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Effects of pH on the kinetics of the interaction between anthracyclines and lipid bilayers

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Abstract In the present study we have analyzed the kinetics of the initial steps (first 10 seconds) of the interaction between the anthracycline daunomycin (DNM) and artificial lipid vesicles bearing opposite surface charge. The process can be monitored by measuring the fluorescence increase of the drug accompanying its association with the lipid bilayers. The results indicated that DNM consistently interacts to a larger extent with the liposomes having negative surface charge than with those having positive surface charge, suggesting the involvement of electrostatic components in the interaction. In contrast, DNM associates with the vesicles bearing positive surface charge 2–3 times faster (in terms of the apparent rate constants describing the process of interaction) than with those having negative surface charge, an observation probably related to the more fluid physical state of the former. Regarding the rate of access of DNM to the vesicles, rather than depending on the surface charge of the vesicles, this is critically affected by the ionization state of the drug, i. e. by the pH. Thus, the rate at which the interaction proceeds is increased nearly 15-fold when the pH of the medium increases from 7.0 to 8.3, regardless of the surface charge of the liposomes. On this basis, and taking into account the fact that the anthracyclines enter the cells by passive diffusion, possible effects of pH on the transport of these drugs through the membranes of tumor cells are discussed.

Key words Anthracyclines · Drug-lipid · Interactions

Abbreviations *DNM* Daunomycin · *PCL* Positively charged liposomes · *NCL* Negatively charged liposomes · *DPH* 1,6-diphenylhexa-1,3,5-triene

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Introduction

The anthracyclines are lipophilic antibiotics commonly employed in clinical anticancer protocols (Arcamone 1985). In addition to the intercalation of these drugs into nucleic acids inhibiting DNA and nuclear RNA synthesis (Powis 1987), it is known that the entry of the anthracyclines into tumor cells takes place by passive diffusion (Siegfried et al. 1985; Willingham et al. 1986; Ruetz and Gros 1994). Moreover, it has been suggested that the cell membrane is a target for drugs (Powis et al. 1990; Tritton and Hickman 1990) and that the anthracyclines can exert their cytotoxic activity solely through interaction with the cell membrane (Tritton 1982). These observations prompted several laboratories to carry out studies devoted to describing the nature of the drug-lipid interactions in natural membranes (Siegfried et al. 1983; Posada et al. 1989; Escriba et al. 1990). Such studies are greatly facilitated by the fact that there is a significant change in the quantum yield of the intrinsic fluorescence of these drugs (Burke and Tritton 1985) accompanying their incorporation into the hydrophobic membrane domains. This property has been used to monitor, under equilibrium conditions, the binding of the anthracyclines to membrane vesicles (Escriba et al. 1990; Burke and Tritton 1985) and more recently, to describe by rapid kinetics (stopped-flow) the early steps of the interaction between DNM and tumor cells (Soto et al. 1992).

On the other hand, the use of artificial lipid vesicles has contributed significantly to the present knowledge of the drug-membrane interactions (Henry et al. 1985; Goormaghtigh et al. 1986; Dupou-Cezanne et al. 1989; Bañuelos et al. 1993; Wadkins and Houghton 1993). Artificial vesicles are good models which are simpler than the more complex biological cell membranes. They permit a detailed analysis of the contribution of the lipid component and that of the dynamics of the membrane bilayer to the interaction with the drugs.

In an attempt to gain new insights into the interaction of anthracyclines with the membranes of tumor cells, we

have exploited the ionizable nature of these drugs to explore the significance of the ionization state of DNM on the kinetics of the initial steps of its incorporation into artificial lipid vesicles bearing different surface charge. The rationale for this approach is based on the presence of a pharmacologically relevant, ionizable amino group (pK_a ~8.25 (Demant et al. 1990)) at the sugar moiety of the anthracyclines, which makes the biological activity of these molecules susceptible to changes in the equilibrium between protonated and deprotonated forms of the drug. Thus, in cancer cells, the protonated species seemingly bind cellular constituents with greater affinity than the deprotonated forms while the latter appear to be more permeant through the membranes (Siegfried et al. 1985; Simon et al. 1994).

