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# Novel halogenated sulfonamide biguanides with anti-coagulation properties

Magdalena Markowicz-Piasecka[a,*], Joanna Sikora[a], Agnieszka Zajda[b], Kristiina M. Huttunen[c]

[a] Laboratory of Bioanalysis, Department of Pharmaceutical Chemistry, Drug Analysis and Radiopharmacy, Medical University of Lodz, ul. Muszyńskiego1, 90-151 Lodz, Poland

[b] Students Research Group, Laboratory of Bioanalysis, Department of Pharmaceutical Chemistry, Drug Analysis and Radiopharmacy, Medical University of Lodz, ul. Muszyńskiego 1, 90-151 Lodz, Poland

[c] School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, Yliopistonranta 1C, POB 1627, 70211 Kuopio, Finland

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ABSTRACT

Apart from its hypoglycaemic properties, metformin also offers beneficial effects for the cardiovascular system resulting in significant reduction of diabetes-related death, and all-cause mortality. The aim of this study was to synthesize nine new benzenesulfonamide derivatives of metformin with a halogen substituent, and estimate their influence on selected parameters of plasma and vascular hemostasis.

The study describes the synthesis of nine benzenesulfonamide biguanides with *o*-, *m*-, and *p*- chloro-, bromo-, and fluoro substituents. All *orto*- derivatives (chloro- (**1**), bromo- (**4**), and fluoro- (**7**)) significantly prolong prothrombin time (PT) and partially activated thromboplastin time (APTT). In addition compounds **4** and **7** slow the process of fibrin polymerization, and contribute to increased TT. Multiparametric CL-test revealed that compounds **1**, **4**, **7** and *p*-fluorobenzenesulfonamide (**9**) significantly prolong the onset of clot formation, decrease initial clot formation velocity, and maximum clotting.

Analysis of human endothelial cell (HUVECs) and human aortal smooth muscle cell (AoSMCs) viability over the entire tested concentration range (0.001–3.0 μmol/mL) indicated that the examined compounds can undergo further tests up to 1.5 μmol/mL concentration without decreasing cellular viability. Furthermore, none of the synthesized compounds exert an unfavourable effect on erythrocyte integrity, and thus do not interact strongly with the lipid-protein bilayer.

In summary, chemical modification of the metformin backbone into benzenesulfonamides containing halogen substituents at the *o*- position leads to the formation of potential agents with stronger anti-coagulant properties than the parent drug, metformin. Therefore, *o*-halogenated benzenesulfonamides can be regarded as an initial promising step in the development of novel biguanide-based compounds with anti-coagulant properties.

## 1. Introduction

Cardiovascular disease (CVD) constitutes the main cause of morbidity and mortality in subjects with type 2 diabetes mellitus (T2DM). According to Alzahrani [1] up to 80% of people suffering from diabetes die as a result of cardiovascular complications. Numerous studies have shown that T2DM is characterised by an impaired balance between the process of coagulation and fibrinolysis [2]. With regard to haemostasis characteristic findings in T2DM might be divided into the following issues: thrombus formation, impaired fibrinolysis, endothelial dysfunction and platelet hyperreactivity [3]. In individuals with T2DM, increased activity of the coagulation factors such as fibrinogen, von Willebrand factor (vWF), FVII can be observed [2]. Increased levels of tissue factor (TF), and thrombin have also been proposed as other important causes of hypercoagulability in diabetic patients [4]. Moreover, there is also increase in plasminogen activator inhibitor type 1 (PAI-1) which decreases fibrinolysis [5]. Platelet hyperreactivity manifests by increased adhesiveness, exaggerated aggregation and changed metabolism [4]. T2DM predisposes also to the development of endothelium dysfunction, which is an initiating factor contributing to the pathogenesis and clinical expression of atherosclerosis [4,6]. Apart from providing a physical barrier between the vessel wall and lumen, the endothelium also secretes a number of mediators that regulate platelet aggregation, coagulation and fibrinolysis, thus actively participating in haemostasis, which is frequently disturbed in T2DM subjects [7].

Although low molecular weight heparins and vitamin K antagonists

* Corresponding author at: Department of Pharmaceutical Chemistry, Drug Analysis and Radiopharmacy, Medical University of Lodz, Muszynskiego 1, 90-151 Lodz, Poland.

*E-mail addresses:* magdalena.markowicz@umed.lodz.pl (M. Markowicz-Piasecka), joanna.sikora@umed.lodz.pl (J. Sikora), agnieszka.zajda@stud.umed.lodz.pl (A. Zajda), kristiina.huttunen@uef.fi (K.M. Huttunen).

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have proven to be effective for the treatment of thromboembolic disorders, the use of these drugs is associated with several drawbacks, including non-specificity and a parenteral mode of administration, which restricts their use. The recent development of direct oral factor Xa and thrombin inhibitors, as well as antidotes to reverse their effects, therefore represented a breakthrough in anticoagulation treatment [8]. Notwithstanding, the risk of bleeding and the subtle balance between bleeding and prevention of coagulation has driven the search for novel agents [9], and there is an ongoing need for new agents with anti-coagulation properties.

Conventional therapy of T2DM usually begins with lifestyle interventions supported by the prescription of a single anti-diabetic drug, usually metformin. If insulin resistance progresses and the monotherapy option becomes insufficient to control the level of plasma glucose, patients are usually switched to a double-drug regimen [10,11]. Metformin, a synthetic biguanide, is also used for the treatment of polycystic ovarian syndrome (PCOS), metabolic syndrome, and diabetes prevention [12,13]. The administration of metformin leads to a decrease in glucose levels through various approaches such as the amelioration of hyperinsulinemia due to increased insulin sensitivity [14], indirect induction of insulin receptor expression, inhibition of gastrointestinal absorption of glucose, and reduction in hepatic gluconeogenesis [13,15,16].

Apart from its glucose-lowering properties, metformin has been shown to exert advantageous effects on lipid metabolism, including decreasing plasma triglyceride (TG) and low-density lipoprotein (LDL) levels [17]. Importantly, metformin treatment constitutes a relevant element of an integrated lifestyle modification-pharmacotherapy to prevent both T2DM and cardiovascular disease [18]. Therefore, metformin is regarded as a valuable drug on account of its anti-diabetic properties and beneficial effects on mortality in the diabetic population. In addition, recent findings suggest that metformin may decrease the process of atherosclerosis, mainly through the inhibition of leukocyte-endothelial interaction, foam cell formation, smooth muscle cell proliferation and platelet aggregation. The researchers indicate that these effects do not depend on plasma lipid levels, which imply that the drug has a vascular effect, mainly based on the prevention of endothelial lesions and damage [18]. Some publications indicate that metformin might have anti-coagulation and profibrinolytic properties [19,20].

Despite its benefits, metformin also possesses unfavourable pharmacokinetic properties associated with its chemical structure. It is characterized by slow and incomplete intestine absorption, resulting in low bioavailability and considerable inter- and intra-individual differences in clinical response to the drug. Therefore, several bio-reversible sulfenyl biguanidine (N-S) prodrugs with improved oral absorption [21–23] and various sulfonamide derivatives have been synthesized [24]. Sulfonamide derivatives are a group of drugs with recognized biological activity, including antibacterial, hypoglycaemic and anti-cancer activity. However, research is ongoing in which the drug structure is modified and a sulfonamide moiety is added to provide new biological properties [25]. Apaydin and Torok [25] state that sulfonamides are increasingly used in the design and development of multi-target drugs. In addition, nitro-benzenesulfonamide (both *p*- and *o*-) derivatives of metformin exert multidirectional effects on some parameters of haemostasis, including plasma, platelet and vascular haemostasis. For example, they are known to prolong the process of platalet-dependent thrombus formation and decrease the amount of vWF released from endothelial cells (ECs) [26], and decrease the overall potential of clot formation and fibrinolysis [27]. In addition both compounds decrease ADP-induced platelet aggregation as well as spontaneous and ADP-induced platelet adhesion to fibrynogen, and significanlty reduce smooth muscle cell migration (data unpublished).

To better understand, and possibly improve on, the pleiotropic properties of metformin and the anti-coagulant activity of its sulfonamide derivatives, this paper describes the design and synthesis of nine novel sulfonamide derivatives of metformin. The synthesized compounds were screened for their anti-coagulation properties by measuring their prothrombotic time (PT), partially activated thromboplastin time (APTT), thrombin time (TT), and overall potential of clot formation and fibrinolysis (CL-test). It also determines the effects of the synthesized compounds on the viability, integrity and morphology of human umbilical vein endothelial cells (HUVECs), and human aortal smooth muscle cells (AoSMCs) using a cellular *in vitro* model. In addition the study evaluates their effects on red blood cell (RBC) integrity, using RBC lysis assay and microscopy studies.

## 2. Materials and methods

### 2.1. General synthetic materials and methods

All reactions were performed with reagents obtained from Sigma-Aldrich (St. Louis, MO, USA), Acros Organics (Waltham, MA, USA) or Merck (Darmstadt, Germany). Reactions were monitored by thin-layer chromatography using aluminum sheets coated with silica gel 60 $F_{245}$ (0.24 mm) with suitable visualization. Purifications by flash chromatography were performed on silica gel 60 (0.063–0.200 mm mesh). $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Fällanden, Switzerland) operating at 500.13 MHz and 125.75, respectively, using tetramethylsilane as an internal standard. Not all pH-dependent protons of the compounds were observed. ESI-MS spectra were recorded by a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization source. Over 97% purities were obtained for the final products by elemental analysis (C, H, N) with a Perkin Elmer 2400 Series II CHNS/O organic elemental analyzer (Perkin Elmer Inc., Waltham, MA, USA).

### 2.2. General procedure for synthesis of sulfonamide derivatives

Metformin (*N*,*N*-dimethylimidodicarbonimidic diamide hydrochloride) (1.0 eq.) in 1 M NaOH (1.5 eq.) was stirred at room temperature for 30 min. Water was evaporated *in vacuo* and the residue was dissolved in MeOH. The solvent was evaporated and the residue was redissolved in cold anhydrous MeOH. NaCl was filtered out of the solution and the filtrate was evaporated to yield basic metformin as a white solid (99%).

Basic metformin (2.0 eq.), and commercial sulfonyl chlorides (1.0 eq.) were dissolved in anhydrous $CH_2Cl_2$ and stirred under argon for three hours. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography eluting with 0–10% MeOH in $CH_2Cl_2$ to obtain the compounds **1**–**9** (34–88%). Some of the final products were triturated with diethyl ether, methanol or acetone to improve their purity.

#### 2.2.1. $N^1$,$N^1$-Dimethyl-$N^4$-(2-chlorobenzenesulfonamide)-biguanidine (1)

Compound **1** was prepared from 2-chlorobenzenesulfonyl chloride to yield off-white solid, 0.27 g, (46%). $^{1}H$ NMR ($(CD_3)_2SO$): $\delta$ ppm 8.09 (bs, 2H), 8.02 (d, $J$ = 7.8 Hz, 1H), 7.58 (d, $J$ = 7.8 Hz, 1H), 7.54 (t, $J$ = 7.7 Hz, 1H), 7.47 (t, $J$ = 7.6 Hz, 1H), 6.98 (bs, 1H), 6.70 (bs, 1H), 2.92 (s, 6H); $^{13}C$ NMR ($(CD_3)_2SO$): $\delta$ ppm 159.45, 158.32, 141.03, 132.84, 131.51, 130.58, 129.26, 127.09, 36.50 (2C). MS ($ESI^+$) for $C_{10}H_{15}ClN_5O_2S$ $(M+H)^+$: Calcd 304.78, Found 304.20. Anal. Calcd for ($C_{10}H_{14}ClN_5O_2S$ *0.10 EtOH): C, 40.14; H, 4.86; N, 22.51; Found: C, 40.17; H, 4.81; N, 22.21.

