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# Comparison of adriamycin and derivatives uptake into large unilamellar lipid vesicles in response to a membrane potential

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The uptake of adriamycin (ADM) and several derivatives into large unilamellar vesicles (LUV) displaying a transmembrane potential and having a lipid composition close to that of the inner mitochondrial membrane has been measured. Drug association to neutral liposomes, made of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (70:30, w/w) was shown to be potential-dependent: in the absence of potential, accumulation of drug was almost undetectable, whereas between 11 and 50 nmol of drug/$\mu$mol phospholipid, depending on the anthracycline used, was associated to LUV exhibiting a membrane potential after 1 h incubation. Association of drugs to LUV with a lipid composition closer to that of the inner mitochondrial (cardiolipin, CL, 20%; PC 50%; PE, 30%, w/w) and displaying a membrane potential is higher than with neutral vesicles (between 40 and 76 nmol of anthracycline/$\mu$mol phospholipid after 1 h incubation). Since it is known that ADM and derivatives have a high affinity for CL, a fraction of the associated drug may bind to CL on the outer side of the vesicles. This was confirmed by the fact that, in the absence of potential, between 40 and 56 nmol of anthracycline/$\mu$mol phospholipid was still associated to LUV containing CL. In order to discriminate between drug adsorbed at the surface of the LUV and drug accumulated inside the LUV, an anthracycline fluorescence quencher ($I^-$) was used. It was shown on neutral LUV displaying a membrane potential, that between 55 and 81% of the associated drug is actually entrapped inside the vesicles, inaccessible to the quencher. These percentages decreased to between 41 and 68%, respectively, in the presence of LUV containing CL and exhibiting a membrane potential, whereas for LUV of the same composition but displaying no membrane potential almost all the associated drug is adsorbed on the outer face of the LUV, accessible to the quencher, and likely bound to CL. This study brings evidence that antitumour anthracyclines despite important structural homologies do not accumulate to the same extent into vesicles mimicking the lipid composition and the membrane potential of mitoplasts. This ability to reach the matrix compartment of mitochondria could partly explain the differences of cardiotoxicities associated to anthracyclines with closely related molecular structure.

## Introduction

Adriamycin (ADM) is widely used in the treatment of a variety of human cancers [1]. However, its cardiotoxicity limits drastically its clinical use [2–4]. ADM and other anthracyclines have been shown to undergo a one-electron reduction to a semiquinone free-radical species, in the presence of NADH and mitochondria [5–14]. This semiquinone radical is reoxidized by molecular oxygen in a process generating superoxide anion and hydroxyl radical. These free radical species peroxidize membrane lipids [15–18], leading to a general disturbance of the structure of the inner mitochondrial membrane and to the impairment of its essential biological functions. ADM was shown to inhibit in vitro and in vivo the activity of complex I–III and IV of the mitochondrial respiratory chain in the inner mitochondrial membrane and to decrease the fluidity of this membrane [6,19–23]. Free radical scavengers were reported to abolish or to delay this cardiotoxicity [15,24–26].

A component of mitochondrial complex I, the NADH dehydrogenase flavin, facing the matrix, has been identified as the mitochondrial target for anthracycline reduction [11,13]. The accessibility of ADM to this site is still a matter of discussion, because of the well known selectivity of the inner membrane towards most of the biological solutes. It is nevertheless worthwhile to mention that an electrochemical potential could facilitate the entry of ADM into the matrix. Comparison of ADM accumulation inside different cell lines indicates a higher accumulation into cells exhibiting a membrane potential [27–29].

In this work, we investigated on the possibility that ADM and derivatives could accumulate into the mitochondrial matrix after passing through the lipid bilayer of the inner mitochondrial membrane. We therefore measured the uptake of anthracyclines into vesicles displaying a transmembrane potential and having a

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lipid composition close to that of the inner mitochondrial membrane.

