Biochimica et Biophysica Acta, 1104 (1992) 111–116

BBAMEM 75522

Different distribution of daunomycin in plasma membranes from drug-sensitive and drug-resistant P388 leukemia cells

Antonio V. Ferrer-Montiel, Jose M. Gonzalez-Ros and Jose A. Ferragut

Department of Neurochemistry and Institute of Neurosciences (School of Medicine), University of Alicante, Alicante (Spain)

(Received 8 May 1991)

Key words: Anthracycline; Cellular drug resistance; Daunomycin; Drug sensitivity; Leukemia cell P388

When the anthracycline daunomycin (DNM) is incorporated into isolated plasma membranes from P388 murine leukemia cells, the drug partitions between 'deep' and 'surface' membrane domains. Such domains have been characterized on the basis of: (1) fluorescence resonance energy transfer between 1,6-diphenylhexa-1,3,5-triene or 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene as energy donors, which are well known in their positioning within the membrane, and daunomycin as the energy acceptor, and (2) quenching of the fluorescence of the membrane-associated drug by the water-soluble quencher iodide. The distribution of DNM between the two plasma membrane domains is different depending on the cellular phenotype. Thus, in membranes from drug-sensitive cells, DNM is preferentially confined to 'surface' domains, while in membranes from drug-resistant cells, the drug distributes more homogeneously between 'surface' and 'deep' domains. Experiments using artificial lipid vesicles suggest that differences in the relative levels of certain lipids in the plasma membranes from drug-sensitive and drug-resistant cells, namely phosphatidylserine and cholesterol, are partly responsible for the observed differences in the distribution of DNM. Since drug–membrane interactions are important in anthracycline cytotoxicity, it is possible that our observations on a different membrane distribution of daunomycin, may be related to the different sensitivity to the drug exhibited by these cells.

Introduction

The anthracycline daunomycin (DNM), is an antineoplastic antibiotic effective against several human neoplasms [1]. In the past few years, the anthracyclines have been shown to be fully cytotoxic without entering the cell [2], indicating that drug–cell surface interactions may be sufficient for antitumoral activity. Furthermore, direct interaction of the anthracyclines with natural and artificial membranes has been demonstrated, thus, further suggesting that the plasma membrane constitutes a cellular target for these drugs [3–8]. Nevertheless, the location of the drugs within the lipid bilayer of the membranes remains to be elucidated.

In the interaction with membranes both, electrostatic and hydrophobic forces seem necessary for the stabilization of anthracyclines into lipid bilayers. Thus, while electrostatic interactions between the sugar residue of the anthracyclines and the phosphate group of the phospholipids are important in determining drug binding to the vesicles [7,9,10], hydrophobic interactions with natural [4,8] and artificial membranes [3,6,11], appear to govern the location of the drugs in the hydrocarbon interior of the vesicles.

In a previous study, we reported that fluorescence resonance energy transfer (FRET) techniques were useful to determine the location of DNM into model natural membranes [4]. We have now employed this technique and used complementary fluorescence quenching procedures, to study possible differences in

Abbreviations: DNM, daunomycin (daunorubicin); DPH, 1,6-diphenylhexa-1,3,5-triene; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; PS, phosphatidylserine; PC, phosphatidylcholine; Cho, cholesterol; NCL, negatively charged liposomes; PCL, positively charged liposomes; FRET, fluorescence resonance energy transfer; P388/S, wild (drug-sensitive) P388 murine leukemia cells; P388/R, multidrug-resistant P388 murine leukemia cells selected for resistance to daunomycin.

Correspondence: J.A. Ferragut, Department of Neurochemistry, University of Alicante, 03080 Alicante, Spain.

the location of DNM into isolated plasma membranes from drug-sensitive and drug-resistant P388 murine leukemia tumor cells.

Materials and Methods

DNM hydrochloride was from Sigma. DNM concentration in solution (below 10 μm) was determined spectrophotometrically [12]. Free choiesterol (Cho) was from Sigma, and egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), positively (PC/Cho/stearylamine, 63:9:18, molar ratio) (PCL) and negatively (PC/Cho/dicetylphosphate, 63:9:18, molar ratio) (NCL) charged liposomes, were from Avanti Polar Lipids.

