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A fluorescence study examining how 14-valerate side chain substitution modulates anthracycline binding to small unilamellar phospholipid vesicles

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The intrinsic fluorescence properties of the anthracycline antitumor antibiotics were studied in an effort to understand how 14-valerate side chain substitution modulates drug associations with small unilamellar phospholipid vesicles (SUVs) under near physiological conditions Drug location and dynamics in fluid-phase dimyristoylphosphatidylcholine (DMPC) bilayers were evaluated for several analogs; accessibilities of bound fluorophores to membrane-impermeable iodide were evaluated in quenching experiments, while the diffusive motions of these agents were studied using lifetime-resolved anisotropy plots. The bulky and hydrophobic valerate substituent was found to further hinder the rotations experienced by a bound drug molecule, with apparent limiting anisotropy (a_∞) values showing increases of 13–82% upon valerate group substitution. In addition, the bimolecular quenching rate constants (unit, 10^9 $M^{-1} \cdot s^{-1}$) for membrane-bound adriamycin (1.4), *N*-trifluoroacetyladriamycin (0 4), and their corresponding valerate-substituted analogs (k_q values of 1.1 and 0.5, respectively) reveal that the side chain is a weak modulator of fluorophore penetration into the bilayer, with stronger modulation being achieved through amino group substitution. Similar results were obtained for drugs bound to negatively-charged dimyristoylphosphatidylglycerol (DMPG) bilayers. Finally, comparison of the equilibrium binding affinities of the various congeners for electroneutral DMPC versus negatively-charged DMPG bilayers demonstrate that positively-charged parent anthracyclines display high levels of selective binding to negatively-charged phospholipids, unlike valerate-substituted analogs which display no such selectivity The modulation of anthracycline–membrane interactions achieved through valerate substitution offers potential explanations, at least in part, for some of the novel biological properties of valerate-containing anthracyclines.

Introduction

Adriamycin® and daunomycin, two anthracycline antibiotics which first gained clinical prominence in the early 1970's as effective antitumor agents, remain today the medications of choice against a wide spectrum of human cancers [1] Because of their preeminence in the cancer chemotherapy field, extensive studies have been carried out to identify the mechanism or mechanisms of biological activity of anthracyclines. Adriamycin is known to bind tightly to DNA and alter several DNA-related functions such as DNA replication and RNA synthesis [1,2] Through experiments utilizing drug molecules immobilized to nonpenetrating polymeric beads, adriamycin has been found to exert cytotoxic effects without entering cells [3,4] Cellular macromolecules such as DNA and membranes can also be damaged by anthracycline-induced oxygen radicals [5–8] Finally, adriamycin is now known to have adverse effects on the strand-passing activity of mammalian DNA topoisomerase II [9]

Even if native anthracyclines are incapable of eliciting their antitumor effect at the cell surface, characteri-

Abbreviations DMPC, dimyristoylphosphatidylcholine, DPPC dipalmitoylphosphatidylcholine; DMPG dimyristoylphosphatidylglycerol SUV, small unilamellar vesicle, AD32, *N*-trifluoroacetyladriamycin-14-valerate, AD48, adriamycin-14-valerate AD198 *N*-benzyladriamycin-14-valerate, AD199, *N,N* dimethyladriamycin-14-valerate PBS phosphate buffered saline.

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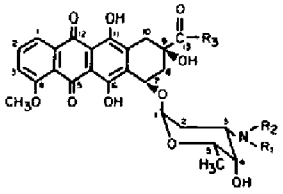
TABLE I

