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Original Article

Application of carbonic anhydrase inhibitors to increase the penetration of doxorubicin and its liposomal formulation into 2D and 3D triple negative breast cancer cell cultures

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Abstract: The aim of our study was to assess the influence of two carbonic anhydrase (CA) inhibitors (methazolamide (MTZ)) and U-104 on weakly basic anticancer drug doxorubicin (DOX) and pegylated liposomal doxorubicin (PLD) delivery into monolayer-cultured 4T1 murine breast cancer cells (2D cultures) and tumor spheroids (3D cultures) at pH 6.0 and 7.4. The effect of compounds on cell viability was evaluated by MTT assay. Spheroids were formed using 3D Bioprinting method. The penetration of DOX and PLD into cells and spheroids was evaluated using fluorescence microscopy. Both MTZ and U-104 increased the DOX (5 μM) and PLD (concentration corresponding to 5 μM DOX) penetration into monolayer-cultured cells at acidic conditions but did not enhance drug delivery at physiological pH. Pretreatment with U-104 inhibitors also increased DOX and PLD delivery into tumor spheroids. Thus, U-104 may be worthy of further studies as possible transport modulator of weakly basic drugs.

Keywords: Drug delivery, extracellular acidity, multidrug resistance, pH modulation, tumor spheroids

Introduction

One of the biggest problems of anticancer chemotherapy is insufficient drug concentration in the tumors. There are many factors affecting drug transport. Among the main ones is tumor pH that plays a very important role in weakly basic drug delivery [1].

Due to certain changes in cancer metabolism, the extracellular tumor environment is often acidic [2]. Meanwhile, most of the anticancer drugs are basic compounds. Once a basic drug gets into the acidic environment, it gets ionized and a positive charge limits its ability to penetrate cell membrane [3]. Thus, the intracellular concentration of a medicine and its efficacy decreases.

It is hypothesized that by modifying tumor pH we may enhance drug delivery into cancer cells, thus increasing its efficacy [3]. One of the ways to achieve this goal is the inhibition of carbonic anhydrase (CA) IX/XII. CA is a family of transmembrane proteins that catalyzes the reversible hydration of carbon dioxide [4]. During this reaction, protons are released into extracellular tissue and extracellular pH decreases [5]. We hypothesize that by inhibiting this protein complex we may prevent extracellular acidification and increase basic drug delivery into a tumor.

16 isoforms of α-CA have been discovered in mammals [6]. It was observed that CA IX and XII expression is highly increased in a broad range of tumors and these isoforms are associated with cancer progression and development [7]. Meanwhile, CA IX expression in normal tissues is restricted to the gastric mucosa, the small intestine, and the biliary tract. CA XII may be found in the gastric mucosa, the large intestine, the renal and pancreatic tissues and eyes [8, 9]. Therefore, CA IX and XII are feasible tar-

gets for anticancer therapy and possibly for the modulation of basic anticancer drug delivery.

CA inhibitors might be advantageous in anticancer therapy not only because of their potential chemosensitizing properties. Since CA IX and XII expression is highly increased in tumors compared to normal cells and these enzymes have extracellular catalytic domains, isoforms IX and XII may serve as drug targets for dual-targeted chemotherapy. In this way anticancer agents may be conjugated with CA inhibitors and directed specifically to tumors, thus minimizing toxicity in healthy tissues [10, 11].

The aim of our research was to evaluate the influence of two CA inhibitors methazolamide (MTZ) and U-104 on doxorubicin (DOX) and its pegylated liposomal form (PLD) delivery into monolayer-cultured 4T1 murine breast cancer cells (2D) and tumor spheroids (3D model) at pH 6.0 and 7.4. U-104, also known as SLC-0111, is a novel specific small-molecule inhibitor of CA IX and XII. Meanwhile, MTZ potently inhibits various isoforms of carbonic anhydrase, especially CA I, II and IV. It was chosen in order to compare the transport modulating efficacy of the inhibitors possessing different selectivity to the CA isoforms. 4T1 cell line was chosen because it is known that CA IX is overexpressed in this type of cells [12]. DOX is one of the principal medications to treat triple-negative breast cancer. It is a weakly basic compound; thus, it tends to be ionized in the acidic environment. Moreover, due to its fluorescence DOX penetration into cell cultures may be easily estimated by fluorescence microscopy. Compared to free DOX, PLD passively targets tumor due to its enhanced permeability. Also, PLD exposure to healthy tissues is reduced. According to our knowledge, this paper is the first study to compare the effects of CA inhibitors on drug transport in both acidic and physiological medium and to test the efficacy of these compounds on drug delivery into tumor spheroids.