#### 2.2.2. $N^1$,$N^1$-Dimethyl-$N^4$-(3-chlorobenzenesulfonamide)-biguanidine (2)

Compound **2** was prepared from 3-chlorobenzenesulfonyl chloride to yield off-white solid, 0.22 g, (37%). $^{1}H$ NMR ($(CD_3)_2SO$): $\delta$ ppm 7.98 (bs, 2H), 7.77 (s, 1H), 7.73 (d, $J$ = 7.5 Hz, 1H), 7.62 (d, $J$ = 8.2 Hz, 1H), 7.55 (t, $J$ = 7.8 Hz, 1H), 6.96 (bs, 1H), 6.79 (bs, 1H), 2.92 (s, 6H); $^{13}C$ NMR ($(CD_3)_2SO$): $\delta$ ppm 159.63, 158.34, 146.17, 133.27, 131.21, 130.90, 125.36, 124.44, 36.54 (2C). MS ($ESI^+$) for $C_{10}H_{15}ClN_5O_2S$ (M

+H)$^+$: Calcd 304.78, Found 304.20. Anal. Calcd for ($C_{10}H_{14}ClN_5O_2S$ *0.10 MeOH): C, 39.52; H, 4.73; N, 22.81; Found: C, 39.92; H, 4.67; N, 22.49.

*2.2.3. $N^1$,$N^1$-Dimethyl-$N^4$-(4-chlorobenzenesulfonamide)-biguanidine (**3**)*

Compound **3** was prepared from 4-chlorobenzenesulfonyl chloride to yield off-white solid, 0.34 g, (58%). $^1$H NMR ($(CD_3)_2SO$): $\delta$ ppm 8.00 (bs, 2H), 7.75 (d, $J$ = 8.7 Hz, 2H), 7.58 (d, $J$ = 8.7 Hz, 2H), 6.99 (bs, 1H), 6.69 (bs, 1H), 2.91 (s, 6H); $^{13}$C NMR ($(CD_3)_2SO$): $\delta$ ppm 159.71, 158.36, 143.11, 136.04, 128.88 (2C), 127.69 (2C), 36.55 (2C). MS (ESI$^+$) for $C_{10}H_{15}ClN_5O_2S$ (M+H)$^+$: Calcd 304.78, Found 304.16. Anal. Calcd for ($C_{10}H_{14}ClN_5O_2S$*0.1 $(CH_3)_2CO$): C, 39.96; H, 4.69; N, 22.62; Found: C, 40.13; H, 4.21; N, 23.07.

*2.2.4. $N^1$,$N^1$-Dimethyl-$N^4$-(2-bromobenzenesulfonamide)-biguanidine (**4**)*

Compound **4** was prepared from 2-bromobenzenesulfonyl chloride to yield off-white solid, 0.50 g, (74%). $^1$H NMR ($(CD_3)_2SO$): $\delta$ ppm 8.10 (bs, 2H), 8.06 (dd, $J$ = 7.7; 1.6 Hz, 1H), 7.77 (dd, $J$ = 8.0; 0.9 Hz, 1H), 7.52 (dt, $J$ = 7.7; 0.8 Hz, 1H), 7.45 (dt, $J$ = 7.8; 1.6 Hz, 1H), 6.97 (bs, 1H), 6.68 (bs, 1H), 2.92 (s, 6H); $^{13}$C NMR ($(CD_3)_2SO$): $\delta$ ppm 159.42, 158.28, 140.64, 134.97, 132.84, 129.44, 127.63, 119.26, 36.51 (2C). MS (ESI$^+$) for $C_{10}H_{15}BrN_5O_2S$ (M+H)$^+$: Calcd 349.23, Found 348.00; 350,00. Anal. Calcd for ($C_{10}H_{14}BrN_5O_2S$ *0.65 MeOH): C, 34.66; H, 4.53; N, 18.98; Found: C, 35.11; H, 4.33; N, 18.72.

*2.2.5. $N^1$,$N^1$-Dimethyl-$N^4$-(3-bromobenzenesulfonamide)-biguanidine (**5**)*

Compound **5** was prepared from 3-bromobenzenesulfonyl chloride to yield off-white solid, 0.59 g, (88%). $^1$H NMR ($(CD_3)_2SO$): $\delta$ ppm 7.97 (bs, 2H), 7.90 (s, 1H), 7.79–7.74 (m, 2H), 7.48 (t, $J$ = 7.9 Hz, 1H), 6.91 (bs, 2H), 2.92 (s, 6H); $^{13}$C NMR ($(CD_3)_2SO$): $\delta$ ppm 159.64, 158.35, 146.32, 134.11, 131.15, 128.16, 124.79, 121.65, 36.55 (2C). MS (ESI$^+$) for $C_{10}H_{15}BrN_5O_2S$ (M+H)$^+$: Calcd 349.23, Found 348.00; 350,00. Anal. Calcd for ($C_{10}H_{14}BrN_5O_2S$ *0.1 $Et_2O$): C, 35.12; H, 4.25; N, 19.69; Found: C, 35.26; H, 4.08; N, 19.36.

*2.2.6. $N^1$,$N^1$-Dimethyl-$N^4$-(4-bromobenzenesulfonamide)-biguanidine (**6**)*

Compound **6** was prepared from 4-bromobenzenesulfonyl chloride to yield off-white solid, 0.46 g, (68%). $^1$H NMR ($(CD_3)_2SO$): $\delta$ ppm 7.98 (bs, 2H), 7.74–7.68 (m, 4H), 6.99 (bs, 1H), 6.69 (bs, 1H), 2.91 (s, 6H); $^{13}$C NMR ($(CD_3)_2SO$): $\delta$ ppm 159.70, 158.37, 143.52, 131.82 (2C), 127.84 (2C), 124.90, 36.55 (2C). MS (ESI$^+$) for $C_{10}H_{15}BrN_5O_2S$ (M+H)$^+$: Calcd 349.23, Found 348;14; 350.14. Anal. Calcd for ($C_{10}H_{14}BrN_5O_2S$*0.1 $(CH_3)_2CO$): C, 34.94; H, 4.10; N, 19.78; Found: C, 35.02; H, 3.74; N, 19.59.

*2.2.7. $N^1$,$N^1$-Dimethyl-$N^4$-(2-fluorobenzenesulfonamide)-biguanidine (**7**)*

Compound **7** was prepared from 2-fluorobenzenesulfonyl chloride to yield off-white solid, 0.40 g, (72%). $^1$H NMR ($(CD_3)_2SO$): $\delta$ ppm 8.03 (bs, 2H), 7.82 (dt, $J$ = 7.6; 1.5 Hz, 1H), 7.62–7.58 (m, 1H), 7.36–7.29 (m, 2H), 7.00 (bs, 1H), 6.70 (bs, 1H), 2.92 (s, 6H); $^{13}$C NMR ($(CD_3)_2SO$): $\delta$ ppm 159.16, 158.97 (d, $J$ = 142.1 Hz), 157.16, 133.94 (d, $J$ = 8.3 Hz), 131.64 (d, $J$ = 15.1 Hz), 128.69, 124.24 (d, $J$ = 3.6 Hz), 117.02 (d, $J$ = 21.6 Hz), 36.52 (2C). MS (ESI$^+$) for $C_{10}H_{15}FN_5O_2S$ (M+H)$^+$: Calcd 288.32, Found 288.10. Anal. Calcd for ($C_{10}H_{14}FN_5O_2S$ *0.15 EtOH *0.05 MeOH): C, 42.63; H, 5.27; N, 23.34; Found: C, 42.82; H, 5.07; N, 23.64.

*2.2.8. $N^1$,$N^1$-Dimethyl-$N^4$-(3-fluorobenzenesulfonamide)-biguanidine (**8**)*

Compound **8** was prepared from 3-fluorobenzenesulfonyl chloride to yield off-white solid, 0.19 g, (34%). $^1$H NMR ($(CD_3)_2SO$): $\delta$ ppm 7.98 (bs, 2H), 7.60 (t, $J$ = 7.7 Hz, 1H), 7.58–7.54 (m, 2H), 7.43–7.38 (m, 1H), 6.97 (bs, 1H), 6.76 (bs, 1H), 2.92 (s, 6H); $^{13}$C NMR ($(CD_3)_2SO$): $\delta$ ppm 157.98 (d, $J$ = 244.52 Hz), 159.67, 158.36, 146.46 (d, $J$ = 6.3 Hz), 131.14 (d, $J$ = 7.6 Hz), 121.92 (d, $J$ = 2.7 Hz), 118.38 (d, $J$ = 21.3 Hz), 112.84 (d, $J$ = 23.8 Hz), 36.53 (2C). MS (ESI$^+$) for $C_{10}H_{15}FN_5O_2S$ (M+H)$^+$: Calcd 288.32, Found 288.10. Anal. Calcd for ($C_{10}H_{14}FN_5O_2S$ *0.10 EtOH): C, 42.38; H, 5.13; N, 23.76; Found: C, 42.59; H, 4.93; N, 23.82.

*2.2.9. $N^1$,$N^1$-Dimethyl-$N^4$-(4-fluorobenzenesulfonamide)-biguanidine (**9**)*

Compound **9** was prepared from 4-fluorobenzenesulfonyl chloride to yield off-white solid, 0.35 g, (63%). $^1$H NMR ($(CD_3)_2SO$): $\delta$ ppm 8.01 (bs, 2H), 7.85–7.80 (m, 2H), 7.36–7.31 (m, 2H), 6.94 (bs, 1H), 6.72 (bs, 1H), 2.91 (s, 6H); $^{13}$C NMR ($(CD_3)_2SO$): $\delta$ ppm 163.43 (d, $J$ = 249.9 Hz), 159.72, 158.35, 140.70 (d, $J$ = 3.3 Hz), 128.53 (d, $J$ = 9.4 Hz, 2C), 115.82 (d, $J$ = 22.2 Hz, 2C), 36.51 (2C). MS (ESI$^+$) for $C_{10}H_{15}FN_5O_2S$ (M+H)$^+$: Calcd 288.32, Found 288.10. Anal. Calcd for ($C_{10}H_{14}FN_5O_2S$ *0.10 EtOH): C, 42.38; H, 5.13; N, 23.76; Found: C, 42.64; H, 5.11; N, 23.42.

### 2.3. Biological material for stability studies

Rat and mouse liver and brain S9 fraction were prepared by collecting fresh tissues from the animals in compliance with the European Commission Directives 2010/63/EU and 86/609 and approved by the Institutional Animal Care and Use Committee of University of Eastern Finland. The liver and brain homogenates were prepared by homogenizing freshly-collected rat or mouse liver or brain with 50 mM Tris-buffered saline (TBS) (pH 7.4) (1:4 w/v). The homogenates were centrifuged at 9 000 rpm for 20 min at 4 °C and the supernatant was collected. Protein concentrations of both fractions were determined by Bio-Rad Protein Assay, based on Bradford dye-binding method (EnVision, Perkin Elmer, Waltham, MA, USA). All biological material was stored at −80 °C until used.

### 2.4. High-performance liquid chromatography (HPLC) analyses

Concentrations of metformin derivatives were determined by HPLC. The apparatus was formed of an Agilent 1100 binary pump (Agilent Technologies Inc., Wilmington, DE, USA), a 1100 micro vacuum degasser, a HP 1050 Autosampler, a HP 1050 variable wavelength detector, operated at 235 nm. The chromatographic separations were achieved on an Agilent Zorbax SB-C18 analytical column (4.6 mm × 150 mm, 5 μm) (Agilent Technologies Inc., Wilmington, DE, USA) by using isocratic elution of water containing 0.1% formic acid (pH ca. 3.0) and acetonitrile containing 0.1% formic acid with a ratio of 75:25 (v/v) for compounds **1**, **4**, **7**–**9** and with a ratio of 70:30 (v/v) for compounds **2**, **3**, **5** and **6**. The retention times of the compounds were ca. 2.8–3.9 min at the flow rate of 1.0 ml/min at room temperature. The lower limit of quantification for the compounds was 0.5 μM. These HPLC methods were also accurate, precise and selective over the range 1–100 μM.

