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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\textsubscript{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.
Key words: metformin, prodrugs, coagulation, fibrinolysis, haemostasis, biguanides

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 prodrugs have been synthesised to improve the bioavailability of metformin (Huttunen et al., 2009; Huttunen et al., 2013, Huttunen et al., 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 hyper-reactivity, increased adhesiveness, exaggerated aggregation and changed metabolism (Soma et al., 2016b; 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; Soma and Pretorius, 2015; Pretorius and Bester, 2016; Pretorius et al., 2013). In the coagulation process in T2DM patients the role of erythrocytes cannot be omitted as Soma and Pretorius (2015) indicated that diabetic erythrocytes are characterized 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; Huttunen et al. 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 x 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; He et al., 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 $\lambda = 405$ 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 – maximum clotting, Tf – plasma clotting time, Fvo – initial plasma clotting velocity, Sf – area under the clot formation curve, Tc – clot stabilization time, Sc – area under the curve of stable clot formation, Lmax – maximum lysis, Tl – fibrinolysis time, Lvo – initial clot fibrinolysis velocity, Sl – area under the fibrinolysis
curve. CL-test allows also for estimation of the overall potential of clot formation and fibrinolysis (CL_AUC) and total time of the process of clot formation and fibrinolysis (T). The method of clot formation and fibrinolysis was validated, and coefficient of variation (W) for single human plasma (n=3) was within the range 1.1 – 9.6 depending on the calculated parameter. Relatively high standard deviations resulted from the individual variability and wide reference values.

2.4. Coagulation assay

General experimental conditions were described previously (Markowicz-Piasecka et al., 2014; Markowicz-Piasecka et al., 2015), and similar to those described above, with measurements taken at λ = 405 nm in Semi-Micro cuvettes (Medlab Products, Poland) using a Cecil CE 2021 spectrophotometer. The tested compounds were added at five concentrations (10 µl volumes) to plasma diluted three times with TBS buffer. The samples were then incubated at 37°C for three min., before thrombin (0.025 IU/ml) and calcium chloride (0.015 mmol/ml) were added in volume of 10 µl. The addition of small amounts of thrombin and calcium chloride does not result in the initiation of coagulation process, but induces a feedback reaction which leads to generation of endogenous thrombin, and coagulation (Sikora et al., 2012, Markowicz-Piasecka et al., 2015). All experiments were repeated five times (n = 5).

The obtained curves were analysed using dedicated software (Kostka et al., 2007) which estimates the following parameters: TGt – thrombin generation time, Fmax – maximum clotting, Tf – plasma clotting time, Fvo – initial plasma clotting velocity, Sf – area under the clot formation curve, Tc – clot stabilization time, S – area under the curve of coagulation.
2.5. Activated Partial Thromboplastin Time (APTT) test

APTT assay (n = 5) was performed using a CoagChrom-3003 coagulometer (Bio-Ksel, Poland) according to the routine laboratory diagnostic method used to monitor the intrinsic coagulation system. PPP was incubated at 37°C then APTT reagent and tested compound in a volume of 10 µl were added followed by a three-minute incubation. The addition of calcium chloride initiated clotting. The APTT is the time taken from the addition of calcium chloride to the formation of a fibrin clot, taken as the point at which the optical density of the mixture exceeds a certain threshold. To validate the method, seven tests were conducted on Bio-Ksel plasma: Normal and Abnormal plasma. The coefficient of variability was counted (W = 0.75 for Normal plasma, W = 2.49 for Abnormal plasma).

2.6. Prothrombin Time (PT), fibrinogen (FBG), International Normalized Ratio (INR)

The PT test (n = 5) was conducted using a CoagChrom-3003 coagulometer (Bio-Ksel, Poland) according to the routine laboratory diagnostic method used to monitor the extrinsic coagulation system. Platelet poor plasma (PPP) was incubated with tested compounds (10 µl) at 37°C for one min. followed by the addition of PT reagent (thromboplastin). The time taken from addition of the reagent to clot formation was counted electronically. The reagent also provides a quantitative measurement of the fibrinogen concentration in the analysed plasma.

