ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Impact of Medium pH on DOX Toxicity toward HeLa and A498 Cell Lines

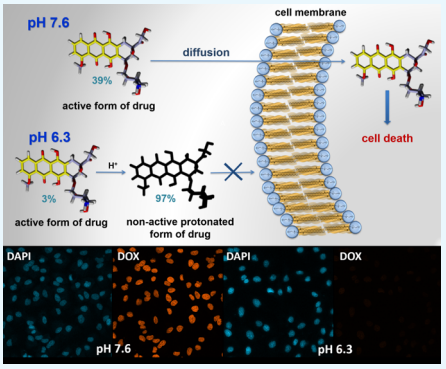
Alicja Trebinska-Stryjewska, Olga Swiech,* Lidia J. Opuchlik, Ewa A. Grzybowska, and Renata Bilewicz*

Cite This: *ACS Omega* 2020, 5, 7979−7986

Read Online

ACCESS | Metrics & More | Article Recommendations

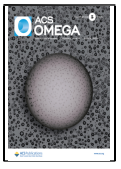
ABSTRACT: The influence of the pH of the multicomponent cell medium on the performance of doxorubicin (DOX), an anticancer drug, was studied on the examples of cervical (HeLa) and kidney (A498) cancer cell lines. The change of pH of the cell medium to more acidic led to a decrease of DOX toxicity on both cell lines due to the change of drug permeability across the cell membrane as a result of drug protonation. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) studies and lactate dehydrogenase (LDH) release tests have shown low toxicity of the drug, especially in the case of A498 cells, which are characterized by an extremely high glycolytic metabolism. The behavior was ascribed primarily to the increased proton concentration in the peripheral blood follicle in the presence of products of the acidic glycolytic metabolism. It is not observed in the measurements performed in commercially available media since they usually have a neutral pH. In earlier reports on kidney cancer, several mechanisms were discussed, including the metabolism of DOX to its less toxic derivative, doxorubicinol, overexpression of ATP binding cassette subfamily B member 1 (ABCB1) transporters, that remove DOX from the inside of cells; however, there was no focus on the simple but very important contribution of drug protonation described in the present study. Drug pH-dependent equilibria in the cell medium should be considered since changes in the drug form may be an additional reason for multidrug resistance.



INTRODUCTION

Cancer is still the top priority issue in the field of medicine, drug delivery, biochemistry, and molecular biology due to the low efficiency of the therapies and many adverse effects assisting the therapy and arising, e.g., from multidrug resistance. Doxorubicin (DOX, Adriamycin) has been used clinically to treat cancer since 1969 and displays an extremely broad spectrum of activity both in experimental tumor models and in human malignancies.[1] Clinical effects of the drug are linked to the modification of the DNA structure.[2−4] Moreover, doxorubicin inhibits topoisomerase II, increasing the stability of a drug−enzyme−DNA cleavable complex during DNA replication and impairing DNA repair. Moreover, doxorubicin activity is directly connected with the pH cancer environment.[5] The tumors exhibit a substantially lower extracellular pH (pH_e) than normal healthy tissues, whereas the intracellular pH (pH_i) of both tissues is quite similar. The low pH_e in tumors can reduce the effectiveness of some chemotherapies due to reduction in the cycling cell fraction,[6] selection for apoptosis-resistant cell phenotypes,[7] and direct effect of ion gradients on drug distribution or ion trapping.[8] The acidic pH_e of tumors will therefore effectively hinder weakly basic drugs, such as doxorubicin, from reaching their intracellular target, thereby reducing cytotoxicity.[9] The toxicity of DOX is strongly influenced by the variation of extracellular pH.[10] The drug is almost impermeable through the membrane in the charged form according to the "ion trapping" hypothesis.[8] Extensive investigation of the mechanism of doxorubicin uptake indicates that passive diffusion of the nonionized form of the drug is the most likely explanation for the pH-dependent cellular drug uptake[11] as well as the uptake in cases of DOX encapsulated in drug carriers.[12−14] HeLa cells are often used for toxicity investigations of DOX-tailored drug delivery systems.[15−18] In comparison to other cancer cell lines, HeLa cells show moderate levels of doxorubicin internalization, but they are very sensitive to DOX treatment.[19,20] The other model of used cancer cells is A498 (renal cell carcinoma).[21] It is characterized by homozygous mutations of the *VHL* (von Hippel-Lindau tumor suppressor) gene that cause the loss of VHL protein activity. It results in improper control of the hypoxia-inducible factor (HIF) that regulates the response of the cells to the decreased oxygen concentration in the cell environment.[21−24] The intracellular pH does not undergo significant changes, while extracellular pH becomes more acidic. The A498 cell line

