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THIOUREA MODIFIED DOXORUBICINE: A NOVEL PERSPECTIVE PH-SENSITIVE PRODRUGS

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ABSTRACT: A novel approach to the synthesis of pH-sensitive prodrugs has been proposed: thiourea drug modification. Resulting prodrugs can release the cytotoxic agent and the biologically active 2-thiohydantoin in the acidic environment of tumor cells. The concept of acid-catalyzed cyclization of thioureas to 2-thiohydantoins has been proven using a FRET model. Dual prodrugs of model azidothymidine, cytotoxic doxorubicin, and 2-thiohydantoin albutoin were obtained, which release the corresponding drugs in the acidic environment. The resulting doxorubicin prodrug was tested on prostate cancer cells, and showed that the thiourea-modified prodrug is less cytotoxic (average IC50 ranging from 0.5584 to 0.9885 μM) than Doxorubicin (IC50 ranging from 0.01258 to 0.02559μM) in neutral pH 7.6, and similar toxicity (average IC50 ranging from 0.4970 to 0.7994 μM) to Doxorubicin (IC50 ranging from 0.2303 to 0.8110μM) under mildly acidic conditions of cancer cells. Cellular accumulation in PC3 tumor cells of Dox prodrug is much higher than accumulation of free Doxorubicin.

INTRODUCTION

Doxorubicin - antitumor antibiotic - is an effective and commonly used chemotherapeutic agent and is able to embed into the double helical structure of cell DNA, inhibiting RNA and DNA synthesis, and thereby leading to apoptosis of cancer cells.[1] Doxorubicin and its modified form Doxil are widely used in cancer therapy.[2] Despite the effectiveness of doxorubicin, there is a high chemotherapeutic failure rate due to its low specificity towards tumor tissues, dose-dependent resistance to therapy, and strong cardiotoxicity. [3]

Development of doxorubicin prodrugs, modified with target-recognizing fragment[4], as well as pH-sensitive prodrugs capable of releasing a cytotoxic agent in a weakly acidic tumor tissues[5], is a relevant task for medical chemistry.

Acidic extracellular pH_e is a major feature of tumor tissue; extracellular acidification is considered to occur in the presence of [6,7] - lactic acid, which accumulates due to anaerobic glycolysis in hypoxic cancer cells; excess of CO_2, which accumulates due to pentose phosphate pathway, and then oxidized by the enzyme carbonic anhydrase (CA), which leads to the increase of the proton concentration.[8,9] Acidic pH_e not only leads to activation of some lysosomal enzymes, but also increases resistance to certain types of chemotherapy[10] through the unforeseen metabolism of therapeutic molecules resulting in high outflow of drugs from tumor cells. In particular, acidic pH_e reduces cytotoxicity of antitumor drugs with weak base pKa: Doxorubicin, Mitoxantrone and Daunorubicin.[11] In the early stages of breast cancer, high CAIX enzyme level is a predictive marker of doxorubicin resistance.[12] Acidic pH_e also plays role in the drug resistance of tumor cells due to the increased p-glycoprotein expression, that increases drug efflux.[13,14]

There are several strategies that can be used to develop pH-sensitive prodrugs capable of cytotoxic drug release in the acidic environment of tumor cells and are described in lots of newest scientific publications. pH-dependent release of Dox from its micellous conjugate with vitamin E, via pH-sensitive hydrazone bond,[15] adamantane-modified Dox via pH-sensitive hynrazone linker, capable to release free Dox at pH 4.5;[16] DOX-conjugated smart polymeric self-assembled micelles, prepared via an imine linkage, which exhibited the pH-triggered charge-conversion property and accelerated drug release at tumor pH;[17] polymeric micelles delivery system based on block copolymers of poly(L-lactic acid)-β-poly(ethylene glycol) β-poly (L- histidine)-TAT (transactivator of transcription) and poly(L-histidine)-β-poly(ethylene glycol). Such micelles have been proven to increase the cytotoxicity of doxorubicin in several multidrug-resistant tumor cell lines through the lifetime increase of the active molecule.[18] Also, conjugation of docosahexaenoic acid (DHA) to Doxorubicin (Dox) with a pH-sensitive hydrazone linker at 13 position formed a lipophilic prodrug,

and demonstrated higher anticancer activity *in vivo* than free doxorubicin, authors suggested a pH-sensitive Dox release and higher cellular accumulation than free Dox, but have not confirmed it experimentally. [19]

We have proposed another approach to the synthesis of pH-sensitive prodrugs: Dox-thiourea drug modification, that would be able to simultaneously release two different therapeutic agents in the acidic pH (pH $\geq$ 6.5/7.5)[20]. Thioureas are perspective prodrugs because in the acidic environment of tumor tissues they are cyclized to corresponding thiohydantoins, releasing free Doxorubicin. At the same time the resulting thiohydantoin, depending on the structure of the initial thiourea, may have different pharmacological properties. 2-thiohydantoins exhibit a wide variety of biological properties: antitumor, [21] antiviral, [22]anti-inflammatory,[23] anticonvulsant.[24, 25] One of the most convenient methods of 2- thiohydantoins synthesis is the acid-catalyzed cyclization of the corresponding thioureas.[26]

In this study, we have chosen thiohydantoin Albutoin as a simple structure model drug - an anticonvulsant [27,28] – that was evaluated by the United States Food and Drug Administration, but not approved . [29]Albutoin, in contrast to the structural similar anticonvulsant diphenylhydantoin, didn't not have toxicity-induced side effects. [30]

Doxorubicin is highly hydrophilic, has short half-life, and its use is associated with severe side effects at high doses. [31] Doxorubicin implements its cytotoxicity by inhibiting Topoisomerase II enzyme.[32] The amino group in the 3'-position is not essential for topoisomerase II-targeting activities, because it can be replaced by a hydroxyl group without reduction of activity (Doxorubicin analog Annamicyn). However, conjugation of 3'-amino group through an amide bond reduced the anticancer activity against the leukemia, breast, ovarian, and colon cancer cell lines, suggesting that the presence of a free amino group is required for anticancer activity of doxorubicin.[33] Thus, we have suggested that the modification of amino group of Doxorubicin with thiourea will reduce the general toxicity of the corresponding prodrug.

