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The Structure of Anthracycline Derivatives Determines Their Subcellular Localization and Cytotoxic Activity

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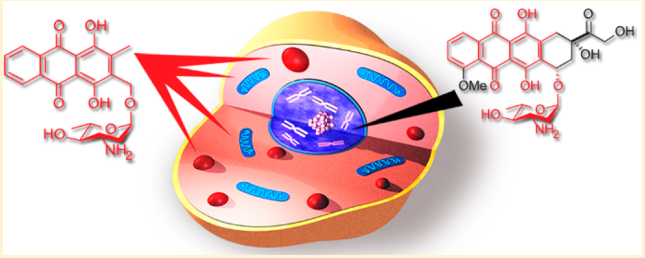
Supporting Information

ABSTRACT: The cytotoxic activities and subcellular localizations of clinically used and synthetic analogues of the anthracycline family of chemotherapeutic agents were studied. The structures of the anthracycline derivatives affected their cytotoxicity and the time required for these compounds to exert cytotoxic effects on tumor cells. Fluorescent DNA intercalator displacement experiments demonstrated that there was no correlation between the DNA intercalation properties and the cytotoxicity of the studied anthracycline derivatives. Confocal microscopy experiments indicated that structural differences led to differences in subcellular localization. All studied anthracycline derivatives were observed in lysosomes, suggesting that this organelle, which is involved in several processes leading to malignancy, may contain previously unidentified molecular targets for these antitumor agents.



KEYWORDS: *antitumor agents, anthracyclines, cytotoxic activity, subcellular localization*

Anthracyclines from both natural and semisynthetic sources (Figure 1) are among the most widely used chemotherapeutic agents and are effective against a broad spectrum of solid tumors and leukemias.[1] The clinical utility of anthracyclines as antitumor agents is compromised by their dose-limiting cardiotoxicitiy[1,2] and rapid evolution of drug resistance.[3] In an attempt to overcome these obstacles, numerous anthracycline analogues have been designed and synthesized over the years, but very few have demonstrated improved clinical properties.[4]

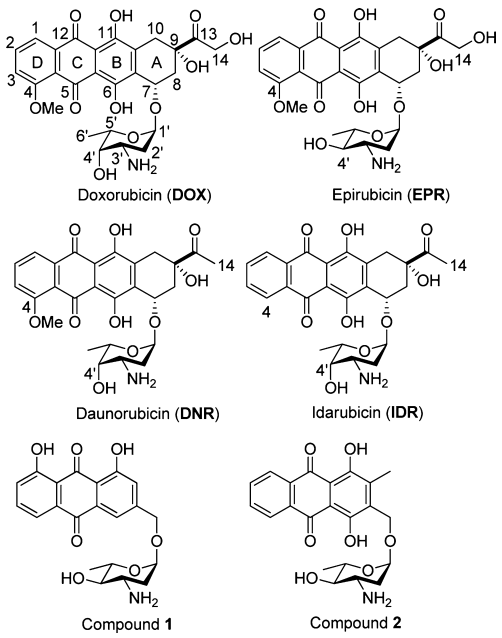


Figure 1. Structures of anthracycline derivatives.

Anthracycline derivatives that differ by the substitution patterns on the anthraquinone segment or by the carbohydrate unit (Figure 1) vary in their spectrum of antitumor activity and dose-limiting cardiotoxicitiy.[4] For example, doxorubicin (DOX) and its semisynthetic C-4′ sugar epimer epirubicin (EPR) demonstrate little difference in antitumor efficacy, but EPR causes less cardiac damage.[5] Removal of the 4-methoxy group from the anthraquinone of daunorubicin (DNR) results in the semisynthetic analogue idarubicin (IDR), which has a broader spectrum of antitumor activity than its parent anthracycline.[4] Even after decades of clinical use as front-line chemotherapy, neither the exact mode of action of anthracyclines nor the pathways leading to their side effects are fully understood. The accepted explanation for anthracycline antitumor activity is that

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these antitumor agents inhibit topoisomerase II activity upon formation of a ternary complex of double-stranded DNA, the enzyme, and the drug; this leads to DNA damage and ultimately to the induction of apoptosis.[6]

