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The role of catechin in electroporation of pancreatic cancer cells – Effects on pore formation and multidrug resistance proteins

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ABSTRACT

Catechin is a bioflavonoid known for its anti-cancer properties. In the present study, we combined theoretical and experimental approaches to reveal the potential of catechin application in the electroporation (EP) or electrochemotherapy (ECT) of pancreatic cancer cells. The molecular dynamics simulations were implemented to examine the interactions of catechin with a model of a membrane, its influence on the membrane's thickness, and the impact of the catechin-membrane interaction on the pore formation. The data were confronted with experimental measurement of the threshold electric field required for permeabilization of pancreatic cancer cells to a fluorescent dye YO-PRO-1. Further, we examined the influence of catechin on cell viability following electroporation with cisplatin or calcium ions. Finally, we investigated the catechin impact on four proteins associated with multidrug resistance: P-glycoprotein, MRP1, BCRP, and LRP. We demonstrated that catechin may boost the effects of electroporation through various mechanisms: i) increasing the cell permeability prior to electroporation ii) increasing the electroporation threshold iii) sensitization of cells to chemotherapeutic compounds. We showed that catechin incubation influences mRNA levels and mitigates the immunoreactivity of Pgp, MRP1, BCRP, and LRP but these changes did not translate to the efficacy of electrochemotherapy.

1. Introduction

1.1. *Electrochemotherapy of pancreatic cancer*

Pancreatic ductal adenocarcinoma (PDAC) is the most prevalent type of pancreatic tumor, accounting for more than 90% of pancreatic cancer cases [1]. It is a highly aggressive and lethal malignancy. Due to a lack of early symptoms and relatively fast progression, 80 to 85% of patients do not qualify for surgical treatment at the time of diagnosis [2,3]. As a consequence, chemotherapy is predominantly the first-choice treatment for this type of tumor. Unfortunately, chemotherapeutic regimens are often ineffective and poor vascularization as well as drug resistance are considered the main causes [4–6]. Therefore, new therapeutic regimens are needed. Electrochemotherapy (ECT) is a treatment method in which electrodes are placed around diseased tissue, and an electric pulse is delivered to tumor cells. As a result, the electroporation (EP) or electropermeabilization phenomenon occurs, allowing for a local administration of therapeutic molecules [7–10]. This approach has been successfully implemented in various cutaneous and subcutaneous tissues [11–13]. ECT of locally advanced pancreatic cancer with bleomycin was shown to be safe and feasible, bringing good results in terms of the local disease control rate and overall survival [14]. However, in the case of PDAC due to legal and equipment limitations, ECT is rarely implemented. Instead, a high electric field is provided to cells in order to evoke cell lysis in a process called irreversible electroporation (IRE). Recently, IRE was found to be more efficient when combined with chemotherapeutic treatment with FOLFIRINOX [15], cisplatin and calcium ions [16]. Although both ECT and IRE produce encouraging results, it is noteworthy that they may evoke various effects in PDAC cells. For example, IRE with FOLFIRINOX increased tumor cell apoptosis [15], whereas the biochemical and morphological changes post ECT with bleomycin, cisplatin, or oxaliplatin were associated with immunogenic cell death that occurs with necroptosis rather than apoptosis [17]. ECT and IRE effectiveness may also be improved by additional interventions

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such as internal electrode cooling [18] or cell incubation with plant-derived compounds [19], Therefore, it seems important to thoroughly examine the action mechanisms of electroporation-based therapies and any additional pretreatment options that may enhance ECT efficacy.

1.2. The anticancer properties of (+)-catechin and (-)-catechin

Catechins are naturally occurring polyphenolic compounds found in various plant-based foods but are traditionally associated with green tea consumption. A single cup of green tea prepared with one gram of tea leaves in 100 ml of boiling water in a three-minute brew usually contains 75–147 mg of catechins and their gallate esters [20]. Structurally, all catechins are composed of two benzene rings (A and B) and a dihydropyran heterocycle (the C-ring). Due to chiral centers on C2 and C3 catechin has four possible diastereoisomers: (+)-catechin and (-)-catechin for *trans* configuration; (+)-epicatechin and (-)-epicatechin for *cis* configuration [21]. According to standards of the International Union of Pure and Applied Chemistry (+)-catechin is referred to as (2R,3S)-Catechin as has a B-ring with clockwise rotation on chiral carbon C2 and hydroxyl group with counterclockwise rotation on chiral carbon C3 (Fig. 1). Analogically, (-)-catechin is labeled as (2S,3R)-Catechin because its B-ring and hydroxyl group have anticlockwise rotation.

Catechins can exert anticancer effects through several mechanisms (Scheme 1). The most studied are antioxidative effects resulting from either direct interactions with free radicals and metal ions or from the indirect catechin influence on cells, such as increasing the activity of antioxidant enzymes, inhibiting the pro-oxidative enzymes, or suppressing the stress-related pathways [22]. The free radical scavenging ability is attributed to catechin phenolic hydroxyl groups, which provide electrons for the reduction of reactive oxygen and nitrogen species [22,23]. Therefore, the measured ferric-reducing antioxidant potential of catechins and their derivatives was as follows: epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), and epicatechin (EC), with the latter being the least potent antioxidant [24]. Conversely, green tea flavonoids can exhibit also prooxidant activity. For example, (+)-catechin showed prooxidant effects when interacting with a low concentration of Cu ions [25]. It has been postulated that the prooxidative action of catechin is mediated by the autoxidation of phenolic hydroxyl groups, that starts with one-electron oxidation of the B ring of catechins generating a semiquinone intermediate and a superoxide anion radical ($O_2^{-}\cdot$), which is in turn further reduced to hydrogen peroxide (H_2O_2) [26,27]. Another trait showing the utility of catechin in anticancer research is its ability to mitigate inflammation. For example, catechins inhibited the TNFα-mediated pro-inflammatory and pro-invasive response in pancreatic cancer cells [28]. Studies on human colon adenocarcinoma cell lines showed that a gallate ester of catechin can inhibit the production of proinflammatory chemokines IL-8 and MIP-3α as well as down-regulate genes involved in inflammatory pathways [29]. In terms of electrochemotherapy, two catechin effects are particularly promising i) interaction of catechin with biological membranes and ii) catechin interaction with membrane proteins, especially with those involved in the acquisition of drug resistance. Moreover, relatively small size and non-complex structure make catechin an attractive molecule for creating theoretical models using molecular dynamics (MD) or molecular docking studies. An MD study on the biophysical catechin interactions with the membrane demonstrated catechin incorporation into a mixed 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC)/1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) bilayer in<50 ns [30]. Experimental studies on liposomal membranes presented that both *cis* and *trans* catechins reduced membrane fluidity at concentrations of 20–500 μM [31]. Altered membrane properties can significantly affect pore formation and, therefore potentially influence the drug penetration through electropermeabilization. If not for increased drug infiltration into cells, the enhancement of ECT could be achieved by the blockage of protein transporters responsible for drug removal and in consequence – drug resistance. There is growing evidence that catechins can play a role in overcoming drug resistance. For instance, green tea flavonoids enhance the antibiotics' effectiveness against *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus,* and drug-resistant *Candida* species [32,33]. This activity is not only limited to microorganisms but also applies to cells, with documented catechins specificity towards Pgp, MRP1, MRP2, MDR1, LRP, and BCRP proteins [34–38].

1.3. The potential of catechin to change the electroporation threshold

From the physical perspective, catechin may act on the electroporation threshold in various ways. First, it may modulate the composition of the membrane and thus alter its properties under the influence of an electric field. It was postulated that flavonoids bound to the cellular membrane can influence the appearance and development of rafts or raft-like membrane domains through various mechanisms including changing the dipole potential and phase segregation, lipid melting, or interaction with integral and surface proteins [39]. The effects triggered by flavonoids rely on their structure. For example, taxifolin and quercetin had a much stronger impact on lipid melting than catechin and myricetin, and no lipid-melting effects were detected for flavonoid glycosides like rutin, hence the lipid-melting effect was correlated with the compound hydrophilicity [39,40] On the other hand, van Dijk *et al.* demonstrated that the more planar configuration of natural compound

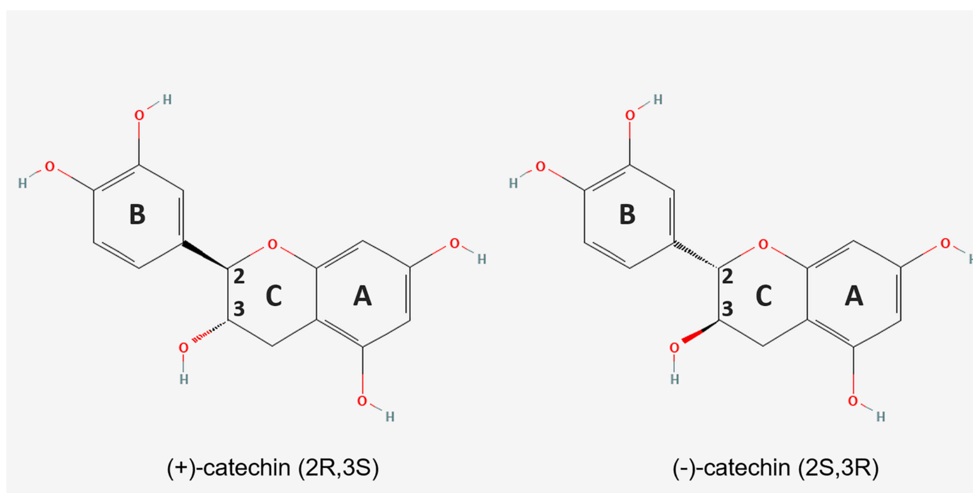
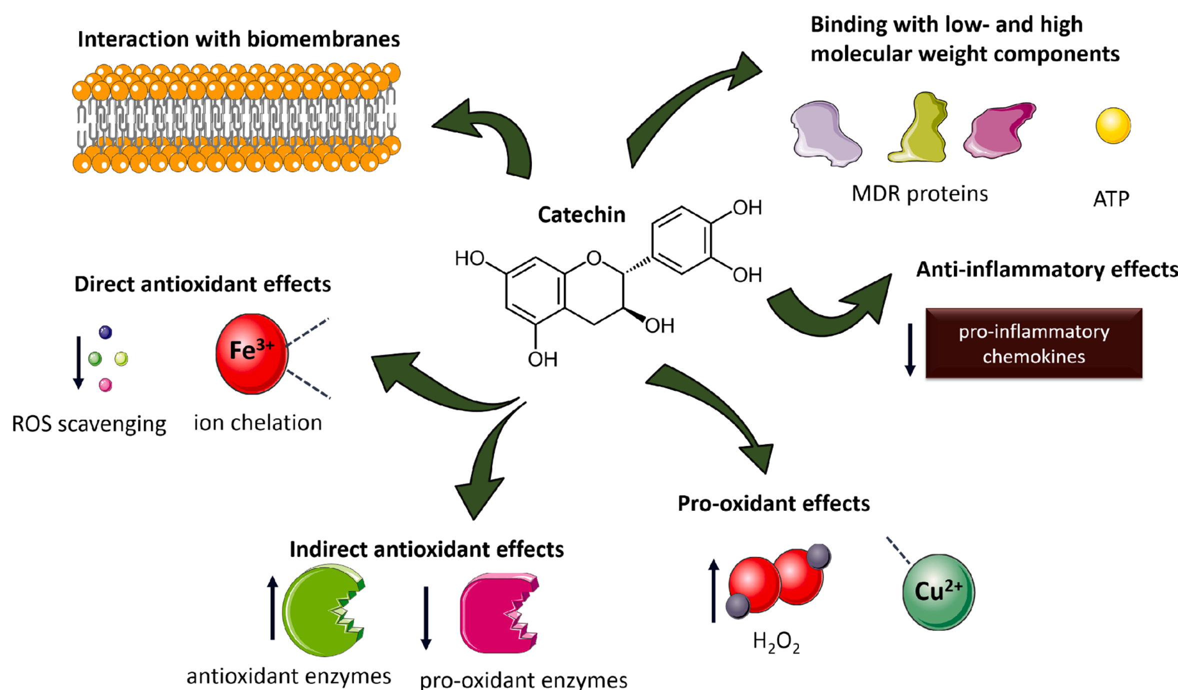