To prove the ability of sterically hindered thioureas to cyclize in a weakly acidic medium, we have synthesized a model of pH-sensitive thiourea: FRET- pair based on naphthalenimide and fluorescein. The present paper reports our initial studies on the synthesis of the novel thiourea derivative of Doxorubicin - the acid-hydrolyzable twin prodrug by the modification of the N- 3' position of Dox through amide bond formation and its anticancer activity. The cytotoxic effects and cellular accumulation of the synthesized compound were evaluated against androgen receptor negative PC3 prostate cancer cell lines.

RESULTS AND DISCUSSION

The studies of pH-sensitive thioureas-based prodrugs using a FRET-pair model

To assess the cyclization of thiourea derivatives in a weakly acidic medium, we have proposed to synthesize a sterically hindered fluorescent derivative of 2-thiohydantoin acid containing the donor naphthalimide and acceptor fluorescein fragments (Scheme 1). In the case of this FRET-pair, the Förster resonance energy transfer must be disrupted by acid-catalyzed cyclization, leading to fragmentation of the initial molecule to naphthalenimide and fluorescein components, which will lead to a significant change in the fluorescence spectrum and can be easily monitored by spectral methods (Fig. 1).
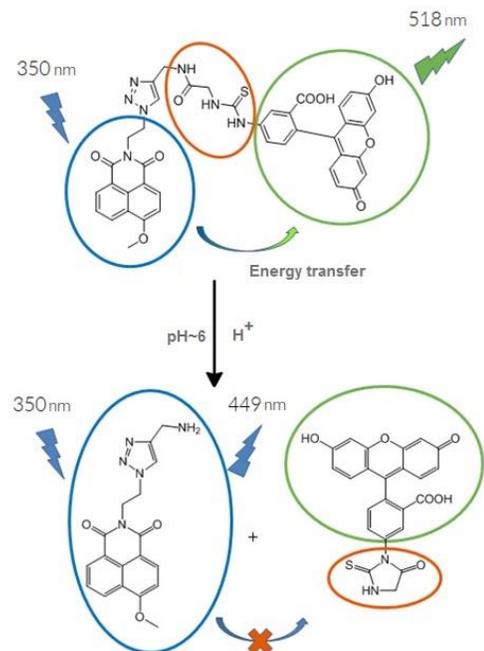
Figure 1. Model pH-sensitive thiourea as naphthalenimide fluorescein FRET-pair

Thus the study of FRET-pair fluorescence, where resonant energy transfer is observed, can show whether fluorophores are part of the same molecule or not. In case the acid catalyzed cyclization occurs, the resonance transfer of energy will cease, clearly demonstrating the acid-catalyzed cyclization of thiourea with the release of the amide fragment and the formation of the 2-thiohydantoin.

The spatial structure of FRET-model optimized by molecular mechanics in the gas phase is shown in Fig 2. The calculated distance between the elements of the FRET pair is about 12.8 Å, and there is also a significant overlap of the emission spectrum of the donor fragment and the absorption spectrum of acceptor moiety (see section 2.1.2.1), which indicates the possibility of resonant transport in this system.

Synthesis of the pH-sensitive thiourea as a FRET-model molecule

The synthesis of the model FRET-pair (thiourea **10**) was carried out according to the Scheme 1.

FRET-pair was synthesized through the ten-stage synthesis, the key stages in which were copper-catalyzed azide-alkyne cycloaddition reaction of propargyl ester **2** with azide **8** in mild conditions and the reduction of the resulting triazole **9** with the formation of thiourea **10**. Target compounds were isolated by high-performance liquid chromatography.

The detailed description of the experimental procedures is given in the Supporting Information.

Study of FRET-model pH- sensitivity

Theoretical study of resonant energy transfer in FRET- model

Effective resonant energy transfer between fluorophores is possible if the distance between them is less than 10 nm, and the emission spectrum of the donor is overlapping with the acceptor absorption spectrum.[34] The superposition of experimental fluorescence spectra of the donor naphthalimide (NI) and acceptor fluorescein isothiocyanate (FITC) absorption are given in Fig. 3.

Scheme 1 structures (reaction schemes with labeled compounds 1–10)
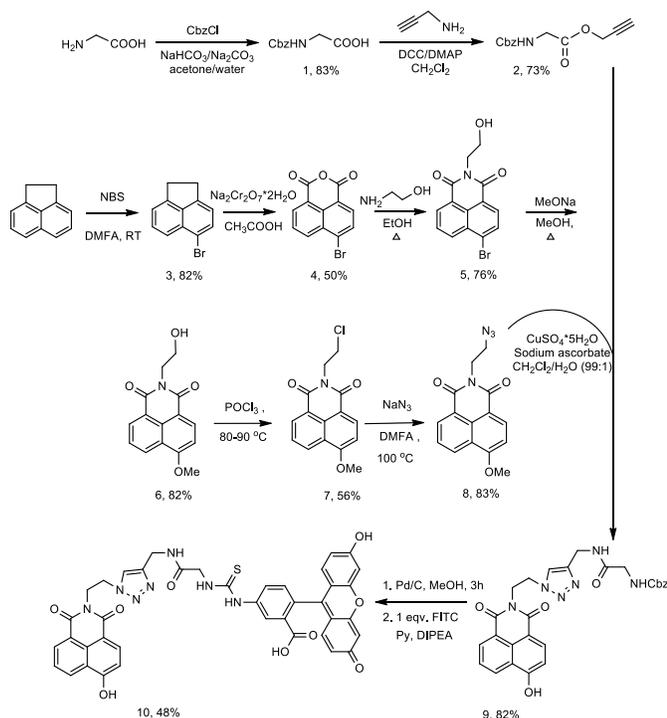
Scheme 1. Synthesis of pH-sensitive thiourea 10 as a FRET-model molecule.

Table 1. Spectral characteristics of individual chromophores NI, FITC and protonated FITC form in methanol-water mixture (v / v = 1: 1).