Materials and methods

DNM hydrochloride was purchased from Sigma-Aldrich Quimica S. A., Madrid, Spain. The concentration of the drug in dilute solutions (below 10 μM) was estimated at 480 nm using a molar extinction coefficient of 11 500 M^{-1} cm^{-1} (Barcelo et al. 1986).

The preparation of liposomes at different pHs has been described previously (Ferrer-Montiel et al. 1992). Briefly, the lipid composition of the liposomes (Avanti Polar Lipids, Inc., Alabama, USA) was: egg phosphatidylcholine (63 μmol), cholesterol (9 μmol) and stearylamine (18 μmol) for PCL, or egg phosphatidylcholine (63 μmol), cholesterol (9 μmol) and dicetyl phosphate (18 μmol) for NCL, respectively. The experiments at different pHs were conducted in buffers containing 100 mM NaCl in addition to either 10 mM HEPES (pH 7.0 and pH 7.6) or 10 mM EPPS (pH 8.3).

Steady-state fluorescence anisotropy measurements were done as previously described (Canaves et al. 1991), using DPH as the fluorescent probe in a SLM-8000 C spectrofluorometer set in the "T format".

The kinetic recordings were obtained in a stopped-flow spectrophotometer (HiTech model SF-51) set up under identical conditions to those described by Soto et al. (1992). The spectroscopic traces were computer fitted by a nonlinear regression method using the algorithm of Marquardt to equations containing one (Eq. (1)) or the sum of two exponential terms (Eq. (2)):

$$F(t) = A_1[\exp(-k_1 t)] + C \qquad \text{(Eq. 1)}$$

$$F(t) = A_1[\exp(-k_1 t)] + A_2[\exp(-k_2 t)] + C \qquad \text{(Eq. 2)}$$

where F(t) is the fluorescence at time t; A_1, A_2, and k_1, k_2, represent the amplitudes and the apparent rate constants, respectively corresponding to each of the exponential terms included in Eqs. (1) and (2); C is the fluorescence intensity at equilibrium.

Results and discussion

The change in the fluorescence quantum yield of the anthracycline DNM accompanying its incorporation into hydrophobic membrane domains (Burke and Tritton 1985; Soto et al. 1992), has been exploited here to describe, by rapid kinetics, how the ionization state of DNM and the membrane surface charge influence the initial steps in the interaction of the drug with the membranes. The studies have been performed using either positively-charged (PCL) or negatively-charged (NCL) liposomes within a reasonably narrow pH range (from 7 to 8.3), in which the net surface charge of each type of liposome remains fairly unaltered, while greatly shifting the equilibrium between protonated and deprotonated forms of the drug (pK_a~8.25, (Demant et al. 1990)). Control experiments demonstrated that the fluorescent properties of free DNM in solution did not change within the range of pH used. Moreover, the fluorescence emission of DNM (as a measure of the fluorescence quantum yield) undergoes an identical change upon incorporation at equilibrium into either PCL or NCL (data not shown).

Representative recordings of the fluorescence increase of DNM upon interaction with NCL and PCL at the pHs 7.0, 7.6 and 8.3 are shown in Fig. 1 (panels A, B and C, respectively). The estimates of the amplitudes and the apparent rate constants as the kinetic parameters defining the process, are summarized in Table 1 and were obtained by computer fitting of the experimental traces to Eqs. (1) and (2) described under Methods.