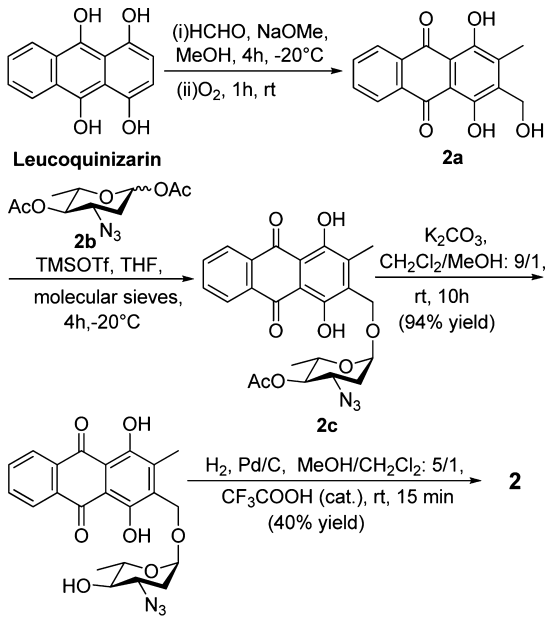
In mitochondria, anthracyclines induce the generation of free radicals, cause mitochondrial membrane disorder, lipid peroxidation, inhibition of the electron chain enzymes, and oxidize mitochondrial DNA.[6,7] Anthracyclines like DOX have been previously shown to accumulate in lysosomes; this organelle was proposed to host additional targets for these chemotherapeutic agents.[8,9] Several lysosomal hydrolases are involved in cell growth control and regulation of cell death.

The multiple cellular processes that are affected by anthracyclines raise the question of whether structural differences between anthracycline derivatives modulate only their cell permeability or their mode of action as well. Answering this question will pave the way to a rational design of new anthracyclines with a defined mode of action.

We therefore studied the associations between the structure and the subcellular distribution and function of clinically used anthracyclines DOX, DNR, and IDR and two synthetic anthracycline analogues (compounds **1** and **2**, Figure 1). The anthraquinones of **1** and **2** lack the substituted cyclohexene ring that is part of the anthraquinone segment of clinically used anthracyclines (represented as ring A in the structure of DOX, Figure 1) and enabled us to study the effects of this ring on the cytotoxic activity and subcellular distribution of these anthracycline analogues. The cytotoxicities of the five anthracycline derivatives were studied using several biological assays, and confocal microscopy analysis was used to determine the effects of the structural differences on the subcellular distribution of these antitumor agents.

Aloe-emodin glycoside **1** (Figure 1) was prepared according to our previously reported procedure.[10] The anthraquinone 3-methyldigiferrol (**2a**, Scheme 1), a truncated version of the anthraquinone of IDR, was obtained as a benzylic dehydroxylation product of the Marschalk reaction starting from leucoquinizarin.[11] The crude product mixture containing the desired **2a** was used directly for glycosylation with the acosamine glycosyl acetate derivative **2b**[12,13] by activation with trimethylsilyl trifluoromethanesulfonate (TMSOTf) in tetrahydrofuran (THF).[14] The protected α-glycoside **2c** was isolated by C-18 reverse-phase HPLC. Removal of the acetyl groups under mildly basic conditions gave compound **2d**, which was purified by size-exclusion chromatography on Sephadex LH-20. Finally, the azido group of **2d** was transformed into the free amine by catalytic hydrogenation. C-18 reverse-phase HPLC purification furnished pure 3-methyldigiferrol glycoside **2**.

Scheme 1. Synthesis of 3-Methyl-digiferrol Glycoside 2



The cytotoxic activities of the anthracycline derivatives were measured in SKOV-3, MCF-7, DA3, and ES-2 cell lines by determining the IC_{50} values using the XTT assay.[15] Results are summarized in Table 1. Of the tested cell lines, DOX was potent only against DA3 cells; the rest of the cell lines had high levels of resistance to this anthracycline. Unlike DOX, IDR and compound **1** were cytotoxic to all of the tested cell lines. These observations may be explained by mode-of-action differences or by the fact that IDR and compound **1** evade the resistance mechanisms that affect DOX.

Table 1. Cytotoxic Activity of Anthracycline Derivatives

	IC_{50} (μM)[a] values in indicated cell lines			
	SKOV-3	MCF-7	DA3	ES2
DOX	55.7 ± 4.3	>60	4.0 ± 0.7	>60
DNR	20.6 ± 1.1	33.9 ± 1.3	1.9 ± 0.4	25.5 ± 0.8
IDR	4.5 ± 0.4	7.3 ± 0.9	2.3 ± 0.7	3.2 ± 0.2
1	11.1 ± 0.9	23.6 ± 1.2	7.9 ± 1.4	6.8 ± 2.4
2	16.3 ± 1.2	17.2 ± 1.7	17.5 ± 1.2	17.7 ± 2.4

[a]Cells (~5×10^3/well) were incubated with concentrations of drug up to 60 μM for 24 h, and viability was determined using an XTT assay.