Structures of the anthracycline congeners



Name	Code	R_1	R_2	R_3
Adriamycin		H	H	CH_2OH
Daunomycin		H	H	CH_3
Adriamycin-14-valerate	AD48	H	H	$CH_2OCO(CH_2)_3CH_3$
N,N-Dimethyladriamycin		CH_3	CH_3	CH_2OH
N,N-Dimethyldaunomycin		CH_3	CH_3	CH_3
N,N-Dimethyladriamycin-14-valerate	AD199	CH_3	CH_3	$CH_2OCO(CH_2)_3CH_3$
N-Trifluoroacetyladriamycin		H	$COCF_3$	CH_2OH
N-Trifluoroacetyldaunomycin		H	$COCF_3$	CH_3
N-Trifluoroacetyladriamycin-14-valerate	AD32	H	$COCF_3$	$CH_2OCO(CH_2)_3CH_3$
N-Benzyladriamycin		H	$CH_2C_6H_5$	CH_2OH
N-Benzyladriamycin-14-valerate	AD198	H	$CH_2C_6H_5$	$CH_2OCO(CH_2)_3CH_3$

zation of anthracycline–membrane interactions is of importance This is because the drug molecule must first interact with and then successfully traverse the plasma membrane in order to gain access to intracellular sites Since plasma membrane alterations are known to accompany neoplastic transformations, differential drug–membrane interactions between host tissue and tumor may serve as a basis of cytotoxic selectivity Also, specific interactions between anthracycline molecules and negatively-charged membrane lipids have been implicated in the adverse toxicity of these drugs to cardiac tissues [10–12] Another compelling reason for pursuing a de ailed understanding of the membrane interactions of the anthracyclines is the recent series of studies suggesting drug involvement with a 170-kDa membrane glycoprotein (gp 170) in the development of multiple drug resistance by tumor cells [13–15]

The intrinsic fluorescence emission of anthracycline molecules has been studied in the past to determine how structural changes modulate binding to small unilamellar vesicles (SUVs) composed of various phospholipids [16–22] In the present study we employ fluorescence methods to examine the effect of 14-valerate side chain substitution on anthracycline associations with electroneutral DMPC and negatively-charged dimyristoylphosphatidylglycerol (DMPG) bilayers Several valerate-containing analogs of adriamycin (Table I) have been the focus of considerable attention in recent years due to their unique biological properties relative to the parent drug molecule (see Ref 23 and references therein), and our model membrane data suggest a physical basis for their biological behavior

Materials and Methods

Chemicals

Adriamycin, *N,N*-dimethyladriamycin and *N,N*-dimethyldaunomycin were the gift of Dr Leonard Kedda of the Division of Cancer Treatment, National Cancer Institute Daunomycin was obtained from Sigma Chemical Adriamycin-14-valerate, *N,N*-dimethyladriamycin-14-valerate, *N*-trifluoroacetyladriamycin, *N*-trifluoroacetyldaunomycin, *N*-trifluoroacetyladriamycin-14-valerate, *N*-benzyladriamycin and *N*-benzyladriamycin-14-valerate were prepared at the University of Tennessee College of Medicine according to previously described synthetic procedures [24–26] All anthracycline compounds were of at least 98% purity as determined by HPLC analysis L-α-Dimyristoylphosphatidylcholine and L-α-dimyristoylphosphatidic acid were obtained from Avanti Polar Lipid Co , Pelham, AL, and were used without further purification All chemicals were reagent grade and were used without further purification

Vesicle preparation

Small unilamellar vesicle suspensions were prepared prior to an experiment as previously reported [18] Briefly, lipid suspensions containing 34 mg/ml lipid in phosphate-buffered saline (PBS) containing 8 mM

Na_2HPO_4, 1 mM KH_2PO_4 and 1.0 M halide (pH 7.4) were prepared by vortex mixing. The dispersions were then sonicated using a bath-type sonicator. The DMPC vesicle preparations were annealed for 30 min at 37°C prior to use.

Fluorescence instrumentation

Fluorescence measurements were obtained by using a SLM model 4800S subnanosecond spectrofluorometer interfaced to a Compaq 286 computer. Lifetimes and anisotropy measurements were computed using SLM software. Lifetimes were measured by phase shift using exciting light modulated at 30 MHz [27]. A glycogen scattering solution was used as reference. Lifetime determinations on anthracycline samples were conducted by using an excitation wavelength of 470 nm and a band-pass of 0.5 nm and a 550 nm long-pass filter (Schott) for each emission channel to isolate fluorescence from scattered light. Since only one frequency (30 MHz) was used in our analysis, we refer to our values as apparent lifetimes. Similarly, nonzero limiting anisotropies (a_∞) and rotational diffusion rates (R) are also designated as apparent values. All experiments were conducted in 1-cm quartz cuvettes. Steady-state anisotropy and intensity measurements were obtained as described previously [18,19].