Cell cultures of DNM-sensitive P388 murine leukemia cells (P388/S) and a 50-fold resistant subline (P388/R) (LD_{50} values for DNM in 'in vitro' proliferation assays were of 0.008 and 0.4 μM, respectively, as well as the isolation of plasma membranes from the two cell lines were carried out as described elsewhere [13]. Neither the P388/S nor the P388/R cells have detectable levels of P-glycoproteins [14], which are frequently overexpressed in drug-resistant tumor cells and are involved in active drug efflux [15].

Aliquots from 1 mM 1,6-diphenylhexa-1,3,5-triene (DPH) or 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) stock solutions (Molecular Probes) dissolved in UV grade tetrahydrofuran or *N,N'*-dimethylformamide, respectively, were added to the membrane fractions at a ratio of 1 molecule of fluorophore for every 1000 phospholipid molecules, and incubated at room temperature during 1 h (DPH) or 15 min (TMA-DPH), respectively [16]. FRET between the donors DPH or TMA-DPH incorporated in the membrane vesicles and the acceptor DNM, was carried out by incubating a fixed amount of donor-containing vesicles, with increasing DNM concentrations during 2 h at 25°C. Experimental conditions to monitor the FRET process have been detailed previously [4].

Quenching of DNM by iodide was performed by mixing aliquots from a 100 mM KI stock solution (2 mM $Na_2S_2O_3$ to prevent I_3^- formation) in 10 mM Hepes (pH 7.4), with either membrane or lipid vesicles containing greater than 90% of the total 2.5 μM added drug incorporated into the bilayer (estimated according to Ref. 13). Excitation and emission wavelengths were 472 nm and 555 nm, respectively. Scattering values represented less than 1% of the total fluorescence signal.

Artificial lipid vesicles were prepared as it follows: Lipid mixtures from stock solutions of PC, PS and Cho in chloroform, were dried under a steam of argon on the inner surface of glass vials. Lipid dispersions in 10 mM hepes buffer (pH 7.4), containing 100 mM NaCl, at the desired lipid phosphorus concentration, were submitted to ultrasounds in a probe-type Soniprep 150 apparatus until a clear solution was obtained. Positively (PCL) and negatively (NCL) charged liposomes were also prepared as indicated above. Experiments at different pH were conducted in buffers containing 100 mM NaCl, in addition to either 10 mM Hepes (pH 8.3); 10 mM Hepes (pH 7.6); 10 mM Hepes (pH 7.0), or 10 mM Pipes (pH 6.1).

Lipid phosphorus determinations were performed by the method of Kyaw et al. [17].

Results

Fig. 1 shows the efficiency of FRET between the energy donors DPH or TMA-DPH (exploring 'deep' and 'surface' hydrophobic membrane domains, respectively [18]) and DNM as the energy acceptor, when incorporated into isolated plasma membranes from P388/S (Panel A) and P388/R (Panel B) cells. The fluorescence emission of both donor probes was quenched by the drug in a dose-dependent manner through a Förster energy transfer mechanism. Control experiments in the presence of Triton X-100 to disrupt the energy transfer (as in Ref. 4), demonstrated that only membrane-associated DNM, and not free drug in solution, was involved in the FRET process. The high efficiency values reported in Fig. 1, indicated that the fluorescence emission of the donors (DPH and TMA-DPH), can be completely quenched by DNM.

The main differences in the FRET process observed in membranes from P388/S and P388/R cells are seen at low drug/phospholipid ratios, which correspond in fact to drug concentrations of pharmacological relevance both 'in vivo' and 'in vitro' [19]. In membranes from P388/S cells (Fig. 1, Panel A), TMA-DPH transfers the fluorescence energy to DNM more efficiently

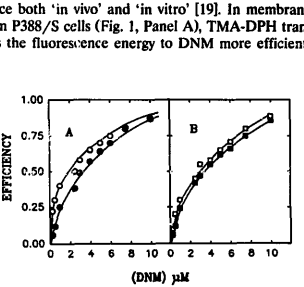

Fig. 1. FRET between the energy donors DPH (●, ■) or TMA-DPH (○, □) and daunomycin (energy acceptor) in plasma membranes from drug-sensitive (Panel A) or drug-resistant (Panel B) P388 cells. Lipid concentration of the membrane vesicles was 20 μM in terms of lipid phosphorus. The concentration of DNM ranged from 0.25 to 10 μM. The efficiency of the energy transfer was determined as described in Ref. 4.