### 2.5. Stabilities of metformin sulfonamide derivatives

Enzymatic and chemical stabilities of metformin sulfonamide derivatives in rat liver and brain S9 fractions, in human plasma or in TBS buffer (pH 7.4) were determined at 37 °C. Stability in 0.1 M NaOH was determined at RT. The incubation mixtures were prepared by mixing liver or brain S9 fraction (final protein concentration 1.0 mg/mL) with TBS buffer (pH 7.4) and 10 mmol/L stock solution of studied compounds in DMSO (the final concentration of compounds were 100 μmol/L and the DMSO concentration 2%). The mixtures were incubated for six hours and the samples (100 μL) were withdrawn at appropriate intervals. The proteins in the samples were precipitated with ice-cold acetonitrile (100 μL) and the samples were centrifuged for 5 min at 12 000 rpm at room temperature. The supernatants were collected and analyzed by the HPLC method described above. In chemical stability study with TBS buffer, the S9 fractions were replaced with same volume of buffer and in study with 0.1 M NaOH, the reaction mixture contained only 0.1 M NaOH and 10 mmol/L studied compound.

### 2.6. Preparation of biological material for basic coagulology studies and RBCs lysis assay

All experiments using human blood were performed in accordance with Polish national guidelines, and the study protocols were approved by the Bioethics Committee of the Medical University of Lodz (Medical University of Lodz, Poland) (approval no. RNN/27/18/KE).

Blood samples were obtained from the Wojewódzki Specjalistyczny Szpital im. Dr W. Biegańskiego w Łodzi (*Voivodship Specialized Hospital in Łódź*), Poland; the tested material was a remnant of routine diagnostic tests intended for recycling. The blood was collected to vacuum tubes containing 3.2% buffered sodium citrate, and centrifuged (3000 rpm, 10 min, room temperature) with a Micro 22R centrifuge (Hettich ZENTRIFUGEN, Germany). Obtained platelet poor plasma (PPP) was stored in small portions 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.

RBCs separated from the plasma were washed three times with 0.9% saline, and used for the studies within 24 h.

### 2.7. Materials for biological studies

Basic coagulation parameters were determined using Bio-Ksel reagents (Poland): APTT reagent, calcium chloride, Bio-Ksel PT plus reagent (tromboplastin and solvent), and thrombin (3.0 UNIH/mL) for TT experiments. Calibrator (Bio-Ksel, Poland), normal plasma (Bio-Ksel, Poland) and water for injection (Polpharma, Poland) were used for calibration and determination of the coefficient of variation (for PT, APTT, TT studies).

The reagents for CL-test were as follows, thrombin was produced by Biomed (Poland) and recombinant tissue plasminogen activator (*t*-PA) by Boehringer-Ingelheim (Germany). Tris-buffered saline (TBS) and sodium chloride were provided by Polish Chemical Reagents (Poland).

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Italy), human aortal smooth muscle cells (AoSMC) were purchased from ScienCell Research Laboratories (US). The reagents for HUVECs maintenance were as follows: EGM-2 - medium + bullet kit (Lonza, Clonetics, Italy), trypsin-EDTA – 0.05% solution (Sigma Aldrich, Germany), trypsin neutralizing solution (Lonza, Italy), and HEPES buffered saline solution (Lonza, Italy). The AoSMC cell culture medium consisted of 500 ml of basal medium (SMC, ScienCell Research Laboratories, US), 10 ml of fetal bovine serum (FBS, ScienCell Research Laboratories, US), 5 ml of smooth muscle cell growth supplement (SMCGS, ScienCell Research Laboratories, US), and 5 ml of penicillin/streptomycin solution (ScienCell Research Laboratories, US). The AoSMC cells were passaged in trypsin/EDTA (ScienCell Research Laboratories, US), Trypsin Neutralizing Solution (ScienCell Research Laboratories, US), and Poly-ʟ-Lysine (ScienCell Research Laboratories, US). Cell viability was assessed using WST-1 assay (Takara, Takara Bio Europe, Saint-Germain-en-Laye, France). E-Plate 16 View (Roche & ACEA Biosciences) and phosphate buffered saline (PBS, Biomed Lublin, Poland) were used for evaluation of integrity of HUVECs in the real-time cell electric impedance sensing system.

The Triton X-100 and 0.9% saline used in the erythrotoxicity test were obtained from Polish Chemical Reagents (Poland).

### 2.8. Basic coagulation tests: PT, INR, FBG, APTT, TT

The measurements of PT, APTT, TT, and fibrinogen concentration were conducted on coagulometer (CoagChrom-3003 Bio-Ksel, Poland) according to the routine procedure, and described elsewhere [28]. In every test, control samples consisting of distilled water were included. The experiments were conducted in multiplicates (n = 5), and the results are presented as mean ± standard deviation (SD).

The methods were validated using Bio-Ksel normal plasma dissolved in water for injection (Polpharma, Poland). Coefficients of variability for all tests were counted (PT: W = 4.56%, FBG: W = 8,16%, INR: W = 4,52%, APTT: W = 1.66%, TT: W = 1.18%). The reference values for each test are as follows: PT: 9.7–14.6 s; APTT: 26.7–40.0 s; TT: 14.0–18.0 s for 3.0 UNIH/mL of thrombin.

### 2.9. Clot formation and lysis test (CL-test)

The CL-test was used to evaluate the effect of synthesized metformin derivatives on the overall potential of clot formation and fibrinolysis, as well as its kinetic parameters. The CL-test is based on the continuous measurement of the changes in optical transmittance over time, as described by Kostka et al. [29] and Sikora et al. [30].

General conditions of the experiments were the same as published previously [30,31]. The process of clot formation was triggered by addition of 10 μL thrombin (0.5 IU/mL), and fibrinolysis by *t*-PA (final concentration in a sample 220 ng/mL). The measurements were conducted on three-fold diluted pooled human citrate plasma (3H Biomedical, Sweden) in Semi-Micro cuvettes (Medlab Products, Poland). The clot formation and lysis curves were recorded at λ = 405 nm, by means of a Cecil CE 2021 (England) spectrophotometer with circulating thermostated water (37 °C) and a magnetic stirrer (Electronic Stirrer Model 300 Rank Brothers Ltd, England). Tested compounds were added in a volume of 10 μL to diluted plasma (470 μL), together with 10 μL of *t*-PA, and the samples were incubated at 37 °C for three minutes, and then 10 μL thrombin was added to initiate clot formation. The experiments were conducted in multiplicates (n = 5–9), and the results are presented as mean ± standard deviation (SD).

The obtained curves were analysed by means of dedicated software [29] used to measure the following parameters of clot formation, its stabilization and fibrinolysis. Tt – thrombin time [s], Fmax – maximum clotting [%T], Tf – plasma clotting time [s], Fvo – initial plasma clotting velocity [%T/min], Sf – area under the clot formation curve [%Txmin], Tc – clot stabilization time [s], Sc – area under the curve of stable clot formation [%Txmin], Lmax – maximum lysis [%T], Tl – fibrinolysis time [s], Lvo – initial clot fibrinolysis velocity [%T/min], Sl – area under the fibrinolysis curve [%Txmin]. The overall potential of clot formation and fibrinolysis ($CL_{AUC}$, [%Txmin]) and total time of the process of clot formation and fibrinolysis (T, [s]) were also estimated.

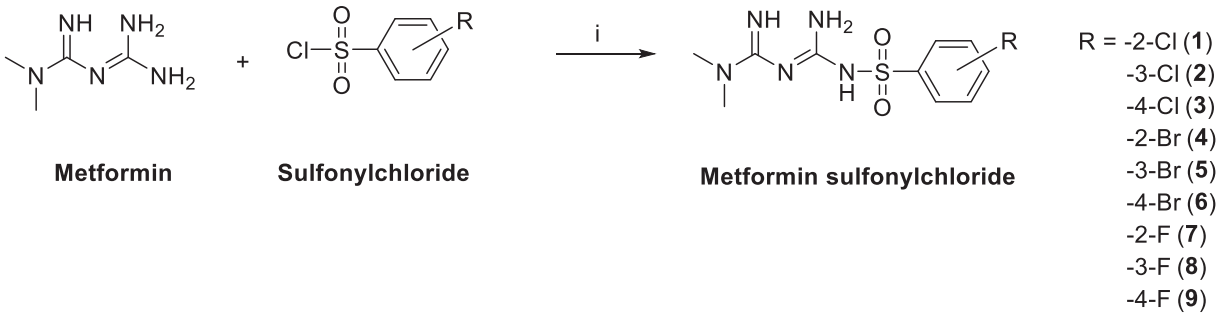
The method of clot formation and fibrinolysis was validated, and coefficient of variation (W) for pooled human plasma (n = 4) was within the range 4.57–9.80 depending on the calculated parameter.

### 2.10. Cell viability assay

WST-1 assay (Takara, Takara Bio Europe, France) was used to assess the effects of synthesized derivatives on the viability of HUVECs and AoSMCs. The experiments were conducted as described previously [32]. Briefly, HUVECs and AoSMCs were seeded on 96-well microplates at a density of 7500 and 5000 cells per well, respectively. After culturing for 24 h, the cells were treated with pure medium (v = 100 μL) or compounds diluted in medium (1 + 9; v = 100 μL). The cells were incubated for 24 h at standard conditions (37 °C, 5% $CO_2$). Afterwards, the cells were washed with culture medium (100 μL) and WST-1 reagent diluted in medium (1 + 9) was added. The plates were incubated at 37 °C in 5% $CO_2$ for additional 90 min, and the absorbance was read at 450 nm using a microplate reader (iMARK, Bio-Rad, US).

The experiments were conducted in multiplicates (n = 8), and the results were presented as mean ± standard deviation (SD). Cellular viability was expressed as a percentage of the control samples which constituted 100% viability. $IC_{50}$ values (the concentration of tested compound inhibitiing cell growth by 50%) were determined using concentration-response curves (GraphPad Prism). The variability coefficient of the method was counted (CV = 4.79%, n = 8).

The influence of synthesized derivatives on morphology of HUVECs and AoSMCs was examined using an inverted microscope with phase contrast (magnification 100x) (Opta-Tech, software OptaView 7).




**Scheme 1.** Synthetic route for the metformin sulfonamide derivatives; (i) $CH_2Cl_2$, RT, 20 h, 34–88%.

### 2.11. HUVEC integrity studies

The influence of biguanide derivatives on integrity of endothelium monolayer was performed using the real-time cell electric impedance monitoring system (Real-Time Cell Analyzer; Roche & ACEA Biosciences). The RTCA-DP system allows cell growth and integrity to be monitored in real time based on tracking electrical impedance signals. The cell condition such as adhesion, integrity, morphology or barrier properties are described by Cell Index (CI) [33].

HUVECs were cultured in RTCA DP system according to the previous description [26]. The cells were seeded at a density of 15 000 cells per well on dedicated 16-well plates (ACEA Biosciences). Once the cells reached plateau phase (the value of CI at the level of 6–8) the medium was extracted from each well and the solutions of tested compounds dissolved in cell culturing medium (100 µL) or pure medium (100 µL, control) were added. After 48 h, the experiment was stopped and the values of CI were collected.

The results were presented as mean ± standard deviation (SD) expressed as 'normalized cell index' (nCI) which is calculated by division of a CI value at a certain time point by the CI value at a reference time point. The experiments were conducted in multiplicates (n = 5). The method was validated and the variability coefficient was counted (CV = 7.5–11.5%, depending on the measured time point, n = 6).

### 2.12. Red blood cells lysis assay

The influence of metformin derivatives (**1–9**) on RBC membrane integrity was performed by lysis assay which was conducted as described previously [31]. Briefly, 2% RBC suspension in NaCl was incubated at 37 °C for one hour with the tested compounds at concentrations ranging from 0.006 to 1.5 µmol/mL or 0.9% NaCl (control). Afterwards, the samples were centrifuged at 3000*g* for 10 min and the absorbance of the supernatant was collected at 550 nm.

The results are presented as the degree of haemolysis which constituted a percentage of released haemoglobin. A sample containing 10 µL of 2.0% v/v Triton X-100 was used as a positive control (100% of haemolysis), whereas a sample of saline solution represented spontaneous haemolysis of RBCs (control).

The experiments were conducted using at least four different biological materials (n = 4). The results are presented as mean ± standard deviation (SD). The coefficient of variability was counted: W = 9.21%, n = 5.