Fig. 1. The chemical structure of (+)-catechin and (-)-catechin; adapted from PubChem.



Scheme 1. Anticancer effects of catechin on cells; ROS – Reactive Oxygen Species, MDR – Multidrug Resistance.

results in higher affinity to the membrane, therefore hydrophobic flavanols exhibited a substantially higher affinity to liposomes than flavanones characterized by the tiled configuration [41]. When studying electroporation, MD simulations provide the most precise information on the mechanisms of pore formation. It was demonstrated that electroporation takes place preferentially in the liquid-disordered regions [42]. However, recent studies by Rems *et al* revealed that poration does not depend solely on local lipid arrangement, but also on membrane mechanical properties and the polarity of the electric field [43]. Nevertheless, the physical properties and state of the membrane have a considerable impact on the electroporation efficacy, which was confirmed in experimental studies on lung cancer and normal cell lines revealing a positive correlation between electroporation and the cell membrane stiffness and resting transmembrane potential [44].

Another consequence of the interaction between the flavonoid and the cell membrane is the modulation of a membrane's surface tension which can alter the electroporation threshold. Actin connected to the cell membrane via zyxin is one of the key regulators of surface tension of the membrane [45]. Catechin has already proved its effects on the modulation of actin fibers' organization [46]. Kim *et al.* demonstrated that the blockage of the actin polymerization with cytochalasin D results in increased cell roughness and diminished membrane stiffness as well as the transmembrane resting potential, which resulted in the decreased dye penetration into electroporated cells [47]. Studies by Szlasa *et al.* using the lipid-lowering drug atorvastatin confirmed that the surface tension regulates the membrane permeability and as a consequence, the electroporation threshold increased following the atorvastatin treatment [45]. The relationship between surface tension and electroporation encourages the search for natural, non-toxic compounds that can modulate the effects of electrochemotherapy.

The third possibility in which catechin may influence the efficacy of electroporation through membrane interaction is the stabilization of the formed pore. Several studies concern the problem of membrane reseal after electroporation and show how the kinetics of resealing influence the efficiency of electroporation [48]. Conversely, from the biological perspective, catechin may modulate the expression of membrane-bound proteins related to drug resistance and thus influence the effects of electrochemotherapy. Moreover, modified catechin was proved to induce the formation of pores in the membrane, thus the permeability can be induced by the standalone incubation with the compound [49].

In our previous research, we observed increased efficacy of cisplatin electroporation on two cell lines of pancreatic cancer when subjected to incubation with catechin before the treatment [19]. We found that short, 2-hour incubation with catechin was the most effective for increased electropermeabilization, which indicated the involvement of the transcriptional-independent mechanisms of catechin action. The present study aims to verify the favorable catechin effects on various PDAC cell lines and further confirm or exclude two possible mechanisms responsible for catechin action: i) direct interaction with the cell membrane or ii) interaction with selected multidrug resistance proteins.

2. Material and methods

2.1. Cell cultures

The study was conducted on four cell lines of pancreatic ductal adenocarcinoma: parental (EPP85-181P), resistant to daunorubicin (EPP85-181RDB), resistant to mitoxantrone (EPP85-181RNOV), and a well-differentiated cells HPAF-II. The EPP85-181 cell lines were kindly shared by Dr. H. Lage (Charité University Hospital, Institute of Pathology, Berlin, Germany), and the HPAF-II cells were purchased from the American Type Culture Collection (ATCC; CRL-1997™). The cultures were maintained at 37 °C under high humidity in the automated CO_2 incubator (Binder) and regularly tested for mycoplasma with a Myco-Blue™ Mycoplasma Detector (Vazyme Biotech Co. Ltd., Nanjing, China). The culture medium for all EPP85-181 cell lines was modified Leibovitz's (L-15) medium (Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin), 1.5% sodium bicarbonate (7.5%, Gibco), 0.1% glucose (45%, Sigma), 2.5 mM ultraglutamine (Lonza, Basel, Switzerland), 0.2% insulin (10 mg/mL, Sigma) and 30 TIU/L aprotinin (BioShop, Canada). HPAF-II cells were cultured in ATCC-formulated Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% antibiotics. Cells were collected from flasks using a TrypLE™ Express Enzyme (ThermoFisher Scientific, Waltham, MA, USA). To sensitize cells to the action of the electric field,

cells were incubated with (±)-catechin (Sigma, cat no. C1788) dissolved in 99% ethanol to 10 mM and further with a culture medium to a final concentration.

2.2. Toxicity of catechin

Cells were seeded on a flat-bottom 96-well plate at a density of 1×10^4 cells per well (150 µl). After 24 h, the medium over cells was replaced with 100 µl of fresh culture medium (control group) or with a medium containing catechin at a concentration of 10 – 200 µM. Cell viability was measured with the Presto Blue assay (ThermoFisher Scientific): after 24 or 48 h of incubation with catechin, 11 µl of PrestoBlue have been added to each well and fluorescence was measured using a GloMax® Discover microplate reader (Promega; Exc. 520 nm/Em. 580–640 nm).

2.3. MD simulations

The MD simulations were performed with GROMACS 2018.3 software [50] on the calculational cluster in the Department of Theoretical Chemistry and Physics at the Lorraine University. The models for simulations were built with CHARMM-GUI web software and visually inspected with VMD software [51,52]. The simulated systems were composed of a membrane in the ionic water solution. The membrane was composed of ~ 690 lipids per membrane layer (Top leaflet: 196 CHL1, 448 DOPC, 42 DOPE; Bottom leaflet: 168 CHL1, DOPC 112, DOPE 350, DOPS 70). Before the simulation, the system was solvated in physiological conditions of NaCl water (TIP3) solution. The whole simulation was performed in the periodic boundary conditions. The simulation proceeded with the CHARMM36 force field [53]. The systems were minimized equilibrated (100 ns, NPT conditions: Nose-Hoover thermostat and Berendsen barostat). Afterward, the system was simulated for 100 ns under various external electric field conditions. The electroporation simulation was carried out under NPT conditions. After the simulation, the system was evaluated if the pore was formed during the time of the simulation. The calculations of membrane thickness were conducted with Membplugin 1.1 Software on the last 10 frames of the simulation of the membrane with catechin [54]. Thickness maps were plotted with VMD software.

2.4. Electroporation protocol

For the pulse delivery, cells were collected from culture vessels and suspended in electroporation buffer (10 mM HEPES, 250 mM sucrose, and 1 mM $MgCl_2$; pH 7.4) in a 4 mm electroporation cuvette (BTX, Holliston, MA). Pulses were applied according to standard ESOPE protocol: 8 electric pulses, duration of 100 µs, delivered at 1 Hz frequency [55], with the electric field intensity ranging from 500 – 1250 V/cm using an ECM 830 Square Wave Electroporation System (BTX).

2.5. Experimental measurement of cell permeability to YO-PRO-1

Cell permeability was assessed by measurements of YO-PRO™-1 Iodide (491/509) (Thermo Fisher Scientific) infiltration into cells with a CyFlow Cube 6 flow cytometer (Sysmex, Warsaw, Poland). After 2 h of incubation with 50 µM catechin or culture medium as a control, cells were suspended in an electroporation buffer containing 1 µM YO-PRO-1 and immediately after subjected to electroporation using the above-mentioned protocol. The dye was excited with a 488 laser and the signal was collected with the 525/50 filter. Data were collected and analyzed by CyView software (Sysmex, Warsaw, Poland).

2.6. Electrochemotherapy with cisplatin and calcium ions

Following 2, 6, or 24 h of incubation with 50 µM catechin cells were collected from flasks and suspended in electroporation buffer alone or with 10 µM cisplatin or 5 mM calcium chloride. Pulses were delivered at an electric field intensity of 1000 V/cm using a protocol described in the *Electroporation protocol.* Next, cells were resuspended in a culture medium and incubated in cuvettes at 37 °C for 10 min in order to allow the cell membrane to reseal. After that time, cells were centrifuged to remove electroporation buffer, resuspended in a culture medium and seeded on a 96-well plate for 24 or 48 h. Cell viability was measured with PrestoBlue assay as described in *Toxicity of catechin.*

2.7. Expression of genes encoding MDR-related proteins

Cells were incubated in a culture medium with 50 µM catechin for 0, 2, 6, 12, or 24 h. Next, cells were harvested from culture flasks, centrifuged for 5 min at 500 × g, stored for up to one week at −20 °C for further analysis. RNA extractions from cells were performed using a NucleoSpin RNA II kit (Macherey-Nagel & Co., Düren, Germany) according to the manufacturer's protocol with DNAse I digestion option. 500 ng of total RNA was used for cDNA synthesis by reverse transcription reaction using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Foster City, USA). Then, 3 µl of three-fold diluted RT products were added into a single real-time PCR analysis complemented with AceQ qPCR Probe Master Mix (Vazyme Biotech, China) and specific TaqMan Assays: Hs00184500_m1 for ABCB1, Hs00219905_m1 for ABCC1, Hs01053790_m1 for ABCG2, Hs00233856_m1 for LRP1 and Hs99999905_m1 GAPDH (Thermo Fisher Scientific). All reactions were performed in triplicate in 96-well plates under the following thermal cycling conditions: 3 min. at 95 °C followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The reactions were run in the TOptical Real-Time PCR Thermocycler (Biometra GmbH, Göttingen, Germany) and the threshold cycle data (Ct) were collected using qPCRsoft (Biometra GmbH, Göttingen, Germany). For relative quantification (RQ) the samples were normalized against the expression of GAPDH mRNA, using the comparative Ct method (2-ΔΔCt).

