Published in final edited form as:

J Control Release. 2018 January 10; 269: 171–176. doi:10.1016/j.jconrel.2017.11.007.

Leakage Kinetics of the Liposomal Chemotherapeutic Agent Doxil: The Role of Dissolution, Protonation, and Passive Transport, and Implications for Mechanism of Action

Luisa M. Russell^{1,2}, Margot Hultz^{1,2}, and Peter C. Searson^{1,2,3}

¹Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, Maryland 21218, USA

²Institute for Nanobiotechnology, Johns Hopkins University, Baltimore, Maryland 21218, USA

³Department of Oncology, Johns Hopkins University, Baltimore, Maryland 21218, USA

Abstract

Doxil, a liposomal formulation of the chemotherapeutic drug doxorubicin, is FDA-approved for multiple indications. Doxil liposomes are designed to retain doxorubicin in circulation, minimize clearance by the mononuclear phagocyte system, and limit uptake in healthy tissue. Although pharmacokinetic data and survival statistics from clinical trials provide insight into distribution and efficacy, many details of the mechanism of action remain unresolved, despite the importance in translating liposome-based drug delivery systems to other molecules and cargo. Therefore, the objective of this study is to quantitatively assess the kinetics of doxorubicin leakage from Doxil liposomes. In contrast to previous studies, we consider three processes: dissolution of solid doxorubicin, protonation/deprotonation of soluble doxorubicin, and passive transport of neutral doxorubicin across the lipid bilayer of the liposomes. Experiments were performed for Doxil, Doxil-like liposomes, and Doxil-like liposomes with reduced cholesterol and pegylation. To mimic physiological conditions, we also performed experiments in serum and under slightly acidic conditions at pH 5. We show that crystalline doxorubicin dissolution can be described by a first order rate constant of $1.0 \times 10^{-9} \text{ cm s}^{-1}$ at 37 °C. Doxorubicin leakage can be described by first order rate constant for transport across the lipid bilayer with values in the range from $1 - 3 \times 10^{-12} \text{ cm s}^{-1}$ at 37 °C. Based on these results we discuss implications for the mechanism of action, taking Doxil pharmacokinetics into account.

Graphical abstract

Corresponding Author: Peter C. Searson, searson@jhu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Doxil; drug delivery; doxorubicin; liposome leakage

Introduction

Doxil is a liposomal formulation of doxorubicin [1, 2] that was FDA-approved in 1995 and has been subsequently approved for other indications including ovarian cancer and multiple myeloma. Doxil liposomes are about 100 nm in diameter and encapsulate 10,000 – 15,000 doxorubicin molecules [3]. PEGylation of the liposomes results in long circulation half-time, small distribution volume, low clearance rate, and high area under the curve [3]. While Doxil liposomes are designed to retain doxorubicin in circulation, surprisingly little is known about the leakage kinetics of doxorubicin or other drugs [2]. Similarly, the mechanism by which doxorubicin enters target cells within a solid tumor is not well understood [2].

There have been relatively few studies of the transport of doxorubicin or other drugs out of liposomes [4–6]. Most of these studies report the fraction of drug leaked out in a specific time. For example, depending on liposome composition, the loss of doxorubicin ranges from no detectable leakage over 24 hours (HSPC:CHOL:mPEG2000-DSPE liposomes (2:1:0.1 mole ratio)) [6] to 40% leakage in 60 minutes (egg PC:cholesterol (1:1)) [5]. While these studies provide useful comparisons, they do not provide insight into the kinetics of transport of doxorubicin. Therefore, the objective of this study is to quantitatively assess the kinetics of doxorubicin transport from Doxil liposomes. We consider three processes: dissolution of solid doxorubicin, protonation/deprotonation of soluble doxorubicin, and passive transport of neutral doxorubicin across the lipid bilayer of the liposomes. We first determine the rate constant for dissolution of solid doxorubicin and then determine the rate constant for doxorubicin leakage from liposomes. These quantitative data provide a foundation for modeling drug distribution and bioavailability.

