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New prodrugs of metformin do not influence the overall haemostasis potential and integrity of the erythrocyte membrane

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ABSTRACT

Although metformin, an oral anti-diabetic drug, has been found to have multidirectional effects over the past decade, it is characterised by unfavourable pharmacokinetic properties. This study discusses the effects of metformin, phenformin and three prodrugs of metformin on the haemostasis and integrity of Red Blood Cells (RBCs).

The influence of examined biguanide derivatives on haemostasis was evaluated spectrophotometrically by clot formation and lysis test (CL-test) at 405 nm. The extrinsic and intrinsic coagulation pathway were examined by measuring the PT (Prothrombin Time) and aPTT (Activated Partial Tromboplastin Time). Haemolysis assay, microscopy and flow cytometry studies were used to assess the effect of the tested compounds on RBCs.

Although none of the tested biguanide derivatives significantly influenced the overall potential of clot formation and fibrinolysis (CL_{AUC} constants), statistically significant changes were seen in the values of the kinetic parameters of fibrinolysis. Furthermore, only prodrug **2**, with an 8-carbon alkyl chain, unfavourably affected RBCs by interaction with the erythrocyte membrane leading to significant haemolysis.

Our results provide a further insight into the effects of metformin and its prodrugs on haemostasis and RBCs and underscore the necessity for further research.

1. Introduction

Conventional therapeutic strategy in the treatment of type 2 diabetes usually begins with lifestyle interventions supported by prescription of one oral anti-diabetic drug. A first-line drug for the treatment of type 2 diabetes is metformin, a drug which demonstrates a multidirectional action: in addition to its hypoglycaemic activity, i.e. its inhibition of hepatic gluconeogenesis, increase of tissue glucose consumption and insulin sensitivity, and reduction of intestinal glucose absorption (Giannarelli et al., 2003), it exerts beneficial effects on mortality rate in diabetic patients, improves serum lipid profile, reduces inflammatory cell adhesion to the endothelium, and stimulates gene expression responsible for cellular antioxidant defences (Bashmakov and Petyaev, 2011; Rizos and Elifas, 2013).

Considering its chemical structure, metformin is a biguanide (1,1-dimethylbiguanide) (Riedmaier et al., 2013). Due to its very polar guanidine structure, metformin is highly hydrophilic base that exists as a cationic species at physiological pH, with a minimal passive diffusion through the cell membranes (Graham et al., 2011).

Metformin is slowly and incompletely absorbed from the intestine, and therefore, the pharmacologically active doses are relatively high (0.5–2.0 g per day); these are associated with adverse gastrointestinal effects, such as nausea, vomiting, diarrhoea, abdominal pain and loss of appetite. These adverse drug reactions frequently contribute to the discontinuation of therapy (Belcher et al., 2005). In addition, the bioavailability of metformin after oral administration has been estimated to be approximately 50–60% and its plasma half-life to be short, only 1.5 to four hours (Riedmaier et al., 2013). Therefore, several

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prodrugs have been synthesised to improve the bioavailability of metformin (Huttunen et al., 2009, 2013, 2012).

Type 2 diabetes is characterised by an impaired balance between the processes of coagulation and fibrinolysis (Kinalska and Telejko, 2003), known as diabetic thrombophilia (Fonseca, 2003). Lipiński and Pretorius (2012) in their extensive review highlight the associations between T2DM and thrombosis and blood coagulation. The main cause of this phenomenon is altered platelet function, including hyperreactivity, increased adhesiveness, exaggerated aggregation and changed metabolism (Soma et al., 2016; Soma and Pretorius, 2015), endothelial dysfunction (Soma and Pretorius, 2015), the increased activity of the coagulation factors such as fibrinogen, von Willebrand factor (vWF), FVII and fibrinolysis disorders (Kinalska and Telejko, 2003). Scientists mention also increased levels of tissue factor (TF), thrombin as other important causes of hypercoagulability in diabetic patients (Pretorius et al., 2015). The authors emphasize also that in diabetic patients fibrin levels and thrombin generation are also changed which contribute to formation of denser fibrin clots with reduced lysability (Pretorius et al., 2015, 2013; Soma and Pretorius, 2015; Pretorius and Bester, 2016). In the coagulation process in T2DM patients the role of erythrocytes cannot be omitted as Soma and Pretorius (2015) indicated that diabetic erythrocytes are characterised by enhanced aggregation, rigid membrane with decreased deformability and increased osmotic fragility.

Several experimental and clinical studies highlight the multidirectional effect of metformin on haemostasis, including platelets and plasma haemostasis with both coagulation and fibrinolysis system (Grant, 2003). However, the exact mechanism of action of metformin on coagulation and fibrinolysis is not fully understood. Therefore, the aim of the study was to assess in vitro the effect of metformin, phenformin and three selected metformin prodrugs (Fig. 1) on the overall potential of clot formation and fibrinolysis. Our study estimates the kinetic parameters of these processes, evaluates the influence of metformin prodrugs on the process of coagulation after the generation of endogenous thrombin and determines the effect of the prodrugs on extrinsic and intrinsic coagulation pathways by determining PT and aPTT. The final part assesses the influence of the compounds on Red Blood Cells (RBCs) using RBC lysis assay, microscopy and flow cytometry studies.