Evaluation of the relative location and dynamics of membrane-bound fluorophores

Using previously developed methodologies [19], iodide quenching experiments at constant ionic strength were used to evaluate the relative accessibilities of bound fluorophores to membrane-impermeable iodide, while steady-state fluorescence anisotropy measurements under iodide quenching conditions were used to investigate the diffusive motions of the drug molecules. Aqueous drug stock solutions were added to aliquots of liposome preparations such that the drug, lipid and salt concentrations were 5.0×10^{-6} M, 30 mg/ml, and 1.0 M, respectively. Samples were allowed to equilibrate for 30 min at 37°C prior to measurement. Quenching solutions contained 2×10^{-3} M sodium thiosulfate to prevent the oxidation of iodide. The modified Stern-Volmer plots ($\tau[I^-]$ versus $(F_0/F) - 1$) were linear for each drug-liposome system studied, the linearity consistent with the present of one class of bound fluorophore.

Evaluation of equilibrium binding constants

As previously described [18], the method of fluorescence anisotropy titration was used to determine the concentrations of free and bound drug in liposome samples containing a total drug concentration of 2×10^{-6} M and varying lipid concentrations. The overall association constants are defined as $K = [A_B]/([A_F][L])$ where $[A_B]$ is the concentration of bound drug, $[A_F]$ represents the concentration of free drug, and [L] equals the total lipid concentration in the sample. Double-reciprocal plots were linear and K values were determined from their slopes [18].

Results and Discussion

Incorporation of a valerate side chain enhances the hindered rotations experienced by a membrane bound drug molecule

The Perrin equation relates a fluorophore's steady-state fluorescence anisotropy value to its rotational rate in the following manner

$$a_0/a = 1 + 6R\tau$$

where a_0 is the limiting fluorescence anisotropy observed in the absence of depolarizing rotations, τ, is the excited-state lifetime, and R is the rotational diffusion rate of the fluorophore in radians per second. This equation is applicable for fluorophores whose depolarizing rotational motions are unhindered and isotropic in nature [28].

In the event that the depolarizing rotations of a fluorophore are isotropic, but hindered such that at times which are long compared with the fluorescence lifetime a nonzero limiting anisotropy (a_∞) is possible, the Perrin equation can be modified as follows [29]

$$a = a_\infty + (a_0 - a)/6R\tau$$

For this type of isotropic but hindered rotator, a plot of a vs $(a_0 - a)/\tau$ permits $1/6R$ and a_∞ to be obtained from the slope and a-axis intercept, respectively.

Steady-state fluorescence anisotropy measurements using oxygen quenching have been used in the past to study the hindered rotations of membrane-bound hydrocarbon probes such as DPH [30] and perylene [31]. Similar lifetime-resolved anisotropy plots generated under iodide quenching conditions have been used to investigate the diffusive motions of anthracyclines in isotropic solvent and in fluid-phase DMPC bilayers [19]. Anthracycline fluorophores free in solution were found to display apparent limiting anisotropy (a_∞) values which delayed to zero at times which were long compared to the excited-state lifetime, evidence that unbound anthracycline drug molecules undergo essentially unhindered rotations in aqueous solution. In contrast, anthracyclines bound to fluid phase DMPC bilayers have been shown to display nonzero a_∞ values [19], with a_∞, and not the mean rotational rate (R), being the main contributor to the steady-state anisotropy value.