Materials and Methods

Reagents

Doxil (liposomal doxorubicin) was purchased from Avanti Polar Lipids (LipoCure). To prepare Doxil-like liposomes, hydrogenated soybean phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]

(DSPE-mPEG2k), and cholesterol were purchased from Avanti Polar Lipids. HEPES buffer, PBS, sodium chloride, ammonium sulfate, doxorubicin HCl, and Sepharose CL-4B were purchased from Sigma-Aldrich.

Crystalline doxorubicin sulfate dissolution

Doxorubicin dissolution was characterized by measuring the concentration of doxorubicin in the solution (see Supplemental Figure S1). Briefly, doxorubicin crystals were formed by slowly pipetting 50 μL of a 5 mg mL^{-1} solution of doxorubicin HCl (Sigma) into 800 μL of a solution containing 10 mM HEPES, 140 mM NaCl, 300 mM ammonium sulfate in a microcentrifuge tube. This solution mimics the solution used for remote loading doxorubicin into liposomes [7]. The solution was left to crystallize for 30 minutes in the dark at room temperature. Dissolution experiments were performed by removing the supernatant and replacing it with 800 μL of 300 mM ammonium sulfate solution pre-incubated to 4, 25, or 37 $^{\circ}\text{C}$. At the prescribed time points, 400 μL of the ammonium sulfate solution were removed for absorbance measurement, and then replaced in the tube. The concentration of doxorubicin in solution was determined from the absorbance using a calibration curve obtained from known concentrations. The saturation concentration was obtained by allowing the crystals and solution to equilibrate at the given temperature over the course of several days. Volumes and amounts were optimized for UV-vis absorbance measurements.

Liposome Leakage

Doxil-like liposomes were formed from 90 mol.% HSPC and 10 mol.% DSPE-PEG2k, with an approximately 1:1 ratio of lipids to cholesterol. 191 μL of 25 mg mL^{-1} HSPC, 63 μL of 25 mg mL^{-1} cholesterol, and 63 μL of 25 mg mL^{-1} DSPE-PEG2k were added to a glass vial. The lipids were then dried to a lipid film under a constant stream of argon and dried under vacuum overnight. The next day, 0.5 mL of 300 mM ammonium sulfate solution was added to the vial containing the lipid film, which then underwent 10 freeze-thaw cycles between liquid nitrogen and room temperature water. The resulting solution was mechanically extruded 10 times through a 100 nm filter. The liposomes were then purified by size exclusion chromatography using a Sepharose CL-4B column equilibrated in HEPES buffered saline. Liposomes containing ammonium sulfate solution were then incubated with 200 μL of 5 mg mL^{-1} doxorubicin HCl at 4 $^{\circ}\text{C}$ overnight. Six days later, free liposomes were transferred to PBS solution (pH = 7.4) via use of a Sepharose CL-4B column. Doxil-like liposomes with 75% cholesterol were prepared using 47.25 μL cholesterol, otherwise the preparation was the same. For further tests of leakage conditions, leakage was also assessed for liposomes with approximately 50% of the standard amount of PEG, and for standard liposomes diluted in 50% serum or PBS pH-adjusted to a pH of approximately 5, both of which were tested to mimic conditions in a tumor environment. All of these liposomes were prepared according to the methods described above. All liposomes were characterized by dynamic light scattering (DLS) using a Malvern Zetasizer.

Doxorubicin leakage was determined by measuring the concentration of doxorubicin remaining in the liposomes (see Supplemental Figure S2). Following transfer of the liposomes into PBS solution (a total solution volume of 3 mL), 150 μL of liposome solution (both Doxil, and Doxil-like liposomes) was removed at every time point and separated from

free doxorubicin using a Zeba filter (Fisher Scientific) equilibrated in PBS. The Zeba filters were centrifuged five times (3 times at 1500 g for 1 minute, and 2 times at 1500 g for 2 minutes) and the liposome band was collected separately from the free doxorubicin that had leaked out. The amount of Doxil remaining in the liposomes was then determined from the absorbance at 500 nm and previously determined calibration curves. In control experiments, we also determined the amount of Doxil in isolated liposomes after rupturing the liposomes. Since there was no difference in the measured amount of Doxil, all absorbance measurements were performed on intact liposomes.