The influence of the vesicle's surface charge on the kinetics of the process can be easily noticed by comparing the behavior exhibited by NCL and PCL samples at any of the pHs used in the experiments (comparison of traces 1 and 2 in each panel of Fig. 1). First, the amplitude of the fluorescence change corresponding to the interaction of DNM with PCL and NCL (see also Table 1) is consistently larger in the latter, regardless of pH. This agrees with previous studies made under equilibrium conditions, in which NCL demonstrated higher capacity to bind drug than PCL (Ferragut et al. 1988), suggesting a possible involvement of electrostatic components in the accommodation of DNM into the negatively-charged lipid bilayer. Second, the rate at which the fluorescence of DNM increases in the presence of PCL is faster than that observed in the presence of NCL, irrespective of the pH. This qualitative conclusion can be substantiated by comparing the k_1 and k_2 rate constant values corresponding to each type of vesicle in Table 1. In an attempt to explain the latter observation, we have determined the apparent fluidity of the hydrophobic lipid matrix in NCL and PCL, since it has been established that penetration and location of anthracyclines in the bilayers can be markedly dependent on the lipid packing (Dupou-Cezanne et al. 1989; Bañuelos et al. 1993). As illustrated in the Fig. 2, at 25° (the temperature at which the stopped-flow experiments were run), the membrane fluidity probe DPH displayed lower anisotropy values when incorporated in PCL than in NCL, suggest-