Received: December 30, 2019
Accepted: March 20, 2020
Published: April 1, 2020



https://dx.doi.org/10.1021/acsomega.9b04479
ACS Omega 2020, 5, 7979−7986

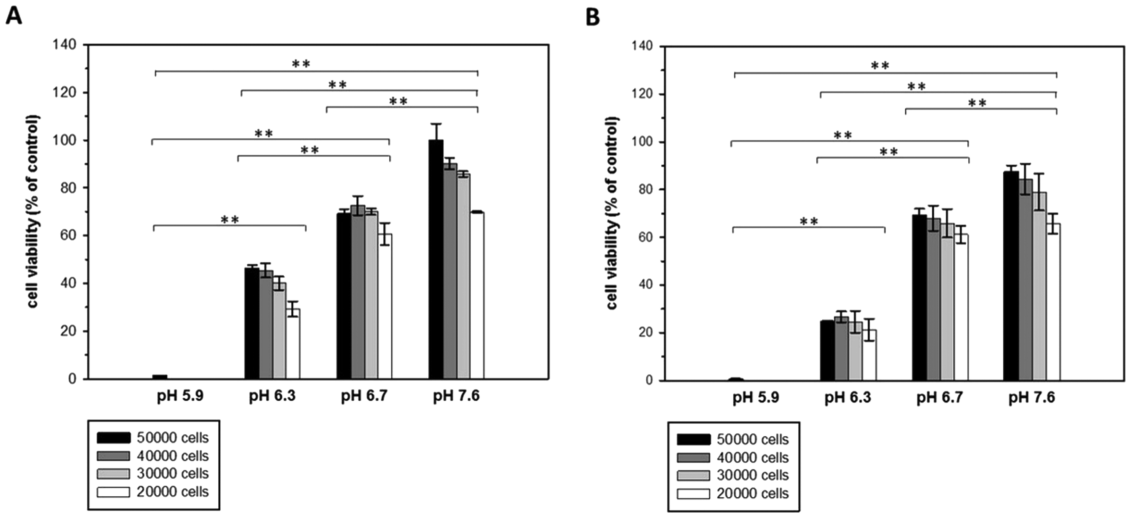


Figure 1. Dependence of the viability of HeLa cancer cells (measured by the MTT test) on the pH of the medium itself for cell populations containing a different number of cells after 24 h (A) and 48 h (B) from the exposure. All results were normalized to a control (5×10^4 cells at 24 h, pH 7.6). Statistical analysis (two-way analysis of variance (ANOVA) followed by the post-hoc Tukey test) showed significant differences between mean cell viability for different pH values of the medium (24 and 48 h: $p < 10^{-4}$) and cell numbers (24 and 48 h: $p = 2.12 \times 10^{-4}$). Significant differences in the post-hoc Tukey test are denoted with ** (for clarity, only differences for pH are shown; only 5×10^4 vs 2×10^4 cells difference was significant in post-hoc analysis).

is less sensitive to doxorubicin treatment. This lower sensitivity is explained by the presence of a faster metabolic path of converting doxorubicin to its less toxic derivative, doxorubicinol.[3] Lee et al. also discovered that A498 cells are characterized by the high level of transporters responsible for the removal of DOX from the cells.

The majority of already published research is conducted with the use of commercially available cell media whose pH is above 7, which is typical for healthy tissue. These experiments do not reflect the real conditions of pathological cell divisions and growth; therefore, to carry out biological experiments in conditions similar to those of the cancer cells, it is important to adjust the pH of cell media to the proper value. The experiments in this work were performed in both neutral (pH 7.6) and acidified (pH 6.3) cell media. The influence of various factors that have an impact on the toxicity results on HeLa and A498 cell lines was evaluated, that is, the pH of the cell media and the size of the cell population. The behavior of these two cell lines in the media with varying acidity has, to our knowledge, never been examined before.

RESULTS AND DISCUSSION

Influence of pH and Cell Population Size on Viability of HeLa Cells. Figure 1 shows the dependence of the viability of HeLa cancer cells measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test on the pH of the medium itself (without drug) for cell populations containing a different number of cells after 24 h (Figure 1A) and 48 h (Figure 1B) from the exposure.

As shown in Figure 1, the pH of the medium has an impact on the viability of HeLa cancerous cells for all sizes of the cell population. For pH 7.6 and 6.7, the dependence of the toxicity of the environment is not profound (although the difference is statistically significant), but below pH 6.7, there is a rapid threshold change in viability. At pH 6.3, after 24 h, half of the initial population is still vital, while after 48 h, around 30% maintains its biological functions. Below pH 6, the cells do not reveal any vital function, which indicates that the environment itself is toxic in 100% for the HeLa cell line. These results are particularly important and interesting. Most of the literature reports that the extracellular pH of cancer cells is lower (ranging from 6.3 to 7.0, as measured *in vivo* by various methods) than the pH of the healthy tissue (pH 7.0−7.6).[25−28] According to this, all of the biological tests should be conducted in acidified media, simulating at least the chemical properties of the natural environment of malignant tissue instead of the healthy one. Due to the described differences, further experiments were performed at two pH values, 7.6 and 6.3.

For pH 6.3, the time-dependent toxicity of the medium was observed. After 48 h, the viability dropped almost twice compared to its value after 24 h, whereas the toxicities above pH 6.3 were quite similar, both after 24 and 48 h. Therefore, further toxicity experiment results are normalized to the control.

Influence of Concentration of DOX on Viability of the HeLa Cell Line after 48 h. The studies were conducted with the aim to investigate the effect of increasing concentrations of DOX on HeLa survival rates measured by the MTT test. At pH 7.6, DOX caused a significant decrease in cell viability at concentrations higher than 5 μM. The respective viability was around 10% and did not change with increasing therapeutic agent concentration (Figure 2, dots). In a more acidic environment, at pH 6.3, the performance of DOX was not as effective as in the neutral medium, and the slope of the curve was smaller (Figure 2, open squares).

Toxicities of DOX toward HeLa and A498 at pH 7.6 and 6.3. Cancerous cell lines, A498 human kidney carcinoma cells, were chosen for comparison in this study because of different metabolisms, an oxidative glycolytic path. These cells are, therefore, better adapted to acidic extracellular pH than HeLa cells, and as a result, they show higher viability at lower pH compared to physiological pH (control) (Figure 3). Statistical analysis showed a significant difference between both cell lines in their response to lowered pH. Furthermore, the experiments revealed that the viability is connected with the

https://dx.doi.org/10.1021/acsomega.9b04479
ACS Omega 2020, 5, 7979−7986

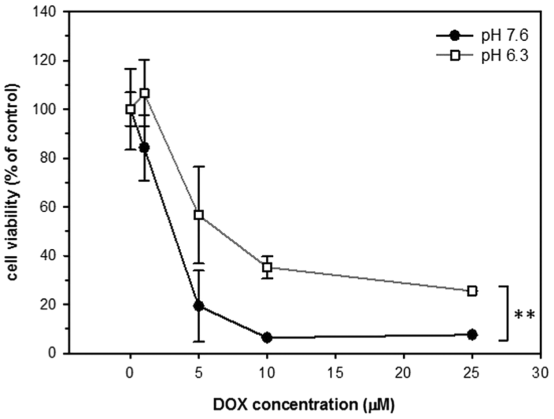


Figure 2. Cell viability dependences on DOX concentration measured by the MTT test at pH 7.6 (black line, dotted circles) and 6.3 (gray line, open squares) for the HeLa cell line after 48 h of treatment. The number of treated cells: 2.5×10^4. Results were normalized to the control (dimethyl sulfoxide (DMSO)-treated cells), separately for pH 7.6 and 6.3. Statistical analysis (two-way ANOVA with the interaction followed by the post-hoc Tukey test) showed significant differences between mean cell viabilities for different pH values of the medium ($p = 1.98 \times 10^{-3}$, denoted with **) and increasing DOX concentrations ($p = 1.40 \times 10^{-6}$).

