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Original Article

Effect of natural flavonoids to reverse P-glycoprotein-related multidrug resistance in breast cancer cell cultures

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Abstract: The aim of the research was to evaluate the influence of two P-glycoprotein (P-gp) inhibitors silymarin and quercetin on anticancer drug doxorubicin (DOX) and pegylated liposomal doxorubicin (PLD) delivery into breast cancer cells (2D cultures) and cancer cell spheroids (3D cultures) at different pH. The cytotoxicity of the compounds was assessed using MTT assay. Spheroids were generated using magnetic 3D Bioprinting method. The uptake of DOX and PLD into monolayer-cultured cells and spheroids was assessed by fluorescence microscopy. Both tested flavonoids did not increase DOX and PLD levels into monolayer-cultured 4T1 cells and 4T1 cell spheroids. However, both silymarin and quercetin enhanced DOX and PLD uptake into JC cell cultures. Silymarin and quercetin may modulate DOX and PLD transport into monolayer-cultured cells and three-dimensional cancer cell cultures depending on P-gp activity.

Keywords: Drug delivery, P-glycoprotein, multidrug resistance, silymarin, quercetin, cell spheroids

Introduction

P-glycoprotein (P-gp), or multidrug resistance protein 1 (MDR1), is an ATP-dependent multidrug transporter that actively effluxes its substrates including many chemotherapeutic drugs out of the cell [1]. Therefore, it plays a very important role in the development of multidrug resistance. It is known that P-gp is overexpressed in many types of tumors [2, 3]. It was also observed that the expression of P-gp may increase after the exposure to chemotherapeutic agents [4]. Many anticancer drugs, such as anthracyclines (doxorubicin, daunorubicin) [5, 6], podophyllotoxins (etoposide, teniposide) [7, 8], taxanes (paclitaxel, doctetaxel) [9, 10], vinca alcaloids (vinblastine, vincristine) [11, 12] are the substrates of this efflux pump. Therefore, P-gp is one of the major obstacles to the efficacy of chemotherapy. It is hypothesized that the inhibition of this transporter may prevent P-gp mediated drug efflux in tumor cells and reverse multidrug resistance [2, 13].

The idea to increase the chemosensitivity of tumor cells to anticancer agents has been extensively investigated for over three decades and many different P-gp inhibitors were developed. According to their specificity and toxicity P-gp inhibitors can be classified in four generations [14]. First generation P-gp inhibitors, e.g. verapamil, are pharmacologically active therapeutic agents that additionally inhibit P-gp. However, their clinical application was limited due to relatively low binding affinity and high toxicity at the required doses [15]. Second generation P-gp inhibitors, such as doxverapamil, were developed after structural modifications of first-generation inhibitors. These compounds exert lower toxicity compared to first generation inhibitors. However, they showed to be potent CYP 3A4 inhibitors, thus causing many pharmacokinetic interactions with other compounds [16]. Third generation P-gp inhibitors, such as tariquidar and zosuiqudar, do not interact with CYP 3A4 and exert high binding affinity to P-gp [17].

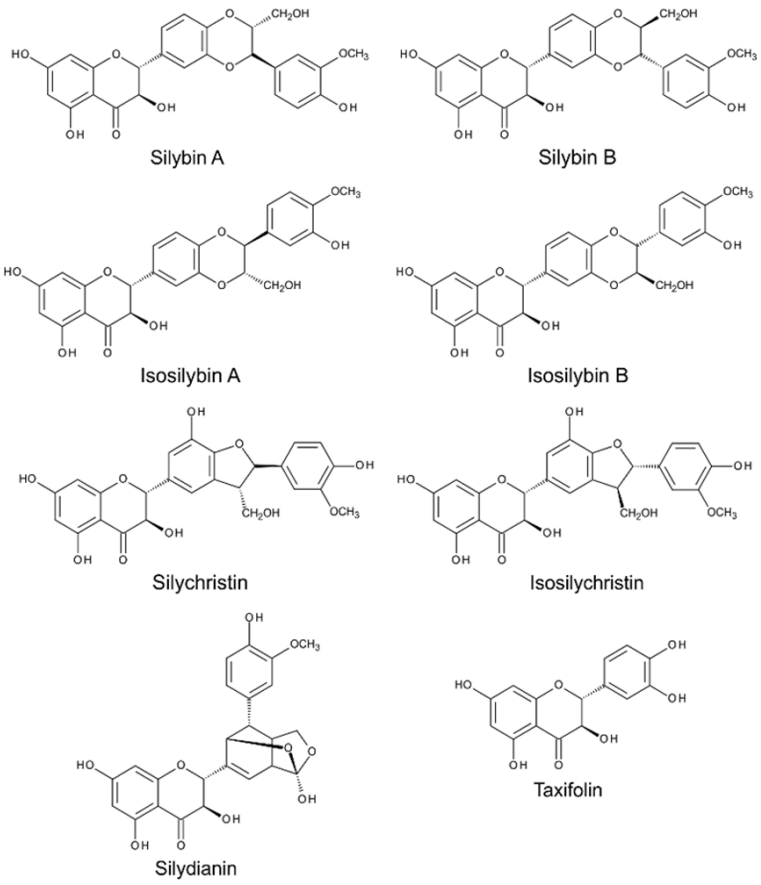

Figure 1. Chemical structures of silymarin compounds.

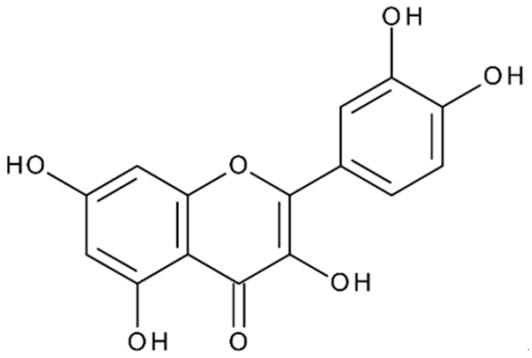

Figure 2. Chemical structure of quercetin.

Unfortunately, these compounds did not show successful results in clinical trials and natural substances started to be investigated. Fourth generation P-gp inhibitors are plant-based compounds, that include flavonoids, coumarins, alkaloids, saponins, and terpenoids. Quercetin and silymarin are flavonoids that belong to the fourth generation P-gp inhibitors. These compounds are derived from fruits, vegetables, some of them are used as food supplements for a long time, therefore it is believed that they should be safer and better tolerated than first, second, or third generation inhibitors [18]. In our work, we selected two fourth-generation P-gp inhibitors - silymarin which is composed of several different flavonolignans and flavonoids (**Figure 1**) and quercetin (**Figure 2**), and evaluated their efficacy in increasing DOX and PLD levels in 4T1 and JC breast cancer cell cultures.

Materials and methods

Materials

DOX hydrochloride and quercetin were bought from Abcam (Cambridge, UK). PLD was bought from FormuMax Scientific Inc. (Palo Alto, CA, USA). Silymarin was purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Cell cultures

4T1 triple-negative murine breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). JC triple-negative murine breast cancer cell line was kindly provided by Prof. Chiara Riganti (University of Torino, Italy). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) GlutaMAX medium supplemented with 10% fetal bovine serum (FBS), 10,000 U/mL penicillin, and 10 mg/mL streptomycin. RPMI 1640, FBS, and antibiotic solution were purchased from Gibco (Carlsbad, CA, USA). Cells were cultured in a humidified atmosphere that contained 5% CO_2 at 37°C.