Materials and methods

Materials

DOX hydrochloride was purchased from Abcam (Cambridge, UK). PLD was bought from FormuMax Scientific Inc. (Palo Alto, CA, USA). MTZ and U-104 were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Cell cultures

A triple-negative murine breast cancer cell line 4T1 was bought from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in Roswell Park Memorial Institute 1640 GlutaMAX medium, which was supplemented with 10,000 U/mL penicillin, 10 mg/mL streptomycin, and 10% fetal bovine serum. Media and supplements were bought from Gibco (Carlsbad, CA, USA). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell viability

The effect of compounds on the viability of cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Co.) method. 5,000 cells were seeded in each well of 96-well plates in a volume of 100 µL. After 24 h preincubation, the cells were treated with 100 µL of different concentrations of compounds (CA inhibitors, DOX or PLD). A medium without cells was used as a positive control, and a medium with 0.5% DMSO (Sigma-Aldrich Co.) served as a negative control. After 4, 8, 12 and 24 h the cells were incubated for 3 h with the MTT solution (Sigma-Aldrich Co.). The absorbance was measured at the wavelengths of 570 and 630 nm.

Drug delivery in monolayer-cultured cells

4T1 cells were seeded on collagen-coated coverslips in 24-well plates in a volume of 500 µL (50,000 cells/well) and incubated for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C. Later the medium was replaced by the new medium of pH 6.0 or 7.4 and put in an incubator for 1 h. Then the cells were incubated with a new medium of the same pH that contained 100 µM MTZ or U-104 or 0.2% DMSO. After 2 h of incubation, the medium was replaced with a new one of the same pH that contained 1 or 5 µM of DOX or relative concentrations of PLD. After 0.5, 1, 2 and 4 h cells were washed with PBS, fixed with 4% paraformaldehyde (Thermo Scientific, Waltham, MA, USA) solution in PBS and stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Scientific). DOX and PLD penetration into whole cells and their nucleus was assessed using fluorescence microscopy and *ImageJ* software (National Institutes of Health).

Drug delivery in tumor spheroids

The spheroids were formed using 3D bioprinting method 13. 4T1 cells were incubated with nanoparticles NanoShuttle™ (Greiner Bio-One North America, Inc., Monroe, NC, USA) for 8 h. Then they were resuspended and seeded into ultra-low attachment 96-well plates in a volume of 100 µL (800 cells/well). The plate was placed on a magnetic drive and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 2 days. After that, the magnetic drive was removed, and the medium was replaced by a fresh one of pH 6.0 or 7.4 and incubated for 1 h. Then, the cells were incubated with a fresh medium of the same pH that contained 100 µM MTZ, U-104 or 0.2% DMSO for 2 hours. In the next step, the medium was replaced with a new medium of the same pH that contained 20 µM of DOX or a relative concentration of PLD. After 1, 2, 4 and 8 h spheroids were washed with PBS and fixed with a 4% solution in PBS. DOX and PLD penetration into spheroids was assessed using fluorescence microscopy and ImageJ software by evaluating the fluorescence intensity each degree from the spheroid center to the edge around the whole spheroid.

Statistical analysis

Statistical analysis was carried out using Microsoft Office Excel 2016 software (Microsoft Corporation, Redmond, WA, USA). All the experiments were performed in at least triplicate independent measurements and the obtained values were reported as mean ± standard deviation. Student's t-test was used, and *p*-values were calculated. A value of $P<0.05$ was considered as the level of significance.