## Materials and Methods

### *Materials*

ADM, 4′-epiADM, 4′-deoxyADM, 4′-deoxy-4′-iodoADM and 4-demethoxydaunorubicin (4-demethoxyDNR) were provided by Farmitalia (Milan). Daunorubicin (DNR), bovine heart cardiolipin (CL), egg yolk L-α-phosphatidylethanolamine (PE), egg yolk L-α-phosphatidylcholine, 1,6-diphenyl-1,3,5-hexatriene (DPH) and valinomycin were purchased from Sigma Chemical Co. (Saint-Louis, USA) and L-α-[$^{14}$C]dipalmitoylphosphatidylcholine ([$^{14}$C]DPPC, 117 mCi/mmol) from Amersham. Pyranine is an Eastman-Kodak product and [$^{3}$H]methyltriphenylphosphonium iodide ([$^{3}$H]MTPPI, 39.7 Ci/mmol) is a NEN product. KOH, KI, NaOH, KCl, NaCl and Triton X-100 are Merck products; glutamic acid and $Na_2S_2O_3$ Aldrich products.

### *Methods*

*Preparation of large unilamellar vesicles (LUV).* Lipids are dissolved in chloroform or methanol. The organic phase is slowly evaporated under $N_2$ to form a thin lipidic layer on the walls of the test tube. Solvent is eliminated under vacuum overnight. The lipidic film containing 0.006 μCi of [$^{14}$C]DPPC/μmol of phospholipid is vortexed in an appropriate aqueous buffer (50 mg of lipid/ml). LUV are prepared by freeze-thawing of the MLV dispersion and extrusion through standard 25 mm polycarbonate filters with 0.1 μm pore size (Lipex Biomembranes, Extruder, Vancouver, Canada). Mean vesicle size and size distribution were determined by quasi elastic light scattering using a Malvern BC201 particle sizer. Mean vesicle diameter was 117 nm for PC/PE LUV and 108 nm for PC/PE/CL LUV. At the temperature used (21°C), all lipids were in the liquid crystal state.

*Membrane potential and pH gradient.* LUV are prepared in a glutamic acid (125 mM)/KOH (150 mM)/KCl (20 mM) buffer, pH 7.5, 280 mosM. The external buffer is exchanged after elution on a Sephadex G-50 column, equilibrated with a glutamic acid (150 mM)/NaOH (150 mM) buffer, pH 6.2, 280 mosM. Addition of valinomycin (0.5 μg/μmol lipid) to the LUV preparation generates a membrane potential measured by the distribution of [$^{3}$H]MTPP$^+$, a lipophilic cation, across the lipid bilayer of LUV containing no [$^{14}$C]DPPC. 1 μCi of [$^{3}$H]MTPP$^+$ in 1 μl ethanol is added to 2 ml of LUV suspension (2 mM lipid). In a set of experiments, ADM (0.2 mM) was added to the reaction mixture. At various times, an aliquot of the solution is filtered on a 2 ml Sephadex G-50 column to remove the untrapped [$^{3}$H]MTTP$^+$ [30]. Amounts of [$^{3}$H]MTPP$^+$ entrapped and phospholipid concentration are determined by liquid scintillation counting (Beckman LS 7500 counter) and by phosphate assay respectively. The membrane potential is calculated from the Nernst equation: $\Delta\psi$ (mV) = $-59 \log([MTPP^+]_i/[MTPP^+]_o)$, where $[MTPP^+]_i$ and $[MTPP^+]_o$ account respectively for the concentration of MTPP$^+$ inside and outside the vesicles.

In order to demonstrate that a pH difference of about one unit is effectively maintained between the internal and the external buffer, prior to addition of the anthracyclines, the intraliposomal pH after buffer exchange is measured using entrapped pyranine as a fluorescent, pH dependent marker [31]. Pyranine ($5 \cdot 10^{-7}$ M) is added to the glutamic acid/KOH/KCl (pH 6.2) buffer. After formation of the LUV and exchange of the external buffer, pyranine fluorescence is measured at 510 nm with excitation wavelengths at 400 nm ($I_{400}$) and 450 nm ($I_{450}$) successively. The logarithm of the $I_{450}/I_{400}$ ratio varies linearly with the pH of the medium in which the probe is dissolved [31]. We measured a pH of 7.2, which corresponds to a ΔpH of one unit between the external and internal buffer.

