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Probing the β-pocket of the active site of human liver glycogen phosphorylase with 3-(C-β-D-glucopyranosyl)-5-(4-substituted-phenyl)-1, 2, 4-triazole inhibitors

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ABSTRACT

Human liver glycogen phosphorylase (hlGP), a key enzyme in glycogen metabolism, is a valid pharmaceutical target for the development of new anti-hyperglycaemic agents for type 2 diabetes. Inhibitor discovery studies have focused on the active site and in particular on glucopyranose based compounds with a β-1 substituent long enough to exploit interactions with a cavity adjacent to the active site, termed the β-pocket. Recently, C-β-D-glucopyranosyl imidazoles and 1, 2, 4-triazoles proved to be the best known glucose derived inhibitors of hlGP. Here we probe the β-pocket by studying the inhibitory effect of six different groups at the *para* position of 3-(β-D-glucopyranosyl phenyl)-5-phenyl-, 1, 2, 4-triazoles in hlGP by kinetics and X-ray crystallography. The most bioactive compound was the one with an amine substituent to show a K_i value of 0.43 μM. Structural studies have revealed the physicochemical diversity of the β-pocket providing information for future rational inhibitor design studies.

1. Introduction

Type 2 Diabetes mellitus (T2D) is one of the most common and serious metabolic disorders. The number of patients with T2D keeps rising and even exceeds the predictions. It was estimated that in 2015, 415 million of people lived with diabetes all around the world, while approximately 193 million were undiagnosed. This number may increase to 642 million in 2040 according to the International Diabetes Federation (IDF). IDF estimated that diabetes was the primary cause of death to almost 5 million people in 2015 [2]. The pharmaceutical recommendations for T2D thus far, include drugs as metformin (a biguanide drug), aiming at optimizing insulin-mediated glucose uptake from the cells and, therefore, leading to lower blood glucose levels [3]. However, in T2D patients a significant β-cell insulin secretory dysfunction and decreased β-cell mass due to increased apoptosis, are observed. As a result, the body can't handle increases in metabolic load [4] and oral medication shows often limited or even insufficient results. Furthermore, some of these treatments may have side effects, like hypoglycaemia, which affects the quality of diabetic patients' life. Therefore, the identification of new compounds that could result in a better management of blood glucose levels is in need. Hepatic enzymes involved in glycogen metabolism are promising targets for T2D treatment and glycogen phosphorylase (GP; EC 2.4.1.1) inhibitors (GPi's) are confirmed to be able to lead to a better hepatic glycogen balance and blood glucose levels control [5]. These results are supported by *in vitro*, *ex vivo* and *in vivo* studies [6–13]. Furthermore, although GPi's exert their action in the liver, they have also been found to induce UCP2 (a mitochondrial inner membrane protein that uncouples mitochondrial proton gradient from ATP production) expression to protect from mitochondrial hydroxyl radicals produced by excess glucose flux [13]. This has also been observed in the muscle [14,15]. Recently, Nagy et al., suggested that GPi's can also target pancreatic cells and hence they can improve β-cell function and survival [16].

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GP is a key enzyme involved in glycogen metabolism by catalyzing the first step of glycogen breakdown towards glucose-1-phosphate (Glc-1-P). GP has at least seven binding sites: the catalytic, allosteric, inhibitor, quercetin, new allosteric, benzamidazole and the glycogen storage binding site [11], as shown in Fig. 1. GP follows the Monod-Wyman-Changeux model of allosteric enzymes and exists in two forms, GPb with low activity and affinity for the substrate (T-state) and GPa with high activity and affinity for the substrate (R-state). The equilibrium between T and R state lies on a flexible loop (280 s, residues 282–286) and is controlled by small molecules (metabolite effectors) that bind on the aforementioned binding sites of the enzyme. The T state is inactive and the substrate cannot reach the catalytic site since it is blocked by the 280 s loop. During the allosteric transition from T to R state, the 280 s loop is displaced and the catalytic site becomes accessible. The biologically active GP is a homodimer and exists in three isoforms, liver (lGP), brain (bGP) and muscle (mGP), depending on the tissue they are expressed. These isoenzymes share approximately 80–83% sequence homology, with no insertions or deletions, and there are no structural differences at the catalytic sites of lGP and mGP.

In the past years structure-based inhibitor design studies have led to the discovery of new inhibitors with high affinity for specific GP binding sites. Most studies focused on the active site using glucose derivatives [1,9,11]. Recently *C*-β-D-glucopyranosyl imidazoles [17] and 1,2,4-triazoles [18] have been studied for their potency against the pharmacologically relevant isoform, human liver GP (hlGP), revealing the best known glucose derived inhibitors thus far, with K_i values in the low nM range [17]. The efficiency of these glucose derivatives as GPi's has been attributed to the exploitation of an extensive network of polar and non-polar interactions with residues that form the β-pocket within the catalytic site, so called because only the β-anomeric substituents of D-glucose can bind there. This cavity extends by the catalytic site and inhibitors binding at this site can form an extensive network of interactions with Asp283, Phe285, and Phe286 on one side and Asp339, His341, and Arg292 on the other side [19]. Our recent discovery [18] showed that 3-(*C*-β-D-glucopyranosyl)-5-phenyl-1,2,4-triazole's (K_i = 156 nM) and 3-(*C*-β-D-glucopyranosyl)-5-(4-methyl phenyl)-1,2,4-triazole's (K_i = 278 nM) significant potency was attributed to their extensive van der Waals interactions within the β-pocket. Herein we probe this pocket's characteristics by studying the effect on the inhibitory potency of six groups (–COO^-, –CF_3, –NO_2,–OCH_3, –OH and –NH_2), different in size and polarity, in the *para* position of the phenyl group. Moreover, we have determined and analyzed the X-ray crystal structures of their GP-complexes to elucidate their protein binding pattern. We also present a new efficient protocol for the production of sufficient amounts of recombinant human liver GP, which are essential for inhibitor binding studies.

