SUMMARY

Glycogen is a branched polymer consisting of glucose monomers and serves as glucose storage for the body. It has been shown that glycogenolysis increases the blood glucose levels in type 2 diabetes (T2D) patients even more and therefore several studies have validated glycogen metabolism enzymes as pharmaceutical targets for T2D treatment. Glycogen degradation occurs mainly, by the action of glycogen phosphorylase (GP) and glycogen debranching enzyme (GDE). GP catalyzes glycogen breakdown but its action halts four residues from a branch point. GDE removes the branched residues and GP can further degrade glycogen.

GP has been studied through structure based inhibitor design to discover small molecules that could serve as anti-hyperglycaemic agents for T2D patients. Among GP's binding sites, the catalytic site has been studied in depth with glucose derivatives but less is known for the inhibitor binding site. Herein, we studied four inhibitor collections targeting the catalytic site and one collection of chrysin analogues targeting the inhibitor binding site. The first collection included (*S*) and (*R*) epimers of glucopyranosylidene-spiro-imidazolinones as inhibitors of human liver GP (hlGP). (*R*) spiro-epimers were found to be more potent than the corresponding (*S*) spiro-epimers. The most potent compound was 2-naphthyl-substituted (*R*)-imidazolinone **30** (K_i = 1.72 μM) and X-ray crystallography revealed that only the (*R*) epimers were bound in the catalytic site of GP. Furthermore, complex structures revealed the structural basis of inhibition in comparison with other glucopyranosylidene-spiro-heterocycles. In the second collection of glucose derivatives; we evaluated C-β-D-glucopyranosyl -thiazoles, -imidazoles and an N-β-D glucopyranosyl –tetrazole as inhibitors of hlGPa. The most potent compound was 2-naphthyl-substituted –imidazole **9** (K_i = 3.2 μM). *C*-β-*D*-glucopyranosyl –thiazoles were less potent than the corresponding –imidazoles whereas N-β-D glucopyranosyl –tetrazole was the less potent inhibitor studied in this section. X-ray crystallography revealed crucial interactions with His377 main chain carbonyl oxygen, located at the catalytic site of GP. In the third collection of glucose derivatives, we tested β-*D*-Glucosaminyl triazoles as inhibitors of hlGPa. In these series, the 2′-OH of the glucose unit was replaced by -NH_2 group (glucosamines). The most potent compound was the 2-naphthyl-substituted ***29b*** (K_i = 7.6 μM) which is classified among the top three most potent inhibitors for the catalytic site of GP with a modified glucopyranosyl ring discovered thus far. Moreover, we observed that glucose modification had a negative effect in the inhibitory potency but these inhibitors where still potent against GP, in comparison with previous reported inhibitors with modifications of the

glucose unit. In the fourth collection, we assessed the characteristics of the *β*-pocket of the catalytic site of GP by the binding of C-*β*-D-glucopyranosyl –triazoles. The most potent compound was ***CK900*** (K_i = 427 *nM*) which was found bound at the catalytic site of GP. X-ray crystallography revealed hydrophilic and hydrophobic regions in the β-pocket that can be used for further rational inhibitor design. Finally, we studied the inhibitor binding site of GP with chrysin analogues (K_i = 7.6 *μM*). Kinetic experiments showed that the most potent compound, **43,** displayed a K_i of 1.0 *μM,* similar to flavopiridol. Kinetic studies and X-ray crystallography revealed that these inhibitors bind at the inhibitor site of GP and offered insights for the next steps in the structure based inhibitor design process.

In addition to GP, we studied human Glycogen Debranching Enzyme (hGDE). There is no reference available for hGDE production in *E. coli* and the only known structure for eukaryotic GDE comes for *Candida glabrata* (CgGDE). We tried to produce and fold the protein in the periplasm and the cytoplasm of bacterial strains of *E. coli*. Regarding periplasmatic localization, the amounts of hGDE folded in that area were not enough for structural studies. Focusing in the cytoplasmatic space, we tried to enhance protein folding, stability and solubility using different *E. coli* strains and lysis buffers. Unfortunately, we didn't manage to produce soluble hGDE. Co-expression with molecular chaperones resulted in soluble protein production. Fast protein liquid chromatography was used to separate the active from the inactive protein form. The molecular chaperone trigger factor led to high soluble protein production but co-expression with molecular chaperones GroES/EL led to less amounts of soluble protein but with higher enzymatic activity. Kinetic studies revealed that hGDE follows the Michaelis-Menten kinetics and it reaches maximum activity at 37 ^{o}C at pH 6.0. Finally, we manage to grow protein crystals at 16 ^{o}C in two different conditions (0.1 *M Tris-HCl pH* 8.8, 10 % *w/v PEG*4000 και 0.1 *M Hepes-NaOH pH* 7.5, 12 % *w/v PEG*8000). Besides hGDE, we also purified CgGDE establishing a new protocol using affinity chromatography and a β-CD sepharose 6B column. Crystals of CgGDE were grown in a novel condition (0.1 M Hepes-NaOH pH 7.5, 10 % (v/v) 2-propanol, 20 % w/v PEG4000).