### 2.13. Red blood cell morphology

A 2% RBCs suspension was incubated at 37 °C for 1 h with various concentrations of tested compounds. Afterwards, erythrocyte morphology was evaluated using a phase contrast Opta-Tech inverted microscope, at 400-times magnification, equipped with software (OptaView 7) for image analysis.

### 2.14. Statistical analysis

Statistical analysis was conducted using commercially-available packages (Statistica 12.0, StatSoft; GraphPad Prism 5).

The normality of the distribution of continuous variables was verified with the Shapiro-Wilk test, while the homogeneity of variances was checked using the Levene test. The paired *t*-test was used to test the dependent variables (e.g. studies on biological material), while statistically significant differences between the means of independent groups were identified using one-way ANOVA. The variables with non-normal distributions were compared using the Wilcoxon signed rank test. The results of all the tests were considered significant at *p*-values lower than 0.05.

## 3. Results

### 3.1. Design, synthesis and stability of novel metformin sulfonamide derivatives

Sulfonamide derivatives **1–9** of metformin were obtained as previously described by coupling commercial sulfonyl chlorides with basic metformin to yield final compounds with moderate to good yields (Scheme 1) [24]. All compounds were stable in TBS buffer pH 7.4, 0.1 M NaOH, human plasma and rat liver and brain subcellular fractions (S9).

### 3.2. Basic coagulation tests: PT, INR, APTT, TT

The effects of synthesized sulfonamides **1–9** and metformin on the basic coagulation parameters are shown in Table 1. Some of the compounds were found to significantly prolong PT and its international equivalent (INR). For instance, *o*-, *m*-chloro derivatives (compounds **1**, **2**) as well as *o*-, *m*-bromosulfonamides (compounds **4**, **5**) at the highest concentration (1.5 µmol/mL) statistically prolong PT, and increase INR. Compounds **1**, **4** and **5** (concentration of 1.5 µmol/mL) induced statistically significant changes of PT that go beyond the range of reference values (9.7–14.6 s), which may be of potential importance in further studies. Furthermore, all compounds possessing a fluoro- substituent in the aromatic ring also increased PT and INR.

All *o*- substituted sulfonamides significantly prolonged APTT. Compound **4** with an *o*-bromine substituent exerted an APTT lengthening effect above 0.3 µmol/mL. Compound **9** was also found to increase APTT. The most complex effect on APTT measurements was demonstrated by compound **7** which significantly shortens APTT at lower concentrations (0.006–0.06 µmol/mL), but significantly prolongs it while at the highest tested concentration (1.2 and 1.5 µmol/mL) (43.96 ± 11.51 s for 1.5 µmol/mL vs. 30.35 ± 4.89 s for control).

The results of the studies showed (Table 1) that compounds **4**, with bromine, and **7**, with fluoro substituents in the *ortho* position, significantly prolong TT. For instance, compound **4** at 1.5 µmol/mL prolonged TT up to 16.30 ± 1.26 s, while the value for control samples was 14.88 ± 1.37 s. Among the synthesized derivatives, compound **8**

**Table 1**
The effects of synthesized compounds **1–9** on basic coagulation parameters.

| Compound | Concentr. [μmol/mL] | PT [s] | INR | APTT [s] | TT [s] |
|---|---|---|---|---|---|
| Control | – | 12.35 ± 0.66 | 0.99 ± 0.05 | 30.35 ± 4.89 | 14.88 ± 1.37 |
| Metformin | 0.06 | 12.40 ± 0.17 | 1.01 ± 0.04 | 29.72 ± 2.83 | 15.11 ± 1.01 |
| | 0.3 | 12.49 ± 1.15 | 1.01 ± 0.11 | 31.05 ± 2.98 | 15.20 ± 1.35 |
| | 0.9 | 12.98 ± 1.25 | 1.08 ± 0.12 | 31.24 ± 2.04 | 15.17 ± 1.09 |
| | 1.2 | 13.15 ± 1.59 | 1.16 ± 0.09 | 31.88 ± 2.92 | 15.44 ± 1.27 |
| | 1.5 | 14.12 ± 1.56 | 1.21 ± 0.12 | 32.49 ± 3.85 | 15.50 ± 1.38 |
| 1 | 0.006 | 12.80 ± 0.75 | 1.02 ± 0.06 | 29.92 ± 3.72 | 14.20 ± 1.21 |
| | 0.06 | 13.16 ± 1.50 | 0.98 ± 0.12 | **26.48 ± 1.87*** | 14.20 ± 1.11 |
| | 0.3 | 14.43 ± 1.30 | 1.17 ± 0.10 | 29.02 ± 3.50 | 14.20 ± 1.47 |
| | 0.6 | 13.95 ± 0.24 | 1.12 ± 0.02 | 31.12 ± 3.52 | 14.85 ± 1.55 |
| | 0.9 | 14.29 ± 0.95 | 1.14 ± 0.09 | 31.55 ± 2.94 | 15.12 ± 1.21 |
| | 1.2 | **15.26 ± 0.88*** | **1.20 ± 0.12*** | 31.69 ± 2.48 | 16.41 ± 1.51 |
| | 1.5 | **15.58 ± 1.89*** | **1.24 ± 0.14*** | **32.86 ± 4.50**** | 17.03 ± 1.93 |
| 2 | 0.006 | 12.16 ± 0.25 | 0.97 ± 0.02 | 26.40 ± 5.40 | 12.90 ± 0.55 |
| | 0.06 | 12.44 ± 0.35 | 1.00 ± 0.03 | 26.72 ± 2.24 | **12.83 ± 0.53*** |
| | 0.3 | 12.28 ± 0.62 | 0.98 ± 0.05 | 26.62 ± 2.19 | 13.75 ± 1.10 |
| | 0.6 | 12.66 ± 0.40 | 1.01 ± 0.03 | 25.70 ± 3.01 | 13.25 ± 1.26 |
| | 1.5 | **13.72 ± 0.33***** | **1.13 ± 0.08***** | 29.96 ± 5.48 | 14.83 ± 1.27 |
| 3 | 0.006 | 13.14 ± 1.27 | 1.05 ± 0.10 | 32.26 ± 3.68 | 15.15 ± 3.80 |
| | 0.06 | 13.60 ± 1.83 | 1.09 ± 0.15 | 35.06 ± 6.82 | 14.70 ± 1.56 |
| | 0.3 | 13.12 ± 1.38 | 1.05 ± 0.11 | 35.96 ± 7.46 | 14.18 ± 0.71 |
| | 0.6 | 13.18 ± 0.88 | 1.06 ± 0.07 | 35.20 ± 5.51 | 14.73 ± 1.69 |
| | 1.5 | 13.38 ± 0.81 | 1.07 ± 0.07 | 35.84 ± 5.34 | 14.85 ± 1.56 |
| 4 | 0.006 | 11.98 ± 0.68 | 0.97 ± 0.06 | 28.04 ± 5.05 | 13.93 ± 1.15 |
| | 0.06 | 12.10 ± 0.64 | 0.97 ± 0.05 | 30.22 ± 4.57 | 14.03 ± 1.24 |
| | 0.3 | 12.35 ± 0.86 | 0.99 ± 0.07 | **37.38 ± 2.85*** | 14.23 ± 1.41 |
| | 0.6 | 12.90 ± 0.77 | 1.04 ± 0.07 | 37.18 ± 5.40 | 15.13 ± 1.58 |
| | 0.9 | 13.15 ± 0.55 | 1.09 ± 0.05 | **37.54 ± 2.28*** | 15.22 ± 1.24 |
| | 1.2 | **16.26 ± 1.09*** | **1.38 ± 0.1*** | **37.69 ± 3.15*** | **14.96 ± 0.98*** |
| | 1.5 | **20.80 ± 1.67***** | **1.66 ± 0.13***** | **37.64 ± 4.30*** | **16.30 ± 1.26**** |
| 5 | 0.006 | 12.98 ± 1.15 | 1.04 ± 0.09 | 32.04 ± 2.86 | 15.05 ± 0.95 |
| | 0.06 | 12.74 ± 0.67 | 1.02 ± 0.05 | 32.24 ± 2.19 | 12.80 ± 1.22 |
| | 0.3 | 12.96 ± 0.66 | 1.04 ± 0.06 | 31.10 ± 5.44 | 13.45 ± 1.02 |
| | 0.6 | 13.00 ± 0.51 | 1.04 ± 0.04 | 31.98 ± 5.76 | 12.88 ± 1.32 |
| | 1.5 | **16.86 ± 0.90***** | **1.35 ± 0.07***** | 37.78 ± 10.89 | 16.50 ± 3.81 |
| 6 | 0.006 | 13.03 ± 0.66 | 1.04 ± 0.05 | 31.18 ± 4.57 | 14.78 ± 0.90 |
| | 0.06 | 12.88 ± 1.24 | 1.03 ± 0.10 | 32.76 ± 4.87 | 14.45 ± 1.44 |
| | 0.3 | 13.28 ± 1.31 | 1.06 ± 0.11 | 31.88 ± 5.10 | 13.95 ± 1.21 |
| | 0.6 | 13.15 ± 0.60 | 1.05 ± 0.05 | 33.32 ± 5.12 | 14.00 ± 1.36 |
| | 1.5 | 13.20 ± 0.62 | 1.06 ± 0.05 | 33.40 ± 4.45 | 15.18 ± 2.84 |
| 7 | 0.006 | 12.04 ± 0.80 | 0.96 ± 0.06 | **25.97 ± 2.97*** | 13.45 ± 1.08 |
| | 0.06 | 12.16 ± 0.63 | 0.97 ± 0.05 | **25.93 ± 3.31*** | 13.73 ± 0.85 |
| | 0.3 | 12.68 ± 0.63 | 1.01 ± 0.05 | 32.78 ± 2.49 | 14.23 ± 1.51 |
| | 0.6 | 12.54 ± 0.36 | 1.00 ± 0.03 | 31.86 ± 2.76 | 14.63 ± 1.78 |
| | 0.9 | 12.59 ± 0.39 | 1.02 ± 0.04 | 34.55 ± 2.95 | 14.85 ± 1.21 |
| | 1.2 | **13.28 ± 0.55*** | **1.09 ± 0.12*** | **37.68 ± 2.11*** | **16.54 ± 1.85*** |
| | 1.5 | **14.30 ± 1.70*** | **1.14 ± 0.13*** | **43.96 ± 11.51***** | **17.20 ± 0.96*** |
| 8 | 0.006 | 13.22 ± 0.62 | 1.06 v 0.05 | 26.78 ± 4.36 | 13.23 ± 1.16 |
| | 0.06 | 12.82 ± 0.70 | 1.03 ± 0.06 | 26.60 ± 3.11 | 12.98 ± 1.20 |
| | 0.3 | 13.04 ± 1.15 | 1.04 ± 0.09 | 27.28 ± 4.09 | 12.65 ± 1.68 |
| | 0.6 | **13.60 ± 0.61*** | **1.09 ± 0.05*** | 25.46 ± 5.41 | 12.28 ± 1.48 |
| | 1.5 | **14.22 ± 0.64***** | **1.18 ± 0.12**** | 29.80 ± 5.88 | **12.90 ± 1.15*** |
| 9 | 0.006 | 12.22 ± 0.54 | 0.99 ± 0.06 | 32.18 ± 3.76 | 14.18 ± 1.33 |
| | 0.06 | 12.52 ± 0.94 | 1.00 ± 0.07 | 29.30 ± 3.86 | 14.33 ± 1.86 |
| | 0.3 | 11.84 ± 0.40 | 0.95 ± 0.03 | 28.82 ± 5.27 | 14.40 ± 1.98 |
| | 0.6 | 12.14 ± 0.53 | 0.97 ± 0.04 | 35.36 ± 5.42 | 15.03 ± 1.54 |
| | 0.9 | 12.52 ± 0.65 | 0.99 ± 0.05 | 35.51 ± 4.95 | 15.01 ± 1.28 |
| | 1.2 | **12.96 ± 0.81*** | **1.02 ± 0.07*** | **36.97 ± 2.37*** | 15.32 ± 1.59 |
| | 1.5 | **13.30 ± 1.06**** | **1.06 ± 0.08**** | **38.22 ± 3.18*** | 15.83 ± 1.04 |

The results are presented as mean ± standard deviation, n = 4–6. Values in bold are statistically significant in comparison to control samples. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. The reference values: PT: 9.7–14.6 s; INR: 0.85–1.15; APTT: 26.7–40.0 s; TT: 14.0–18.0 s for 3.0 UNIH/mL of thrombin.

at 1.5 μmol/mL significantly shortened TT (12.90 ± 1.15 s vs. 14.88 ± 1.37 s for control). In the case of other compounds, no significant changes in TT value were reported.