*Uptake of anthracyclines.* At time $t = 0$, 0.2 mM of antimitotic agent and valinomycin are added to the LUV suspension (2 mM lipids, 21°C). At times $t = 5$, 10, 25, 40, 60, 90 and 120 min, an aliquot (150 μl) of the solution is filtered on a 2 ml Sephadex G-50 column to remove the untrapped drug [30]. Anthracycline concentration is measured spectrophotometrically at 492 nm on a Titertek Multiskan Plus MK II spectrophotometer after lysis of the vesicles (Triton X-100, final concentration 0.5%). Lipid concentration is determined by liquid scintillation counting of [$^{14}$C]DPPC (Beckman LS 7500 counter).

*Iodide quenching.* To establish that $I^-$ does not enter the membrane core, DPH fluorescence quenching by iodide is measured on LUV. LUV prepared in the $K^+$ containing buffer are incubated 1 h at room temperature in the dark with DPH (stock solution 3 mM in tetrahydrofuran), at a molar ratio of 1 DPH molecule for 500 phospholipid molecules. LUV at a final concentration of 0.1 mg/ml are then incubated with KI and KCl in various proportions. A total halide concentration ($I^-$ and $Cl^-$) of 0.165 M allows to maintain an osmolarity of about 300 mosM. The KI stock solution (1 M) contains 2 mM $Na_2S_2O_3$ to prevent $I_3^-$ formation. DPH fluorescence is monitored for increasing iodide concentrations at 431 nm, using an excitation wavenlength of 360 nm (Jobin-Yvon JY3 B spectrofluorimeter, 4 nm slit).

In order to establish the accessibility to iodide of ADM entrapped into LUV, PC/PE (70/30, w/w) vesicles were prepared in the $K^+$ containing buffer, in the presence of ADM ($10^{-5}$ M). ADM outside the LUV was discarded by passage of the suspension on Sephadex G-50 column equilibrated with the $K^+$ buffer.

Fluorescence measurements were performed in the same conditions as those described for the assays of DPH fluorescence quenching by iodide.

In another set of experiments, drugs are incubated with LUV displaying a membrane potential in the conditions described above. After 1 h, the untrapped drug is removed by running an aliquot through a 2 ml Sephadex G-50 column. 500 $\mu$l of an aqueous solution containing KI and KCl in various proportions are added to 50 $\mu$l of effluent. Fluorescence of the anthracyclines is measured (excitation wavelength 470 nm, emission 554 nm for all derivatives except 4-demethoxyDNR, emission 538 nm) at various KI concentrations. Accessibility of the anthracyclines to iodide is determined graphically as described in Ref. 32. Fluorescence quenching constants of anthracyclines are calculated as described in Ref. 33.

## Results

The membrane potential was measured by determining the distribution of tritiated methyltriphenylphosphonium ($MTPP^+$), a lipophilic cation, across the lipid bilayer of LUV displaying a transmembrane $K^+$ gradient ($K^+$inside) and a pH gradient (outside acidic), in the presence of valinomycin. The potential gradually increases with time, from $-100$ to $-120$ mV for class I LUV (70% PC and 30% PE w/w) after 1 h, whereas it decays from $-140$ to $-105$ mV for class II LUV (50% PC, 30% PE and 20% w/w), after 1 h (Fig. 1). After addition of ADM (0.2 mM), the membrane potential remains fairly constant around $-90$ mV for class I LUV, whereas it decays from $-147$ to $-110$ mV for class II LUV after 1 h (Fig. 2).