In order to validate the method, six tests were conducted on Bio-Ksel plasma: Normal and Abnormal plasma. The coefficient of variability was counted (W = 2.56 for Normal plasma, W = 4.10 for Abnormal plasma).
2.7. Red blood cells (RBC) lysis assay

The material for the RBC lysis assay constituted the blood obtained from the Regional Blood Bank in Łódź, Poland (Regionalne Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi). The blood from healthy donors was collected to tubes containing a solution of potassium EDTA. RBCs were separated from the plasma by centrifugation (3000 x g, 10 min) at 4 °C and washed three times with 0.9% saline (Markowicz-Piasecka et al., 2015). The influence of metformin prodrugs on RBCs was evaluated according to Markowicz-Piasecka et al. (2015). Briefly, RBC suspension (2% in NaCl) was incubated at 37°C with 10-µl volumes of the tested compounds at appropriate concentrations; the final volume of the sample was 1 ml. After a one-hour incubation, the samples were centrifuged at 1000 x g for 10 min. and the absorbance of the supernatant was measured at 550 nm. The degree of haemolysis was expressed as a percentage of released haemoglobin. With regard to controls, a sample of 2.0% v/v Triton X-100 represented 100% of haemoglobin release (positive control), whereas a sample of saline solution represented spontaneous haemolysis of RBCs (negative control), W = 4.8 – 6.5% (Markowicz-Piasecka et al., 2015). The experiments were conducted 5 times (n = 5).

2.8. Microscopy studies

A 2% erythrocyte suspension was incubated at 37°C for 60 min. with various concentrations of tested compounds. After this time, the suspension was diluted two-fold on titration plates. The morphology of the RBCs was evaluated using a phase
contrast Opta-Tech inverted microscope, at 400-times magnification, equipped with software (OptaView 7) for image analysis.

2.9. Flow cytometry – FSC parameter

Erythrocyte size was measured using flow cytometry (FMC), performed using a FACS Canto II (Becton Dickinson) cytometer. The FMC gate was established on the erythrocytes for data acquisition and the cell size was determined by the detection of low angle (FSC) light scattering. The results were displayed as a diagram of the cell number versus the light scattered. The study was limited to 20 000 events and lasted 20 – 30 s. The studies were performed on 2% erythrocyte suspensions prepared in the same way as the RBC lysis assay and microscope studies. The tested compounds were added to the RBC suspension at concentrations of 0.6 µmol/ml and 3.0 µmol/ml, and the samples were incubated for one hour at 37° C. Afterwards the samples were diluted four-fold and analysed by flow cytometry (n = 3).

2.10. Statistics

Statistical analysis was conducted with a commercially-available package (Statistica 12.0, StatSoft). The normal distribution of continuous variables was verified with the Shapiro-Wilk test. Variance homogeneity was verified with Levene’s test. For all variables with a normal distribution, means ± S.D. were given, and paired t-test, one or two-way Anova and subsequent post hoc tests were used for intergroup comparisons. The variables with non-normal distributions were compared using the non-parametric Kruskal-Wallis test. The results of all the tests were considered significant at p-values lower than 0.05.

3. RESULTS
3.1. Clot formation and fibrinolysis assay CL-test

The aim of this study was to investigate in vitro the effect of biguanide derivatives on the overall potential of clot formation and fibrinolysis (CL\textsubscript{AUC}) using the CL-test (Markowicz-Piasecka et al., 2014).

The study showed that both metformin, phenformin and synthesized prodrugs did not significantly influence the overall potential of clot formation and fibrinolysis (constant CL\textsubscript{AUC}, Fig. 2), which may indicate that the tested compounds can be regarded as biocompatible towards plasma haemostasis. In addition, two-way Anova analysis did not record any statistically significant changes with regard to the total duration of clot formation and fibrinolysis (T constant) (Fig. S1 Supplementary materials).

No statistically significant changes in the length of thrombin time (Tt) as a result of action of the compounds studied with the exception of the lowest concentrations (0.06 μmol/ml, P =0.035 and 0.3 μmol/ml, P =0.014) of prodrug 1 (↓ Tt) were reported.

As the test compounds were found to have no statistically significant effect on FBG (Fig. S2 Supplementary materials), it may be concluded that the prodrugs do not contribute to the disintegration or aggregation of this protein. This fact was also confirmed by the lack of visual changes in turbidity of the samples after the addition of prodrugs. The two highest concentrations of prodrug 3 were associated with a reduction of the maximum clotting (↓ Fmax, P =0.028 and P =0.010) (Fig. 3), which can be attributed to its effect on the structure of the formed clot.