In this report we again employ lifetime-resolved anisotropy plots (see Fig. 1) to study anthracycline dynamics in membranes, this time to ascertain the conse-

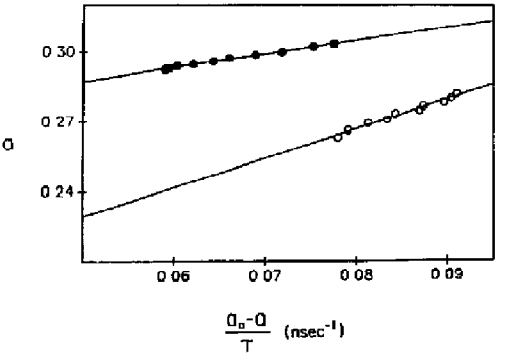


Fig 1 Lifetime-resolved anisotropy plots for *N*-trifluoroacetyladriamycin (○) and its corresponding 14-valerate substituted analog AD32 (●) bound to DMPC bilayers at 37°C Iodide quenching at constant ionic strength was used to vary the excited state lifetime (τ) of the fluorophore as previously described [19] The terms a and a_0 refer to the steady-state and limiting fluorescence anisotropies respectively Both plots were found to have nonzero y-intercepts (indicative of hindered rotations) the diminished slope of the AD32 data relative to that for the parent molecule suggest the valerate moiety further restricts rotational motions (see Results and Discussion)

quences of valerate side chain incorporation Table II compares the fluorescence lifetimes, iodide quenching rate constants, steady-state anisotropies and rotational rates for parent anthracyclines and their corresponding valerate-substituted analogs free in solution and bound to SUVs composed of DMPC A more complete discussion of the dependence on anthracycline drug structure of relative membrane location and dynamics may be found elsewhere [19]

Comparison of the rotational rates for anthracyclines free in PBS shows that substitution of the valerate moiety slows the Perrin rotational rate of a drug molecule by 10–15% Table II also shows that drug association with DMPC bilayers typically results in a 2–3-fold reduction in rotational rates at 37°C All anthracyclines listed exhibit nonzero a_∞ values when bound to DMPC bilayers, consistent with our previous finding that membrane-bound anthracyclines are hindered rotators [19] For each parent anthracycline, incorporation of a bulky valerate side chain increases the a_∞ value, these results indicate that the presence of the valerate side chain further hinders the rotations experienced by bound drug molecules For each of the membrane-bound anthra-

TABLE II

Summary of apparent lifetimes iodide quenching rate constants steady state anisotropies, apparent limiting anisotropies and apparent rotational rates for free and membrane bound anthracyclines

All fluorescence experiments were conducted at 37°C in phosphate-buffered saline (PBS) (pH 7 4) at constant ionic strength (1 0 M halide) and small unilamellar DMPC vesicles were used Experiments on drugs free in solution were conducted at concentrations of 1 10^{-6} M while studies of membrane-bound fluorophores were conducted at fluorophore concentrations of 5 0 10^{-6} M and lipid concentrations of 30 mg/ml, thereby assuring essentially complete binding of all drugs Lifetime values (τ) were determined at a chloride concentration of 1.0 M and have uncertainties of ±0 04 ns Steady-state anisotropy (a) values for bound drugs have not been corrected for background scatter, which did not exceed 2% of the total signal The k_q values for membrane-bound drugs were obtained from the slopes of modified Stern-Volmer plots as described previously [19] Rotational rates (R) and limiting anisotropies (a_∞) for membrane-bound compounds were obtained from lifetime-resolved anisotropy plots, the a values being subject to 3% uncertainty a_0 values of 0 39 were previously measured for various anthracyclines [18]

Compound	Code	Octanol/PBS partition coefficient [a]	Free in PBS			Bound to DMPC bilayers				
			τ (ns)	a	R (10^8 s^{-1})	τ (ns)	k_q (10^9 M^{-1} s^{-1})	a	R (10^8 s^{-1})	a_∞
Adriamycin [b]		1 2	1 04	0 057	9 5	1 52	1 4	0 260	3 5	0 236
Daunomycin [b]		9 0	1 06	0 052	10 2	1 55	1 1	0 271	3 6	0 245
Adriamycin-14-valerate	AD48	35	1 05	0 063	8 2	1 55	1 1	0 294	3 7	0 267
N,N-Dimethyladriamycin		1 6	1 06	0 057	9 2	1 53	1 8	0 248	2 4	0 137
N N-Dimethyldaunomycin [b]		16	1 07	0 053	10 0	1 53	1 3	0 256	3 5	0 221
N,N-Dimethyladriamycin-14-valerate	AD199	67	1 07	0 064	8 0	1 63	1 1	0 276	3 6	0 250
N-Trifluoroacetyl-adriamycin		> 99	1 05	0 059	9 0	1 63	0 4	0 263	1 3	0 166
N-Trifluoroacetyl-daunomycin		> 99	1 06	0 056	9 4	1 59	0 4	0 280	1 5	0 202
N-Trifluoroacetyl-adriamycin-14-valerate	AD32	> 99	1 00	0 068	7 9	1 66	0 5	0 292	2 8	0 258
N-Benzyladriamycin		> 99	1 08	0 059	8 7	1 60	0 8	0 266	5 2	0 245
N-Benzyladriamycin-14-valerate	AD198	> 99	1 07	0 066	7 6	1 47	0 7	0 293	3 7	0 264