### 3.3. CL-test

Following on from the results of basic coagulation tests, compounds, **1**, **4**, **7** and **9** were examined using the CL-test. This assay allows the *in vitro* effects of compounds on the overall potential of clot formation and fibrinolysis to be estimated ($CL_{AUC}$), together with several kinetic

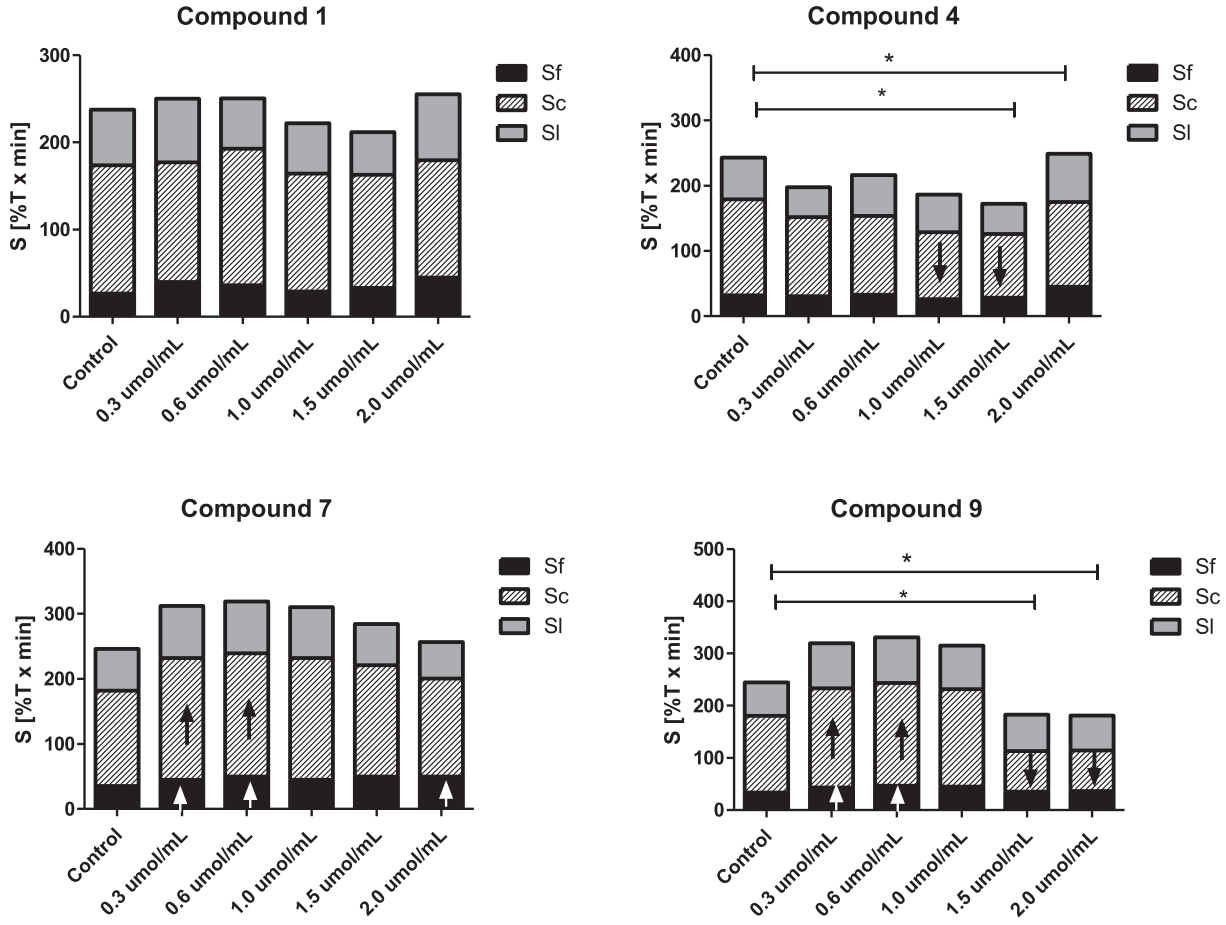



**Fig. 1.** Influence of sulfonamides **1**, **4**, **7**, and **9** on the overall potential of the clot formation and fibrinolysis ($CL_{AUC}$), expressed as area under the clot formation and fibrinolysis curve (S) (data are presented as means, n = 5–8) after 3 min incubation in plasma; final volume 500 µL. S is a sum of Sf – area under the clot formation curve, Sc – area under the curve of stable clot formation, and Sl – area under the fibrinolysis curve. Up and down arrows (black and white) indicate a significant difference in the parameters S for Sc (increase or decrease) in comparison with control. * denotes the significant difference ($p < 0.05$) in $CL_{AUC}$ in plasma treated with compound **4** and **9** and respective controls. In our previous studies [31] metformin over the entire concentration range (0.006–3.0 µmol/mL) did not affect the overall potential of the clot formation and fibrinolysis ($CL_{AUC}$). $CL_{AUC}$ for metformin at the concentration of 3.0 µmol/mL was 397.26 ± 68.82 %T × min versus control 422.01 ± 55.29 %T × min [31].

parameters of clot formation phase, clot stabilisation and fibrinolysis [31].

Compounds **4** and **9,** at a concentration of 1.5 and 2.0 µmol/mL, significantly decreased the overall potential of clot formation and fibrinolysis (↓$CL_{AUC}$, expressed as the area under the curve (S), Fig. 1, Fig. S1, Table S1, Supplementary materials). However, all examined compounds at 1.5 and 2.0 µmol/mL significantly prolonged the total time of clot formation and fibrinolysis (↑T, Table S1, Supplementary materials).

Compounds **1** and **4** at 1.5 and 2.0 µmol/mL, and **7** and **9** at 2.0 µmol/mL induced significant changes in the length of thrombin time (↑Tt); however, the other compounds tended to prolong Tt.

Incubation of human plasma with synthesized sulfonamides (**1**, **4**, **7**, **9**) at 1.0–2.0 µmol/mL was associated with a reduction of maximum clotting (↓Fmax) (Fig. 2), which can be attributed to their effects on the structure of the formed clot. They were also found to lengthen plasma clotting time (↑Tf), which may be due to a decrease in the initial velocity of plasma clotting (↓Fvo). Compounds **7** and **9** demonstrated altered the area under the curve values for plasma clot formation, which may have been associated with changes in the kinetic parameters of clot formation (↑Sf) at lower concentrations. This phenomenon stem from prolonged Tf, and decreased Fvo without affecting Fmax. Compounds **1** and **4** did not induce any change in Sf, and their use in the proposed concentration range was not associated with any increased risk of clot formation.

Compound **1** did not significantly affect the second phase i.e. the clot stabilization phase (constant Tc), however, at 2 µmol/mL it significantly prolonged Tc (Table S1, Supplementary materials). Additionally, no changes were observed in the area under the clot formation (Sc constant). In turn, the other tested compounds contributed to the significant prolongation of clot stabilization time (↑ Tc), which was correlated with an increase in Sc. The value of Sc for compounds **4** and **9** at 1.5 and 2.0 µmol/mL was decreased due to the decrease in Fmax.

The reduction in maximum fibrinolysis (↓ Lmax) observed for compounds **4** and **9** (concentration of 1.5 and 2.0 µmol/mL) occurred as a consequence of the reduced maximum clotting value (↓Fmax), and suggests complete lysis of previously-formed clots. With regard to fibrinolysis time (Tl), statistically significant changes were observed for the highest concentrations (1.5 and 2.0 µmol/mL) of compound **4**, **7** and **9** (↑ Tl). These compounds also contributed to a significant decrease in the initial velocity of fibrinolysis (↓ Lvo). In contrast, compound **1** did not influence the kinetic parameters of fibrinolysis (apart from 2.0 µmol/mL) or the area under the fibrinolysis curve (Sl constant).

### *3.4. Viability of HUVEC and AoSMC cells*

The effects of the compounds **1**–**9** on the viability of two human cell lines, HUVEC and AoSMC, were determined using WST-1 assay. The

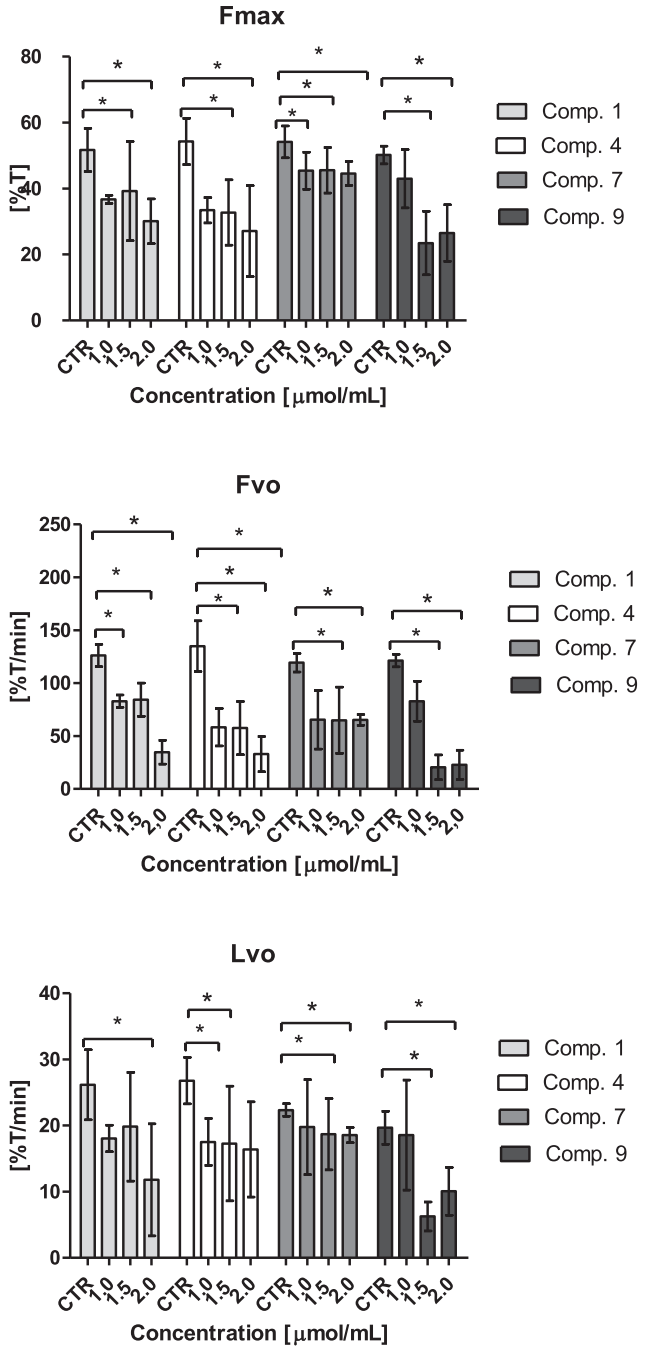



**Fig. 2.** The effects of sulfonamides **1**, **4**, **7**, and **9** on the selected parameters of clot formation and fibrinolysis process: A) maximum clotting (Fmax); B) initial plasma clotting velocity (Fvo); C) initial clot fibrinolysis velocity (Lvo). The results are presented as mean ± standard deviation (SD), n = 5–8. * denotes the significant difference ($p < 0.05$) between sample with tested compound and respective controls. Metformin was not found to affect Fmax (at 3.0 μmol/mL 66.3 ± 10.65 %T vs. control 71.4 ± 3.06 %T, ns), Fvo (at 3.0 μmol/mL 149.87 ± 31.05 %T vs. control 137.53 ± 38.50 %T, ns), and Lvo (at 3.0 μmol/mL 33.23 ± 9.24 %T vs. control 34.95 ± 6.73 %T, ns) in our previous studies [31]. Ns – non-significant, $p > 0.05$.

cells were stimulated with the test compounds at various concentrations ranging from 0.001 μmol/mL to 3.0 μmol/mL. The results are presented in Table 2.