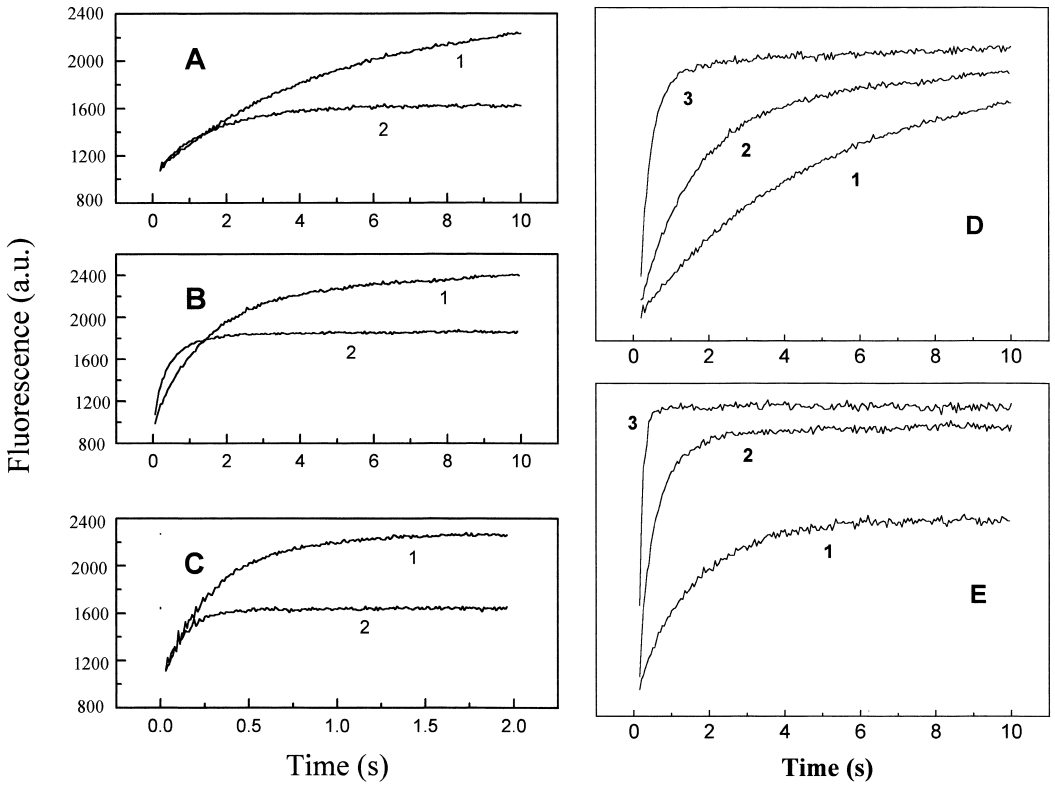

Fig. 1 A–E *Left*, Stopped-flow recordings corresponding to the fluorescence increase of DNM observed upon mixing 0.1 ml of 10 μM DNM with 0.1 ml of the liposome suspensions (250 μM in terms of lipid phosphorus) of: (*1*): Negatively charged liposomes (NCL); (*2*): Positively charged liposomes (PCL). Units on the Y axis are expressed as arbitrary fluorescence units (a. u.). **A**: Experiments made at pH 7.0; **B**: pH 7.6 and **C**: pH 8.3. *Note* the different time scale in panel C as compared to panels A or B. Temperature was 25°. *Right*, scaled traces for a better comparison of the effect of pH on the interaction of DNM with NCL (*Panel* **D**) and PCL (*Panel* **E**). Recordings at pH 7.0 (*1*), 7.6 (*2*) and 8.3 (*3*), respectively

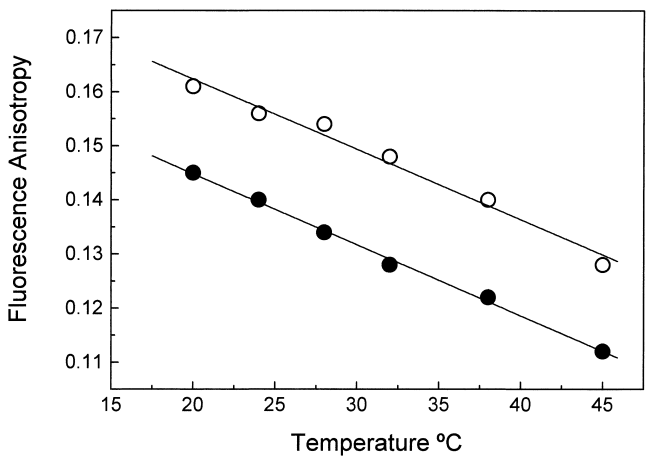

Fig. 2 Representative temperature-dependent steady-state fluorescence anisotropy of DPH incorporated into NCL (O) or PCL (●) at pH 7.6. The lipid concentration of the vesicles was 100 μM in terms of lipid phosphorus and the molar ratio of DPH to the lipid was 1 : 500. Detailed experimental conditions are given in Canaves et al. (1991)

ing a more rigid physical state of the lipid bilayer in the latter, which presumably causes a decrease in the rate of incorporation of DNM to NCL with respect to that in PCL.

In spite of the above observations, the rate of interaction between DNM and the liposomes appears mainly governed by the state of protonation of the drug rather than by the surface charge of the vesicles. This can be concluded by comparing the time courses of the interaction (fluorescence increase) shown in panels A, B and C of Fig. 1, corresponding to the assays performed at pH 7.0, 7.6 and 8.3, respectively. The profound effect of pH on the rate of interaction appears most clearly illustrated at pH 8.3 (Fig. 1 C), in which the time scale required to describe the interaction of DNM with either NCL or PCL has been reduced to only 2 s, versus the 10 s needed for completion of the same process at the lower pHs 7.0 or 7.6. In order to better visualize the effect of pH on the kinetics of the process, scaled traces corresponding to the interaction of DNM with NCL and PCL at the three pHs are shown in the Fig. 1, panels D and E, respectively. The comparison of the scaled traces reinforces the notion that, regardless of the surface charge, the interaction of the drug with the liposomes becomes much faster as the pH increases. The progressive shift in the equilibrium from protonated to deprotonated forms of DNM on increasing the pH, which parallels the concomitant increase in the rate of the interaction of DNM with the liposomes, strongly suggests that the deprotonated DNM (based on a pK_a of ~8.25, the deprotonated species accounts for a calculated 5.4, 18 and 53% of the total DNM, at pH values of 7.0, 7.6 and 8.3, respectively), is the form