While plasma clotting time was generally not significantly affected by the tested compounds (constant Tf), the highest concentration of prodrug 3 was found to lengthen the time (Tf ↑, P =0.024), which may be due to a decrease in the initial velocity of plasma clotting.
Two-way Anova Welch analysis (heterogeneity in variances) confirmed statistically significant differences in this regard between prodrug 3 and all other tested compounds at 3.00 µmol/ml. The other examined compounds exerted no effect on the initial velocity of plasma clotting (constant Fvo) (Fig. 4). The observed changes in the parameters of the kinetic phase of clot formation did not notably affect the area under the curve of plasma clot formation (constant Sf), indicating that the prodrugs did not contribute to the changes in the overall potential of clot formation, and their use in the proposed concentration range was not associated with increased risk of clot formation.

Tested compounds did not significantly affect the second phase i.e. the clot stabilization phase (constant Tc), (Fig. S3 Supplementary materials). No changes were observed in the area under the clot formation (Sc constant).

The reduction in maximum fibrinolysis (↓ Lmax) observed for compound 3 (concentration of 1.5 and 3.0 µmol/ml, P =0.052 and P =0.049, respectively) occurred as a consequence of the reduced maximum clotting value (↓ Fmax). No statistically significant effect on the value of Lmax was observed for the other compounds. The two-way Anova analysis for data with heterogeneous variance did not reveal any statistically significant differences between the compounds. All compounds exhibited complete lysis of previously-formed clots.

With regard to fibrinolysis time (Tl), statistically significant changes were observed only for the highest concentration of prodrug 1 (↑ TI, P =0.022). The two-way Anova analysis for data with heterogeneous variance showed statistically significant differences between (i) metformin at concentrations of 0.06 – 1.5 µmol/ml and subsequent concentration of prodrug 1 and 3; (ii) phenformin over the whole concentration range and prodrug 3, and (iii) prodrug 2 and 3 also in the case of all concentrations tested. Prodrug 3 at concentrations of 1.5 – 3.0 µmol/ml significantly decreased the initial velocity of fibrinolysis (↓ Lvo; P
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=0.0354 and \( P =0.044 \), respectively), which was also insignificantly correlated with the prolongation of fibrinolysis time (TI). Changes in these fibrinolysis parameters (compounds 1 and 3) did not significantly affect the area under the fibrinolysis curve (SI).

### 3.2. Coagulation assay

In order to determine the effects of the metformin prodrugs on the process of coagulation after the generation of endogenous thrombin, calcium chloride (0.05 mmol/ml) and thrombin (0.025 IU/ml) were added to previously three-fold diluted PPP.

Fig. 6 depicts the effects of tested compounds on the thrombin generation time (TGt). None of the compounds were shown to induce any significant increase in thrombin generation time (TGt) when added over the whole concentration range. Even at the highest concentrations, none of the prodrugs demonstrated any significant effect on the value of maximum clotting (Fmax) (Fig. S4, Supplementary materials). This lack of changes in Fmax indicates that the studied compounds do not affect the structure of the clot after the generation of endogenous thrombin. When added at concentrations between 0.06 and 3.00 µmol/ml, the metformin prodrugs did not cause any significant changes in plasma clotting time (constant Tf) or concomitant changes in the initial plasma clotting velocity (constant Fvo) (Fig. S5, Supplementary materials).

The abovementioned results suggest that metformin prodrugs do not affect the kinetics of the coagulation process initiated by generation of endogenous thrombin. This was confirmed by the unchanged value of the overall coagulation potential (constant S) (Fig. S6, Supplementary materials).

### 3.3. APTT

The influence of biguanide derivatives on APTT is illustrated on Fig. 7.
Generally, exposure to the test compounds was shown not to significantly influence the value of APTT over the whole concentration range. However, prodrug 2 significantly affected the intrinsic coagulation system by shortening APTT at a concentration of 3.0 \( \mu \text{mol/ml} \) (38.87 s for control vs. 33.37 s for concentration 3.0 \( \mu \text{mol/ml} \)) \( P < 0.005 \).

### 3.4. PT, INR, FBG

The tested biguanides appeared to have no significant effect on PT values (Fig. 8). Similarly, no effect was observed in the case of INR (Fig. S7, Supplementary materials), and none of the tested compounds had a statistically significant effect on FBG (Fig. S2, Supplementary materials).