Drug delivery in monolayer-cultured cells

The cells were seeded on collagen-coated coverslips in 24-well plates in a volume of 500 µL (50,000 cells/well) and placed in the incubator for 48 h. After the incubation, the medium was

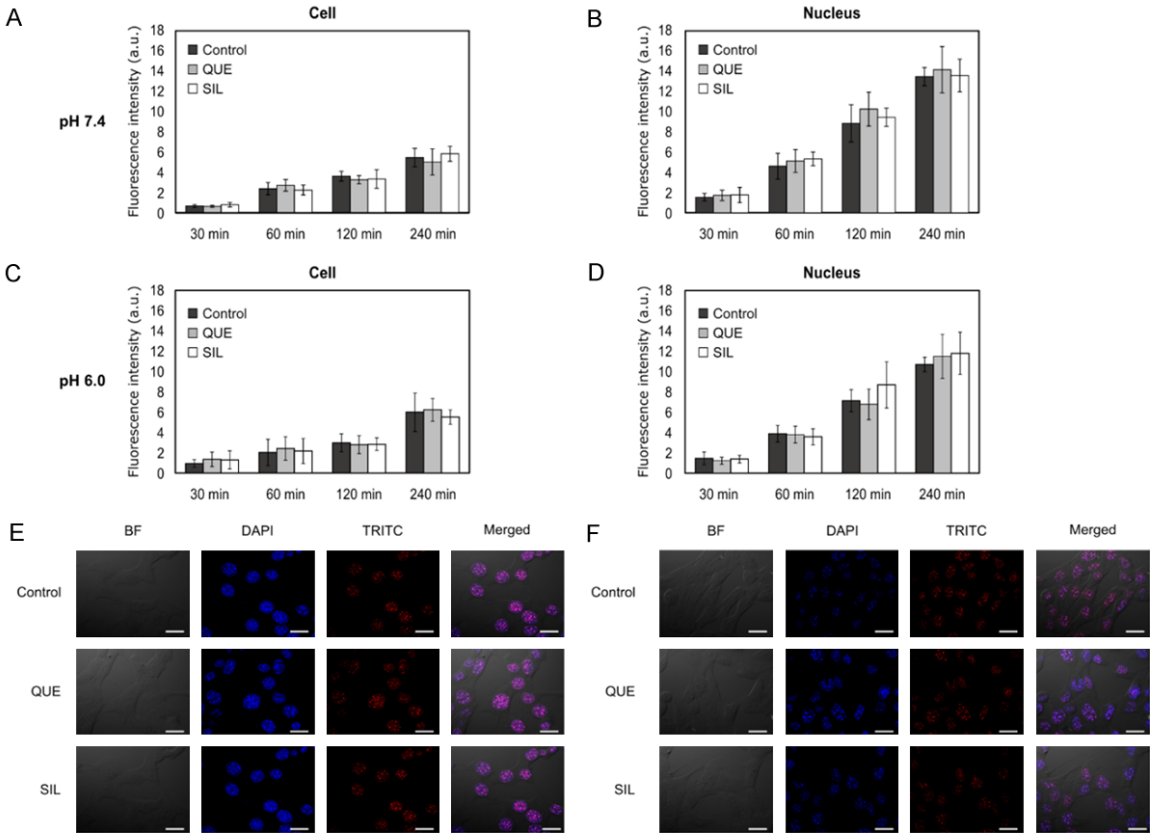

Figure 3. The influence of P-glycoprotein inhibitors on doxorubicin (DOX) levels into 4T1 cells and their nuclei in medium of different pH. A. Fluorescence intensity of DOX in 4T1 cells at different time points at physiological pH; B. Fluorescence intensity of DOX in 4T1 cell nuclei at different time points at physiological pH; C. Fluorescence intensity of DOX in 4T1 cells at different time points at weakly acidic pH; D. Fluorescence intensity of DOX in 4T1 cell nuclei at different time points at weakly acidic pH; E. Photos of 4T1 cells after 4 h of incubation with DOX at physiological pH; F. Photos of 4T1 cells after 4 h of incubation with DOX at weakly acidic pH. Scale bar =50 μm. Abbreviations: QUE, quercetin; SIL, silymarin.

changed by a new medium of pH 6.0 or 7.4 and maintained in the incubator for 1 h. Later the medium was removed and the solutions of the same pH that contained 100 μM quercetin or silymarin or 0.2% DMSO were poured into the wells and incubated for 2 h. After that, the medium was replaced with the solutions of the same pH that contained 1 or 5 μM of DOX or PLD and placed in the incubator for 0.5, 1, 2 and 4 h. After that, the wells were rinsed with PBS, fixed with 4% paraformaldehyde (Thermo Scientific, Waltham, MA, USA) solution, and stained using 4',6-diamidino-2-phenylindole (DAPI; Thermo Scientific). The photos were taken using fluorescence microscope Olympus FLUOVIEW FV1000 (Olympus Corp., Japan). Fluorescence intensity of DOX into cells and cell nuclei was calculated using ImageJ software (National Institutes of Health).

Drug delivery in cell spheroids

The spheroids were made using magnetic 3D Bioprinting method as described by Tseng et al. [19]. The spheroids were incubated with the medium of pH 6.0 or 7.4 for 1 h. After that, the medium was replaced with the solutions of the same pH that contained 100 μM quercetin or silymarin or 0.2% DMSO and incubated for 2 h. After that, the medium was replaced with 20 μM DOX or PLD solutions of the same pH. After 1, 2, 4 and 8 h of incubation the spheroids were rinsed with PBS and fixed with paraformaldehyde. The photos were taken using fluorescence microscope Olympus IX73. DOX and PLD delivery into spheroids was estimated using ImageJ software. Fluorescence intensity was assessed by tracing a radius from the center of the spheroid to the edge each degree around

