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Analysis of the biological activity of new metformin analogues with a sulfonamide structure

PhD dissertation based on a series of scientific publications

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Summary

Diabetes mellitus is currently ranked as one of the most widespread chronic metabolic diseases worldwide. In the last two decades, the number of patients diagnosed with diabetes has been increasing at an alarming rate which currently constitutes one of the most serious threats to global health as well as the greatest health challenges of the current century. According to the present definition provided by the World Health Organization (WHO), diabetes mellitus constitutes a devastating, multi-factorial chronic cluster of metabolic disorders manifested by high blood glucose concentrations (state of chronic hyperglycaemia) arising from impaired insulin secretion and/or defects in insulin action. Out of all types of diabetes, type 2 diabetes mellitus (T2DM) accounts for almost 95% of all diagnosed diabetes cases. In the initial stage of T2DM, treatment begins with comprehensive lifestyle modification, i.e. changing dietary habits, introducing moderate physical effort, abstaining from alcohol, as well as smoking cessation. If the aforementioned measures fail to achieve optimal metabolic control of this disease, it is necessary to incorporate appropriate pharmacotherapy into the behavioral therapy. In pursuance of the latest recommendations of the American Diabetes Association, the European Association for the Study of Diabetes, and the Polish Diabetes Society, *N*,*N*-dimethylbiguanide hydrochloride, well-known as metformin, constitutes the first-line drug of choice, used both as a monotherapy in the early stages of T2DM and as combined drug therapy with insulin or other hypoglycaemic drugs in further steps of treatment, if there are no contraindications to its administration.

T2DM is quite often directly bound up with a number of co-morbidities, such as cardiovascular diseases, hypertension, obesity, hyperlipidaemia, chronic kidney disease or non-alcoholic fatty liver disease. These diseases are rated among the main contributing factors to the prothrombotic state in diabetics. In view of the high risk of atherothrombotic events, clinically manifested as coronary artery disease and also peripheral or cerebrovascular disease; cardiovascular diseases constitute the most prevalent cause of mortality in this group of patients. It is worth mentioning that metformin has been the top-prescribed medicine for the management of T2DM for over 60 years, and its clinical application is supported by a vast database of both pre-clinical and clinical studies, which also includes significant evidence for reducing cardiovascular events in diabetes patients. The United Kingdom Prospective Diabetes study revealed landmark results indicating that this molecule significantly reduces diabetes-related deaths by 42%, all-cause mortality by 36%, and the risk of myocardial infarction by 39% in patients diagnosed with T2DM. These beneficial properties of metformin arise from its multidirectional

effect on glycaemia and the pleiotropic mechanism of action, including a beneficial effect on the plasma lipid profile, amplification of plasma fibrinolytic activity and reduction of oxidative stress as well as its protective effect on the cardiovascular system, covering the reduction of myocardial remodelling, and anti-atherosclerotic properties. The good safety profile of metformin, long-term use in humans, and all the above multidirectional pharmacological properties of metformin justify its leading position in the treatment of patients with T2DM, as well as open new possibilities for the using this unique molecule in the prevention and treatment of many complex disease entities. As a result, metformin has emerged as a promising candidate in drug repositioning strategy.

Although metformin exerts numerous advantageous pharmacological properties, it unfortunately possesses unfavourable pharmacokinetic properties due to its chemical structure and strongly hydrophilic character. This results in low permeability through biological membranes, leading to poor drug absorption and ultimately limiting its bioavailability. Considering the above, this molecule has aroused particular interest among researchers in recent years and encouraged them to modify the structure of biguanide. The scientists at the University of Eastern Finland synthesized several metformin analogues aimed at improving the pharmacokinetic and physicochemical characteristics of the original drug. Among these derivatives, sulfonamide derivatives of metformin have become promising candidates. To obtain metformin analogue with a potential dual action, including the hypoglycaemic and anti-coagulation properties, one of the terminal nitrogen atoms in the metformin backbone was substituted with sulfonamide moiety possessing an alkyl group or an aromatic ring in its structure.

Taking the aforementioned premises into consideration, the purpose of a thematically coherent collection of scientific publications, which serves as the foundation of the present dissertation, was to carry out an analysis of the biological activity of **14** novel promising metformin analogues with a sulfonamide structure. Hence, their effect on selected aspects of hemostasis, including plasma and vascular parameters as well as coagulation and fibrinolysis process, was assessed. In the paper presented herein, a detailed analysis of the therapeutic potential of metformin as a promising molecule with anti-ageing properties; including the molecular mechanisms of its action in the context of its potential use as a multi-target drug, was conducted.

The first step of this studies included the biological evaluation of **14** sulfonamide derivatives of metformin for their effect on vascular hemostasis. The cytotoxic activity of studied metformin analogues was determined using experimental *in vitro* models of the vasculature: human umbilical vein endothelial cells (HUVEC) and human aortal smooth muscle cells (AoSMC).

Previous studies prove that metformin does not affect the viability of both HUVEC and AoSMC over the entire concentration range of 0.006–3.0 μmol/mL. The research presented here suggests that among all the analyzed compounds, alkyl derivatives of metformin (**1**–**3**), *m*-nitrobenzenesulfonamide (**11**) as well as *o*-aminobenzenesulfonamide (**12**) reveal the greatest safety profile in both HUVEC and AoSMC cell lines at concentrations up to 1.5 μmol/mL. Chemical modification of the metformin molecule into benzenesulfonamides substituted with –CN group in the *orto*, *meta* or *para* position resulted in a significant increase in the cytotoxicity of benzenesulfonamide biguanides, which was manifested by both a significant reduction in HUVEC cell viability and a decrease in their integrity to values close to zero. The remaining compounds, depending on the concentration, decreased the integrity of HUVEC cells; however, they did not contribute to increased cell death.