than DPH does. This observation indicates that at low drug/phospholipid ratios, DNM locates preferentially at 'surface' domains in membranes from P388/S cells. However, when increasing the DNM/phospholipid ratio, the drug seems to distribute more homogeneously between 'deep' and 'surface' membrane domains, as indicated by the similar efficiencies of energy transfer from the two donors to DNM. On the other hand, in membranes from P388/R cells (Fig. 1, Panel B), transfer efficiencies between both donor pairs and the drug were basically identical regardless of the drug/phospholipid ratio tested.

The location of DNM at different domains in plasma membranes from P388 cells was also assessed by quenching experiments with iodide, a water soluble collisional quencher of DNM [6]. Fig. 2 shows representative Stern-Volmer plots for the quenching of DNM by iodide under conditions of low DNM/phospholipid ratios (drug incorporation > 90% of the total drug present), thus resembling those previously used in FRET experiments. A downward deviation of the plots at quencher concentrations greater than 40 mM, suggests heterogeneity in the drug population incorporated into the membranes [20]. The fraction of membrane-bound DNM accessible to iodide, f_A, was estimated from the Y-axis intercept of the linear plot represented in Fig. 3, by using a modified Stern-Volmer equation [21]. Approximately 11% and 41% of the total membrane-bound DNM was unaccessible to the quencher in membranes from P388/S and P388/R cells, respectively. Since interaction between iodide ions and DNM occurs at the lipid/solvent interface, the result suggests that DNM is located in a more interfacial environment in drug-sensitive membranes than in drug-resistant membranes.

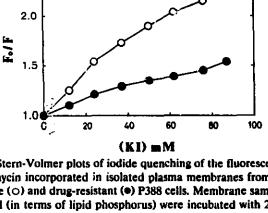

Fig. 2. Stern-Volmer plots of iodide quenching of the fluorescence of daunomycin incorporated in isolated plasma membranes from drug-sensitive (○) and drug-resistant (●) P388 cells. Membrane samples at 500 μM (in terms of lipid phosphorus) were incubated with 2.5 μM of DNM in 10 mM Hepes buffer (pH 7.4), 100 mM NaCl. Under these conditions, more than 90% of the drug was incorporated into the lipid bilayer [13]. F_0 and F represent the fluorescence intensity of daunomycin in the absence and in the presence of iodide, respectively.

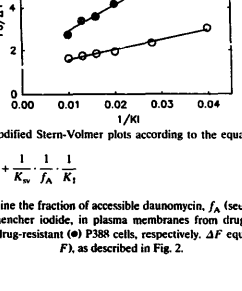

Fig. 3. Modified Stern-Volmer plots according to the equation [21]:

$$\frac{F_0}{\Delta F} = \frac{1}{f_A} + \frac{1}{K_{sv}} \cdot \frac{1}{f_A} \cdot \frac{1}{K_I}$$

to determine the fraction of accessible daunomycin, f_A (see Results), to the quencher iodide, in plasma membranes from drug-sensitive (○) and drug-resistant (●) P388 cells, respectively. ΔF equals ($F_0 - F$), as described in Fig. 2.

Collisional quenching constant (K_{sv}) values of approximately 13.8 M^{-1} and 24 M^{-1} are obtained from the slope of the plots shown in Fig. 3, corresponding to plasma membranes from P388/R and P388/S cells, respectively, while a K_{sv} of 14 M^{-1} was determined for the iodide quenching of free DNM in solution (not shown). Lifetime values (τ_0) for DNM in solution [12] or for the drug bound to fluid phase DMPC bilayers [6] (which partly resemble the physical state of biological membranes) have been reported to be 0.8 and 1.8 ns, respectively. According to the equation $K_{sv} = K_q \cdot \tau_0$, where K_q represents the bimolecular quenching constant, K_q values of 1.75, 1.33 and $0.7 \cdot 10^{10}$ M^{-1} s^{-1} are obtained for iodide quenching of free DNM in solution and for DNM bound to drug-sensitive and drug-resistant membranes, respectively. This indicates that incorporation of DNM into the membranes decreases its susceptibility to quenching by iodide, mainly in the case of drug-resistant membranes. Furthermore, the above K_q values are higher than those expected for a pure collisional quenching mechanism [21], suggesting that a static component on the quenching of DNM by iodide is also present.