### 3.5. RBC lysis test

The effects of metformin, phenformin and the three metformin prodrugs on RBC haemolysis are presented in Fig. 9. Neither metformin or compound 3 was found to have any effect on the erythrocyte membrane over the whole concentration range. A statistically significant increase in the rate of haemolysis was documented for the highest concentrations of phenformin (1.5 and 3.0 \( \mu \text{mol/ml} \)) and compound 1 (3.0 \( \mu \text{mol/ml} \)); however, the percentage values of haemolysis did not exceed 10%. Of the tested compounds, prodrug 2 appeared to have the strongest influence on disintegration of RBC membrane. Even at the concentration of 0.6 \( \mu \text{mol/ml} \) a statistically significant increase in RBC lysis reaching 3.70% was observed. The degree of haemolysis increased with prodrug 2 concentration, up to a value of 92.32% for the highest concentration (3.0 \( \mu \text{mol/ml} \)).

### 3.6. RBC morphology
The microscope studies enabled the effects of biguanide derivatives on erythrocytes to be visualized (Fig. 10A and 10B). While both reference compounds (metformin and phenformin) contributed to the formation of echinocytes, the echinocytosis was visible even at the lowest tested concentration in the case of metformin (0.6 μmol/ml). In the case of metformin as well as phenformin at concentration of 3.0 μmol/ml RBCs membrane deformability can also be observed which might be attributed to eryptosis. Prodrug 1 did not exert any effects on the morphology of erythrocytes over the whole concentration range. In the case of prodrug 2, an insignificant transformation of discocytes into stomatocytes was observed at a concentration of 0.6 μmol/ml, and haemolysis of most erythrocytes at 3.0 μmol/ml. As presented in Fig. 10 B prodrug 3 at concentration of 0.6 μmol/ml contributed to the extensive eryptosis, while in the case of higher concentration single eryptotic erythrocytes can be observed.

3.7. Flow cytometry – FSC parameter

The analysis was conducted on a few separated gates (Fig. 11 A). On the basis of SSC-A (side scattered light), an indicator of cell granularity or internal complexity, one subpopulation marked as light grey and separated by gate P5 was segregated from all analysed erythrocytes. In the case of controls, this population constituted approximately 20% of all erythrocytes. Among all events, the erythrocytes were divided into P3 and P4 gates varying with regard to the FSC parameter value, corresponding to the size of the measured objects. Similarly, the erythrocytes of gate P5 were divided into P6 and P7 according to the FSC parameter.

The FSC scan clearly viewed the flow of ellipsoid, biconcave RBCs (control) as essentially two populations of cells, and FSC histograms showed a typically bimodal
distribution of RBCs (Fig. 11 B.). Therefore the present study estimates the percentage of RBCs in gates P3 and P4, and P6 and P7, for the samples treated with metformin, phenformin and three prodrugs at two concentrations. Fig. 12 A depicts the percentage of all RBCs divided into gates P3 and P4. The statistical analysis found that only the highest tested concentrations (3.0 μmol/ml) of prodrugs 1 and 2 contributed to significant changes in the amount of RBCs within these gates. For instance, in the case of prodrug 2, 14.6 % of all erythrocytes were collected in gate P3 and 85.4 % in gate P4, whereas for control samples the 44.07 % were collected in P3 and 55.93 % in P4. These results are also confirmed by the histograms presented in Fig. 11 B: a typical bimodal distribution of RBCs can be seen in the case of metformin, phenformin and prodrug 3, whereas significant changes can be seen in the distribution of RBCs for prodrugs 1 and 2.

The study showed that compound 2 also induced significant changes in the morphology of RBC subpopulation P5 (Fig. 12 B). The percentage of RBCs in the P6 gate decreased from 63.1 % to 29.17 % after one hour incubation with compound 2 at a concentration of 3.0 μmol/ml, caused by the destruction of the erythrocyte membrane. Inversely, the percentage of RBCs in gate P7 increased from 36.9 % to 70.83 %.

4. DISCUSSION

As there have been no systematic studies on the effects of metformin on plasma haemostasis, the present study uses the CL-test to examine the effects of biguanide derivatives on the overall potential of clot formation and fibrinolysis (CL \(_{AUC}\)). The CL-test is used for in vitro drug screening and for ex vivo clinical experiments (Sikora et al., 2012). By using a computer application that allows continuous recording of the transmittance changes taking place during the processes of clot formation and fibrinolysis, and the estimation of a number of kinetic
parameters, it was possible to conduct a comprehensive and accurate analysis of the obtained curves. Greater overall haemostasis potential (OHP), which is equivalent to $\text{CL}_{\text{AUC}}$, correlates with an increased risk of cardiovascular events (He et al., 2001). As the tested compounds were not found to affect the value of $\text{CL}_{\text{AUC}}$ across the whole range of tested concentrations, they might be regarded as having no risk associated with plasma haemostasis.

