The modulating effect of lipid bilayer/p-coumaric acid interactions on electrical properties of model lipid membranes and human glioblastoma cells

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ABSTRACT

Biological membranes are one of the most important elements of living cells determining their permeability to the active compounds. Still, little is known about the drug-membrane interactions in terms of pharmacological properties of potential drugs. Chemoprevention based on natural compounds is becoming a strong trend in modern oncopharmacology, and p-coumaric acid (p-CoA) is one such compound with tentative anticancer activity. The microelectrophoretic mobility measurements and electrochemical impedance spectroscopy were applied to study the effects of p-CoA on electrical properties of liposomes, spherical bilayers, and human glioblastoma cell membranes. Our results demonstrated that after treatment with p-CoA, the surface charge of LBC3, LN-229 and LN-18 cell lines was significantly changed in alkaline pH solutions, but not in acidic pH solutions. In contrast, no changes in surface charge density values were registered for phosphatidylethanolamine liposomal membranes and A172 cell membranes after p-CoA treatment. The impedance data showed an increase in values of both the electrical capacitance and the electrical resistance, indicating that p-CoA can be partially inserted into the phosphatidylcholine bilayers. The MTT assay showed cell line-dependent cytotoxic effect of p-CoA. Further molecular analyses revealed the ATP depletion and gene transcription modulation, which might indicate organelle membrane-crossing potential of p-CoA. These results suggest, that changes in surface charge of membranes of living cells not only might be potential predictor of membrane permeability, but also indicate differential composition of cell membranes in various cell lines. Thus further multidirectional analyses are required to implement electrochemical methods as standard testing procedures during drug development process.

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1. Introduction

Biological membranes are crucial elements of the living systems. They are known to be the interface between extracellular space and the inside of the cell. It is readily apparent, that biological membranes play an essential role in regulation of electrochemical processes occurring in living organisms, and that this regulation is dependent on their structure. However, mechanisms underlying these processes are complicated and yet, not completely understood [1]. It has been evident, that lipid bilayer is widely recognized as the major structural component of biological membranes. Thus, great deal of attention is now shifted towards investigation of the organization and properties of these structures concerning both experimental and theoretical aspects. Because systematic examinations are impeded by the complexity of the natural membranes, the best approach to perform detailed physical and chemical studies of biological membranes is to use simplified well-defined model lipid membranes [2]. These models can be exploited to acquire information on the properties of actual biological membranes and associated electrochemical reactions. Electrochemical methods, in particular electrochemical impedance spectroscopy (EIS) and microelectrophoresis, may be very helpful in characterization of properties of model lipid membranes such as liposomes or so called black lipid membranes (BLMs) [1].

Since cell membrane is one of the primary obstacle to overcome during drug transportation, knowledge concerning membrane-dependent processes may become novel trend in contemporary...
pharmacological and medical studies. The direct interaction between drug and the cell membrane if frequently neglected when it comes to studying drug effects [3].

Taking into account a constant need of developing novel drugs and finding novel drug targets, multidirectional analyses are required to unravel all possible aspects of drug functioning. Nowadays, a strong emphasis is put on the development of pharmaceutics of natural origin. Thus, the intake of phytochemicals has become a novel trend in prevention and treatment of various diseases. For decades, their diverse health benefits have increasingly been discovered pointing an interesting avenue in pharmacological and medical research. Most phytochemicals are characterized by their antioxidant and free radical scavenging abilities, as well as anti-inflammatory capacity. These features are thought to be correlated with alleviation of the incidence of some chronic diseases, such as, metabolic diseases, viral infections, neurodegeneration and cancer [4,5]. Some of the biggest contributions in terms of pure molecules come from polyphenols, which are the most abundant secondary metabolites of plants with well-recognized beneficial effects in vitro and in vivo [6]. Polyphenols are widely distributed in plant kingdom, and therefore are an essential part of human diet [7]. Chemically, these substances are amphipathic molecules, containing both hydrophilic hydroxyl groups and hydrophobic aromatic rings.

Polyphenols are divided into some subclasses according to their chemical structures: simple phenols, phenolic acids, flavonoids, lignans, flavonolignans, tannins and depsides [8]. Biological activity of polyphenols could be better understood by examination of the polyphenols-biomembranes interactions. This might bring some information about the solubility of these molecules in membrane bilayer which is essential for predicting their permeation abilities. The capability of polyphenols to interact with biological membranes is an important factor determining their transport, action, distribution, toxicity and selectivity [9].

Small organic acids can possibly interact with and penetrate across biological membranes [10], however, this is strongly affected by the substituents present in their main structure. Consequently, compounds sharing structural similarity, but possessing diverse functional groups influencing their lipophilicity, should interact differently with biomembranes. Therefore, these interactions may be affected by pH changes, especially for acidic compounds. Phenolic acids are a diverse group of compounds that includes hydroxybenzoic and hydroxycinnamic acids [11,12]. The hydroxycinnamates occur usually in a form of simple esters with hydroxy carboxylic acids or D-glucose [13].

These compounds have attracted a great deal of attention due to their broad biological and pharmacological activities [14–16]. One such compound presenting various biological functions such as antioxidant, anti-inflammatory, antimicrobial and anti-cancer activities, is p-coumaric acid (p-CoA) [17].

p-CoA was shown to suppress proliferation and growth of several cancer cell lines such as colorectal, tongue squamous cell, and hepatocellular carcinoma cell lines [18–21]. However, limited amount of data reports p-CoA efficiency in brain tumors [22]. Unlike other cancers, brain tumors are particularly resistant to pharmacological treatment due to the blood-brain barrier, and a number of other factors limiting the efficiency of available therapies. Thus, glioblastoma remains a therapeutic challenge with poor overall prognosis [23,24]. To address the need for novel therapeutic approaches, application of natural compounds such as p-CoA might be considered in the management of brain malignancies. Nevertheless, any advanced studies need to be preceded by an extensive chemical and biological analysis, in order to get insight into all aspects of the potential drug functioning. In this respect, the systematic research investigating the effect of p-CoA on electrical properties of model biological membranes and the membranes of living cells were performed. In order to gain new insights into the anticancer mechanisms of p-CoA, the cytotoxic potential of p-CoA in four human glioblastoma cell lines was evaluated. The anti-glioblastoma effect was assessed by analyzing cell viability, ATP production, caspases 3/7 and 9 activities and expression of main apoptosis-related genes. Moreover, to obtain information on the actual ability of p-CoA to permeate the aqueous layer surrounding the lipid bilayer or to pass through the membrane itself, a comparison between values of the electrical parameters obtained from experiments conducted on BLMs, liposomes and cell lines need to be performed. In an attempt to cover all
aspects of membrane/p-CoA interactions, these three different experimental approaches were applied. The chemical structures of p-CoA and lipid components of model membranes: phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) are presented in Fig. 1.