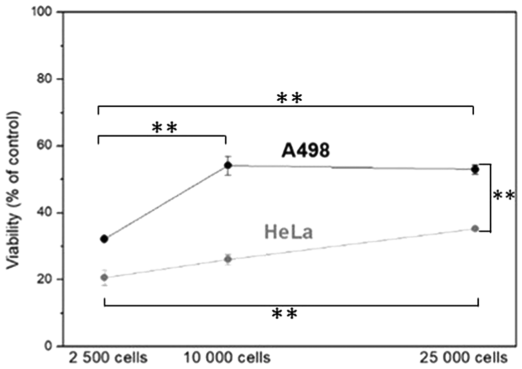


Figure 3. Dependence of the viability of HeLa and A498 cancer cells (measured by the MTT test at pH 6.3) on the cell population after 48 h of exposure to lower pH compared to control (cells grown at pH 7.6). Statistical analysis (two-way ANOVA with interaction) showed significant differences ($p = 2.0 \times 10^{-6}$) between both cell lines in their response to lowered pH. There were also differences in how populations of different cell numbers responded to pH 6.3 (two-way ANOVA, cell number: $p = 2.9 \times 10^{-5}$, post-hoc Tukey test: significant results denoted with **).

size of the population of cancerous cells. The following tendency was observed: the bigger the population, the better the viability. It is not unexpected and can be explained by the decreasing ratio of H^+ ions to the number of cells. However, this relationship was more pronounced for smaller cell populations, below 2×10^4, as it was not clearly observed for cell populations with greater numbers (Figure 1).

Treatment of A498 cells with increasing concentrations of DOX (Figure 4) showed their overall greater resistance to the drug in comparison to HeLa cells (Figure 2). However, similar to HeLa, there was a significant difference in cell viability between pH 6.3 and 7.6.

The measurements allowing to compare the toxicities of DOX on two cell lines, HeLa and A498, were performed at two pH values, 7.6 and 6.3, after 24 and 48 h. The concentration of DOX used for HeLa cells was equal to 5 μM, while for A498, it

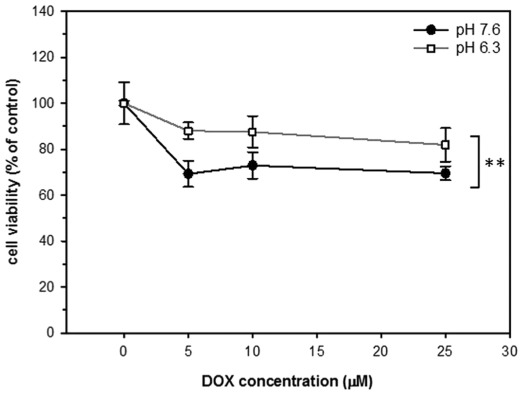


Figure 4. Dependence of cell viability on the concentration of DOX measured by the MTT test at pH 7.6 (dotted joints) and 6.3 (open square joints) for the A498 cell line after 24 h of treatment. The number of treated cells: 2.5×10^4. Results were normalized to the control (DMSO-treated cells), separately for pH 7.6 and 6.3. Statistical analysis (two-way ANOVA with interaction followed by the post-hoc Tukey test) showed significant differences between mean cell viabilities for different pH values of the medium ($p = 4.4 \times 10^{-3}$, denoted with **) and increasing DOX concentrations ($p = 1.4 \times 10^{-3}$).

was five times higher (25 μM) due to the reported resistance of this cell line to anthracycline drugs.[3,29] The population size was equal to 2.5×10^4 cells per treatment.

The toxicity of DOX differed between two tested pH values (Figure 5). It decreased with the decrease of the pH value. This effect was especially enhanced in the case of the A498 cell line. HeLa cancerous cells exhibited higher sensitivity toward DOX treatment. The viability values at pH 7.6 after 24 h were 28.0 ± 12.4 and 77.9 ± 19.0% for HeLa and A498, respectively. After 48 h, these values decreased more than twice and were equal to 10.9 ± 4.3% for HeLa and 32.7 ± 6.3% for A498. At a lower pH (6.3), two cell lines behaved differently. For HeLa cells exposed to DOX, the viability decreased in time. A498 cells did not reveal any sensitivity to DOX at this pH over time. In contrast, they were completely resistant to the DOX treatment. The viability values at pH 6.3 after 24 h were 94.4 ± 10.4 and 104.2 ± 9.6% for HeLa and A498, respectively. After 48 h, these values were equal to 59.3 ± 7.1% for HeLa and 101.2 ± 8.3% for A498 (Figure 5A).

The LDH release test allowed to directly measure the toxicity of DOX (Figure 5B). At pH 7.6, DOX showed significantly higher toxicity toward HeLa and A498 cells than the vehicle control. High (25 μM) concentration induced a massive cell damage after 48 h of treatment in A498 cells treated with DOX. The acidic environment (pH 6.3) itself was toxic to HeLa cells, which is consistent with the results presented in Figure 1. The addition of DOX resulted in a minimal increase in toxicity in comparison with DMSO. A498 showed complete resistance to DOX at pH 6.3, as indicated by similar values of % LDH release as DMSO-treated cells.

The differences in toxicities in normal and acidic pH may be ascribed to the level of protonation of doxorubicin. Neutral, uncharged, form of DOX is easily permeable through the cell membrane, while the protonated, ionized, form does not efficiently penetrate it, due to the diffusional character of the drug transport[9,10] (Scheme 1). Accordingly, we observed an at least 3-fold lower amount of DOX accumulated in HeLa and A498 cells at lower pH after 4 h (Table 1).