Results

Doxil liposomes contain doxorubicin in the solid and liquid phases. In addition, doxorubicin can be ionized at low pH ($pK_a = 8.3$). Therefore, the release of doxorubicin from liposomes involves three processes: dissolution of the solid doxorubicin crystal, protonation/deprotonation of the soluble doxorubicin, and passive transport of neutral doxorubicin across the lipid bilayer of the liposomes (Figure 1). Here we report on the kinetics of dissolution of solid doxorubicin and the kinetics of leakage of neutral doxorubicin in solution from liposomes. We assess the leakage kinetics in Doxil and Doxil-like liposomes. To assess the role of cholesterol, we assessed leakage in Doxil-like liposomes with 75% cholesterol.

Dissolution of solid doxorubicin

The dissolution of solid doxorubicin sulfate was studied at 4 °C, the temperature at which it is stored, 25 °C, the temperature at which it is handled in the clinic, and 37 °C, the temperature at which it is administered. On removing the supernatant from the solution containing the doxorubicin crystals and replacing it with ammonium sulfate solution, the doxorubicin concentration in solution increased with time until equilibration at the solubility limit (Figures 2A–C). The solubility limit was dependent on temperature, increasing from 0.12 mM at 4 °C to 0.4 mM at 37 °C. These values are consistent with reported values of the solubility of doxorubicin sulfate in water of around 0.2 mg mL⁻¹ (0.34 mM) at pH 7 and 20 °C [7].

Doxorubicin dissolution can be represented by the equilibrium $D_{crys} \leftrightarrow D_{lip}$ where D_{crys} and D_{lip} denote doxorubicin in the solid and liquid phases, respectively. To analyze doxorubicin dissolution, we assume that dissolution and precipitation can be described by the first order rate constant, k_d . From integration of the rate equations (see Supplemental Information, Section 1), we obtain:

$$c_{lip} = c_{sat} \left(1 - \exp \left(- \frac{v_{lip} + v_{crys}}{v_{crys}} k_d t \right) \right) \quad (1)$$

where c_{lip} is the concentration of doxorubicin in the liquid phase, c_{sat} is the saturation concentration, v_{lip} is the liposome volume, v_{crys} is the volume of the crystal, and k_d is the dissolution rate constant. Rearranging:

$$\log \left(\frac{c_{\text{sat}} - c_{\text{lip}}}{c_{\text{sat}}} \right) = - \left(\frac{v_{\text{lip}} + v_{\text{cryst}}}{V_{\text{cryst}}} \right) k_d t \quad (2)$$

Replotting the dissolution curves as $\log((c_{\text{sat}} - c_{\text{lip}})/c_{\text{sat}})$ versus time (Figure 2D–F) shows a linear dependence for the three different temperatures, verifying first order kinetics. From the slopes of the plots we can calculate the dissolution rate constant, k_d , which is in the range from $5 \times 10^{-5} \text{ s}^{-1}$ at 4°C to $6 \times 10^{-4} \text{ s}^{-1}$ at 37°C . To compare this to rate constants we might expect for membrane permeability, we can convert this value to cm s^{-1} using liposome geometry. We then calculate that at 37°C , the rate constant for dissolution is $1.0 \times 10^{-9} \text{ cm s}^{-1}$. An Arrhenius plot of the rate constants is linear (Figure 2H), confirming that the dissolution process is thermally activated, and from which we calculate an activation energy of 44.6 kJ mol^{-1} (0.58 eV). The activation energy of doxorubicin is similar to calcite (40 kJ mol^{-1}) and quartz (64 kJ mol^{-1}) in distilled deionized water [8].