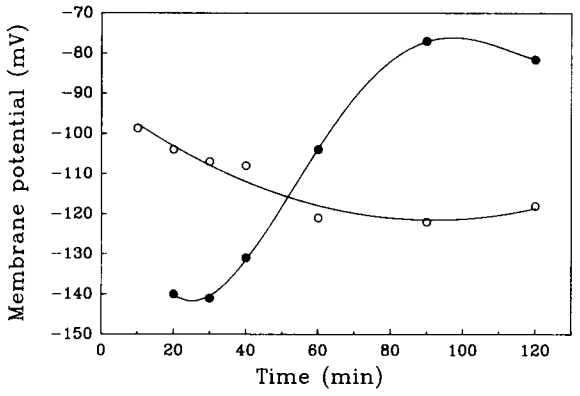


Fig. 1. Time-course of the membrane potential of PC/PE (70:30, w/w) (○) and PC/PE/CL (50:30:20, w/w) (●) LUV (2 mM lipid) experiencing a $Na^+/K^+$ transmembrane electrochemical gradient and a pH gradient, in the presence of valinomycin (0.5 $\mu$g/$\mu$mol lipid). LUV were formed in a glutamic acid (125 mM)/KOH (150 mM)/KCl (20 mM) buffer (pH 7.5, 280 mosM) and subsequently eluted through a Sephadex G-50 column equilibrated with a glutamic acid (150 mM)/NaOH (150 mM) buffer (pH 6.2, 280 mosM). The membrane potential was determined by measuring the transmembrane distribution of $[^3H]MTTP^+$. Non-specific binding of the probe to LUV was determined in control experiments with $K^+$ containing buffer present on both sides of the LUV membrane. Experimental points were corrected in consequence.

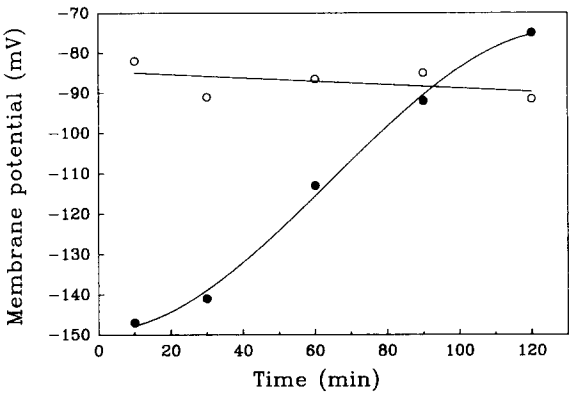


Fig. 2. Time-course of the membrane potential of PC/PE (○) and PC/PE/CL (●) LUV (2 mM lipid) experiencing a $Na^+/K^+$ electrochemical gradient and a pH gradient, in the presence of valinomycin (0.5 $\mu$g/$\mu$mol lipid) and ADM (0.2 mM). For details, see Fig. 1 and Materials and Methods.

The amount of drug associated with class I LUV displaying a membrane potential increases progressively and reaches 40 to 50 nmol anthracycline/$\mu$mol phospholipids after 1 h (Fig. 3A). Only 4'-deoxy-4'-iodoADM is weakly associated (11 nmol/$\mu$mol phospholipids). The low uptake observed for each antimitotic in the absence of membrane potential, as illustrated for ADM in Fig. 3B, provides evidence that the membrane potential favours the association.

