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Redox-related Molecular Mechanism of Sensitizing Colon Cancer Cells to Camptothecin Analog SN38

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Abstract. *Background/Aim: The aim of this study was to elucidate the possibility of sensitizing colon cancer cells to the chemotherapeutic drug SN38 and investigate its mechanism of action after combined treatment with electroporation (EP). Materials and Methods: Cells were treated with SN38, EP and their combination for 24/48 h. The cell viability, actin cytoskeleton integrity, mitochondrial superoxide, hydroperoxides, total glutathione, phosphatidyl serine expression, DNA damages and expression of membrane ABC transporters were analyzed using conventional analytical tests. Results: The combination of EP and SN38 affected cell viability and cytoskeleton integrity. This effect was accompanied by: (i) high production of intracellular superoxide and hydroperoxides and depletion of glutathione; (ii) increased DNA damage and apoptotic/ferroptotic cell death; (iii) changes in the expression of membrane ABC transporters – up-regulation of SLCO1B1 and retention of SN38 in the cells. Conclusion: The anticancer effect of the combined treatment of SN38 and EP is related to changes in the redox-homeostasis of cancer cells, leading to cell death via apoptosis and/or ferroptosis. Thus, electroporation has a potential to increase the sensitivity of cancer cells to conventional anticancer therapy with SN38.*

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Colorectal cancer is one of the most aggressive cancers (1). It is a widespread malignant disease in industrialized countries. The clarification of its mechanism and the development of new therapeutic methods and techniques are of great interest nowadays.

Chemotherapy with the semisynthetic pro-drug irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin; CPT-11) is one of the common therapeutic strategies in colorectal cancer. In 1996, irinotecan was approved for clinical use both in the United States and Japan (2, 3). So far, it is widely used in first- and second-line treatment of advanced colorectal cancer. Irinotecan is a water-soluble camptothecin analogue – alkaloidal derivative from Chinese tree plant (*Camptotheca acuminata*) (4). Bioactivation of CPT-11 in cells to its active metabolite SN38 is performed *via* a carboxylesterase-catalyzed reaction (4). SN38 has a 100- to 1,000-fold greater anticancer activity than irinotecan (4, 5). Furthermore, SN38 is a potent DNA topoisomerase I (Topo-I) inhibitor, acting *via* formation of a stable Topo-I-DNA cleavable complex, subsequently causing DNA damage (resulting in cell cycle arrest and/or cell death by apoptosis) (4).

It is widely accepted that apoptosis is an energy-dependent process, which is characterized by early release of mitochondrial cytochrome c; activation of apoptotic protease activating factor 1 (APAF-1); and activation of caspase-9, ending with degradation of cellular proteins, such as PARP, laminin, and β-actin (hallmarks for programmed cell death). Induction of apoptosis is dependent on the cellular redox-status, which is essential for cell viability. Cellular redox-status is defined as a balance between the main endogenous

pro-oxidants [reactive oxygen species (ROS); reactive nitrogen species (RNS); transition metals] and reducing equivalents [antioxidant systems; thiol-containing proteins; and cofactors such as NADH and NADPH], which maintain ROS/RNS within physiological concentrations. Under physiological conditions, the intracellular level of ROS has a key role in the regulation of cell response through modulation of signaling pathways, thereby influencing the synthesis of antioxidant enzymes, repair processes, inflammation, and proliferation. ROS have been implicated also in the anticancer effects of conventional chemotherapeutics (6). It is well-known that cancer is a free radical disease, which could be explained by a number of phenomena as: induction of oxidative stress, mitochondrial dysfunction, impairment of redox-signaling, *etc*. (7). Recent studies have reported that SN38 is characterized by cell cycle-dependent cytotoxicity and induces caspase-dependent apoptotic death *via* a ROS-mediated mechanism (4, 8, 9).

Another conventional therapeutic technique in cancer is electrochemotherapy. It is used to increase the concentration of drugs in cancer lesions by electroporation (10, 11). In the last 3 years, several studies have shown a successful application of electroporation for local treatment of solid tumors, which are inoperable because of a high risk of massive blood loss, and/or influence on life-threatening organs (11, 12). The efficiency of the applied electrical pulses is due mainly to the increased internalization and accumulation of the anticancer drug in cancer cells and the promoted cytostatic and cytotoxic effects.

It has been proven that electrical pulses perturb membranes of cultured mammalian cells in a dose-dependent manner, thus, affecting their viability (13). The field intensity and duration can either reversibly open pores on the cell membrane after treatment or irreversibly damage membrane integrity. In cancer treatment, the reversible electroporation has been exploited to increase internalization of chemotherapeutics into the target cells (10). Apart from the effect on the cell membrane (opening of membrane pores), the applied external electrical pulses can alter the cytoskeletal integrity. The cytoskeleton is essential for cellular shape, intracellular biochemical networks and cellular viability (14).

One of the major and contemporary challenges in cancer therapy is the development of multidrug resistance (MDR) to conventional and/or new generation chemotherapeutics. Ninety percent of failures in conventional cancer chemotherapy result from rapid invasion and metastases due to MDR (15). MDR is advanced by triggering a wide range of cellular mechanisms, such as: inactivation of the active drug ingredients; modification of drug target; quantitative reduction of drug uptake into the cancer cells; increased drug efflux; activation of different detoxifying/neutralizing systems; activation of DNA repair mechanisms; overcoming the drug-induced apoptosis/cell death, and others (16, 17). Acceleration of drug efflux, and respectively, reduction in drug accumulation, is considered to be one of the main mechanisms of MDR in various cancers (16). A family of transmembrane proteins called ATP-binding cassette (ABC) transporters is responsible for this process (18). In most cases, the development of MDR is associated with increased expression of ABC transporters, leading to accelerated elimination of the drug from cancer cells, as well as antibiotics from resistant bacterial strains. Despite extensive studies on MDR and its characterization *in vitro*, this knowledge has not been successfully transferred to the clinic practice. Therefore, exploring new approaches to manipulate MDR underlying mechanisms, is crucial to predicting the development of resistance and effectiveness of chemotherapy.

Based on the above-mentioned, our study was designed to investigate: (i) the impact of SN38 on the cellular redox-status and oxidative stress-related mechanism(s) of its anticancer effect; and (ii) the ability of electroporation to decrease MDR and sensitize cancer cells to SN38 by modulating cytoskeleton integrity and expression of ABC transporters.