As the measurements of the process of clot formation were taken under stable conditions, at a constant concentration of exogenous thrombin, the lack of changes in the length of thrombin time ($\text{Tt}$) probably reflects the lack of changes in the activity of the latter. In addition, initial plasma clotting velocity (constant $\text{Fvo}$) and plasma clotting time (constant $\text{Tf}$) remained unchanged when using the tested compounds, which suggests that the examined biguanides do not affect the phase of clot formation. However, according to our present findings, prodrug 3 administration was found to decrease the maximum clotting value ($\downarrow \text{Fmax}$) despite not having any significant effect on FBG concentration in plasma. As the maximum clotting value ($\text{Fmax}$) is likely to be influenced by the diameter of individual fibrin fibres, thicker fibres are more likely to be associated with lower plasma permeability and a greater difference in the transmittance values. Higher concentrations of FBG in plasma contribute to the formation of thicker and longer fibres, while lower concentrations of FBG lead to the formation of clots with a more densely-packed structure (Standeven et al., 2005). Noticeably, since Fmax depends also on the diameter of the individual fibrin filaments (Standeven et al., 2005) changes in Fmax values, without changes in FBG level, might imply that the prodrug influences the structure of the clot. Our findings indicate a significant increase in the differences in transmittance values, which may suggest that the thickness of fibrin filaments changed.

Moreover, the unchanged clot stabilization time (constant $\text{Tc}$) identified in the present study also indicates that biguanide derivatives do not influence the structure of the clot. An
increase in Tc may lead to a delay in the process of fibrinolysis and prolong the time elapsed between the initiation of the clotting and complete lysis of the clot (Sikora et al., 2012). As a result, the clot may persist longer in the vessel lumen; this may have important clinical implications, such as an increased risk of cardiovascular events (Antovic, 2010). Therefore, due to the fact that the tested compounds do not change the parameters of clot stabilization phase, we may regard the prodrugs biocompatible. These results are crucial in the view of the fact that diabetes is associated with resistance to fibrinolysis and denser clot structure ((Soma and Pretorius, 2015).

In the case of fibrinolysis, it should be highlighted that all reported changes in maximum fibrinolysis stem from changes in maximum clotting; e.g. prodrug 3 was associated with ↓Fmax and ↓Lmax. This is an important finding, as it demonstrates the complete lysis of a previously formed clot. Of the tested compounds, compound 3 exhibited the most significant influence on the parameters of fibrinolysis. When added at a concentration of 1.5 – 3.0 µmol/ml, compound 3 caused a decrease in the initial clot fibrinolysis velocity (↓Lvo); however, it was not accompanied by an increase in the fibrinolysis time (↑Tl) due to lower Lmax (↓Lmax). Further studies on the effect of compound 3 on plasmin activity should be performed to elucidate the mechanism of its influence on the process of fibrinolysis.

Our evaluation of the influence of biguanide derivatives on the generation of endogenous thrombin did not reveal any significant influence on thrombin generation time (TGt constant). It is also important to note that other kinetic parameters of coagulation (Fmax, Tf, Fvo) are remain unaltered. Our findings imply that the tested prodrugs do not significantly interfere with the process of coagulation, which may confirm their biocompatibility in terms of plasma haemostasis.

PT and APTT allow the respective evaluation of extrinsic and intrinsic coagulation pathway. Both of these parameters depend on the Factor X, prothrombin (II), Factor V and
fibrinogen levels. PT also depends on the Factor VII, whereas APTT on VIII, IX, XI and XII Factor (Hood and Aby, 2008). The Diabetes Prevention Program Research Group found that diabetic patients are generally characterised by greater levels of fibrinogen than multi-ethnic populations. The overall level of fibrinogen reduction in patients who underwent metformin therapy was 0.3%: a modest, yet significant value (Haffner et al., 2005). Our in vitro studies did not reveal any effect of metformin on the concentration of FBG, which seems to be in accordance with other studies which suggest either a small fall or no change in fibrinogen levels associated with metformin use (Grant, 2003; Ghatak et al., 2011). It has been reported that metformin pre-treatment at various doses prolonged both APTT and PT in an animal model, which indicates that metformin has anticoagulant properties. The greatest prolongation of APTT was observed with 300 mg/kg, with a dose-dependent reduction observed at 400 mg/kg and 500 mg/kg. In the present study, biguanide prodrugs used at concentration ranges equivalent to therapeutic doses of metformin, were found to have no effect on the length of APTT; however, further studies based on decreasing doses may offer further clarification. Another study comparing the effects of metformin-fenofibrate combination treatment with those of fenofibrate monotherapy on haemostasis found that metformin tended to increase INR and prolong the partial thromboplastin time (PT) (Krysiak et al., 2013).

