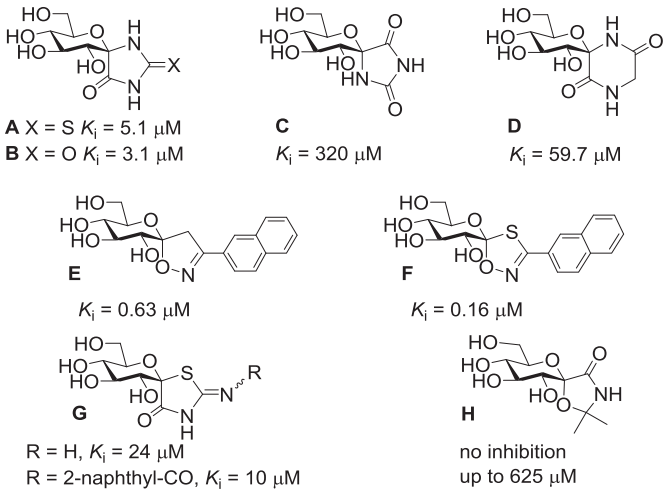


**Fig. 1.** Various types of spiro-anomeric carbohydrate derivatives: structures, bioactivities, or inhibitory properties against rabbit muscle glycogen phosphorylase *b* (RMGP*b*).

GP inhibition is a therapeutic approach to limit the pathogenic consequences of chronic hyperglycaemia in T2DM but also possibly tumor growth [28,29], or cerebral ischemia [30]. Nevertheless, little is known about the potential of glucose-based molecules that bind at the catalytic site at cellular level, and only a few have been studied in detail in hepatocytes [27,31,32]. This is why on the basis of our preliminary studies [33,34], the synthesis of additional glucose-based spiro-isoxazolines followed by kinetic investigations were performed. The more potent molecules ($K_i$ in the low μM range) were selected for cellular assays with primary cultures of rat or human hepatocytes. The most promising molecules identified from these *in vitro* cellular assays were further investigated in an animal model (i.e. Zucker hyperinsulinemic rat).

The glucopyranose-based analog **B** of hydantocidin (Fig. 1) prepared by Fleet's group was found to be a potent inhibitor of glycogen phosphorylase [35]. This was an incentive for investigating chemical synthesis, kinetic measurements and crystallographic analysis of enzyme–ligand complexes. The kinetic and crystallographic data obtained showed that spiro-compounds **A** [36], **B** [35], **C** [35], and **D** [37] are competitive inhibitors and are bound at the enzyme catalytic site through a network of stabilizing interactions. By exploiting stereoselective 1,3-dipolar cycloadditions, we have also synthesized glucopyranose-based spiro-isoxazolines [33,34] (e.g. **E**) and spiro-oxathiazoles [38–40] (e.g. **F**) which were found among the best inhibitors of GP targeting the catalytic site [39]. Glucopyranosylidene-spiro-iminothiazolidinone derivatives **G** were reported recently as good GP inhibitors [41], while spiro-oxazolidinones **H** proved practically inactive against the enzyme [42].

The present report discloses further synthetic and kinetic, as well as *in vitro* and *in vivo* pharmacological evaluation of new representatives of type **E** compounds to determine the properties