[a] Several of the octanol/PBS coefficients were reported previously [22]
[b] Fluorescence data for these drugs at 28°C have been reported previously [19]

cyclines studied, the limiting anisotropy was found to be the main contributor to the steady-state anisotropy

Valerate side chain is only a weak modulator of anthracycline penetration into the bilayer

Iodide quenching data at constant ionic strength were obtained as part of this study in order to evaluate the consequences of valerate side chain incorporation on the penetration of the anthracycline fluorophore into electroneutral DMPC and negatively-charged DMPG model membranes Iodide has an immeasurably small penetration into the bilayer [32,33] and is therefore able to discriminate between molecules bound to the hydrophilic surface from those in the hydrophobic interior of a bilayer The Stern-Volmer equation is the classical relationship used to describe the collisional quenching process

$$F_0/F = \tau_0/\tau = 1 + K_{sv}[Q]$$

where F_0, F and τ_0, τ are the fluorescence intensities and lifetimes in the absence and presence of Q, respectively. [Q] represents the quencher concentration, and K_{sv} is the collisional or dynamic quenching constant which equals $k_q\tau_0$, where k_q equals the experimentally observed rate constant for the collisional quenching process

Iodide quenching studies [19] have been used in the past in our laboratory to examine the relative location of several chromophore-modified anthracyclines in fluid-phase DMPC bilayers at 28°C For a set of anthracyclines where the aglycone portion of the drug molecule was varied systematically (positions 1 through 12 as depicted in Table I) while the aminosugar portion was held constant, k_q values ranging from 0 6 10^9 to $1.2 \cdot 10^9$ M^{-1} s^{-1} were obtained. Since these k_q values are much higher than the negligible values observed for DPH and anthracene embedded in lipid bilayers [33,34], more interfacial binding sites are indicated relative to those for hydrocarbon probes For these chromophore-modified anthracyclines, the more hydrophobic drugs were found to be buried the deepest in the DMPC bilayer

Table I summarizes the structures of several anthracycline congeners which are the subjects of this study This time the aglycone portion of the parent adriamycin or daunomycin has been held constant, and either one or both of the following modifications have been made (1) incorporation of a valerate side chain at position 14 and (2) substitution of the aminosugar Table II shows that both of these structural changes have dramatic effects on the aqueous solubility of an anthracycline molecule For example, incorporation of a valerate side chain into adriamycin or *N,N*-dimethyladriamycin increases the octanol/PBS partition coefficient from values of 1 2 to 35 and 1 6 to 67, respectively

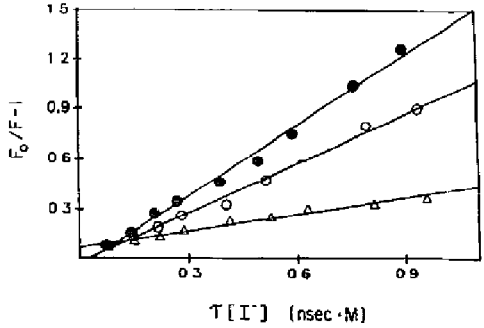


Fig. 2 Modified Stern-Volmer plots for the iodide quenching under constant ionic strength of the following drugs bound to DMPC bilayers at 37°C adriamycin (●) adriamycin-14-valerate (○), and *N* trifluoroacetyladriamycin (△) Lipid and drug concentrations of 4 42 10^{-2} M and 5 10^{-6} M respectively assured essentially complete binding for each analog [19] F_0 equals the initial fluorescence intensity F equals the fluorescence intensity in the presence of a given iodide concentration ($[I^-]$) and τ equals the initial excited state lifetime Collisional rate constant (k_q) values were obtained from the slopes of these plots, and these values are summarized in Table II Note that both valerate- and *N*-trifluoroacetyl- substituents decrease the accessibility of the membrane-bound fluorphore to iodide