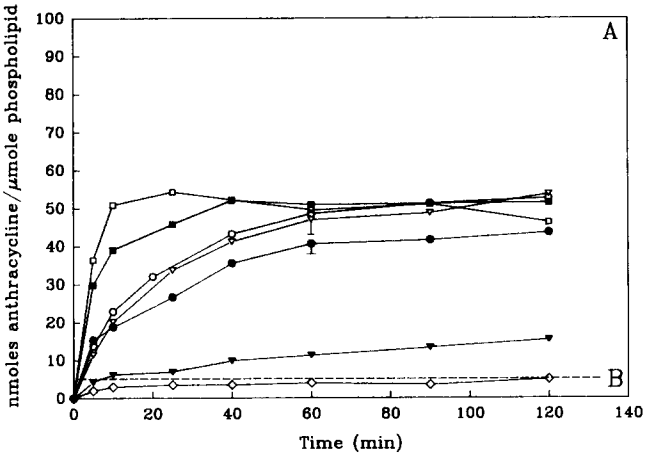


Fig. 3. (A) Association rate of anthracyclines to PC/PE (70:30, w/w) LUV exhibiting a membrane potential. ○, ADM; ●, 4'-epi-ADM; ▽, 4'-deoxyADM; ▼, 4'-deoxy-4'-iodoADM; □, DNR; ■, 4-demethoxyDNR. Standard deviations do not exceed those displayed at $t = 60$ min. ($n = 8$ for ADM, 4 for the other compounds). The incubation mixture contained 2 mM lipid, 0.2 mM antimitotic and valinomycin (0.5 $\mu$g/$\mu$mol lipid). At different times, an aliquot was filtered on a 2 ml Sephadex G-50 column to remove the free drug. Anthracycline concentration is measured spectrophotometrically after lysis of the vesicles with Triton X-100. For further details, see Materials and Methods. (B) Association rate of ADM (◇) to PC/PE LUV exhibiting no membrane potential ($K^+$ containing buffer on both sides of the LUV membrane) in the presence of valinomycin (0.5 $\mu$g/$\mu$mol lipid).

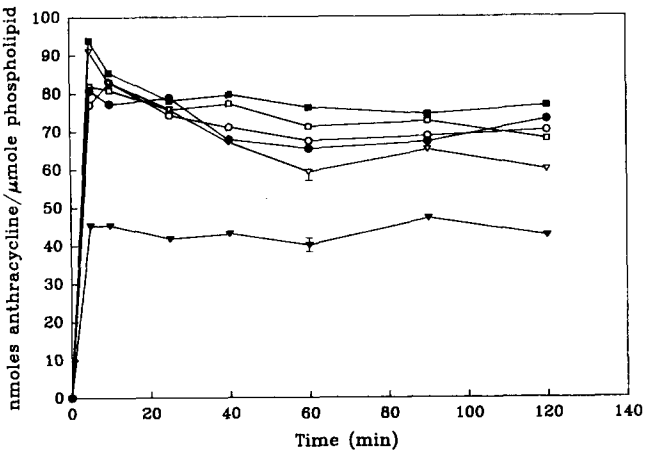


Fig. 4. Association rate of anthracyclines to PC/PE/CL (50:30:20, w/w) exhibiting a membrane potential. ○, ADM; ●, 4′-epiADM; ▽, 4′-deoxyADM; ▼, 4′-deoxy-4′-iodoADM; □, DNR; ■, 4-demethoxyDNR. Standard deviations do not exceed those displayed at $t = 60$ min ($n = 8$ for ADM, 4 for the other compounds). For experimental details, see Fig. 3 and Materials and Methods.

In order to better mimic the lipid composition of the inner mitochondrial membrane, 20% of PC in class I LUV have been replaced with CL (class II LUV). ADM and derivatives uptake increases in LUV displaying a membrane potential as compared to LUV without CL (Fig. 4). The uptake is maximal after 5 or 10 min, depending on the anthracycline. 4′-deoxy-4′-iodoADM binding is weak as compared to the other compounds. The uptake of anthracyclines is always weaker into LUV exhibiting no membrane potential (Fig. 5).