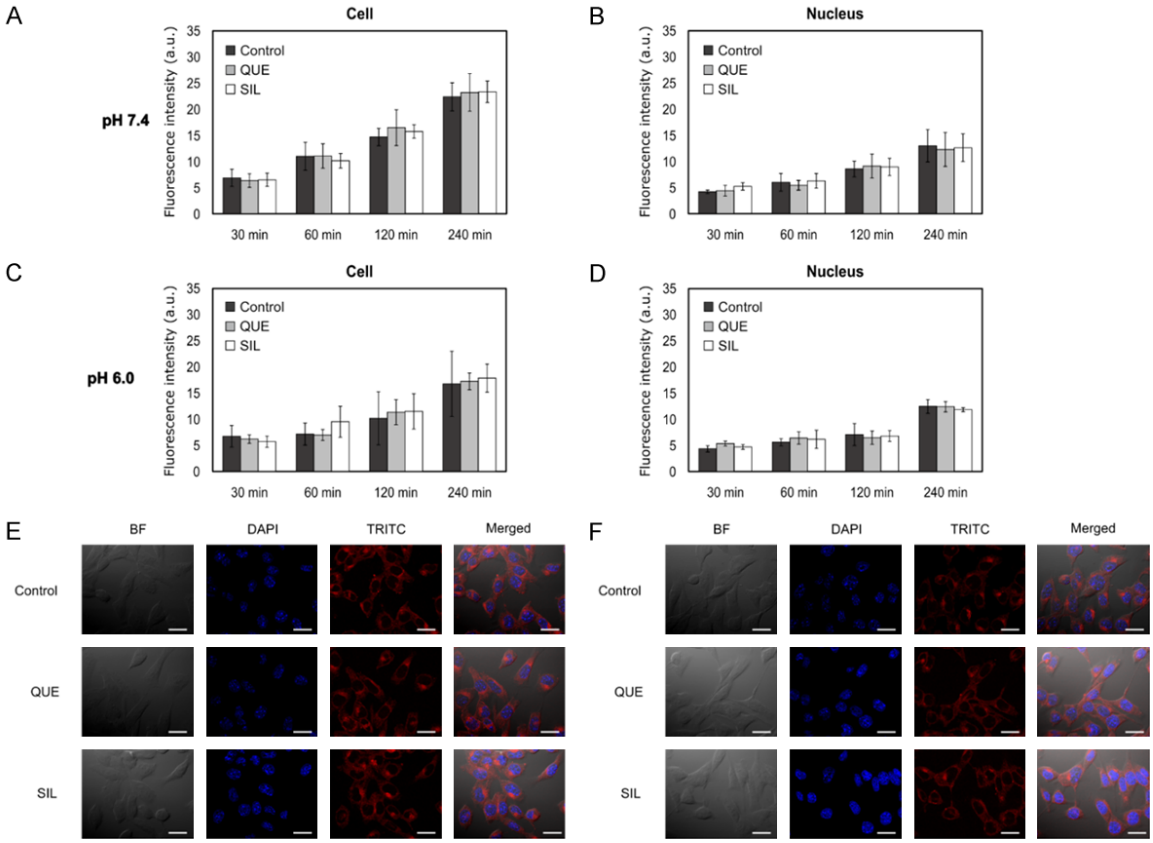

Figure 4. The influence of P-glycoprotein inhibitors on pegylated liposomal doxorubicin (PLD) levels in 4T1 cells and their nuclei in medium of different pH. A. Fluorescence intensity of PLD in 4T1 cells at different time points at physiological pH; B. Fluorescence intensity of PLD in 4T1 cell nucleus at different time points at physiological pH; C. Fluorescence intensity of PLD in 4T1 cells at different time points at weakly acidic pH; D. Fluorescence intensity of PLD in 4T1 cell nuclei at different time points at weakly acidic pH; E. Photos of 4T1 cells after 4 h of incubation with PLD at physiological pH; F. Photos of 4T1 cells after 4 h of incubation with PLD at weakly acidic pH. Scale bar =50 μm. Abbreviations: QUE, quercetin; SIL, silymarin.

the spheroid. For analytical purposes, the spheroids were partitioned into three zones: edge (0-50 μM), middle (100-150 μM), and central zone (200-225 μM).

Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, WA, USA). Each experiment was repeated at least thrice independently. The results were shown as mean ± standard deviation. The analysis was done using Student's t-test. A *P*-value less than 0.05 was considered as a significant difference.

Results

Effect of P-gp inhibitors on cell viability

Silymarin EC_{50} for both 4T1 and JC cell lines after 8 h were >250 μM. Quercetin EC_{50} for 4T1 and JC cells after 8 h were 40.3 μM and 161.2 μM, respectively. However, after 2 h of incubation quercetin EC_{50} for 4T1 was also >150 μM. Therefore, silymarin and quercetin concentrations of 100 μM were used in the following experiments. EC_{50} values of DOX and PLD after 8 h were >150 μM. It shows that 1, 5 and 20 μM concentrations of DOX and corresponding concentrations of PLD used in the research did not reduce cell viability during the experiments.

Effect of P-gp inhibitors on DOX and PLD delivery in cancer cells (2D cultures)

Neither silymarin, nor quercetin increased DOX delivery into 4T1 cells or their nuclei at pH 7.4 and 6.0. No significant difference in DOX fluorescence intensity between control group and cell affected with silymarin or quercetin was observed (**Figure 3**).

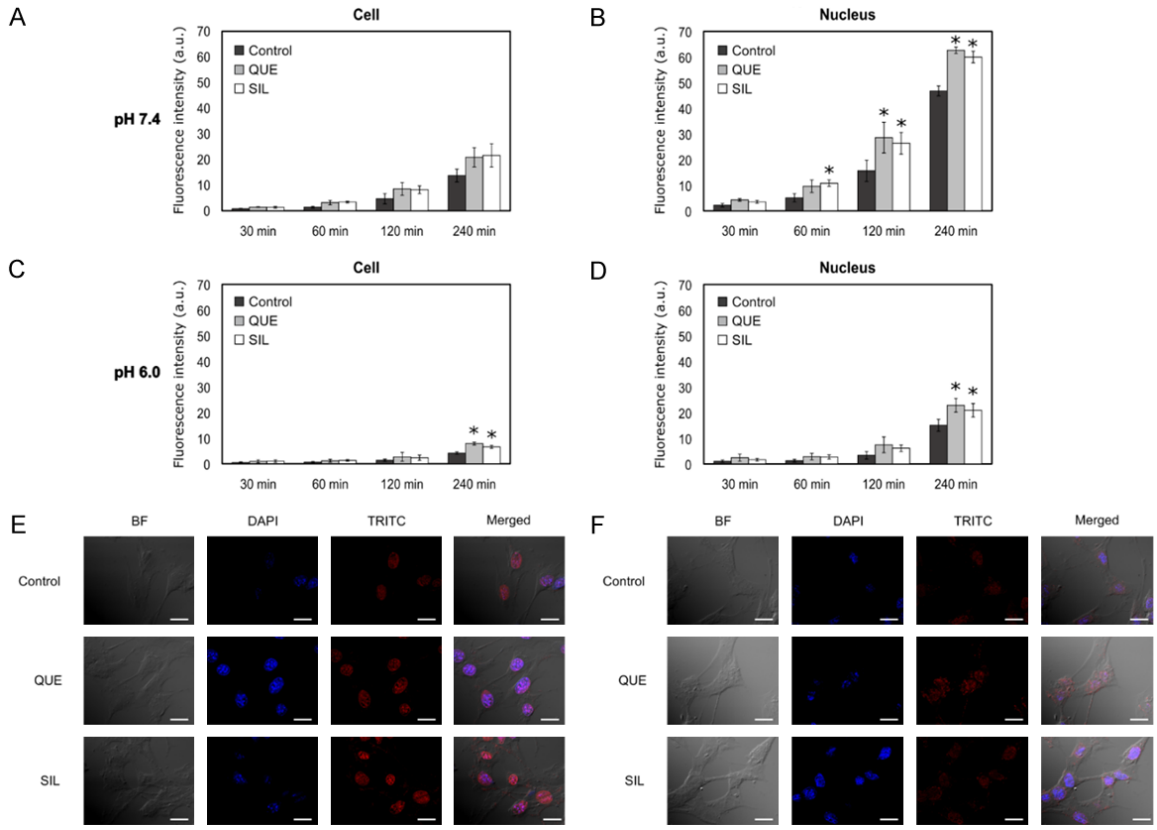

Figure 5. The influence of P-glycoprotein inhibitors on doxorubicin (DOX) levels in JC cells and their nuclei in medium of different pH. A. Fluorescence intensity DOX in JC cells at different time points at physiological pH; B. Fluorescence intensity of DOX in JC cell nuclei at different time points at physiological pH; C. Fluorescence intensity of DOX in JC cells at different time points at weakly acidic pH; D. Fluorescence intensity of DOX in JC cell nuclei at different time points at weakly acidic pH 6.0; E. Photos of JC cells after 4 h of incubation with DOX at physiological pH; F. Photos of JC cells after 4 h of incubation with DOX at weakly acidic pH. Scale bar =50 μm. The asterisks (*) indicate $P<0.05$. Abbreviations: QUE, quercetin; SIL, silymarin.