The octanol/PBS partition coefficient data found in Table II also display the strong effects which amino group substitution can have on the aqueous solubility of an anthracycline Adriamycin with a pK_a of approximately 8 2 [35], is a very soluble drug molecule as evidenced by its octanol/PBS partition coefficient of 1 2. Likewise, *N,N*-dimethyladriamycin with its partially ionized amino group at pH 7 4 [36], is also quite water soluble with a partition coefficient of 1 6 In contrast to the hydrophilic adriamycin and *N,N*-dimethyladriamycin congeners, our data show that both *N*-trifluoroacetyladriamycin and *N*-benzyladriamycin, with octanol/PBS partition coefficients in excess of 99, are very hydrophobic drug molecules The hydrophobic nature of *N*-trifluoroacetyladriamycin is due to the reduced state of ionization of its amino group, caused by the strong electron withdrawing inductive effects of the *N*-trifluoroacetyl group The decreased water solubility of *N*-benzyladriamycin relative to adriamycin is thought to be due, not to a decreased pK_a value, but rather to the hydrophobic character of the benzyl moiety

Modified Stern-Volmer plots, as depicted in Fig. 2, were used to determine the accessibilities of bound fluorophores to membrane-impermeable iodide The k_q values listed in Table II allows us to evaluate how 14-valerate side chain substitution and aminosugar substitution influence drug penetration into DMPC bilayers The presence of the hydrophobic valerate side chain, in general, promotes deeper bilayer penetration of the drug molecules For example, adriamycin, *N,N*-

dimethyladriamycin, and *N*-benzyladriamycin exhibit k_q values (10^9 M^{-1} s^{-1}) of 1 4, 1 8, and 0 8, respectively, while the corresponding valerate-containing analogs showed values of 1 1, 1 1, and 0 7, respectively However, it is clear from k_q values which appear in Table II that valerate substitution is not nearly as strong a modulator of drug location in the bilayer as is amino group substitution Whereas the k_q value for membrane-bound adriamycin is only 27% greater than that for adriamycin-14-valerate, adriamycin exhibits a k_q value 250% greater than that for its deeply-buried, amine-substituted analog *N*-trifluoroacetyladriamycin Replacement of the *N*-trifluoroacetyl moiety of this drug with *N*,*N*-dimethyl or *N*-benzyl moieties results in increased k_q values of 350% and 100%, respectively

We have also employed iodide quenching studies here to examine the relative location of drugs bound to negatively-charged DMPG bilayers in PBS at 37°C The k_q values (10^{-9} M^{-1} s^{-1}) for anthracyclines bound to DMPG bilayers are as follows adriamycin (0 22) adriamycin-14-valerate (0 12), and *N*-trifluoroacetyladriamycin (0 09) Because iodide is a negatively-charged quencher, it is of interest to compare quenching constants for anthracyclines bound to negatively-charged DMPG bilayers with those for drugs bound to electroneutral DMPC bilayers The quenching rate constants (10^9 M^{-1} s^{-1}) determined in DMPC bilayers are as follows adriamycin (1 4), adriamycin-14-valerate (1 1), *N*-trifluoroacetyladriamycin-14-valerate (0 4) For each anthracycline studied, our data indicate that anthracyclines are significantly more accessible (from 4- to 9-fold) to membrane-impermeable iodide when bound to electroneutral DMPC bilayers than when bound to negatively-charged DMPG bilayers These results are not unexpected since unfavorable charge-charge interactions between negatively-charged iodide and the negatively-charged DMPG headgroup should reduce the quencher's accessibilities to the binding sites of the drug molecules As was observed for anthracyclines bound to DMPC bilayers, accessibilities of iodide to anthracycline fluorophores bound to DMPG membranes decreased in the following order adriamycin > adriamycin-14-valerate > *N*-trifluoroacetyladriamycin-14-valerate