As it has been shown that ADM and other anthracyclines have a high affinity for CL [34–36], a significant amount of drug is expected to bind to the outside of LUV of class II displaying a membrane potential. To distinguish between the fraction of the drug entrapped inside the vesicles and the one merely adsorbed at their surface, we used an anthracycline fluorescence quencher, $I^-$ which does not penetrate the lipidic bilayer [37,38]. This was verified by measurement of DPH and ADM fluorescence quenching in the presence of iodide. DPH was incorporated into the LUV lipid bilayer and its fluorescence monitored for increasing $I^-$ concentrations. Stern-Volmer plot (Fig. 6A) provides evidence that iodide does not penetrate into the core of the bilayer of class I and class II LUV, as described earlier with small unilamellar vesicles [38]. LUV (PC/PE, 70/30) were prepared in the presence

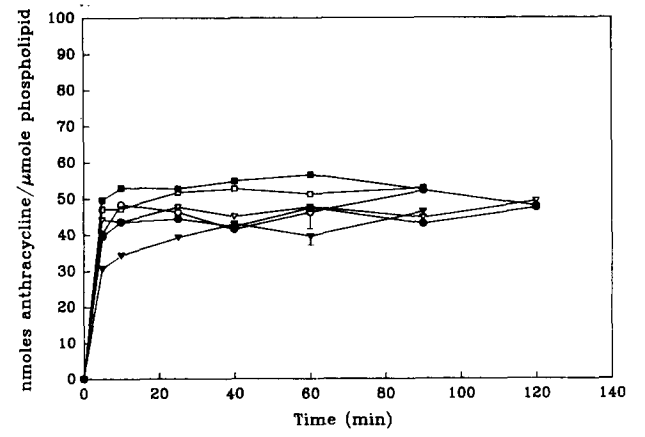


Fig. 5. Association rate of anthracyclines to PC/PE/CL (50:30:20 w/w) LUV exhibiting no membrane potential ($K^+$ containing buffer present on both sides of the LUV membrane, valinomycin present). ○, ADM; ●, 4′-epiADM; ▽, 4′-deoxyADM; ▼, 4′-deoxy-4′-iodoADM; □, DNR; ■, 4-demethoxyDNR. Standard deviations do not exceed those displayed at $t = 60$ min ($n = 8$ for ADM, 4 for the other compounds). For further experimental details, see Fig. 3 and Materials and Methods.

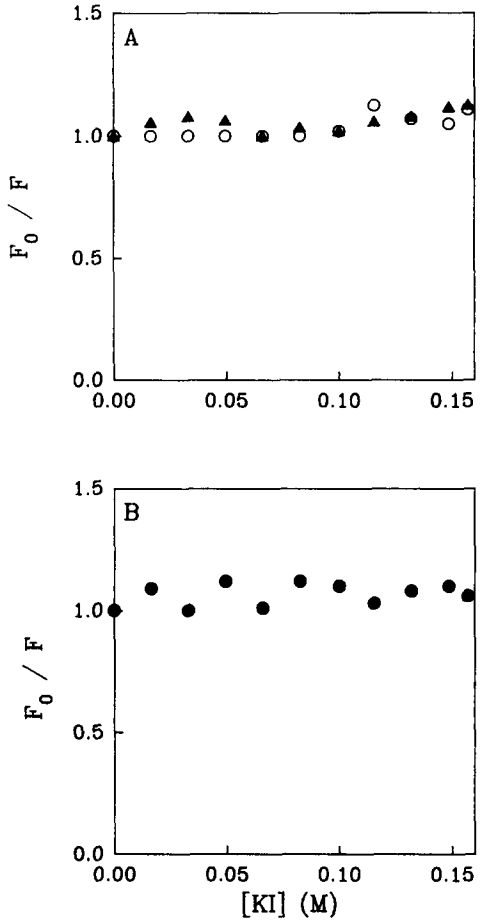


Fig. 6. (A) Stern-Volmer plots. Iodide quenching of DPH fluorescence. ○, PC/PE (70:30, w/w) LUV; ▲, PC/PE/CL (50:30:20, w/w) LUV. DPH was incorporated into the LUV membrane after 1 h incubation in the presence of the vesicles at a molar ratio of 1 molecule for 500 lipid molecules. Lipid concentration during the fluorescence measurements was 0.1 mg/ml. Halide concentration was kept constant at 0.165 M. Further experimental details are in Materials and Methods. (B) Stern-Volmer plot. Iodide quenching of ADM entrapped into LUV. PC/PE (70:30, w/w) LUV were prepared in the $K^+$ buffer containing ADM ($10^{-5}$ M). Untrapped ADM was separated by elution of the LUV suspension on a Sephadex G-50 column. Lipid and halide concentrations were those described for DPH quenching measurements.