Results

Effect of CAIs on 4T1 cell viability

Neither MTZ nor U-104 did not reduce 4T1 cell viability at 100 µM and lower concentrations at different time periods from 4 h to 24 h, hence in further experiments 100 µM CAIs were used. EC50 values of DOX and PLD after 12 hours of incubation were >150 µM. Therefore 1, 5 and 20 µM concentrations of DOX and corresponding concentrations of PLD were chosen to use in the research, considering that these concentrations are below the toxicity level.

Effect of CAIs on DOX and PLD delivery in cancer cells (2D cultures)

None of the tested CA inhibitors enhanced the delivery of DOX into monolayer-cultured cells at physiological conditions (**Figure 1A**, **1B**). On the other hand, both MTZ and U-104 increased the amount of DOX into cancer cells and nucleus at acidic conditions (**Figure 1C-E**). The positive effect of MTZ on DOX delivery into 4T1 cells and nucleus was observed after 1, 2 and 4 hours of incubation. U-104 increased DOX delivery into cells and nucleus during the time period from 30 min. to 4 h. At the end of the experiment DOX fluorescence intensity in the nucleus of 4T1 cells, incubated with MTZ or U-104 was about 1.5-fold higher compared to the control group. Meanwhile, DOX fluorescence intensity in whole cells affected with CA inhibitors increased 1.7-1.8-fold compared to control. Nevertheless, the transport enhancing effect of MTZ and U-104, DOX fluorescence intensity in 2D cell cultures at pH 6.0 was lower than at pH 7.4.

Similar tendencies were observed on the transport of PLD into 2D cell cultures. None of tested CA inhibitors increased PLD delivery into monolayer-cultured cells (**Figure 2A**, **2B**). MTZ enhanced the delivery of PLD into cells and nucleus after 2 and 4 hours of incubation. Meanwhile, U-104 increased the fluorescence intensity of PLD into cells and nucleus after 1, 2 and 4 hours of incubation (**Figure 2C-E**). After 4 hours of incubation MTZ and U-104 enhanced PLD delivery into monolayer-cultured cells about 1.3 and 1.6-fold, respectively.

Effect of CAIs on DOX and PLD delivery in spheroids (3D cultures)

In order to evaluate the effect of CA inhibitors on the delivery of DOX and PLD into 3D cancer cell cultures, we divided tumor spheroids into three zones-edge zone (0-50 µM), middle zone (100-150 µM) and center zone (200-225 µM).

As in 2D cell cultures, neither MTZ, nor U-104 did not enhance the delivery of DOX and PLD into tumor spheroids at pH 7.4 (**Figure 3A**, **3B**). MTZ did not increase the fluorescence intensity of DOX and PLD into 3D cultures at pH 6.0, as well. Meanwhile U-104 enhanced DOX delivery into edge zone of spheroids at the time period from 1 to 8 h and into the middle zone after 4 and 8 h of incubation (**Figure 3C**, **3D**). After 8 h