The observed differences in the location of DNM at surface domains in membranes from drug-sensitive and drug-resistant cells, can partly be explained by the differences in the relative levels of certain lipids in the two types of membranes. Drug-sensitive and drug-resistant membranes differ mainly in their PS and Cho contents [13]. We have analyzed how the presence of these two lipids affects the location of DNM, by using model lipid bilayers made from egg PC supplemented with PS or Cho at different molar fractions. Increasing the content of the anionic PS into the liposomes, increases the efficiency of the energy transfer to DNM

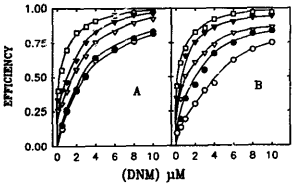

Fig. 4. Effect of increasing the PS content on the efficiency of the FRET process between DPH (Panel A) or TMA-DPH (Panel B) and DNM, in artificial lipid vesicles made from egg yolk PC/PS mixtures at PS molar percentages of 0 (○), 5 (●), 10 (▽), 20 (▼) and 40 (□). The lipid concentration of the vesicles was 20 μM in terms of lipid phosphorus.

from each donor (Fig. 4, panels A and B), the transfer from 'surface' domains being more sensitive to the presence of the acidic phospholipid than the transfer from the domains explored by DPH. FRET is optimal when the mole percent of PS into the PC vesicles is 20–25% or higher (Fig. 4, panels A and B). This is likely the result of an increased incorporation of DNM into the vesicles, as suggested from experiments of equilibrium binding of DNM to lipid vesicles containing increasing amounts of PS [13]. The above effects of PS on the efficiency of the energy transfer, further suggest the occurrence of ionic interactions between the negative charge of the polar head group of the phospholipid and protonated forms of DNM. This is also supported by FRET experiments using liposomes with positive or negative surface charge and at different pH. Negatively charged liposomes (NCL), facilitate FRET between DPH and DNM (Fig. 5), therefore emphasizing the importance of electrostatic interactions between the drug and the negatively charged surface of the vesicles [22]. Also, assays with positively charged liposomes (PCL), result in a dramatic reduc-

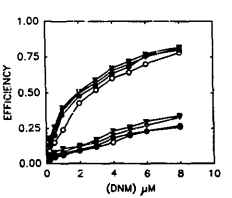

Fig. 5. Effect of the surface charge of liposomes on the efficiency of FRET from DPH to DNM, at pH: 6.1 (○), 7.0 (●), 7.6 (▽) and 8.3 (▼). The upper and the lower set of curves correspond to FRET between the donor-acceptor pair in NCL and PCL, respectively.

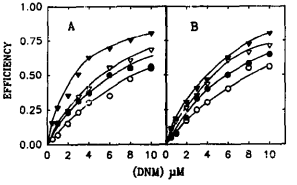

Fig. 6. Changes in the efficiency of FRET between DPH (Panel A) or TMA-DPH (Panel B) and DNM, as a function of the Cho content in egg yolk PC/Cho liposomes. The Cho molar percentage in the PC/Cho mixtures was 0 (▼), 9.1 (▽), 20 (■) and 28.6 (○).

tion on the efficiency of the energy transfer process (Fig. 5). TMA-DPH could not be used in these experiments because of its poor incorporation into PCL. The contribution of pH, although quantitatively less important than the membrane surface charge effect on the efficiency of FRET between DPH and DNM (Fig. 5), reveals that the ionization state of DNM (the pK of DNM ranges from 7.6 to 8.2, depending upon the ionic strength [23]), modulates also the efficiency of the energy transfer, regardless of the surface charge of the liposomes.