Liposome leakage

Having determined the rate constant for dissolution of solid doxorubicin, we next assess the kinetics of leakage across the liposome membrane. To determine the rate constant for leakage, we measured the amount of doxorubicin remaining in the liposomes for up to two weeks. Before leakage experiments, liposomes were characterized by DLS and zeta potential (at 37°C). Commercial Doxil liposomes had a Z-average size of 75 nm and a polydispersity index (PDI) of 0.18, while all synthesized Doxil-like liposomes had a Z-average of approximately 140 nm, with a PDI of 0.18. No significant differences in size were observed between batches. The zeta potential for the commercial liposomes was -5 mV , and for synthesized liposomes was about -20 mV .

Representative plots show a progressive increase in the amount of doxorubicin leaked out, up to 20 – 30% over two weeks (Figure 3A–C). The amount lost is greatest for the Doxil liposomes and slightly lower for the Doxil-like liposomes (Fig. 3A, B). Decreasing the amount of cholesterol in the liposomes, results in a further small increase in the amount leaked out (Fig. 3C).

To quantitatively analyze the leakage kinetics (Fig. 3A–C), we assume that transport across the lipid bilayer is described by the first order rate constant, k_m (see Supplementary Information, Section 3). Since dissolution of solid doxorubicin is much faster than transport across the lipid bilayer (verified below), we assume that in the liposome, the crystal and free doxorubicin are in equilibrium. Therefore, the concentration in the aqueous core is assumed to be the saturation concentration, $\approx 0.4 \text{ mM}$ at 37°C . This assumption is reasonable as long as any solid doxorubicin remains in the liposome.

Doxorubicin is a weakly basic molecule with a pK_a of 8.3 [9] and hence neutral doxorubicin (D^0) becomes protonated (D^+) in neutral or weakly acidic environments. Although neutral doxorubicin can passively diffuse across cell membranes, protonated doxorubicin has extremely low permeability due to the excess charge [10]. At the pH inside the liposomes ($\text{pH} = 7$), 22% of the doxorubicin is in the neutral state and available for transport.

At the start of the experiments, the liposomes are isolated and transferred to PBS so that the concentration of doxorubicin in solution (outside the liposomes) is initially zero. The leakage of neutral doxorubicin across the lipid bilayer (see Supplementary Information, Section 3) can be written as:

$$C_{out} = \frac{N_T}{1.06V_{out}} \left(1 - e^{-\frac{0.23k_m A t}{V_{in}}} \right) \quad (3)$$

where C_{out} is the concentration that has leaked out of the liposomes, N_T is the total amount of doxorubicin, V_{out} is the volume of liquid outside each liposome, A is the surface area of a liposome, t is time, V_{in} is the volume inside the liposome, and k_m is the leakage rate constant.

The fractional amount of doxorubicin leaked out of the liposomes as a function of time was fit to equation (3) using a linear least squares method (Figs. 3D–F), from which we obtain rate constants for doxorubicin transport k_m of $2.24 \pm 0.76 \times 10^{-12} \text{ cm s}^{-1}$ for Doxil liposomes, and $1.41 \pm 0.26 \times 10^{-12} \text{ cm s}^{-1}$ for Doxil-like liposomes. There is no statistical significance between the two rate constants ($p = 0.058$). Scatter in the data could be due to lipid age, variations associated with extrusion conditions, and slight differences in yield, leading to differences in the ratio between liposome volume and solution volume.

To assess the role of cholesterol on doxorubicin leakage, we performed experiments with Doxil-like liposomes with 75% cholesterol. It is not possible to study a broader concentration range since liposomes with lower concentrations are not sufficiently stable. The permeability of Doxil-like liposomes with 75% cholesterol was $1.58 \pm 0.13 \times 10^{-12} \text{ cm s}^{-1}$, which is not statistically significant from Doxil-like liposomes.

To assess the influence of the solution in which the liposomes are suspended, we performed experiments to determine the rate constant for doxorubicin leakage in 50% serum (Fig. 4A) and buffer at pH 5 (Fig. 4B). The rate constant for the Doxil-like liposomes in 50% serum was $2.82 \pm 0.73 \times 10^{-12} \text{ cm s}^{-1}$, higher than in buffer at pH 7.4 ($p = 0.036$). In pH 5 buffer, the rate constant was $3.02 \pm 0.95 \times 10^{-12} \text{ cm s}^{-1}$, larger than the value at pH 7.4 ($1.41 \pm 0.26 \times 10^{-12} \text{ cm s}^{-1}$; $p = 0.024$).

