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Biocompatible sulfenamide and sulfonamide derivatives of metformin can exert beneficial effects on plasma haemostasis

Magdalena Markowicz-Piasecka[a,*], Kristiina M. Huttunen[b], Elżbieta Mikiciuk-Olasik[c], Joanna Sikora[a]

[a] Laboratory of Bioanalysis, Department of Pharmaceutical Chemistry, Drug Analysis and Radiopharmacy, Medical University of Lodz, ul. Muszyńskiego1, 90-151 Lodz, Poland
[b] School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, Yliopistonranta 1C, POB 1627, 70211 Kuopio, Finland
[c] Department of Pharmaceutical Chemistry, Drug Analysis and Radiopharmacy, Medical University of Lodz, ul. Muszyńskiego 1, 90-151 Lodz, Poland

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ABSTRACT

As the pharmacokinetic properties of metformin are unfavourable, several analogues and prodrugs have been synthesised to improve its bioavailability. The aim of this study was to assess the plasma stability of sulfenamide and sulfonamide derivatives of metformin and establish their effects on plasma haemostasis and integrity of red blood cells (RBCs).

The overall haemostasis potential was evaluated spectrophotometrically by clot formation and lysis test (CL-test). PT (Prothrombin Time) and APTT (Activated Partial Tromboplastin Time) were used to evaluate the effects if the compounds on the extrinsic and intrinsic coagulation pathway. Haemolysis assay, microscopy and flow cytometry studies were conducted to determine the effect of the compounds on RBCs.

Two sulfonamide and one sulfenamide derivatives of metformin were associated with a statistically significant decrease in the overall potential of clot formation and fibrinolysis (↓ CL_{AUC}), suggesting that these compounds may exert beneficial effects regarding plasma haemostasis, which is frequently impaired in diabetic patients. *p*- and *o*-Nitrobenzene sulfonamides contributed to the beneficial change in kinetic parameters of clot formation and fibrinolysis. *o*-Nitrobenzene sulfonamide significantly increased thrombin generation time (↑ TGt) and was also found to prolong both APTT and PT. All compounds did not exert any effects on the integrity of RBCs over the concentration range 0.006–0.6 μmol/mL which constitutes the expected therapeutic concentration.

In conclusion, sulfonamide derivatives of metformin present potentially beneficial properties in terms of plasma haemostasis which is frequently impaired in T2DM patients. Therefore, metformin sulfonamides may become a prototype for further design and synthesis of novel metformin analogues and prodrugs with improved pharmacokinetic properties.

1. Introduction

All newly synthesized drug molecules, various natural and synthetic polymers (cellulose, chitosan, dendrimers etc.) used in biomedical applications have to be tested for their blood compatibility, known also as hemocompatibility [1]. The potential immediate interactions between drug molecules or biomaterials and blood tissue include activation of the clotting cascade, platelet adhesion and activation of the fibrinolytic system to remove fibrin deposits of the thrombus, protein absorption, antibody production and toxicity towards erythrocytes [2,3]. Therefore, the development of hemocompatible drugs is regarded as one of the most challenging problems in the field of pharmaceutical sciences.

The blood coagulation assay is one of the most frequently used tests to evaluate the hemocompatibility of a novel compound or biomaterial [1]. Blood coagulation is a local cascade process whereby soluble plasma proteins become activated in response to vascular injury, leading to the formation of a fibrin clot [3]. Coagulation can be triggered either by surface-mediated reactions (intrinsic pathway), or by exposure to factors derived from damaged tissue (extrinsic pathway). The two processes merge into a common path leading to the formation of a clot [3]. For the evaluation of these two coagulation pathways, prothrombin time (PT) and Activated Partial Thromboplastin Time

* Corresponding author.
E-mail addresses: magdalena.markowicz@umed.lodz.pl (M. Markowicz-Piasecka), kristiina.huttunen@uef.fi (K.M. Huttunen), elzbieta.mikiciuk-olasik@umed.lodz.pl (E. Mikiciuk-Olasik), joanna.sikora@umed.lodz.pl (J. Sikora).

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(APTT) assays are performed routinely. Besides the interaction between a material and plasma proteins, a series of interactions with blood cells might be initiated. Within this field, biocompatibility refers to the quantification of cellular and plasma components of the blood. Generally, hematology is evaluated through hemolysis of red blood cells (RBCs), which is considered a simple and reliable measurement for estimating blood biocompatibility [4].

Metformin is one of the most frequently used oral anti-diabetic drugs. However, it is slowly and incompletely absorbed from the gastrointestinal tract, resulting in low bioavailability and considerable inter- and intra-individual differences in clinical response to the drug [5]. Therefore, several attempts, including metformin analogues as well as prodrugs, have been applied to improve the pharmacokinetic profile of metformin [6–8]. Huttunen's team has synthesized several bio-reversible sulfenyl guanidine (N-S) prodrugs with improved oral absorption in relation to metformin absorption [6–8]. *In vivo* studies of these prodrugs have revealed accumulation into the RBCs and sustained-release of metformin, which subsequently extended the plasma half-life for metformin after oral administration [7]. The prodrug was concluded to be converted to metformin by free thiols in the RBCs, such as reduced glutathione (GSH), which then slowly released metformin back into plasma [7].

