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Sulfenamide derivatives can improve transporter-mediated cellular uptake of metformin and induce cytotoxicity in human breast adenocarcinoma cell lines

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

[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

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ABSTRACT

Metformin, the most frequently administered oral anti-diabetic drug, is a substrate for organic cation transporters (OCTs). This determines not only its pharmacokinetic properties but also its biochemical effects in humans, including its recently-discovered antiproliferative properties. The aim of the study was to verify the hypothesis whether chemical modification of its biguanide backbone may increase the cellular uptake and antiproliferative efficacy of metformin.

The study examines five sulfenamide derivatives of metformin with differing lengths of alkyl chains. It determines their cellular uptake and the role of OCTs in their transport in human breast adenocarcinoma cells (epithelial-like MCF-7, and MDA-MB-231). It also evaluates whether increased cellular uptake of metformin derivatives is associated with their cytotoxic properties.

Sulfenamide derivatives were characterized by a greater ability to bind to OCTs than metformin. Compound **2** with *n*-octyl alkyl chain was found to possess the greatest affinity towards OCTs, as measured by determination of [^{14}C]choline uptake inhibition (IC_{50} = 236.1 ± 1.28 µmol/L, and 217.4 ± 1.33 µmol/L, for MCF-7 and MDA-MB-231 respectively). Sulfenamides were also found to exhibit better cellular uptake in comparison with the parent drug, metformin. For instance, the uptake of cyclohexyl derivative **1** was 1.28 ± 0.19 nmol/min/mg of proteins and thus was 12-fold higher than the metformin in MCF-7 cells. Furthermore, higher uptake was associated with the greatest antiproliferative properties expressed as the lowest IC_{50} value i.e. inhibiting the growth of 50% of the cells (IC_{50} = 0.72 ± 1.31 µmol/L).

Collectively, chemical modification of metformin into sulfenamides with different alkyl substituents obtains better substrates for OCTs, and subsequently higher cellular uptake in MCF-7 and MDA-MB-231 cells. Additionally, the length of alkyl chain introduced to the sulfenamides was found to influence selectivity and transport efficiency via OCT1 compared to other possible transporters, as well as potential intracellular activity and cytotoxicity.

1. Introduction

Metformin is the 'gold standard' and drug of choice to treat patients with Type 2 Diabetes Mellitus (T2DM) [1], with over 120 million people using it worldwide [2]. The major clinical advantages of using metformin includes its low potential for hypoglycaemia induction, combined with its potential for weight loss and amelioration of blood glucose levels with remarkable cardiovascular safety [3]. Metformin use is also associated with decreased cancer risk and improved cancer prognosis [4,5].

Metformin is a highly hydrophilic drug which exists in a positively-charged form under physiological conditions. These unfavourable physicochemical properties contribute to slow and incomplete absorption from the small intestine and unable passive diffusion of the drug through cell membranes [4]. Transport of metformin involves an active uptake process [4]. Numerous studies have shown that metformin is a substrate for several organic cation transporters (OCTs), which determine its oral absorption, distribution and elimination (hepatic uptake and renal excretion) as well as biochemical effects in humans [6,7]. Apart from OCTs, metformin absorption is mediated also by other

* Corresponding author.
E-mail addresses: magdalena.markowicz@umed.lodz.pl (M. Markowicz-Piasecka), kristiina.huttunen@uef.fi (K.M. Huttunen).

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carriers such as the plasma membrane monoamine transporter (PMAT), and multidrug and toxin extrusion 1 and 2 (MATE 1, 2), depending on the tissue [4,8].

OCT transporters (three subtypes OCT 1, OCT2, OCT3) constitute multispecific uptake transporters expressed in numerous epithelia throughout the body, and have crucial functions in the tissue distribution of a wide variety of positively-charged molecules, including exogenous and endogenous compounds exhibiting Km values in the micro- and millimolar range [9,10]. Among these compounds are catecholamines, monoamine neurotransmitters, choline and drugs, including biguanides and antiviral drugs [10,11].

Curiously, most of the available literature on the antiproliferative properties of metformin in cancer cell lines neglects the role of transporters or suggests that a single transporter is responsible for metformin uptake into cancer cells or tissues. However, taking into consideration the number of transporters engaged in cellular uptake of metformin into various tissues it is likely that the presence of transporters, and the interactions between them, may affect the antiproliferative efficacy of metformin by influencing its uptake into tumor cells. These assumptions have recently been confirmed by Cai et al. [12], who have demonstrated that expression levels of cation-selective transporters correlate with the antiproliferative and antitumor efficacy of metformin in breast cancer [12].

Taking these findings into account, the present study attempts to verify the hypothesis that certain chemical modifications to the biguanide backbone may increase cellular uptake and antiproliferative efficacy of metformin. It examines five sulfenamide derivatives of metformin characterized by different lengths of alkyl chains, and displaying varying physico-chemical properties, lipophilicity and plasma stability [13–15]. The paper assesses the uptake of metformin derivatives in two the most commonly-studied human breast cancer cell lines: MCF-7 and MDA-MB-231. These cell lines were selected based on the two main subtypes of breast cancer, namely luminal and basal. MCF-7 cell line is a good representative of cells expressing estrogen receptors (ER), while MDA-MB-231 cells are triple negative. Thus, this is the first comprehensive study to examine how the chemical modification of the metformin backbone can contribute to greater transporter-mediated cellular uptake of metformin. Furthermore, it establishes explicit relationships between the cellular uptake and antiproliferative efficacy of the studied compounds.

2. Materials and methods

2.1. Cell culturing

MCF-7 human breast adenocarcinoma cells (HTB-22) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 human breast adenocarcinoma cells were provided by Sigma Aldrich (European Collection of Authenticated Cell Cultures (ECACC, Public Health England, Salisbury, UK)).

MCF-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, UK) supplemented with L-glutamine (2 mM) (Gibco, UK), heat-inactivated fetal bovine serum (10%) (Gibco, UK), penicillin (50 U/mL), and streptomycin (50 µg/mL) (Gibco, UK) at standard conditions (37 °C, 5% CO_2). MCF-7 cells were seeded at a density of 100,000 cells/well on 24-well plates. The cells were grown for 24 h before the experiments.

For characterization of OCT transporters, the MDA-MB-231 cells were grown in two different conditions. The first one according to the supplier instructions, which were as follows: 37 °C (without additional CO_2) in Leibovitz's L-15 (Sigma Aldrich, Germany) medium containing 2 mM glutamine (Gibco, UK) and supplemented with 10% foetal bovine serum (FBS) (Gibco, UK). The second batch was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, UK) supplemented with L-glutamine (2 mM) (Gibco, UK), heat-inactivated FBS (10%) (Gibco, UK), penicillin (50 U/mL), and streptomycin (50 µg/mL) (Gibco, UK) at 37 °C, 5% CO_2. MDA-MB-231 cells were seeded at a density of 125,000 cells/well on 24-well plates. The cells were grown for 24 h before the uptake experiments.

2.2. OCT1-3 gene expression and function in MCF-7 and MDA-MB-231 cells

The RNA was extracted by using the E.Z.N.A.® Total RNA Kit I (Omega Bio-tek, Norcross, Georgia). The RNA (0.5 µg) was converted into cDNA by using M-MuLV reverse transcriptase (400 U), random hexamers (20 µg) and dNTPs (10 mM) (Fermentas, Hanover, MD, USA). Quantification of the OCT1, OCT2 and OCT3 genes was performed by employing a Prism 7500 sequence detection system (Applied Biosystems, Inc., Foster City, CA, USA). Briefly, 6 µL of each sample was mixed with 10 µL of PCR reagent mixture containing 0.5 µL of primer probe mix (TaqMan Gene Expression assay, Applied Biosystems), 0.5 µL of sterile water and 5 µL of TaqMan master mix (Applied Biosystems). Transporter gene expression was determined by real-time polymerase chain reaction (RT-PCR) and normalized to endogenous cyclophilin A. The used primer probe mixes were Hs00427552_m1 (OCT1, *SLC22A1*), Hs01010726_m1 (OCT2, *SLC22A2*) and Hs01009571_m1 (OCT3, *SLC22A3*).

For characterization of OCT transporters the cell lines were grown for 24 h on 24-well plates. After removal of the culture medium, the cells were carefully washed with pre-warmed HBSS (Hanks' balanced salt solution) containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.6 mM glucose, and 25 mM HEPES (pH 7.4). The cells were then pre-incubated in 500 µL of prewarmed HBSS at 37 °C for 15 min before adding radiolabelled [^{14}C]choline (PerkinElmer, US) (250 µL in HBSS) for the uptake experiment.

For the concentration dependency characterization, choline chloride was used at concentrations of 10–400 µmol/L together with [^{14}C]choline at 18.1 or 9.05 µmol/L for MCF-7 and MDA-MB-231 cells respectively. The uptake time was 10 min. The concentration dependent uptake of [^{14}C]choline was calculated from the standard curve prepared by spiking known amounts of [^{14}C]choline to the cell lysate. Time-dependent radiolabelled choline uptake was conducted between two and 240 min for MCF-7 cells and 5 min–24 h for MDA-MB-231 cells.

The cells were washed three times with ice-cold HBSS (500 µL). The cells were then lysed with 250 µL of 0.1 M NaOH on the ice bath, and the lysate was mixed with 1.0 mL of emulsifier safe cocktail (PerkinElmer, Waltham, MA, USA). The radioactivity was measured by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland).

Function of different OCT transporters (OCT 1–3) was checked using three known OCT inhibitors: disopyramide (Sigma Aldrich, Germany), naringin (Sigma Aldrich, Germany) and cimetidine (Sigma Aldrich, Germany). The protocol was the same as described above, the cells were incubated with 250 µL of uptake medium (HBSS) containing 18.1 µmol/L [^{14}C]choline (MCF-7 cells) or 9.05 µmol/L [^{14}C]choline (MDA-MB-231 cells) and OCT inhibitors at concentrations of 10–800 µmol/L.

2.3. Tested compounds

Sulfenamide derivatives tested within the paper are presented in Fig. 1. The synthesis and basic properties were described earlier [13–15].