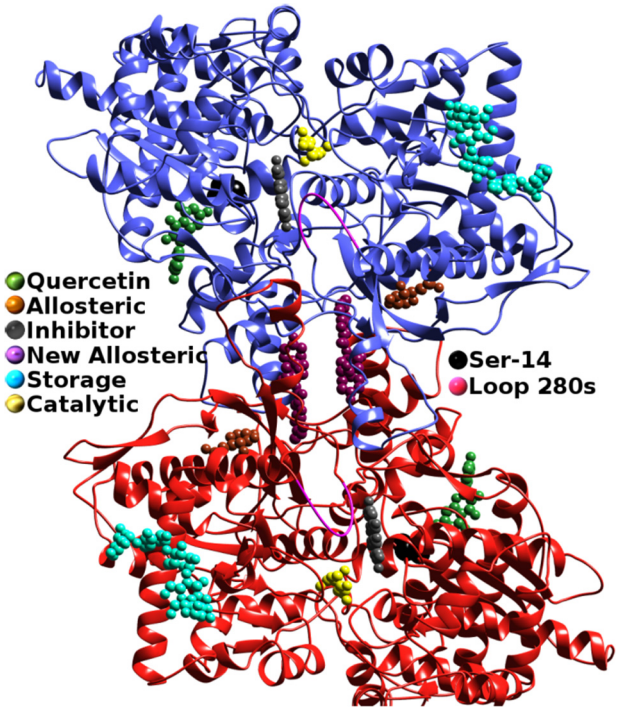

Fig. 1. The biologically active homodimer of GP in the T-state conformation with the different binding sites [1].

2. Materials and methods

The investigated compounds were synthesized and chemically characterized as described previously [20].

2.1. Protein production and purification

Rabbit muscle GPb (rmGPb) was purified from rabbit skeletal muscle following the protocol developed by Fischer and Krebs [21] with a slight modification (L-cysteine was replaced with 2-mercaptoethanol) [22]. The optimized gene sequence encoding human liver glycogen phosphorylase (hlGPa, optimized by Eurofins Genomics, Ebersberg, Germany) was cloned on a pET-M11 vector. The N-terminus site was followed by a His-tag (6xHis) and a TEV protease prescission site. The *E. coli BL21-GOLD (DE3)* strain was the host strain for the heterologous protein expression. Large cultures were produced by inoculating 1 L of LB broth (plus 20 μg/mL kanamycin, 100 mg/L pyridoxine, and 600 mg/L $MnCl_2$) with 10 mL overnight cultures (growth in the same conditions). Cultures were grown until reaching OD_{600}: 0.5–0.6, at 37 °C, 210 rpm and then protein expression was induced using 0.5 mM IPTG. Cultures were incubated for a further 16 h at 18 °C, 210 rpm. Bacterial cells were harvested with centrifugation and the pellet was resuspended in lysis buffer containing 20 mM β-glycerol phosphate pH 7.0, 500 mM NaCl, 20 mM Imidazole and protease inhibitor cocktail (Roche). The mixture was incubated at 4 °C, for 15 min in the presence of benzonase and then sonicated at 70% amplitude, 4 °C for total 3 min. Finally, the lysate was centrifuged and the supernatant containing the soluble protein was filtered through a 0.45 μm filter and purified using IMAC (HiTrap Talon Crude, GE Healthcare) and Ion exchange (Resource Q, GE Healthcare) chromatography, applied on ÄKTA purifier (GE Healthcare). The chelating Sepharose resin was equilibrated with buffer A (20 mM β-glycerol phosphate pH 7.0, 500 mM NaCl, 20 mM imidazole) before the addition of the crude extract. The column was washed with buffer A and then the protein eluted with buffer B (20 mM β-glycerol phosphate pH 7.0, 500 mM NaCl, 500 mM imidazole) followed by overnight dialysis against a buffer containing 20 mM Tris HCl pH 8.0 and 1 mM DTT using dialysis tubing (10 kDa MWCO, Sigma). The protein was then loaded on an anion exchange column (Resource Q, GE Healthcare) which was pre-equilibrated in buffer A (20 mM Tris HCl pH 8.0, 1 mM DTT). The column was washed with buffer A and the protein eluted with gradient concentration of buffer B (20 mM Tris HCl pH 8.0, 1 mM DTT, 1 M NaCl). Afterwards the protein incubated overnight in presence of TEV protease (His-tagged) at 4 °C for 16 h. TEV protease and the uncleaved protein were separated, applying a HiTrap Talon Crude (GE Healthcare) column, from the cleaved protein which was collected (flow through) and concentrated. This procedure led to 250 μg of pure hlGPb sample per Lt of bacterial culture based on SDS-PAGE.