Modern biomedical and pharmacological research concentrate on involving multidirectional analyses for maximal improvement of the therapeutic outcomes. Therefore, the combination of physicochemical and cell-based methods should potentially be used to investigate the effects of drug molecules on membrane structure, and to understand how these interactions can translate into effect while predicting drug efficiency in vivo.

2. Materials and methods

2.1. Reagents and chemicals

p-Coumaric acid (≥98%), 1,2-diacyl-sn-glycerol-3-phosphocholine (99%), 1,2-diacyl-sn-glycerol-3-phospho-l-serine (≥97%), 1,2-diacyl-sn-glycerol-3-phosphoethanolamine (≥98%), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased (99%), 1,2-diacyl-sn-glycero-3-phosphocholine (≥97%), 1,2-diacyl-sn-glycero-3-phosphoethanolamine (≥98%), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma. The Dulbecco’s modified Eagle’s medium (DMEM), containing glucose at 4.5mg/cm³ with GlutaMax™, trypsin-EDTA, penicillin, streptomycin and fetal bovine serum Gold (FBS Gold) were provided by Thermo Fisher Scientific (Waltham, MA, USA). A high-capacity RNA-to-cDNA kit was purchased from Thermo Fisher Scientific. The ReliaPrep RNA Cell Miniprep system, Caspase-Glo 3/7 assay, Caspase-Glo 9 assay, CellTiter-Glo luminescent assay were provided by Promega (Fitchburg, WI, USA). Other reagents were of the best quality commercially available and they have been employed as received. The solutions and all cleaning procedures were performed using deionized water purified to a resistance of 18.2 MΩ (HLP5 UV System, Hydrolab, Hach Company, Loveland, CO, USA) and filtered by a 0.2-μm membrane filter to remove any impurities. All experiments were carried out at room temperature (20 ± 2 °C).

2.2. Cell cultures

Human glioblastoma cell lines A172, LN-229 and LN-18 were provided by American Type Culture Collection (ATCC). The LBC3 cell line was developed from glioblastoma multiforme tissue taken from 56-year-old female patient subjected to surgical tumor resection, and was kindly given to us by Prof. Cezary Marcinkiewicz (Department of Neuroscience, Temple University, Philadelphia, PA, USA) [25]. Cells were cultured in high-glucose DMEM with addition of 10% of heat-inactivated fetal bovine serum Gold (FBS Gold), streptomycin (100 μg/cm³), penicillin (100 U/cm³), and 2 mmol/dm³ l-glutamine. The cells were cultivated in Falcon flasks (BD Pharmingen™, San Diego, CA, USA) in a 5% CO₂ incubator Galaxy S+ (RS Biotech, Irvine, UK) at the temperature of 37 °C. Cells reaching sub-confluence were detached from the culture dishes using 0.05% trypsin, 0.02% EDTA in calcium-free phosphate-buffered saline (PBS) and counted in a Scepter cell counter (Millipore, Billerica, MA, USA). p-CoA was dissolved in ethanol as 1 mol/dm³ stock solution and subsequently diluted with growth medium to desired final concentrations.

2.3. Cell viability

The viability of the cells was measured according to the method of Carmichael et al. [26] using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, cells were seeded in 96-well plates at a density of 2 × 10⁴ cells per well. Cultured cells were exposed to various concentrations of p-coumaric acid (0.5–10 mmol/dm³) for 24 and 48 h. Then, the cells were washed twice with PBS and incubated with 0.2 cm³ of MTT solution (0.25 mg/cm³ in PBS) at 37 °C in a humidified 5% CO₂ atmosphere for 4 h. The medium was removed and formazan products were solubilized in 0.2 cm³ of 0.1 mol/dm³ HCl in absolute isopropanol. The absorbance of a converted dye in living cells was read at λ = 570 nm using a microplate reader (Tecan). The viability of p-coumaric acid-treated A172, LBC3, LN-229 and LN-18 cells was calculated as a percentage of control non-treated cells. All experiments were run in triplicate in at least two cultures.

2.4. Determination of cellular ATP levels

Measurement of cellular ATP levels in control and p-CoA-treated A172 and LBC3 cells was performed using the CellTiter-Glo assay following the supplier’s specifications. Briefly, A172 and LBC3 cells were seeded in a white-walled 96-well culture plate (Nunclon) at a density of 1×10⁴ cells/well. Cells were allowed to attach, and then incubated with medium containing 0.5–10 mmol/dm³ of p-CoA at 37 °C for 24 h and 48 h. After incubation, 100 μmol/dm³ staining solution (CellTiter-Glo reagent) was added to each well and mixed for 2 min on an orbital shaker to induce cell lysis. Cells were incubated at room temperature for 10 min to stabilize the luminescence signal, which was recorded using the microplate reader. The experiment was run in triplicate.

2.5. Caspase 3/7 and caspase 9 activity

Measurement of caspase 3/7 and caspase 9 activities after p-coumaric acid treatment was performed using the luminescent Caspase-Glo 3/7 and Caspase-Glo 9 assays following the manufacturer’s instructions. Briefly, A172 and LBC3 cells were seeded in white-walled 96-well culture plates (Nunclon; Thermo Fisher Scientific) at a density of 1×10⁴ cells/well. Subsequently, cells were incubated with medium containing 0.5–10 mmol/dm³ of p-coumaric acid at concentrations of 5 mmol/dm³ and 8 mmol/dm³ for 48 h. After incubation, 100 μmol/dm³ Caspase-Glo 3/7 or Caspase-Glo 9 reagent was added to each sample. Cells were mixed using a plate shaker at 300 rpm for 45 s and left in the dark at room temperature for 40 min, followed by measurement of luminescence with a microplate reader (Tecan). The experiment was run in triplicate.