2.4. Inhibition of [^{14}C]choline uptake by metformin and its derivatives

The ability of metformin and its derivatives to inhibit the uptake of a OCT substrate, [^{14}C]choline, was examined as described above. MCF-7 and MDA-MB-231 cells were incubated at 37 °C for 10 min with 250 µL of uptake medium (HBSS) containing 18.1 µmol/L [^{14}C]choline (MCF-7 cells) or 9.05 µmol/L [^{14}C]choline (MDA-MB-231 cells) and 10–2000 µmol/L metformin or its derivatives. Afterwards, the cells

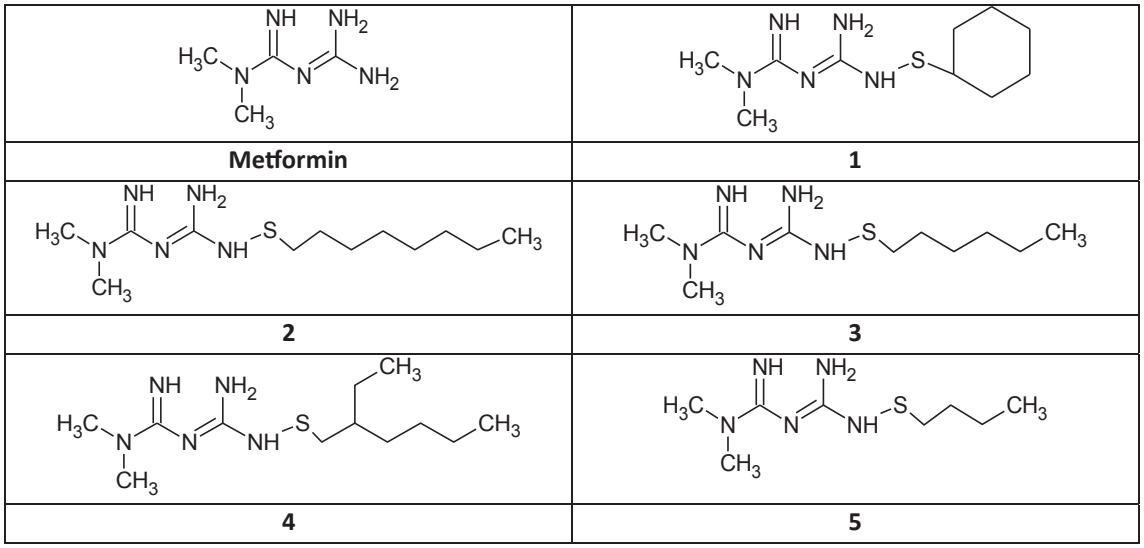

Fig. 1. Chemical structure of tested biguanide derivatives – metformin and its sulfenamide derivatives.

were washed three times with ice-cold HBSS and then lysed with 0.1 M NaOH and analyzed as described above. The studies were conducted at minimum of three replicates from the same cell passage.

2.5. Uptake studies

Before commencing uptake studies, the stability of tested compounds was determined in 0.1 mol/L NaOH. The incubation mixtures were prepared by adding studied compounds to NaOH to achieve a concentration of 100 μmol/L. The mixture was incubated for two hours at room temperature, centrifuged for 5 min (12,000g) and the samples (150 μL) were collected. The samples were kept on ice until HPLC analysis (described below).

The transport of metformin and its derivatives in MCF-7 and MDA-MB-231 cells was studied by the addition of 10–2000 μmol/L of compounds in 250 μL of pre-warmed HBSS buffer on the top of the cell layer and incubating the solution at 37 °C for 10 min. Subsequently, the cells were then washed three times with 500 μL of ice-cold HBSS and lysed with 250 μL of 0.1 M NaOH. The samples were collected, centrifuged (5 min, 1400 rpm) and the supernatants were analyzed by HPLC method. The concentration of each compound in the cells was calculated from the standard curve that was prepared by spiking the cell layer with known amounts of each compound in 250 μL of 0.1 M NaOH.

The concentrations of the examined compounds were determined by HPLC, consisting of an Agilent 1100 binary pump (Agilent Technologies Inc., Wilmington, DE), a 1100 micro vacuum degasser, an HP 1050 Autosampler, an HP 1050 variable wavelength detector (operated at 235 nm). The chromatographic separations of compounds **2**, **3** and **4** were conducted on an Agilent Zorbax SB-C18 analytical column (4.6 mm × 150 mm, 5 μm) by using isocratic elution with acetonitrile (containing 0.1% (v/v) formic acid) and water (with 0.1% (v/v) formic acid, pH 3.0) with a changing ratio depending on the compounds (from 75:25 to 40:60) (v/v) at a flow rate of 1.0 mL/min at room temperature. The analysis of metformin, butyl sulfenamide (**5**) and cyclohexyl sulfenamide (**1**) was conducted by using a Supelco Supercosil LC-Si analytical column (4.6 mm × 250 mm, 5 μm) (Supleco, Inc. Bellefonte, PA) with a mobile phase of acetonitrile and water (with 0.1% (v/v) phosphoric acid at pH = 5) with a ratio of 70:30 at a flow rate of 1.9 mL/min at room temperature. The studies were conducted in quadruplicate.

The HPLC evaluation of biguanide uptake was linear within the range 1–20 μmol/L, specific (no interfering peaks), accurate (QC, i.e. quality control, samples were within the range 90–110%) and precise: RSD% (relative standard deviation) for LLOQ (lower limit of quantification) < 20% and for other QQ samples < 15%.

The protein concentrations on each plate were assessed using Bio-Rad protein assay according to Bradford (EnVision, PerkinElmer, Inc., Waltham, MA, USA).

2.6. Uptake of metformin derivatives in the presence of OCT inhibitors

The uptake of metformin and its derivatives in MCF-7 and MDA-MB-231 cells was studied also in the presence of disopyramide and cimetidine (OCT inhibitors). The cells were incubated (37 °C, 10 min) with metformin or its derivatives (100, 200 and 400 μmol/L) and disopyramide (400 μmol/L) or cimetidine (400 μmol/L) in 250 μL of pre-warmed HBSS buffer. Further stages of research were the same as described above. The studies were conducted in quadruplicates from the same cell passage.

2.7. Cell viability assay

The cell viability was determined using WST-1 assay (Takara, Takara Bio Europe, Saint-Germain-en-Laye, France). The test enables cell proliferation and cell viability to be measured with a colorimetric assay, based on cleavage of tetrazolium salts by mitochondrial dehydrogenase in viable cells. The amount of soluble formazan dye formed directly correlates to the number of live cells. The experiment was conducted using 96-well plates and MCF-7 and MDA-MB-231 cells seeded at the density of 7500 cells per well. The cells were cultured for 48 h to obtain 80% confluency, following by addition of compounds at the concentration ranges 1–3000 μmol/L. The cells were incubated with tested compounds or pure medium (control) for 24 h (37 °C, 5% CO_2). Afterwards, the cells were washed with culture medium (100 μL) and the reagent dissolved in medium was added (100 μL). The plates were incubated under standard conditions for one hour and the absorbance was read at 450 nm using a microplate reader (iMARK, Bio-Rad). The cell viability was expressed as a percentage of the control samples which constituted 100% viability. The data were presented as mean ± standard deviation (SD), n = 8. IC_{50} values (the concentration of tested compound that inhibited cell growth by 50%) were calculated using concentration-response curve (GraphPad Prism).

2.8. Cell morphology

The cells (MCF-7 and MDA-MB-231) were seeded at the density of 20,000 per well on 48-well plates and allowed to reach 70%

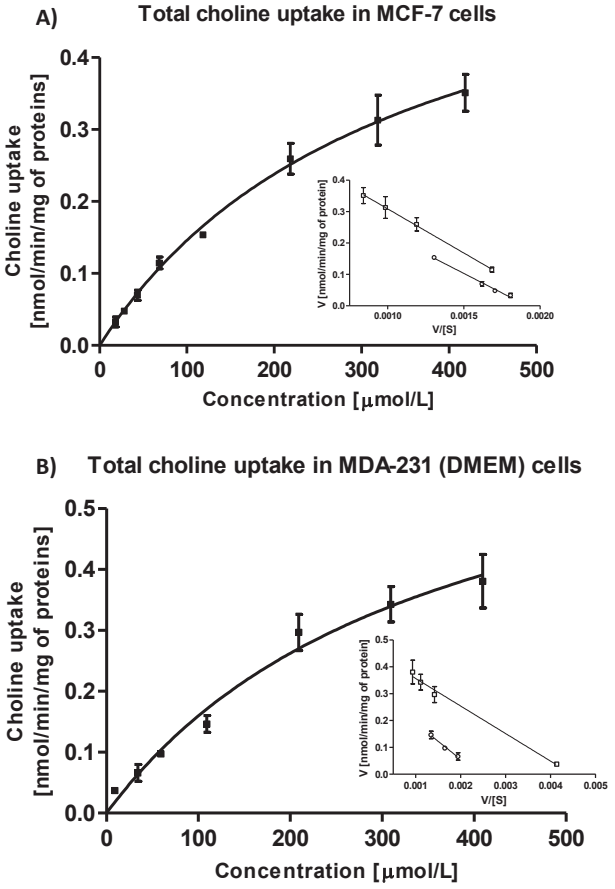

Fig. 2. Total choline uptake (unlabeled + [^{14}C] choline] in the presence of increasing choline concentration in MCF-7 cells (A), and MDA-MB-231 cells cultured in DMEM (B) medium. Choline chloride was used at the concentrations of 10–400 µmol/L, while [^{14}C]-choline at 18.1 µmol/L for MCF-7 and 9.05 µmol/L in MDA-MB-231. The uptake time was 10 min. The curves were analyzed by non-linear regression. Eadie-Hofstee analysis (inserts) revealed 2 transporters engaged in choline transport into the studied cells.

confluency. Then the medium (control) or medium with tested compounds at appropriate concentrations was added. The cells were incubated with compounds for 24 h. Cell morphology was examined after 10 min and then 24 h after the introduction of compounds using an inverted microscope with phase contrast (Opta-Tech, software OptaView 7).