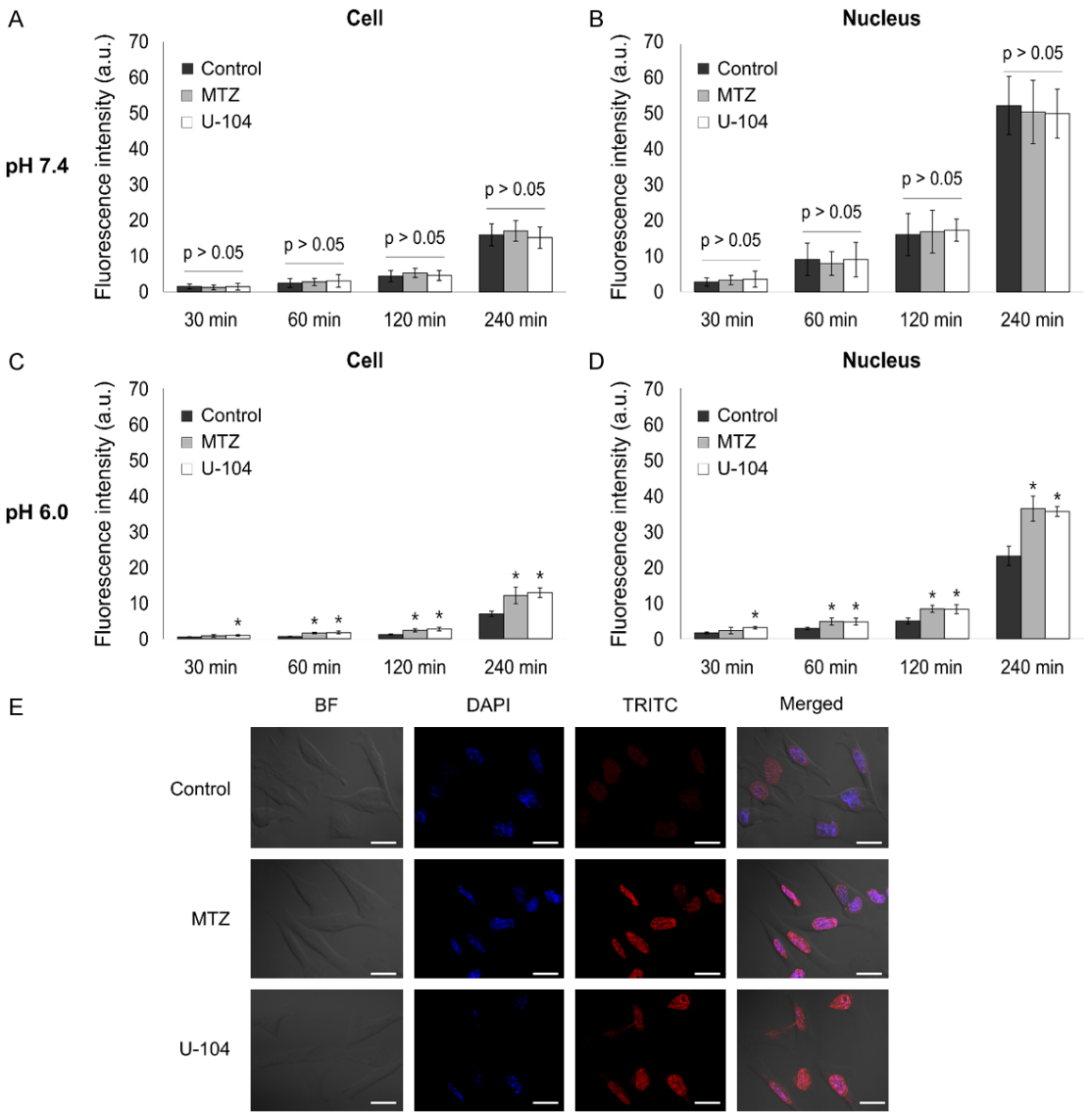

Figure 1. The effect of carbonic anhydrase inhibitors on doxorubicin (DOX) delivery into monolayer-cultured cells at different pHs. A. DOX fluorescence intensity in cells at different time periods at pH 7.4. B. DOX fluorescence intensity in the cell nucleus at different time periods at pH 7.4. C. DOX fluorescence intensity in cells at different time periods at pH 6.0. D. DOX fluorescence intensity in the cell nucleus at different time periods at pH 6.0. E. Images of cells after 4 h of incubation with DOX at pH 6.0. Magnification 600×. Scale bar = 50 μm. The asterisks (*) indicate $P<0.05$. Abbreviations: MTZ, methazolamide.

of incubation DOX fluorescence intensity into edge and middle zones increased 2.4- and 2.3-fold, respectively.

At acidic conditions U-104 increased the penetration of PLD into edge and middle zones of spheroids only after 4 and 8 h of incubation (**Figure 4A**, **4B**, **4D**) but did not enhance PLD delivery into spheroids at physiological pH (**Figure 4C**). At the end of the experiment the fluorescence of PLD into edge and middle zones of spheroids was 1.3- and 2.0-fold more intense, respectively.