2.6. RNA isolation and gene expression analysis

Total RNA was isolated using the ReliaPrep system with DNase I treatment according to the manufacturer’s instructions. Spectrophotometric measurements were performed to evaluate the quality (A260/A280) and quantity of the purified RNA (NanoPhotometer; Implen, Munich, Germany). Synthesis of cDNA was performed using the high-capacity RNA-to-cDNA Kit following the supplier’s recommendations. Briefly, 0.5 μg purified total RNA was used in a 20 μmol/dm³ 1. reaction mixture containing oligo(dT)16 primers, random octamers, dNTPs, and murine leukemia virus reverse transcriptase (RT). cDNA (2 μmol/dm³) served as a template for real-time RT quantitative polymerase chain reaction (qPCR). Amplification of the product was performed using 2 × HS-PCR Master Mix SYBR A (A&A Biotechnology, Gdynia, Poland). Primer sequences for Chop, Bax, Noxa, Casp3, Casp9 and housekeeping Rpl13A have been described in previous papers [24,27,28]. Additional evaluation of primer accuracy was done using Primer-BLAST software. The following reaction parameters were applied: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 1 min, 60 °C for 30 s, and 72 °C for 45 s. The CFX Connect realtime PCR system (Bio-Rad Laboratories, Hercules, CA, USA) was used to perform a real-time qPCR assay. Reactions were run in triplicates and expressions were analyzed using the relative quantification method modified by Pfaffl [29].

2.7. Bilayer lipid membranes formation

The bilayer-forming solutions, contained PC (20 mg/cm³ of solvent system) or a PC/p-CoA mixture (concentrations of p-coumaric acid: 5
and 8 mmol/dm³ with respect to the lipid) and were measured by electrochemical impedance spectroscopy. First, both substances were dissolved in chloroform and mixed in appropriate proportions. Next, the solvent was evaporated in a stream of argon, and the residues were dissolved in a n-hexadecane-n-butanol mixture (10:1 by volume). All of the solutions were stored in darkness and refrigerator at 4 °C for at least four days after preparation.

BLMs were prepared by method of extruding the solution that allows to create spherical bilayers dividing two aqueous solutions. A solution of 0.155 mol/dm³ sodium chloride was used as an supporting electrolyte. During membrane creation, the solvent mixture was removed from the lipid phase resulting in a bilayer composed of substances in the same ratio as the stock solution. Bilayer formation was controlled both electrically and optically. Membrane images were captured by color CCD camera using the WinFast PVR program. The BLMs areas were calculated from the photograph using the Makroaufmassprogramm program (http://ruedig.de/tmp/messprogramm.htm), additionally regarding the spherical nature of the surface. The area of the bilayer membranes was about 6 × 10⁻² cm².

More details concerning the procedure of membrane formation have been reported in our previous works [30,31].

2.8. Electrochemical impedance spectroscopy measurements

Electrochemical impedance spectroscopy was performed using Autolab PGSTAT302N potentiostat (Metrohm, Poland), equipped with FRA2 module. A four-electrode vessel was used with two identical current platinum electrodes and the two identical reversible silver-silver chloride electrodes. The design of the vessel dedicated for formation of bilayer membranes was described and illustrated previously [32,33]. EIS was measured in the frequency range of 0.1-10,000 Hz employing AC amplitude of 4 mV (five points per decade were recorded). Data obtained from EIS measurements were analyzed using the software package NOVA 1.10. The reported values represent the average of six independent measurements and are presented in relation to the bilayer surface-area unit.

2.9. Preparation of the phospholipids vesicles

Phospholipid vesicles were prepared according to the method proposed by Huang [34]. 10 mg of phospholipid (PC, PE or PS) was dissolved in 1–2 ml chloroform and mixed in appropriate proportions with p-CoA to achieve the desired molar final concentration (5 and 8 mmol/dm³). The solvent was evaporated under a gentle stream of argon to obtain dry film. Then, the film was hydrated with 0.155 mol/dm³ NaCl. The last stage of liposome formation was sonication the suspension five for 90 s each time using an ultrasound generator UD 20 (Techpan, Poland). The sample was kept ice-cold during the sonication.

2.10. Microelectrophoretic mobility measurements

Microelectrophoretic mobility measurements of liposomes and cell lines were performed with a Zetasizer Nano ZS (Malvern Instruments, UK) apparatus. The measurements were carried out as a function of pH (in pH range 2–10), with 0.155 mol/dm³ NaCl as a supporting electrolyte. The samples were suspended in an electrolyte solution and titrated to the desired pH using HCl or NaOH. Each result is a mean of six measurements at the given pH value and at the given concentration of sodium chloride value.

Based on electrophoretic mobility measurements, the surface charge density δ was determined using following formula [35]:

\[
d = \frac{\eta \cdot u}{4 \cdot \varepsilon_0 \cdot R \cdot T \cdot I} \sqrt{\frac{1}{2} \cdot F \cdot I}
\]

(2)

in which: \(I\) - the ionic strength of 0.9% NaCl, \(\varepsilon\) - the relative permittivity of electrolyte, \(\varepsilon_0\) - the permittivity of vacuum (8.854 · 10⁻¹² F m⁻¹), and other symbols bear their usual meaning.

2.11. Statistical analysis

Electrochemical data are expressed as mean ± SD from six independent measurements, and their statistical analysis was performed with STATIST prol. Cell-based results from three replicated experiments are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using Statistica Data Miner (StatSoft, Poland). Differences between controls and sample treated cells were analyzed by using one-way ANOVA with Tukey’s post hoc test. The half maximal inhibitory concentration (IC₅₀) values were calculated using the GraphPad Prism 5 software (GraphPad Software, Inc., USA). A p value less than 0.05 was set for statistical significance.