2.8. Immunoreactivity of MDR proteins

For the examination of the immunoreactivity of Pgp, MRP1, LRP, and BCRP proteins, cells were seeded on 10-well diagnostic slides (Thermo Fisher Scientific). After 24 h, the medium above cells were replaced with a fresh culture medium containing 50 µM catechin. The cells were incubated at 37 °C for 6 more hours, subsequently fixed in 4% paraformaldehyde (Roth, Germany) and stained with the EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (Abcam, United States; cat. No ab80a36). For the overnight incubation, the following antibodies were used: anti-BCRP/ABCG2 antibody (cat. no ab3380, Abcam, Cambridge, United Kingdom), anti-P Glycoprotein antibody (cat no. ab235954, Abcam), Anti-LRP antibody (cat no. sc-23917, Santa Cruz Biotechnology, Heidelberg, Germany), anti- MRP1 antibody (cat no. MA5-16112, ThermoFisher Scientific) diluted 1:150 with the Antibody Diluent (Millipore, Poland). Samples were analyzed in a double-blinded manner with the upright microscope (Olympus BX51, Japan).

2.9. Statistical analysis

Numerical data were analyzed and presented with Microsoft Excel and GraphPad Prism 7 (La Jolla, CA).

All experiments were performed in triplicates unless noted otherwise. The results are presented as a mean +/- standard error (SE). Statistical analyses were performed using a two-tailed *t*-test or one-way ANOVA followed by Dunnett's multiple comparisons tests, with $p < 0.05$ considered statistically significant.

3. Results

3.1. The cytotoxicity of catechin

For three out of four tested cell lines (HPAF-II, EPP85-181RNOV, and EPP85-181P), catechin concentrations below 100 μM appeared to be non-toxic (Fig. 2). Only one cell line (EPP85-181RDB) presented toxicity after incubation with the lower catechin concentrations; however, this result was relevant only for 24-hour incubation (Fig. 2A). After the next 48 h, only 200 μM catechin significantly decreased cell viability suggesting fast cell recovery after exposition to the lower catechin concentrations. Based on this data, we selected 50 μM concertation for further experiments as the highest tested non-toxic dose with the best potential for cell stimulation.

3.2. The influence of catechin on permeabilization - MD simulations

Catechin is an aromatic molecule that localizes in the water-membrane interphase and interacts with head groups of the membrane lipids (Fig. 3E).

Fig. 3A reports the formation of the pore throughout the membrane during electroporation induced by the externally applied electric field. The presented model shows that there might be no shift of catechin molecules through the pore during the simulation. Interestingly, catechin molecules localize outlining the thinnest membrane region, which can be observed in Fig. 3C. Catechin molecules may localize in the thick regions of the membrane but never localized in the middle of the thin regions. Importantly, as shown in Fig. 3B catechin has no effect on the membrane's electroporation threshold. MD studies prove that catechin may modulate the membrane without the effects on its permeabilization, showing the potential of the molecule in the sensitization of the cell to the potential therapy.

3.3. The influence of catechin on permeabilization – Experimental studies

To experimentally confirm MD simulations, we examined the permeabilization threshold to fluorescent YO-PRO-1 dye in electroporation-treated cells stimulated with 50 μM catechin (Fig. 4). Flow cytometry measurements revealed that catechin incubation did not significantly affect the permeabilization threshold in EPP85-181P and EPP85-181RNOV cells, but enhanced YO-PRO-1 penetration into HPAF-II and EPP85-181RDB cells (Fig. 4 A-D).

Interestingly, 2-hour incubation with 50 μM catechin triggered a shrinkage of HPAF-II cells (Fig. 4E), suggesting a release of molecules from the cell interior. This effect was not apparent in other tested cell lines.

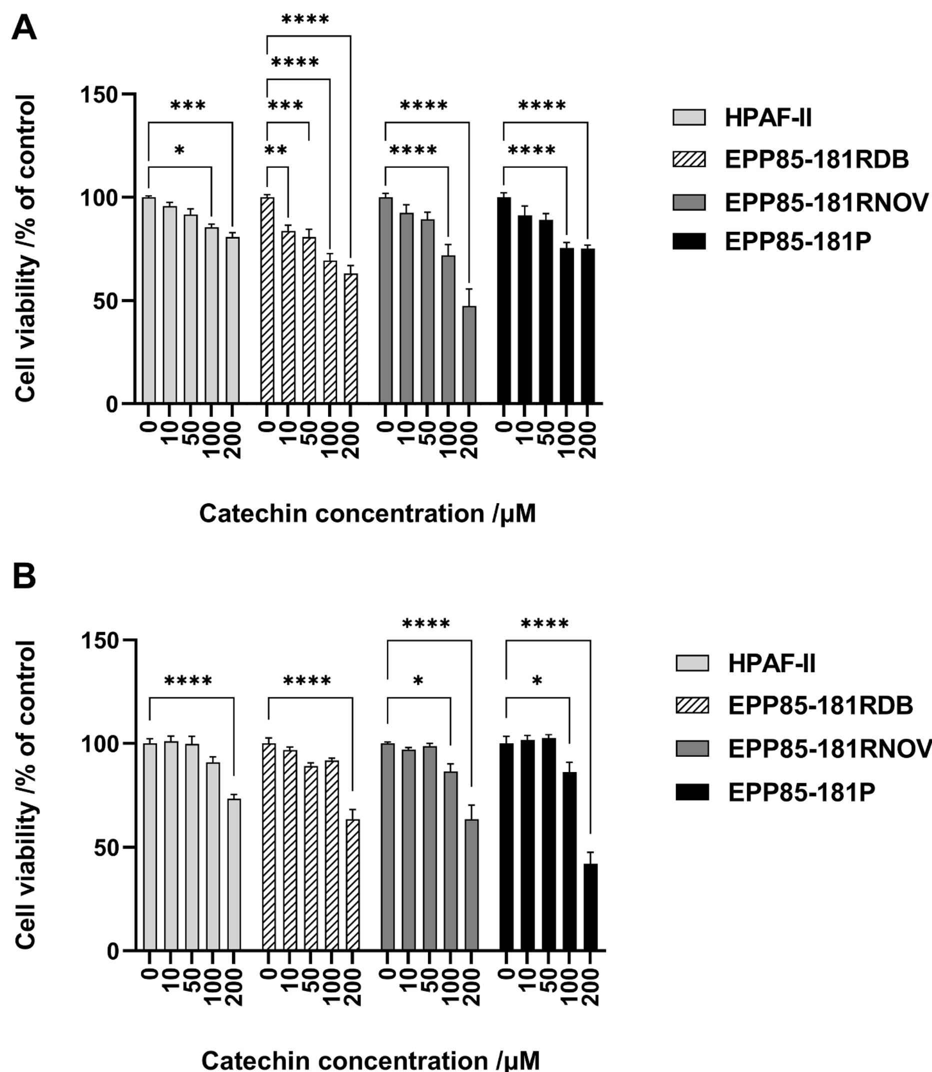
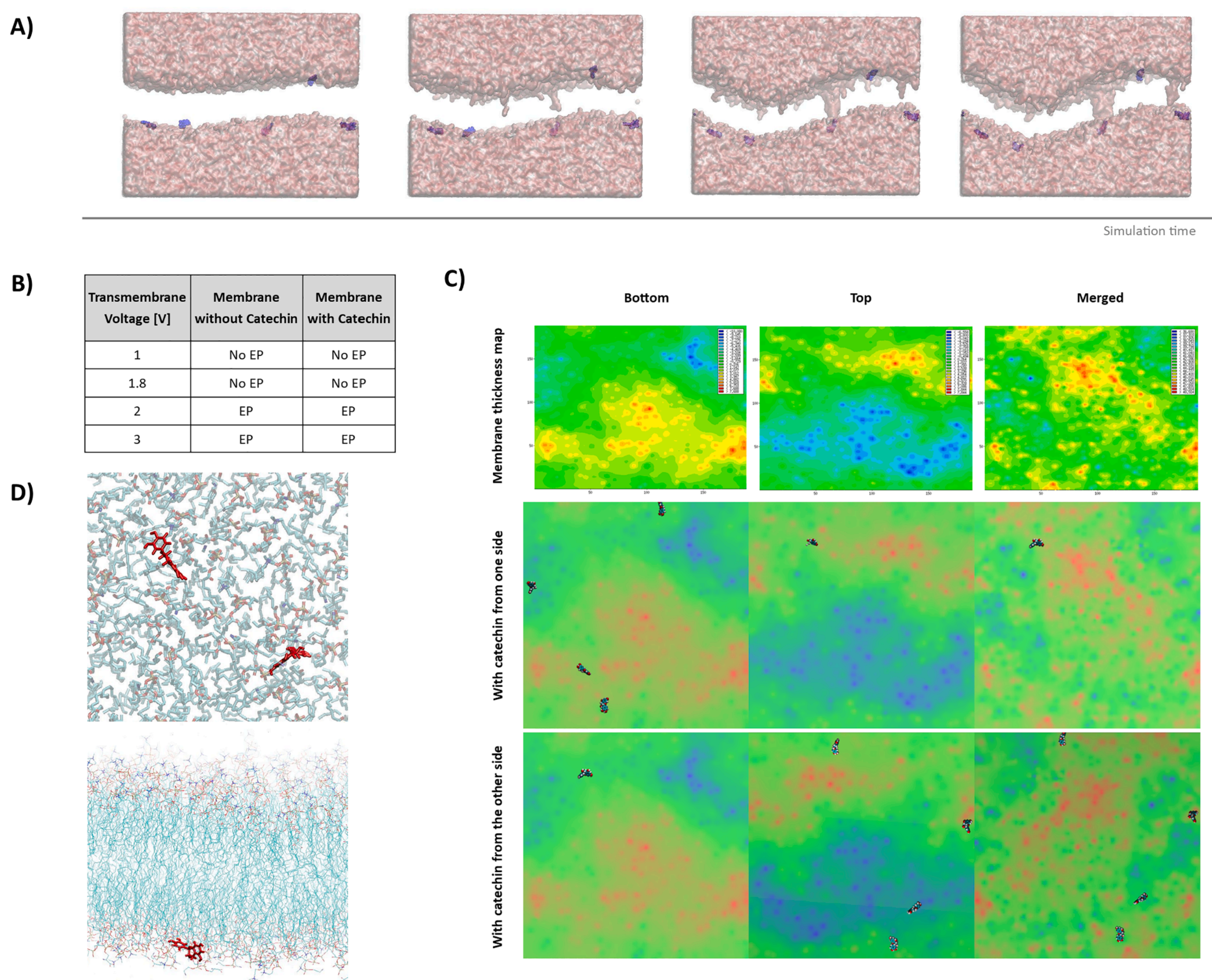


Fig. 2. The cytotoxicity of catechin after 24 h (A) or 48 h (B) of incubation in four cell lines of pancreatic cancer: HPAF-II, EPP85-181RDB, EPP85-181RNOV, and EPP85-181P. One-way ANOVA with Dunnett's corrections was used for comparison between Control (0 μM catechin) and catechin-treated groups (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ****<0,0001).