Finally, to test the role of DSPE-PEG on doxorubicin leakage, we performed experiments on Doxil-like liposomes in pH 7.4 buffer with half the standard amount of PEG (Fig. 4C). The leakage rate constant was $1.0 \pm 0.35 \times 10^{-12} \text{ cm s}^{-1}$, not statistically different from the Doxil-like liposomes with the standard PEG concentration. These rate constants are compared in Figure 5.

Discussion

Although Doxil has been FDA-approved for multiple indications, several questions remain over the mechanism of action [11]. Here we focus on the kinetics of doxorubicin leakage from Doxil liposomes. Previous studies reported the amount of doxorubicin leaked out of liposomes at one or two time points, and hence provide useful comparisons, but do not

provide details of the kinetics [4–6]. Here we consider three processes: doxorubicin dissolution, ionization, and transport across the liposome lipid bilayer.

The two most important design features of Doxil are the ability to load a large number of drug molecules and the long circulation half-time [1]. Sequestration of doxorubicin in liposomes minimizes the concentration of free drug in circulation, thereby minimizing the adverse side effects associated with uptake in healthy tissue [3, 12]. The long circulation half-time, compared to free drug, increases tumor accumulation by increasing the number of opportunities for extravasation from circulation [3, 12].

Doxorubicin loading in liposomes results in supersaturation and precipitation of solid doxorubicin sulfate. The average concentration doxorubicin in Doxil liposomes (solid and liquid phases) is about 50 mM, larger than the concentration that can be achieved by passive loading, which is limited by the solubility limit of doxorubicin HCl in water (10 mg mL⁻¹ or about 20 mM) [1]. At 37 °C, the rate constant for dissolution of solid doxorubicin sulfate k_d was $6 \times 10^{-4} \text{ s}^{-1}$, which is equivalent to $1 \times 10^{-9} \text{ cm s}^{-1}$ when taking into account liposome geometry. This is three orders of magnitude faster than the calculated leakage rate constant of about $10^{-12} \text{ cm s}^{-1}$. Therefore, the kinetics of leakage are dominated by the rate constant for transport across the liposome bilayer and the pK_a .

Doxil liposomes are optimized for stability and hence the ability to test how changes in the amount of DSPE-PEG or cholesterol is limited to a relatively narrow range. In all cases, we verified that the liposomes maintained colloidal stability during the leakage experiments. We also confirmed that there was no measurable leakage of doxorubicin during storage at 4°C. Here we show that doxorubicin leakage from liposomes can be modeled using a first order rate constant for transport across the lipid bilayer with $k_m = 1 - 3 \times 10^{-12} \text{ cm s}^{-1}$. In studies of passive diffusion of various molecules across red blood cells or lipid vesicles, a linear relationship was found between molecular weight and permeability [13]. Based on the molecular weight for doxorubicin, the value for k_m predicted by this relationship is close to the experimental value reported here [13]. A rate constant of $1 \times 10^{-12} \text{ cm s}^{-1}$ corresponds to around 2% leakage over 24 hours, or 10 – 15% leakage over 10 days.

To assess whether the local microenvironment influences leakage kinetics, we performed experiments with synthesized Doxil-like liposomes in 50% serum to mimic circulation, and in pH 5 buffer to mimic the slightly acidic tumor microenvironment. The rate constants for leakage under these conditions were around $3 \times 10^{-12} \text{ cm s}^{-1}$, approximately 2-fold larger than in pH 7.4 buffer. This rate constant corresponds to loss of 5% over 24 hours, or around 30% leakage over 10 days. The presence of serum in the solution has been shown to increase leakage of calcein from liposomes prepared from either egg PC, DPPC, or DSPC, an effect attributed to membrane destabilization due to serum phospholipid exchange [14]. Under acidic conditions doxorubicin becomes progressively protonated, reducing the concentration of the neutral molecule. Therefore, the driving force for leakage increases with decreasing pH [15].