Fig. 6 shows how increasing amounts of Cho (the other differentiating lipid between membranes from P388/S and P388/R cells) into artificial PC vesicles, reduces the efficiency of the energy transfer between DPH or TMA-DPH, and DNM (Fig. 6, Panels A and B, respectively). The most prominent effect of Cho has been observed when its content in the vesicles is above 25%, which is close to the concentration of the sterol in isolated membranes from the P388 cells (28 and 31% for drug-sensitive and drug-resistant membranes, respectively [13]).

Discussion

The anthracycline DNM partitions into membrane 'surface' and 'deep' domains in plasma membranes from murine leukemia P388 cells. More interestingly, such partitioning seems to be different depending on whether plasma membranes from drug-sensitive or drug-resistant P388 cells, are used in the studies. These conclusions are based on results obtained from two different techniques: FRET and fluorescence quenching. The former technique uses DPH or TMA-DPH as energy donors and DNM as the energy acceptor, and has been shown to be useful to determine the distribution of the drug in model natural membranes [4]. It must be pointed out that the two energy donors DPH and TMA-DPH, occupy extensive membrane domains which partly overlap with each other [18,24]. Further-

more, partial overlapping of these domains should also be expected from the fact that the R_0 value (the Förster distance at which the transfer efficiency is 50%) for these two donors and DNM as the acceptor, is about 2.9 nm [4,8], versus an estimated 6–7 nm for the bilayer total thickness. Consequently, even small differences observed in the efficiency of the energy transfer from each donor to DNM, reflect a significant alteration in the relative location of the drug. Thus, our FRET results indicate that DNM incorporated into membranes from drug-sensitive cells, is preferentially located at 'surface' domains. On the other hand, DNM incorporated in membranes from drug-resistant cells, is distributed more homogeneously throughout the membrane. These conclusions are further confirmed by the iodide quenching experiments, which indicate that the drug incorporated into membranes from drug-sensitive cells, is significantly more accessible to the aqueous environment than that incorporated into membranes from drug-resistant cells.

Differences in the relative contents of certain lipid classes between drug-sensitive and drug-resistant membranes, seem to partly account for the different distribution of DNM in these membranes. Thus, FRET experiments with liposomes containing increasing amounts of PS or Cho (drug-sensitive membranes contain more PS and slightly less Cho than drug-resistant membranes [13]), reveal an increase or a decrease, respectively, on the efficiency of transfer between the two donors and DNM (Figs. 4 and 6, respectively).

Stabilization of the drug at membrane surface domains seems to involve electrostatic forces, as indicated by FRET experiments with liposomes in which the presence of either, the anionic phospholipid PS (see above) or dicetylphosphate in NCL (Fig. 5), increased the efficiency of FRET mainly between TMA-DPH and the cationic DNM. Electrostatic interactions have also been reported to be responsible for the formation of stable complexes between anionic phospholipids such as cardiolipin or phosphatidic acid, and the cationic amino group of the daunosamine moiety of the anthracyclines [7,9,10].

On the other hand, the occurrence of an also efficient FRET process between DPH (deeply located into the lipid bilayer) and DNM, suggests the involvement of 'deep' hydrophobic sites on the location of DNM. In fact, hydrophobic interactions between the anthracycline ring and the hydrocarbon interior of the lipid bilayers, have been proposed to be important for the association of these drugs to vesicles [6,8,11,25].

Since the report by Tritton and Yee [2] on the activity of polymer-immobilized anthracyclines, it is suggested that cell surface interactions may be sufficient to elicit cytotoxicity. In this regard, it is possible that the observations reported here on a different distribution of DNM in the plasma membranes from P388/S and P388/R cells, could be relevant to the different sensitivity to the drug exhibited by both cell lines. Furthermore, the large efficiencies of FRET between DPH and DNM, along with the previously observed binding between the drug and the lipid component of the plasma membranes from the P388 cells [13], lend further support to the hypothesis that the transport of anthracyclines through membranes, may occur by passive diffusion [26,27]. In this regard, it can be speculated that the observed accommodation of a significant DNM population into 'deep' plasma membrane domains in the drug-resistant cells, could result in an easier diffusion of the drug through these membranes, as compared to that in membranes from drug-sensitive cells.