2. Materials and methods

2.1. Materials

The design and syntheses of selected prodrugs 1–3 (Fig. 1) was carried out at the University of Eastern Finland and reported elsewhere (Huttunen et al., 2009, 2013).

For the CL-test, thrombin was produced by Biomed (Poland) and recombinant tissue plasminogen activator (t-PA) by Boehringer-Ingelheim (Germany). Tris-buffered saline (TBS, cat. no. SRE0032) was purchased from Sigma Aldrich, sodium chloride (cat. no. 794121116) and calcium chloride (cat. no. 26224) was provided by Polish Chemical Reagents (Poland).

The Triton X-100 used in the erythrotoxicity test (cat. no. 841810492) was obtained from Polish Chemical Reagents (Poland).

The APTT assay used Bio-Ksel System APTTs reagent and calcium chloride (Bioksel, Poland). Bio-Ksel PT plus reagent (tromboplastin and solvent, Bioksel Poland) was used in PT tests.

2.2. Plasma preparation for CL-test, APTT and PT

Blood samples were obtained from healthy donors from the Regional Blood Bank in Łódź, Poland (*Regionalne Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi*). The blood was collected to vacuum tubes containing 3.2% buffered sodium citrate. Platelet poor plasma (PPP) was obtained by centrifugation (3000×*g*, 20 min, room temperature) with a Micro 22R centrifuge (Hettich ZENTRIFUGEN). Small portions of PPP were stored for up to one month at −30 °C. Before each experiment, PPP was restored at 37 °C for 15 min. Once thawed, the PPP was not frozen again nor used for retesting.

The studies on biological material were approved by the Bioethics Committee of the Medical University of Lodz (RNN/109/16/KE).

2.3. Clot formation and fibrinolysis assay (CL-test)

The CL-test, described previously by Kostka et al. (2007), was used to evaluate the effect of the metformin prodrugs on the overall potential of clot formation and fibrinolysis as well as its kinetic parameters. The test is based on the evaluation of the global assay of coagulation and fibrinolysis by measuring the changes in optical transmittance over time (Kostka et al., 2007; Markowicz-Piasecka et al., 2014). The CL-test is a modification of the optical measurement of coagulation and blood fibrinolysis previously described by Glover et al. (Glover and Warner, 1975) and He et al. (He et al., 1999, 2001).

General experimental conditions were the same as published previously (Sikora et al., 2012; Markowicz-Piasecka et al., 2015). In brief, the measurements were taken at λ = 405 nm, in Semi-Micro cuvettes (Medlab Products, Poland), by means of a Cecil CE 2021 spectrophotometer with circulating thermostated water (37 °C) and a magnetic stirrer (Electronic Stirrer Model 300 Rank Brothers Ltd, England). Tested compounds at five concentrations (in a 10 µl volume) and t-PA (220 ng/ml) were added to plasma diluted three times with TBS buffer. Afterwards, the samples were incubated at 37 °C for three min, and then 10 µl thrombin (0.5 IU/ml) was added to initiate clot formation. The final volume of the sample was 500 µl. The number of samples in CL-test was 8.

The obtained curves were analysed by means of dedicated software (Kostka et al., 2007) used to measure the parameters of clot formation (phase I), stabilization (phase II) and fibrinolysis (phase III). The kinetic parameters are as follows: Tt – thrombin time, Fmax –

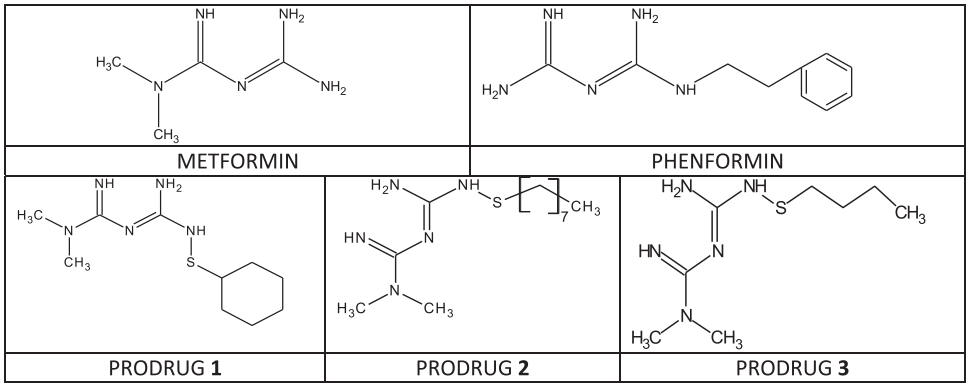

Fig. 1. Chemical structure of biguanide derivatives: metformin, phenformin, prodrugs **1** – **3**.