Compound	λ_{max}^{abs} /нм	$\varepsilon \cdot 10^{-3}$ / M^{-1} · cm^{-1}	λ_{max}^{fl} /нм	φ^{fl_*}	τ_S / нс
Naphthalimide NI (Donor)	371	8.0	449	1.0	9.0
FITC (Acceptor)	492	31.4	518	0.23	3.6
FITC+HCl (10^{-4})	453;480	7.8;7.2	514	0.11	2.4

The most important spectral characteristics of fluorophores were determined by the Förster model to calculate the characteristics of resonant transport: the absorption maximum wavelength, the maximum fluorescence wavelength, the extinction coefficient at the absorption maximum, the quantum yield fluorescence, lifetime of the excited state.[35] The absorption and fluorescence spectra of individual naphthalimide and FITC are shown in Fig S1 and S2 in Supporting Information. Spectral characteristics of individual chromophores NI, FITC and protonated FITC form in methanol-water mixture (v / v = 1: 1), are presented in Table1.
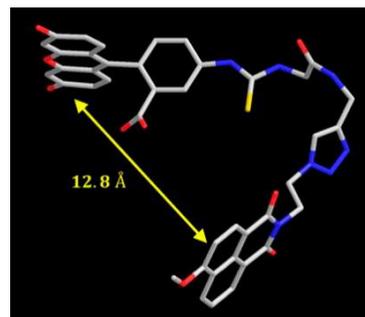
Figure 2. Geometry of naphtalineimide-fluorescein FRET-model optimized by MM2 – method

Results of the calculations based on the experimentally measured spectral characteristics of fluorophores are given in Table 2.

Table 2. Theoretical calculation of the resonance energy transfer process characteristics according to the Förster model in the NI-FIC compound 10 and in the protonated form of NI-FITC

| Compound	$J(\lambda)$ $l \cdot nm^4$ / $mol \cdot cm$	$R0$/Å	r/Å	k_{FRET}/c^{-1}	Φ_{FRET}
FRET-model	$4.35 \cdot 10^{14}$	44.7	12.8	$2.0 \cdot 10^{11}$	0.9994
FRET-model +H^+	$2.08 \cdot 10^{14}$	39.5	12.8	$9.6 \cdot 10^{10}$	0.9988

Also, we have studied the resonant energy transfer in FRET-model in comparison of absorption and fluorescence spectra of model thiourea **10** and equimolar amount of the free corresponding donor (NI) and acceptor (FITC). The data obtained indicate the high efficiency of resonant transport in the FRET-pair 10, consistent with the theoretical calculation. For experimental details, see Supporting Information (Figure S3).

*Study of acid-catalyzed cyclization of FRET-model **10** using stationary and time-resolved fluorescence spectroscopy*

To investigate the cyclization process of FRET-model **10** a series of solutions at acidic pH was prepared ($5*10^{-6}$M of **10**) in a water: methanol (1 : 1) mixture with HCl in 0 M, $1*10^{-5}$ M, and $1*10^{-4}$ M concentrations. The absorption spectra of these mixtures are presented in Fig. 4.

The results of stationary fluorescence and fluorescence kinetics analysis are shown in Fig. 5, Fig. S4. Fluorescence kinetics (Fig. S4) analysis allowed to calculate the contribution of an exponent with a characteristic time of 6.0 ns corresponding to free naphthalimide–which was 52% (calculated from the known values[36] of the pre-exponential factors = 619 /(619 + 561) = 0.52),

i.e. there is an increase in contribution of this component if compared to neutral pH, where it was 37%. In the stationary spectra a significant decrease in the fluorescence intensity of the acceptor (λ = 518 nm), and the growth of donor fluorescence in-
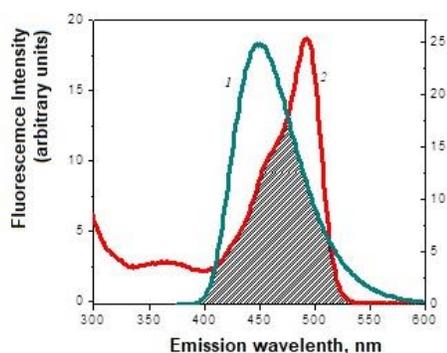
Figure 3. Superposition of the experimentally observed fluorescence spectra of the donor (NI, 1) and acceptor (FITC, 2).

tensity ($\lambda = 449$ nm) are also observed, confirming the proposed hypothesis about compound **5** decomposition in a weakly acid medium with the interruption of resonance energy transfer and a change in spectrum of fluorescence.
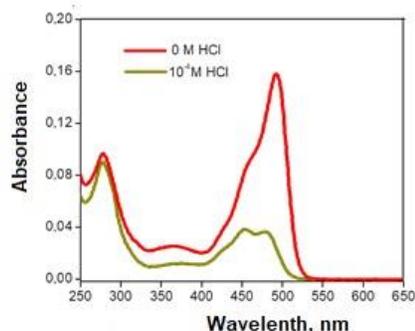
Figure 4. Absorption spectra of Fret-pair 10 (5×10^{-6} M) in a methanol-water mixture (v / v = 1: 1) in the presence and absence of hydrochloric acid.

Thereby, in studied FRET-model of pH-sensitive thiourea an effective resonance energy transfer was observed, but decomposition of thiourea **10** took place in acidic medium (pH = 5) which was proved by a change in the fluorescence spectrum.

Thus, the hypothesis about the ability of sterically hindered derivatives of 2-thiohydantoin acid to cyclize in a weakly acidic medium was confirmed. Taking this into account it can be assumed the principle possibility of working out of pH-sensitive prodrugs of cytostatic molecules based on the same derivatives, capable of releasing the biologically active substance in the acidic medium of tumor tissue.
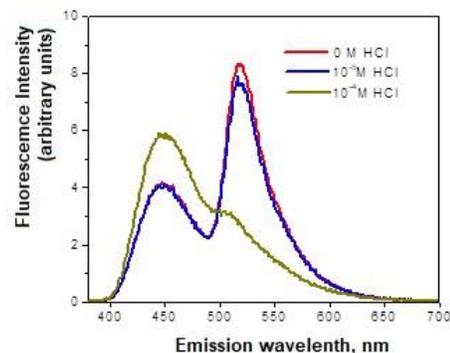

Figure 5. Fluorescence spectra of the FRET-model 10 in methanol-water mixture (v/v = 1: 1) in the presence and absence of hydrochloric acid.

pH sensitive thioureas prodrugs based on 3'-amino-3'- deoxythymidine

To develop synthetic approaches to the acid-sensitive thiourea derivatives with sugar moieties we synthesized a model conjugate of the Albutoin precursor with 3'- amino-3'-deoxythymidine.