2.9. Cell apoptosis assay

The cells were seeded at a density of 50,000 per well on a 24-well plate and incubated for 48 h (37 °C, 5% CO_2) to reach 70% confluency. Then the medium (control) or medium with tested compound in a volume of 250 µL was added and incubated for the next 24 h. The cells were trypsinized, collected to Eppendorf tubes, and centrifuged (1100 rpm, 5 min). Afterwards, the cells were resuspended in cold cell staining buffer (Biolegend, United Kingdom), centrifuged once again, and the cells pellets were suspended in 100 µL of binding buffer. The cell suspensions were mixed with propidine iodide (PI) and FITC-Annexin solutions (FITC Annexin V Apoptosis Detection Kit with PI: Biolegend, United Kingdom). The analysis was conducted on a FACS Canto II cytometer (Becton Dickinson).

Annexin V(−) and PI (−) cells were considered as living cells, Annexin V (+) and PI (−) as early-apoptotic cells, Annexin V(+) and PI (+) as late-apoptotic cells, and Annexin V (−) and PI (+) as necrotic cells. The experiments were conducted in triplicate (n = 3). The coefficients of variation for the assay were found to range from 1.42 to 5.84%, depending on the measured parameter.

2.10. Data analysis

All statistical calculations were performed using the commercially-available Prism 5 package (GraphPad, San Diego, USA). The results are presented as the mean ± standard deviation (SD) for variables with a normal distribution of values. Statistical differences between groups were tested using one-way ANOVA, followed by a subsequent *post hoc* test (Dunnett's or Tukey's test). The results of all the tests were considered significant at *p*-values lower than 0.05.

Half of maximum inhibitory concentration (IC_{50}) values were calculated by nonlinear regression analysis (fitting the curve to log (concentration) vs. cellular response).

3. Results and discussion

3.1. Expression and function of OCTs in MCF-7 and MDA-MB-231 cells

The expression of OCTs in MCF-7 and MDA-MB-231 cells was determined using RT-PCR (Fig. S1, Supplementary materials). The studies revealed that while the predominant transporter in MDA-MB-231 cells was OCT3, no OCT transporters were identified in MCF-7. Similar results were obtained by Cai et al. [12] who found type OCT3 to be the predominant OCT transporter in MDA-MB-231 cells: relative transporter expression normalized to 18 s rRNA was ca. 0.00100, while no OCT gene expression was detected in the case of MCF-7. However, it should also be kept in mind that this assay only measures the transporter gene expression levels at the given time point, and does not give any information about functional transporter on the cell surface. The results of the gene expression experiments are reflected by the levels of protein identified by Cai et al. [12]. Western blot analysis showed also a faint line for OCT1 in MDA-MB-231 cells. On this basis, it can be stated that our RNA expression experiments confirmed that the cells that we were using were in line to previous literature [12].

The function of OCTs in MCF-7 and MDA-MB-231 cells was assessed using radiolabeled [^{14}C]choline. The uptake of choline into cells is facilitated by various transporters, among them are (i) the high-affinity choline transporter family (CHT), (ii) the family of OCTs, and (iii) the choline transporter–like family (CTL). Increased choline uptake was reported in several human breast cancer cells, including MCF-7 and MDA-MB-231 cells. Choline was chosen for the present study, as were cell lines MCF-7 and MDA-MB-231, and its uptake in these cell lines has already been studied, with v_{max} of saturable transport in these both cell line being approximately 20 nmol/mg protein [16]. On the basis of this knowledge we decided to characterize the function of OCTs in these cell line. Choline uptake was measured as a function of time. Based on the obtained results (Fig. S2, Supplementary materials) the incubation time for studies with metformin and its derivatives was chosen to be 10 min.

Choline uptake in MCF-7 and MDA-MB-231 cells was also characterized according to increasing concentration (Fig. 2). The concentration-dependent uptake of [^{14}C]choline was calculated from the standard curve that was prepared by spiking the cell lysate with known amounts of [^{14}C]choline. The Eadie-Hofstee analysis of the choline uptake curves showed that in all experiments, two transporters are engaged in choline transport into the MCF-7 and MDA-MB-231 cells (Fig. 2, Fig. S3). Kinetic parameters of choline uptake in MDA-MB-231 cells cultured in L15 and DMEM media appear to be comparable (Table 1). Therefore, for easier comparison of results with MCF-7 cells, and to eliminate the influence of culturing conditions on the obtained results, the MDA-MB-231 cells were cultured in DMEM medium and with 5% CO_2 in further studies with metformin and its sulfenamide derivatives.

[^{14}C]choline uptake in MCF-7 and MDA-MB-231 cells was also determined in the presence of OCTs inhibitors: disopyramide (the highest

Table 1
Comparison of kinetic parameters of Eadie-Hofstee graphs for the choline uptake in studied cell lines.

Transporter	MCF-7		MDA in L15		MDA in DMEM	
	Km [μmol/L]	Vmax [nmol/min/mg of proteins]	Km [μmol/L]	Vmax [nmol/min/mg of proteins]	Km [μmol/L]	Vmax [nmol/min/mg of proteins]
I	248.8 ± 9.96	0.4761 ± 0.02	131.1 ± 18.32	0.4267 ± 0.04	135.1 ± 15.54	0.325 ± 0.02
Vmax/Km	0.00191		0.00325		0.00240	
II	281.6 ± 19.94	0.5902 ± 0.02	114.0 ± 12.98	0.4882 ± 0.03	102.7 ± 6.68	0.4594 ± 0.01
Vmax/Km	0.00209		0.00428		0.00447	

affinity for OCT1), naringin (OCT2) and cimetidine (OCT3) [10,17]. The influence of these inhibitors at concentrations of 200–800 μmol/L on the [^{14}C]choline uptake in MCF-7, and MDA-MB-231 is depicted in Fig. S4 (Supplementary materials). In both cell lines, a significant decrease in [^{14}C]choline uptake was reported in the presence of all inhibitors. Therefore, it can be concluded that OCTs are present in both cell lines. However, taking into account that the inhibitors are not specific, it cannot be excluded that other transporters such as PMAT and MATE1 may facilitate the uptake of choline into the cells. Although these transporters are considered as efflux transporters, they can function bi-directionally [12].

Available data suggests that variability exists in metformin transporter expression profiles among several breast cancer cell lines. Therefore, we may expect that the cells within breast cancer tissues are also likely to show heterogeneity in metformin transporter expression [12] which may limit the antiproliferative effects of metformin. Based on this finding, the next stage of the study examined whether the chemical modification of metformin structure can result in stronger affinity towards OCTs and higher cellular uptake irrespective of OCT expression, and how it affects the biological response of cells.

3.2. Inhibition of [^{14}C]choline uptake by metformin and its derivatives

The ability of metformin and its derivatives to bind to OCT1-3 transporters was determined by means of a competitive inhibition assay with natural substrate for OCT, [^{14}C]choline. In the first step of the studies [^{14}C]choline uptake in MCF-7 and MDA-MB-231 cells was examined in the presence of metformin. The maximal inhibition of [^{14}C]choline uptake was reported for the highest concentration of metformin (2400 μmol/L), and constituted 34.3% for MCF-7 cells and 29.9% for MDA-MB-231 cells cultured in DMEM (Fig. S5, Supplementary materials). As the inhibition of [^{14}C]choline uptake was similar in MDA-MB-231 cells cultured in both media (L15 and DMEM) all other studies were conducted in DMEM to provide the same conditions (including CO_2) for MCF-7 and MDA-MB-231 growth.

The ability of metformin derivatives to bind to OCT1-3 transporters is presented in Table 2 and is expressed as the half maximal inhibitory concentration (IC_{50}). Sulfenamide derivatives were characterized by a greater ability to bind to OCTs than metformin (Fig. S5, Supplementary materials). The maximal inhibition of [^{14}C]choline uptake for metformin was 34.3% and 29.9% for MCF-7 and MDA-MB-231 cells respectively, therefore the exact IC_{50} values could not be calculated (> 2.4 mmol/L). The half maximal inhibitory concentration value for derivative **2**, with an octyl alkyl chain, was 236.1 ± 1.28 μmol/L (estimated by nonlinear regression analysis from results at seven concentrations between 50 and 1200 μmol/L). This indicates that the compound has the highest affinity for OCTs as well as the potential to inhibit choline uptake in MCF-7 cells. Similar properties were found for this derivative in MDA-MB-231 cell line, in which 50% of [^{14}C]choline transport was inhibited at 217.4 ± 1.33 μmol/L. Cyclohexyl derivative **1** was characterized by moderate OCT binding properties, but stronger towards transporters in MDA-MB-231 cells. Compound **3** with a hexyl alkyl chain has higher affinity towards OCTs in MDA-MB-231 cells whereas branched chain derivative **4** presents slightly higher affinity towards these transporters in MCF-7 cells. Of the tested sulfenamides compound **5**, with an *n*-butyl alkyl chain, showed the lowest affinity towards OCTs in MCF-7 cells.

Table 2
The effectiveness of metformin derivatives on inhibition of [^{14}C] choline uptake in MCF-7 and MDA-MB-231 cells. The results (IC_{50} values, μmol/L) are presented as mean ± SD (n = 3).

Derivative	MCF-7 cells [μmol/L]	MDA-MB-231 cells [μmol/L]
1	676.2 ± 1.31	417.1 ± 1.34
2	236.1 ± 1.28	217.4 ± 1.33
3	950.6 ± 1.36	740.0 ± 3.63
4	825.7 ± 2.39	885.6 ± 4.39
5	992.0 ± 2.83	781.5 ± 2.76

3.3. Cellular uptake of metformin derivatives

All the studied compounds were stable in 0.1 mol/L NaOH (92.17–99.32% compounds left after two-hour incubation at room temperature), which was the most harsh condition that was used in the following studies and therefore tested before these studies. The detailed results of stability studies in NaOH are presented in Table S1 (Supplementary materials).