To test the kinetics of the cytotoxic effect, DA3 cells were treated at a concentration of 20 μM anthracycline (significantly higher than the IC_{50}), and XTT assays were performed after 60, 120, and 180 min of incubation. We compared DOX, IDR, and compound **1** since these three anthracycline derivatives were the most potent in DA3 cells. The time dependence of cell viability for each of these compounds is shown in Figure 2. Under these experimental conditions, DOX did not cause any measurable reduction in cell viability after 180 min; the effect of DOX on cell viability could be observed after longer incubation

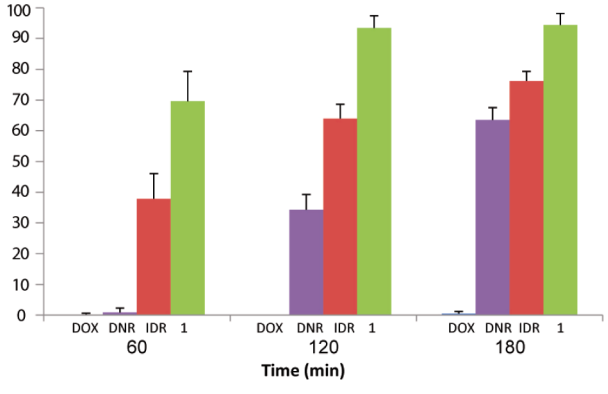


Figure 2. Time-dependent cell viability assay. DA3 cells were incubated in 20 μM DOX, DNR, IDR, or compound **1**, and the percentage of cell death was determined relative to untreated control cells (100% viability).

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periods (~12% after 6 h). Both IDR and aloe-emodin glycoside **1** dramatically reduced cell viability even after a 60 min incubation. Unlike DOX, after 180 min, IDR and aloe-emodin glycoside **1** caused the death of approximately 80 and 100% of cells, respectively. DNR, which differs from its semisynthetic analogue IDR by its anthraquinone 4-methoxy group, affected cell viability after a longer incubation time than IDR; yet, this anthracycline acted significantly faster than DOX (Figure 1). These observations strongly suggest that the clinically used IDR and DOX do not share a similar mode of action and that the synthetic anthracycline derivatives **1** and IDR may exert their cytotoxic effect by acting on other cellular targets that are different from those inhibited by DOX. The average cell doubling time of DA3 cells is approximately 21 h;[16] therefore, unlike DOX, both the clinically used IDR and the synthetic aloe-emodin glycoside **1** acted in a cell cycle-independent manner under the experiment conditions.

The significantly faster cytotoxic effects of IDR and compound **1** suggest that these two anthracycine derivatives differ from DOX in their mode of action and that these differences enable IDR and compound **1** to overcome DOX resistance mechanisms. Similar to the observed kinetics of the cytotoxic effect on DA3 cells, IDR and compound **1** rapidly affected the viability of ES2 cells as well, therefore demonstrating that the rapid cytotoxic effect of these compounds is not specific to DA3 cells (see Figure S1 in the Supporting Information).

As it is very well established that DOX and IDR interact strongly with double-stranded DNA, we tested whether this was also true for the two synthetic anthracycline derivatives **1** and **2**. DNA intercalating properties of anthracyclines such as DOX have been studied extensively in the past using a variety of different methods.[10,16−18] We studied the DNA intercalation properties of the five anthracycline derivatives using the fluorescent DNA intercalator displacement (FID) assay.[19] Rather than using ethidium bromide, which was used in the original assay, we used DNA intercalating dyes SYBR safe (SYBR) and YOYO-1 for the FID experiments. Upon DNA intercalation, ethidium bromide fluorescence quantum yield increases by ~10 fold; the increase for YOYO-1 is ~400 fold and that of SYBR is over 1500-fold.[20] Furthermore, the fluorescence quantum yield of free ethidium bromide is almost 40-fold higher than that of free YOYO-1 and close to 100-fold higher than that of SYBR. These two dyes offer enhanced sensitivity and are also less toxic than ethidium bromide.