Most compounds exhibited moderate effects on the viability of both HUVECs and AoSMC cells within the entire concentration range, therefore the concentrations inducing a 50% decrease of cell viability were not calculated. However, all tested compounds at 3.0 μmol/mL significantly ($p < 0.001$) decreased cell viability. Among the synthesized compounds derivatives **3** and **6**, i.e. with *p*-chloro and *p*-bromo substituents, appear to exert the most unfavourable effects on cell growth. For instance, compound **6** at the concentration of 3.0 μmol/mL contributed to the decrease of HUVECs viability by approximately 48%. Only in the case of compound **3** were $IC_{50}$ values obtained: compound **3** inhibited the growth of 50% of HUVECs at 1.77 ± 0.12 μmol/mL, and AoSMCs at 1.89 ± 0.11 μmol/mL.

**Table 2**
The effects of sulfonamides **1–9** on HUVEC and AoSMC cells growth. The results express the percentage of cell viability at the compound concentration of 3.0 μmol/mL in comparison to control samples. The results are presented as mean ± SD (n = 6).

| Compound | HUVEC cells [μmol/mL] | AoSMC cells [μmol/mL] |
|---|---|---|
| **CTR** | 100.03 ± 3.16 | 100.00 ± 4.79 |
| **1** | 60.02 ± 3.16 | 60.53 ± 3.00 |
| **2** | 78.66 ± 2.26 | 81.50 ± 1.51 |
| **3** | 24.26 ± 3.73[#] | 28.13 ± 4.17[‡] |
| **4** | 69.01 ± 4.03 | 72.17 ± 3.76 |
| **5** | 61.44 ± 5.88 | 67.20 ± 6.64 |
| **6** | 52.46 ± 2.58 | 65.56 ± 5.67* |
| **7** | 69.26 ± 4.74 | 73.20 ± 3.51 |
| **8** | 69.91 ± 2.90 | 73.27 ± 3.78 |
| **9** | 65.55 ± 5.49 | 82.34 ± 5.36* |

[#] $IC_{50}$ = 1.77 ± 0.12 μmol/mL.
[‡] $IC_{50}$ = 1.89 ± 0.11 μmol/mL.
* Denotes the significant difference between the viability in HUVEC and AoSMC cells ($p < 0.01$) obtained in two-way Anova analysis. Viability of HUVEC and AoSMC cell after 24-h stimulation with metformin at 3.0 μmol/mL was 108.91 ± 6.05%, and 95.34 ± 7.51%, respectively (data not published).

As presented in Table 2, compounds **1–9** exhibited comparable effects on the viability of HUVEC and AoSMC cells. Two-way Anova analysis found that compounds **6** and **9** elicited significantly different cellular responses, with more toxic effects being reported in the case of HUVEC cells.

### 3.5. HUVEC and AoSMC morphology

The effects of compounds **1–9** on HUVEC and AoSMC cell viability were also monitored using light and phase-contrast microscopy. The effects of 2.0 μmol/mL sulfonamide treatment on the morphology of both cell lines are depicted in Fig. 3. The images were taken after 24 h of co-treatment with tested compounds.

In the case of compound **1** and **2,** morphological examination of HUVECs showed slight compound-mediated changes manifested by increased numbers of rounded cells. In contrast, compound **3** contributed to membrane disruption, cell shrinkage and rounding. A similar effect, although to a lesser extent, was also observed for compound **6**. Sulfonamides with fluorine substituents also induced slight changes in morphology in HUVECs. For instance, compound **8** showed a tendency for cell lengthening. In addition, greater numbers of dead cells were observed in comparison with control samples.

Similarly to HUVECs, incubation of the AoSMC cells with compound **1** or **2** did not reveal substantial morphological changes but they decreased the number of viable cells. In turn, compound **3** at 2.0 μmol/mL exhibited an unfavourable effect on cell viability, manifested as cell shrinkage, rounding and clumping. Compounds **4–9** did not contribute to any profound morphological changes in AoSMC cells; however, a substantial decrease in the number of attached cells and an increased number of shrinked and rounded cells were reported.

### 3.6. HUVEC integrity and adherence

The results of stimulation of HUVECs with test compounds at

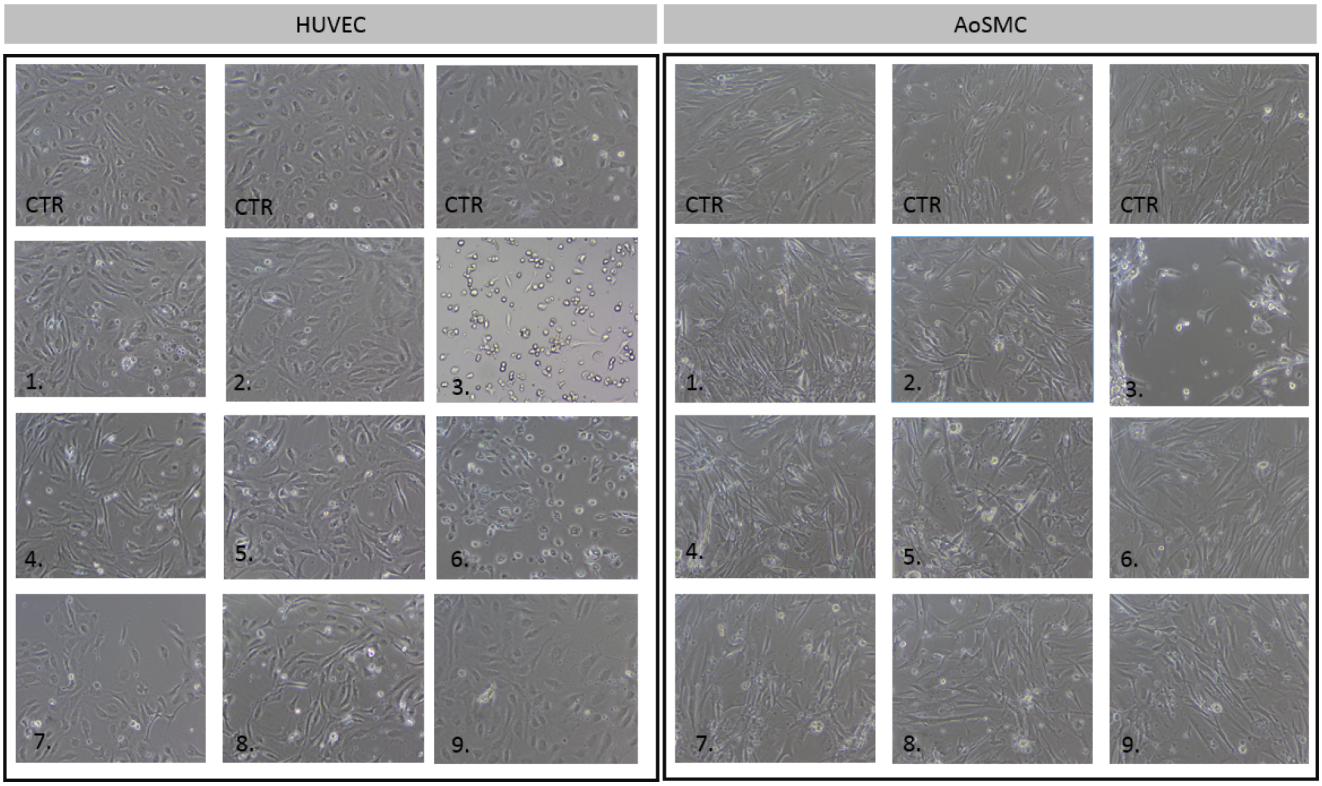



**Fig. 3.** The effect of synthesized compounds **1–9** on endothelial cells (HUVECs) and smooth muscle cells (AoSMC) viability and morphology after 24-h incubation. HUVECs and AoSMCs were cultured without (control, CTR) and in the presence of compounds **1–9** at concentration of 0.006–3.0 μmol/mL. Representative cell images are shown for concentration 2.0 μmol/mL (100-fold magnification). Metformin in our previous study was not found to affect the viability and morphology of HUVEC and AoSMC cells [26].

concentrations of 0.3 and 1.0 μmol/mL for 36 h are presented in Fig. 4 (compounds **1–3**) and Table S2 (compounds **4–9**) (Supporting materials). The value of the normalized cell index (nCI) reflecting the integrity and barrier properties of the cells was affected by the concentration of examined compounds. All compounds at a concentration of 1.0 μmol/mL caused a significantly greater decrease in nCI than 0.3 μmol/mL. Statistical analysis indicates that compound **1**, having a chlorine atom in the aromatic ring in the *orto* position, has the least effect on the function of HUVEC cells: no statistically significant reduction of nCI was observed after 36 h of stimulation. Similar properties were exhibited by compound **3**, for which also did not significantly reduce cell integrity after 36-h incubation. Compound **2**, with the *m*-chlorine substituent did not influence nCI during the first six hours of incubation. The other compounds at both 0.3 and 1.0 μmol/mL significantly reduced the integrity of endothelial cells during 36-h incubation.

### 3.7. Red blood cell lysis assay

The effects of synthesized sulfonamide derivatives of metformin on the integrity of the erythrocyte membrane are presented in Fig. 5. Neither sulfonamides with the fluorine substituent (compounds **7–9**) nor sulfonamides **3** and **5** were found to have any effect on the erythrocyte membrane over the entire concentration range. A statistically significant increase in the rate of haemolysis was observed for compounds **1**, **2**, **4** and **6.** However, the degree of RBC hemolysis accounted for approximately 5%, which is not regarded as clinically important. For instance, compound **6** contributed to hemolysis of 4.53 ± 1.09% of erythrocytes, while the degree of RBC haemolysis after incubation with compound **4** at a concentration of 1.5 μmol/mL reached 5.27 ± 1.75%.

### 3.8. Red blood cell morphology

In plasma at physiological pH 7.4, the erythrocytes were observed in the form of discocytes resembling a two-concave disk. In this configuration, the erythrocytes have a larger surface area, which allows better gas exchange and easier ability to deform during transport through the capillaries. Under the influence of chemical and physical factors, the disks are reversibly transformed into physiological forms: echinocytes or stomatocytes [34].

Morphological evaluation of erythrocytes under the influence of the synthesized compounds was performed using a phase contrast microscope. It was observed that the majority of tested compounds did not contribute to changes in the morphological structure of erythrocytes during incubation (Fig. S1, Supporting materials). The cells tended to form echinocytes was under the influence of compounds **5**, **7** and **9**. During the microscopic analysis, no pathological forms of erythrocytes were observed, which may indicate that the tested derivatives may only have interacted to a small degree with the erythrocyte membrane.