Acknowledgements

This work has been partly supported by Grants PB87-0790 (to J.M.G.-R.) and PB87-0791 (to J.A.F.) from the DGICYT of Spain.

References

1 Neidle, S. and Sanderson, M.R. (1983) in Molecular Aspects of Anticancer Drug Action (Neidle, S. and Waring, M.J., eds.), Topics in Molecular and Structural Biology, Vol. 3, pp. 35–55, Verlag-Chemie, Weinheim.
2 Tritton, T.R. and Yee, G. (1982) Science 217, 248–250.
3 Dupou-Cezanne, L., Sautereau, A.M. and Tocanne, J.F. (1989) Eur. J. Biochem. 181, 695–702.
4 Ferrer-Montiel, A.V., Gonzalez-Ros, J.M. and Ferragut, J.A. (1988) Biochim. Biophys. Acta 937, 379–386.
5 Yee, G., Carey, M. and Tritton, T.R. (1984) Cancer Res. 44, 1898–1903.
6 Burke, T.G. and Tritton, T.R. (1985) Biochemistry 24, 5972–5980.
7 Henry, N., Fantine, E.O., Bolard, J. and Garnier-Suillerot, A. (1985) Biochemistry 24, 7085–7092.
8 Griffin, E.A., Vanderkooi, J.M., Maniara, G. and Erecinska, M. (1986) Biochemistry 25, 7875–7880.
9 Goormaghtigh, E., Brasseur, R., Huart, P. and Ruysschaert, J.M. (1987) Biochemistry 26, 1789–1794.
10 Nicolay, K., Sautereau, A.M., Tocanne, J.F., Brasseur, R., Huart, P., Ruysschaert, J.M. and De Kruiff, B. (1988) Biochim. Biophys. Acta 940, 197–208.
11 Burke, T.G. and Tritton, T.R. (1985) Biochemistry 24, 1768–1776.
12 Barcelo, F., Barcelo, I., Gavilanes, F., Ferragut, J.A., Yanovich, S. and Gonzalez-Ros, J.M. (1986) Biochim. Biophys. Acta 884, 172–181.
13 Escriba, P.V., Ferrer-Montiel, A.V., Ferragut, J.A. and Gonzalez-Ros, J.M. (1990) Biochemistry 29, 7275–7282.
14 Garcia-Segura, L.M., Ferragut, J.A., Ferrer-Montiel, A.V., Escriba, P.V. and Gonzalez-Ros, J.M. (1990) Biochim. Biophys. Acta 1029, 191–195.
15 Endicott, J.A. and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171.
16 Kuhry, J.-G., Fonteneau, P., Duportail, G., Maechling, C. and Laustriat, G. (1983) Cell Biophys. 5, 129–140.
17 Kyaw, A., Maung-V, K. and Toe, T. (1985) Anal. Biochem. 145, 230–234.
18 Prendergast, F.G., Haugland, R.P. and Callahan, P.J. (1981) Biochemistry 20, 7333–7338.

19 Bielack, S.S., Erittmann, R., Winkler, K. and Landbeck, G., Eur. J. Cancer Cli. Oncol. (1989) 25, 873–882.
20 Lehrer, S.S. (1971) Biochemistry 10, 3254–3263.
21 Lakowicz, J.R. (1983) in Principles of Fluorescence Spectroscopy, pp. 257–301, Plenum Press, New York.
22 Ferragut, J.A., Gonzalez-Ros, J.M., Ferrer-Montiel, A.V. and Escriba, P.V. (1988) Ann. N.Y. Acad. Sci. 551, 443–445.
23 Skovsgaard, T. and Nissen, N.I. (1986) in Membrane Transport of Antineoplastic agents (Goldman, I.D., eds.), pp. 195–213, Pergamon Press, Oxford.
24 Engel, L.W. and Prendergast, F.G. (1981) Biochemistry 20, 7338–7345.
25 Samuni, A., Chong, P., L.-G., Barenholz, Y. and Thompson, T.E. (1986) Cancer Res. 46, 594–599.
26 Siegfried, J.M., Burke, T.G. and Tritton, T.R. (1985) Biochem. Pharmacol. 34, 593.
27 Dalmark, M. and Storm, H.H. (1981) J. Gen. Physiol. 78, 349–355.