Synthesis of 3'-amino-3'-deoxythymidine analogs of pH-sensitive thioureas

After multiple optimizations, we have proposed to introduce the drug fragment at the last stage of the synthesis, through the ester formation of the corresponding thiourea in the reaction with N-hydroxysuccinimide as an approach to twin 3'-amino-3'-deoxythymidine-based prodrug (Scheme 2):
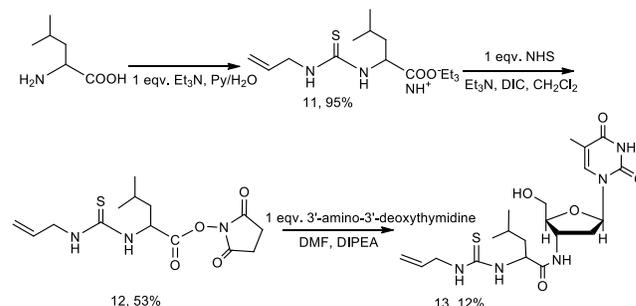

Scheme 2. Synthesis of pH-sensitive 3'-amino-3'-deoxythymidine-Albutoin prodrug 13.

Product **13** was isolated by preparative chromatography on silica gel and characterized by ^{1}H NMR spectroscopy and high-resolution mass spectrometry. For a detailed description of the synthesis see Supporting Information.

Evaluation of pH- sensitivity of twin 3'-amino-3'- deoxythymidine - based prodrug 13 by LC-MS.

The thiourea-based prodrugs should release of biologically active substance and thiohydantoine in a weakly acidic medium. The model drug release from the conjugate of 3'-amino-3'- deoxythymidine with Albutoin precursor **13** was studied *in vitro* under physiological conditions at different pH.

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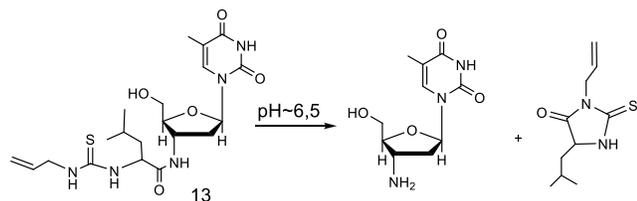
Scheme 3. The proposed way of acid-catalyzed decomposition of the model conjugate 13 in a weakly acid medium.

Compound **13** was dissolved in formate buffer solutions with a pH of 5.5; 6.5; 7.4. The resulting mixtures were incubated at 37° C for 24 hours. Samples for analysis were taken at different time intervals: 0, 20, 40, 60, 90, 120, 240, 480, 1440 minutes, and analyzed by the LC-MS method. Relative rates of hydrolysis of the compound **13** over a 48h period were calculated using the percent of prodrug **13** hydrolysis in acidic condition; the relative rate of free aminothymidine accumulation was also measured. Conjugate **13** flow rate is shown in Fig. 6, the accumulation rate of the cyclization product 3'- amino-3'-deoxythymidine - in Fig. 7. We have suggested the following scheme for the release of 3'-amino-3'- deoxythymidine and Albutoin, which proceeds in a slightly acidic solution pH ~ 6.5 (Scheme 3).

We also have determined the kinetic parameters of this process. The reaction order for both the product and the reagent is zero. The ratio of the rate constants of the process at pH 7.4 (k_1), pH 6.5 (k_2), pH 5.5 (k_3) was k_1: k_2: k_3 = 1: 6.6: 8.5. This data indicates the almost 10-fold increase in the rate of aminothymidine release at pH = 5.5 compared to neutral medium (pH = 7.4).

It should be noted that during the LC-MS analysis of compound **13** the signal of Albutoin (m/z = 211.0911) was observed in all cases, which confirms that the cyclization reaction proceeds according with the Scheme 3.
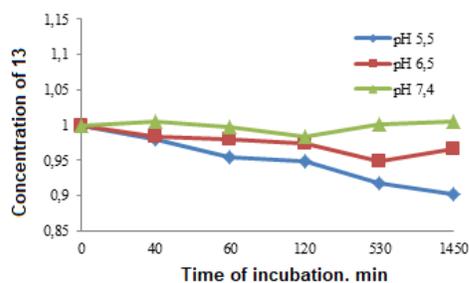
Figure 6. The dependence of prodrug 13 concentration from the time of incubation at in medium with different pH.

Thus, the resulting conjugate **13** is capable to release of the starting drug and Albutoin in a weakly acidic medium, but practically is not disintegrating under neutral conditions. This suggests that such compounds in *in-vivo* models would be selectively released in tumor (pH~6,5),[37] while in healthy tissues release of the drug should not occur.

Based on our result, we conclude that the cyclization of thioureas prodrug can occur under conditions corresponding
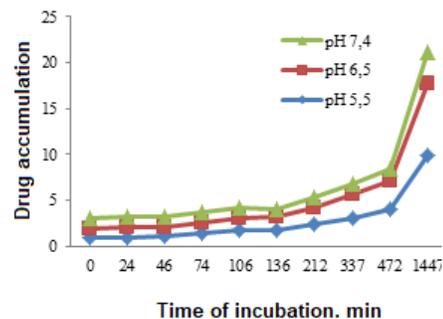
Figure 7. The dependence of 3'-amino-3'-deoxythymidine concentration from the time of incubation in medium with different pH.

to the tumor tissue. Next step is the synthesis of the anti-cancer drug (Doxorubicin)-based thioureas.

pH-sensitive thiourea prodrug based on Doxorubicin

We have proposed a synthesis of twin Doxorubicin-Albutoin prodrug that can possess both antitumor and anticonvulsant properties. It was suggested that the introduction of an Albutoin precursor fragment would reduce the toxicity in the neutral pH due to a significant change in the structure of the initial cytotoxic agent, but such molecule will be able to release the initial drug in a weakly acidic pH of the tumor tissue medium, also, higher lipophilicity of the thiourea-doxorubicin prodrug should improve cell penetration compared to unmodified Doxorubicin, thereby lowering the effective drug dose reducing toxicity.

Synthesis of Doxorubicin derivative of pH-sensitive thiourea

To obtain a twin Doxorubicin-based prodrug **14** we have used a previously described three-steps approach with intermediate preparation of the N-hydroxysuccinimide ester **12**. The synthesis was carried out according to the Scheme 4:
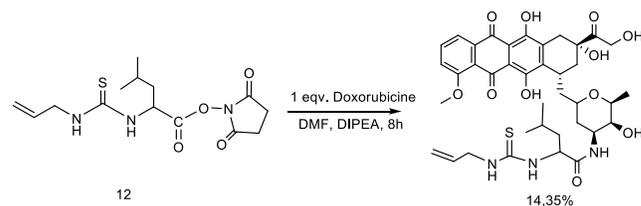
Scheme 4. Synthesis of pH-sensitive Doxorubicin-Albutoin prodrug 14.