3. Results

A series of experiments was performed to establish whether p-coumaric acid is able to alter the electrical properties of biological membranes. In this respect, two experimental approaches including models of artificial cell membranes, and membranes of living cells were applied. These results were additionally combined with the preliminary analysis of molecular changes occurring in glioblastoma cells under the influence of p-CoA. Firstly, an evident cytotoxic activity of p-CoA was found in all tested cell lines: A172, LBC3, LN-229, and LN-18. Further measurements were carried out in concentrations exceeded IC₅₀ values to intensify the outcome of the applied treatment and enhance the visibility of the observed results. Subsequently, the electrical parameters of BLMs formed from pure PC and PC/p-CoA system were obtained based on the impedance data. Next, the surface charge density of phospholipid liposomes (PC, PS and PE), pure and modified by p-CoA, was calculated based on the electrophoretic mobility measurements. Afterwards, the surface charge density of glioblastoma cells, the intact and those incubated with p-CoA was determined. Moreover, the influence of p-CoA on certain molecular markers of apoptosis, such as ATP production, caspase 3/7 and 9 activities, and expression of main pro-apoptotic genes was determined.

3.1. The effect of p-coumaric acid on cell viability

The MITT test was used to assess the anti-proliferative effect of p-coumaric acid on four glioblastoma cell lines: A172, LBC3, LN-18, and LN-229. Cells were exposed to increasing concentrations of p-CoA for 24 and 48 h. For all four tested cell lines concentrations of 0.5–10 mmol/dm³ caused dose- and time-dependent reductions in glioblastoma cell viability (Fig. 2). However, in A172, and LBC3 cells the cytotoxic effect of p-CoA was clearly the strongest of all examined cell lines (Fig. 2a, b). In A172 cells stimulated with 10 mmol/dm³ p-CoA, the percentage of unviable cells was 51.51% (± 4.31%) after 24 h and 67.68% (± 5.31%) after 48 h (Fig. 2a). In comparison, LBC3 cells exposed to the highest concentration (10 mmol/dm³) of p-CoA showed more pronounced cytotoxic effects, approaching nearly 95% (± 2.01%) unviable cells, after 48 h of treatment. In this case, the loss of viability was most apparent (Fig. 2b). The p-CoA had relatively low ability to limit LN-229 cell proliferation, with only 44.5% (± 5.57%) of cells losing viability at the highest concentration after 48 h (Fig. 2d), moreover it reduced cell viability to a similar extent after 24 and 48 h of treatment. The greatest time-dependent differences in response to p-coumaric acid treatment were observed in LN-18 cells (Fig. 2c). Here, the percent of unviable cells were 24.4% (± 3.09%), and 78.8%
In order to compare the sensitivity of glioblastoma cells to p-CoA, the GraphPad Prism 5 software was used to calculate the IC₅₀ values of its activity after 48 h of treatment. The results showed significantly different IC₅₀ values of approximately 4.56, 2.43, 5.26, and 13.39 mmol/dm³ for A172, LBC3, LN-18 and LN-229, respectively. Based on the MTT results, we chose to proceed with A172 and LBC3 cells for further examination. The concentrations of 5 and 8 mmol/dm³ p-CoA were chosen to perform more detailed physicochemical and molecular analysis.

3.2. The effect of p-coumaric acid on the impedance parameters of bilayer lipid membranes

In order to understand the possible effect of p-CoA on the electrical properties of phospholipid bilayer the EIS method was first utilized. This non-destructive technique processed under small amplitude was used over a wide range of frequencies from 10⁻² Hz to 10⁵ Hz. The spectra were registered in 0.155 mol/dm³ NaCl electrolyte solution. p-CoA was introduced to the lipid model membrane solution at final concentrations of 5 and 8 mmol/dm³. Three different phospholipids i.e. PC, PS and PE were used to prepare model membranes. Unfortunately, only bilayers composed from PC had the ability to form sufficiently stable membranes for the impedance measurements.

The measured EIS data registered for PC BLMs and PC BLMs modified with p-CoA are presented in Fig. 3 in a form of Nyquist plots together with the proposed equivalent circuit inserted in the top right corner. The elements of the circuit include the resistance of the electrolyte solution together with the resistance of the cables and connections (Rs). The electric parameters of the lipid bilayer are given by parallel connection of its resistance (Rₘ) and capacitance (Cₘ). This simple circuit is characteristic for a model lipid membrane when specific channels, pores, ionophore systems, and adsorption process are absent [37]. The assumed electrical equivalent circuit was validated with experimental data and used to determine values of the resistance and the capacitance of analyzed BLMs. The Fig. 3 indicates that the experimental results match well the fitted ones, which manifests that the fitting results are credible. The Rs was constant for all measured conditions and was equal to (5.55 ± 0.15)·10³ Ω. The obtained Rₘ and Cₘ values for bilayers formed from pure PC were (2.57 ± 0.07)·10⁵ Ω cm² and (0.513 ± 0.039) μF cm⁻², respectively, which is in line with values reported previously [38].

The EIS results presented in Nyquist diagrams demonstrate pronounced variation of membrane resistance during introduction of p-CoA into the membrane forming solution, therefore confirming p-CoA activity within the examined concentration range. The values of Rₘ extracted from the fit were (2.71 ± 0.06)·10⁵ Ω cm² for PC BLMs modified with lower amount of p-CoA, and (2.84 ± 0.04)·10⁵ Ω cm² for PC BLMs modified with higher acid content. An increase in phospholipid membrane capacity was also observed after the addition of p-CoA to the bilayers. Here, the values of Cₘ obtained by fitting the impedance diagrams were found to be (0.517 ± 0.033) μF cm⁻² for PC
BLMs containing smaller acid content and \((0.522 \pm 0.035)\, \mu F\, cm^{-2}\) for PC BLMs containing higher acid content.