Materials and Methods

Chemicals. Anticancer drug - SN38, propidium iodide, trypan blue (0.4% solution), triton-X-100, bovine serum albumin (BSA), paraformaldehyde (PFA), cell cultured medium - DMEM, fetal bovine serum (FBS) and trypsin-EDTA solution (0.5% of trypsin, 0.2% of EDTA) were purchased from Sigma-Aldrich (Weinheim, Germany). BODIPY558/568-phalloidin was purchased from Invitrogen. The following kits and reagents were used in this study: CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit - purchased from Promega; OxiSelect™ Intracellular Assay Kit (Green Fluorescence), OxiSelect™ Total Antioxidant Capacity (TAC), OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit and OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation) - from Cell Biolabs, Inc.; Human Solute Carrier Organic Anion Transporter Family Member 1B1(SLCO1B1) ELISA Kit - My Biosource Inc., Human Multidrug Resistance-Associated Protein 1 (ABCC1) ELISA Kit - Cusabio Technology LLC., Annexin V-FITC Apoptosis Detection Kit - BioVision, Inc. and Dihydroethidium from AAT Bioquest® Inc. All the reagents used in the experiments were „HPLC-grade".

Cell culture. All experiments were performed with Colon26 cell line (Cell Line Service, Heidelberg, Germany), established *in vitro* from the Colon26 tumor of female mice, as a model of colon carcinoma. The cell line was grown as a monolayer in DMEM medium, supplemented with 2 mM L-glutamine and 10% FBS (at 37˚C in a 5% CO_2 incubator). Cells were passaged two times weekly by trypsinization.

Treatment protocol. The described study was conducted by usage of the active metabolite SN38 of the anticancer prodrug irinotecan. For drug treatment, SN38 was diluted from a stock solution of 5 mM in dimethyl sulfoxide (DMSO). The stock was kept at -20˚C and diluted in cell culture medium (without antibiotics) of appropriate

concentration range (0.5-10 μM) before use. The final concentration of DMSO in all treated samples was below 1%. At this concentration, DMSO did not influence cell viability. Colon26 cells were trypsinized, counted, plated (1×10^5 cells per well) in duplicates, and then incubated for full adhesion (overnight). Then, the cells were treated with selected concentrations of SN38 and harvested after 24 h and 48 h incubation, respectively. Untreated cells were used as a control. Each experiment was repeated three times.

Electroporation (EP) protocol. In the electro-treatment experiments an electroporator Chemopulse IV was used. Bipolar pulses were applied using flat parallel stainless-steel electrodes with a 1 cm intra-electrode distance. Chemopulse IV is equipped with a large voltage control in the limits of 100-2200 V, simplified operations, locking against illegal manipulations and enhanced protection against electrical hazards. For the electrotreatment 16 biphasic pulses were used, each of them with a 50+50 μs duration and a 20 μs pause between both phases and an 880 μs pause between bipolar pulses.

Colon26 cells (1×10^5 cells per well) were seeded 24 h before electroporation to allow cells adherence. The anticancer drug SN38 (0.5, 2.5, and 5 μM) was added immediately before pulse application. In this study, electric pulses with an intensity of 100, 200, 500, and 1000 V/cm were applied. For fluorescent microscopy experiments, the cells were cultivated on cover glasses. The controls were treated under the same conditions, but without electric pulse application and/or addition of SN38.

Cell viability/cytotoxicity assays. Cell viability of Colon 26 cells was determined using MTS [Owen's reagent: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]. This colorimetric test is based on the conversion of tetrazolium salt (MTS) to a water-soluble product (formazan) from mitochondrial dehydrogenases. The presence of formazan represents metabolic activity and thus, viable cells. The MTS-solution was added after treatment of cells with/without electrical pulses in the absence or presence of different concentrations of SN38 after 24 h/48 h incubation. IC_{50} and cell viability were calculated after measurement of optical density values for each sample (multiplied by 8 repeats) with a spectrophotometer ("Tecan Infinite F200 PRO", Tecan, Austria).

Additionally, all cells stained with probes were further counted with Trypan blue test by Countess® Automated Cell Counter (Invitrogen) for expressing the data relative to the number of cells. Briefly, after trypsinization, 10 μl of cell suspension was added to 10 μl of trypan blue (0.4%) and incubated for 20 s. Then, 10 μl from each sample was placed in a Countess® (Invitrogen) glass chamber. The number of live/dead cells in the suspension was counted automatically. The linear range to operate with the Countess® counter was 1×10^4-5×10^6 cells/ml, and the optimal cell size was in the range of 5-60 μm.

Propidium iodide (PI) staining. Colon cancer cells (1.5×10^5 cells/ml) were seeded on cover glasses (18/18 mm) in 6-well plates. After 24-h incubation, the cells were treated in basal medium with 100 μM propidium iodide (stock solution 1 mg/ml) for 5 min at 37°C (in an incubator, at humid atmosphere and 5% CO_2) and then, electroporated with different voltages – 100, 200, 500 and 1000 V/cm. After a 15 min incubation, the samples were washed three times with phosphate-buffered saline (PBS, pH 7.4). The adhered cells were then fixed with 1 ml of 3% solution of PFA for 15 min at room temperature, followed by washing – three times with PBS and once with distilled water. The samples were installed on objective glasses by Mowiol.

Actin staining. Colon cancer cells (1.5×10^5 cells/ml) were cultivated on cover glasses (18/18 mm) placed in 6-well plates. After 24-h incubation, the cells were treated in a basal cell medium and cultivated additionally for 24 h in full cell medium. After the incubation period, non-adhered cells were removed by triple rinsing with PBS. The adhered cells were fixed with 1 ml of 3% solution of PFA for 15 min at room temperature. The fixed cells were permeabilized using 1 ml of 0.5% solution of Triton X-100 for 5 min and then incubated with 1 ml of 1% solution of BSA for 15 min. The samples were then washed three times with PBS and incubated for 30 min at room temperature with BODIPY$^{558/568}$-phalloidin. Again, the samples were washed three times with PBS and once with distilled water, and then installed on objective glasses by Mowiol.

Optical imaging in vitro. For *in vitro* imaging, the cells were grown on coverslips. Twenty-four hours after treatment, cells were fixed by 4% PFA. The coverslips were then mounted onto microscope slides using mounting media. Imaging analysis was performed *via* laser scanning confocal microscopy (Leica DM 2500, Leica Microsystems, Germany).