https://dx.doi.org/10.1021/acsomega.9b04479
ACS Omega 2020, 5, 7979−7986

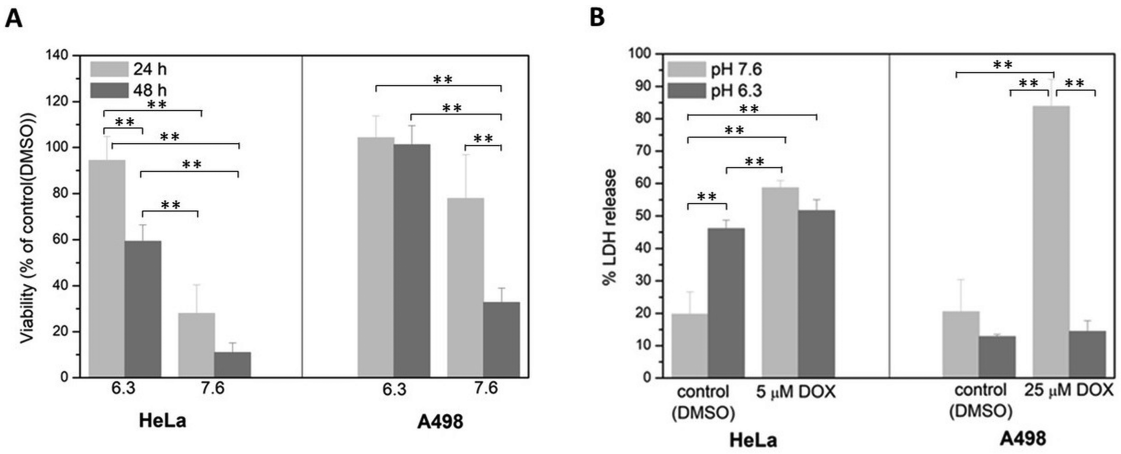


Figure 5. MTT (A) and lactate dehydrogenase (LDH) (B) test results obtained for DOX treatment of both cell lines, HeLa and A498, at two pH values, 7.6 and 6.3. The concentration of DOX was 5 μM for HeLa and 25 μM for A498 cells. Results of the MTT test were normalized to the control (DMSO-treated cells). Statistical analysis (one-way ANOVA followed by a post-hoc Tukey test) showed significant differences between groups indicated with **.

Scheme 1. Scheme of Transport of Doxorubicin through the Biological Membrane

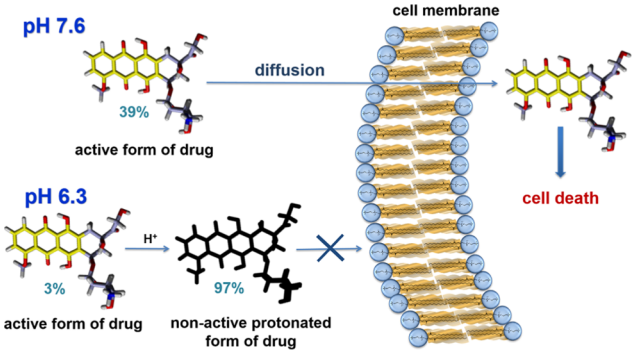


Table 1. Fluorescence of Free DOX Accumulated in Cell, Dependent on pH for HeLa and A498 Cancer Cell Lines after 4 h of Treatment[a]

cell line	pH 7.6	pH 6.3
HeLa	264.8 ± 8.0	74.8 ± 1.4
A498	170.0 ± 6.9	43.4 ± 1.4

[a]DOX fluorescence was measured on a plate reader and relative fluorescence values were normalized for 1 μM of DOX.

This trend was confirmed by performing confocal microscopy measurements. The fluorescence level registered after 4 h of exposure to the therapeutics revealed the decrease of the accumulation of DOX in the nucleus at pH 6.3 compared to pH 7.6. While for the HeLa cell line, the fluorescence of DOX was still observable even at lower pH values (Figure 6), in the case of the A498 cell line, at pH 6.3, DOX did not give a fluorescence signal anymore (Figure 6), confirming the lack of permeability of the drug through the nucleus membrane. The accumulation of DOX in the nucleus at pH 7.6 was similar for both cell lines.

Weaker DOX toxicity toward the A498 cell line at pH 7.6, compared to HeLa cells, can be explained taking into account several processes: metabolism of DOX to its less toxic/active derivative, doxorubicinol, overexpression of ATP binding cassette subfamily B member 1 (ABCB1) transporters that

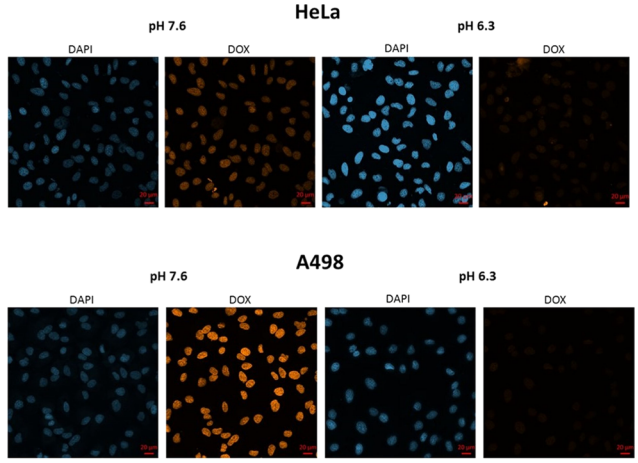


Figure 6. Confocal images of HeLa and A498 cells after 4 h of treatment with DOX (orange) at pH 7.6 and 6.3. Cell nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI) (blue).

remove DOX from the inside of cells, and the effect of pH-dependent DOX protonation. At lower, more acidic pH of the medium, the last one becomes dominant, since it blocks the performance of the drug. It explains, e.g., the lack of efficacy of doxorubicin in the case of kidney cancer. Metabolic over-acidification of the tumor environment by glycolytic nutrition of renal cancer cells promotes the protonation of doxorubicin that turns into an inactive ionic form, preventing drug transport across the cell membrane and inhibiting DOX toxic action (Scheme 1).

Doxorubicin contains three prototropic groups in their structure: one ammonium group (C3′) and two hydroxyl groups (C6 and C11) (Scheme 2). Due to the possibility of protonation and deprotonation of individual groups, the aqueous solutions of DOX contain a mixture of four forms of the drug, whose concentrations depend on the presence of H^+ in the solution.

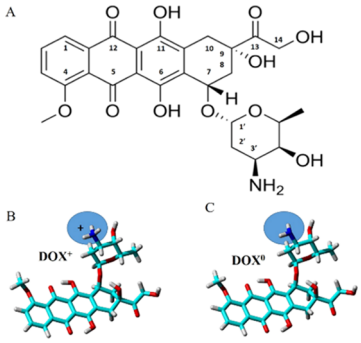
$$DOX^+ \leftrightarrow DOX^0 + H^+ \quad (1)$$

$$DOX^0 \leftrightarrow DOX^- + H^+ \quad (2)$$

$$DOX^- \leftrightarrow DOX^{2-} + H^+ \quad (3)$$

https://dx.doi.org/10.1021/acsomega.9b04479
ACS Omega 2020, 5, 7979−7986

Scheme 2. Structure of Doxorubicin (A) and its Two Forms—Protonated (B) and Neutral (C)



The ionization constants are defined as

$$K_1 = \frac{[DOX^0]\cdot[H^+]}{[DOX^+]} \quad (4)$$

$$K_2 = \frac{[DOX^-]\cdot[H^+]}{[DOX^0]} \quad (5)$$

$$K_3 = \frac{[DOX^{2-}]\cdot[H^+]}{[DOX^-]} \quad (6)$$

where DOX^+ is the protonated form of the drug at the C3′ position, DOX^- and DOX^{2-} are the first and second deprotonated forms of the drug at C6 and C11 hydroxyl groups, DOX^0 is an uncharged form of DOX.