To evaluate whether metformin derivatives were transported into MCF-7 and MDA-MB-231 cells or only bound to the cell surface, their concentration-dependent uptake was examined with the corresponding cell lines. Fig. 3 presents the uptake of metformin and its sulfenamide derivatives at a concentration of 800 μmol/L. As seen in Fig. 3, the greatest uptake in MCF-7 cells was reported for cyclohexyl and *n*-butyl derivatives, which reached over 1.2 nmol/min/mg of protein at concentration of 800 μmol/L, which is approximately 11-times higher than the reference drug, metformin. Similarly, in the case of MDA-MB-231 cells, the highest uptake was reported for cyclohexyl derivative (0.956 ± 0.065 nmol/min/mg of proteins). The results of metformin uptake in MCF-7 and MDA-MB-231 cells (0.11 ± 0.006 and 0.12 ± 0.010 nmol/min/mg of proteins, respectively) are mostly in agreement with the results obtained by other authors [12,18]. Some

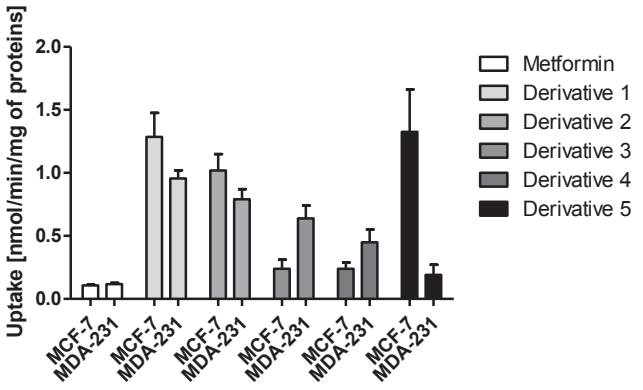

Fig. 3. The uptake of metformin and sulfenamides into MCF-7 and MDA-MB-231 cells at 800 μmol/L concentration after 10 min incubation at 37 °C.

authors report an approximately 10-fold greater metformin uptake in various cell lines, but these results concern OCT transfected cells [19,20].

Cyclohexyl derivative (**1**) was characterized by moderate affinity to OCTs in both cell lines but higher affinity in MDA-MB-231 (Table 2). This might suggest that derivative **1** in MCF-7 is transported with the aid of other transporters than OCTs. According to the data in Table 2, the *n*-butyl derivative demonstrated the lowest affinity towards OCTs in MCF-7 cells (**5**); however, it was also readily taken up by MCF-7 cells. This may mean that derivative **5** may also use another transport mechanism than OCTs, which is present in MCF-7 but not in MDA-MB-231 cells which use PMAT [12]. PMAT is a recently-discovered polyspecific organic cation transporter that transports a variety of biogenic amines and xenobiotic cations, including metformin. Wang [21] suggests that many of the OCT substrate drugs are likely to be PMAT substrates; therefore, it is possible that derivative **5** might be transported into MCF-7 by PMAT.

However, it should be noticed that in many cases, OCTs work in conjunction with MATE (multidrug and toxin extrusion) transporters to mediate the uptake or elimination of an array of structurally diverse molecules including drugs, toxins and endogenous compounds [18]. MATE transporters are responsible for the efflux of organic cations from cells [22]. Two MATE substrates of particular interest are metformin and phenformin. Hence, it is possible that the differences observed in the uptake of metformin derivatives in these both cell lines might stem from their various affinity to MATEs.

The derivative with *n*-octyl alkyl chain (**2**) was characterized by high affinity towards OCTs in both cell lines, as expressed by the lowest IC_{50} value (Table 2) and an approximately 10-fold higher uptake than metformin. Derivatives **3** and **4** with the *n*-hexyl and 2-(ethyl)hexyl chains demonstrate higher calculated IC_{50} values and lower uptake than derivatives **1** and **2**, suggesting they are not good OCT substrates.

In the first stage of analysis, the relationship between the concentration of the test compound and its uptake in cells was established; the obtained curves were then transformed into Eadie-Hofstee plots, and analyzed to calculate Km and Vmax values. The summary of obtained results is presented in Table 3.

Metformin uptake was found to be slightly better in MDA-MB-231 than MCF-7. Eadie-Hofstee analysis of metformin uptake showed that both cell lines two transporters engaged in cellular uptake of the drug in both cell lines (Fig. 4). Based on Nies et al. [23], it can be concluded that at lower concentrations, metformin is carried into the cells by OCT1, while at higher concentrations OCT3 is used.

Our present findings indicate that the Vmax and related Km values are higher for the MDA-MB-231 cell line than MCF-7 (Table 3), indicating that at lower concentrations uptake of metformin is more efficient in MDA-MB-231 cells than in MCF-7. In contrast, metformin uptake was greater in the MCF-7 cells at higher concentrations, as the Vmax was higher (0.7692 vs. 0.489 nmol/min/mg of proteins). The reported differences in metformin uptake stem from the shape of uptake curves: MDA-MB-231 being more logarithmic and MCF-7 curve being more linear. Hence the transport of metformin saturates faster in MDA-MB-231 cells than in MCF-7.

The participation of OCTs in biguanide uptake was determined using OCT inhibitors. Metformin uptake in the presence of disopyramide, a preferential OCT1 inhibitor, is presented in Fig. 5. Both cell lines demonstrated significantly lower uptake of metformin at 100 μmol/L in the presence of disopyramide, indicating that OCTs, presumably OCT1, participate in the transport of metformin at lower concentrations. At higher concentrations, another transporter not inhibited by disopyramide is probably used. The presence of cimetidine did not appear to have any significant influence on uptake (0.053 ± 0.011 vs. 0.068 ± 0.021 nmol/min/mg of proteins).

Our findings represent the first comparison of the uptake of metformin and its sulfenamide derivatives in two unmodified human breast carcinoma cell lines, MCF-7 and MDA-MB-231. Many previous studies have examined metformin uptake in various cell lines transfected with rOCT1 [19,24], hOCT2 [7] or hOCT3 [12]. Following on from Cai et al. [12], it is possible that an increase in the uptake of metformin derived compounds may translate into greater potency, such as an enhanced antiproliferative effect.

The uptake of cyclohexyl sulfenamide (**1**) was found to be over 12-fold and 8-fold higher than metformin in MCF-7 and MDA-MB-231 cells, respectively. Compound **1** was taken up slightly more readily in

Table 3
Eadie-Hofstee analysis of the uptake of metformin derivatives in MCF-7 and MDA-MB-231 cells (Km and Vmax values).

		Kinetic parameters of derivatives uptake			
		MCF-7 cells		MDA-MB-231 cells	
Derivative	Transp.	Km [μmol/L]	Vmax [nmol/min/mg]	Km [μmol/L]	Vmax [nmol/min/mg]
Metformin	I	136.3 ± 15.17	0.065 ± 0.003	467.7 ± 49.1	0.212 ± 0.01
	Vmax/Km	0.0005		0.0004	
	II	4760.0 ± 987.3	0.769 ± 0.13	2432.0 ± 325.2	0.489 ± 0.05
	Vmax/Km	0.0002		0.0002	
1	I	738.9 ± 44.86	2.436 ± 0.11	2445.0 ± 224.7	3.054 ± 0.31
	Vmax/Km	0.0033		0.0016	
	II	874.1 ± 144.1	2.398 ± 0.30	1946.0 ± 148.1	3.264 ± 0.18
	Vmax/Km	0.0027		0.0017	
2	I	467.81 ± 36.44	1.155 ± 0.09	1211.94 ± 56.6	1.927 ± 0.09
	Vmax/Km	0.0025		0.0016	
	II	1642.86 ± 154.16	3.197 ± 0.30	3092.17 ± 321.7	3.556 ± 0.37
	Vmax/Km	0.0019		0.0012	
3	I	2036.0 ± 203.0	1.167 ± 0.09	1775 ± 215.8	2.041 ± 0.17
	Vmax/Km	0.0006		0.0012	
	II	1365.0 ± 208.6	0.652 ± 0.07	1082.0 ± 297.6	0.977 ± 0.20
	Vmax/Km	0.0005		0.0009	
4	I	1799.0 ± 127.4	0.859 ± 0.05	195.5 ± 29.2	0.382 ± 0.03
	Vmax/Km	0.0005		0.0019	
	II	1154.0 ± 243.8	0.532 ± 0.08	435.1 ± 53.3	0.693 ± 0.04
	Vmax/Km	0.0005		0.0016	
5	I	871.6 ± 278.7	1.985 ± 0.56	2230.0 ± 449.3	0.671 ± 0.10
	Vmax/Km	0.0023		0.0003	
	II	3308.0 ± 646.7	7.857 ± 1.19	3551.0 ± 796.1	0.774 ± 0.15
	Vmax/Km	0.0024		0.0002	

MCF-7 than in MDA-MB-231 cells (Fig. 2). The Eadie-Hofstee analysis of cyclohexyl sulfenamide uptake (Fig. 4) found two transporters to be engaged its cellular uptake in both cell lines. According to the Vmax/Km ratios, both transporters appeared to be more efficient in the MCF-7 cells than in MDA-MB-231 cells; derivative **1** also possessed higher affinity towards the transporters in MCF-7 cells as the respective Km values were lower (Table 3). In turn, in MDA-MB-231 cells, derivative **1** was characterized by a higher Vmax value but a higher Km value, making these cells less efficient than MCF-7. Both transporters had higher capacity and lower affinity for **1** in MDA-MB-231 cells, while in MCF-7 they had higher affinity and possessed lower capacity. Their high capacity suggests that MCF-7 cells saturate more quickly than in MDA-MB-231 cells, as transporters such as OCT3 start to transport derivative **1** at lower concentrations. In both cell lines, **1** has equal affinity for both transporters, as indicated by Km values, and therefore is not selective. The uptake of **1** in the presence of disopyramide and cimetidine is presented on Fig. 6 (6A and 6B, respectively). No statistically significant changes in the uptake of **1** in the presence of both OCT inhibitors was reported between MCF-7 and MDA-MB-231 cells, which suggests that some other transporter than OCTs is a predominant carrier in its cellular uptake. This phenomenon might be explained by the fact that the compound has affinity for both OCT1 and OCT3: when