Dihydroethidium assay (DHE-assay). Dihydroethidium (Hydroethidine) is a fluorogenic probe, which enters the plasma membrane of living cells and interacts selectively with the intracellular superoxide, resulting in an easily detectable fluorescent product. The major advantage of this probe is its ability to distinguish between superoxide and hydrogen peroxide. According to the manufacturer's instructions, after treatment, Colon26 (1×10^6 cells/ml) cells were incubated at room temperature for 15 min with 10 μl DHE (working solution) per 100 μl cell suspension sample. Hydroethidine had been previously, dissolved in DMSO to a 65 mM stock solution (kept at –40˚C) and then, diluted with PBS to prepare 50 μM working solution in the day of experiment. After incubation, fluorescence intensity was detected immediately at λ_{ex}=518 nm and λ_{em}=605 nm by "Tecan Infinite F200 PRO" spectrofluorimeter in 96-well black microplates. The experiment was multiplied three times and the level of superoxide expressed as mean±S.D.

OxiSelect™ Intracellular ROS Assay (DCF-assay). In this assay the 2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) fluorogenic probe was utilized. It penetrates into the live cells and reacts with ROS-predominantly with hydrogen peroxide, resulting in the fluorescent product 2',7'-dichlorodihydrofluorescin (DCF). Briefly, after drug treatment and the consequent incubation of Colon26 cells with DCFH-DA, fluorescence intensity for each sample was measured at λ_{ex}=480nm and λ_{em}=530nm by "Tecan Infinite F200 PRO" spectrofluorimeter in 96-well black microplates. The ROS content in the samples was determined by comparison with the predetermined DCF standard curve. Three parallels were prepared for each sample and hydroperoxide levels were expressed as mean±S.D.

Analysis of total glutathione. The total glutathione content (GSH/GSSG) in Colon26 cells after drug treatment was

quantitatively measured with the OxiSelect™ Total Glutathione (GSSG/GSH) Assay. In the presence of NADPH, glutathione reductase reduces oxidized glutathione (GSSG) to glutathione (GSH) in a simple enzymatic recycling reaction. Subsequently, chromogen was added into each sample, which reacted with the thiol group of GSH to produce a spectrophotometrically detectable compound at 405 nm. The total glutathione content was determined by comparison with the predetermined glutathione standard curve. The concentration of glutathione in the samples is was proportional to the rate of chromophore production, which was determined from the absorbance change over time. Three parallels were prepared for each sample and antioxidant level was expressed as mean±S.D.

Detection of apoptosis. Expression of phosphatidylserine on the cell surface soon after initiation of apoptosis is a main principle in apoptosis detection by Annexin V-FITC Apoptosis Detection Kit. After treatment, cells were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide for 5 min at room temperature, according to the manufacturer's instructions (BioVision, Inc.). Spectrofluorimetric detection was performed with the following fluorescence parameters: λ_{ex}= 488nm, λ_{em}=530nm, using "Tecan Infinite F200 PRO" (Tecan). The experiment was performed three times and the ratio of apoptotic cells was expressed as mean±S.D.

Detection of oxidative DNA damages. To detect whether SN38 treatment with or without EP leads to DNA damage in Colon26 cells the OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation) was utilized. This immunoassay quantitatively measures 8-hydroxydeoxyguanosine (8-OHdG) – a marker of oxidative stress. After drug treatment, samples were added into an 8-OHdG/BSA conjugate preabsorbed microplate and incubated for 10 min at room temperature. Next steps included the addition of anti-8-OHdG antibody, and after that, the HRP-conjugated secondary antibody to complete the reaction. Absorbance of samples was read by a microplate spectrophotometer at λ_{abs}=450 nm. The 8-OH-dG content was calculated by comparison with the predetermined 8-OH-dG standard curve. The experiment was multiplied three times and the level of DNA damage was expressed as mean±S.D.

Quantitative determination of ABCC1 - protein. The Human Multidrug Resistance-Associated Protein 1 (ABCC1) ELISA Kit was used to measure the ABCC1 protein levels, according to the manufacturer's protocol (Cusabio Technology LLC.), improved for cell lysate samples. This assay represents the quantitative sandwich enzyme immunoassay technique. Briefly, after electro-assisted drug treatment, all samples and standards were added into microplate wells (pre-coated with specific for ABCC1 protein antibody) and incubated (2 h at 37˚C) for immobilization. After removing any unbound substances, a biotin-conjugated antibody (specific for ABCC1 protein) was added to each well for one h incubation. After washing, the avidin-conjugated horseradish peroxidase (HRP) was added to the wells for 1 h. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to each sample/standard (15-30 min incubation of microplate, protected from light). The color was developed in proportion to the amount of ABCC1 bound to the initial step. Finally, stop solution was added (color development was stopped) and intensity of the color was analyzed by microplate reader "Tecan Infinite F200 PRO" at at λ_{abs}=450 nm. All results were determined by comparison with the predetermined standard curve and normalized by the total protein (Bradford protein assay). The ABCC1-protein levels were expressed as mean±SD from three independent experiments.

Quantitative determination of SLCO1B1 protein. SLCO1B1 protein levels were estimated by the Human Solute Carrier Organic Anion Transporter Family Member 1B1 (SLCO1B1) ELISA Kit, according to the manufacturer's protocol (My Biosource Inc.), modified for cell lysate samples. The first step included electro-assisted drug treatment and preparation of samples. Every sample and the standard were added into microplate wells pre-coated with an antibody specific for SLCO1B1 protein). Immediately after this step, the HRP-conjugated reagent was added to each well, except for the blank wells. The plate was covered with closure plate membrane and incubated for 60 min at 37˚C. After washing, the procedure continued with the addition of Chromogen Solution A and B (provided with kit). After reaction with Chromogen B the plate was protected from light and incubated for 15 min at 37˚C. The procedure ended with the addition of Stop solution to every well, followed by measurement of optical density at λ_{abs}=450 nm by a microplate reader - "Tecan Infinite F200 PRO". All results were calculated by comparison with the predetermined standard curve and normalized by the total protein levels (Bradford protein assay). The quantity of SLCO1B1 protein was expressed as mean±SD from three independent experiments.

Statistical analysis. All statistical analyses were performed using Microsoft Excel Software. Data are reported as means±S.D. To evaluate the statistical significance of experimental data, a comparison between treated and control probes was performed by Student's *t*-test. Each *p*-value lower than 0.05 was considered statistically significant.