## 4. Discussion

The metabolic abnormalities associated with T2DM have been linked to haemostatic distrubances, including plasma, platelet and vascular haemostasis. Mosty importantly, diabetic subjects present symptoms of hypercoagulability and hypofibrinolysis. As presented by Soares et al. [35] approximately 80% of diabetics die from thrombotic events with 75% to 80% of these deaths resulting from cardiovascular events. Vascular endothelial cells are exposed to the negative effects of hyperglycaemia, which leads to endothelial injury through irreversible glycation of collagen and other subendothelial structural proteins, forming advanced glycation end products (AGEs). These molecules contribute to the changes in structure and properties of the basement

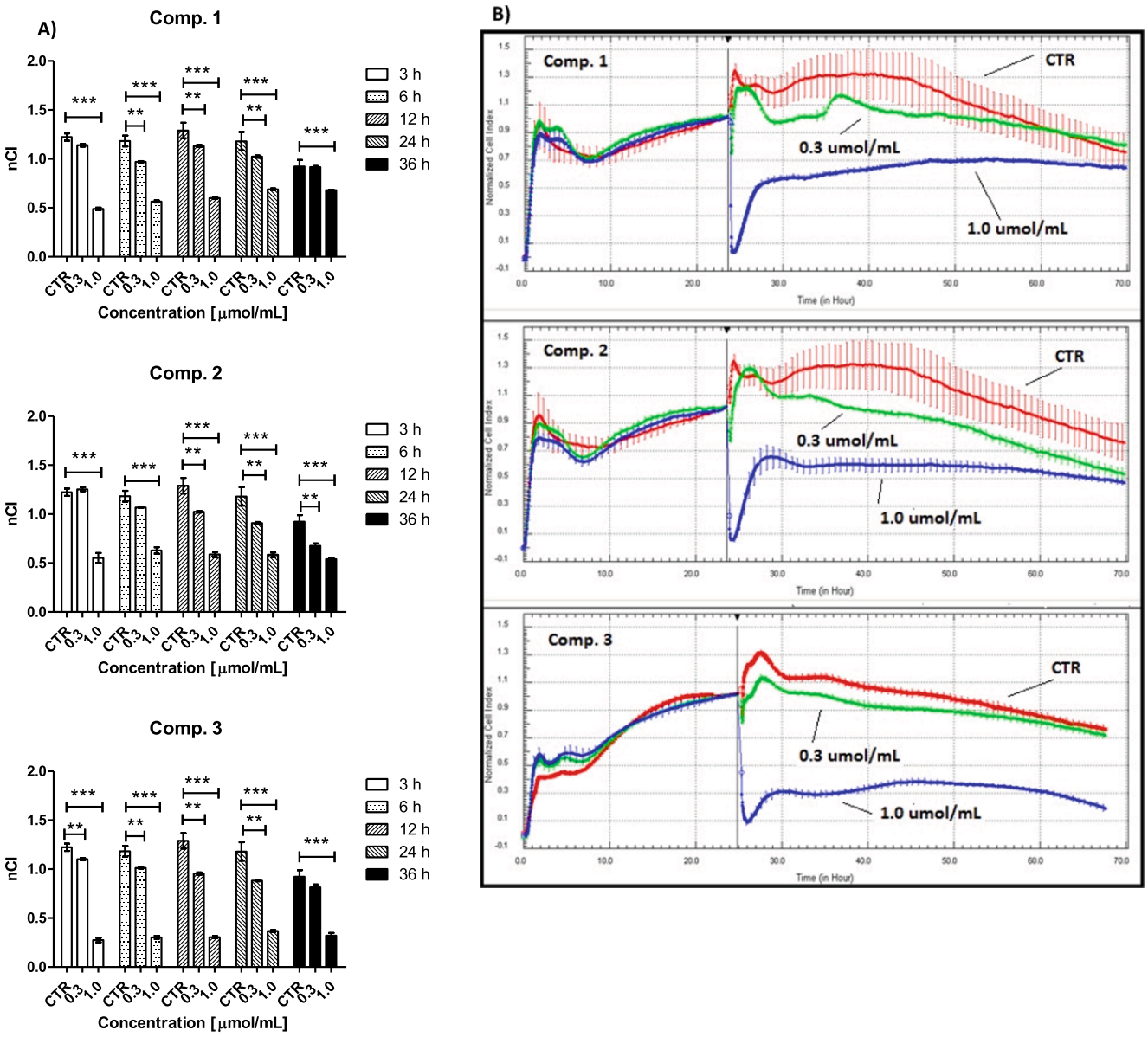



**Fig. 4.** The effect of the exposure of compounds **1–3** on human vascular endothelial cells (HUVECs) measured by RTCA-DP system. A) The effect of the exposure of compounds **1–3** on normalized Cell Index (nCI) at selected time points (3, 6, 12, 24 and 36 h). The results are presented as mean ± SD, n = 5–6. ** $p < 0.01$; *** $p < 0.001$. B) The pictures present representative plots of one experiment conducted in duplicates (the results are presented as a mean (solid line) ± standard deviation). For the statistical analysis there were conducted three independent experiments. Red line – control (unstimulated cells); green line – compounds at the concentration of 0.3 μmol/mL; navy blue line – 1.5 μmol/mL. Metformin was not found to affect the integrity and adherence of HUVEC cells over the entire concentration range (0.006–1.5 μmol/mL) in our previous study [26]. For instance, after 24 h of co-stimulation with metformin at 1.5 μmol/mL nCI was 0.95 ± 0.08 versus control 1.01 ± 0.07) [26].

membrane which ultimately affect the permeability and vasodilation of blood vessels. Additionally, various metabolic abnormalities associated with diabetes such as hypertension, dyslipidemia and hyperinsulinemia may cause endothelial injury resulting in microvascular lesions. Plasma coagulation abnormalities most commonly include increased activity of certain coagulation factors, such as FVII, FVIII and fibrinogen, and hypercoagulability markers, such as thrombin-antithrombin complex (TAT) and fibrinopeptide A (FPA) [35].

Several studies have confirmed that metformin demonstrates multidirectional activity with regard to the cardiovascular system. For instance, metformin was found to decrease endothelial apoptosis, vascular remodelling and fibrosis; it also enhances smooth muscle cell relaxation and possesses anti-oxidative and anti-inflammatory properties [36]. Unfortunately, the pleiotropic properties of metformin are diminished due to the unfavorable pharmacokinetic properties of the drug. Due to its basic biguanidine structure, metformin exists in a protonated form when at physiological pH [37], and in this highly hydrophilic state (logP octanol: water = –2.6) the drug is unable to diffuse pasively through cellular membrane, resulting in moderate and slow intestinal absorption, with only 50–60% bioavailability [13]. This may be due to the fact that metformin is a substrate for several organic cation transporters (OCTs), which determine its oral absorption, distribution and elimination, as well as its biochemical effects in humans. Our previous studies confirm that sulfenamide derivatives of metformin are also actively transported into cells by OCTs [32]. There is hence a need to develop novel approaches to improve the bioavailability of metformin.

One of these strategies is the development of novel pharmaceutical formulation, for example immediate-release (IR) and extended-release (ER) formulations [37]. Metformin ER has a slower absorption rate than

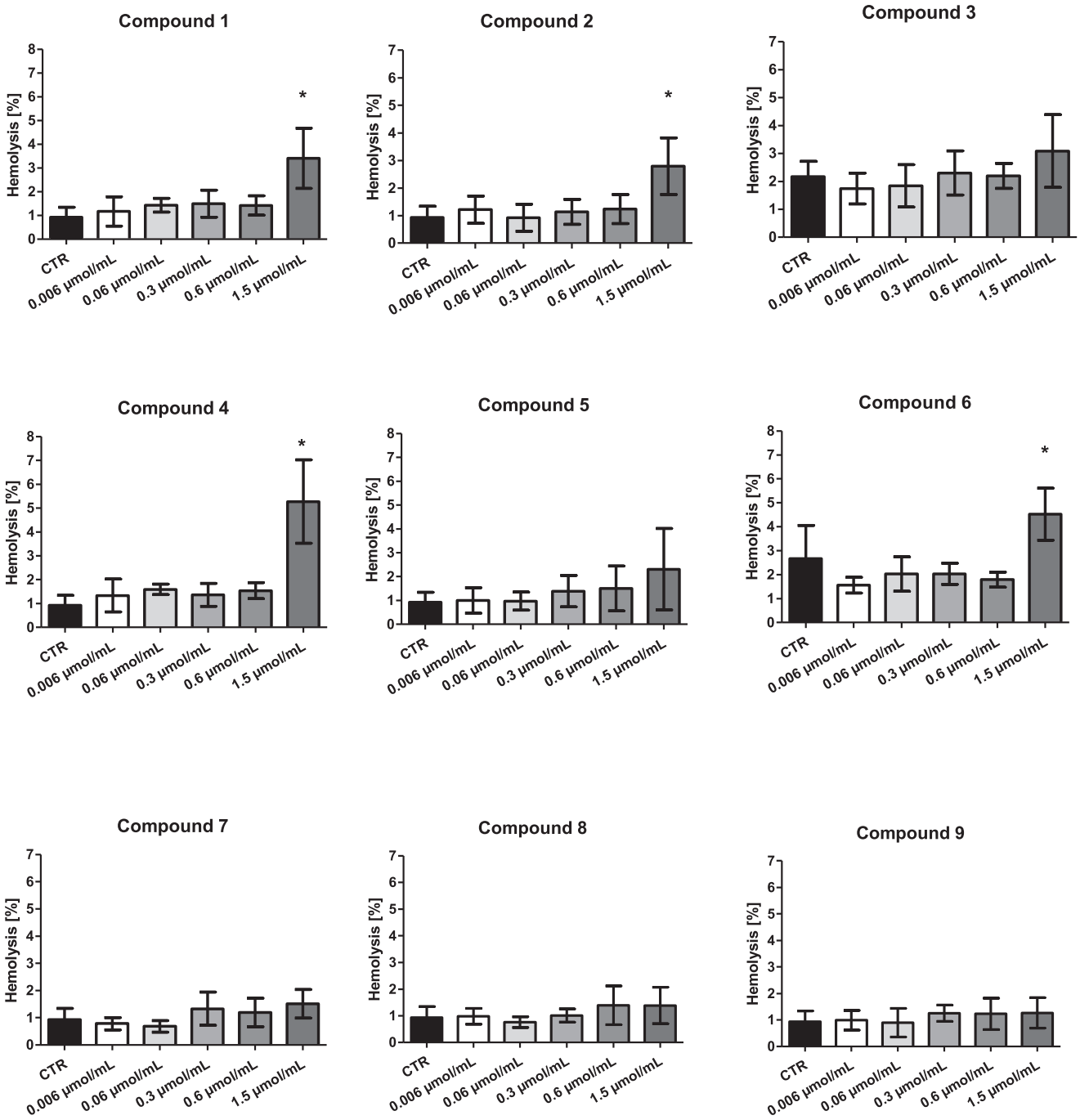



**Fig. 5.** Percentage of hemolysis obtained from the interaction of compounds **1–9** with 2% RBCs suspension, compared to the positive control Triton X-100 at 0.2% (100% haemolysis). The results are presented as mean ± S.D.; n = 4, *$p < 0.05$ vs. control (with 0.9% NaCl). Exposure to compounds **1**, **2**, **4** and **6** at 1.5 μmol/mL contributed to the significant increase in erythrocyte hemolysis. The other compounds were shown not to affect the erythrocytes membrane over the whole concentration range. In our previous studies metformin did not affect the integrity of erythrocyte membrane over the entire concentration (0.006–3.0 μmol/mL) (percentage of hemolysis was 1.81 ± 0.78% for 3.0 μmol/mL versus 1.11 ± 0.24 for control) [31].

metformin IR, reaching a maximum plasma concentration after seven hours. In general, administration of ER formulation is associated with fewer side effects, and has the potential to improve patient adherence with a simpler dosing regimen and increased tolerability [38].

Another way of improving of metformin pharmacokinetic properties is synthesis of novel derivatives or analogues. A review of state-of-the-art literature shows that most of this data concern anti-neoplastic properties. For instance, Koh et al. [39] synthesized a novel metformin derivative HL010183 and a series of metformin salts which exerted more potent inhibitory effects on the proliferation and invasiveness of Hs578T breast carcinoma cells than the parent drug, metformin. In turn, Cheng et al. synthesized a series of metformin analogues containing alkyl chains of various lengths and a triphenylphosphonium (TPP) cation. Metformin modified with a 10-carbon aliphatic chain and $TPP^+$ was approximately 1000- fold more effective than metformin with regard to inhibition of pancreatic ductal adenocarcinoma proliferation [40]. Gutierrez-Lara et al. [41] reported that N-benzylbiguanide, a novel analogue of metformin increases the vasopressor responses to sympathetic stimulation without modifying it to noradrenaline, and is capable of increasing insulin sensitivity. Our

previous studies indicate that modification of metformin into sulfonamides can allow compounds with greater anti-coagulant properties to be obtained [26].

A crucial stage in the process of drug development is the structural optimization of lead compounds. Therefore, we attempted to modify the structure of metformin to synthesize new metformin derivatives with improved anti-coagulation properties. Biguanide-based glucose-lowering drugs such as metformin, phenformin and buformin differ in the number and identity of the substituents on one of the terminal nitrogens; however, the other terminal nitrogen is always unsubstituted. The administration of phenformin and buformin, with alkylated amino groups, have been withdrawn from the pharmaceutical market, due to significant adverse side effects.