Both DOX and IDR had similar IC_{50} values for displacement of SYBR; however, DNR displaced this dye 5−6-fold better than DOX and IDR (Table 2). In the YOYO-1-based assay, DOX and DNR had similar IC_{50} values that were approximately 3-fold higher than that of IDR (IC_{50} values 0.60 ± 0.06 μM for DOX, 0.84 ± 0.06 for NDR, and 1.97 ± 0.29 μM for IDR). However, compounds **1** and **2** did not have any significant effect on the fluorescence of either dye even at a concentration of 40 μM. FID assay results indicated that anthracycline derivatives **1** and **2** are unlikely to exert their biological activity by intercalating with double-stranded DNA. It is important to note that the lack of significant DNA intercalation that was observed for compounds **1** and **2** does not rule out the possibility that these anthracycline derivatives may still act as topoisomerase II inhibitors and generate double-stranded DNA breaks.

Moreover, the observed small differences between the DNA dye displacement potencies of DOX and IDR suggest that the differences in the cytotoxic activities between IDR and DOX may not result from DNA intercalation properties but from mode-of-action differences between these two anthracyclines.

Table 2. Fluorescent DNA Intercalator Displacement (FID) Assay[a]

	IC_{50} (μM)[a] in assay with indicated dye	
compd	YOYO-1	SYBR
DOX	0.60 ± 0.06	0.73 ± 0.07
IDR	1.97 ± 0.29	0.89 ± 0.16
DNR	0.84 ± 0.06	0.14 ± 0.01
1	>40	>40
2	>40	>40

[a]Experiments were performed in 96-well plates at 37 °C; each well contained 500 ng of phage lambda DNA (48 Kb), SYBR at final dilution of 1:10000, or YOYO-1 at a ratio of one YOYO-1 molecule per 40 base pairs and the tested compound in concentrations up to 40 μM.

Taking advantage of the natural fluorescence of anthracyclines and that of specific subcellular markers, we determined the subcellular localization of the studied anthracycline derivatives. DA3 cells were incubated with the different anthracycline derivatives and subjected to live cell confocal microscopy analysis prior to addition of anthracycline and after 30 min of incubation with the anthracycline derivatives. To reduce cross-talk between the dyes and the compound fluorescence, we performed spectral analysis of each compound and of the subcellular marker (emission fingerprints) followed by linear unmixing. To study the subcellular localization, colocalization analysis was performed between the compound fluorescence and the subcellular localization marker, and the Returns Pearson's correlation coefficient (R-coloc) was calculated (ImageJ macro colocalization threshold class function).[21]

Figure 3 shows confocal laser scanning microscopy analysis of anthracycline derivatives in live cells in the presence of markers for mitochondria, lysosomes, and nuclei. DOX was detected in the nucleus (41.43 ± 9.35%, R-coloc values ranged between 0.2838 and 0.4321, $p < 0.0001$) and in the lysosomes (27.55 ± 2.35%, R-coloc 0.5260−0.5977, $p < 0.0001$) but not in the mitochondria (Figure 3, images d, c, and b, respectively). Because DOX required more than 30 min to affect DA3 cells viability, we studied its subcellular distribution after prolonged incubation (10 h) and found that the distribution pattern was essentially the same as in cells that were exposed to this anthracycline for only 30 min (see S2 in the Supporting Information). Unlike IDR, DNR localized in the nucleus (65.13 ± 5.23%, R-coloc values ranged between 0.2379 and 0.4786, $p < 0.0001$) and in the lysosomes (72.11 ± 11.47%, R-coloc 0.1110−0.3300, $p < 0.0001$) but not in the mitochondria (Figure 3, images h, g, and f, respectively). The single structural difference between IDR and DNR (IDR is the 4-demethoxy derivative of DNR) led to a dramatic effect on the observed subcellular distribution of this anthracycline: IDR was not detected in the nucleus (images i and l). Treatment of the cells with IDR dramatically reduced the fluorescence levels of the LysoTracker green; this phenomenon may result from intracellular pH changes induced by high lysosomal concentrations of this anthracycline. Hence, colocalization of IDR with LysoTracker green was analyzed from images obtained with a sensitive Hybrid Detection System for Photon Counting (Leica HyD); these analyses demonstrated that a high percentage of