3.3. The effect of p-coumaric acid on the surface charge of phospholipid liposomal membranes

The measurements of the electrophoretic mobility of liposome membranes formed of PC, PS and PE, pure and modified by p-CoA at concentrations of 5 and 8 mmol/dm³, as a function of electrolyte solution’s pH (0.155 mol/dm³ NaCl) were implemented. The obtained values of electrophoretic mobility were converted into a surface charge density using Eq. (1) given in Section 2.

Experimental data referring to surface charge densities versus pH values for PC, PS, and PE liposomal membranes non-treated and treated with p-CoA are presented in Fig. 4. There was an increase in positive surface charge density at low pH values, however only up to a certain point. In contrast, at high pH values the negative charge of the liposomal membranes was rising until the plateau was reached. The current results demonstrated that no apparent changes were observed in the surface charge density values in the PC, PS or PE model lipid membranes after treatment with p-CoA at low pH values. Interestingly, at high pH values the presence of p-CoA caused significant alterations of surface charge densities of the PC and PS liposomal membranes (decrease in negative charge of the membranes), while no significant changes in values obtained for PE membranes were observed. Thus, as displayed on experimental curves for the PC membranes, evident drop in the surface charge at pH ~ 8.5 is probably related to destruction of membrane structures at such a high pH, which is consistent with previously published data [39].

3.4. The effect of p-coumaric acid on the surface charge of glioblastoma cell membranes

The surface charge densities of the analyzed glioblastoma cell lines were determined analogously to what was described for the liposomes. The pH dependencies of the surface charge of the A172, LBC3, LN-18, and LN-229 cell membranes are plotted in Fig. 5. Data are presented for untreated control cancer cells and cells treated with 5 and 8 mmol/dm³ of p-coumaric acid at incubation time points \(t = 24\) and 48 h. The dependencies obtained as a result of conducted experiments are of similar shape for all cell lines. The decrease in pH values was followed by an increase in positive surface charge density, but only up to a certain point. Conversely, along with an increase in pH values, the negative charge of the liposomal membranes increased until the plateau was achieved. The data presented in Fig. 5a demonstrate that for A172 glioblastoma cells treated with 5 and 8 mmol/dm³ of p-CoA for 24 and 48 h, the value of surface charge density is independent on the dose and time of treatment. It is noteworthy, that no visible changes were also found in the surface charge density values of the LBC3 (Fig. 5b), LN-229 (Fig. 5c) or LN-18 (Fig. 5d) cell membranes incubated with p-CoA at low pH values. However, at high pH values the presence of p-CoA affected the increase in negative charge of the cell membranes in comparison to the untreated control cells. This effect was evidently dose dependent but not time dependent.

Furthermore, no noticeable changes were observed in the isoelectric point values in all analyzed glioblastoma cell membranes cultured with p-CoA (independently on the dosage and the time of treatment).

3.5. The effect of p-coumaric acid on ATP production

In order to determine the influence of p-CoA on mitochondria functioning in A172 and LBC3 cells, the ability of ATP production was evaluated (Fig. 6). In A172 cells the content of ATP was slightly, but significantly reduced as early as 24 h after treatment, however only in high concentrations of p-CoA (8 and 10 mmol/dm³) (Fig. 6a). More pronounced inhibition of ATP synthesis was observed after 48 h of incubation with p-CoA, and this effect was clearly dose-dependent (Fig. 6a). Here, the ATP production was dropped up to 68.26% and 55.32% of the control level in 5 and 8 mmol/dm³ p-CoA respectively (Fig. 6a). Even stronger ATP generation-inhibiting effect was observed in LBC3 cells (Fig. 6b). In these cells, 5 mmol/dm³ concentration was already enough to significantly lower the ATP level after 24 h, and this effect was even more pronounced after 48 h, achieving 67.28% and 28.37% of the control level in 5 and 8 mmol/dm³ concentrations,
3.6. The effect of p-coumaric acid on caspase 3/7 and caspases 9 activities

To take a closer look at the mechanism of p-coumaric acid cytotoxicity, the possibility of apoptotic death of A172 and LBC3 cells was investigated (Fig. 7). For evidence of caspase-dependent apoptosis caspase 3/7 and caspases 9 activity was assayed. Based on the previous results of MTT and ATP tests only 48 h treatment was applied in order to observe possibly the most pronounced changes. Significant elevation respectively (Fig. 6b).
of caspase 9 activity was observed in A172 cells treated with both 5 and 8 mmol/dm³ p-CoA (Fig. 7a). However, in LBC3 cells only incubation with 5 mmol/dm³ p-CoA evoked marked increase in caspases 9 activity, while 8 mmol/dm³ concentration did not elevate such activity significantly (Fig. 7b). Similarly, in A172 cells we observed nearly twofold increases in caspase 3/7 activity in both tested concentrations of p-CoA (Fig. 7c). Unexpectedly, caspase 3/7 activity was diminished in both 5 and 8 mmol/dm³ concentrations of p-CoA in LBC3 cells (Fig. 7d). This effect together with simultaneous decline in cell viability might indicate that events other than apoptotic cell death might be responsible for anti-proliferative effect of p-CoA in LBC3 cells.

3.7. The effect of p-coumaric acid on the expression of main apoptotic-related genes

In order to check if p-CoA not only is able to enter cells through cellular membrane, but also to penetrate into other membrane-bound organelles such as nucleus, deregulation of gene transcription was studied. It is known, that apoptotic changes in cells are accompanied by altered gene expression. For instance, in cells undergoing apoptosis the level of pro-apoptotic genes is markedly elevated. In this respect, RT-qPCR analysis of certain of genes involved in apoptotic cell death was carried out (Fig. 8).