Results and Discussion

We first evaluated the effect of the combined treatment with SN38 and EP on Colon26 cells. Cells were treated with different concentrations of SN38 alone (from 0.5 to 10 μM) and cell viability was analyzed by trypan blue assay after 24 and 48 h of incubation (Figure 1A). SN38 manifested anti-proliferative and cytotoxic effects in a dose-independent manner. Its cytotoxicity was slightly higher at 48 h compared to 24 h of incubation. The aim of this experiment was to select the optimal concentration range (red arrows on Figure 1A) of SN38, inducing a ~30-50% decrease of cell proliferation compared to control (untreated) cells, to be able to analyze potentially synergistic, additive or antagonistic effects of the combined treatment with EP on cell viability and cytoskeletal response. The concentrations of 0.5 and 5 μM SN38 that decreased cell proliferation by ~40-45% were used in further experiments.

The electroporation technique was used to examine whether the cytotoxic effect of SN38 on colon cancer cells was affected by electrical pulses in the presence of the drug (combine treatment). The application of SN38 for 48 h alone at concentrations 0.5-5 μM reduced cell viability up to 50%

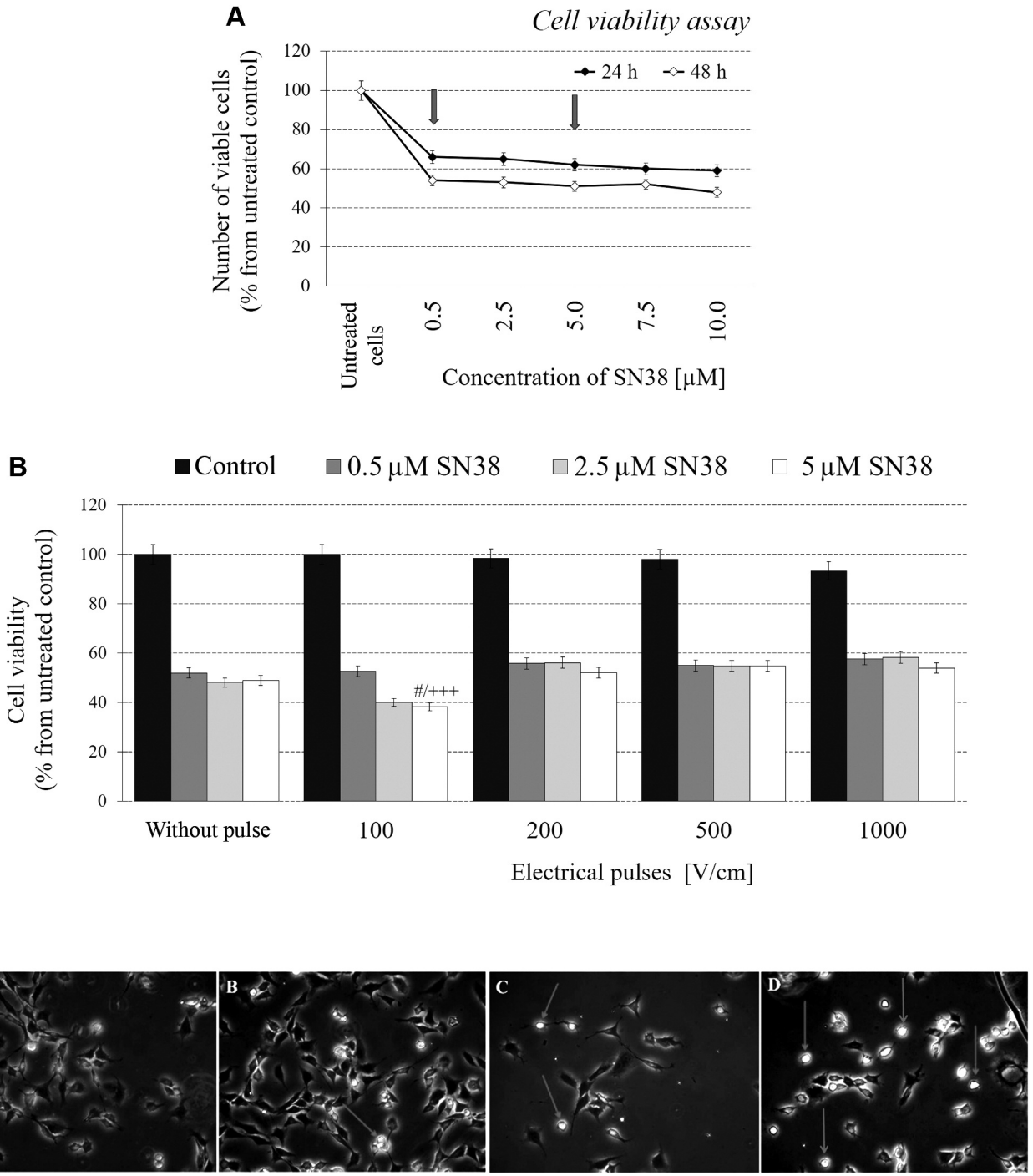

Figure 1. *Effects of SN38, electroporation and their combination on cell viability and cytoskeleton integrity of colon cancer cells. (A): Number of viable Colon26 cells after 24 and 48 h of incubation with different concentrations of SN38, presented as a percentage of control (untreated) cells. Cell viability was analyzed by trypan blue staining and was in the range of 94-99%. All data are means±SD from three independent experiments. The arrows indicate the selected concentrations for further experiments. (B): Cell viability of Colon26 cells after 48 h of treatment with SN38 in combination with electroporation, analyzed by the MTS assay. All data are means±SD from three independent experiments.* $^{+++}p<0.001$ *versus cells with 100 V/cm,* $^{\#}p<0.05$ *versus cell treated with 5 μM SN38 alone, all other differences are significant versus respective control. (C): Propidium iodide staining and fluorescence imaging of Colon26 cells after electroporation: (a) – Control (untreated cells); (b) – 100 V/cm; (c) – 500 V/cm; (d) – 1000 V/cm. The arrows indicate electroporated cells. Magnification – 63×.*

(Figure 1B). The maximum reduction of cell viability (over 60%) was achieved after the combined application of 5 μM SN38 and low voltage electrical pulse (100 V/cm), which suggest a synergistic effect. Any further increase in the applied EP intensity reduced the effect of SN38 on cell viability. One possible explanation is that the substance is amphiphilic and may enter the cells and released from the cells if the electro-induced pores are long lasting (19).