The amount of DSPE-PEG (10%) in Doxil-like liposomes, is around 5000 PEG groups per liposome. To assess whether the PEG groups influence leakage, we performed experiments

with half the DSPE-PEG concentration, but found no difference in rate constant. These results imply that the PEG groups do not influence leakage. Since liposomes with lower concentrations of DSPE-PEG have relatively poor stability, we did not study lower concentrations.

The extremely slow leakage profile has several implications for the mechanism of action of Doxil, which remains unknown [11]. First, we consider leakage of the free drug into circulation. From the leakage kinetics it is evident that Doxil is not a reservoir for release of doxorubicin in circulation. Following administration of a typical dose of Doxil (100 mg) the average concentration in circulation during the first 24 hours is about 15 mg L^{-1} (75 mg) [1]. From this concentration and the rate constant k_m , we calculate that only 3% of the doxorubicin (about 2 mg) will leak into circulation within the first 24 hours post-injection [1]. This is consistent with the dogma that most doxorubicin in circulation is contained within the liposomes.

Next, we consider leakage of doxorubicin from liposomes accumulated in a solid tumor. The relatively long circulation half-time of PEGylated liposomes, typically 1 – 4 days, results in a tumor accumulation of up to 0.1 – 1 % ID g^{-1} tumor [12]. For a typical 100 mg dose of Doxil, a tumor accumulation of 0.1 – 1% corresponds to 10^{13} – 10^{14} liposomes (for a 1 g tumor). Since the leakage rate from the liposomes is very slow, this implies that the mechanism of action is not due to slow release of doxorubicin once liposomes accumulate in the tumor. Accumulation of 10^{13} liposomes would result in leakage of only about 3 μg over 24 hours post-injection. Even if we assume a 2-fold increase in leakage in serum and at pH 5, we still expect a small difference in accumulated doxorubicin, up to 6 μg of leaked doxorubicin over 24 hours post-injection. Therefore, the mechanism of action of Doxil is expected to involve uptake of liposomes, possibly by endocytosis, as suggested in the literature [11]. In comparison to Doxil, it has been estimated that the tumor accumulation of free doxorubicin 24 hours post-injection is around 0.02 % ID g^{-1} tumor, which for a single 100 mg dose, corresponds to a tumor accumulation of approximately 20 μg over 24 hours. This is almost 10-fold larger than the estimate of 3 μg leaked into the tumor from Doxil liposomes.

A 1 cm tumor contains approximately 10^9 cells [16], therefore, the number of liposomes accumulating in the tumor is far in excess of the number of tumor cells. Intravital microscopy studies in mouse models show extravasation of liposomes and other nanoparticles with diameters up to around 500 nm due to the enhanced permeability and retention effect [17]. However, the diffusion rate of Doxil into the tumor is expected to be very slow, certainly much slower than for a small molecule such as doxorubicin [18]. The penetration depth of free doxorubicin was found to be about 50 μm in a mouse xenograft model [19]. Therefore, it is the tumor cells closer to the vasculature that will initially take up Doxil liposomes.

The release of doxorubicin from one Doxil liposome into a cell (assuming a cell volume of $10^3 \mu\text{m}^3$) corresponds to a concentration of about 10 ng mL^{-1} (1.7 nM). Doxorubicin can induce cell death either through mitotic catastrophe at continuous low doses (50 ng mL^{-1}) or apoptosis at high doses ($10 \mu\text{g mL}^{-1}$) [20]. Therefore, uptake of only a small number of

liposomes would be sufficient to induce cell death. In this scenario, even though the number of liposomes extravasating from circulation is much larger than the total number of cells, cell death is induced only in cells that are close to the vasculature. Exposure of cells deeper into the tumor can occur by two mechanisms: (1) apoptosis of tumor cells close to the vasculature allowing uptake of Doxil liposomes by underlying cells, or (2) apoptosis of tumor cells close to the vasculature releasing the relatively large concentration of free doxorubicin within the cytosol. More recent work implies increased leakage at the tumor site due to an increase in ammonia in the tumor microenvironment [21].