Transmembrane Voltage [V]	Membrane without Catechin	Membrane with Catechin
1	No EP	No EP
1.8	No EP	No EP
2	EP	EP
3	EP	EP

Fig. 3. A) Evolution of the pore in the membrane in the presence of catechin in the simulated system, for convenience, the membrane was removed from visualization and only red water and blue catechin remained; B) Table summarizing the effects of catechin presence on the electroporation threshold; C) Membrane thickness maps as the function of catechin molecules' localization on both sides of the membrane; D) Visualization of catechin localization in the water-membrane interphase from both the top and side view perspectives.

3.4. The influence of catechin on the efficacy of ECT in vitro

Studies of cell viability following catechin incubation and electroporation revealed that catechin can exert various effects depending on a cell line, even though most of the cell lines originated from pancreatic cancer tumor. In HPAF-II catechin incubation had no effect on electrochemotherapy with calcium or cisplatin, however, long incubation with catechin had a slight simulative effect on cell proliferation (Fig. 5A) and decreased cell viability in the group subjected to electroporation without drugs (Fig. 5B). For the daunorubicin-resistant EPP85-181RDB cell line at only 6- and 24-hour incubations had a negative impact on viability (Fig. 5C). After another 24-hours, statistically significant differences were also detected after short 2-hour catechin incubation in cells subjected to electroporation alone or with calcium ions (Fig. 5D). It should be noted that this cell line is characterized by a higher sensitivity to the electric field itself when compared to other PDAC cell lines, causing a drop in viability even without additional drugs. Most promising catechin effects were obtained for multidrug-resistant cell line EPP85-181RNOV in which incubation with catechin substantially increased the cytotoxicity of ECT with both cisplatin and calcium (Fig. 5 E, F). The shortest incubation time with catechin (2 h) was the most effective one, causing a significant decrease in cell viability after 24 h and further after 48 h, which demonstrates that the cell could no longer proliferate. Conversely, in parental, drug-sensitive cell line EPP85-181P long-term catechin incubation (6 h or more) played a protective role against electroporation-delivered calcium and cisplatin (Fig. 5G). However, short 2-hour incubations increased cell mortality evoked by the action of the electric field alone or together with cisplatin, which was visible after 48-hours (Fig. 5H). Altogether, catechin was able to boost the effects of electroporation and electrochemotherapy *in vitro,* but its action cannot be simply explained by membrane effects alone and mechanisms induced by catechin depend on its incubation length.

3.5. The influence of catechin on drug resistance – Gene expression

Before catechin stimulation, we examined the tested cell lines for the basal mRNA expression of genes involved in the drug resistance (Fig. 6). The most prominent differences applied to *ABCB1* gene encoding glycoprotein P – with the highest expression in EPP85-181RDB cell line (resistant to daunorubicin), moderate expression in EPP85-181P (parental) and HPAF-II cells, and no expression in EPP85-181RNOV (multidrug-resistant). The expression of *ABCC1* (encoding MRP1 protein), as well as LRP (encoding LRP protein) genes, was the highest in HPAF-II cells. *ABCG2* gene expression (encoding BCRP protein) was

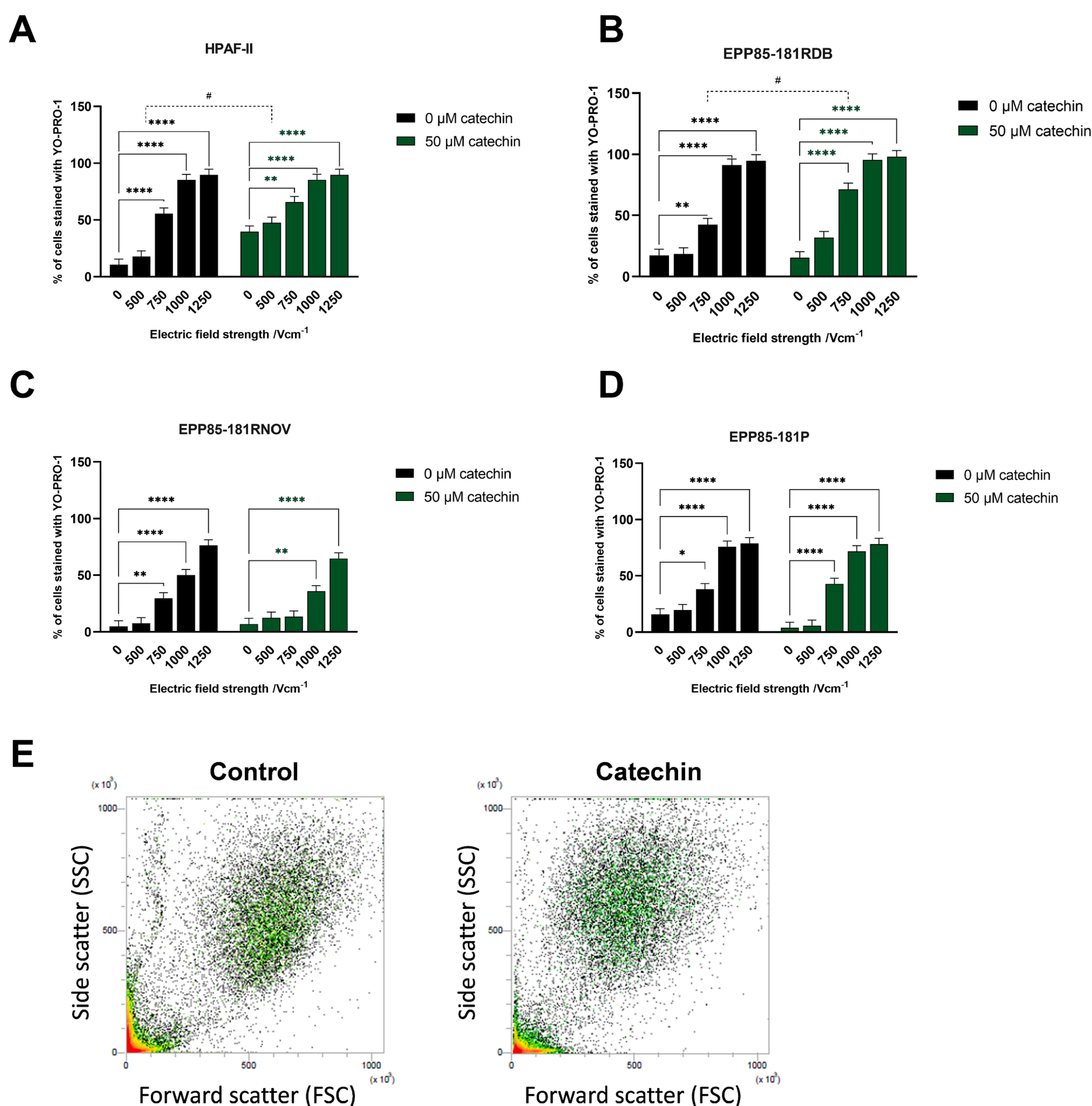


Fig. 4. The uptake of YO-PRO-1 dye in cells without catechin incubation (black) or with catechin incubation (green) in four PDAC cell lines subjected to electroporation: HPAF-II (A), EPP85-181RDB (B), EPP85-181RNOV (C) and EPP85-181P (D). One-way ANOVA with Dunnett's corrections was used for comparison between control group (0 V/cm) and electroporated groups (500–1250 V/cm) (* $p \leq 0.05$, ** $p \leq 0.01$, ****≤0,0001) or between groups untreated with catechin vs incubated with catechin (# $p \leq 0.005$). E – HPAF-II non-electroporated cells untreated (left) vs treated with catechin (right).

similarly high for HPAF-II and EPP85-181P and similarly low in EPP85-181RDB and EPP85-181RNOV cells.

The measurements of the mRNA expression following catechin incubation revealed specific trends. During the first two hours of incubation, the expression of all examined genes increased in all PDAC cell lines (Fig. 7). Surprisingly, after the next 6 h, expression dropped in almost all cases except for *ABCB1* in EPP85-181P and EPP85-181RDB cell lines which either remained stable or rose, respectively (Fig. 7A). Effects of catechin incubation longer than 6 h varied depending on the examined gene and the cell line. The gene expression of *ABCC1* and *ABCG2* was gradually raising in all EPP85-181 cell lines (Fig. 7 B, C) but in HPAF-II cells after initial bounce mRNA levels were lowered; however; only *ABCC1* gene expression after 24 h was significantly lower when compared to time 0 ($p = 0{,}0005$). The expression of the *LRP* gene slightly increased in all cell lines except for EPP85-181RNOV; however, neither of these changes were statistically significant. As the 6-hour incubation caused the most promising changes in mRNA expression of drug resistance-associated proteins, for that incubation time we also examined the level of the protein by the immunocytochemical staining.