The expression levels of Chop, Bax, Noxa, Casp3, and Casp9 were determined in A172 and LBC3 cells subjected to p-coumaric acid treatment. As presented in Fig. 8 the expression of analyzed genes was deregulated after treatment with p-CoA in both A172 as well as LBC3 cells. Gene expression-modulatory effect was visible as early as 24 h after stimulation with p-CoA (Fig. 8a, c). After 48 h, general up-regulation of pro-apoptotic genes was demonstrated only in case of A172 cells (Fig. 8b), while in LBC3 cells only Chop was markedly overexpressed (Fig. 8d).
4. Discussion

The main interest of pharmaceutical research focus on designing novel drugs or improving the properties and bioavailability of already available ones. Proper drug investigations require complex interactions between different fields of science including medicinal chemistry, pharmacology, biochemistry, biotechnology, and physiology [40]. One of the primary causes of drug failure in clinical trials is either its adverse effects or poor absorption mostly due to the limited ability of active agent to cross biological membranes and/or other barriers [40]. Physicochemical characteristics of substances are crucial factors determining the fate of the drug in the organism from its administration and absorption to its excretion [41]. These properties are responsible for its efficient ligand-target conjunction mediated by hydrophobic interactions and hydrogen bonding. Additionally, they determine physicochemical processes such as ionization, lipophilicity, solubility, and permeability all of which are of high importance to the absorption of most orally administered drugs [41].

In this respect, one of the parameters needed to be assessed is the interaction of the compound with cellular membrane. Such interaction is a key regulator of biological activity of certain compounds such as phenolic agents, which has currently been extensively studied in terms of anti-cancer properties. Effects of selected phenolic acids on the rigidity and dynamics of the model membrane in the gel state was investigated by fluorescence polarisation measurements [42]. The authors used p-coumaric, caffeic and ferulic acids belonging to the hydroxycinnamic acid class of polyphenols and the lipid bilayers of liposomes prepared from 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). Based on the obtained data, they concluded that the phenolic acids, which are negatively charged, have very little effect on the structure of DPPC membranes because these compounds cannot cross the lipid bilayer of the zwitterionic DPPC lipids in the gel state. The greatest effect on the stability of lipid membranes was detected for p-CoA, the least polar acid among the tested ones, suggesting that the aromatic ring of this polyphenol is likely to be partially inserted into the cell membrane structure [42]. The interactions between DPPC serving as a model of cell membrane with other bioflavonoids e.q. apigenin were also examined and the ability of tested compounds to alter permeability of the DPPC bilayer was as well addressed [9]. On the other hand, Castelli et al. examined the interaction of cinnamic and p-coumaric acids with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) liposomes in the disordered state using differential scanning calorimetry [43]. A deeper interaction of cinnamic acid with lipidic membranes was shown in comparison to p-CoA. This may suggest a higher capability of cinnamic acid to collocate inside the bilayer by traversing it, while for p-CoA to remain anchored only at the bilayer surface without perturbing the lipidic structure. These differences were believed to occur essentially due to existence of an additional hydroxyl group in the structure of the p-CoA, which in consequence resulted in weaker interaction with lipid membranes in comparison to cinnamic acid.

Techniques employed to investigate drug-membrane interactions represent useful tools to obtain preliminary information about membrane permeation pathways or bioavailability of a drug [44]. Moreover, by means of modulation of certain experimental conditions (for example, by lowering pH) they can allow us to make interesting considerations about the importance of physicochemical properties of a bioactive compound in determining its permeation across biological membranes.
barriers [44]. Electrochemical methods have been applied for pharmaceutical and drug analysis since 1960s. However, most of them deal with only the voltammetric analysis [45,46]. In the present study, we made an attempt to implement other electrochemical methods such as electrochemical impedance spectroscopy and microelectrophoresis, to investigate the effects of chemical structure of p-CoA on its ability to interact with biomembranes employing electrical parameters of model lipid membranes and cell lines as experimental modules.

The EIS method is a non-destructive technique processed under sinusoidal voltage with small amplitude which is specifically suitable and useful for characterization of surface with excellent dielectric properties and high electrical or electrochemical impedance [47,48]. It is an important tool in the study of self-assembled monolayers [49], bilayers [9,50] or biosensors [51].

Although according to certain reports, reliability of BML as accurate models of biological membranes is still an issue of concern, it has been demonstrated that a number of their properties resemble biological membranes rather closely. For example, their capacitance is 0.3–1.3 μFcm−2 and their resistance is 103–109Ωcm2 as compared to physiological membranes rather closely. For example, their capacitance is 0.3–1.3 μFcm−2 and their resistance is 103–109Ωcm2 as compared to biological membranes.

The impedance parameters of the bilayers constructed in a spherical form dividing two aqueous solutions were examined herein. Since our bilayers were made from the mixture of 1,2-diacyl-sn-glycero-3-phosphocholine, 1,2-diacyl-sn-glycero-3-phospho-l-serine, and 1,2-diacyl-sn-glycero-3-phosphoethanolamine, a varying and unidentified chain lengths and degrees of saturation of utilized phospholipids were received. It is currently apparent, that the amphiphilic nature of phospholipids leads to the formation of bilayers. However, even when two chains are identical, the tilted orientation of the glycerol group to which they are attached means that one chain extends further from the phosphate group than the other (by about 1.3 methylene groups), and this effect may be larger with the many phospholipids where the two chains are different. When such molecules form a condensed monolayer, the outer surface would be irregular if the chains were orientated perpendicular to the plane of the head groups. With a floating monolayer this would not matter, however, in a bilayer the chains must pack without leaving voids in the acyl chain layer. The maintenance of the energetically favorable structure of the bilayer is possible in two different ways, one of which is tilting of the molecules relative to the surface normal, and second is interdigititation of the chains from the two sides of the bilayer [54]. Unfortunately, in case of the phospholipid mixtures used to create BMLs in our study, only PC allowed us to achieve membranes stable enough to dilute them up till proper bilayers were formed, and further impedance measurements could have been performed.

The EIS data obtained in 0.155 mol/dm3 NaCl electrolyte solution (pH equals 6.59) demonstrated a slight increase in the determined electrical parameters after p-CoA addition to the bilayer-forming solution. The values of the membrane capacitance as well as the membrane resistance tend to grow with the increase in the amount of p-CoA.