SN38 is a small amphiphilic molecule, which displays increased rate of transmembrane diffusion. It is known that EP opens temporally pores on the cell membrane and the number, diameter and lifetime of these pores are strongly dependent on pulse intensity (13). We hypothesize that after application of high voltage electroporation (over 500 V/cm), an equilibrium of molecules outside and inside the cells is achieved, and additional increase of intensity can lead to the opening of more pores with higher diameter and long lifetime followed by leakage of molecules outside the cells (19, 20). According to Neumann`s theory of electroporation, the first step is the formation of narrow hydrophobic pores with a diameter lower than the thickness of the membrane (20). If the field strength reaches the critical value for a given pulse duration, a different pore quality appears – broader hydrophilic pores. The formation of these pores is slower because the conversion of hydrophobic to hydrophilic pores involves rotation of lipid molecules in the pore wall (20). To confirm that the electrical treatment really induces the formation of membrane pores, we stained cells with propidium iodide (PI) and imaged fluorescence using a confocal microscopy. The data in Figure 1C show that the number of electroporated cells increases by increasing the applied voltage. We suppose that cell viability is maintained higher after high-intensity electrotreatment due to the impact on specific group of transmembrane proteins, responsible for SN38 transport. A number of studies have reported increased expression of transmembrane proteins after SN38 treatment (21, 22), but not after electro-assisted delivery, which should to be clarified.

In this study, we used an adherent cell line as a model to simulate the *in vivo* conditions. We hypothesize that cellular elasticity based on the actin cytoskeleton is a contributing parameter, and alteration of cytoskeleton integrity could sensitize cancer cells to conventional anticancer drugs, potentiating their cytotoxicity and minimizing side-effects. Considering the foregoing conception, we further examined the changes of actin cytoskeleton integrity after treatment with SN38 and/or EP (Figure 2). The applied electrical pulses induced reversible changes. Untreated (control) cells had a typical shape and a lot of stress fibers (Figure 2A – white arrows). The application of electrical pulses led to changes in the cytoskeleton that were dependent on field intensity. A reorganization of actin filaments is was visible at 100 V/cm, but still stress fibers could be observed. Completely different is situation after application of 1000 V/cm: the cells retained their morphology, but stress fibers cannot be detected and diffuse organization of actin filaments was observed. The reduced cell viability after application of 1000 V/cm alone can also be partially explained by the destruction of cell cytoskeleton. However, the application of 5 μM SN38 alone led to the slight reorganization of the actin cytoskeleton, which was expected because the anticancer effect of SN38 is due to topoisomerase inhibition. The cross-talk between cytoskeletal integrity and topoisomerase activity has been described (23). Recently, Wang *et al*. (23) have reported that SN38 affects microtubule structure of cancer cells (HeLa and U2OS) *via* inhibition of tubulin polymerization and reduction of the mass of actin filaments. This is accompanied by cell-cycle arrest in the S-phase and reduction of cell population. The combined treatment of Colon26 cells with 100 V/cm EP and 5 μM SN38 led to an interesting cytoskeletal response (Figure 2E). We found that the cells were elongated and oriented towards the applied electric field. With regard to cytoskeleton changes, the results obtained showed that electrotreatment with low-voltages is most conducive for the sensitization of cells to SN38.

One of the basic mechanisms of the anticancer activity of conventional chemotherapeutics is related to redox-signaling modulation and induction of oxidative stress in cancer cells, leading to apoptosis and necrosis (24). Moreover, cellular redox-signaling has a crucial role in carcinogenesis, the effectiveness of anticancer therapy and the development of resistance to anticancer drugs (25). In this context, our further experiments were focused on clarifying the potential redox-related molecular mechanism of SN38/EP combination.

The level of oxidative stress in Colon26 cancer cells after treatment with SN38, EP or their combination was determined, using two conventional methods: (i) DHE-assay, which specifically detects intracellular superoxide, and (ii) DCF-assay, which specific detects intracellular hydroperoxides. The results clearly showed that in comparison to untreated control cells, both types of ROS, superoxide (Figure 3A) and hydrogen peroxides (Figure 3B), were increased 48 h after the combined treatment. The results suggest a redox-related mechanism of SN38 in cancer. EP increased superoxide levels, but did not reduce cell viability, which is a promising effect for the development of a combined electro-assisted chemotherapeutic strategy. Low intensity electrical treatment (100 V/cm) alone may increase the levels of superoxide by releasing different ions from the electrodes (26). In 2019, Ruzgys *et al*. (27) have investigated the effect of electrode material on the efficiency of EP on the molecular transport and generation of ROS. The authors compared three different types of electrodes – aluminum, copper and stainless-steel and it was determined that the high EP efficiency was related with increased ROS generation in the order: aluminum < stainless-