3.6. The influence of catechin on drug resistance-associated proteins

The immunocytochemical staining revealed that a non-toxic concentration of catechin can efficiently mitigate the immunoreactivity of tested drug resistance-associated proteins (Fig. 8). However, if the mitigation occurred or how strong it was, varied between the tested

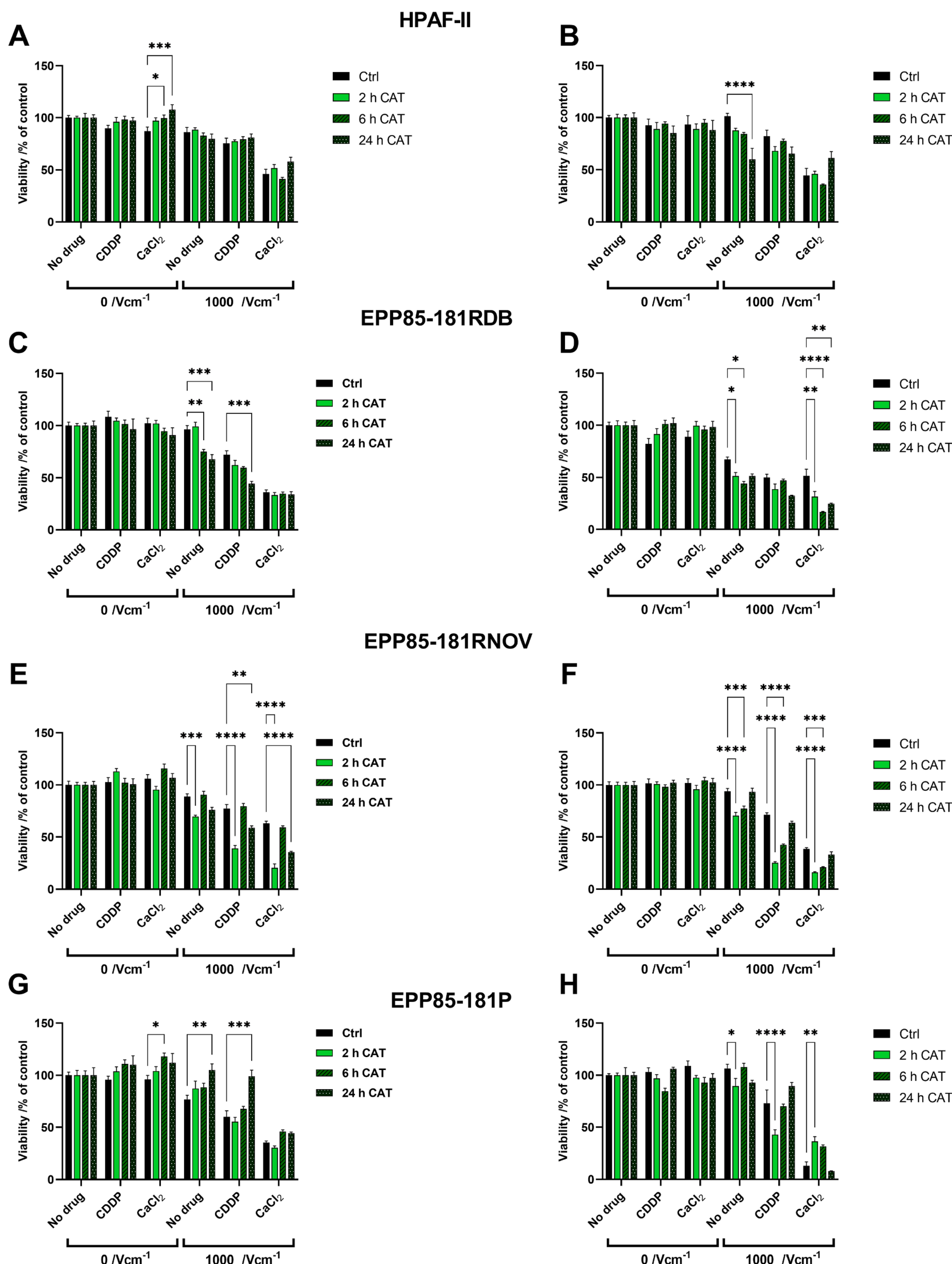


Fig. 5. The effects of catechin (CAT) incubation on the viability of cells 24 h (left column) or 48 h (right column) after electrochemotherapy *in vitro* with 10 μM cisplatin (CDDP) or 5 mM calcium ($CaCl_2$) in four cell lines of pancreatic cancer: HPAF-II (A, B), EPP85-181RDB (C, D), EPP85-181RNOV (E, F) and EPP85-181P (G, H). One-way ANOVA with Dunnett's corrections was used for comparison between Control (Ctrl, non-incubated with catechin) and groups treated with catechin for 2 h (light green), 6 h (medium green) and 24 h (dark green) (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ****<0,0001).

PDAC cell lines. The level of Pgp lowered in EPP85-181P, EPP85-181RDB, and HPAF-II cells. EPP85-181RNOV showed no Pgp immunoreactivity prior to and after catechin treatment, which is in agreement with previously tested mRNA levels (Fig. 6). Catechin incubation lowered MRP1 levels in all three EPP85-181 cell lines but had no impact on MRP1 in HPAF-II cells. BCRP levels were lowered only moderately and only in EPP85-181RDB and EPP85-181RNOV cells. The catechin effects on LRP were also weaker when compared to Pgp and MRP1,

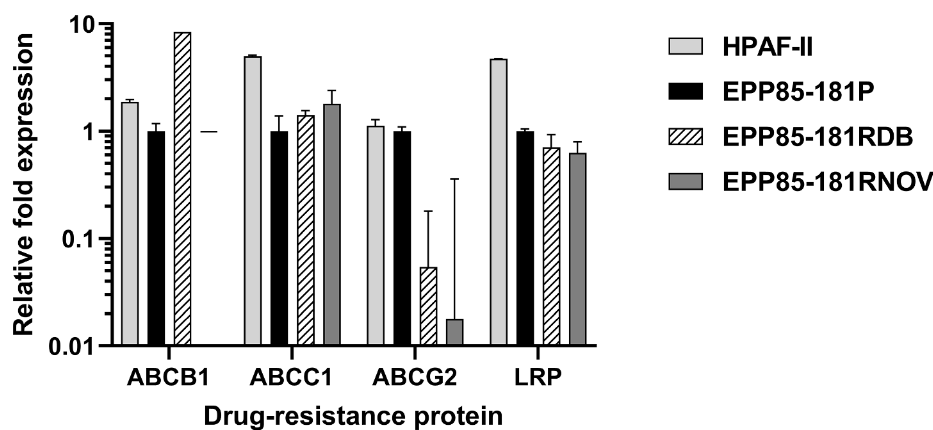


Fig. 6. Relative mRNA expression of genes associated with drug resistance: *ABCB1*, *ABCC1*, *ABCG2,* and *LRP* in four cell lines of pancreatic cancer: HPAF-II, EPP85-181P, EPP85-181RDB, and EPP85-181RNOV. No expression of *ABCB1* was detected in EPP85-181RNOV which was demonstrated with a thin line.

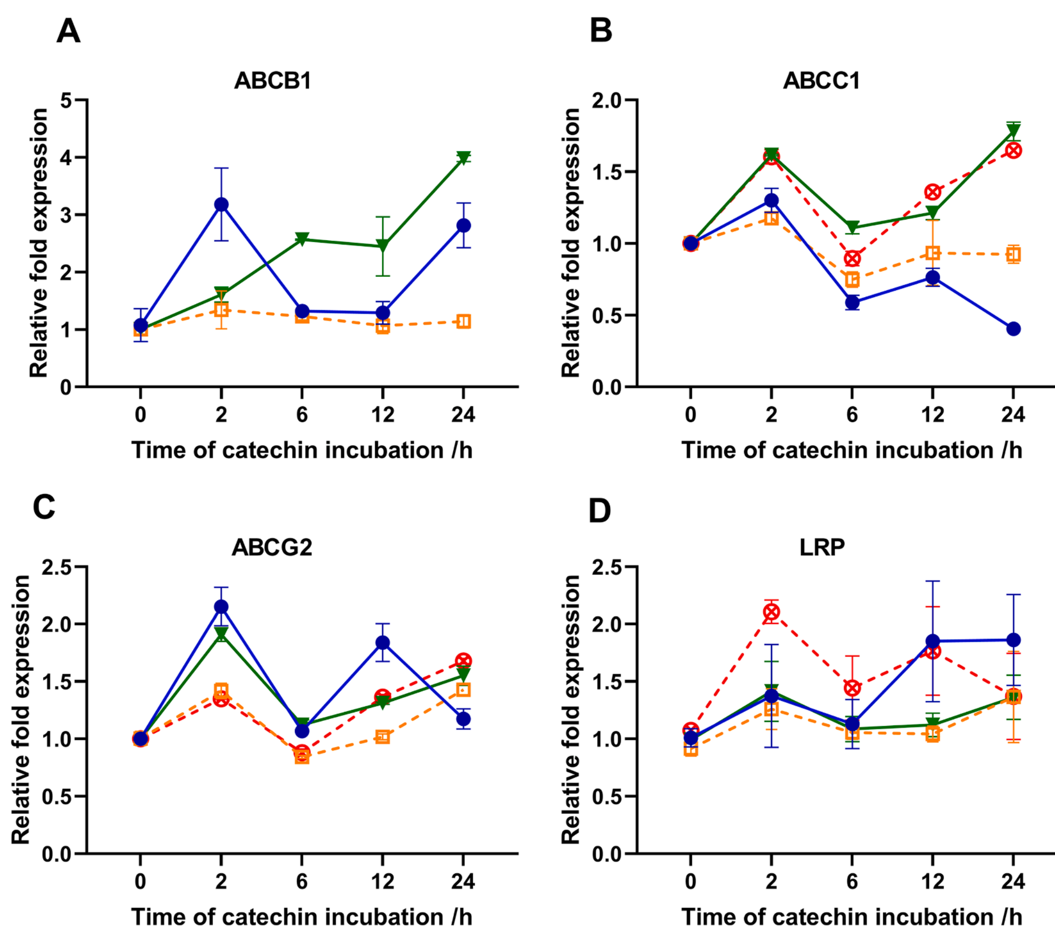


Fig. 7. Relative mRNA expression of genes associated with drug resistance: ABCB1, ABCC1, ABCG2, and LRP following the incubation with 50 μM catechin in four cell lines of pancreatic cancer: HPAF-II (respresented by a blue, solid line and filled circles), EPP85-181P II (represented by an orange, dashed line and empty squares), EPP85-181RDB (represente by a green, solid line and filled triangles) EPP85-181RNOV (respresented by a red, dashed line and empty circles).

causing a slight decrease in LRP immunoreactivity in EPP85-181P and EPP85-181RNOV cell lines.

4. Discussion

In the current study, we demonstrated that catechin is a low toxic compound with multidirectional action on pancreatic cancer cells. The catechin concentration of < 100 μM was nontoxic, which stands in agreement with other *in vitro* studies [35,56]. Our MD simulations show that the catechin molecule binds to the membrane in the lipid-water interphase. Similar to our previous studies, the transmembrane transport of such compounds is not enhanced via electroporation [57]. However, the additional molecules in the cell membrane may modulate its integrity and stability and thus affect the efficiency of electroporation.