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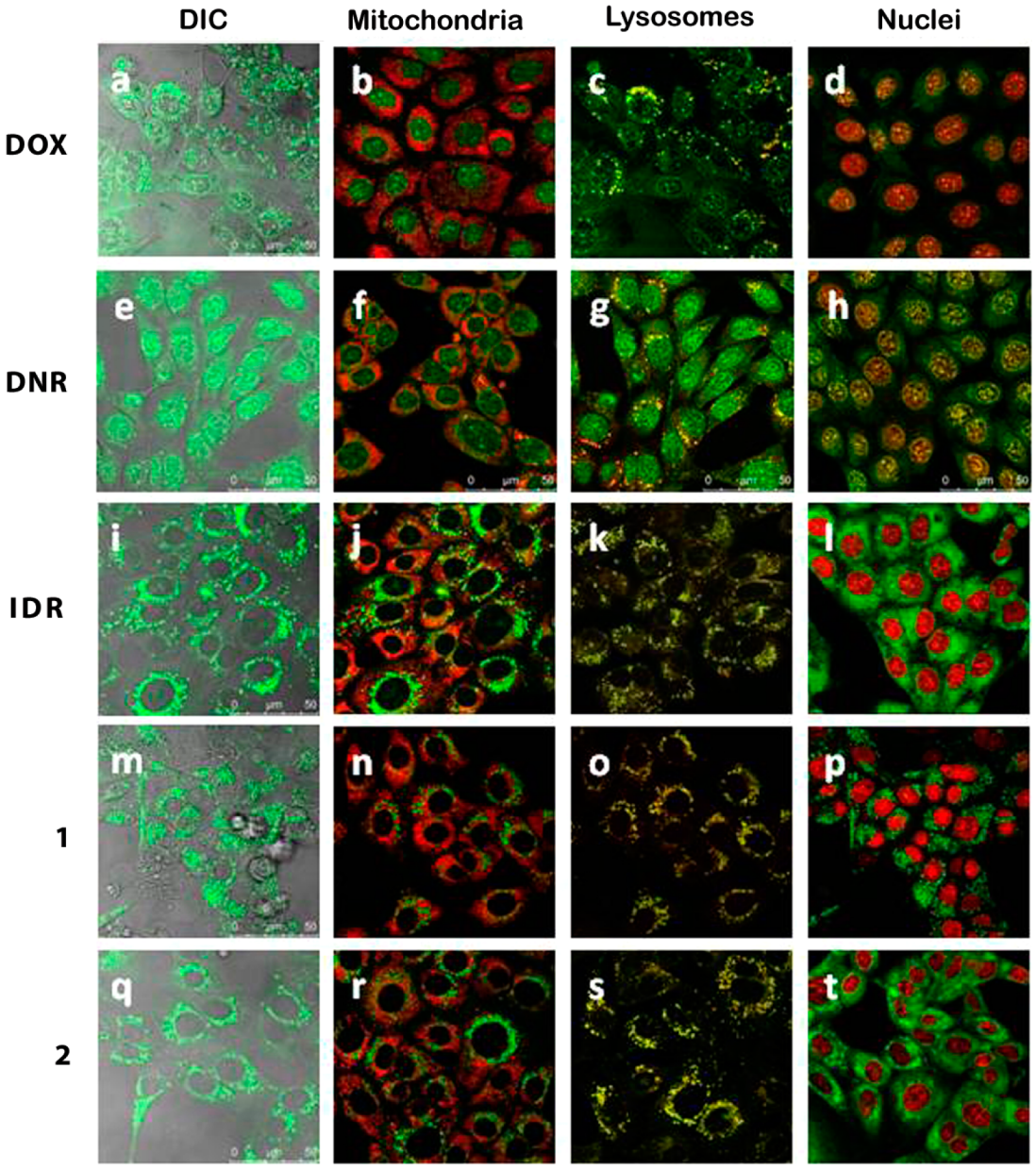

Figure 3. Confocal laser scanning microscopy analysis of anthracycline derivatives in live cells. DA3 cells were treated with 10 μM DOX (images a–d), DNR (images e–h), IDR (images i–l), compound **1** (images m–p), or compound **2** (images q–t) for 30 min. Subcellular colocalization was evaluated using the mitochondrial dye MitoTracker deep red or the nuclear dye DRAQ-5 deep red. Lysosome colocalization experiments were performed using the lysosome dye LysoTracker green for DOX, DNR, and IDR and LysoTracker red for compounds **1** and **2**. In each of the images, the anthracycline derivative appears in green and the dye in red. DIC overlaid images a, e, i, m, and q show the subcellular distribution of each compound. Images b, f, j, n, and r show the mitochondrial colocalization. Images c, g, k, o, and s show the lysosomal colocalization of each compound. Images d, h, l, p, and t show the nuclear colocalization of each compound.