Due to the balance between all DOX forms described by equilibrium constants K_1−K_3, the total doxorubicin concentration (DOX_{total}) can be represented as follows

$$DOX_{total} = DOX^+ + DOX^0 + DOX^- + DOX^{2-} \quad (7)$$

By the combination of eqs 4−6 with eq 7, we can determine the concentrations of each doxorubicin form from the following equations

$$[DOX^+] = \left(\frac{[H^+]^3}{[H^+]^3 + [H^+]^2K_1 + [H^+]K_1K_2 + K_1K_2K_3}\right)DOX_{total} \quad (8)$$

$$[DOX^0] = \left(\frac{[H^+]^2K_1}{[H^+]^3 + [H^+]^2K_1 + [H^+]K_1K_2 + K_1K_2K_3}\right)DOX_{total} \quad (9)$$

$$[DOX^-] = \left(\frac{[H^+]K_1K_2}{[H^+]^3 + [H^+]^2K_1 + [H^+]K_1K_2 + K_1K_2K_3}\right)DOX_{total} \quad (10)$$

$$[DOX^{2-}] = \left(\frac{K_1K_2K_3}{[H^+]^3 + [H^+]^2K_1 + [H^+]K_1K_2 + K_1K_2K_3}\right)DOX_{total} \quad (11)$$

The concentrations of individual forms of the drug in a given environment depend on the pH, and the total concentration of doxorubicin added to the solution. Values of K_1−K_3 at 37 °C, the standard cell culture temperature, are not available. The literature reports provide the values of constants only for 25 and 50 °C. Therefore, K_1−K_3 values for 37 °C were calculated using the van Hoff equation under the assumption that the reaction enthalpy ΔH is constant

$$\frac{d\ln K}{dT} = \frac{\Delta H^\theta}{RT^2} \quad (12)$$

where ΔH^θ is the reaction enthalpy, R is the gas constant, and T is the temperature.

The concentration values calculated for the experimental conditions used based on literature values of K_1−K_3 for 25 and 50 °C and calculated values of K_1−K_3 for 37 °C are collected in Table 2.

The concentration of the active form of doxorubicin drops significantly when the pH is changed from 7.6 to 6.3. At room temperature, we observe 18−36 times lower concentration of DOX^0 at the pH of cancer cells compared to the pH of cell media used commercially in the studies of toxicity and mechanistic studies of the drug−cell interactions.

The increase in temperature from 25 to 50 °C causes a slight decrease in the value of pK_1 (exothermic reaction). As a consequence, at pH 7.6, the concentration of the active form of the drug DOX^0 is slightly higher than the protonated form DOX^+. However, at pH 6.3, even at a higher temperature, the concentration of active DOX^0 is not larger than 6% of the total concentration of doxorubicin in the solution.

CONCLUSIONS

The experiments performed in both neutral (pH 7.6) and acidified (pH 6.3) cell media showed that the pH of the medium has a crucial impact on the viability of HeLa and A498 cancerous cell lines. At pH 7.6 and 6.7, the toxicity of the environment is not significant, but below these values, there is a rapid threshold change of the toxicity, which finally increases to 100% below pH 6.0 (for HeLa). On the other hand, A498 cells are more resistant to lower pH than HeLa cells as a result of possessing different metabolisms, the oxidative glycolytic path.

Furthermore, the viability of cancerous cells is connected with the size of the cancerous cell population. The larger the population, the better the viability. However, this relationship was more pronounced for smaller cell populations, below 2×10^4, as it was not clearly observed for cell populations with greater numbers. The drug toxicities decreased with the decrease of the pH value. The toxicity of DOX toward both cell lines was completely different. Due to the high resistance of the A498 cell line to anthracyclines, the effective concentration that gave a pronounced effect was equal to 25 μM and was five times larger than needed for the HeLa cell line. The pH value also had an impact on therapeutic performance. LDH release and MTT tests revealed that at pH 7.6, both cell lines exhibited sensitivity to the therapeutics, while at pH 6.3, only the HeLa cell line was vulnerable to the therapy. In contrast, A498 cells were completely resistant to treatment at lower pH values. It was confirmed by confocal microscopy.

Weaker DOX toxicity toward the A498 cell line at pH 7.6, compared to HeLa cells, could result from the overlapping of several processes: metabolism of DOX to its less toxic/active derivative, doxorubicinol, transporters that remove DOX from the inside of cells or DOX protonation effect. At a lower, more acidic pH of the medium, the last one is dominant, and it completely blocks the performance of the drug. The conducted experiment may explain the lack of efficacy of doxorubicin over kidney cancer. Metabolic overacidification of the tumor environment by glycolytic nutrition of renal cancer cells promotes the protonation of doxorubicin that turns into an

https://dx.doi.org/10.1021/acsomega.9b04479
ACS Omega 2020, 5, 7979−7986

Table 2. Concentration Values of DOX^+, DOX^0, DOX^-, and DOX^{2-} Calculated for Experimental Conditions (pH 7.6 and 6.3) for 25, 37, and 50 °C