Our study demonstrates that catechin molecules are located in the membrane between thick and thin regions, just before the formation of the pore. The possible explanation of the curve-inducing effect of

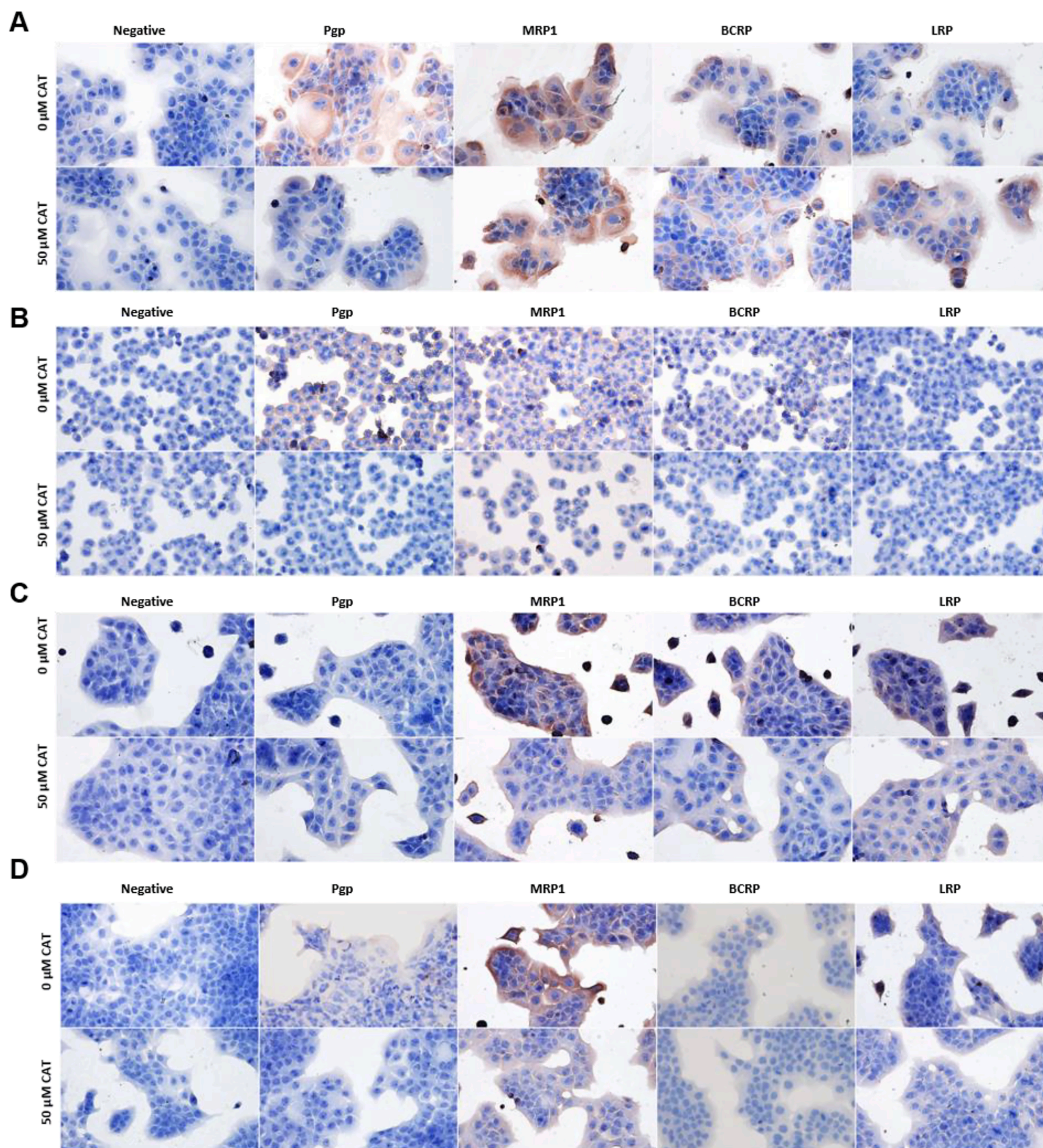


Fig. 8. Representative photographs of the immunoreactivity of Pgp, MRP1, BCRP, and LRP proteins in four cell lines of pancreatic cancer: HPAF-II (A), EPP85-181RDB (B), EPP85-181RNOV (C), and EPP85-181P (D) following a 6-hour incubation with 50 μM catechin.

catechin is the release of vesicles by the cell. Indeed, a similar fact was observed by Tamba *et al.* who proved that the binding of catechin to the external monolayer of a giant unilamellar vesicle increased its membrane area, simultaneously inducing an increase in the membrane's surface tension [49]. Namely, catechin localizes between thick and thin regions, showing the curvature-inducing properties of the compound. According to our previous studies, under normal conditions, the higher the surface tension, the lower the transmembrane voltage required to induce the formation of the pore. The same tendency is observed independently in the cholesterol content of the membrane [45]. Moreover, Casado *et al.* proved the effects of modified catechin in perturbing the effect of gel-to-fluid transition of the membrane [58]. Our flow cytometry studies show that catechin may influence the electroporation threshold but this applied only to half of the tested cell lines hence it cannot be the only mechanism of catechin action that supports ECT efficacy. The observed change in HPAF-II size suggests that catechin may trigger the release of intracellular molecules, which corresponds with the differential scanning calorimetry (DSC) studies by Selvaraj et al. showing that catechins, similar to other flavonoids - rutin, epicatechin, and epigallocatechin - can control liposome vesicles [59].

The possible implication of the vesicles release from cells is decreasing the content of membrane-bound antigens, including proteins

responsible for drug resistance. There is abundant evidence that natural compounds such as green tea flavonoids have an impact on drug resistance and may sensitize cells to chemotherapeutics [60]. Nozhat *et al.* showed a promising potential of apigenin to overcome drug resistance and enhance the anti-cancer properties [61]. Ye *et al.* demonstrated flavonoids as multi-functional agents as key factors contributing to the decreased expression of MDR genes [62]. Following real-time PCR analysis, we found that catechin incubation exerts effects on the mRNA levels of genes associated with drug resistance: ABCB1, ABCC1, ABCG2, and LRP. The initial upsurge in mRNA levels after 2 h of catechin incubation may be related to cell compensation of the antigen loss following exocytosis. Whilst catechin incubation affected the size and cell granularity only in HPAF-II cells, it cannot be ruled out that the exocytosis phenomenon also occurred in other cell lines. However, the extent of exocytosis, what type of vesicles are released, and their exact content remains a matter of a separate investigation.

Using the immunocytochemical staining we found that 6-hour incubation with catechin can attenuate the immunoreactivity of proteins encoded by the aforementioned genes, namely: Pgp, MRP1, BCRP, and LRP. Similar effects on Pgp, BCRP, and LRP were shown by Przystupski *et al.* (2018) on multidrug-resistant ovarian cancer SKOV-3 cells [38]. Many studies focused on the effects of catechin gallate esters on drug resistance. The suppression of Pgp by ECG or EGCG was confirmed *in vitro* in human hepatocellular carcinoma [63], breast cancer [64], gastric cancer [65], and finally, pancreatic cancer [66]. In our study, there was no clear link between mRNA levels of tested drug resistance proteins and outcomes of ECT with cisplatin. 2-hour incubation with catechin caused an increase in relative mRNA levels in all of the tested cell lines, but at the same time, the efficacy of ECT with cisplatin in EPP85-181P and EPP85-181RNOVcells increased. The increase in mRNA levels of genes encoding for drug resistance proteins may result from the loss of these proteins after the treatment with catechin, however another possible mechanism is the physical blockage of drug resistance proteins causing its malfunction. The recently synthesized stereoisomers of methylated catechin and its esters were shown to inhibit the Pgp mediated drug efflux leading to a restoration of the intracellular drug concentration to a cytotoxic level [35]. They appeared to be specific towards Pgp but not towards MRP-1 and BCRP. Hong et. al. (2003) demonstrated that EGCG and its methyl metabolites are substrates for MRP1 and MRP2, but not for Pgp [67].

In the present study, we examined if the catechin incubation sensitizes PDAC cells to electrochemotherapy with cisplatin and calcium ions. The most favorable effects were obtained for the multidrug-resistant cell line EPP85-181RNOV, however, catechin incubation was equally effective for cisplatin and calcium electroporation. The main limitation of the study is that ECT is performed with calcium chloride and cisplatin. Both are small compounds that can be easily transported through electropores and already found clinical application in the treatment of pancreatic cancer [16]. However, the effect of catechin on calcium channels is unknown and there might be possible interaction between catechin and calcium transporters that may influence ECT efficacy. Calcium ions allow to track permeabilization, but their penetration is independent of drug resistance proteins. On the other hand, drug resistance to cisplatin in pancreatic cancer is multifactorial. Therefore, even silencing the expression of tested proteins may result in a lack of increased ECT efficacy and conversely – ECT still may be boosted without catechin influence on drug resistance proteins. Shervington *et al.* showed that treatment with EGCG sensitized glioma cells to cisplatin via a change in the expression of telomerase [68], which is also considered a target for pancreatic cancer treatment [69]. In all tested cell lines, short catechin incubation affected cell mortality after electroporation – 48 h after incubation viability for cells incubated with CAT for 2 h dropped in all tested cell lines. At the same time, MD simulations showed that catechin impacted the membrane properties. Altogether, it may be concluded that catechin can sensitize cell membrane to electroporation but increased permeability is not the only mechanism involved. It cannot also be excluded that increased toxicities of cisplatin and calcium are achieved through separate membrane-related mechanisms such as influence on membrane repair or ionic channels.

In summary, using the theoretical and experimental approach we demonstrated that catechin molecules interact with the plasma membrane, influencing its properties. Catechin was found to reduce the reactivity of Pgp, MRP1, BCRP, and LRP proteins, but this reduction did not influence the efficacy of ECT on PDAC cells. On a contrary, we observed the increased efficacy of ECT with calcium and cisplatin, which was linked with the increased permeabilization and the increased sensitization of PDAC cells to a drug following catechin treatment.

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Declaration of Competing Interest

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

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References

[1] P. Sarantis, E. Koustas, A. Papadimitropoulou, A.G. Papavassiliou, M. V. Karamouzis, Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvironment and immunotherapy, World J. Gastrointest. Oncol. 12 (2) (2020) 173–181, https://doi.org/10.4251/wjgo.v12.i2.173.

[2] A. Vincent, J. Herman, R. Schulick, R.H. Hruban, M. Goggins, Pancreatic cancer, in, Lancet (2011) 607–620, https://doi.org/10.1016/S0140-6736(10)62307-0.

[3] A. McGuigan, P. Kelly, R.C. Turkington, C. Jones, H.G. Coleman, R.S. McCain, Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes, World J. Gastroenterol. 24 (2018) 4846–4861, https://doi.org/10.3748/wjg.v24.i43.4846.