Table 1 Kinetic parameters corresponding to the initial interaction of DNM with negatively (NCL) or positively (PCL) charged liposomes obtained by fitting the traces obtained at different pHs to Eqs. (1) or (2) (see Methods)

	pH	A_1	k_1	A_2	k_2	C	χ^2
NCL	7.0	1353±5	0.210±0.002	–	–	802±5	81
		1019±1764	0.25 ±0.15	480±895	0.078±0.42	662±882	80
	7.6	1336±7	0.57 ±0.006	–	–	830±3	389
		955±75	0.877±0.046	512±66	0.243±0.036	761±13	115
	8.3	1220±6	3.18 ±0.003	–	–	939±2	252
		1116±127	3.65 ±0.28	162±91	0.96 ±1.22	905±44	219
PCL	7.0	502±14	0.532±0.03	–	–	1568±5	1534
		464±74719	0.54 ±7.7	38±74721	0.54 ±77	1571±19	1552
	7.6	783±9	1.97 ±0.03	–	–	1344±1	158
		606±29	3.41 ±0.18	266±32	0.97 ±0.07	1339±1	61
	8.3	690±10	8.54 ±0.16	–	–	1560±1	139
		694±16	8.54 ±0.16	–2607±112871	196±1454	1560±1	140

Values expressed as the mean ±S.E. of triplicate determinations, are given as fluorescence arbitrary units (A_1, A_2, C) and s^{-1} (k_1, k_2), respectively

of the drug which is largely responsible for determining the kinetics.

The change in pH also affects the number of components needed to fit the experimental kinetic traces. Thus, at pH 7.0 (Fig. 1 A), the process was satisfactorily described by a single exponential term (Eq. (1)) since fitting of the traces to the sum of two exponentials (Eq. (2)) did not improve the statistics of the fit, yielding S. E. values greater than the mean in most of the calculated kinetic parameters (see Table 1). At pH 7.6 and using either NCLs or PCLs, the traces (Fig. 1 B) had a much better fit to Eq. (2), indicating the need for an additional exponential component to adequately describe the interaction process. Finally when the assays were done at pH 8.3, again only one exponential component was required but, in contrast to the situation at pH 7.0, it is now defined by a large apparent rate constant value. In fact, fitting of data obtained at pH 8.3 to Eq. (2) yields large S. E. values for those parameters associated with a putative second component (A_2 or k_2) as in the case of NCL, or even nonsensical negative values as in the case of A_2 in the PCL (Table 1).

Based on the idea that the equilibrium between neutral and protonated forms of DNM is greatly shifted in the range of pH employed and on the idea that the deprotonated species interact more rapidly with the liposomes, we propose the following interpretation for the observed pH dependence of the DNM-liposome interaction. First, we assume that the calculated fast kinetic component accounts for the interaction of the deprotonated form of DNM with the vesicles. Likewise, the slow kinetic component is assigned to the interaction of the protonated form of the drug. At pH 7.0 the relative content of the rapidly interacting, deprotonated form of DNM (5.4%) would be too small to contribute significantly to the overall process, which would then be defined by the contribution of the slowly interacting, protonated drug form. Indeed, as indicated in Table 1, the drug-liposome interaction at this pH can be described by only one kinetic component characterized by a relatively small apparent rate constant ($k_1 = 0.21\ s^{-1}$ and $0.53\ s^{-1}$ for NCL or PCL, respectively).

At pH 7.6, the coexistence of significant amounts of deprotonated (18%) and protonated drug forms, would be expected to increase the complexity in the kinetics of interaction with the liposomes and, indeed, two exponential terms are needed to describe the process. Interestingly, the slow kinetic component retains apparent rate constant values fairly close to those previously determined at pH 7.0 ($k_2 = 0.24\ s^{-1}$ and $0.97\ s^{-1}$ for NCL or PCL, respectively), which is consistent with the idea that this component reflects the contribution of the still predominant protonated form of DNM at this pH. As stated above, we assume that the fast kinetic component detected as this pH results from the interaction of the liposomes with the deprotonated form of DNM. Moreover, the latter drug form accounts for more than 50% of the total DNM at pH 8.3 and under these conditions, the rapid interaction of these species with the vesicles could mask the contribution of the slowly interacting, protonated form of DNM, thus making it possible to define the overall process by only one kinetic component.