The experiments with PLD yielded similar results. Both P-gp inhibitors did not increase PLD delivery into 4T1 cells and their nuclei, as well (**Figure 4**).

However, different results were observed in experiments with JC cells. In this case both silymarin and quercetin increased DOX concentration in JC cells and their nuclei (**Figure 5**). At pH 7.4, quercetin increased DOX uptake into cell nuclei after 1, 2, and 4 h of incubation, meanwhile SIL enhanced DOX delivery after 2 and 4 h of incubation. After 4 h of incubation the fluorescence intensity of DOX in the nuclei of cells affected with silymarin or quercetin was approximately 1.3-fold higher compared to the control group. At pH 6.0 positive quercetin and silymarin effect on DOX delivery in JC cells was observed only at the end of the experiment (after 4 hours of incubation). At that point in time DOX fluorescence intensity in JC cell affected with P-gp inhibitors nuclei was 1.4 (quercetin group) and 1.5 (silymarin group) higher compared to control group.

Both tested P-gp inhibitors increased PLD delivery into JC cells, as well (**Figure 6**). Quercetin improved PLD uptake after 2 and 4 h of incubation at weakly acidic and physiological pH. Silymarin increased PLD fluorescence intensity in JC cell nuclei only after 4 h of incubation.

Effect of P-gp inhibitors on DOX and PLD delivery in tumor spheroids (3D cultures)

Silymarin and quercetin did not enhance DOX uptake into 4T1 spheroids at physiological and weakly acidic pH (**Figure 7**). No positive effect of these compounds was observed on PLD uptake in 4T1 spheroids, as well (**Figure 8**).

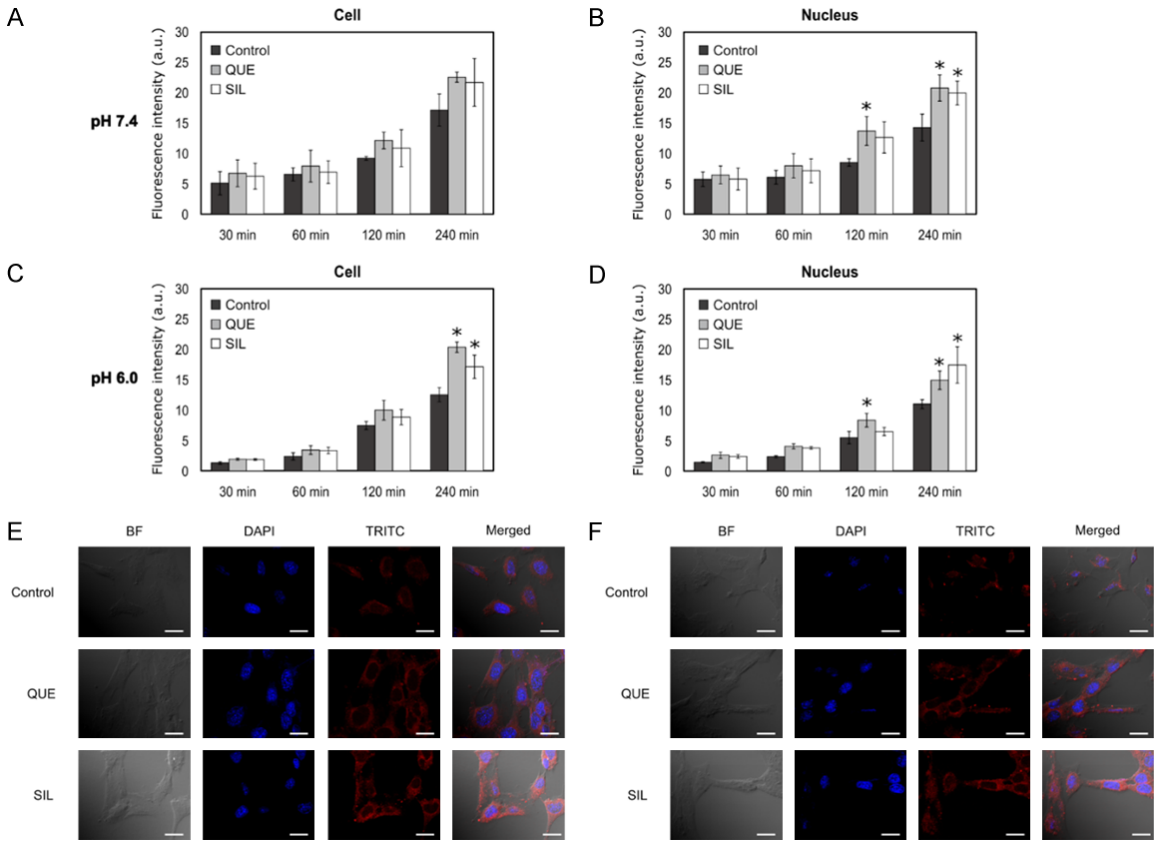

Figure 6. The influence of P-glycoprotein inhibitors on pegylated liposomal doxorubicin (PLD) delivery JC cells and their nuclei in medium of different pH. A. Fluorescence intensity of PLD in JC cells at different time points at physiological pH; B. Fluorescence intensity of PLD in JC cell nuclei at different time points at physiological pH; C. Fluorescence intensity of PLD in JC cells at different time points at weakly acidic pH; D. Fluorescence intensity of PLD in JC cell nuclei at different time points at weakly acidic pH; E. Photos of JC cells after 4 h of incubation with PLD at physiological pH; F. Photos of JC cells after incubating for 4 h with PLD in weakly acidic at pH. Scale bar =50 μm. The asterisks (*) indicate $P<0.05$. Abbreviations: QUE, quercetin; SIL, silymarin.

However, during experiments with JC spheroids it was observed that after 1 and 8 h of incubation quercetin increased DOX uptake into the edge zone of spheroids at pH 7.4 (**Figure 9**). At pH 6.0 quercetin increased DOX uptake into the edge zone of JC spheroids after 1, 2, 4 and 8 h of incubation. Data obtained with silymarin was very similar, however, in weakly acidic medium silymarin increased DOX fluorescence intensity in the edge zone of spheroids only after 4 and 8 h of incubation.

Results from experiments with PLD showed that quercetin increased PLD fluorescence intensity in the edge zone of JC spheroids only at the end of the experiment - after 8 h of incubation (**Figure 10**). Silymarin was more effective in this case. It enhanced PLD uptake into the edge zone of spheroids after 1, 2, 4 and 8 h of incubation at both physiological and weakly acidic conditions. At pH 7.4 this P-gp inhibitor slightly increased PLD uptake also into middle zone of JC spheroids after 4 and 8 h of incubation.