One crucial measure of the biocompatibility of a drug is its influence on RBCs, since enhanced haemolysis can be detrimental for the cardiovascular system. Another important reason for undertaking these experiments is the fact that metformin accumulates in erythrocytes, which contributes to longer half-life in erythrocytes than in plasma (23.4±1.9 hours versus 2.7±1.2 hours) (Briet et al., 2012). It should also be mentioned that RBCs with altered morphology can be typically seen in a blood samples of patients suffering from systemic inflammatory diseases such as T2DM, thromboembolic stroke as well as neurodegenerative diseases (Pretorius et al. 2016 a).
Our spectrophotometric evaluation of the *in vitro* response of RBCs to various concentrations of biguanide derivatives revealed a significantly greater rate of haemolysis for two highest concentrations of phenformin and compound 1; however, the percentage values of haemolysis did not exceed 10%. These compounds, together with metformin and prodrug 3, are unlikely to be toxic to erythrocytes over the whole tested concentration range. Fischer et al. report that haemolysis should not be considered toxic when its rate does not exceed 10% of total haemolysis in the positive control (0.2% Triton X-100) (Fischer et al., 2003). Only prodrug 2 appears to exert the most unfavourable effect on the RBC membrane, as a statistically significant increase in RBC haemolysis was observed even for the lowest concentration used. This was confirmed by microscope studies, in which total haemolysis of erythrocytes was observed at a concentration of 3.0 μmol/ml.

Flow cytometry studies identified significant changes in the percentage of RBCs of prodrug 2 within the gates P3/P4 (↓% in P3, ↑% in P4) and P6/P7 (↓% in P6, ↑% in P7), suggesting that the erythrocytes lost their discoidal shape and adopted more of a circular shape. These results suggest that compound 2 destroys the erythrocyte membrane without incorporation into lipid bilayer. Metformin contributed to the formation of echinocytes at a concentration of 3.0 μmol/ml. The transformation of erythrocytes to echinocytes occurs naturally in blood vessels. As this is a reversible transformation, one caused by a series of chemical and physical factors including increased ion strength, alkaline pH and decreased ATP level, it may be assumed that the analysed compounds do not affect RBCs in a pathological manner (Stasiuk et al., 2009). Changes in the shape of erythrocytes have been proposed to arise from the differential expansion of the two monolayers of the membrane lipid bilayer, and echinocytes are formed during the insertion of xenobiotics into the outer monolayer.
(Sheetz and Singer, 1974). In the case of metformin, phenformin and prodrug 3 the RBCs undergoing eryptosis could be seen. Eryptosis is defined as a coordinated process of programmed cell death and is similar to apoptosis (Lang et al, 2012; Lang and Qadri, 2012; Pretorius et al., 2016 a). Eryptosis is characterized by cell shrinkage and RBCs membrane scrambling and might be caused by calcium entry triggered by several factors including hyperosmotic shock, energy reduction, inflammatory markers (e.g. protein C, coagulation factors, prostaglandin E2 etc.) leading to oxidative stress.

In the comprehensive review of Pretorius et al. (2016, b) a list of various molecules and drugs leading to eryptosis formation can be found. Among the compounds causing eryptosis are well-known drug molecules such as terfenadine, fluoxetine, anti-cancer drugs (topotecan, cisplatin), monoclonal antibodies (sunitinib, sorafenib, lipatinib), and anti-viral drugs (ribavirin, efavirenz. In turn, according to Lang et al. (2010) resveratrol is a natural inhibitor of eryptosis. The consequences of eryptosis eg. caused by drugs, if not compensated by enhanced erythropoiesis, may lead to erythropenia, and anemia. Eryptotic erythrocytes are further known to stimulate blood clotting which may result in impede microcirculation (Land et al. 2012).