The product **14** was isolated by preparative chromatography on silica gel and characterized by 1H NMR spectroscopy and high-resolution mass spectrometry. For a detailed description see Supporting Information.

Evaluation of pH-sensitivity of twin Doxorubicin-Albutoin prodrug 14 of by LC-MS.

Relative rates of compound **14** hydrolysis over a 48 h period were evaluated using the experimentally determined percentage of the initial Doxorubicin-Albutoin prodrug **14** under the hydrolyzes in acidic conditions at different pH.

Prodrug **14** were incubated in aqueous formate buffer at pH 5.5, 6.5, and 7.4 at 37°C over a 20 h. LC-MS technique was employed to analyze how much of compound **14** was hydrolyzed. We have demonstrated the ability of the derivative **14** to enter the acid-catalyzed cyclization reaction with the formation of Albutoin and

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Doxorubicin in a weakly acid solution. Sampling for LC-MS analysis was carried out at 0, 70, 1210 minutes. The content of the initial conjugate was evaluated; the resulting concentration/time dependence in semilogarithmic coordinates is shown in Figure 8.
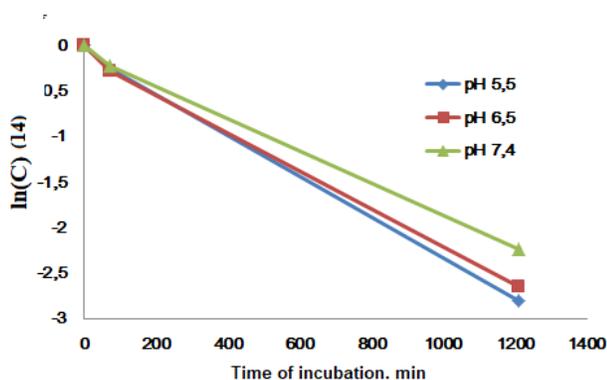
Figure 8. Semi-logarithmic dependence concentration of compound 14 on time incubation at various pH conditions

We have suggested the following scheme for the release of Doxorubicin and Albutoin, which proceeds in a slightly acidic solution pH ~ 6.5 (Scheme 5):
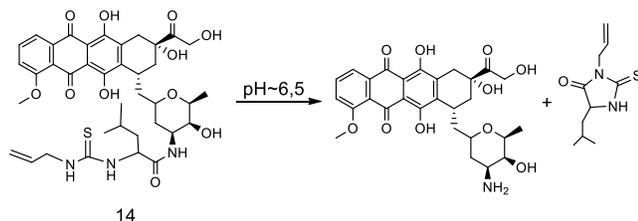
Scheme 5. The proposed way of acid-catalyzed decomposition of the twin Doxorubicin-Albutoin prodrug 14 in aweakly acid medium.

The release of free Doxorubicin reaches a value of 94% at pH = 5.5; 93% at pH = 6.5; 89% at pH = 7.4. Analysis of the data in semilogarithmic coordinates allows to assume the first order of the reaction for the conjugate **14**. On this base, the half-transformation times at different pH values were calculated, which are 300 min at pH = 5.5; 320 min at pH = 6.5; 380 min at pH = 7.4.

Thus, the hypothesis of faster release of conjugate **14** in an acid medium was confirmed.

Previously, the fatty acid derivatives of Doxorubicin have been synthesized .[38] These prodrugs are amides, and they possess generally lower toxicity compared to Doxorubicin, which was explained by the high stability of amides to non-enzymatic hydrolysis under physiological conditions. Also, recently, adamantane-modified doxorubicin via amide and ester linker was developed, and, also, this conjugates were found to be non-toxic and didn't show the ability to release free Dox in weakly acid medium.[16]

Thereby, previous attempts to obtain pH-sensitive prodrugs of Doxorubicin, by modifying the 3'-NH2-position with an amide bond, did not give satisfactory results on drug release and toxicity. The proposed concept of thiourea modification shows a proton-sensitive release even at pH 6.5. Due to the special structure of the introduced thiourea fragment, resulting prodrug does not undergo hydrolysis according to the classical mechanism of amide hydrolysis, instead initiating the formation of a 2-thiohydantoin derivative with the release of the amide fragment. This reaction, unlike the

hydrolysis of amides, easily occurs in weakly acid media. The next step was to study the effect of thiourea modification on cytotoxicity.

In vitro studies of compound 14 cytotoxicity against PC3 cell line under pre-established acidic cancer cell culture medium (pH < 7.0).

After the studying the pH-sensitive dual prodrug **14** ability to release Doxorubicin and Albutoin in acidic environment, we studied the effect of compound **14** on tumor cells of prostate cancer PC3. The cells treated with same concentration of free Doxorubicin were used as negative control.

We have prepared two different cell cultures for comparison: one was incubated in standard nutrient medium with pH = 7.6, and the other one was adapted to conditions of tumor tissue with pH = 6.6. Previously it has been shown that a pH decrease of the nutrient medium from 7.4 to 6.7 does not affect the growth rate and the population of cells.[39] This means that the cytotoxicity can be attributed exclusively to the effect of the test drug, but not to the change in external conditions.

Doxorubicin prodrug **14** and free Doxorubicin were added to the aforementioned cultures. The MTT method[40] was used to study cell survival after incubation for 48 hours.

We have detected a decrease in the toxicity of the conjugate **14** under study in a weakly alkaline medium corresponding to healthy tissues, compared with its toxicity in more acidic medium. IC50 values for Doxorubicin prodrug **14** and Doxorubicin in various media also were calculated, and were found to be dramatically different (Table 3). The cytotoxicity curve of **14** and Dox at different concentrations are given in Supporting information (Fig. S5).

Table 3. IC50 values of Doxorubicin prodrug 14 and free Doxorubicin against PC3 cells.

| IC50,мM	pH = 6.6		pH = 7.6	
Average	Doxorubicin	**14**	Doxorubicin	**14**
	0,4322	0,6303	0,01794	0,7430
95% Confidence Intervals				
	0.2303 to 0,8110	0.4970 to 0,7994	0,01258 to 0,02559	0,5584 to 0,9885

Such a different IC50 values for Doxorubucin in different pH can be explained by low cellular penetration, which occurs due to protonation. In addition to lipophilicity, the degree of molecular ionization, which is dependent on pH and pKa, determines the transport action. Doxorubicin act as weak base (pKa 8.34).[41] Therefore, a decreasing extracellular pH leads to an increasing ionization of drug molecules and, hence, drug transport into cells is rendered more difficult.[42]

Thus, due to pKa value, Doxorubicin shows increased toxicity to pH-neutral healthy tissues compared with pH-acidic tumor tissues, therefore, more hypophilic and penetrating prodrugs are promising analogues. Comparison of the cellular penetration of prodrug **14** with free doxorubicin is given in 2.3.4.