Discussion

Our results showed that both tested P-gp inhibitors are not effective DOX and PLD transport modulators in 4T1 cell cultures. However, both quercetin and silymarin enhanced DOX and PLD uptake in monolayer-cultured JC cells and their spheroids. This difference can be explained by the different P-gp activity in 4T1 and JC cell lines. It is known that JC cells overexpress P-gp and do not express multidrug resistance protein 1 which makes this cell line a good option to study P-gp inhibitors [20, 21]. Meanwhile in 4T1 cells P-gp expression is lower. Liu et al. found that the EC_{50} value of liposomal

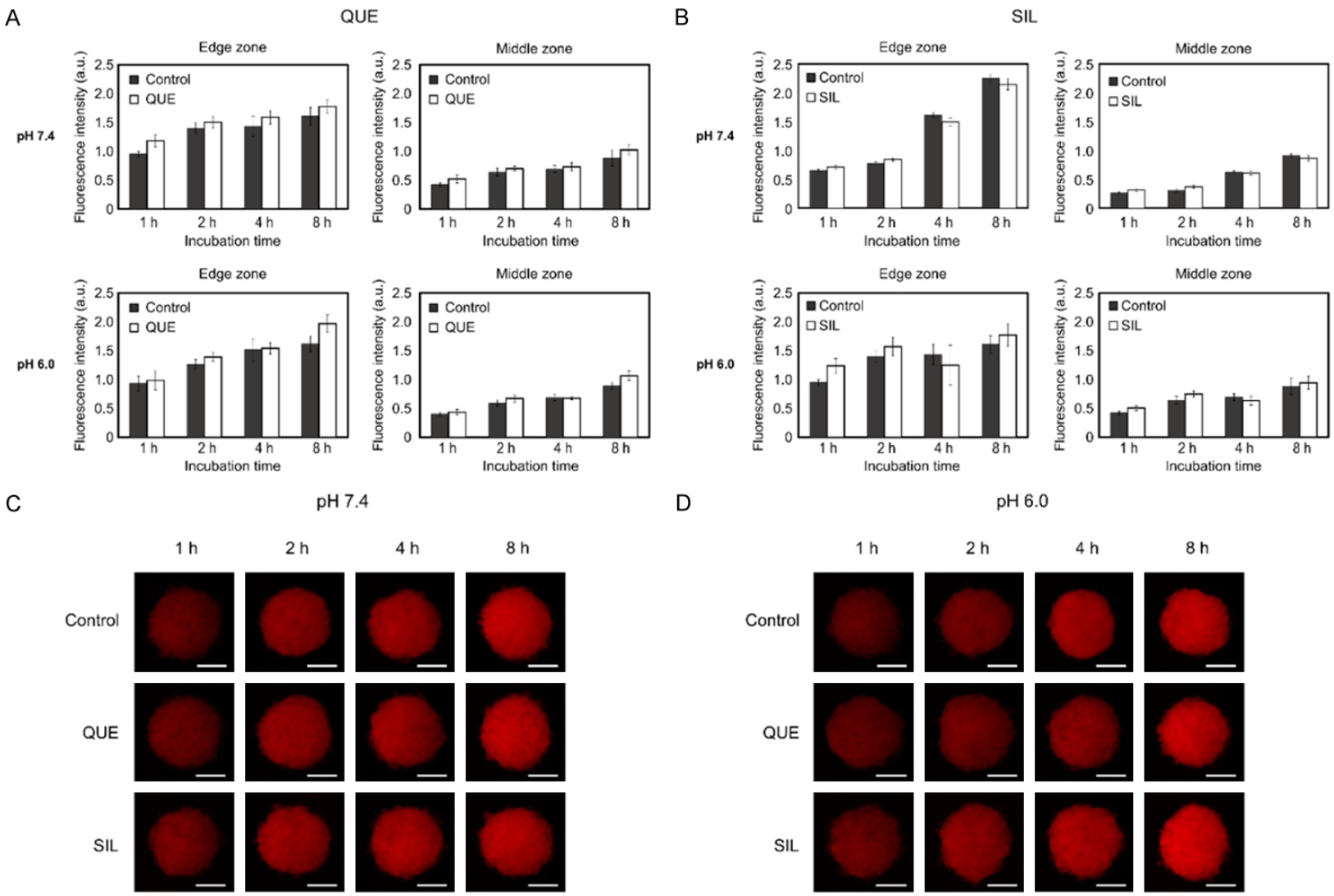

Figure 7. The influence of P-glycoprotein inhibitors on doxorubicin (DOX) levels in 4T1 cell spheroids at physiological and weakly acidic pH. A. Fluorescence intensity of DOX in edge and middle zones of 4T1 cell spheroids incubated with quercetin at physiological and weakly acidic pH; B. Fluorescence intensity of DOX in edge and middle zones of spheroids incubated with silymarin at physiological and weakly acidic pH; C. Photos of 4T1 cell spheroids after the incubation with DOX at physiological pH; D. Photos of 4T1 cell spheroids after the incubation with DOX at weakly acidic pH. Scale bar =200 μm. Abbreviations: QUE, quercetin; SIL, silymarin.