Eryptosis accomplishes the removal of defective erythrocytes, which would otherwise die from hemolysis, which is regarded as a necrosis-like death (Lang et al. 2012). According to Lang et al. (2012), a father of eryptosis, in a wide variety of conditions, eryptosis precedes and therefore prevents hemolysis. Another beneficial aspect of eryptosis is the fact that it may accomplish clearance of infected erythrocytes from circulating blood, as a result some drugs contributing to eryptosis may favourably influence the clinical course of malaria (Lang and Lang, 2015; Waibel et al., 2016). The results of conducted microscopy studies, indicating that both phenformin and prodrug 3 may lead to eryptosis can be regarded as more sensitive in measuring hemocompatibility of drug candidates for erythrocyte injuries.
5. CONCLUSIONS

The presented study was designed to conduct preliminary studies whose aim was to determine the biocompatibility of metformin, phenformin and three metformin prodrugs with a particular emphasis on haemostasis and interactions with erythrocyte membranes. Our findings suggest that none of the tested biguanide derivatives significantly affect the overall potential of clot formation and fibrinolysis (constant $CL_{AUC}$), which may indicate that the tested compounds can be regarded as biocompatible towards plasma haemostasis. Studies on the generation of endogenous thrombin also revealed that these compounds do not change the value of the overall potential of coagulation (constant $S$) and thrombin generation time (constant $TGt$). The obtained PT, APTT and fibrinogen concentrations demonstrate that the tested compounds do not interfere with the extrinsic and intrinsic coagulation pathways.

Only compound 2 unfavourably affected RBCs, causing a high percentage of RBC haemolysis by interacting with the erythrocyte membrane. Prodrug 1 did influence the morphology of erythrocytes. However, it was also found that metformin, phenformin and prodrug 3 contributed to eryptosis of erythrocytes.

Despite their documented interference with the kinetic parameters of clot formation, stabilization and fibrinolysis, and their influence on RBCs, the tested metformin prodrugs, apart from prodrug 2 should be generally considered safe for use. They do not affect the overall potential of clot formation or fibrinolysis, and do not significantly contribute to haemolysis or changes in RBC morphology. On the basis of our obtained results, we may conclude that of the studied compounds, prodrugs 1 and 3 are suitable for further studies on cellular or animal models in designated concentration range.