In summary, this report documents the following concerning anthracycline drug structure: (1) valerate substitution in general promotes slightly deeper membrane penetration of the fluorophore, and (2) amino group substitution strongly modulates the depth of fluorophore penetration, with drug molecules with nonbasic moieties such as *N*-trifluoroacetyl groups buried the deepest.

TABLE III

A summary of the equilibrium binding affinities of several anthracycline congeners for electroneutral DMPC and negatively charged DMPG bilayers

As described in Materials and Methods, experiments were conducted in PBS under near physiological conditions of pH, temperature and ionic strength The method of fluorescence anisotropy titration [18] was used to determine the concentrations of free and bound drug Overall association constants were determined from the inverse of the slope of a double-reciprocal plot under conditions where the concentration of free lipid was in great excess over the concentration of bound drug Using binding data for a minimum of six samples of varying lipid concentration a linear-least-squares analysis was used to determine the binding constant from the slope of the plot *K* values are subject to uncertainties of 10%

Analog	K_{DMPC} (M^{-1})	K_{DMPG} (M^{-1})	Selectivity ratio (K_{DMPG}/K_{DMPC})
Adriamycin [a]	200	3400	17
Daunomycin	1400	9000	6 4
Adriamycin-14-valerate	12000	8000	0 7
N N-Dimethyladriamycin [a]	90	1600	18
N N-Dimethyldaunomycin	450	5000	11
N N-Dimethyldaunomycin-14-valerate [a]	8000	9000	1 1
N Trifluoroacetyl-adriamycin [a]	1600	600	0 4
N-Trifluoroacetyl-daunomycin	6000	3000	0 5
N-Trifluoroacetyladriamycin-14-valerate [a]	38000	25000	0 7
N-Benzyladriamycin [a]	1500	10000	6 7
N-Benzyladriamycin-14-valerate [a]	36000	45000	1 2

[a] Binding affinities for these analogs have been reported elsewhere [22]

Incorporation of a valerate side chain strongly modulates the type and extent of phospholipid binding by anthracyclines

Membrane alterations are known to be important morphological features of adriamycin cardiotoxicity, and the formation of stable complexes between positively-charged drug molecules and negatively-charged phospholipids, such as cardiolipin, are thought to play a key role in the disturbance of cardiac mitochondrial function [10–12] Thus it is of interest to understand how structural changes in the anthracycline molecule modulate binding selectivity for various types of headgroups In this report we have used the previously developed method of fluorescence anisotropy titration to study the effect of 14-valerate substitution on anthracycline affinities for negatively-charged DMPG and electroneutral DMPC vesicles Both DMPC and DMPG bilayers are fluid-phase at 37°C Comparison of the DMPC and DMPG binding data presented in Table II allows us to evaluate the manner in which the selectivities of adriamycin and daunomycin for negatively-charged phospholipids are modulated by 14-valerate side chain incorporation as well as amino group substitution

Table III summarizes the equilibrium binding affinities of several anthracycline congeners for the two types

of bilayers Of the various amino group modifications studied (parent, *N*-trifluoroacetyl, *N*-benzyl, *N,N*-dimethyl), the *N*-trifluoroacetyl moiety was found to promote DMPG binding to the least extent *N*-Trifluoroacetyl derivatives, which are uncharged at pH 7 4 are not expected to be attracted electrostatically to negatively-charged phospholipids, and this notion is corroborated by the very low K_{DMPG}/K_{DMPC} selectivity ratios exhibited by *N*-trifluoroacetyladriamycin and *N*-trifluoroacetyldaunomycin In contrast, positively-charged anthracyclines such as adriamycin, daunomycin, *N,N*-dimethyladriamycin, *N,N*-dimethyldaunomycin, and *N*-benzyladriamycin all display strong binding preferences for the negatively-charged DMPG bilayers (K_{DMPG}/K_{DMPC} values of 17, 6 4, 18, 11 and 6 7, respectively)