cell line	pH	pK_1	pK_2	pK_3	total DOX [μM]	DOX^+ [μM]	DOX^0 [μM]	DOX^- [μM]	DOX^{2-}	[%] DOX^+	[%] DOX^0
HeLa 50 °C	7.6	7.5[30]	9.4[30]	10[30]	5	2.18	2.78	4.4×10^{-2}	$1{,}8 \times 10^{-4}$	43.6	55.5
	6.3				5	4.70	0.30	2.4×10^{-4}	4.8×10^{-8}	94.0	6.0
A498 50 °C	7.6				25	10.9	13.9	2.2×10^{-1}	8.8×10^{-4}	43.6	55.5
	6.3				25	23.5	1,50	1.2×10^{-1}	2.5×10^{-7}	94.0	6.0
HeLa 25 °C	7.6	7.84[31]	9.5[34]	10.1[33]	5	3.16−4.08	0.91−1.82	1.2×10^{-2}−2.3×10^{-2}	3.4×10^{-5}−7.2×10^{-5}	63.2−81.5	18.3−36.4
	6.3	8.2[32]	10[30]		5	4.86−4.94	0.14−0.055	3.5×10^{-5}−8.8×10^{-5}	5.5×10^{-9}−1.4×10^{-8}	97.2−98.9	1.11−2.80
A498 25 °C	7.6	8.25[33]	10.2[31]		25	15.8−20.0	4.56−9.09	5.7×10^{-2}−1.1×10^{-1}	1.8×10^{-4}−3.6×10^{-4}	63.2−81.5	18.3−36.4
	6.3				25	24.3−24.7	0.27−0.70	1.8×10^{-4}−4.4×10^{-4}	2.8×10^{-8}−7.0×10^{-8}	97.2−98.9	1.11−2.80
HeLa 37 °C	7.6	7.8[a]	9.65[a]	10.05[a]	5	3.05	1.93	1.7×10^{-2}	6.2×10^{-5}	61.0	39.0
	6.3				5	4.85	0.15	6.9×10^{-5}	1.2×10^{-8}	96.9	3.08
A498 37 °C	7.6				25	15.2	9.67	8.7×10^{-2}	3.1×10^{-4}	61.0	39.0
	6.3				25	24.2	0.77	3.5×10^{-4}	6.2×10^{-8}	96.9	3.08

[a]Based on eq 12, the mean values of reported pK_1 and pK_2 at 25 °C were taken to calculate the values at 37 °C.

inactive ionic form, preventing drug transport across the cell membrane and inhibiting DOX toxic action.

These results are particularly important due to the fact that the extracellular pH of the cancer cell is lower than the pH of the healthy tissue, the biological test should be conducted in acidified media simulating the chemical properties of the natural environment of malignant tissue instead of that of the healthy one. Unfortunately, the commercially available media that are used in common measurements exhibit neutral or slightly basic pH, hence do not reproduce the conditions of the environment resulting from the presence of metabolic products of cancer cell glycolysis. The above results indicate that the concentrations of hydrogen ions in the cell media have a significant effect on the measurement results and the conclusions drawn and, therefore, should be carefully considered in designing meaningful biological/biomedical experiments.

EXPERIMENTAL SECTION

Chemicals and Reagents. The doxorubicin hydrochloride salt was purchased from LC Laboratories (Woburn). Roswell Park Memorial Institute (RPMI) medium, fetal bovine serum, penicillin, streptomycin, and *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) buffer were purchased from Thermo Fisher Scientific. Other compounds used in this work were purchased from Aldrich, Fluka. The pH was measured using a pH-Meter E2 (Mettler Toledo).

Cell Lines. Two human cancer cell lines were used: cervical cancer HeLa (American Type Culture Collection, ATCC) and renal carcinoma A498 (ATCC). The cells were routinely grown in an RPMI medium supplemented with 10% fetal bovine serum, penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 °C, 5% CO_2 in a humidified incubator. For the experiments, 25 mM of HEPES buffer was added to the cell medium to stabilize the pH independent of CO_2/$NaHCO_3$ buffer. HCl (PPH Standard) was used to acidify cell medium.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Test, Lactate Dehydrogenase (LDH) Release Tests and Accumulation of Doxorubicin in Cells. Indicated numbers of HeLa or A498 cells were seeded onto 96-well plates in 100 μL of standard cell culture medium 24 h before experiments. Free doxorubicin was diluted to a final concentration of 1.25 mM, with DMSO. Doxorubicin was incubated for 18 h at 4 °C, then for 1 h at 22 °C and diluted with the cell culture medium with pH 7.6 or 6.3 (with 25 mM HCl) to a final doxorubicin concentration of 5 μM (HeLa) or 25 μM (A498). The standard cell culture medium was replaced with 100 μL of medium containing tested compounds, and cells were treated for 24 or 48 h. The cell culture medium at pH 7.6 or 6.3 was used for untreated controls and medium with 0.1% DMSO (HeLa) or 0.5% DMSO (A498) was used as vehicle control.

MTT Test. After 24 and 48 h of treatment, the cell culture medium was replaced with 100 μL of medium containing 0.5 mg mL^{-1} MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) and cells were incubated at 37 °C, 5% CO_2. After 2 h of incubation, the medium was replaced with 100 μL DMSO to dissolve formazan crystals. Plates were incubated for 1 h at room temperature in the dark, and absorbance at 540 nm (1 s) was measured using Wallac 1420 Multilabel Counter (Perkin Elmer).

LDH Release Test. The LDH release test was performed after 48 h of treatment using the Pierce LDH Cytotoxicity

https://dx.doi.org/10.1021/acsomega.9b04479
ACS Omega 2020, 5, 7979−7986

Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Absorbance at 490 nm (1 s) for spontaneous and maximum (after cell lysis) LDH activity was measured for all types of treatment using the Wallac 1420 Multilabel Counter. The background absorbance of the cell culture medium with pH 7.6 and 6.3 at 490 nm was assessed independently. % LDH release was calculated for each condition as described by Smith et al.[35] using the following formula 1

$$\%\ \text{LDH release} = 100 \times \frac{A_{s\,(490\ nm)} - A_{b\,(490\ nm)}}{A_{m\,(490\ nm)} - A_{b\,(490\ nm)}} \qquad (13)$$

where $A_{(490\ nm)}$ is the absorbance at 490 nm, s is the spontaneous release, b is the background (cell culture medium), and m is the maximum release (after cell lysis).

Accumulation of Doxorubicin in Cells. After 4 h of treatment, the cells were washed twice with 100 μL phosphate-buffered saline, pH 7.4 (PBS, 137 mM NaCl, 2.7 KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), 100 μL of PBS was added to the wells, and doxorubicin fluorescence was measured with the Wallac 1420 Multilabel Counter from the bottom of the plate using excitation/emission: 485/600 nm (0.1 s).

Confocal Microscopy. After 4 h of treatment with DOX, the cells were fixed as described previously,[36] stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), mounted and observed using the Zeiss LSM 800 confocal microscope with a maximally opened pinhole.