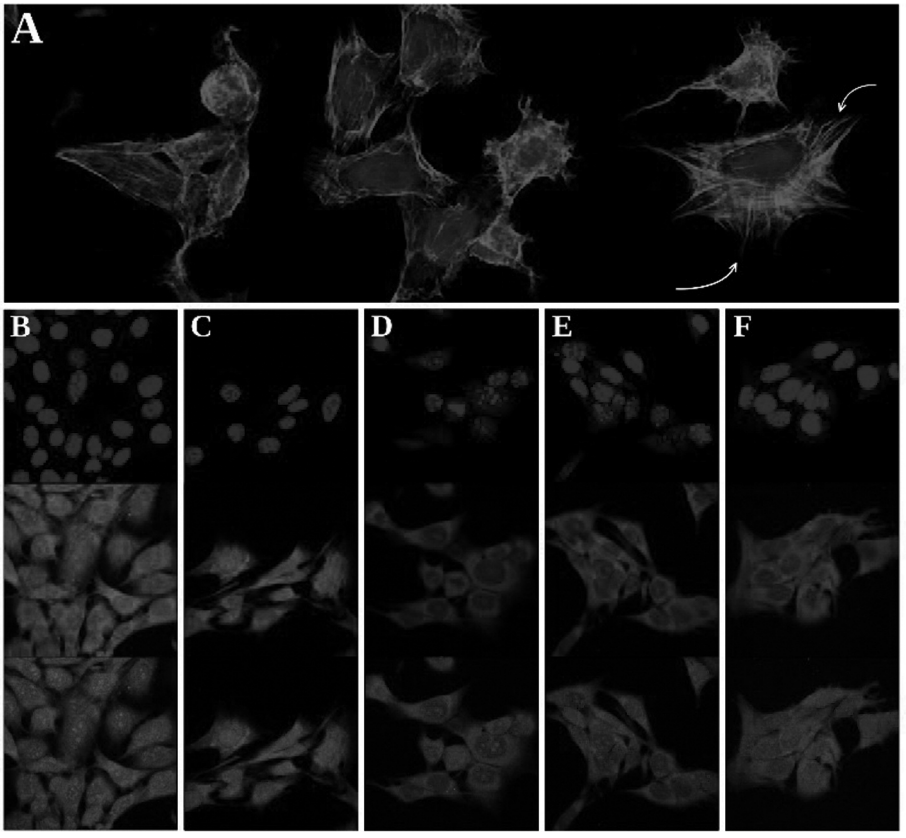

Figure 2. *Immunofluorescence imaging of actin cytoskeleton: (A) – control; (B) – 100 V/cm; (C) – 1000 V/cm; (D) – 5 μM SN38; (E) – 5 μM SN38 and 100 V/cm; (F) – 5 μM SN38 and 1000 V/cm. The arrows indicate stress fibers. Magnification – 63×.*

steel < copper. Their conclusion was that metal ions, not the pH fronts near the electrodes, play a major role in the generation of ROS during electroporation (27).

It should be noted that, in comparison with untreated controls, hydroperoxides are slightly elevated 48 h after electrotreatment (100 V/cm) (Figure 3B). We suggest that the observed results could be also explained by electrode's material. We have used stainless-steel electrodes and this resulted in the release of ferrous ions during electrotreatment procedure. Moreover, we assumed that the lower levels of hydroperoxides were due to their rapid chemical reaction with electrode-derived ferrous ions *via* the Fenton`s reactions (28).

In addition, we analyzed the induction of apoptosis by measuring phosphatidylserine (PhSer) exposure and oxidative DNA damages as markers of oxidative stress and cytotoxicity of SN38, EP and their combination (Figure 3C, D). SN38-treated cells had approximately 2.5 times higher PhSer exposure compared to untreated control cells. The combined treatment (SN38 and EP 100 V/cm) increased the expression of the apoptotic marker in comparison with the control cells, but lower than SN38-treatment alone. In cells treated with EP alone, PhSer exposure was even lower than the basal (control) levels. Data about oxidative DNA damage showed the same tendency as for apoptosis assay. We assume that application of SN38 and electrical pulses, in the presence of free Fe^{2+} ions leads to induction of apoptosis as well as ferroptosis. Ferroptosis is a non-apoptotic regulated cell death, characterized by iron-dependent accumulation of lipid peroxides (29). This regulated cell death type has been implicated in the pathological cell death associated with degenerative diseases (*i.e.*, Alzheimer's, Huntington's, and Parkinson's diseases), carcinogenesis, stroke, intracerebral hemorrhage, traumatic brain injury, ischemia-reperfusion injury, and kidney degeneration in mammals and is also implicated in heat stress in plants (30). It is known that ROS, iron ions and lipid peroxides are the main activators of this type of cell death (29). The reported high levels of superoxide and decreased amount of hydroperoxides are fully consistent with the hypothesis of a ferroptotic mechanism. According to Toyokuni *et al.* (31), ROS generation, produced *via* iron-catalyzed Fenton`s reactions, induces ferroptosis. Ferroptosis-related mechanisms of