The interaction between DNM and lipid vesicles described here by rapid kinetic methods resembles the "adsorption component" defined by Skovsgaard (1978) as an early step involved in the uptake of anthracyclines by the cells, which takes place during the first five seconds of exposure to the drugs. The observed pH-dependent increase in the rate of incorporation of DNM into the liposomes would be expected to increase the local concentration of drug in the lipid bilayers, which could, in turn, facilitate the entrance of DNM into the vesicles, since the anthracyclines are thought to enter the cells by a passive diffusion mechanism (Siegfried et al. 1985; Willingham et al. 1986; Ruetz and Gros 1994).

In this regard, our results consistently support the view that while electrostatic components seem to influence the extent to which the anthracyclines interact with the lipid bilayers (Ferragut et al. 1988; Gallois et al. 1996), it is the drug charge which mainly dictates the kinetics of the interaction. From this, it is expected that deprotonated species of the anthracyclines, though not exclusively, represent the more diffusable forms of the drugs (Skovsgaard and Nissen 1986). Transport and intracellular retention of anthracyclines by cancer cells has been proposed to be dependent on microenvironmental pH in studies with P388 murine leukemia (Alabaster et al. 1989; Soto et al. 1993),

human myeloid (Vasanthakumar and Ahmed 1986) and human non-small cell lung carcinoma cells (Mülder et al. 1992).

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References

Alabaster O, Woods T, Ortiz-Sanchez V, Jahangeer S (1989) Influence of microenvironmental pH on adriamycin release. Cancer Res 49:5638–5643

Arcamone F (1985) Properties of antitumor anthracyclines and new developments in their application; Cain Memorial Award Lecture. Cancer Res 45:5995–5999

Bañuelos S, Arrondo JLR, Canaves JM, Ferragut JA, Muga A (1993) The interaction of daunomycin with model membranes. Effect of the lipid physical state and the lipid composition. Eur J Biochem 213:1269–1275

Barcelo F, Barcelo I, Gavilanes F, Ferragut JA, Yanovich S, Gonzalez-Ros JM (1986) Interaction of anthracyclines with nucleotides and related compounds studied by spectroscopy. Biochim Biophys Acta 884:172–181

Burke TG, Tritton TR (1985) Structural basis of anthracycline selectivity for unilamellar phosphatidylcholine vesicles: an equilibrium binding study. Biochemistry 24:1768–1776

Canaves JM, Ferragut JA, Gonzalez-Ros JM (1991) Verapamil prevents the effects of daunorubicin on the thermotropic phase transition of model lipid bilayers. Biochem J 279:413–418

Demant EJF, Sehested M, Jensen PB (1990) A model for computer simulation of P-glycoprotein and transmembrane ΔpH-mediated anthracycline transport in multidrug-resistant tumor cell. Biochim Biophys Acta 1055:117–125

Dupou-Cezanne, Sautereau AM, Tocanne JF (1989) Location of adriamycin in model and natural membranes. Influence of lipid molecular packing. Eur J Biochem 181:695–702

Escriba PV, Ferrer-Montiel AV, Ferragut JA, Gonzalez-Ros JM (1990) Role of membrane lipids in the interaction of daunomycin with plasma membranes from tumor cells: implications in drug resistance phenomena. Biochemistry 29:7275–7282

Ferragut JA, Gonzalez-Ros JM, Ferrer-Montiel AV, Escriba PV (1988) The surface charge of membranes modulates the interaction with the anthracycline daunomycin. Ann NY Acad Sci 551:443–445

Ferrer-Montiel AV, Gonzalez-Ros JM, Ferragut JA (1992) Different distribution of daunomycin in plasma membrane from drug-sensitive and drug-resistant P388 leukemia cells. Biochim Biophys Acta 1104:111–116