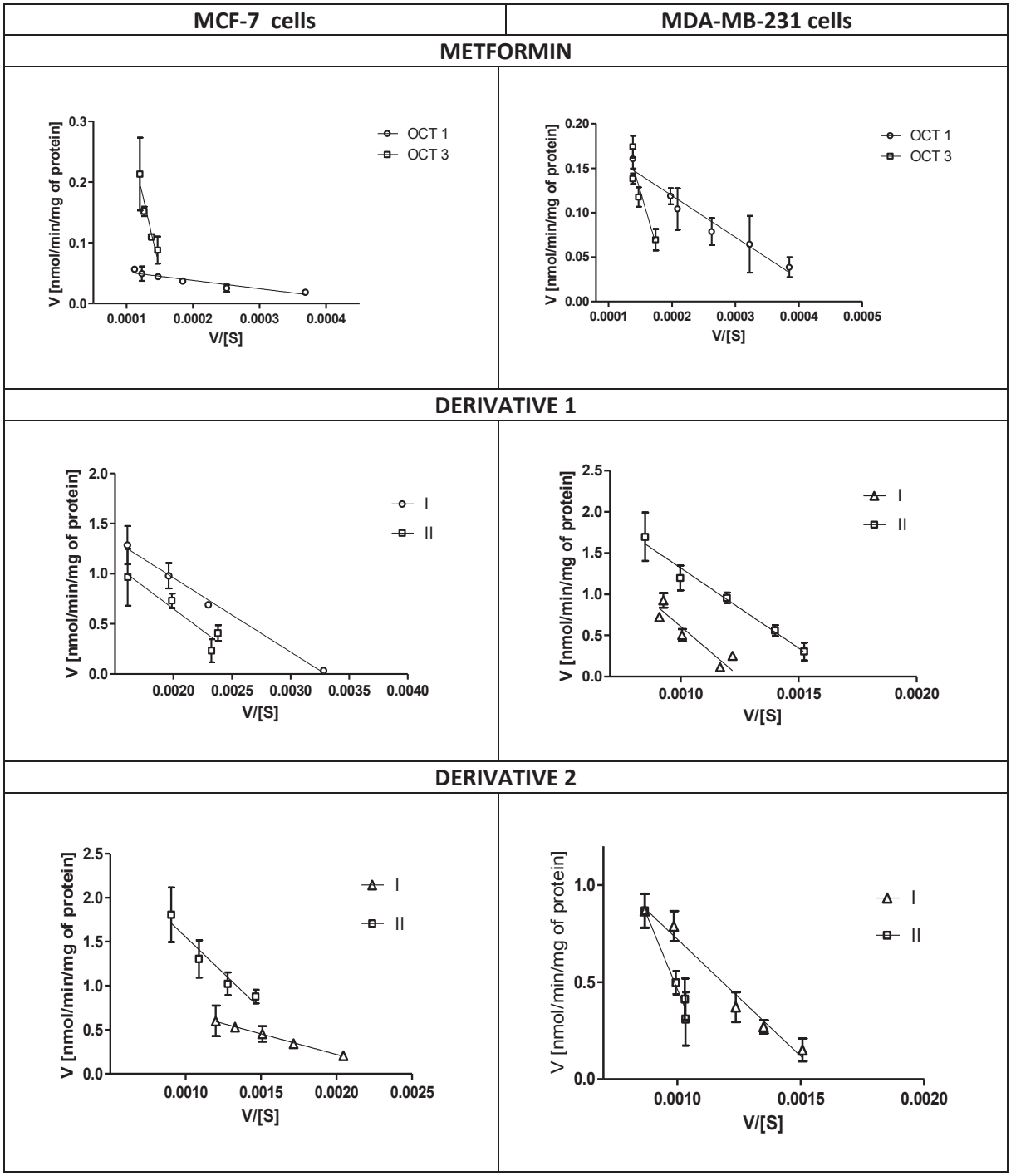

Fig. 4. Eadie–Hofstee plots for OCTs mediated transport of metformin and derivatives 1–5 at the concentration of 10–2000 μmol/L in MCF-7 cells and MDA-MB-231 cells.

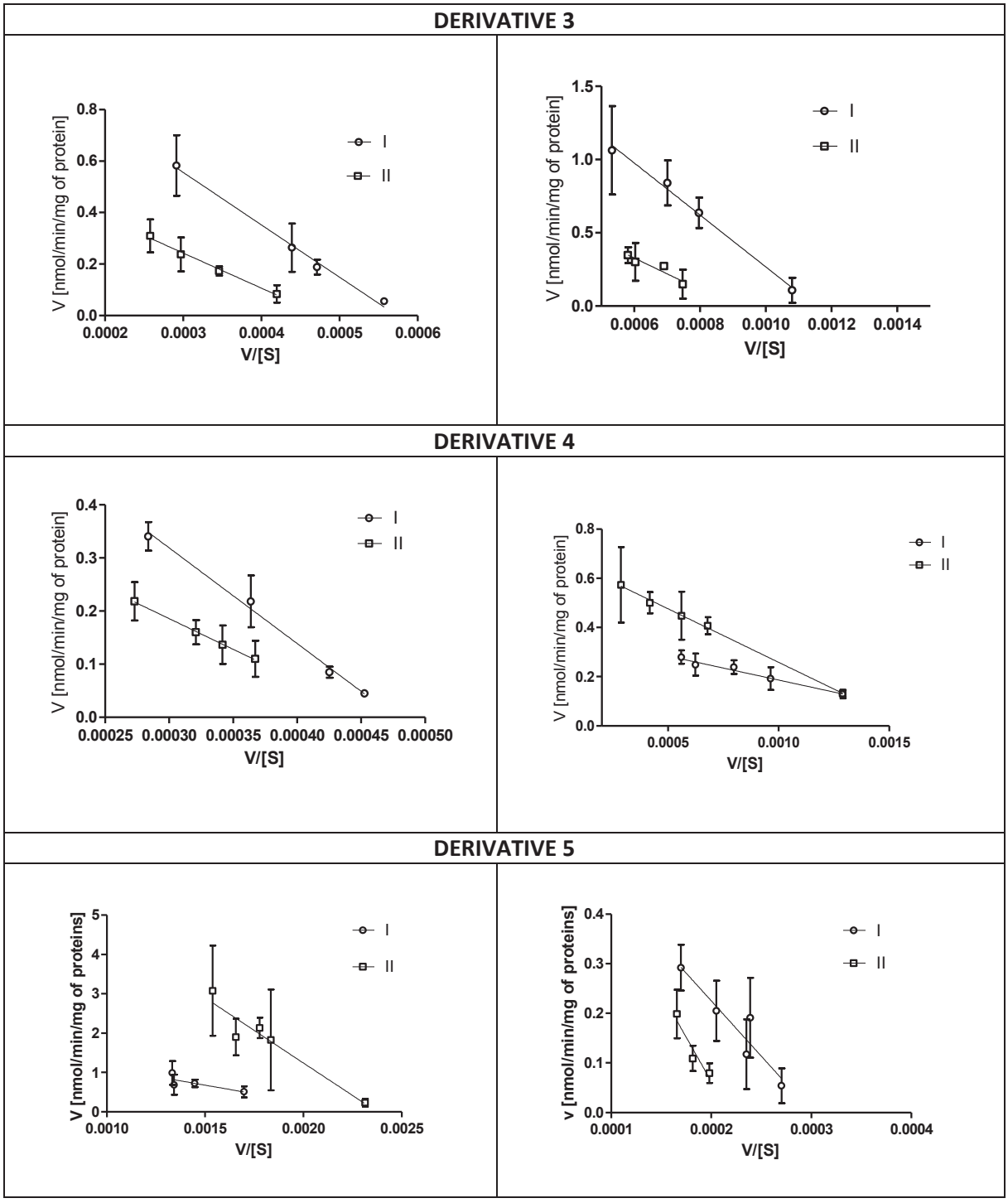

Fig. 4. (*continued*)

one is inhibited, the other compound can bind to another one, which can transport it with higher capacity.

Uptake studies showed that *n*-octyl sulfenamide (**2**) was transported more efficiently than metformin in both cell lines. Its uptake is slightly better in MCF-7 than MDA-MB-231 cells (Fig. 2).

Eadie-Hofstee analysis of *n*-octyl sulfenamide uptake is presented in Fig. 4, and shows two transporters were engaged in cellular uptake in both cell lines. Both transporters appeared to be more efficient in MCF-7 cells than in MDA-MB-231 cells, and derivative **2** possessed higher affinity towards the transporters in MCF-7 cells, indicated by lower respective Km values (Table 3). Km value was high for **2** in MCF-7, so it is likely that similarly to metformin, **2** is more selective for OCT1 than other transporters in MFC-7. Comparing the uptake profile of derivatives **1** and **2** in both cell lines, we may conclude that the octyl tail favours OCT1-selectivity, while the cyclohexyl tail does not.

The uptake of compound **2** was also studied in the presence of OCT inhibitors (Fig. 6). In the case of MCF-7 cells, derivative **2** displayed significantly lower uptake in the presence of disopyramide at all concentrations tested (100–400 μmol/mL), while in the case of MDA-MB-231, a significant decrease was observed only for the highest concentration (400 μmol/L) (Fig. S6, Supporting materials). In the presence of cimetidine (Fig. 6B) compound **2** uptake was also significantly

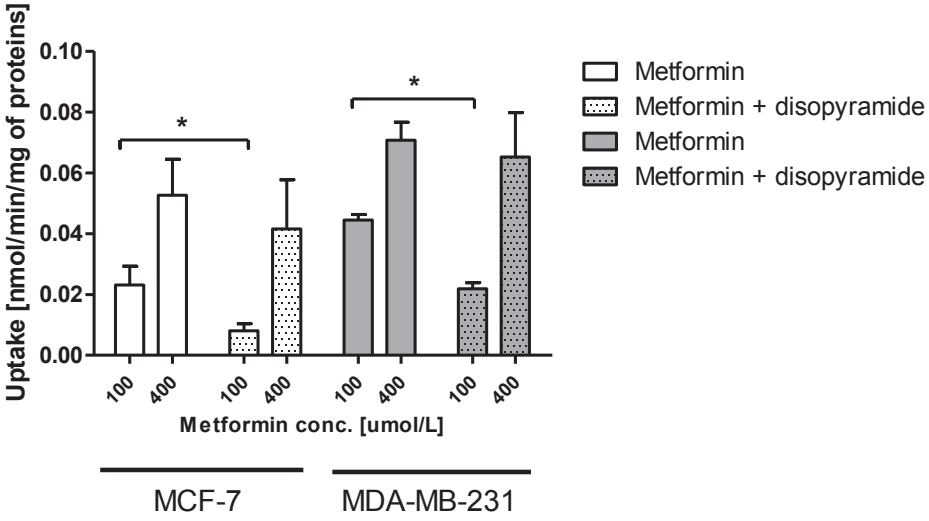

Fig. 5. The uptake mechanism of metformin (100; 400 μmol/L) into MCF-7 cells and MDA-MB-231 cells. The uptake was determined in the presence of OCT1 inhibitor, disopyramide at 37 °C. One-way Anova analysis revealed significant changes between the uptake of metformin at.