Discussion

In the present study, we demonstrated that pretreatment with U-104 enhanced DOX and PLD delivery into 2D and 3D cell cultures at acidic conditions. Meanwhile MTZ improved DOX and

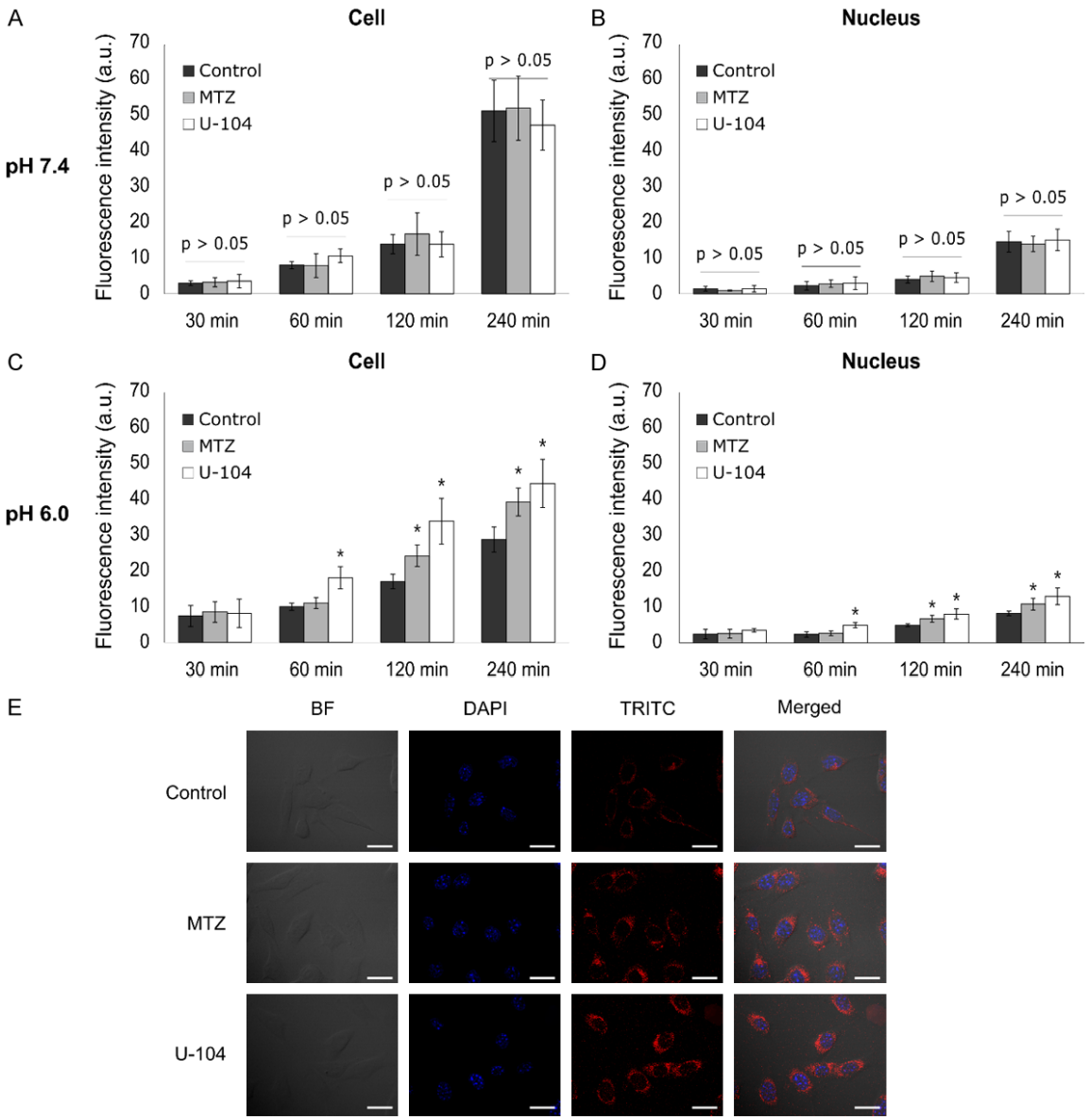

Figure 2. The effect of carbonic anhydrase inhibitors on pegylated liposomal doxorubicin (PLD) delivery into monolayer-cultured cells at different pHs. A. PLD fluorescence intensity in cells at different time periods at pH 7.4. B. PLD fluorescence intensity in the cell nucleus at different time periods at pH 7.4. C. PLD fluorescence intensity in cells at different time periods at pH 6.0. D. PLD fluorescence intensity in the cell nucleus at different time periods at pH 6.0. E. Images of cells after 4 h of incubation with PLD at pH 6.0. Magnification 600×. Scale bar = 50 μm. The asterisks (*) indicate P<0.05. Abbreviations: MTZ, methazolamide.