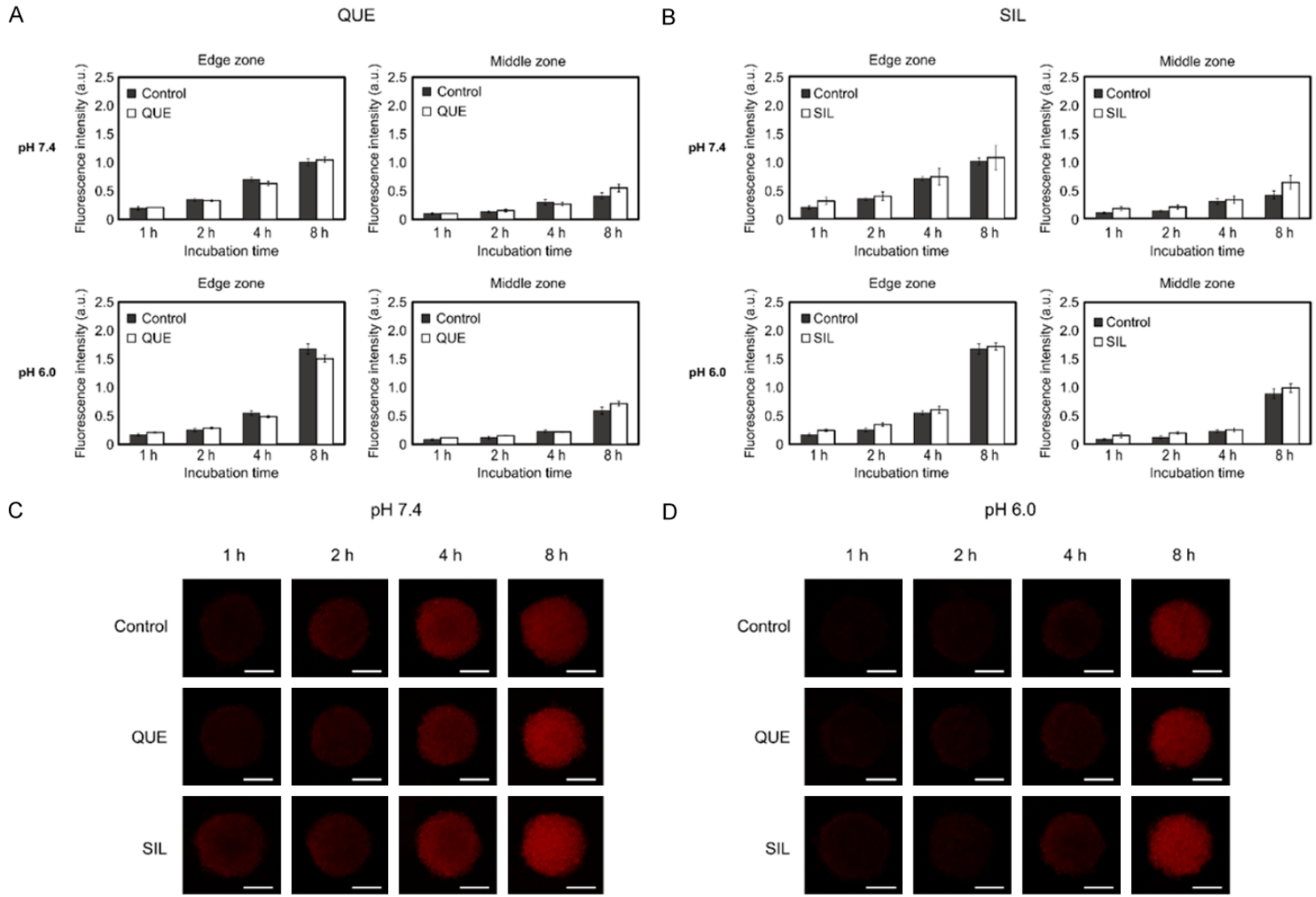

Figure 8. The influence of P-glycoprotein inhibitors on pegylated liposomal doxorubicin (PLD) levels in 4T1 cell spheroids at physiological and weakly acidic pH. A. Fluorescence intensity of PLD in edge and middle zoned of 4T1 cell spheroids incubated with quercetin at physiological and weakly acidic pH; B. Fluorescence intensity of PLD in spheroids incubated with silymarin at physiological and weakly acidic pH; C. Photos of spheroids after the incubation with PLD at physiological pH; D. Photos of spheroids after the incubation with PLD at weakly acidic pH. Scale bar =200 μm. Abbreviations: QUE, quercetin; SIL, silymarin.

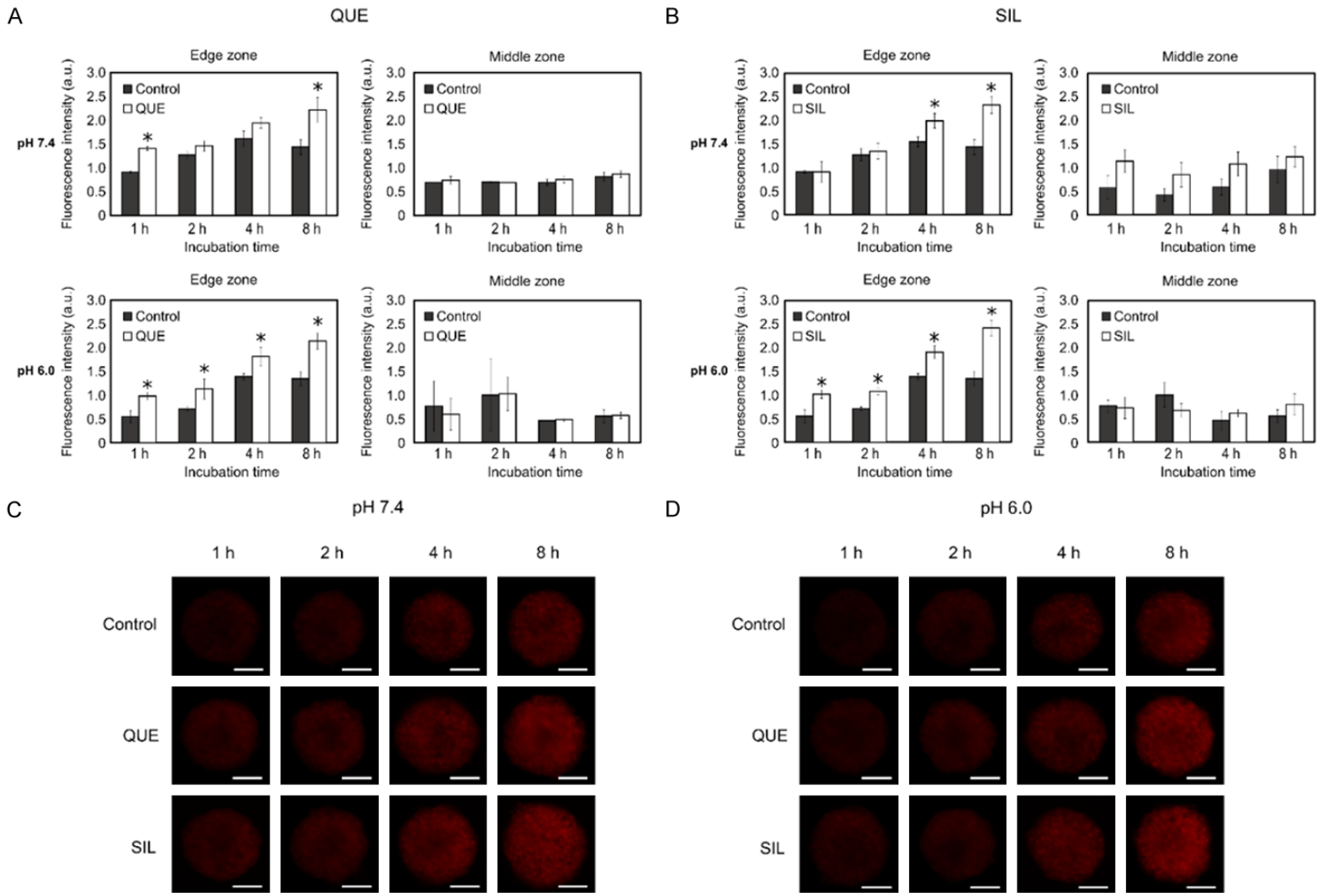

Figure 9. The influence of P-glycoprotein inhibitors on doxorubicin (DOX) levels into JC cell spheroids at physiological and weakly acidic pH. A. Fluorescence intensity of DOX in edge and middle zones of spheroids incubated with quercetin at physiological and weakly acidic pH; B. Fluorescence intensity of DOX in edge and middle zones of spheroids incubated with silymarin at physiological and weekly acidic pH; C. Photos of JC cell spheroids after the incubation with DOX at physiological pH 7.4; D. Photos of JC cell spheroids after the incubation with DOX at weakly acidic pH. Scale bar =200 μm. The asterisks (*) indicate $P<0.05$. Abbreviations: QUE, quercetin; SIL, silymarin.