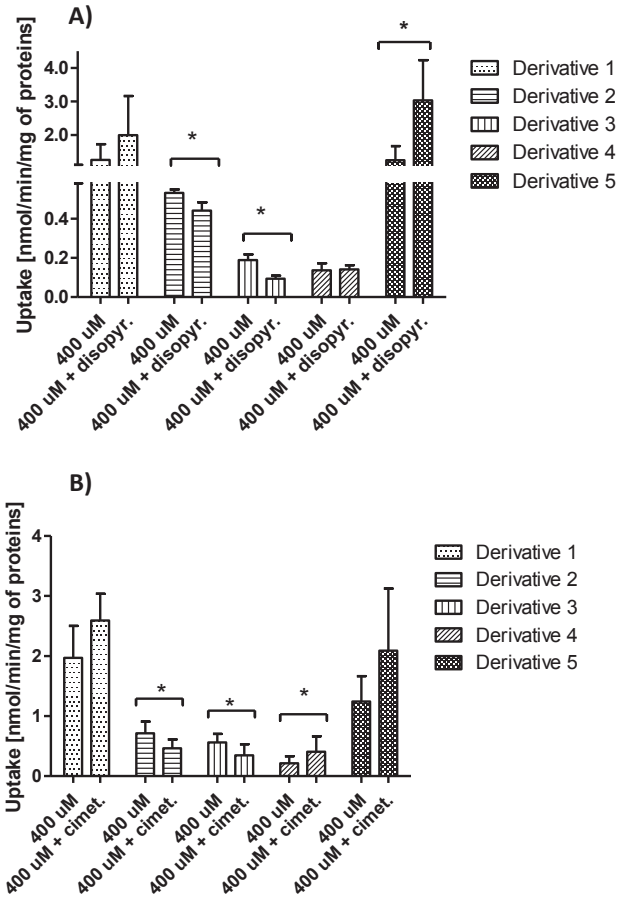

Fig. 6. The uptake mechanism of derivatives 1–4 (400 μmol/L) into MCF-7 cells in the presence of (a) disopyramide and (b) cimetidine, at 37 °C. One-way Anova analysis revealed significant changes in the uptake of compound 2 and 3 at concentration of 400 μmol/L in the presence of disopyramide in comparison with respective control, and compounds 2, 3 and 4 in the presence of cimetidine (* indicates $p < 0.05$).

lowered. These results suggest that in MCF-7 cells, OCT1 performs most of the uptake but OCT3 may also participate. In the case of MDA-MB-231 cells, the other transporter is the main carrier at lower concentrations, and OCT1 is also engaged at 400 μmol/mL.

Derivatives **3** and **4** were characterized by moderate uptake; however, this uptake was still better than metformin. Both derivatives demonstrated better uptake in the MDA-MB-231 cells (Fig. 2).

Eadie-Hofstee analysis of the *n*-hexyl and the branched *n*-hexyl derivatives showed that two transporters were engaged in their uptake in both cell lines (Table 3, Fig. 4). Both transporters appeared to be more efficient in MDA-MB-231 cells than in MCF-7 cells, and both possessed higher affinity towards the transporters in MDA-MB-231 cells as indicated by lower Km values and higher Vmax values (Table 3).

It can be concluded that the *n*-hexyl derivative (**3**) is not a good substrate for either of the recognized transporters in either cell line (Table 3). Similarly to the *n*-hexyl derivative, the 2-(ethyl)-hexyl derivative (**4**), also favoured the other transport mechanism in MCF-7 cells; however, both capacities were low. These findings and the relatively low capacities of both transporters in both cell lines suggest that compound **4** is also not a good substrate for OCTs.

The uptake of compounds **3** and **4** in the presence of disopyramide is presented on Fig. 6. A Compound **3** uptake was significantly different when administered at a concentration of 400 μmol/L with and without disopyramide and cimetidine in MCF-7 cells; this suggests that OCTs are involved in the transport of derivative **3** into MCF-7 cells (Fig. 6A and 6B). No statistically significant changes were observed in the uptake of derivative **3** in the presence of disopyramide (MDA-MB-231 cells), however, lower cellular uptake was observed. In the case of derivative **4,** two-way Anova analysis revealed a significant change between the uptake of derivative with and without disopyramide at concentrations of 100 and 400 μmol/L in MDA-MB-231 cells (Fig. S6, Supporting materials). This observation suggests that OCT, probably OCT1 is involved in the transport of derivative **4** into MDA-MB-231 cells. Conversely, no statistically significant changes in the uptake of derivative **4** in the presence of disopyramide (MCF-7 cells) were obtained.

Butyl sulfenamide (**5**) was taken up into MCF-7 cells approximately 12 fold more readily than metformin (1.325 ± 0.338 vs. 0.11 ± 0.01 nmol/min/mg of proteins). In contrast, its uptake in MDA-MB-231 cell was much lower (Fig. 2) which might be explained by the fact that transport efficiency (Vmax/Km) in MDA-MB-231 is approximately seven-times lower. This may suggest that derivative **5** uptake uses a different transport mechanism, such as PMAT or MATE1, which is only present in MCF-7 cells [12].

Eadie-Hofstee analysis found two transporters to be engaged in cellular uptake of *n*-butyl sulfenamide in both cell lines (Fig. 4). Both transporters were more efficient (Vmax/Km ratios) in the MCF-7 cells than in the MDA-MB-231 cells, and derivative **5** possessed higher

affinity towards the transporters in MCF-7 cells, as indicated by lower respective Km values (Table 3). Derivative **5** seemed to have poor affinity for both transporters, and therefore low transport capacity, in MDA-MB-231 cells; however, due to its relatively low affinity for OCTs, it was difficult to state whether the OCTs were involved in its transport in MCF-7 cells (Table 2). Two transport mechanisms were observed also for the *n*-butyl derivative in MCF-7, one being high affinity-low capacity and the other low affinity-high capacity (Table 3). These findings indicate that the "addition" of a butyl tail to the metformin backbone contributed to a substantial decrease in affinity for OCTs and a subsequent transfer to different transporters. This was confirmed by experiments with OCT inhibitors (Fig. 6 A and B), as a significant increase in cellular uptake of *n*-butyl derivative was observed in the presence of 400 µmol/L of disopyramide and cimetidin. The uptake of *n*-butyl sulfenamide in the presence of the OCT inhibitors, disopyramide and cimetidin, was increased because they redirected compound **5** from OCTs to other transporters. This phenomenon was also observed in the case of LAT1 transporters (L-type amino acid transporter) [25].

In summary, chemical transformation of metformin into sulfenamides differing in the length of alkyl chain or the presence of a saturated hexyl ring contributed to the obtaining of better substrates for OCTs and related transporters, and higher cellular uptake in MCF-7 and MDA-MB-231 cells. The longer alkyl chain introduced to the sulfenamides resulted in higher selectivity for OCT1 over other transporters and greater transport efficacy. Although additional experiments using OCTs transfected cell lines would be desirable to study the direct role of OCTs in the uptake of metformin and its derivatives, this would be outside the scope of the present study, whose ultimate goal was to identify biguanide-derived compounds that could display greater antiproliferative activity through improved cellular OCT uptake.

3.4. Cell viability assay

The effects of the metformin sulfenamide derivatives on the viability of MCF-7 and MDA-MB-231 cells were analyzed using WST-1 assay, which predominantly reflects mitochondrial function. Metformin was found not to significantly affect cell growth up to a concentration of 3 mmol/L (Fig. S7, Supplementary materials). Further analysis showed that metformin induced similar response patterns in both cell lines. Broad ranges of concentrations were used to verify whether the effects of metformin were restricted to clinically-relevant concentrations or to higher or experimental concentrations. It was found that at lower concentrations, metformin only demonstrated weak cytotoxic properties, or none at all.

A review of current literature brings ambiguous conclusions: some papers report metformin to have antiproliferative properties [26,27], while others do not; for example, Queiroz et al. [28] found 10 mmol/L metformin for 24 h to display significant antiproliferative activity. These discrepancies might stem from variations in experimental conditions, incubation time and methods of application. In addition, researchers may not estimate OCT expression in studied cell lines; this is an important issue as metformin uptake has been found to be over 13-fold higher in OCT3-BT20 cells compared to BT20 cells (BT-20 cell line): the former differing only in greater expression of OCT 3 transporters [12]. Similar conclusions were drawn by Checkley et al. [29] who found that intratumoral metformin accumulation was highly correlated with OCT2 positivity, and responsive tumors had significantly greater OCT2 protein expression. Our results seem to confirm these findings, as the highest concentration of metformin contributed to a weak, yet significant decrease in cell viability in MDA-MB-231 cells (Fig. S7, Supplementary materials). Therefore, metformin uptake and its antiproliferative effects in breast cancer cell lines is appear to be transporter dependent.

For the other tested compounds, a concentration-response analysis was performed to determine the concentration inducing a 50% decrease of cell viability (IC_{50}) (Table 4). These sulfenamide derivatives were originally designed as a prodrugs, and were supposed to release metformin. However, during the later studies it occurred that most of them were too stable to be regarded as prodrugs. Butylsulfenamide is the only prodrug in this publication, however, according to the stability studies (Table S1, Supplementary materials) it is stable enough in the experiments conditions. Taken into consideration metformin's molecular mechanism of action, including effects on AMPK, mTOR and other signalling pathways [2,3] it would also be highly valuable to verify whether the chemical modification of metformin backbone can result in the same mechanism of antiproliferative activity.

Table 4
The effects of metformin derivatives on inhibition of MCF-7 and MDA-MB-231 cells viability. The results (IC_{50} values, µmol/L) are presented as mean ± SD (n = 6–8).