Rm was found to be slightly elevated after membrane modification, nevertheless it has stayed within the same order of magnitude. It is widely recognized that the resistance may vary by at least one order of magnitude between different lipid membranes. However, Rm of a single BLM reconstituted from the same forming solution and under the same conditions is usually constant, therefore any changes in resistance value caused by the presence of additional substances incorporated into the membrane can be determined with a relatively high degree of accuracy [55]. Consequently, it might be concluded that p-CoA can be partially inserted into the PC bilayers causing an increase in their ordering and a decrease in the dynamics of their phospholipid alkyl chains in the liquid form what is in line with the findings reported by Ota et al. [42].

As documented in previous studies, the values of normalized capacitance of BLMs usually range between 0.3 and 1 μFcm−2 depending on the lipid bilayer composition and physicochemical properties of the surrounding solution [56,57]. Fitting revealed that the Cm values of the BLMs analyzed in the present paper fall into this range. As the change in capacitance can be affected by change of the membrane thickness, the thickness for pure PC BLMs and PC BLMs modified with p-CoA were calculated using the formula [58]: Cm = ε ε0 d, where ε refers to the dielectric coefficient of the hydrocarbon interior of the BLM, which is usually taken to be 2–2.2 [58,59], and d stands for the thickness of the BLM. As the dielectric constant remains intact, the thickness of membranes can be easily calculated using the normalized capacitance values obtained from the fitting. We demonstrated that this value ranged between 3.53 nm and 3.74 nm for pure PC BLMs what is in agreement with previous reports showing corresponding values to be 3.3–4.1 nm for BLMs formed from PC headgroup lipids with hydrocarbon chains of various length and degree of unsaturation [60].

The thickness determined for PC BLMs containing lower acid content changed in range from 3.54 nm to 3.66 nm and for PC BLMs containing higher acid content varied between 3.50 nm and 3.64 nm. This barely noticeable decrease in the bilayer thickness occurring with increasing amount of p-CoA can be attributed to the interactions between the PC head groups and p-CoA molecules located within these headgroups. These interactions may include: induction of orientational changes of polar headgroups, increase in the mutual penetration of alkyl chains or tilting of phospholipids towards the bilayer interior [61].

Experiments conducted with a series of hydroxycinnamic acids and different lipids, suggested that their interaction with membranes may rely on the establishment of hydrogen-bonds among the hydroxyl groups of the acids and the polar headgroups of phospholipids [43]. The ability to interact with and cross the membranes can be modified by protonation/deprotonation equilibrium. Thus, our experiments were carried out in different pHs (from 2 to 10) and additionally changes in values of the surface charge density induced by membrane interactions with p-CoA were studied by electrophoretic mobility measurements using model liposomes formed from PC, PS or PE. At physiological pH, PC and PE are neutral molecules that behave as dipolar ions or zwitterions, whereas PS has a net negative charge and acts as an acid. According to the results detected by microelectrophoresis, addition of the p-CoA caused the change in PC and PS liposomal surface charge in electrolyte solutions with pH above 4, while there were no significant alterations at acidic pH solutions. Moreover, the effect of p-CoA on the PS surface charge was dose-dependent. Detection of the specific interactions of p-CoA with PS is very valuable information, because the displacement of PS to the cell surface is the most pronounced effect of the collapse of membrane asymmetry, maintenance of which is very important for many cellular processes. Uncontrolled loss of PS asymmetry contributes to the perturbations in cell functioning and is observed in many pathological changes. Frequently, this process occurs before any other morphological symptoms associated with cell death are visible. Our results obtained from electrophoresis are in agreement with those reported in [62], where the existence of specific interactions of polyphenols with negatively-charged phospholipids rather than zwitterionic ones were suggested. The lack of significant changes in the surface charge density values obtained for PE membranes is not surprising taking into account that this phospholipid occurs in the gel phase at the temperature at which the tests were carried out. Compatible conclusions were also given by Ota et al. [42], who implied that the phenolic acids cannot cross the bilayer formed from zwitterionic lipids existing in the gel state.

In order to dispel doubts concerning membrane permeability of p-CoA, we decided to perform an in vitro study exploring cytotoxic properties of this acid. Since current dietary and epidemiological studies have implied that food-derived polyphenols may display potentially beneficial effects on prevention, alleviation or even inhibition of oncogenesis [20], we decided to use cell lines of human glioblastoma in
our experiment.

Glioblastoma is the most frequently diagnosed and highly aggressive form of primary brain malignancies, with poor prognosis of survival [63]. Thus, alternative therapies to prevent and effectively treat glioblastoma are highly requested. p-Coumaric acid is one such micronutrient with tentative anticancer potential [64]. Until now, in vitro studies have shown that p-CoA evokes its anti-cancer activity through reduction of proliferation, diminished adhesion, and suppressed migration of human cancer cell lines such as lung (A549), colon (HT29-D4, HCT-15) and probably glioblastoma cells [18,22,65].