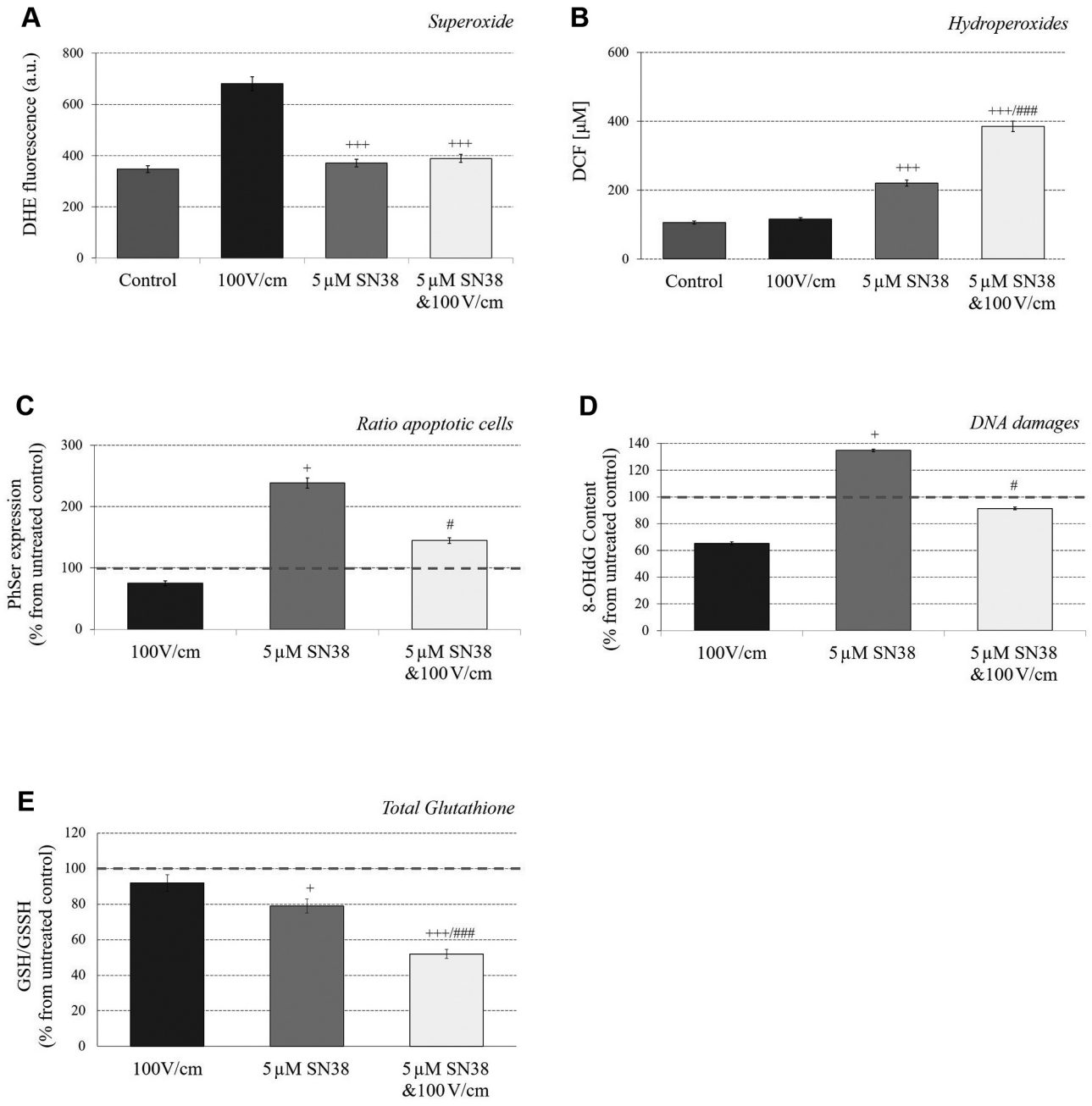

Figure 3. *Effects of SN38, electroporation and their combination on the parameters of oxidative stress in colon cancer cells. (A) Intracellular levels of superoxide, analyzed by the DHE-assay. The data were normalized to 1×10^6 cells/ml. All data are means±SD from three independent experiments; $^{+++}$p<0.001 versus cells treated with 100 V/cm, all differences are significant versus control. (B) Intracellular levels of hydroperoxides, analyzed by the DCF-assay. The data were normalized to 1×10^6 cells/ml. All data are means±SD from three independent experiments. $^{+++}$p<0.001 versus cells with 100 V/cm, $^{###}$p<0.001 versus 5 µM SN38. (C) Induction of apoptosis in SN38/EP-treated Colon26 cells, analysed by phosphatidylserine (PhSer) exposure on the cell surface. The data were normalized to 1×10^6 cells/ml. All data are means±SD from three independent experiments: $^{+}$p<0.05 versus cells with 100 V/cm, $^{#}$p<0.05 versus 5 µM SN38, all other differences are significant versus control. The dotted line indicates PhSer exposure in control (untreated) cells. (D) Oxidative DNA damages in SN38/EP-treated Colon26 cells, analyzed by the OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation). All data are the means±SD from three independent experiments: $^{+}$p<0.05 versus cells with 100 V/cm, $^{#}$p<0.05 versus 5 µM SN38, all other differences are significant versus control. The dotted line indicates the content of 8-OHdG in control (untreated) cells. (E) Intracellular levels of total glutathione, analyzed by the OxiSelect™ Total Glutathione (GSSG/GSH) Assay. The data were normalized to 1×10^6 cells/ml. Data are shown as a percent from untreated control (basal level – dotted line) and are the means±SD from three independent experiments: $^{+}$p<0.05 versus cells with 100 V/cm, $^{+++}$p<0.001 versus cells with 100 V/cm, $^{###}$p<0.001 versus 5 µM SN38, all differences are significant versus control.*

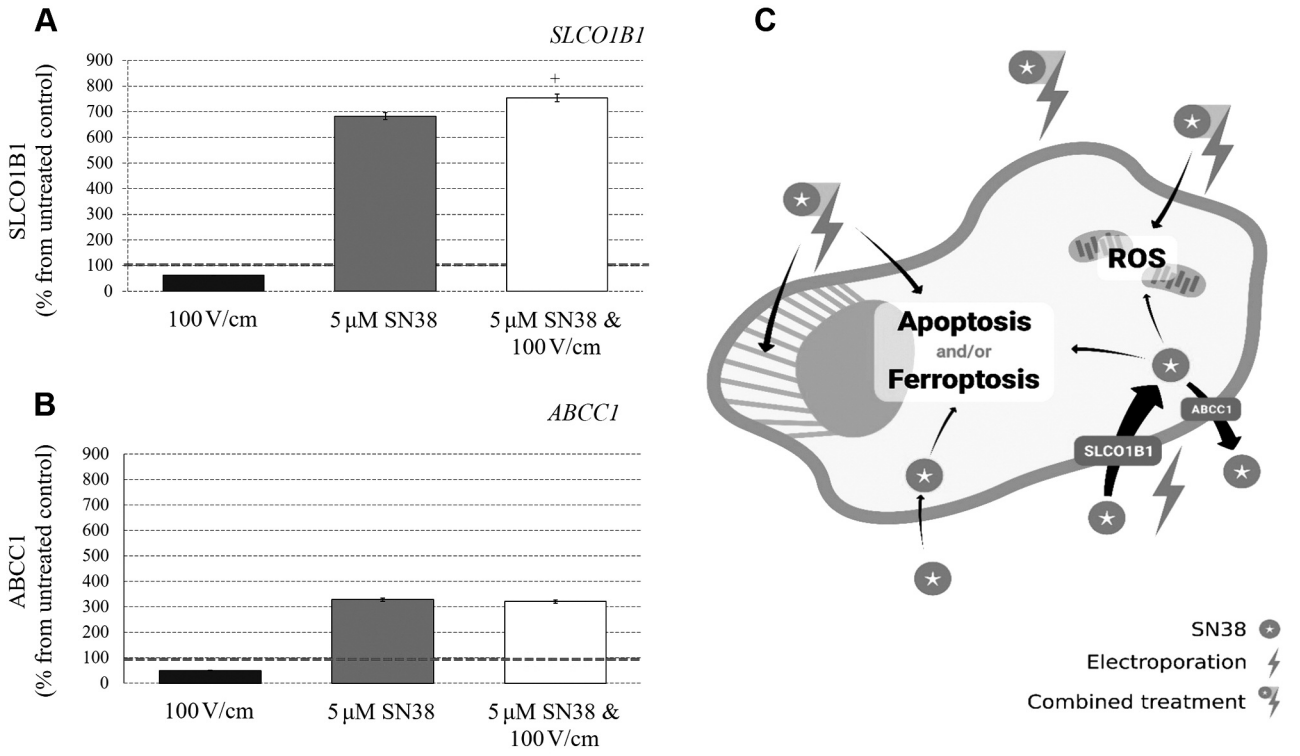

Figure 4. *Effects of SN38, electroporation and their combination on the expression of ABC transporters. (A) Levels of SLCO1B1 protein expression (as a % from untreated control) in Colon26 cells after 48 h of incubation, analyzed by Human the Solute Carrier Organic Anion Transporter Family Member 1B1 (SLCO1B1) ELISA Kit. The dotted line indicates the levels of SLCO1B1 expression in untreated cells. The data were normalized to 1×10^6 cells/ml and total protein quantitation (Bradford protein assay). All data are the means±SD from three independent experiments: $^+p<0.05$ versus cells with 100V/cm, all other differences are significant versus control and versus cells with 100 V/cm. (B) Levels of ABCC1 protein expression, (as a % from untreated control) in Colon26 cells after 48 h of incubation, analyzed by the Human Multidrug Resistance-Associated Protein 1 (ABCC1) ELISA Kit. The dotted line indicates the levels of ABCC1 expression in untreated cells. The data were normalized to 1×10^6 cells/ml and total protein quantitation (Bradford protein assay). All data are means±SD from three independent experiments: all differences are statistically significant versus control and versus cells with 100 V/cm. (C) Schematic representation of the effects of SN38, electroporation (EP) and their combination (SN38/EP) on colon cancer cells.*