Derivative	MCF-7 cells [µmol/L]	MDA-MB-231 cells [µmol/L]
1	0.72 ± 1.31	39.3 ± 1.18
2	442.6 ± 1.27	524.4 ± 12.28
3	1027.5 ± 12.78	> 2000
4	376.7 ± 1.33	959.9 ± 13.16
5	880.3 ± 12.30	832.3 ± 14.54

In WST-1 assay the cells were stimulated with the test compounds at concentrations within the same range as the uptake studies (10 µmol/L to 2000 or 3000 µmol/L). As depicted in Table 4, derivative **1** with cyclohexyl ring was found to be the most cytotoxic towards MCF-7 cells, with an IC_{50} value equal to 0.72 ± 1.31 µmol/L. The IC_{50} value was higher in the case of MDA-MB-231 cells: 39.3 ± 1.18 µmol/L. We presume that relatively low IC_{50} values and profound cytotoxic properties of cyclohexyl sulfenamide result from its high uptake in both cell lines, which were 12-fold and 8-fold higher than metformin in MCF-7 and MDA-MB-231 cells, respectively.

Equivalently, compound **2** was characterized by high cellular uptake in both cell lines, which was reflected by moderate growth inhibitory properties (IC_{50} values 442.6 ± 1.27 and 524.4 ± 12.28 µmol/L for MCF-7 and MDA-MB-231 cells, respectively). Both sulfenamides **3** and **4** were reasonably taken up by both cell lines; however, they showed different potential towards cell growth inhibition: compound **3** exhibited poor cytotoxic properties, while compound **4** demonstrated a low IC_{50} value, especially for MCF-7 cells. Similar cytotoxic properties, manifested by comparable IC_{50} values, were reported for compound **5**, despite the large differences in cellular uptake.

As a particular compound may also contribute to primary cell death by exerting cytotoxic properties towards cancer cell lines, our studies included several experiments using human endothelial cells (human umbilical vein endothelial cells, HUVECs) which showed, for instance, that compound with *n*-butyl alkyl chain (**5**) did not induce any changes in cell viability or integrity up to 1.5 mmol/L (data not shown). However, recently performed studies on human Aortal Smooth Muscle Cells (AoSMC) showed that most of the sulfenamides contributed to the significant decrease in viability and integrity of AoSMCs at the highest concentration (1.5 mmol/L) (data not shown). Therefore, in further studies, it is necessary to consider the possibility of a toxic effect of the test compounds if studied at a concentration of 1 mmol/L or higher.

Bearing in mind the fact that metformin uptake and its antiproliferative efficacy are based on the action of multiple transporters [12], our results indicate a novel solution for improving metformin cytotoxicity. On the other hand, Obianom et al. [18] present a reverse modification by inclusion of a biguanide functionality in non-substrates of OCTs resulting in the production of compounds that can potentiate uptake by OCT1 and OCT2. Such an approach may improve the delivery of various compounds to tissues with significant expression of OCT transporters.

Our results indicate that chemical modification of metformin structure into sulfenamides increases their cytotoxic properties.

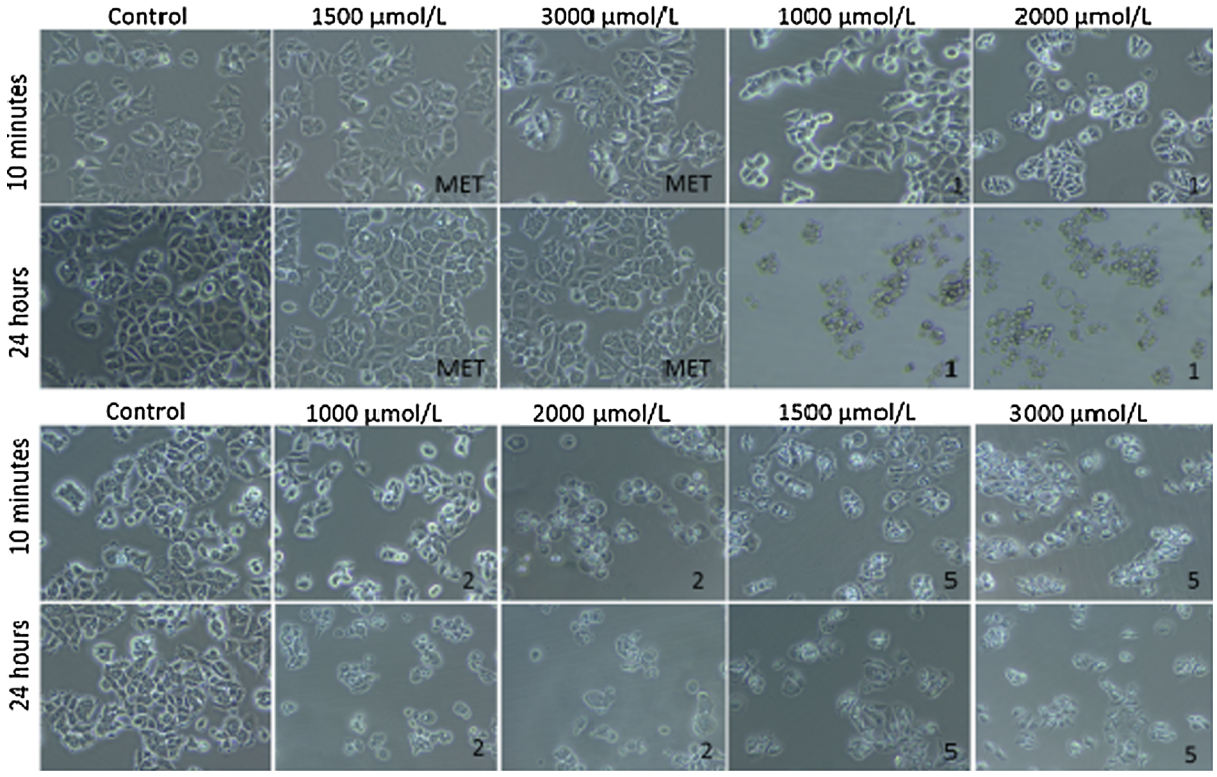

Fig. 7. Dose-dependent effect of metformin (MET) and derivatives 1, 2, 5 on MCF-7 cells viability and morphology after 10 min and 24 h incubation. MCF-7 were cultured without (control) and in the presence of biguanides at concentration of 10–2000 µmol/L. Representative cell images are shown for the highest tested concentrations 1000 and 2000 (comp. 1, 2) or 1500 and 3000 (MET, comp. 5) µmol/L (100-fold magnification).

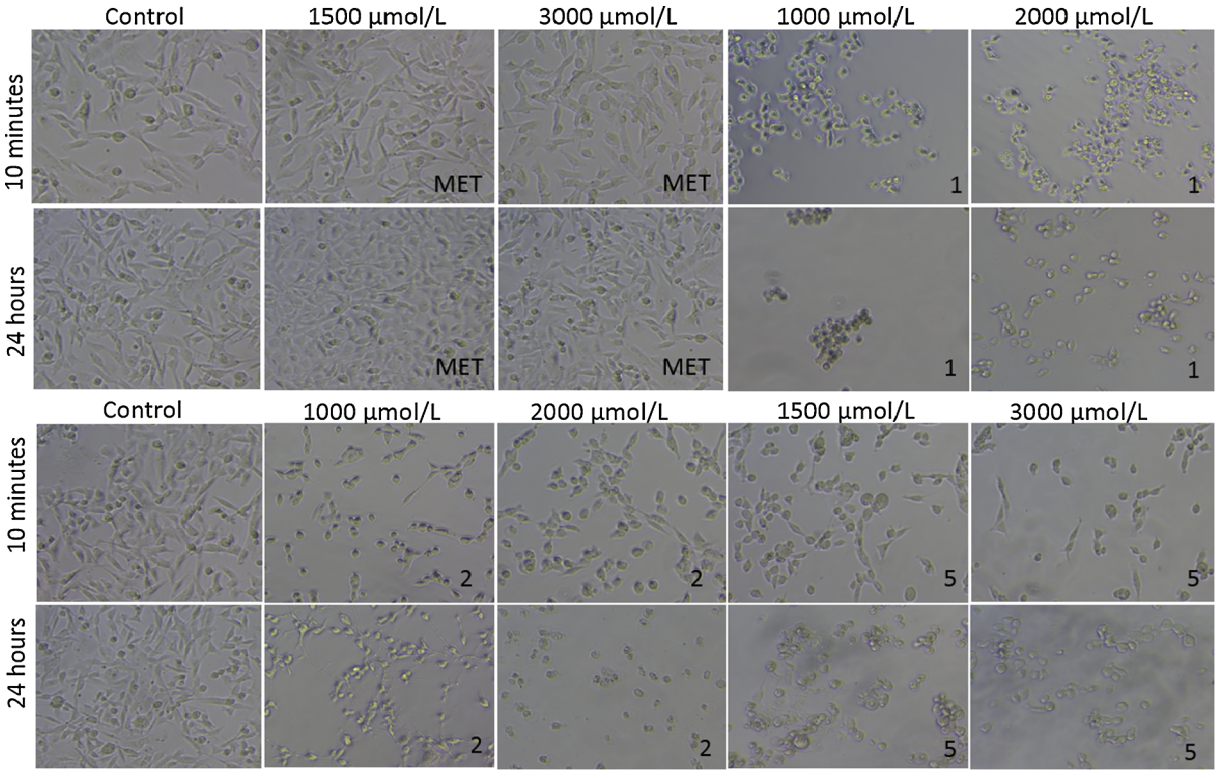

Fig. 8. Dose-dependent effect of metformin (MET) and derivatives 1, 2, 5 on MDA-MB-231 cells viability and morphology after 10 min and 24 h incubation. The cells were cultured without (control) and in the presence of biguanides at concentration of 10–2000 µmol/L. Representative cell images are shown for the highest tested concentrations 1000 and 2000 (comp. 1, 2) or 1500 and 3000 (MET, comp. 5) µmol/L (100-fold magnification).