According to the previous studies, other polyphenols such as quercetin were demonstrated to be present not only in the cell membrane, but also in the cytoplasm and nucleus of the cells [38,66]. This observation might be indirectly translated into our results, as reflected by reduced viability of all tested glioblastoma cell lines. Here, the cytotoxic effect of p-CoA may be treated as an indicator of its ability to enter cells, which in turns results in perturbed proliferation of A172 and LBC3 cell lines in particular. To further confirm membrane permeability of p-CoA the ATP biosynthetic capacity and alterations in gene expression pattern were examined. As clearly evidenced, the term biological membrane not exclusively refers to the cellular membrane. Inside the plasma membrane that surrounds eukaryotic cells, there are many other membranes defining the intracellular compartments or organelles [67]. The examples of such organelles are mitochondria and nucleus, which are known to be key regulators of cell functioning. The ATP biogenesis is a key function of mitochondria, while gene transcription is a hallmark of nucleus functioning [66]. Therefore, to confirm mitochondrial toxicity, ATP generation was assayed. Indeed, results showed decreased ATP levels in both A172 and LBC3 cells. Furthermore, RT-qPCR analysis showed altered gene expression pattern, suggesting permeation through the nuclear envelope and possible interaction of p-CoA with the DNA strand. The results of MTT assay combined with ATP analysis may suggest apoptotic cell death occurring after p-CoA treatment. This assumption is in agreement with data available from other p-CoA studies [18,22,65]. Since apoptosis is a controlled death program activated to remove defective cells without any harm to neighboring cells, it is preferred way of cancer cell elimination [68–70]. Apoptosis can be triggered via death receptors located on the cell surface or by intrinsic signals from the mitochondria [69]. Nevertheless, still little is known about p-CoA-dependent molecular mechanisms correlated with cellular signaling pathways. Some reports suggest that p-CoA induces apoptosis through ROS-mitochondrial pathway [18,70], while other reveal that p-CoA inhibits Grp78 up-regulation through the activation of PERK-Elf2α-ATF4-CHOP pathway resulting in apoptotic cell death [19]. Finally, induction of apoptosis via p53-mediated up-regulation of caspase-8 mRNA was identified in N2a neuroblastoma cells [70]. One of the general hallmarks of apoptotic cell death is activation of caspase 3, while mitochondrial-mediated apoptosis is mostly evidenced by an increase in caspase 9 activity. Thus, to further confirm possible apoptotic implications of p-CoA on glioblastoma cells, additional analyses of caspasas activity were performed. Interestingly, A172 cells showed significant up-regulation of Casp3 and Casp9 mRNA, which was confirmed by enhanced enzymatic activity of these caspases after p-CoA treatment. In line with this, other pro-apoptotic genes such as Chop and Noxa were significantly overexpressed. Surprisingly, caspases-activating effect was not observed in LBC3 cells which may suggest events other than apoptosis to be responsible for cell elimination. This was also suggested by Shailasree et al., who implied that autophagy and necrosis might also be minor processes triggered by p-CoA in neuroblastoma cells [70].

In order to align the results of cellular and molecular analyses with electrochemical measurements, ζ-potential of four glioblastoma cell lines was evaluated. All biochemical processes, which take place within membranes, constitute the proper functioning of living cells. Any changes in cellular homeostasis translate into cell membrane functioning, which influences physicochemical properties of membranes (e.g. resistance, capacitance, electric charge). An essential property of the electric double layer is its electric charge. For this reasons, studies of the electric charge can provide information on the equilibrium existing within the membrane as well as within the membrane and its environment, under both physiological and nonphysiological conditions [71,72]. According to the microelectrophoretic mobility results, the change in surface charge of LBC3, LN-229 and LN-18 cell membranes after their incubation with p-CoA was detected only in alkaline pH solutions, while there were no significant alterations in p-CoA-treated cells compared to untreated controls at acidic pH solutions. These results might be particularly relevant since cells are typically cultured in pH = 7, thus certain retention of p-CoA in cell membrane under these conditions might be suggested. Due to the high complexity of biological membranes, model membrane systems such as BLMs or liposomes, are helpful in interpretation and understanding of the processes that occur at the membrane surface or that are associated with cell membranes. Microelectrophoretic experiments performed on liposomes have shown changes in both PC and PS membrane surface charge values after incubation with p-CoA in alkaline pH solutions. Analogous data were obtained for membranes of tested glioblastoma cells LBC3, LN-229 and LN-18. These results may suggest, that p-CoA interacts with both phospholipids, PC as well as PS. However, to fully unravel the nature of these interactions, further investigations concerning quantitative descriptions of the equilibria occurring between the components of membranes, are necessary.

Interestingly, collected results have shown that the electrical properties of A172 cell membranes were not affected by p-CoA, implying complete transition of this acid through cell membrane followed by strong cytotoxic effect. Compared with data obtained for both PC as well as PS liposomes, it can be concluded that one of the reasons explaining the lack of effect of p-CoA on membrane charge density of A172 cells, are the interactions of p-CoA with membrane proteins, which is probably why we do not observe changes in the surface charge values. This suggests probable differences in membrane composition of various cancer cell lines. In fact, it has already been confirm, that the lipid composition of human gliomas differs from that found in non-malignant brain tissue [73,74]. Modifications in membrane compositions cause modifications in cell membrane charge because the amount of functional groups at the membrane surface is changing. In low pH, the phospholipid charge is due to the amino groups, whereas in high pH it is due to the phosphate and carboxy groups. Nevertheless, when considering the influence of p-CoA on the electrical properties of cancer cell membranes, the existence of various proteins should also be kept in mind. In this respect, electrical parameters of living cells will be the resultant of mutual interactions between their components. The data presented here indicate that the dynamic and structural properties of membranes can be altered by p-CoA, however how deep these alterations would be is probably dependent on the type of membrane itself.

Multidirectional analysis of potential active compounds is gradually becoming a strong trend in modern pharmaceutical and medical sciences. Our studies were designed to evaluate the properties of p-CoA combining physicochemical and molecular investigational approaches. Here, we observed that jointed electrochemical and cellular analysis of p-CoA may provide novel information about the modulatory effect of this acid on surface charge density of model membranes and give preliminary prediction of membrane permeability in living cells. Thus, further studies are required to confirm the accuracy and improve the versatility of these analyses, before they are routinely used as method of choice when predicting membrane permeability for novel drug candidates.

5. Conclusions

In the present paper, we demonstrated the modulating effect of p-coumaric acid on electrical properties of the liposomes, bilayer lipid membranes, and human glioblastoma cell membranes. Additionally, an
attempt to combine the electrochemical measurements with cellular and molecular analyses was undertaken. Our results demonstrate that p-coumaric acid changes electrochemical parameters such as surface charge density, electrical capacitance or electrical resistance of almost all analyzed systems via insertion into the polar headgroup region of the membranes. Simultaneously, reduction in cell viability, ATP depletion, increase in caspases activity, and deregulation of gene transcription was noticed, suggesting cell and organelle membrane-permeable potential of p-CoA. These results suggest, that changes in surface charge of membranes of living cells not only might be potential predictor of membrane permeability, but also indicate differential composition of cell membranes in various cell lines. These are one of the first studies linking physicochemical and cell-based studies combined together to unravel the complicated nature of membrane-related functioning of p-coumaric acid. The significance of such analysis may be specifically valuable for modern pharmacological and chemical studies, where engaging interdisciplinary analytical approaches contribute to the huge progress in translating chemical theories into pharmacological practice.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References