Incorporation of the hydrophobic valerate side chain, which decreases the water solubility of an anthracycline molecule considerably (see Table II), is found not unexpectedly in Table III to promote drug binding to both negatively-charged as well as electroneutral membranes The membrane affinities of AD32 and AD198 are impressively high For example, in previous work we determined the consequences of removing the polar aminosugar portion of the anthracycline molecule on membrane binding, the resultant 7-deoxydaunomycinone molecule displayed K values of approx 10000 M^{-1} [18], values significantly less than those for AD32 and AD198 The K_{DMPG} and K_{DMPC} values of 45000 M^{-1} and 36000 M^{-1} for AD198 make this agent the most avid binder of membranes in the anthracycline family to yet be identified [18,20]

Another striking feature in our data is the finding that valerate side chain substitution can completely eliminate the high levels of preferential binding which positively-charged parent anthracyclines (e g , adriamycin, daunomycin, *N,N*-dimethyladriamycin, *N,N*-dimethyldaunomycin, *N*-benzyladriamycin) exhibit for negatively-charged DMPG bilayers over DMPC bilayers This trend is exemplified by the 24-fold reduction in K_{DMPG}/K_{DMPC} selectivity ratio which occurs for the adriamycin molecule upon incorporation of the valerate side chain It thus appears that 14-*O*-acyl substitution may prove to be a useful synthetic modification in preventing the selective accumulation of positively-charged anthracyclines in tissues or membrane domains rich in negatively-charged lipid

Conclusion

In this report we have used small unilamellar phospholipid vesicles to serve as models in order to determine what special membrane properties are imparted to an anthracycline drug molecule through valerate side chain incorporation Concerning this topic, our studies have successfully identified three consequences of valerate side chain incorporation (1) further hindrance of the rotations experienced by a bound drug molecule, (2) enhancement of the depth of membrane penetration of the fluorescent aglycone portion of an anthracycline molecule (an effect which is only slight relative to the large changes observable through amino group substitution), and (3) promotion of binding affinities for both electroneutral and negatively-charged phospholipids, with an essentially complete loss of the high levels of the preferential binding of positively-charged anthracycline congeners for negatively-charged membranes

Having achieved a reasonable level of understanding of the manner in which valerate side chain incorporation modulates drug interactions with liposomes, it is of interest to examine the possibility that some of the unique biological features of valerate-containing drugs like AD32 and AD198 may be based upon their unusual membrane properties relative to adriamycin Like its parent analog adriamycin, AD32 has been shown to display significant activity against human disease, however, in contradistinction to adriamycin, no patient treated with AD32 exhibited clinical or electrocardiographic evidence of cardiac dysfunction, with the absence of cardiac dysfunction confirmed by endomyocardial biopsies [23] Since the formation of stable drug-cardiolipin complexes is thought to promote drug localization in cardiac tissue, AD32's lack of preferential drug binding to negatively-charged phospholipids documented in this report (K_{DMPG}/K_{DMPC} value of 0 7, compared to a value of 17 for adriamycin) offers a potential explanation for the drug's lack of cardiotoxicity

AD198 is another example of a valerate-containing anthracycline which has distinguished itself in direct experimental comparisons with adriamycin Israel et al [23] have shown that exposure of human-derived CEM lymphocytes in vitro to 5 μM AD198 for 3 h results in a gross change in cellular morphology, with degeneration of the plasma membrane and cell death Exposure of cells to the same levels of either adriamycin or *N*-trifluoroacetyladriamycin, even for a longer time, does not produce a similar effect The authors speculate that the compound's high membrane-active properties may offer a potential explanation for the lack of cross-resistance seen with AD198 against various adriamycin-resistant and multiple drug-resistant tumor cell lines [37,38] This report documents that AD198 is far and away the most impressive binder of phospholipids in the anthracycline family yet to be identified Accordingly, we conclude that exceptionally high drug concentrations present in plasma membranes may, at least in part, account for the unique effects of AD198 on tumor cell surfaces

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