Statistical Analysis. The results from three independent experiments are presented as means ± standard deviations. Comparisons between more than two groups were performed using one-way or two-way ANOVA with or without interaction followed by the post-hoc Tukey test. P values adjusted to account for multiple comparisons are reported. All tests were two-sided, and α was set to 0.05. Statistical analysis was performed using Statistica version 13.3 (TIBCO Software Inc.). The differences were considered to be not significant (not marked in the figures) or very significant when $p < 0.01$ (denoted with ** in the figures).[37]

AUTHOR INFORMATION

Corresponding Authors

Olga Swiech − *Faculty of Chemistry, University of Warsaw, 02-093 Warsaw, Poland;* orcid.org/0000-0003-3322-2497; Email: oswiech@chem.uw.edu.pl

Renata Bilewicz − *Faculty of Chemistry, University of Warsaw, 02-093 Warsaw, Poland;* orcid.org/0000-0003-0058-3691; Email: bilewicz@chem.uw.edu.pl

Authors

Alicja Trebinska-Stryjewska − *Institute of Optoelectronics, Biomedical Engineering Centre, Military University of Technology, 00-908 Warsaw, Poland; Department of Molecular and Translational Oncology, Maria Sklodowska-Curie National Research Institute of Oncology, 02-781 Warsaw, Poland*

Lidia J. Opuchlik − *Faculty of Chemistry, University of Warsaw, 02-093 Warsaw, Poland*

Ewa A. Grzybowska − *Department of Molecular and Translational Oncology, Maria Sklodowska-Curie National Research Institute of Oncology, 02-781 Warsaw, Poland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.9b04479

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Science Center through Project DEC-2014/13/B/ST5/04117. O.S. also thanks the National Science Center for the support through Project O. Swiech DEC-2011/01/N/ST5/05550.

REFERENCES

(1) Arcamone, F.; Franceschi, G.; Penco, S.; Selva, A. Adriamycin (14-hydroxydaunomycin), a novel antitumor antibiotic. *Tetrahedron Lett.* **1969**, *10*, 1007−1010.

(2) Beretta, G. L.; Zunino, F. Molecular Mechanisms of Anthracycline Activity. In *Anthracycline Chemistry and Biology II: Mode of Action, Clinical Aspects and New Drugs*; Krohn, K., Ed.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2008; pp 1−19.

(3) Bains, O. S.; Szeitz, A.; Lubieniecka, J. M.; Cragg, G. E.; Grigliatti, T. A.; Riggs, K. W.; Reid, R. E. A Correlation between Cytotoxicity and Reductase-Mediated Metabolism in Cell Lines Treated with Doxorubicin and Daunorubicin. *J. Pharmacol. Exp. Ther.* **2013**, *347*, 375−387.

(4) Quigley, G. J.; Wang, A. H.; Ughetto, G.; Marel, G.; van der Boom, J. H.; van and Rich, A. Molecular structure of an anticancer drug-DNA complex: daunomycin plus d(CpGpTpApCpG). *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 7204−7208.

(5) Kleeberger, L.; Rottinger, E. M. Effect of pH and moderate hyperthermia on doxorubicin, epirubicin and aclacinomycin A cytotoxicity for Chinese hamster ovary cells. *Cancer Chemother. Pharmacol.* **1993**, *33*, 144−148.

(6) Newell, K.; Wood, P.; Stratford, I.; Tannock, I. Effects of agents which inhibit the regulation of intracellular pH on murine solid tumours. *Br. J. Cancer* **1992**, *66*, 311−317.

(7) Ohtsubo, T.; Igawa, H.; Saito, T.; Matsumoto, H.; Park, H. J.; Song, C. W.; Kano, E.; Saito, H. Acidic environment modifies heat- or radiation-induced apoptosis in human maxillary cancer cells. *Int. J. Radiat. Oncol., Biol., Phys.* **2001**, *49*, 1391−1398.

(8) Raghunand, N.; Gillies, R. J. pH and drug resistance in tumors. *Drug Resist. Updates* **2000**, *3*, 39−47.

(9) Mahoney, B. P.; Raghunand, N.; Baggett, B.; Gillies, R. J. Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents in vitro. *Biochem. Pharmacol.* **2003**, *66*, 1207−1218.

(10) Born, R.; Eichholtz-Wirth, H. Effect of different physiological conditions on the action of adriamycin on Chinese hamster cells in vitro. *Br. J. Cancer* **1981**, *44*, 241−246.

(11) Roos, A.; Boron, W. F. Intracellular pH. *Physiol. Rev.* **1981**, *61*, 296−434.

(12) Ruan, S.; Yuan, M.; Zhang, L.; Hu, G.; Chen, J.; Cun, X.; Zhang, Q.; Yang, Y.; He, Q.; Gao, H. Tumor microenvironment sensitive doxorubicin delivery and release to glioma using angiopep-2 decorated gold nanoparticles. *Biomaterials* **2015**, *37*, 425−435.

(13) Chen, K.; Cai, H.; Zhang, H.; Zhu, H.; Gu, Z.; Gong, Q.; Luo, K. Stimuli-responsive polymer-doxorubicin conjugate: Antitumor mechanism and potential as nano-prodrug. *Acta Biomater.* **2019**, *84*, 339−355.

(14) Nazaruk, E.; Szlęzak, M.; Górecka, E.; Bilewicz, R.; Osornio, Y. M.; Uebelhart, P.; Landau, E. M. Design and Assembly of pH-Sensitive Lipidic Cubic Phase Matrices for Drug Release. *Langmuir* **2014**, *30*, 1383−1390.

(15) Swiech, O.; Dutkiewicz, P.; Wójciuk, K.; Chmurski, K.; Kruszewski, M.; Bilewicz, R. Cyclodextrin Derivatives Conjugated with Aromatic Moieties as pH-responsive Drug Carriers for Anthracycline. *J. Phys. Chem. B* **2013**, *117*, 13444−13450.