In the further part of the studies, all sulfonamide-based analogues of metformin were underwent screening to assess their glucose utilization properties in HUVECs as well as their potential anti-coagulation properties in the biological model of human plasma. Prothrombin Time (PT), Partially Activated Thromboplastin Time (APTT), and Thrombin Time (TT) were measured. Afterwards, their influence on the activity of coagulation X factor, thrombin, and antithrombin III (AT) was also assessed. Furthermore, the analyzed compounds were subjected to further in-depth studies in terms of their influence on the process of clot formation and its lysis (CL-test). Prior research demonstrates that metformin does not affect both the intrinsic, and extrinsic coagulation pathway (APTT, PT), as well as the process of fibrin polymerization (TT), the kinetic parameters of clot formation or fibrinolysis (CL-test) and the activity of factor X over the entire concentration range 0.006–3.0 μmol/mL. However, metformin statistically significantly increases AT activity, but only at a concentration of 1.5 μmol/mL. Based on the obtained results, it was found that both alkyl analogues of metformin **1**–**4** and its aromatics derivatives **5**, **9**, **10**, **13**, **14**, similarly to the parent drug, metformin, elevated glucose uptake in HUVECs. Unlike the original drug, all its aromatic analogues (**5**, **9**–**14**) significantly prolonged both APTT, and TT. Owing to the structural modification of a metformin backbone into *m*-substituted sulfonamides (**9**, **11**, **13**) resulted in compounds that also affect the extrinsic coagulation pathway (↑PT). These data were further proven experimentally in the factor X activity test, which found that all sulfonamides induced a decrease in its activity, thus confirming their anti-coagulant activity. In turn, an increase in the activity of AT was also reported in the presence of derivatives **9**, **11**, and **14**. The attachment of the nitro group to the *meta* position of the benzene ring (derivative **11**) determines its effect on the process of clot formation (↓ initial clot

formation velocity (↓Fvo), ↓ maximum clotting velocity (↓Fmax), ↑ plasma clotting time (↑Tf)), and the time it takes to start the clot formation process (↑Tt). Moreover, the coagulation assay proved that both alkyl metformin analogues **2**, **4** and its aromatic derivatives **5**, **9**, **11**, **12**, **14** contributed to a prolonged thrombin generation time (TGt), plasma clotting time (↑Tf), and a decreased initial velocity of coagulation (↓Fvo), which might be related to the inhibition of the amidolytic activity of thrombin. From the structural point of view, the chemical modification of a metformin skeleton into benzenesulfonamide substituted with an amino moiety in the *para* position in the aromatic ring resulted in a compound **14** with the most potent inhibitory effect on the maximum activity of thrombin (Amax) and initial velocity of the reaction (dA/dt) over the entire concentration range, implying its anti-coagulant properties.

The last purpose of this research was to assess the effect of new metformin derivatives on red blood cell (RBC) hemolysis and the morphology of these blood cells. The biocompatibility of these substances in a model of human red blood cells was assessed through microscopic examination and RBC lysis test. Indubitably, none of the analyzed sulfonamide-based analogues of metformin besides cyanobenzenosulfonamides (**6**–**8**) revealed an adverse effect on erythrocyte integrity as well as their morphology (the degree of RBC hemolysis < 10%); thus all the newly synthesized metformin analogues do not interact strongly with the lipid-protein bilayer. Only physiological changes, i.e. stomatocytes and echinocytes, were observed in the microscopic images. On the basis of these results, it was concluded that compounds **1**–**5**, **9**–**14** are hemocompatible in the entire tested concentration range: 0.006–1.5 μmol/mL. The cytotoxicity analysis of the tested compounds **1**–**5**, **9**–**14** using HUVEC and AoSMC cells, as well as erythrocytes, suggest the safety of their potential use. Nevertheless, to obtain a comprehensive model of the effectiveness and safety of the present sulfonamide-based metformin analogues, it is crucial to conduct additional *in vivo* research.

The results of this dissertation indicate that newly synthesized metformin analogues with a sulfonamide structure, containing both an alkyl substituent (**1**–**4**) or an aromatic ring in their chemical structure (**9**–**14**), have a more favourable effect on individual parameters of plasma hemostasis than metformin. Furthermore, the obtained outcomes suggest that *m*-nitrobenzenesulfonamide (**11**) reveals highly desirable anti-coagulant activity; while *m*-methoxybenzenesulfonamide (**9**), *m*-aminobenzenesulfonamide (**13**) and *p*-aminobenzenesulfonamide (**14**) exert both anti-hyperglycaemic properties and more potent anti-coagulant activity than the parent drug, metformin. On this basis, it was inferred a conclusion that the chemical modification of the metformin scaffold into benzenesulfonamides substituted especially in the *meta* position (**9**,

11, **13**) leads to molecules with stronger anticoagulant properties than the reference drug, metformin. The above-mentioned derivatives can be regarded as valuable candidates as prototypes for further design and development of new potential therapeutic representatives with highly desirable anti-coagulant properties or with multimodal action – antidiabetic agents with a favourable effect on hypercoagulability.