[4] M. Hidalgo, Pancreatic cancer, N. Engl. J. Med. 362 (17) (2010) 1605–1617, https://doi.org/10.1056/NEJMra0901557.

[5] Z. Wang, Y. Li, A. Ahmad, S. Banerjee, A.S. Azmi, D. Kong, F.H. Sarkar, Pancreatic cancer: Understanding and overcoming chemoresistance, Nat. Rev. Gastroenterol. Hepatol. 8 (2011) 27–33, https://doi.org/10.1038/nrgastro.2010.188.

[6] J. Long, Y. Zhang, X. Yu, J. Yang, D.G. LeBrun, C. Chen, Q. Yao, M. Li, Overcoming drug resistance in pancreatic cancer, Expert Opin. Ther. Targets. 15 (2011) 817–828, https://doi.org/10.1517/14728222.2011.566216.

[7] R. Heller, R. Gilbert, M.J. Jaroszeski, Clinical applications of electrochemotherapy, Adv. Drug Deliv. Rev. 35 (1) (1999) 119–129, https://doi.org/10.1016/S0169-409X(98)00067-2.

[8] L.M. Mir, S. Orlowski, Mechanisms of electrochemotherapy, Adv. Drug Deliv. Rev. 35 (1999) 107–118, https://doi.org/10.1016/S0169-409X(98)00066-0.

[9] G. Sersa, D. Miklavcic, M. Cemazar, Z. Rudolf, G. Pucihar, M. Snoj, Electrochemotherapy in treatment of tumours, Eur. J. Surg. Oncol. 34 (2008) 232–240, https://doi.org/10.1016/J.EJSO.2007.05.016.

[10] L.M. Mir, S. Orlowski, The Basis of Electrochemotherapy, Electrochemother. Electrogenether. Transdermal Drug Deliv. (2000) 99–117, https://doi.org/10.1385/1-59259-080-2:99.

[11] U. Reinhold, Electrochemotherapy for primary skin cancer and skin metastasis related to other malignancies, Anticancer, Drugs. 22 (2011) 711–718, https://doi.org/10.1097/CAD.0b013e32834618da.

[12] M.J. Jaroszeski, P. Illingworth, C. Pottinger, M. Hyacinthe, R. Heller, Electrically mediated drug delivery for treating subcutaneous and orthotopic pancreatic adenocarcinoma in a hamster model, Anticancer Res. 19 (1999) 989–994.

[13] J. Gehl, G. Sersa, L.W. Matthiessen, T. Muir, D. Soden, A. Occhini, P. Quaglino, P. Curatolo, L.G. Campana, C. Kunte, A.J.P. Clover, G. Bertino, V. Farricha, J. Odili, K. Dahlstrom, M. Benazzo, L.M. Mir, Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases, Acta Oncol, (Madr) 57 (7) (2018) 874–882, https://doi.org/10.1080/0284186X.2018.1454602.

[14] F. Izzo, V. Granata, R. Fusco, V. D'Alessio, A. Petrillo, S. Lastoria, M. Piccirillo, V. Albino, A. Belli, S. Tafuto, A. Avallone, R. Patrone, R. Palaia, Clinical phase I/II study: Local disease control and survival in locally advanced pancreatic cancer treated with electrochemotherapy, J. Clin. Med. 10 (6) (2021) 1305, https://doi.org/10.3390/jcm10061305.
[15] N. Bhutiani, Y. Li, Q. Zheng, H. Pandit, X. Shi, Y. Chen, Y. Yu, Z.R. Pulliam, M. Tan, R.C.G. Martin, Electrochemotherapy with Irreversible Electroporation and FOLFIRINOX Improves Survival in Murine Models of Pancreatic Adenocarcinoma, Ann. Surg. Oncol. 27 (11) (2020) 4348–4359, https://doi.org/10.1245/s10434-020-08782-2.
[16] J. Rudno-Rudzińska, W. Kielan, M. Guziński, M. Płochocki, A. Antończyk, J. Kulbacka, New therapeutic strategy: Personalization of pancreatic cancer treatment-irreversible electroporation (IRE), electrochemotherapy (ECT) and calcium electroporation (CaEP) – A pilot preclinical study, Surg. Oncol. 38 (2021) 101634, https://doi.org/10.1016/j.suronc.2021.101634.
[17] P. Fernandes, T.R. O'Donovan, S.L. McKenna, P.F. Forde, Electrochemotherapy causes caspase-independent necrotic-like death in pancreatic cancer cells, Cancers (Basel). 11 (8) (2019) 1177, https://doi.org/10.3390/cancers11081177.
[18] T.J. O'Brien, M. Bonakdar, S. Bhonsle, R.E. Neal, C.H. Aardema, J.L. Robertson, S. N. Goldberg, R.V. Davalos, Effects of internal electrode cooling on irreversible electroporation using a perfused organ model, Int. J. Hyperth. 35 (1) (2018) 44–55, https://doi.org/10.1080/02656736.2018.1473893.
[19] O. Michel, D. Przystupski, J. Saczko, A. Szewczyk, N. Niedzielska, J. Rossowska, J. Kulbacka, The favourable effect of catechin in electrochemotherapy in human pancreatic cancer cells, Acta Biochim. Pol. 65 (2018) 173–184, https://doi.org/10.18388/abp.2018_2602.
[20] C. Braicu, M.R. Ladomery, V.S. Chedea, A. Irimie, I. Berindan-Neagoe, The relationship between the structure and biological actions of green tea catechins, Food Chem. 141 (3) (2013) 3282–3289, https://doi.org/10.1016/j.foodchem.2013.05.122.
[21] R. Tsao, Chemistry and biochemistry of dietary polyphenols, Nutrients. 2 (12) (2010) 1231–1246, https://doi.org/10.3390/nu2121231.
[22] J. Bernatoniene, D. Kopustinskiene, The Role of Catechins in Cellular Responses to Oxidative Stress, Molecules. 23 (4) (2018) 965, https://doi.org/10.3390/molecules23040965.
[23] W. Bors, W. Heller, C. Michel, M. Saran, Flavonoids as antioxidants: Determination of radical-scavenging efficiencies, Methods Enzymol. (1990), https://doi.org/10.1016/0076-6879(90)86128-I.
[24] I. Kalai Selvi, S. Nagarajan, Separation of catechins from green tea (Camellia sinensis L.) by microwave assisted acetylation, evaluation of antioxidant potential of individual components and spectroscopic analysis, LWT - Food Sci. Technol. 91 (2018) 391–397, https://doi.org/10.1016/j.lwt.2018.01.042.
[25] S.A. Cherrak, N. Mokhtari-Soulimane, F. Berroukeche, B. Bensenane, A. Cherbonnel, H. Merzouk, M. Elhabiri, J. Chamani, In vitro antioxidant versus metal ion chelating properties of flavonoids: A structure-activity investigation, PLoS One. 11 (10) (2016) e0165575, https://doi.org/10.1371/journal.pone.0165575.
[26] M. Mochizuki, S.-I. Yamazaki, K. Kano, T. Ikeda, Kinetic analysis and mechanistic aspects of autoxidation of catechins, Biochim. Biophys. Acta - Gen. Subj. 1569 (1–3) (2002) 35–44, https://doi.org/10.1016/S0304-4165(01)00230-6.
[27] S. Shishido, R. Miyano, T. Nakashima, H. Matsuo, M. Iwatsuki, K. Nakamura, T. Kanno, H. Egusa, Y. Niwano, A novel pathway for the photooxidation of catechin in relation to its prooxidative activity, Sci. Rep. 8 (2018), https://doi.org/10.1038/s41598-018-31195-x.
[28] C. Kürbitz, D. Heise, T. Redmer, F. Goumas, A. Arlt, J. Lemke, G. Rimbach, H. Kalthoff, A. Trauzold, Epicatechin gallate and catechin gallate are superior to epigallocatechin gallate in growth suppression and anti-inflammatory activities in pancreatic tumor cells, Cancer Sci. 102 (4) (2011) 728–734, https://doi.org/10.1111/j.1349-7006.2011.01870.x.
[29] D. Porath, C. Riegger, J. Drewe, J. Schwager, Epigallocatechin-3-gallate impairs chemokine production in human colon epithelial cell lines, J. Pharmacol. Exp. Ther. 315 (3) (2005) 1172–1180, https://doi.org/10.1124/jpet.105.090167.
[30] T.W. Sirk, E.F. Brown, A.K. Sum, M. Friedman, Molecular Dynamics Study on the Biophysical Interactions of Seven Green Tea Catechins with Lipid Bilayers of Cell Membranes, J. Agric. Food Chem. 56 (17) (2008) 7750–7758, https://doi.org/10.1021/jf8013298.
[31] H. Tsuchiya, Stereospecificity in membrane effects of catechins, Chem. Biol. Interact. 134 (2001) 41–54, https://doi.org/10.1016/S0009-2797(00)00308-2.
[32] F.M.S. Gomes, J. da Cunha Xavier, J.F.S. dos Santos, Y.M.L.S. de Matos, S. R. Tintino, T.S. de Freitas, H.D.M. Coutinho, Evaluation of antibacterial and modifying action of catechin antibiotics in resistant strains, Microbial Pathogenesis 115 (2018) 175–178, https://doi.org/10.1016/j.micpath.2017.12.058.
[33] Y. Ning, J. Ling, C.D. Wu, Synergistic effects of tea catechin epigallocatechin gallate and antimycotics against oral Candida species, Arch. Oral Biol. 60 (10) (2015) 1565–1570, https://doi.org/10.1016/j.archoralbio.2015.07.001.
[34] S. Kitagawa, T. Nabekura, S. Kamiyama, Inhibition of P-glycoprotein function by tea catechins in KB-C2 cells, J. Pharm. Pharmacol. 56 (2004) 1001–1005, https://doi.org/10.1211/0022357044003.
[35] I.L.K. Wong, X.-K. Wang, Z. Liu, W. Sun, F.-X. Li, B.-C. Wang, P. Li, S.-B. Wan, L.M. C. Chow, Synthesis and evaluation of stereoisomers of methylated catechin and epigallocatechin derivatives on modulating P-glycoprotein-mediated multidrug resistance in cancers, Eur. J. Med. Chem. 226 (2021) 113795, https://doi.org/10.1016/j.ejmech.2021.113795.
[36] G. Li, tea catechins augment the antitumor activity of doxorubicin in an in vivo mouse model for chemoresistant liver cancer, Int. J. Oncol. 37 (2010), https://doi.org/10.3892/ijo_00000659.
[37] H.-H. Tang, M. Zhou, G. Liang, Impact of epigallocatechin gallate on gene expression profiles of human hepatocellular carcinoma cell lines BEL7404/ADM and BEL7402/5-FU, Ai Zheng. 27 (2008) 1056–1064.
[38] D. Przystupski, O. Michel, J. Rossowska, S. Kwiatkowski, J. Saczko, J. Kulbacka, The modulatory effect of green tea catechin on drug resistance in human ovarian cancer cells, Med. Chem. Res. 28 (5) (2019) 657–667, https://doi.org/10.1007/s00044-019-02324-6.
[39] Y.S. Tarahovsky, E.N. Muzafarov, Y.A. Kim, Rafts making and rafts braking: How plant flavonoids may control membrane heterogeneity, Mol. Cell. Biochem. 314 (2008) 65–71, https://doi.org/10.1007/S11010-008-9766-9/FIGURES/5.
[40] H.A. Scheidt, A. Pampel, L. Nissler, R. Gebhardt, D. Huster, Investigation of the membrane localization and distribution of flavonoids by high-resolution magic angle spinning NMR spectroscopy, Biochim. Biophys. Acta. 1663 (2004) 97–107, https://doi.org/10.1016/J.BBAMEM.2004.02.004.
[41] C. Van Dijk, A.J.M. Driessen, K. Recourt, The uncoupling efficiency and affinity of flavonoids for vesicles, Biochem. Pharmacol. 60 (2000) 1593–1600, https://doi.org/10.1016/S0006-2952(00)00488-3.
[42] R. Reigada, Electroporation of heterogeneous lipid membranes, Biochim. Biophys. Acta - Biomembr. 2014 (1838) 814–821, https://doi.org/10.1016/J.BBAMEM.2013.10.008.
[43] L. Rems, X. Tang, F. Zhao, S. Pérez-Conesa, I. Testa, L. Delemotte, Identification of electroporation sites in the complex lipid organization of the plasma membrane, Elife. 11 (2022). doi:10.7554/ELIFE.74773.
[44] H.B. Kim, S. Lee, Y. Shen, P.D. Ryu, Y. Lee, J.H. Chung, C.K. Sung, K.Y. Baik, Physicochemical factors that affect electroporation of lung cancer and normal cell lines, Biochem. Biophys. Res. Commun. 517 (2019) 703–708, https://doi.org/10.1016/J.BBRC.2019.07.119.
[45] W. Szlasa, A. Kiełbik, A. Szewczyk, V. Novickij, M. Tarek, Z. Łapińska, J. Saczko, J. Kulbacka, N. Rembiałkowska, Atorvastatin modulates the efficacy of electroporation and calcium electrochemotherapy, Int. J. Mol. Sci. 22 (20) (2021) 11245, https://doi.org/10.3390/ijms222011245.
[46] Y.S. Hwang, K.-K. Park, W.-Y. Chung, Epigallocatechin-3 gallate inhibits cancer invasion by repressing functional invadopodia formation in oral squamous cell carcinoma, Eur. J. Pharmacol. 715 (1–3) (2013) 286–295, https://doi.org/10.1016/j.ejphar.2013.05.008.
[47] H.B. Kim, S. Lee, J.H. Chung, S.N. Kim, C.K. Sung, K.Y. Baik, Effects of Actin Cytoskeleton Disruption on Electroporation In Vitro, Appl. Biochem. Biotechnol. 191 (2020) 1545–1561, https://doi.org/10.1007/s12010-020-03271-4.
[48] G. Saulis, M.S. Venslauskas, J. Naktinis, Kinetics of pore resealing in cell membranes after electroporation, J. Electroanal. Chem. 321 (1) (1991) 1–13, https://doi.org/10.1016/0022-0728(91)85564-6.
[49] Y. Tamba, S. Ohba, M. Kubota, H. Yoshioka, H. Yoshioka, M. Yamazaki, Single GUV method reveals interaction of tea catechin (2)-epigallocatechin gallate with lipid membranes, Biophys. J. 92 (2007), https://doi.org/10.1529/biophysj.106.097105.
[50] M.J. Abraham, T. Murtola, R. Schulz, S. Páll, J.C. Smith, B. Hess, E. Lindahl, Gromacs: High performance molecular simulations through multi-level parallelism from laptops to supercomputers, SoftwareX. 1-2 (2015) 19–25, https://doi.org/10.1016/j.softx.2015.06.001.
[51] W. Humphrey, A. Dalke, K. Schulten, VMD: Visual molecular dynamics, J. Mol. Graph. 14 (1) (1996) 33–38, https://doi.org/10.1016/0263-7855(96)00018-5.
[52] S. Jo, T. Kim, V.G. Iyer, W. Im, CHARMM-GUI: A web-based graphical user interface for CHARMM, J. Comput. Chem. 29 (11) (2008) 1859–1865, https://doi.org/10.1002/jcc.20945.
[53] K. Vanommeslaeghe, E. Hatcher, C. Acharya, S. Kundu, S. Zhong, J. Shim, E. Darian, O. Guvench, P. Lopes, I. Vorobyov, A.D. Mackerell, CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields, J. Comput. Chem. (2009), https://doi.org/10.1002/jcc.21367.
[54] R. Guixà-González, I. Rodriguez-Espigares, J.M. Ramírez-Anguita, P. Carrió-Gaspar, H. Martinez-Seara, T. Giorgino, J. Selent, MEMBPLUGIN: Studying membrane complexity in VMD, Bioinformatics. 30 (10) (2014) 1478–1480, https://doi.org/10.1093/bioinformatics/btu037.
[55] S. Romeo, A. Sannino, M.R. Scarfì, P.T. Vernier, R. Cadossi, J. Gehl, O. Zeni, ESOPE-Equivalent Pulsing Protocols for Calcium Electroporation: An In Vitro Optimization Study on 2 Cancer Cell Models, Technol. Cancer Res. Treat. 17 (2018), https://doi.org/10.1177/1533033818788072.
[56] M. Zor, S. Aydin, N.D. Güner, N. Başaran, A.A. Başaran, Antigenotoxic properties of Paliurus spina-christi Mill fruits and their active compounds, BMC Complement. Altern. Med. 17 (2017), https://doi.org/10.1186/s12906-017-1732-1.
[57] W. Szlasa, A. Szewczyk, M. Drąg-Zalesińska, H. Czapor-Irzabek, O. Michel, A. Kiełbik, K. Cierluk, A. Zalesińska, V. Novickij, M. Tarek, J. Saczko, J. Kulbacka, Mechanisms of curcumin-based photodynamic therapy and its effects in combination with electroporation: An in vitro and molecular dynamics study, Bioelectrochemistry. 140 (2021) 107806, https://doi.org/10.1016/j.bioelechem.2021.107806.
[58] F. Casado, J. Teruel, S. Casado, A. Ortiz, J. Rodríguez-López, F. Aranda, Location and effects of an antitumoral catechin on the structural properties of phosphatidylethanolamine membranes, Molecules. 21 (7) (2016) 829, https://doi.org/10.3390/molecules21070829.
[59] S. Selvaraj, S. Krishnaswamy, V. Devashya, S. Sethuraman, U.M. Krishnan, Influence of membrane lipid composition on flavonoid–membrane interactions: Implications on their biological activity, Prog. Lipid Res. 58 (2015) 1–13, https://doi.org/10.1016/J.PLIPRES.2014.11.002.
[60] A. Liskova, M. Samec, L. Koklesova, A. Brockmueller, K. Zhai, B. Abdellatif, M. Siddiqui, K. Biringer, E. Kudela, M. Pec, L.K. Gadanec, M. Šudomová, S.T.S. Hassan, A. Zulli, M. Shakibaei, F.A. Giordano, D. Büsselberg, O. Golubnitschaja, P. Kubatka,