PLD delivery only in monolayer-cultured cells. The lack of efficacy of MTZ on DOX and PLD delivery into tumor spheroids may be explained by its lower selectivity to CAIX and XII compared with other CA isoforms. Inhibition constants of MTZ against CAI and II are 0.78 nM and 14 nM, respectively; meanwhile its Ki against CA IX is 27 nM [13].

Thus, MTZ possess higher selectivity against CAI and II rather than against CAIX. On the contrary, U-104 is a selective CAIX and XII inhibitor; its Ki against CAI, II, IX and XII is 5,080 nM, 9,640 nM, 45.1 nM and 4.5 nM, respectively [14].

As we hypothesized, at physiological pH both tested CAIs did not enhance drug transport neither in 2D cell cultures, nor in tumor spheroids. Since DOX is a weakly basic compound, at pH 7.4 most of the molecules of DOX stay in non-ionized form and do not possess charge that

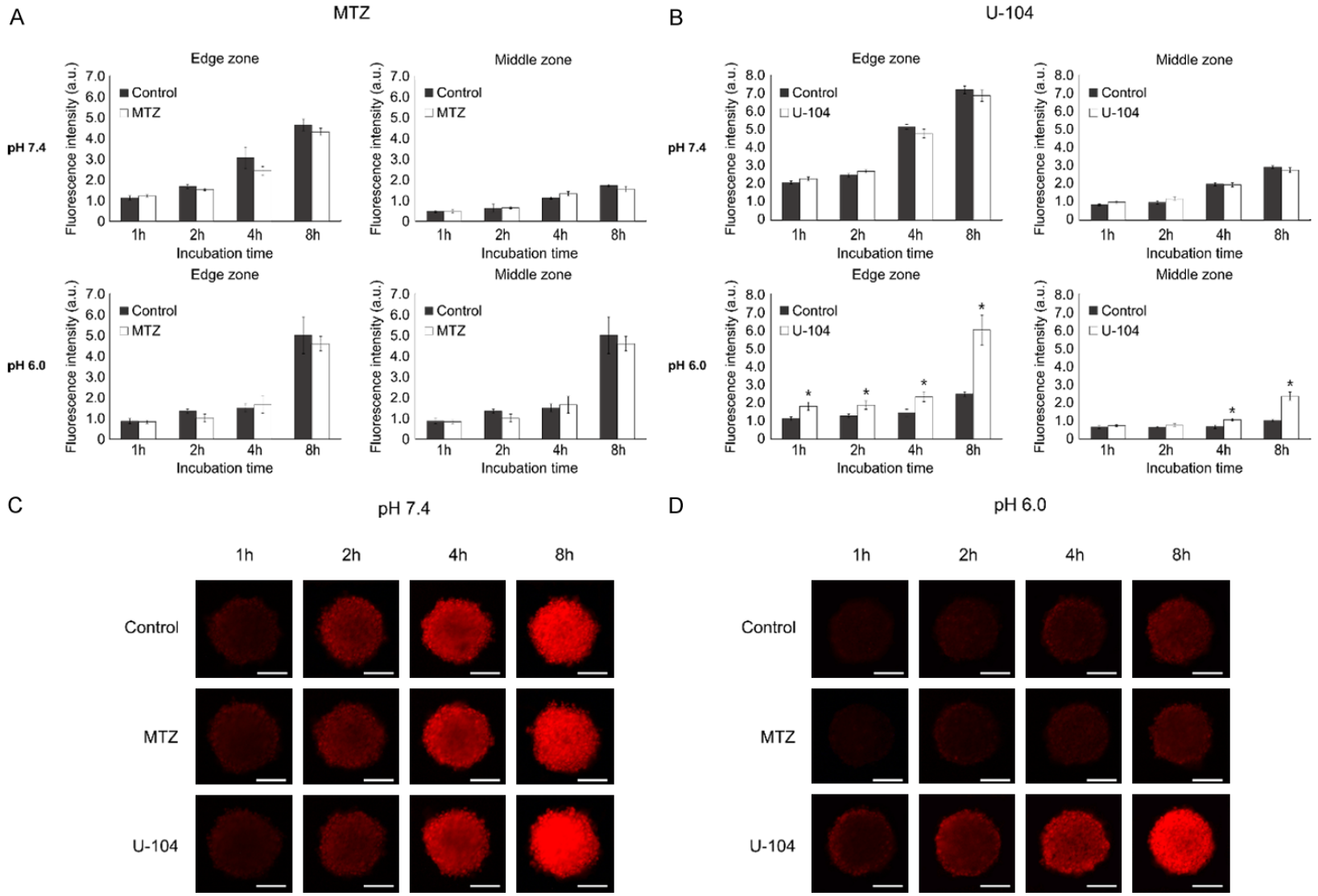

Figure 3. The effect of carbonic anhydrase inhibitors on doxorubicin (DOX) delivery into tumor spheroids at different pHs. A. Fluorescence intensity of DOX in spheroids affected with methazolamide at pH 7.4 and 6.0. B. Fluorescence intensity of DOX in spheroids affected with U-104 at pH 7.4 and 6.0. C. Images of spheroids after different time of incubation with DOX at pH 7.4. D. Images of spheroids after different time of incubation with DOX at pH 6.0. Magnification 100×. Scale bar = 200 μm. The asterisks (*) indicate $P<0.05$. Abbreviations: MTZ, methazolamide.