At pH = 7.6 high-lipophilic conjugate **14** penetrates into the cells, but Dox release does not take place sufficiently. However, a rather high toxicity even in a neutral medium compared to the low toxicity of prodrugs incapable of release[16] suggests Dox releasing inside the cell in a weakly acidic environment of the endosomes. (pH~6.0). This explains the similarity of the cytotoxicity values of **14** at different acidity of the medium - in both experiments, one substance acts by one mechanism - most likely, Topoisomerase II inhibition.

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Thus, the resulting conjugate **14** has toxicity similar to Doxorubicin in a tumor tissue environment and is 30 times less toxic in a healthy tissue environment.

*Cellular accumulation studies of compound **14** in PC3 cell line*
To study the cell penetration ability of **14**, and comparison of penetration with doxorubicin, we have investigated intracellular accumulation in PC3 cells after 2 hours of incubation with 14 and Dox. The results are shown in Figure 9 (PC3 cells), 10 (PC3 cells labeled with DAPI)
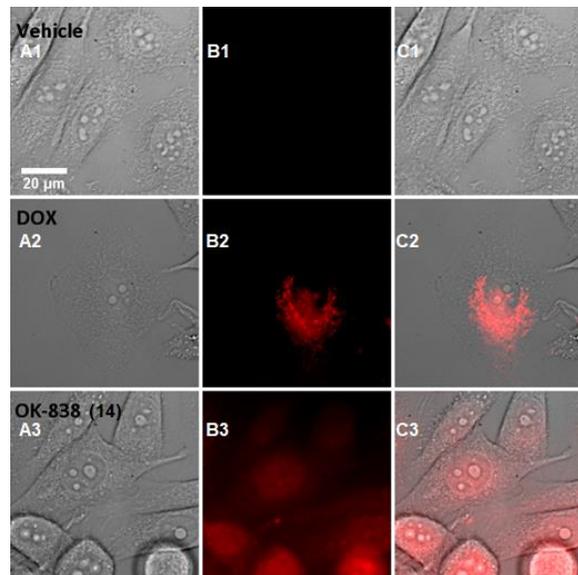
Figure 9. Cellular accumulation of 14 in PC3cells (A) PC3 cells treated with water (A1), Doxorubicin (A2), 14 (A3); (B) visualization of drugs in the red fluorescent channel (561nm) (C) merge of A and B.
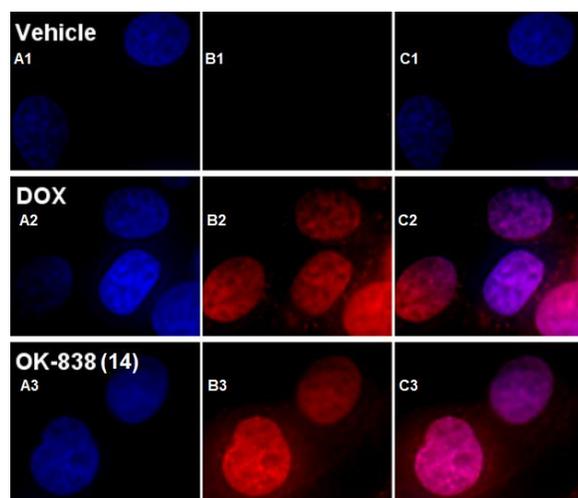
Figure 10. Cell nucleous accumulation of 14 in DAPI-labeled PC3 cells (A) DAPI-labeled PC3 cells treated with water (A1), Doxorubicin (A2), 14 (A3), vizualization of DAPI in blue fluorescent channel (450 nm); (B) visualization of drugs in the red fluorescent channel (561nm) (C) merge of A and B.

Transport of anthracyclines is essentially influenced by the parameters pKa and polarity of the molecule. The intracellular drug concentration at steady state increases with increasing lipophilicity in the order doxorubicin, epirubicin, and aclacinomycin.[42] As we

expected, **14** penetrates the cell much better than doxorubicin due to increased lipophilicity.
After penetrating the cell membrane and releasing from the endosome, most likely we observe a mixed signal of Dox, which have released from **14**, and the conjugate **14**. (Figure 9, C3).
Also, high nucleous accumulation was observed. ((Figure 10, C3). It is well-known, that Dox is capable of nuclear accumulation, resulting in Topoisomerase II inhibition.[43] Prodrug **14**, as well as higher cellular penetration, shows higher nuclear penetration in comparison with free Doxorubicin (Figure 10, C3), which indicates that the prodrug 14, which did not undergo hydrolysis in the proteasome, penetrates into the cell nucleus as easily as into the cell, due to its lipophilicity. Also, cell nuclei accumulation was quantified, average fluorescence signals for prodrug **14** and Doxorubicin are presented in Supporting Information (Fig. S6, Table S1).
It should be noted that Doxorubicin is not protonated in neural pH 7.4, and posess a strong cytotoxic effect (Table 3), but its penetration into the cell and nuclei is still extremely low (Figure 9, 10, C2). In the acidic pH of the tumor cells *in vivo*, Doxorubicin will be protonated, and show even lower cellular accumulation. This would leads to a low effective dose of the drug, and high toxicity.
Bioconjugation – is an attach a bioactive molecule to another molecule via a covalent bond, leads to the formation of a novel chemical structure with may have enhanced properties compared to those of the original molecule. At the same time, as can be seen from the literature data, the bioconjugation of various molecules to Doxorubicin often leads to a rapid decline in cytotoxic activity, and biodistribution failure. Conjugating the dexamethasone molecule to Dox results in a more lipophilic conjugate, which also, like prodrug **14**, more easily penetrates into the cell compared to the original Doxorubicin. However, the conjugate with dexomethasone showed no ability to accumulate in the cell nuclei, and also, as in the examples described above, shows toxicity to MCF-7 cells more than 20 times less compared to Dox[44]. Prodrug **14**, due to the pH-sensitive thiourea fragment, which increases the lipophilicity of the molecule, distribution rate, but which at the same time can be easily removed by cyclization, easily penetrates both into the cell and into cell nuclei.
Conjugate **14** is less toxic than doxorubicin in neutral pH (Table 3), however, it exhibits better cellular accumulation even at this pH value, at pH 6.5 **14** shows toxicity similar with doxorubicin (Table 3), due to a combination of high cellular penetration and pH-sensitive release Doxorubicin and Albutoin.