Huttunen et al. [8] have also synthesized sulfonamide metformin prodrugs with increased permeability across the Caco-2 cell monolayer [8]. The sulfonamide prodrugs have been demonstrated to be bioconverted to metformin *in vitro* in the liver subcellular fraction, most likely by glutathione-S-transferase (GST) [9], and requiring a highly electrophilic structure in the sulfonyl promoiety (such as strong electron withdrawing groups attached to the ortho- or para-position of the aromatic ring) for efficient bioconversion by GST. Since GSTs are mainly expressed in the hepatocytes, the prodrugs are intended to stay intact in the bloodstream until they are delivered into the hepatocytes. Therefore, it is important to test their biocompatibility *in vitro* before more advanced *in vivo* studies. Some of these sulfenamide prodrugs have previously shown promising results regarding haemostatic potential and toxicity towards erythrocytes [10]. Thus, in the present study, we explored the effects of 2 previously unstudied sulfenamides (N-S-) with varying numbers of carbon atoms in alkyl chain and a series of sulfonamides (N-SO_2-) of metformin (Fig. 1) on selected parameters of plasma haemostasis.

Before commencing these studies the stability of these compounds in plasma was determined. The influence of metformin derivatives on the overall potential of clot formation and fibrinolysis as well as the process of coagulation after the generation of endogenous thrombin was assessed. The study also describes the effect of the compounds on the extrinsic and intrinsic coagulation pathways by determining PT and APTT. In addition, in this study we evaluated the effects of metformin derivatives on RBCs, to which the sulfenamides are known to be accumulated. This may change the physiological redox balance of the RBCs, which as a consequence, may lead to hemoglobin denaturation and hemolysis. Therefore, we decided to establish the effects of metformin derivatives on the integrity of RBCs membrane using RBC lysis assay, microscopy and flow cytometry studies. Finally, on the basis of conducted studies we draw the conclusion which approach sulfenamide or sulfonamide would be better for further metformin prodrug design.

2. Materials and methods

2.1. Materials

Compounds **1–5** (Fig. 1) were designed and synthesised at the University of Eastern Finland as reported elsewhere [9,10]. On the basis of estimated therapeutic plasma concentrations of metformin (0.129–90 mg/L which is 0.8 nmol/mL–0.6 μmol/mL) and our previously conducted studies decided to use metformin and its derivatives in the range of 0.006–1.5 μmol/mL [5,10,11].

For the High Performance Liquid Chromatography (HPLC) analysis, acetonitrile and formic acid were high purity of analytical grade purchased from J.T.Baker (The Netherlands) and Riedel-de Haën (Germany), respectively, and water was purified using a Milli-Q Gradient system (Millipore, Milford, MA).

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 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.

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

2.2. Stability of compunds 1-5

Stabilities of compounds **1–5** were determined in human plasma and Tris-HCl buffer (pH 7.4) at 37 °C. The incubation mixtures were prepared by adding 1 mM stock solution of the studied compound in Tris-HCl buffer (prepared from 5 mM DMSO stock) to plasma in a ratio of 1:10. In blank reactions, plasma was replaced with same volume of isotonic Tris-HCl buffer (the final concentration of compounds was 100 μM and DMSO concentration less than 2%). The mixture was incubated for 2 h and the samples (50 μL) were withdrawn at appropriate intervals. Ice-cold acetonitrile (50 μL) was added to the samples, which were then centrifuged for 5 min at 12000 × *g* at room temperature and kept on ice until the supernatants were analyzed by the HPLC method described below. The stability of compounds in RBCs suspension was conducted at 37 °C for 1 h. Afterwards the samples were centrifuged

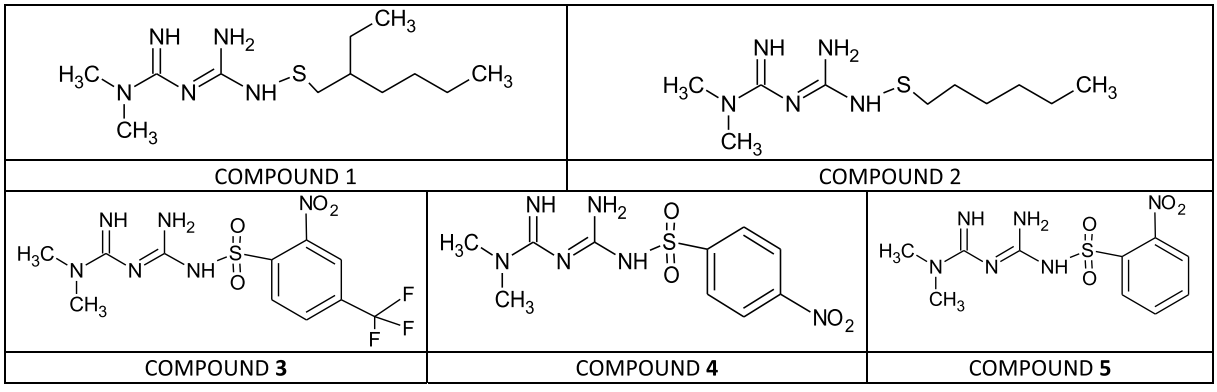

Fig. 1. Chemical structure of tested biguanide derivatives - compounds **1–5**. All compounds were prepared in form of hydrochlorides.