IDR localized in lysosomes (83.19 ± 5.76%, R-coloc range of 0.6645–0.8841, $p < 0.0001$,; Figure 3, image k).

Like IDR, the synthetic derivatives **1** and **2** were detected in the cytoplasm and not in the nucleus (Figure 3, images m and q, respectively). No colocalization of either **1** or **2** with the nuclear dye DRAQ-5 was detected (images p and t, respectively). No mitochondrial localization was observed for these compounds (images n and r, respectively). A high percentage of **1** (67.12 ± 10.02%, image o) and of **2** (84.48 ± 2.85%, image s) was present in lysosomes. The calculated R-coloc range for **1** and LysoTracker red was 0.4727–0.7430 ($p < 0.0001$), and the calculated range for **2** and LysoTracker red was 0.8366–0.8827 ($p < 0.0001$).

It has been previously reported that the incubation of anthracyclines with increasing concentrations of double-stranded DNA quenches their fluorescence in a concentration-dependent manner. This experiment indicated that there was a small difference between the effect of DNA concentration on the degree of fluorescence quench of DOX and IDR.[22] Hence, fluorescence quench cannot account for the fact that DOX was clearly detected in the nucleus of DA3 cells, whereas IDR was not. Taken together with the differences in the spectrum and kinetics of cytotoxicity between DOX and IDR, the results of the confocal microscopy colocalization experiments further demonstrate that even small structural differences between anthracyclines may lead to major differences in their

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subcellular localization and mode of action. Because both IDR and DOX demonstrated potent DNA intercalation properties, it is possible that the higher hydrophobicity of the anthraquinone segment of IDR as compared to that of DOX increases its relative affinity for other cellular targets; however, the reason for the absence of IDR from the nucleus remains unclear. In contrast to DOX and IDR, FID experiments demonstrated that synthetic derivatives **1** and **2** do not interact with double-stranded DNA, a fact that may explain why these compounds were not detected in the cell nucleus.

It is well established that several mitochondrial processes are directly affected by clinically used anthracyclines;[7] however, under the conditions of our confocal microscopy colocalization experiments, none of the tested compounds demonstrated mitochondrial localization. It is possible that the studied compounds target mitochondrial enzymes at concentrations not detectable in our experiments. Our confocal microscopy observations clearly indicate that all tested anthracyclines were present in lysosomes. Hence, our observations support the possibility that lysosomal pathways may serve as important targets for some members of the anthracycline family such as IDR or the three ring anthraquinone-based derivatives **1** and **2**.

In conclusion, structural differences among anthracycline derivatives impacted their cytotoxic activity and subcellular localization. DOX had no measurable effect on DA3 cells after 3 h of incubation, whereas a short exposure to IDR DNR or **1** caused a dramatic reduction in cell viability, suggesting that, unlike DOX, these anthracycline derivatives do not target the cell division mechanism. The DNA intercalator displacement assay indicated that the substituted cyclohexene ring, which is part of the anthraquinones of DOX and IDR and is missing on the anthraquinones of **1** and **2**, is important for DNA intercalation. Confocal microscopy experiments indicated that DOX was the only tested anthracycline derivative that localized in the cell nucleus. Significant lysosomal colocalization was detected for IDR, DNR, and the two synthetic derivatives **1** and **2**, indicating that lysosomes may contain previously unexplored anthracycline targets that may explain the difference in the biological activities between these athracycline derivatives and DOX. Finally, this study demonstrates that even small structural differences between members of the anthracycline family of antitumor agents have a significant effect on their subcellular distribution, cytotoxic activity, and mode of action. On the basis of our observations of the effects of modifying the substation of the anthraquinone or by using three ring anthraquinone-based anthracycline derivatives such as **1** and **2**, novel families of antitumor agents that do not act by targeting the cell division process may be developed.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, cell cytotoxicity test protocol, FID assay protocol, confocal microscopy protocol, and ^{1}H, ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥These authors contributed equally.

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Notes

The authors declare no competing financial interest.

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