In the present study, one of the terminal nitrogen atoms was substituted with two methyl groups, similarly to metformin, while the other was modified with halogenated benzenesulfonamide groups. Thus, nine novel biguanide-based sulfonamides differing in the type and position of halogen atom in aromatic ring were synthesized. This stategy was aimed to establish the optimal functional groups for greater anti-coagulation properties. Although the aim of the present study was not to determine the anti-diabetic properties of the synthesized compounds, this is an important consideration for future studies.

Since nowadays advances are being made in the development of more convenient and more specific drugs, with the aim of substantially increasing their anti-coagulation properties, the first step of our work was to assess the plasma stability of nine sulfonamide metformin derivatives. All synthesized compounds were stable in human plasma for at least two hours. In addition, they did not undergo any metabolic transformation in the liver subcellular fraction.

The plasma haemostasis evaluation included measurements of basic coagulation parameters PT and APTT to evaluate the extrinsic and intrinsic coagulation pathways, respectively. Our previous study [27] showed that sulfonamide with an *o*-nitro substituent presents highly beneficial anti-coagulation properties, manifested by prolonged PT and APTT. Our present findings showed that all synthesized *orto*- derivatives are characterized by prolonged both PT and APTT. In addition compounds **4** (*o*-bromo) and **7** (*o*-fluoro) significantly prolong the process of fibrin polymerization, expressed as increased TT. Among the synthesized compounds, compound **9**, with the *p*-fluoro substituent, also significantly prolonged PT and APTT. Based on these results, and those of a previous study [31] in which metformin was found not to affect APTT and PT, it appears that benzenesulfonamides, especially with *o*-substituents, contribute to a significant slowing of both the extrinsic and intrinsic coagulation pathways, and thus may exert beneficial effects on plasma haemostasis. In the case of TT measurement fibrinogen is converted by thrombin into insoluble threads (fibrin) that crosslink together to form a fibrin net. TT measures the time required for a fibrin clot to form following the addition of a standard amount of thrombin to plasma bypassing the rest of the coagulation factors. Our results suggest that only compounds **4** and **7** affect the activity of fibrynogen expressed by prolonged TT. Collectively, we presume that the synthesized sulfonamides influence extrinsic and/or coagulation pathway (expressed by prolonged PT, and/or APTT) to the greater extent than fibrinogen, and the process of fibrin polymerization.

Considering these results, compounds **1**, **4**, **7** and **9** were chosen for further in-depth studies using a multiparametric CL-test which allows a number of kinetic parameters of clot formation and fibrinolysis to be measured. Regarding the clot formation phase, a significant prolongation of thrombin time (↑Tt) was reported, suggesting that the tested compounds affect the process of fibrin polymerization, and might alter the activity of thrombin. This supposition can be also confirmed by decreased initial clot formation velocity (↓Fvo) in samples treated with examined compounds. The data from the maximum clotting revealed that all tested compounds at the highest concentration contributed to a significant decrease in Fmax (↓Fmax). Due to the fact that the experiments were conducted on a stable FBG level, and the compounds did not lead to any changes in this protein concentration, we presume that tested derivatives influence the structure of the clot. According to Standeven et al. [42] Fmax depends on the diameter of individual fibrin fibres, and thicker fibres are more likely to be associated with lower plasma permeability and a greater difference in the transmittance values, suggesting that sulfonamide derivatives contribute to the formation of thicker fibres, thus decreasing the susceptibility of a previously-formed clot to fibrinolysis. This phenomenon was reported for compounds **7** and **9,** which means that these compounds might delay the process of fibrinolysis and prolong the time elapsed between the initiation of the clotting and complete lysis of the clot. In addition, all compounds apart from compound **1** significantly decreased the initial velocity of fibrinolysis (↓Lvo), and concomitantly prolonged fibrinolysis time (↑Tl). These results are unfortunately adverse when considering the lower lysis rate and resistance to fibrinolysis in diabetic subjects [3].

It should be also mentioned that the incubation of human plasma with examined compounds did not affect the overall potential of clot formation and fibrinolysis ($CL_{AUC}$ constant) apart from the highest concentrations (1.5 μmol/mL), which contribute to a significant decrease in $CL_{AUC}$. This is an important finding, since according to He et al. [43], greater overall haemostasis potential (OHP), which constitutes an equivalent to $CL_{AUC}$, correlates with an increased risk of cardiovascular events. To sum up this part of the study, we presume that the application of tested compounds might be regarded as having no risk associated with plasma haemostasis. In addition, the examined compounds (**1**, **4**, **7**, **9**) decrease the velocity of the clot formation process. While compound **1** appears to be neutral with regard to fibrinolysis, the other compounds decrease the rate of this process.

The next part of our studies evaluated the effects of compounds **1–9** on the function of two human cell lines: endothelial cells (HUVECs), and smooth muscle cells (AoSMCs). The endothelial monolayer plays a pivotal role in the regulation of the haemostatic balance. The function of endothelial cells provides not only a non-thrombogenic inner layer of the vascular wall but also participates in maintaining the balance between pro-coagulant and anti-coagulant factors [44], and prevents thrombosis in several mechanisms. In the case of vascular injury the endothelium is involved in major haemostatic pathways and limits clot formation to the areas where vascular integrity is disturbed. In turn, vascular smooth muscle cells constitute the medial layer of the artery wall, and play a crucial role not only in vasoconstriction and vasodilatation, but also in the pathogenesis of vascular diseases: particularly hypertension and atherosclerosis [33].

The results of viability studies showed that all compounds exert comparable effects on HUVEC and AoSMC cells. It was found that compound **3,** with a *p*-chloro substituent in the aromatic ring, exerted the most unfavourable effects on the viability of both cell lines: the $IC_{50}$ values were 1.77 ± 0.12 μmol/mL for HUVECs and 1.89 ± 0.11 μmol/mL for AoSMCs. All examined compounds were tested up to 3.0 μmol/mL, and the viability of the cells incubated with other compounds at this concentration decreased to 60–70% depending on the compound. Analysis of the cellular viability over the entire concentration range indicates that the tested compounds can undergo further tests up to 1.5 μmol/mL, which can be regarded as a safe concentration. These results appear to be in agreement with studies conducted on sulfonamides with *o*- and *p*-nitro sulfonamides. In the case of HUVECs, the $IC_{50}$ values were 2.742 ± 0.119 μmol/mL for *o*-nitrosulfonamide and 2.407 ± 0.203 μmol/mL for *p*-nitrosulfonamide (unpublished data). The parent drug did not demonstrate any effects on HUVEC or AoSMC viability up to 1.5 μmol/mL. These results were also confirmed by careful microscopic examination of the cells. No significant changes in HUVEC or AoSMC morphology were noticed for most of the tested compounds up to 1.5 μmol/mL, apart from compound **3**, for which membrane disruption, cell shrinkage and rounding were observed.

To further characterize the effects of compounds **1–9** on the

function of HUVECs, studies using the RTCA-DP system were performed. This system allows non-invasive evaluation of the status of adherent cells by continuous measurements of their integrity and adhesion. In contrast to metformin [26], synthesized sulfonamides **1–9** with halogen atoms in aromatic ring significantly decreased HUVEC integrity at a concentration of 1.0 μmol/mL. In the case of previously described *o*- and *p*-nitro sulfonamides, nCI values also decreased profoundly immediately after administration; however, they returned to control values after several hours of co-treatment [26]. This observation might mean that the examined compounds affect the immediate cellular response and adhesion, which is partially aligned during stimulation time. The data of WST-1 experiments and morphology analysis acquired after 24 h incubation indicate that decreased adhesion and integrity manifested by reduced nCI is not associated with profound changes in cellular viability.

As erythrocytes participate in the pathogenesis of microvascular complications of diabetes [3], the next part of the study examined the effects of the synthesized compounds on the integrity of the RBC membrane. Spectrophotometric evaluation of the *in vitro* response of RBCs to various concentrations of synthesized sulfonamides **1–9** revealed a significantly greater rate of haemolysis in comparison with spontaneous controls for compounds **1**, **2**, **4** and **6** at 1.5 μmol/mL; however, the highest percentage of haemolysis reported for compound **4** did not reach 6%. As a hemolysis rate below 10% is not regarded as significant [45], we suggest that these compounds may be safe regarding erythrocytes. The other compounds are unlikely to exert unfavourable effects towards erythrocytes over the whole concentration range. The compounds synthesized for the current paper are characterized by more beneficial effects towards erythrocytes than previously synthesized *o*- and *p*-nitro substituted sulfonamides, which demonstrated approximately 15 and 35% hemolysis rates [27].

The erythrocyte membrane is a protein-lipid structure that separates the inside of erythrocytes from the external environment. The transformation of discocytes to echinocytes, a spherical-shaped blood cell with many small, evenly-spaced thorny projections, can be enhanced by various environmental factors, such as basic pH, the presence of amphipathic compounds that preferentially accumulate in the outer monolayer of the membrane lipid bilayer, lowered ATP levels or excess cholesterol. In the current study the tendency to form echinocytes was observed after incubation with compounds **5**, **7** and **9**. Formation of echinocytes is reversible, therefore we presume that the tested sulfonamides does not interact strongly with the erythrocyte membrane.

The authors are aware that the current paper has a few limitations. We have used experimental *in vitro* models to assess the biological effects of newly synthesized biguanide based compounds on selected parameters of plasma and vascular haemostasis. Some of the results of this study appear to be promising, however, we are not able to transfer them into *in vivo* conditions, because the properties of isolated biological material and primary cells are different from those present in a living organism. In addition, within this paper we did not evaluate glucose-lowering properties of synthesized derivatives. Nevertheless, our ongoing studies focus on the cellular uptake of biguanide-based compounds in HUVECs, and their affinity to Organic Cation Transporters (OCTs), thus offering an insight into their effects on intracellular targets such as AMP kinase (AMPK).

## 5. Conclusions

This paper describes the design and synthesis of nine new sulfonamide derivatives of metformin with halogen substituents. It evaluates the effects of the synthesized compounds on a few markers of plasma haemostasis and endothelial cell function. Furthermore, it also evaluates the influence of sulfonamides **1–9** on erythrocyte membrane integrity.

Our novel findings show that all synthesized *orto*- derivatives (chloro- (**1**), bromo- (**4**), and fluoro- (**7**)) significantly prolong both PT and APTT. In addition, compounds **4** and **7** slow the process of fibrin polymerization, and contribute to increased TT. These compounds were subjected to further, in-depth studies using multiparametric CL-test which allows the measurement of several kinetic parameters of clot formation and fibrinolysis. Compounds **1**, **4**, **7** and **9** were found to significantly prolong the onset of clot formation (↑ Tt), decrease initial clot formation velocity (↓Fvo), and maximum clotting (↓Fmax). Taken together, these data suggest that sulfonamides **1**, **4**, **7** and **9** might decrease the activity of thrombin and affect the clot structure. Combined with those of a previous study [31] in which metformin was found not to affect basic coagulation parameters (eg. APTT, PT, TT) nor the kinetic parameters of clot formation and fibrinolysis (CL-test), our present findindgs suggest that *o*-substituted benzenesulfonamides present advantageous anti-coagulant properties.

Our studies using human endothelial cells (HUVECs) and human aortal smooth muscle cells (AoSMCs) revealed that when applied at the most effective concentration (1.5 μmol/mL), the compounds do not alter the morphology of the cells and maintain cellular viability at approximately 70–80%.

Furthermore, erythrocytotoxicity studies exhibited that none of the synthesized compounds exert an unfavourable effect on erythrocyte integrity, and thus do not interact strongly with the lipid-protein bilayer.

In conclusion, chemical transformation of the metformin scaffold into a benzenesulfonamide containing halogen substituents at the *o*-position leads to the formation of potential agents with stronger anti-coagulant properties than the parent drug, metformin, and can be regarded as an initial promising step in the development of novel biguanide-based compounds with anti-coagulant properties. Particularly, compound **1** (*o*-chlorobenzenesulfonamide) was characterised by advantageous features, including prolonged PT and APTT, and a decreased velocity of plasma clot formation, without affecting the fibrinolysis rate.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103444.

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