Gallois L, Fiallo M, Laigle A, Priebe W, Garnier-Suillerot A (1996) The overall partitioning of anthracyclines into phosphatidyl-containing model membranes depends neither on the drug charge nor the presence of anionic phospholipids. Eur J Biochem 241: 879–887

Goormaghtigh E, Chatelain P, Caspers J, Ruysschaert JM (1986) Evidence of a specific complex between adriamycin and negatively charged phospholipids. Biochim Biophys Acta 861:83–94

Henry N, Fantine EO, Bolard J, Garnier-Suillerot A (1985) Interaction of adriamycin with negatively charged membranes: evidence of two types of binding sites. Biochemistry 24:7085–7092

Mülder HS, Grondelle RV, Westerhoff HV, Lankelma J (1992) A plasma membrane "vacuum cleaner" for daunorubicin in non-P-glycoprotein multidrug-resistant SW-1573 human non-small cell lung carcinoma cells. Eur J Biochem 218:871–882

Posada J, Vichi P, Tritton TR (1989) Protein kinase C in adriamycin action and resistance in mouse sarcoma 180 cells. Cancer Res 49:6634–6639

Powis G (1987) Anthracycline metabolism and free radical formation. In: Powis G, Prough RA (eds) Metabolism and action of anti-cancer drugs. Taylor & Francis, London, pp 211–246

Powis G, Hickman J, Worman P, Tritton TR, Abita JP, Berdel WE, Gescher A, Moses HL, Nicolson GL (1990) The cell membrane and cell signals as targets in cancer chemotherapy. Cancer Res 50:2203–2211

Ruetz S, Gros P (1994) A mechanism for P-glycoprotein action in multidrug resistance: are we there yet? TIPS 15:260–263

Siegfried JA, Kennedy KA, Sartorelli AC, Tritton TR (1983) The role of membrane in the mechanism of action of the antineoplastic agent adriamycin. J Biol Chem 258:339–343

Siegfried JM, Burke TG, Tritton TR (1985) Cellular transport of anthracyclines by passive diffusion. Biochem Pharmacol 34:593–598

Simon S, Roy D, Schindler M (1994) Intracellular pH and the control of multidrug resistance. Proc Natl Acad Sci USA 91: 3497–3504

Skovsgaard T (1978) Carrier mediated transport of daunorubicin, adriamycin and rubidazone in Ehrlich ascites tumor cells. Biochem Pharmacol 27:1221–1227

Skovsgaard T, Nissen NI (1986) Membrane Transport of Anthracyclines. In: Goldman ID (ed) Membrane transport of antineoplastic agents. Pergamon Press, Oxford, pp 195-209

Soto F, Canaves JM, Gonzalez-Ros JM, Ferragut JA (1992) Rapid kinetics of the interaction between daunomycin and drug-sensitive or drug-resistant P388 leukemia cells. FEBS Lett 301: 119–123

Soto F, Planells-Cases R, Canaves JM, Ferrer-Montiel AV, Aleu J, Gamarro F, Castanys S, Gonzalez-Ros JM, Ferragut JA (1993) Possible coexistence of two independent mechanisms contributing to anthracycline resistance in leukaemia P388 cells. Eur J Cancer 29A:2144–2150

Tritton TR, Yee G (1982) The anticancer agent adriamycin can be actively cytotoxic without entering cells. Science 217:248–250

Tritton TR, Hickman J (1990) How to kill cancer cells: membranes and cell signaling as targets in cancer chemotherapy. Cancer Cells 2:95–105

Vasanthakumar G, Ahmed NK (1986) Comparative uptake, retention and cytotoxicity of daunorubicin by human myeloid cells. Biochem Pharmacol 35:1185–1190

Wadkins RM, Houghton PJ (1993) The role of drug-lipid interactions in the biological activity of modulators of multi-drug resistance. Biochim Biophys Acta 1153:225–236

Willingham MC, Cornwell MM, Cardarelli CO, Gottesman MM, Pastan I (1986) Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and sensitive KB cells: effects of verapamil and other drugs. Cancer Res 46:5941–5946