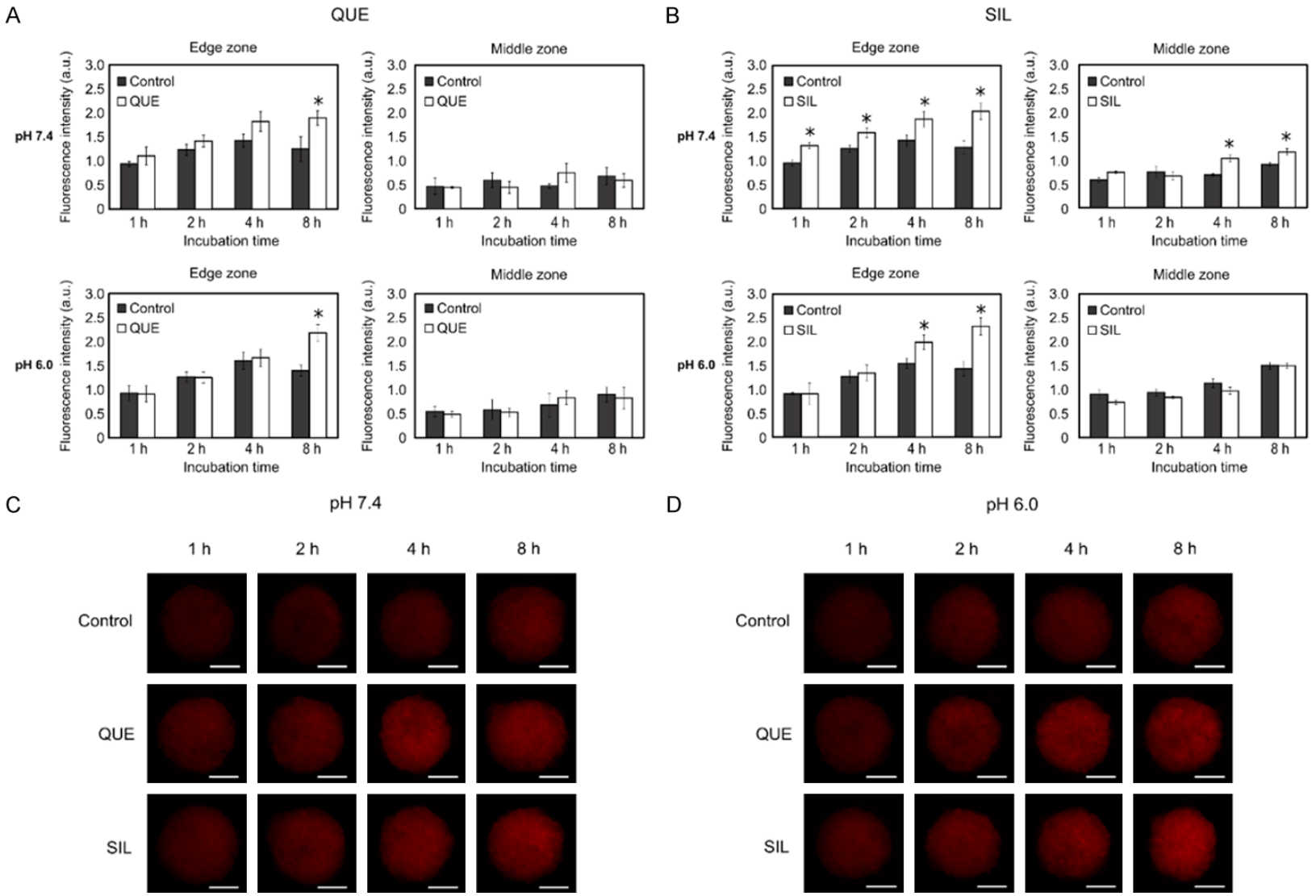

Figure 10. The influence of P-glycoprotein inhibitors on pegylated liposomal doxorubicin (PLD) levels in JC cell spheroids at physiological and weakly acidic pH. A. Fluorescence intensity of PLD in edge and middle zones of spheroids incubated with quercetin at physiological and weakly acidic pH; B. Fluorescence intensity of PLD in edge and middle zones of spheroids incubated with silymarin at physiological and weakly acidic pH; C. Photos of JC cell spheroids after the incubation with PLD at physiological pH; D. Photos of JC cell spheroids after the incubation with PLD at weakly acidic pH. Scale bar =200 μm. The asterisks (*) indicate $P<0.05$. Abbreviations: QUE, quercetin; SIL, silymarin.

DOX for the JC cell line is about four times higher than the EC_{50} value for the 4T1 cell line [22]. This means that JC cells are about four times more resistant to DOX than 4T1.

Different results obtained from the experiments performed in cells with different P-gp activity support the hypothesis that DOX and PLD transport modulation demonstrated by tested compounds quercetin and silymarin acts through a P-gp inhibition mechanism. In 4T1 cell line that shows lower P-gp activity both quercetin and silymarin did not enhance DOX and PLD uptake into cells and their nuclei or cell spheroids. Meanwhile in JC cell line which overexpresses P-gp both flavonoids enhanced DOX and PLD uptake in monolayer-cultured cells, their nuclei and cell spheroids. It is important to note that the pH of the medium affected the uptake of DOX into cells, as well. Under acidic conditions, the uptake of DOX and PLD into cells is lower than at physiological pH. This can be explained by the ion retention mechanism, according to which in acidic environment weakly basic DOX ionizes and the positive charge limits its ability to penetrate the cell membrane.

Our findings are in line with the results published by other researchers. Desrini et al. found that cell incubation for 25 days with 750 nM quercetin increased the susceptibility of the derived DOX-resistant MCF-7 breast cancer cell line to DOX by two to three times [23]. To our knowledge, the efficacy of quercetin in the wild-type MCF-7 cell line has not been studied, so it can be hypothesized that in this case the efficacy of quercetin in improving DOX cell uptake would be significantly lower or DOX efficacy would not be improved at all, as shown by our experiments with 4T1 cell line. This is confirmed by the results published by Borska et al., which showed that quercetin increased the cytotoxicity of daunorubicin to daunorubicin-resistant pancreatic cancer EPP85-181RDB cells, but not to the daunorubicin-sensitive pancreatic cancer cell line EPP85-181P. In another study done by Li et al., it was also shown that quercetin increased the cytotoxicity of DOX in DOX-resistant breast cancer MCF-7 and MDA-MB-231 cells that overexpress P-gp [24]. Furthermore, Wang et al. found that quercetin increased DOX cytotoxicity against liver cancer SMMC7721 and QGY7701 cells [25].

There are limited data on the efficacy of silymarin as a P-gp inhibitor in modulating DOX levels in the cell. Zhang et al. showed that 100 μM silymarin increases DOX cytotoxicity to DOX-resistant MCF-7/ADR and MDA435/LCC6 breast cancer cells [26]. Silibinin, one of the most active compounds in silymarin, has also been shown to significantly increase the cytotoxicity of DOX against the DOX-resistant breast cancer cells MDA-MB-435/DOX [27]. On the other hand, a high concentration (200 μM) of silibinin was used in this study and the authors themselves showed that such a concentration of silibinin is toxic to cancer cells. Therefore, these results do not necessarily imply that silibinin increased DOX concentration in the cells; cell viability may have decreased because of the P-gp inhibitor silibinin.

There are very little published data on studies with P-gp inhibitors in cancer cell spheroids. Hassan et al. found that quercetin at a concentration of 50 μM increases DOX cytotoxicity against pancreatic cancer ASPC-1 and liver cancer HepG2 monolayer cells characterized by high P-gp activity and their spheroids by up to 60% [28]. Barros et al. found that resveratrol, which also exert a P-gp inhibitory effect, increases DOX cytotoxicity in PANC-1 pancreatic cancer cell spheroids [29]. Although the data published by these researchers and our results obtained with JC cells support the hypothesis that silymarin and quercetin may be effective chemosensitizers, they did not demonstrate this activity in 4T1 cells. In view of this, it can be stated that the efficacy and potential applicability of the tested flavonoids is conditional and depends on the activity of P-gp in the cell line.

Conclusions

Quercetin and silymarin do not improve DOX and PLD uptake into 4T1 breast cancer cells with low P-gp expression and their spheroids but increase DOX and PLD levels in JC breast cancer cells that overexpress P-gp and their spheroids. In monolayer-cultured cells, both inhibitors show similar results on enhancing DOX and PLD levels. However, silymarin is slightly more effective in increasing DOX and PLD uptake in JC cell spheroids.