Although the mechanism of action of Doxil remains unknown, its efficacy is unquestionable. Quantifying the kinetics of leakage of doxorubicin provides some important insight onto the mechanism of action. More experiments are required to definitively identify the drug release mechanism at work. This analysis lends some support to the cascading layers of cell death hypothesis posited above.

Conclusion

Liposomal formulations of doxorubicin have many advantages over free drug administration, including reduction of the necessary dose and the debilitating side effects, such as cardiotoxicity. Although it has been used routinely in the clinic for many years since its FDA-approval in 1995, and has been known to be effective for patients suffering from various cancers with solid tumors, its mechanism of action remains unknown. Here, we quantified liposome drug delivery parameters including drug availability and leakage of doxorubicin across a PEGylated liposome. This analysis yielded extremely low permeability values on the order of $10^{-12} \text{ cm s}^{-1}$, consistent with literature for other liposome compositions [13]. This extremely slow availability of passively diffusing drug leads us to several possibilities when it comes to the possible mechanism of action of Doxil, effectively eliminating any processes that may be based in leakage alone for drug availability. Instead, it is far more likely that Doxil is endocytosed, releasing drug into the tumor when the cells nearest to the vasculature die.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge support from DTRA (HDTRA1-15-1-0046) and NIH (U54CA151838).

References

1. Gabizon A, Shmeeda H, Barenholz Y. Pharmacokinetics of pegylated liposomal doxorubicin - Review of animal and human studies. *Clin Pharmacokinet.* 2003; 42:419–436. [PubMed: 12739982]
2. Barenholz YC. Doxil®—the first FDA-approved nano-drug: lessons learned. *J Control Release.* 2012; 160:117–134. [PubMed: 22484195]
3. Dawidczyk CM, Kim C, Park JH, Russell LM, Lee KH, Pomper MG, Searson PC. State-of-the-art in design rules for drug delivery platforms: Lessons learned from FDA-approved nanomedicines. *J Control Release.* 2014; 187:133–144. [PubMed: 24874289]