CONCLUSION

Summarizing the results, we have developed a methodology for the preparation of a naphthalimide-fluorescein FRET pair, which contains in the structure the fragment of thiohydantoin acid and is capable of an acid-catalyzed cyclization. The presence of resonant energy transfer in this system was predicted theoretically and confirmed experimentally, and the hypothesis about the FRET pair cyclization in a weakly acidic medium was proved.

The method for the synthesis of new pH-sensitive twin Doxo-rubicin-thiourea prodrug was proposed. The ability of the resulting prodrug **14** to release cytotoxic and thiohydantoin components in a weakly acidic medium was confirmed. Using *in vitro* cytotoxicity studies, the selectivity of the pH-sensitive twin Doxorubicin-Albutoin prodrug towards PC3 prostate cancer cell line was compared to free Doxorubicin. It was shown that the obtaining pH-sensitive twin Doxorubicin-Albutoin prodrug has more than 30-fold selectivity increase towards healthy tissues compared to the free Doxorubicin. Despite the fact that in neutral pH the toxicity of Dox and prodrug **14** is dramatically different, in acidic pH it is almost equal.

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Thiourea-modified Doxorubicin **14** have showed better cellular and nuclei penetration in comparison with free Dox. Thus, the proposed approach to the modification of Doxorubicin with pH-sensitive thioureas opens up possibilities for obtaining drugs of the same pharmacological efficacy on tumor tissues having a lower effective dose due to higher cellular penetration and the possibility of adding a second pharmacological action depending on the structure of the released thiohydantoin.

MATERIALS AND METHODS

Chemicals Unless otherwise noted, all preparations were carried out in reagent grade solvents. All chemicals used in the synthesis were obtained from Acros or Sigma-Aldrich and were used without further purification. Solvents were deoxygenated/distilled/purified by bubbling through a stream of argon or by conventional methods and dried over molecular sieves. Column chromatography was performed using silica gel 60-120 mesh, 100-200 mesh.

1H NMR spectra were recorded on a Brucker-Avance instrument (operating at 400 MHz for 1H). As the solvent, deuterochloroform (CDCl$_3$) and dimethylsulfoxide-d6 (DMSO-d$_6$) were used. Chemical shifts are given in parts per million on a scale δ with respect to hexamethyldisiloxane as an internal standard.

High resolution mass spectra (HRMS) were recorded on an Orbitrap Elite (Thermo Scientific) mass spectrometer with an IRET. To inject solutions with a concentration of 0.1 to 9 μg/ml (in 1% formic acid in acetonitrile), direct injection into the ion source using a syringe pump (5 μl / min) was used. The spray voltage is ± 3.5 kV, the temperature of the capillary is 275ºC. Mass spectra were recorded using an Orbitrap analyzer with a resolution of 480,000 (1 microscan). The maximum input time is 900 ms, averaging over 9 spectra, the mass range is 90-2000 Da, in some cases 200-4000 Da. For internal calibration, DMSO and diisooctylphthalate signals (m / z 157.03515 and 413.26623) were used in the positive mode and the dodecyl sulfate signal (m / z 265.14790) in the negative mode.

LCMS For purification and analysis of samples we used Shimadzu Prominence LC-20 system with column oven and fraction collector coupled to single quadrupole mass-spectrometer Shimadzu LCMS-2020 with dual DUIS-ESI-APCI ionization source. Analytical and preparative column was Phenomenex Luna 3u C18 100A (150 x 4.6 mm). Mobile phases: A - 0.1% formic acid in water, B - 10 mM ammonium formate in water, D -acetonitrile.

LCMS parameters for analyses were: gradient flow of 1 ml/min (0-0.5 min - 5% D, 0.5 -10.5 min - 5% to 100% D, 10.5-12 min - 100% D, 12-14.5 min - 100% to 5% D), column oven temperature 40 C, optional UV detection of some compounds.

MS parameters: drying gas 15.0 L/min, nebulizing gas 1.5 L/min, DL temperature 250 C, heat block temperature 400 C, interface voltage -3.5 kV, corona needle voltage -3.5 kV. Positive (mass range 250-2000 Da, in some cases 155-2000 Da) and negative ions (mass range 215-2000 Da) were registered. For hydrolysis kinetics study SIM mode was used with registration of the molecular ions and adducts [M+H]+, [M+Na]+, [M+K]+, [M-H]-, [M+HCOO]-.

HPLC For purification of Fret-pair **10** we used identical LC parameters except gradient which was tailored for this compound (0-0.5 min - 35% D, 0.5-5.5 min - 35% to 55% D, 5.5-9.5 min - 55% to 100% D, 9.5-10.5 min - 100% D, 10.5-14 min - 100% to 35% D). Fractionation was based on UV detection only (absorbance on 485 nm), fractions were collected based on UV signal level and slope.

Analytical thin-layer chromatography TLC was performed on Merck silica gel aluminium plates with F-254 indicator. Compounds were visualized by irradiation with UV light or iodine staining.

Geometry estimation of naphtalineimide-fluorescein FRET-model 10 optimized by PM6 semiempirical method

The three dimentional structure of **10** was built with MOPAC 2016 program package using PM6 semiempirical method.[45] The calculations were performed at optimized geometries, which reached gradient variations less than 0.01 kcal/mol. The solvent effect was included in geometry optimizations following the «Conductorlike Screening Model» (COSMO) implemented in MOPAC. A dielectric constant of $\varepsilon = 60$ and a refraction index of solvent (n) such that $n2 = 2$ were used