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Fig. 1. Chemical structure of biguanide derivatives: metformin, phenformin, prodrugs 1 – 3.
Fig. 2. Influence of biguanide derivatives (A. metformin, phenformin; B. prodrugs 1 – 3) on the overall potential of the clot formation and fibrinolysis ($CL_{AUC}$) (mean ± S.D., n = 7 - 8) after 3 min. incubation in plasma; final volume 500 µl. Metformin, phenformin and prodrugs did not affect in a statistically significant way the values of $CL_{AUC}$ in comparison with control. Two-way Welch’s Anova test for heterogenous groups (two independent variables: concentration and compound) did not reveal any other differences between the groups.
Fig. 3. Influence of biguanide derivatives (A. metformin, phenformin; B. prodrugs 1 – 3) on maximum clotting (Fmax) (mean ± S.D., n = 7 - 8) after 3 min. incubation in plasma; final volume 500 µl. *P < 0.05 vs. control. Decrease in Fmax for the two highest concentrations of prodrug 3 in comparison with control was reported. Two-way Welch’s Anova test for heterogenous groups (two independent variables: concentration and compound) did not reveal any other statistically significant changes between the groups.
Influence of biguanide derivatives (A. metformin, phenformin; B. prodrugs 1 – 3) on initial plasma clotting velocity (Fvo) (mean ± S.D., n = 7 - 8) after 3 min. incubation in plasma; final volume 500 µl. No changes in Fvo values were reported in comparison with control. Two-way Anova for homogenous groups (two independent variables: concentration and compound) did not reveal any differences between the groups.
**Fig. 5.** Influence of biguanide derivatives (A. metformin, phenformin; B. prodrugs 1 – 3) on initial clot fibrinolysis velocity (Lvo) (mean ± S.D., n = 7 - 8) after 3 min. incubation in plasma; final volume 500 µl. *P < 0.05 vs. control. Prodrug 3 at concentrations of 1.5 – 3.0 µmol/ml significantly decreased Lvo. Two-way Anova for homogenous groups (two independent variables: concentration and compound) revealed the following statistically significant differences: (i) Control of metformin and prodrug 1, 2 and 3 which then contributed to the differences between these groups with analysed concentrations; (ii) phenformin and prodrug 3 over the concentration range; (iii) prodrug 1 and 2 for two highest concentrations.
Fig. 6. Results of coagulation assay. Influence of biguanide derivatives (A. metformin, phenformin; B. prodrugs 1 – 3) on thrombin generation time (TGt) (mean ± S.D., n = 5) after 3 min. incubation in plasma; final volume 500 µl. Compounds did not induce a significant increase in TGt when added over the whole concentration range when compared to control. Two-way Anova (homogenity of variance; two independent variables: concentration and compound) did not reveal statistically significant differences between the tested compounds.
Fig. 7. Influence of biguanide derivatives (A. metformin, phenformin; B. prodrugs 1 – 3) on Activated Partial Thromboplastin Time (APTT) (mean ± S.D.; n = 6) after 3 minute incubation in plasma; final volume 160 µl. *P < 0.05 vs. control. Exposure to the tested compounds even at the highest concentrations was shown not to significantly influence the value of APTT over the whole concentration range apart from prodrug 2 for which statistically significant shortening of APTT was observed. Two-way Anova (homogenity of variance; two independent variables: concentration and compound) did not reveal any other differences between the groups.
Fig. 8. Influence of biguanide derivatives (A. metformin, phenformin; B. prodrugs 1 – 3) on Prothrombin Time (PT) (mean ± S.D.; n = 5) after 3 min. incubation in plasma; final volume 160 µl. Metformin, phenformin and prodrugs did not affect in a statistically significant way the values of PT in comparison with control. Two-way Anova (homogeneity of variance; two independent variables: concentration and compound) did not reveal any differences between the groups.
Fig. 9. Percentage of haemolysis obtained from the interaction of biguanide derivatives (A. metformin, phenformin; B. prodrugs 1 – 3) of with 2% RBCs suspension, compared to the positive control Triton X-100 at 0.2% (100% hemolysis) (mean ± S.D.; n = 5), *P < 0.05 vs. control. Exposure to metformin and prodrug 3 was shown not to affect the erythrocytes membrane over the whole concentration range. A statistically significant increase in the rate of haemolysis was documented for the highest concentrations of phenformin, prodrugs 1 and 2. Two-way Anova Welch’s test for heterogenous groups (two independent variables: concentration and compound) revealed the following statistically significant differences: (i) prodrug 2 at concentration of 1.5 and 3.0 µmol/mL and metformin, phenformin, prodrug 1, prodrug 3 at respective concentrations; (ii) prodrug 1 at the highest concentration and all other tested biguanides; (iii) prodrug 3 at the highest concentration and prodrug 1 and 2.
Fig. 10 A. Effects of biguanide derivatives on erythrocytes morphology. 2% erythrocyte suspension was treated at 37°C for 60 min. with indicated concentrations of metformin/phenformin. Representative phase-contrast images are shown (magnification of 400 times). Red line on the white background is 10 µm. 

Fig. 10 B. Effects of biguanide derivatives on erythrocytes morphology. 2% erythrocyte suspension was treated at 37°C for 60 min. with indicated concentrations of prodrugs 1 - 3. Representative phase-contrast images are shown (magnification of 400 times). Red line on the white background is 10 µm.
Fig. 11. Effects of biguanide derivatives on erythrocytes (flow cytometry studies). A. On the basis of SSC-A (side scattered light) parameter, subpopulation of erythrocytes marked in grey and separated by gate P5 was distinguished. B. Bimodal distribution of RBCs – control and samples treated with biguanide derivatives (3.0 μmol/ml). Erythrocytes were divided into P3 and P4 gates differing in the value of FSC parameter corresponding to the size of measured objects. The erythrocytes of gate P5 were divided into P6 and P7 according to FSC parameter.
Fig. 12. Effects of biguanide derivatives on erythrocytes. A. Percentage of all RBCs (mean ± S.D., n = 3) divided into P3 and P4 gates. The highest tested concentrations (3.0 μmol/ml) of prodrugs 1 and 2 contributed to significant changes in the amount of RBCs within these gates. B. Percentage of all RBCs divided into P6 and P7 gates. *P < 0.05 vs. control. Two-way Anova Welch’s test for heterogenous groups (two independent variables: concentration and compound) revealed the following statistically significant differences: (i) P3 and P4 gates – Prodrug 2 at 0.6 and 3.0 μmol/ml with all other compounds at respective concentrations; (i) P 6 and P7 gates – Prodrug 2 at 3.0 μmol/ml with all other compounds.