4. Li X, Hirsh DJ, Cabral-Lilly D, Zirkel A, Gruner SM, Janoff AS, Perkins WR. Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 1998; 1415:23–40. [PubMed: 9858673]
5. Frezard F, Santaella C, Montisci MJ, Vierling P, Riess JG. Fluorinated Phosphatidylcholine-Based Liposomes - H⁺/Na⁺ Permeability, Active Doxorubicin Encapsulation and Stability in Human Serum. *Bba-Biomembranes*. 1994; 1194:61–68. [PubMed: 8075142]
6. Ishida T, Okada Y, Kobayashi T, Kiwada H. Development of pH-sensitive liposomes that efficiently retain encapsulated doxorubicin (DXR) in blood. *Int J Pharm*. 2006; 309:94–100. [PubMed: 16364578]
7. Fritze A, Hens F, Kimpfler A, Schubert R, Peschka-Süss R. Remote loading of doxorubicin into liposomes driven by a transmembrane phosphate gradient. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 2006; 1758:1633–1640. [PubMed: 16887094]
8. Brantley, S., Kubicki, J., White, A. *Kinetics of Water-Rock Interaction*. Springer; 2007.
9. Skovsgaard T. Transport and binding of daunorubicin, adriamycin, and rubidazone in Ehrlich ascites tumour cells. *Biochemical pharmacology*. 1977; 26:215–222. [PubMed: 14644]
10. Frezard F, Garnier-Suillerot A. Determination of the osmotic active drug concentration in the cytoplasm of anthracycline-resistant and-sensitive K 562 cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 1991; 1091:29–35. [PubMed: 1995065]
11. Seynhaeve AL, Dicheva BM, Hoving S, Koning GA, ten Hagen TL. Intact Doxil is taken up intracellularly and released doxorubicin sequesters in the lysosome: evaluated by in vitro/in vivo live cell imaging. *J Control Release*. 2013; 172:330–340. [PubMed: 24012486]
12. Dawidczyk CM, Russell LM, Searson PC. Nanomedicines for cancer therapy: state-of-the-art and limitations to pre-clinical studies that hinder future developments. *Frontiers in chemistry*. 2014; 2
13. Saltzman, WM. *Drug delivery: engineering principles for drug therapy*. Oxford University Press; USA: 2001.
14. Allen TM, Cleland LG. Serum-Induced Leakage of Liposome Contents. *Biochim Biophys Acta*. 1980; 597:418–426. [PubMed: 7370258]
15. Lee RJ, Wang S, Turk MJ, Low PS. The effects of pH and intraliposomal buffer strength on the rate of liposome content release and intracellular drug delivery. *Bioscience reports*. 1998; 18:69–78. [PubMed: 9743475]
16. Del Monte U. Does the cell number 109 still really fit one gram of tumor tissue? *Cell Cycle*. 2009; 8:505–506. [PubMed: 19176997]
17. Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK. Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proceedings of the National Academy of Sciences*. 1998; 95:4607–4612.
18. Barua S, Mitragotri S. Challenges associated with penetration of nanoparticles across cell and tissue barriers: a review of current status and future prospects. *Nano today*. 2014; 9:223–243. [PubMed: 25132862]
19. Primeau AJ, Rendon A, Hedley D, Lilge L, Tannock IF. The distribution of the anticancer drug Doxorubicin in relation to blood vessels in solid tumors. *Clin Cancer Res*. 2005; 11:8782–8788. [PubMed: 16361566]
20. Eom Y-W, Kim MA, Park SS, Goo MJ, Kwon HJ, Sohn S, Kim W-H, Yoon G, Choi KS. Two distinct modes of cell death induced by doxorubicin: apoptosis and cell death through mitotic catastrophe accompanied by senescence-like phenotype. *Oncogene*. 2005; 24:4765–4777. [PubMed: 15870702]
21. Silverman L, Barenholz Y. In vitro experiments showing enhanced release of doxorubicin from Doxil® in the presence of ammonia may explain drug release at tumor site, *Nanomedicine: Nanotechnology, Biology and Medicine*. 2015; 11:1841–1850.

Figure 1.

Schematic illustration of doxorubicin leakage from liposomes. The leakage of neutral doxorubicin across the lipid bilayer is described by the rate constant k_m . Doxorubicin dissolution from the solid state is described by the rate constant k_d . The release of doxorubicin from liposomes results in partial dissolution of the solid phase to maintain the saturation concentration. The pK_a for dissociation of doxorubicin is 8.3.

Figure 2.

Dissolution of solid doxorubicin in water at (A) 4 °C, (B) 25 °C, and (C) 37 °C. Dissolution of solid doxorubicin plotted as $(c_{\text{sat}} - c_{\text{lip}})/c_{\text{sat}}$ at (D) 4 °C, (E) 25 °C, and (F) 37 °C. The solid lines represent fits to the model. (G) The saturation concentration of doxorubicin in solution at these temperatures. (H) Arrhenius plot of the dissolution rate constant.

Figure 3.

Liposome leakage in buffer (pH 7.4). Representative leakage curves of doxorubicin from liposomes: (A) Doxil liposomes, (B) Doxil-like liposomes, and (C) Doxil-like liposomes with 75% cholesterol. (D-F) Mean fractional leakage of the same three liposome types, respectively. $N = 3 - 5$. Bars represent mean $\pm$ SE. Solid lines shows fits to the kinetic model described in the text.

Figure 4.

Liposome leakage for test conditions. Mean fractional leakage curves of doxorubicin from liposomes. (A) Doxil-like liposomes in 50% serum solution. (B) Doxil-like liposomes in pH 5 PBS, and (C) Doxil-like liposomes with 50% of the standard amount of DSPE-PEG2k in pH 7.4 buffer. $N = 3$. Bars represent mean $\pm$ SE. Solid lines show fits to the kinetic model.

Figure 5.
Doxorubicin leakage rate constants from liposomes.