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Disclosure of conflict of interest

None.

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References

[1] Karthika C and Sureshkumar R. P-glycoprotein efflux transporters and its resistance its inhibitors and therapeutic aspects. Biomarkers and Bioanalysis Overview: IntechOpen; 2021.

[2] Waghray D and Zhang QH. Inhibit or evade multidrug resistance P-glycoprotein in cancer treatment. J Med Chem 2018; 61: 5108-5121.

[3] He C, Sun ZY, Hoffman RM, Yang ZJ, Jiang YH, Wang L and Hao YQ. P-glycoprotein overexpression is associated with cisplatin resistance in human osteosarcoma. Anticancer Res 2019; 39: 1711-1718.

[4] Budde T, Haney J, Bien S, Schwebe M, Riad A, Tschöpe C, Staudt A, Jedlitschky G, Felix SB, Kroemer HK and Grube M. Acute exposure to doxorubicin results in increased cardiac P-glycoprotein expression. J Pharm Sci 2011; 100: 3951-3958.

[5] Patel KJ and Tannock IF. The influence of P-glycoprotein expression and its inhibitors on the distribution of doxorubicin in breast tumors. BMC Cancer 2009; 9: 356.

[6] Mccormick JW, Vogel PD and Wise JG. Multiple drug transport pathways through human P-glycoprotein. Biochemistry 2015; 54: 4374-4390.

[7] Leu BL and Huang JD. Inhibition of intestinal P-glycoprotein and effects on etoposide absorption. Cancer Chemother Pharmacol 1995; 35: 432-436.

[8] Huang J, Guo L, Tan RX, Wei MJ, Zhang J, Zhao Y, Gong L, Huang ZH and Qiu XH. Interactions between emodin and efflux transporters on rat enterocyte by a validated ussing chamber technique. Front Pharmacol 2018; 9: 646.

[9] Sparreboom A, van Asperen J, Mayer U, Schinkel AH, Smit JW, Meijer DK, Borst P, Nooijen WJ, Beijnen JH and van Tellingen O. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc Natl Acad Sci U S A 1997; 94: 2031-2035.

[10] Shirakawa K, Takara K, Tanigawara Y, Aoyama N, Kasuga M, Komada F, Sakaeda T and Okumura K. Interaction of docetaxel ("taxotere") with human P-glycoprotein. Jpn J Cancer Res 1999; 90: 1380-1386.

[11] Shalinsky D, Jekunen A, Alcaraz J, Christen R, Kim S, Khatibi S and Howell S. Regulation of initial vinblastine influx by P-glycoprotein. Br J Cancer 1993; 67: 30-36.

[12] Meyers MB, Scotto KW and Sirotnak FM. P-glycoprotein content and mediation of vincristine efflux: correlation with the level of differentiation in luminal epithelium of mouse small intestine. Cancer Commun 1991; 3: 159-165.

[13] Callaghan R, Luk F and Bebawy M. Inhibition of the multidrug resistance P-glycoprotein: time for a change of strategy? Drug Metab Dispos 2014; 42: 623-631.

[14] M F Gonçalves B, S P Cardoso D, U Ferreira MJ. Overcoming multidrug resistance: flavonoid and terpenoid nitrogen-containing derivatives as ABC transporter modulators. Molecules 2020; 25: 3364.

[15] Thomas H and Coley HM. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting P-glycoprotein. Cancer Control 2003; 10: 159-165.

[16] Kolitz JE, George SL, Marcucci G, Vij R, Powell BL, Allen SL, Deangelo DJ, Shea TC, Stock W, Baer MR, Hars V, Maharry K, Hoke E, Vardiman JW, Bloomfield CD and Larson RA; Cancer and Leukemia Group B. P-glycoprotein inhibition using valspodar (PSC-833) does not improve outcomes for patients younger than age 60 years with newly diagnosed acute myeloid leukemia: cancer and leukemia group B study 19808. Blood 2010; 116: 1413-1421.

[17] Abdallah HM, Al-Abd AM, El-Dine RS and El-Halawany AM. P-glycoprotein inhibitors of natural origin as potential tumor chemo-sensitizers: a review. J Adv Res 2015; 6: 45-62.

[18] Mohana S, Ganesan M, Agilan B, Karthikeyan R, Srithar G, Beaulah Mary R, Ananthakrishnan D, Velmurugan D, Rajendra Prasad N and Ambudkar SV. Screening dietary flavonoids for the reversal of P-glycoprotein-mediated multidrug resistance in cancer. Mol Biosyst 2016; 12: 2458-2470.

[19] Tseng H, Gage JA, Shen T, Haisler WL, Neeley SK, Shiao S, Chen J, Desai PK, Liao A, Hebel C, Raphael RM, Becker JL and Souza GR. A spheroid toxicity assay using magnetic 3D bioprinting and real-time mobile device-based imaging. Sci Rep 2015; 5: 13987.

[20] Riganti C, Gazzano E, Gulino GR, Volante M, Ghigo D and Kopecka J. Two repeated low doses of doxorubicin are more effective than a single high dose against tumors overexpress-

ing P-glycoprotein. Cancer Lett 2015; 360: 219-226.
[21] Lee BD, French KJ, Zhuang Y and Smith CD. Development of a syngeneic in vivo tumor model and its use in evaluating a novel P-glycoprotein modulator, PGP-4008. Oncol Res 2003; 14: 49-60.
[22] Liu YR, Fang JX, Joo KI, Wong MK and Wang P. Codelivery of chemotherapeutics via cross-linked multilamellar liposomal vesicles to overcome multidrug resistance in tumor. PLoS One 2014; 9: e110611.
[23] Sholikhah EN, Mustofa and Desrini S. The effect of quercetin and doxorubicin combination in inhibiting resistance in mcf-7 cell. Bangladesh Journal of Medical Science 2017; 16: 91-97.
[24] Li SZ, Yuan S, Zhao Q, Wang B, Wang XY and Li K. Quercetin enhances chemotherapeutic effect of doxorubicin against human breast cancer cells while reducing toxic side effects of it. Biomed Pharmacother 2018; 100: 441-447.
[25] Wang GY, Zhang JW, Liu LY, Sharma S and Dong QH. Quercetin potentiates doxorubicin mediated antitumor effects against liver cancer through p53/Bcl-xl. PLoS One 2012; 7: e51764.
[26] Zhang SZ and Morris ME. Effects of the flavonoids biochanin a, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. J Pharmacol Exp Ther 2003; 304: 1258-1267.
[27] Molavi O, Narimani F, Asiaee F, Sharifi S, Tarhriz V, Shayanfar A, Hejazi M and Lai R. Silibinin sensitizes chemo-resistant breast cancer cells to chemotherapy. Pharm Biol 2017; 55: 729-739.
[28] Hassan S, Peluso J, Chalhoub S, Idoux Gillet Y, Benkirane-Jessel N, Rochel N, Fuhrmann G and Ubeaud-Sequier G. Quercetin potentializes the respective cytotoxic activity of gemcitabine or doxorubicin on 3D culture of AsPC-1 or HepG2 cells, through the inhibition of HIF-1α and MDR1. PLoS One 2020; 15: e0240676.
[29] Barros AS, Costa EC, Nunes AS, de Melo-Diogo D and Correia IJ. Comparative study of the therapeutic effect of Doxorubicin and Resveratrol combination on 2D and 3D (spheroids) cell culture models. Int J Pharm 2018; 551: 76-83.