(16) Swiech, O. A.; Opuchlik, L. J.; Wojciuk, G.; Stepkowski, T. M.; Kruszewski, M.; Bilewicz, R. Doxorubicin carriers based on Au nanoparticles − effect of shape and gold-drug linker on the carrier

toxicity and therapeutic performance. *RSC Adv.* **2016**, *6*, 31960−31967.
(17) Tai, X.; Cai, X. B.; Zhang, Z.; Wei, R. In vitro and in vivo inhibition of tumor cell viability by combined dihydroartemisinin and doxorubicin treatment, and the underlying mechanism. *Oncol. Lett.* **2016**, *12*, 3701−3706.
(18) Hu, T.; Qahtan, A. S. A.; Lei, L.; Lei, Z.; Zhao, D.; Nie, H. Inhibition of HeLa cell growth by doxorubicin-loaded and tuftsin-conjugated arginate-PEG microparticles. *Bioact. Mater.* **2018**, *3*, 48−54.
(19) Kibria, G.; Hatakeyama, H.; Akiyama, K.; Hida, K.; Harashima, H. Comparative Study of the Sensitivities of Cancer Cells to Doxorubicin, and Relationships between the Effect of the Drug-Efflux Pump P-gp. *Biol. Pharm. Bull.* **2014**, *37*, 1926−1935.
(20) Pérez-Escuredo, J.; Dadhich, R. K.; Dhup, S.; Cacace, A.; Hée, V. F. V.; Saedeleer, C. J. D.; Sboarina, M.; Rodriguez, F.; Fontenille, M. J.; Brisson, L.; Porporato, P. E.; Sonveaux, P. Lactate promotes glutamine uptake and metabolism in oxidative cancer cells. *Cell Cycle* **2016**, *15*, 72−83.
(21) Brodaczewska, K. K.; Szczylik, C.; Fiedorowicz, M.; Porta, C.; Czarnecka, A. M. Choosing the right cell line for renal cell cancer research. *Mol. Cancer* **2016**, *15*, No. 83.
(22) Yang, O. C. Y.; Maxwell, P. H.; Pollard, P. J. Renal cell carcinoma: translational aspects of metabolism and therapeutic consequences. *Kidney Int.* **2013**, *84*, 667−681.
(23) Huber, V.; Camisaschi, C.; Berzi, A.; Ferro, S.; Lugini, L.; Triulzi, T.; Tuccitto, A.; Tagliabue, E.; Castelli, C.; Rivoltini, L. Cancer acidity: An ultimate frontier of tumor immune escape and a novel target of immunomodulation. *Semin. Cancer. Biol.* **2017**, *43*, 74−89.
(24) Spugnini, E. P.; Sonveaux, P.; Stock, C.; Perez-Sayans, M.; De Milito, A.; Avnet, S.; Garcìa, A. G.; Harguindey, S.; Fais, S. Proton channels and exchangers in cancer. *Biochim. Biophys. Acta, Biomembr.* **2015**, *1848*, 2715−2726.
(25) Samouilov, A.; Efimova, O. V.; Bobko, A. A.; Sun, Z.; Petryakov, S.; Eubank, T. D.; Trofimov, D. G.; Kirilyuk, I. A.; Grigor'ev, I. A.; Takahashi, W.; Zweier, J. L.; Khramtsov, V. V. In Vivo Proton−Electron Double-Resonance Imaging of Extracellular Tumor pH Using an Advanced Nitroxide Probe. *Anal. Chem.* **2014**, *86*, 1045−1052.
(26) Chen, L. Q.; Howison, C. M.; Jeffery, J. J.; Robey, I. F.; Kuo, P. H.; Pagel, M. D. Evaluations of extracellular pH within in vivo tumors using acidoCEST MRI. *Magn. Reson. Med.* **2014**, *72*, 1408−1417.
(27) Chen, M.; Chen, C.; Shen, Z.; Zhang, X.; Chen, Y.; Lin, F.; Ma, X.; Zhuang, C.; Mao, Y.; Gan, H.; Chen, P.; Zong, X.; Wu, R. Extracellular pH is a biomarker enabling detection of breast cancer and liver cancer using CEST MRI. *Oncotarget* **2017**, *8*, 45759−45767.
(28) Garcia-Martin, M. L.; Martinez, G. V.; Raghunand, N.; Sherry, A. D.; Zhang, S.; Gillies, R. J. High resolution pHe imaging of rat glioma using pH-dependent relaxivity. *Magn. Reson. Med.* **2006**, *55*, 309−315.
(29) Lee, W. K.; Chakraborty, P. K.; Thévenod, F. Pituitary homeobox 2 (PITX2) protects renal cancer cell lines against doxorubicin toxicity by transcriptional activation of the multidrug transporter ABCB1. *Int. J. Cancer* **2013**, *133*, 556−567.
(30) Beijnen, J. H.; van der Houwen, O. A. G. J.; Underberg, W. J. M. Aspects of the degradation kinetics of doxorubicin in aqueous solution. *Int. J. Pharm.* **1986**, *32*, 123−131.
(31) Sanli, S.; Altun, Y.; Guven, G. Solvent Effects on pKa Values of Some Anticancer Agents in Acetonitrile−Water Binary Mixtures. *J. Chem. Eng. Data* **2009**, *54*, 3014−3021.
(32) Mayer, L. D.; Bally, M. B.; Cullis, P. R. Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient. *Biochim. Biophys. Acta, Biomembr.* **1986**, *857*, 123−126.
(33) Raval, G. Thermodynamic and Spectroscopic Studies on the Molecular Interaction of Doxorubicin (DOX) with Negatively Charged Polymeric Nanoparticles. M.Sc. Thesis; Department of Pharmaceutical Sciences, University of Toronto, 2012; p 19.
(34) Skovsgaard, T. Transport and binding of daunorubicin, adriamycin, and rubidazone in ehrlich ascites tumour cells. *Biochem. Pharmacol* **1977**, *26*, 215−222.
(35) Smith, S. M.; Wunder, M. B.; Norris, D. A.; Shellman, Y. G. A Simple Protocol for Using a LDH-Based Cytotoxicity Assay to Assess the Effects of Death and Growth Inhibition at the Same Time. *PLoS One* **2011**, No. e26908.
(36) Grzybowska, E. A.; Zayat, V.; Konopiński, R.; Trębińska, A.; Szwarc, M.; Sarnowska, E.; Macech, M.; Korczyński, J.; Knapp, A.; Siedlecki, J. A. HAX-1 is a nucleocytoplasmic shuttling protein with a possible role in mRNA processing. *FEBS J.* **2018**, *280*, 256−272.
(37) Han, H.; Hou, Y.; Chen, X.; Zhang, P.; Kang, M.; Jin, O.; Ji, J.; Gao, M. Metformin-Induced Stromal Depletion to Enhance the Penetration of Gemcitabine-Loaded Magnetic Nanoparticles for Pancreatic Cancer Targeted Therapy. *J. Am. Chem. Soc.* **2020**, *142*, 4944−4954.

https://dx.doi.org/10.1021/acsomega.9b04479
ACS Omega 2020, 5, 7979−7986