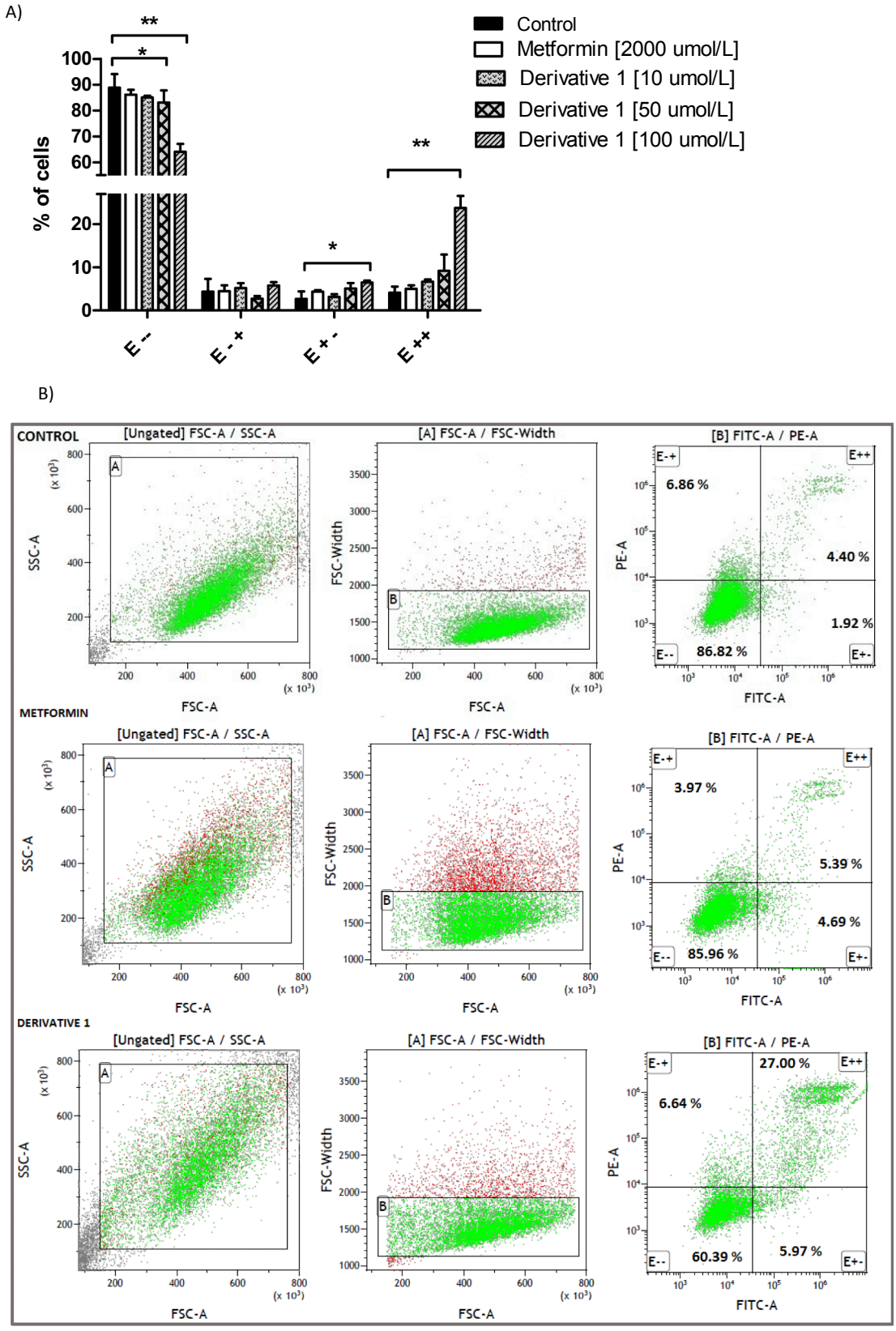

Fig. 9. The effect of selected biguanide derivatives on MDA-MB-231 cells death. (A) The effects of metformin used at 2000 µmol/L and derivative 1 at concentration of 10, 50 and 100 µmol/L on the living cells (E − −), early apoptotic cells (E + −), late-apoptotic (E + +), and necrotic cells (E − +) expressed as the percentage of the cells gathered in gate B. (B) Representative histograms of unstimulated MDA-MB-231 cells (control), metformin (2000 µmol/L) and derivative 1 (100 µmol/L) displaying the percentage of cells gathered within established gates. FSC-A vs SSC-A plots were used for gating cells and to identify any changes in the scatter properties of the cells. Annexin V FITC-A (x-axis) vs Propidium Iodide (y-axis) plots from the gated cells show the populations corresponding to living cells (Annexin V(−) and PI (−)) (D− −); early apoptotic cells (Annexin V (+) and PI (−)) (D + −), late-apoptotic cells (Annexin V(+) and PI (+)), and necrotic cells (Annexin V (−) and PI (+)) (D − +).

However, it should be highlighted that any evaluation of anti-proliferative properties of biguanide derivatives must be accompanied by a determination of their affinity for OCT transporters. This evaluation must include transporters regarded as efflux transporters, as many of them can function as bi-directional carriers. Another issue which should be kept in mind is the fact that cytotoxic properties do not translate directly into anticancer properties, since there are many cytotoxic compounds which do not have anti-neoplastic properties. The applied cell proliferation WST-1 test is used for the quantification of cell proliferation, growth, viability, and chemosensitivity in cell populations in response to various chemical compounds, growth factors, or drugs. Therefore, further studies elucidating the mechanism of cytotoxic activity of biguanide derivatives should be performed. For instance, DNA damage and the consequent induction of apoptosis might be regarded as an example of cytotoxic mechanism of anticancer agent; therefore, these studies should also be carried out.

3.5. Cell morphology

Light and phase-contrast microscopy studies were performed to identify changes in MCF-7 and MDA-MB-231 cell morphology. As presented in Fig. 7, sulfenamides exerted diversified influence on the morphology of MCF-7 at two time-points: 10 min, corresponding to uptake studies, and 24 h relating to cytotoxicity studies. Images in Fig. 7 show that metformin did not affect cell viability, shape or morphology up to 3000 μmol/L following a 24-hour incubation. In the case of compound **1** with a cyclohexyl ring, the numbers of viable cells, their density and changes in morphology were found to decrease at a concentration of 2000 μmol/L following 10 min incubation. After 24 h of co-treatment with derivative **1,** severe compound-mediated changes were reported, manifested by membrane disruption, cell shrinkage and rounding, together with cytoplasm leakage. Similar results; however, to a lesser extent, were also observed for the highest concentration of compound **2** (2000 μmol/L). The MCF-7 cells did not exhibit any profound morphological changes following treatment with sulfenamide **5** up to a concentration 1500 μmol/L; however, cell rounding, membrane disruption and inhibition of growth were reported at the highest concentration. In addition, significant changes in MCF-7 cell morphology were reported for the highest concentrations of sulfenamides **3** and **4** (1000 and 2000 μmol/L).

Fig. 8 depicts the dose-dependent effects of metformin and sulfenamides on MDA-MB-231 growth after 10 min and 24 h of incubation. Similarly to MCF-7 cells, metformin did not influence cell viability or morphology. In the case of compound **1**, significant morphological abnormalities such as cell shrinkage and membrane disintegration were observed even after 10 min of co-treatment. The other compounds (**2–5**) contributed to significant morphological changes only at the highest concentrations tested. In conclusion, the results of the microscopy studies confirm the those of the cytotoxicity assay since the majority of analyzed compounds exhibited antiproliferative properties at their highest concentration.

3.6. Apoptosis assay

Since inhibition of cell growth can cause apoptosis, the next part of the study examined whether metformin and its most cytotoxic derivative (**1**) affect breast cancer cell growth by induction of apoptosis. Apoptosis is a fundamental biological process which is characterized by various biochemical and morphological changes such as DNA fragmentation, plasma membrane blebbing and loss of cell volume. Alternatively, cell death can occur by necrosis, which is a passive, catabolic, pathological process generally occurring in response to external toxic factors such as inflammation, ischaemic or toxic injury [30].

Our present findings indicate that co-treatment of MDA-MB-231 cells with metformin contributed to a significant decrease in the number of the cells gathered within gate B (58.21 ± 1.57% vs. 69.48 ± 4.06% for control samples, $p < 0.001$; Table S2, Supplementary materials); however, it did not significantly change the percentage of viable cells (AV-PI-), nor the fractions of early-, late-apoptotic and necrotic cells. These results seem to be in accordance with those of Sahra et al. [31], who report that metformin does not induce apoptosis in human prostate cancer cells, but blocks cell cycle in G(0)/G(1). However, some studies report that meftormin has the opposite effect on apoptosis [28,32]. For instance, Queiroz et al. [28] found that 10 mM metformin inhibited proliferation of breast cancer cells (MCF-7 cell line) by several mechanisms including cell cycle arrest in the G_0-G_1 phase, inhibition of cyclin D1 and induction of apoptosis and necrosis.

Stimulation of the cells with derivative **1** at a concentration of 10 μmol/L did not affect any of the registered MDA-MB-231 cell population (Fig. 9); however, the percentage of viable cells significantly decreased (↓ AV− PI−), and the percentage of early-, and late-apoptotic cells increased (↑ AV+ PI−; ↑ AV+ PI +) up to 23.77 ± 2.80% at a concentration of 100 μmol/L. To further determine the cytotoxic properties of this compound additional studies regarding the possibility of cell cycle arrest should conducted.

4. Conclusions

In conclusion, our findings present the affinity of metformin and its sulfenamide derivatives towards OCT transporters and their cellular uptake. They confirm that chemical modification of metformin into sulfenamides differing in the length of alkyl chain or presence of saturated hexyl ring facilitates greater binding to OCTs, as well as higher cellular uptake, in MCF-7 and MDA-MB-231 cells. Additionally, the introduction of an alkyl chain to the biguanide backbone results in higher selectivity for OCT1 over other transporters, but also greater transport efficacy. Of the tested derivatives, the greatest cellular uptake for both cell lines was reported for derivative **1**, i.e. with a cyclohexyl ring; this derivative was also associated with the greatest antiproliferative properties expressed as the lowest IC_{50} value, i.e. the concentration resulting in growth inhibition among 50% of the tested cells. The cytotoxic properties of derivative **1** were associated with apoptosis induction. Collectively, these findings provide the direct evidence that a new drug design strategy, including sulfenamide derivatives, was successfully developed and contributed to better cellular uptake and antiproliferative properties of the parent drug.

Declaration of interest

The authors declare no conflict of interest.

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.2019.03.036.

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