conventional and natural anticancer substances are described in the literature (32), but this is the first study about SN38/Irinotecan-induced ferroptosis, especially after combined application with low intensity EP.

The balance ROS/reducing equivalents in the cells (termed as "intracellular redox-status") is an important marker and therapeutic target for all pathologies accompanied by a disturbance of redox-signaling. Glutathione (GSH) is one of the most important endogenous reducers and vital component of the antioxidant defenses system. Moreover, it is well known that inhibition of the glutathione peroxidase 4 (GPX4) enzyme is the critical step in ferroptosis (30). GPX4 is a phospholipid hydroperoxidase that protects cells against membrane lipid peroxidation and ferroptosis by eliminating intracellular lipid hydroperoxides in the presence of GSH as a cofactor. Thus, reduced GSH levels are also indirectly interpreted as an indicator of ferroptosis.

We investigated intracellular glutathione (GSH/GSSH) in Colon26 cells after treatment with SN38, EP and their combination (Figure 3E). The levels of total glutathione decreased below the control baseline in all cases, but the most significant decrease was detected after the combined application of SN38 and EP (100 V/cm). Other investigators have also reported that SN-38 down-regulates intracellular glutathione by a ROS-mediated mechanism (8). They have concluded that the decreased levels of intracellular reduced GSH are a consequence of increased ROS levels and redox imbalance that may trigger DNA damages and may also regulate the cysteine /cysteine cycle (8). Based on our results, we suggest a similar mechanism of action after the combine treatment. According to Dodson *et al*. (33), ferroptosis is characterized by similar morphological changes, cytoskeletal rearrangements and destabilization of plasma membrane, which were partially observed following actin-staining (Figure 2).

The last part of the study aimed to examine whether EP has an effect on the ABC transporters, responsible for import/export of SN38 in cancer cells. This is tightly related to SN38 cytotoxicity and development of multidrug resistance. In addition, such results could explain, at least partially, why there is no synergistic anticancer effect when SN38 is combined with high-voltage EP (>500 V/cm) (Figure 1B).

SLCO1B1 transporter is responsible for the internalization SN38 into colon cancer cells, while ABCC1 protein is one of the major efflux-mediating proteins (34). The results from the ELISA assays showed up to 50% reduced expression for both proteins in EP only treated samples, which is explained by the influence of electrical pulses on the cell membrane (the black columns - Figure 3F and G). In addition, we observed about 7-fold higher expression of SLCO1B1 protein after SN38-treatment and slightly higher levels after combined treatment in comparison to the basal levels of the protein – synergistic effectiveness (Figure 3F). Most literature data show increased expression of efflux proteins after treatment with SN38 (21, 22). SN38 (alone or in combination with EP) up-regulated the ABCC1 expression too, but the effect was significantly lower compared to SLCO1B1. Taken together, the data of this part of the study suggest that, unlike the conventional case where EP usually shows synergism with anticancer drugs that depends on pulse intensity, the SN38 combination with lower voltages leads to a more pronounced effect. We hypothesize that the observed synergistic effectiveness in the case of combine treatment (SN38/EP) is due to: (i) cytoskeleton reorganization and subsequent higher retention rate of SN38 inside the colorectal cells; (ii) influence of the electro-treatment on the transporters that probably changes membrane polarization.

The ABC transporters are of great interest, because the data about the effect of EP on the development of MDR to conventional agents are scanty (35-37). Kulbachka *et al*. (35) have demonstrated that the expression of P-glycoproteins (one of the main MDR-related efflux proteins) decreased after electrochemotherapy with Doxorubicin in resistant human colon (LoVo/Dx) and gastric carcinoma (EPG85-257/P and EPG85-257/RDB) cells. Kambe *et al*. (36) have also investigated gastric cancer cells treated with doxorubicin in combination with EP and showed that the anticancer effect depends on P-glycoprotein expression. Drag-Zalesińska *et al*. (37) have demonstrated that high density electrical pulses potentiate the cytotoxicity of low doses of cisplatin and vinorelbine in resistant small cell lung cancer cells (SCLC) *in vitro*. Recently, Candeil *et al*. (22) have reported that resistance to SN38 is the result of increased expression of ABC-family proteins. Other study suggested that electrical stimulations impair the translocation of MDR proteins to their functional locations at the plasma membrane (38). Thus, EP can increase the intracellular delivery of substances and decrease the efflux rate *via* their transporters.

In summary, our data on the treatment of colorectal cells with SN38/EP combination suggest that (Figure 4):

Electrotreatment with low voltage (100 V/cm) sensitizes colorectal cells to SN38 in a synergetic manner *via* modulation of intracellular redox-status and induction of oxidative stress.

Electro-assisted sensitization of colon cancer cells to SN38 is most likely related to induction of apoptosis and ferroptosis. The mechanism includes depletion of glutathione, which ultimately results in higher levels of lipid peroxidation that cause cell death.

These affects are preceded by cytoskeletal rearrangements and destabilization of plasma membrane, partially observed by actin-staining.

Electro-assisted sensitization of colon cancer cells to SN38 is also accompanied by up-regulation of SLCO1B1 transporter, which is responsible for intracellular drug delivery.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

SS, BN, ZZ and RB conceived the idea for the study. SS and BN produced the first draft. SS, BN, ES, DL and IT were involved in the experiments, PL was involved in image preparation and improvements. RB, ZZ, IA and TH were involved in critical review of subsequent drafts. All Authors read and approved the final manuscript.

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