Experimental study of resonant energy transfer in FRET-model To confirm the presence of resonant energy transfer in FRET-model, the absorption and fluorescence spectra of the equimolar mixture naphthalimide + fluorescein isothiocyanate (NI $5*10^{-6}$M + FITC $5*10^{-6}$M) and the solution of FRET-model $5 * 10^{-6}$M were compared. (excitation wavelength 370 nm). (Figure S3). Due to the instability of FRET-model, there was a partial decay during transportation to the place of investigation according to Fig S3. This resulted in the destruction of the FRET pair with the release of the compound containing the naphthalimide moiety. This process causes the appearance of an additional peak of fluorescence at λ = 449 nm. The time-resolved fluorescence spectra of FRET-model and naphthalimide were also recorded. The results are presented in the form of spectral-temporal maps and a plot of fluorescence intensity at different wavelengths versus time (Fig S3). It was found that the lifetime of the excited impurity state is 5.6 ns, and that of the test compound is 3.0 ns. The contribution of the exponential with the characteristic time 5.6 ns corresponding to the naphthalimide-containing impurity is 37% (calculated from the known values of the pre-exponential factors as 976 / (976 + 1612) = 0.37). Taking into account the difference between the quantum yields of fluorescence FRET-model and naphthalimide, it was found that the impurity content is approximately 15%. Fluorescence of the acceptor-fluorescein appears almost immediately after photoexcitation; fast relaxation of naphthalimide with a simultaneous rapid increase in the fluorescence intensity of the acceptor is not observed. The obtained data indicate the high efficiency of resonant transport in the system under investigation, consistent with the theoretical calculation. Spectral-temporal maps of fluorescence FRET-model and naphthalimide, fluorescence kinetics of FRET-model is show in Supporting information. (Figure S3).

Adaptation of PC3 culture cells to pH = 6.6. The PC3 line cells were incubated in the medium at t = 37 ° C until spontaneous pH = 6.7. The cells were then transferred twice to RPMI-1640 medium with pH = 6.7 to adapt to a weakly acidic medium. After adaptation for 24 hours, the pH of the medium was adjusted to 6.6 using KH$_2$PO$_4$. Cells were incubated in acid medium for 48 hours.

Cytotoxicity study of 14. PC3 line cells incubated at pH 6.6 were suspended and transferred to 2 plates per 96 wells. Similarly, the culture of PC3 cells incubated at pH = 7.6 was reported. Subsequently, solutions of **14** and Doxorubicin in media with a corresponding acid growth medium in concentrations of $1*10^{-4}$ g/L, $1*10^{-3}$ g/L, $1*10^{-2}$ g/L, $1*10^{-1}$ g/L and 1 g/L. Cells were incubated for 48 hours. Then, using the MTT method (using 3- (4,5-dimethyl-thiazol-2-yl) -2,5-diphenyltetrazolium bromide as a colorant, the percentage of surviving cells was determined by the formula , where x is the proportion of surviving cells, - the optical density of the solution of the test sample, - the optical density of the control sample at λ = 700 nm. As a control sample, cells not treated with cytotoxic drugs were used.

PC3 cellular/nucleus accumulation study PC3 cells (10^5 cells/ml) were seated on Petri dishes with a glass bottom with a thickness of 0.17 mm. The experiment was carried out next day (for the complete spreading of the cells and the acquisition of

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characteristic morphology by them). There were 3 samples: cells incubation with DOX (C ~ 0.14 uM), cells incubation with **14** (C ~ 1.192 uM), and incubation with a solvent (H2O) for 2 hours. During incubation, cells were in phosphate-buffered saline (PBS) with the addition of 10 mM HEPES (to reduce autofluorescence due to components of the complete nutrient medium). Living cells were kept in a chamber with a maintained level of CO_2, temperature and humidity. DIC and DOX/**14** fluorescence images (in the red fluorescent channel (561 nm) were obtained using a motorized inverted fluorescent microscope Eclipse Ti-E (Nikon) equipped with an iXon cooled EM-CCD camera (Andor), PerfectFocus (Nikon) autofocus system and Plan Apo 40x lens (NA = 0.95). For DAPI vizualization, the cell preparations were fixed in 3.7% formaldehyde (Sigma-Aldrich, USA) prepared on PBS. Washing from the retainer - 3 times for 5 minutes. Next, 0.2 ug/ml DAPI (Cayman Chemical Company, USA) was added. Washing - 3 times for 5 minutes (PBS). Further shooting in 2 channels - 450 nm for DAPI and 561 for DOX and **14**. Image processing was performed in the NIS-elements imaging software and in ImageJ.

ASSOCIATED CONTENT

Supporting Information

Synthesis details, structural data, materials and methods.
The Supporting Information is available free of charge on the ACS Publications website.

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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

ABBREVIATIONS

Dox, Doxorubicin; FRET, Förster resonance energy transfer.
FITC, fluorescein isothiocyanate.

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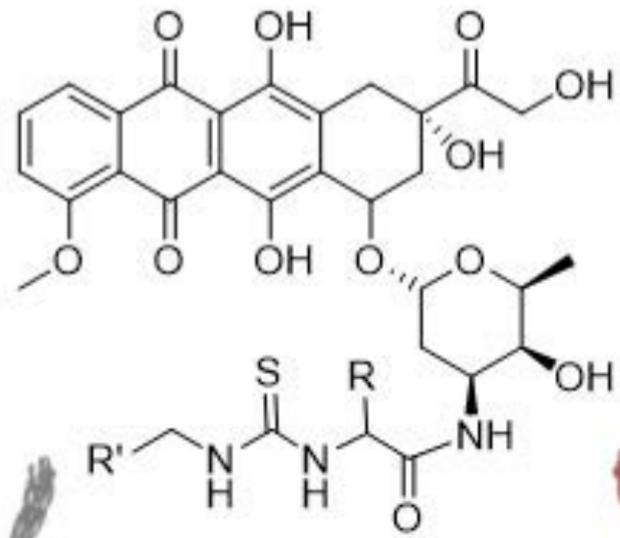
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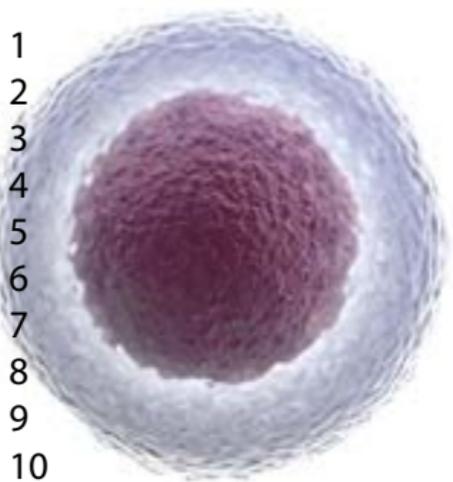
pH$_e$~7,4
Normal cell

pH$_e$~6,5
Cancer cell

Dox-thiourea conjugate stability
Low toxicity

pH-sensitive thiourea cyclization
Dox release
High toxicity