Flavonoids as an effective sensitizer for anti-cancer therapy: insights into multi-faceted mechanisms and applicability towards individualized patient profiles, EPMA J. 2021 122. 12 (2021) 155–176. doi:10.1007/S13167-021-00242-5.
[61] Z. Nozhat, S. Heydarzadeh, Z. Memariani, A. Ahmadi, Chemoprotective and chemosensitizing effects of apigenin on cancer therapy, Cancer Cell Int. 2021 211. 21 (2021) 1–26. doi:10.1186/S12935-021-02282-3.
[62] Q. Ye, K. Liu, Q. Shen, Q. Li, J. Hao, F. Han, R.W. Jiang, Reversal of Multidrug Resistance in Cancer by Multi-Functional Flavonoids, Front. Oncol. 9 (2019) 487, https://doi.org/10.3389/FONC.2019.00487.
[63] G. Liang, S. Zhang, Z.M. Huang, A.Z. Tang, MDR-reversing effect of two components of catechin on human hepatocellular carcinoma BEL-7404/Adr in vitro, Ai Zheng (2004).
[64] A. Zhu, X. Wang, Z. Guo, Study of tea polyphenol as a reversal agent for carcinoma cell lines ' multidrug resistance (study of TP as a MDR reversal agent), Nucl. Med. Biol. 28 (6) (2001) 735–740, https://doi.org/10.1016/S0969-8051(00)90202-6.
[65] H. Tang, L. Zeng, J. Wang, X. Zhang, Q. Ruan, J. Wang, S. Cui, D. Yang, Reversal of 5-fluorouracil resistance by EGCG is mediate by inactivation of TFAP2A / VEGF signaling pathway and down- regulation of MDR-1 and P-gp expression in gastric cancer, Oncotarget. 8 (47) (2017) 82842–82853, https://doi.org/10.18632/oncotarget.20666.
[66] Z. Zhu, Y. Wang, Z. Liu, F. Wang, Q. Zhao, Inhibitory effects of epigallocatechin-3-gallate on cell proliferation and the expression of HIF-1α and P-gp in the human pancreatic carcinoma cell line PANC-1, Oncol. Rep. 27 (2012), https://doi.org/10.3892/or.2012.1697.
[67] J. Hong, J.D. Lambert, S.H. Lee, P.J. Sinko, C.S. Yang, Involvement of multidrug resistance-associated proteins in regulating cellular levels of (-)-epigallocatechin-3-gallate and its methyl metabolites, Biochem. Biophys. Res. Commun. 310 (2003) 222–227, https://doi.org/10.1016/j.bbrc.2003.09.007.
[68] A. Shervington, V. Pawar, S. Menon, D. Thakkar, R. Patel, The sensitization of glioma cells to cisplatin and tamoxifen by the use of catechin, Mol. Biol. Rep. 36 (5) (2009) 1181–1186, https://doi.org/10.1007/s11033-008-9295-3.
[69] N. Sato, K. Mizumoto, E. Nagai, M. Tanaka, Telomerase as a new target for pancreatic cancer treatment, J. Hepatobiliary. Pancreat. Surg. 9 (3) (2002) 322–327, https://doi.org/10.1007/s005340200036.