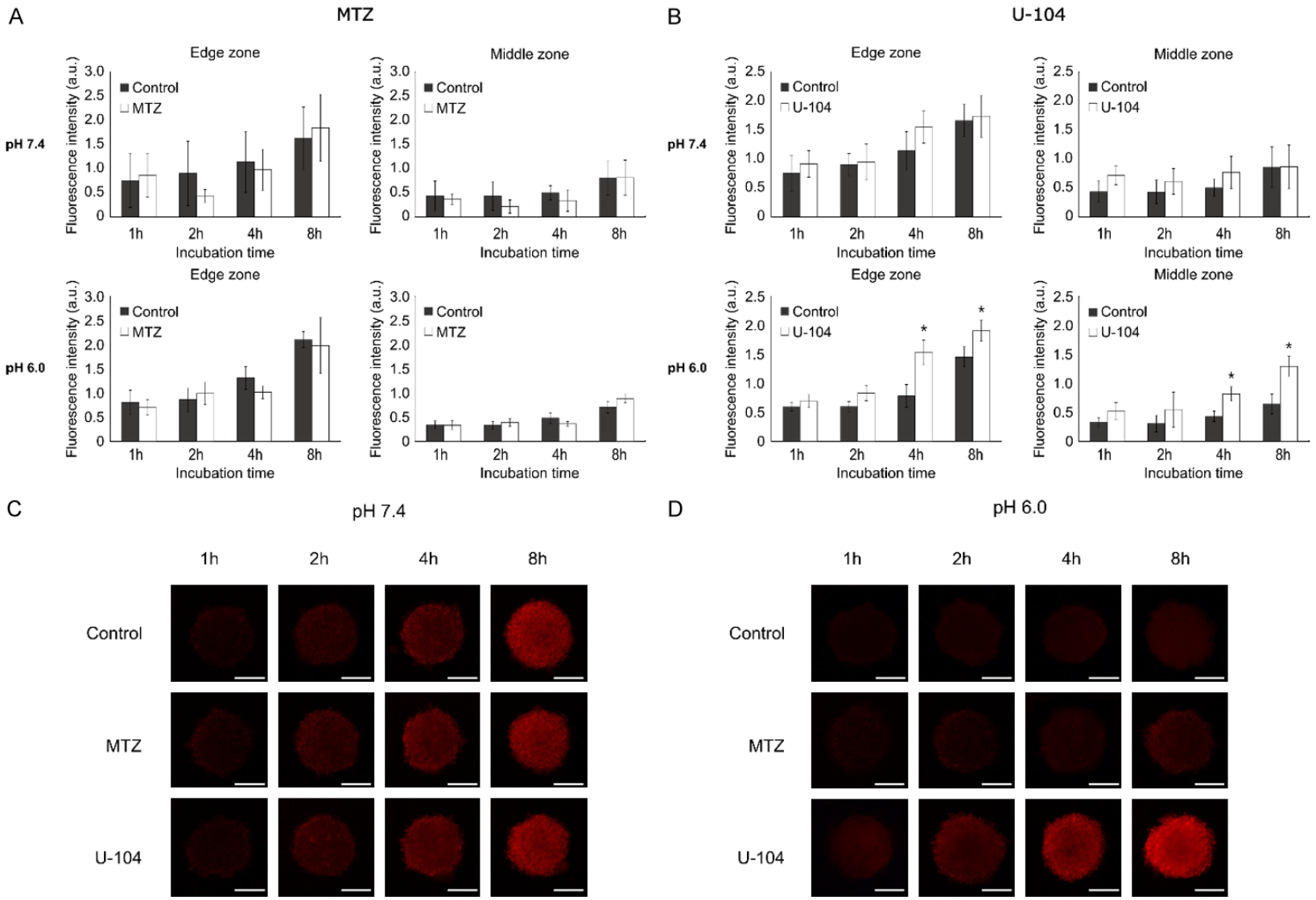

Figure 4. The effect of carbonic anhydrase inhibitors on pegylated liposomal doxorubicin (PLD) delivery into tumor spheroids at different pHs. A. Fluorescence intensity of PLD in spheroids affected with methazolamide at pH 7.4 and 6.0. B. Fluorescence intensity of PLD in spheroids affected with U-104 at pH 7.4 and 6.0. C. Images of spheroids after different time of incubation with PLD at pH 7.4. D. Images of spheroids after different time of incubation with PLD at pH 6.0. Magnification 100×. Scale bar = 200 μm. The asterisks (*) indicate $P<0.05$. Abbreviations: MTZ, methazolamide.

would limit DOX ability to penetrate cell membrane.

Our data agrees with the results previously reported by other authors. Andreucci et al. showed that the addition of 100 μM U-104 to cells treated with temozolomide augmented the percentage of melanoma cell death and increased cytotoxicity of doxorubicin in MCF7 breast cancer cells in acidic conditions [15]. It is known that the activity of temozolomide is related to environmental pH [16]. Gieling et al. investigated the effect of an isoform non-specific CA inhibitor acetazolamide on DOX cytotoxicity and uptake in HT29 colon carcinoma and MDA435 melanoma cells, transfected to overexpress CA IX. They demonstrated that 50 μM acetazolamide increased DOX toxicity from 4.3- to 5.1-fold in HT29 cells, and 12.3-fold in MDA435 cells under anoxic conditions [17].

Other studies also support the hypothesis that the delivery of weakly basic drugs to tumors may be enhanced by modulation of tumor pH using various compounds. In our previous research we showed that proton pump inhibitors may increase the amount of DOX and PLD into monolayer-cultured cancer cells and spheroids at acidic pH [18]. Chen et al. demonstrated that the inhibitors of Na+/H+ exchanger-1 enhance DOX delivery into MDF-7 breast cancer cells and increase the sensitivity for DOX in mice xenograft models [19]. According to our knowledge, none of those classes of compounds are used to modulate the transport of anticancer agents yet. However, the results of in vitro and in vivo experiments support their application for clinical practice.

Conclusions

Both MTZ and U-104 increase DOX and PLD penetration into 2D cancer cell cultures at acidic pH. However, only selective CA IX and XII inhibitor U-104 enhances DOX and PLD delivery into tumor spheroids at acidic conditions. U-104 is a promising transport modulator of weakly basic drugs in triple-negative breast cancer models.

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Disclosure of conflict of interest

None.

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