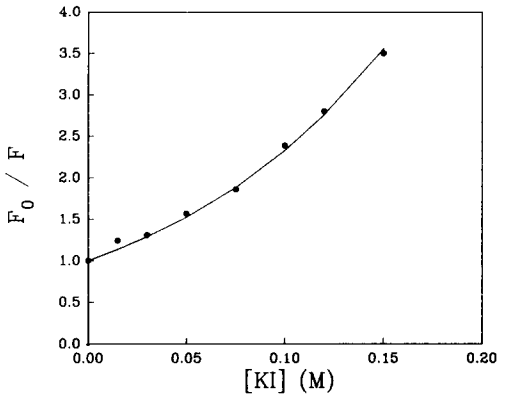


Fig. 7. Stern-Volmer plot. Iodide quenching of 4-demethoxyDNR fluorescence at a constant halide concentration (0.165 M). Anthracycline final concentration was $5 \cdot 10^{-6}$ M.

of ADM ($10^{-5}$ M) and untrapped ADM was eliminated by elution on a Sephadex G-50 column. Iodide quenching of entrapped drug was negligible (Fig. 6B), indicating the poor access of $I^-$ to the internal aqueous compartment of the vesicles.

Fig. 7 illustrates the 4-demethoxyDNR fluorescence quenching for increasing $I^-$ concentrations. This quenching process is described by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}[A]$$

where [A] is the quencher concentration, $F_0$ and $F$ the fluorescence in the absence and in the presence of the quencher and $K_{SV}$ the dynamic quenching constant. This relation is verified only for a dynamic quenching process. The graphic upward curvature indicates that a static quenching process is associated to the dynamic one. The combination of these two types of quenching is described by relation [39]:

$$\frac{F_0}{Fe^{V[A]}} = 1 + K_{SV}[A]$$

TABLE I

*Fluorescence quenching constants of ADM and derivatives*

$V$, static quenching constant; $K_{sv}$, dynamic quenching constant. Anthracycline and halide concentrations were respectively $5 \cdot 10^{-6}$ and 0.165 M.

| Drug | $V$ ($M^{-1}$) | $K_{sv}$ ($M^{-1}$) |
|---|---|---|
| ADM | 2.7 | 10 |
| 4'-EpiADM | 2.4 | 9.3 |
| 4'-DeoxyADM | 2.7 | 9.3 |
| 4'-Deoxy-4'-iodoADM | 2.7 | 9.2 |
| DNR | 2.5 | 9 |
| 4-DemethoxyDNR | 2.7 | 9.5 |

where $V$ is the static quenching constant. $K_{SV}$ and $V$ are calculated according to the method described in Ref. 33. The calculated values of both constants were identical for all derivatives in the limit of the experimental error (Table I, mean $K_{SV} = 9.4$ $M^{-1}$ and mean $V = 2.6$ $M^{-1}$). $I^-$ fluorescence quenching must therefore be considered as a measure of accessibility of the drug to the quencher and not as an intrinsic difference related to the quenching process.

The fraction of anthracycline quenched by iodide is calculated according to the modified Stern-Volmer equation:

$$\frac{F_0}{\Delta F} = \frac{1}{f_a} \cdot \frac{A}{A-1}$$

with $A = (1 + K_{SV}[I^-])\, e^{V[I^-]}$

$f_a$ is the fraction of anthracycline accessible to iodide; it is estimated from the slope of the straight line corresponding to the plot of $F_0/\Delta F$ vs. $A/A - 1$ (Fig. 8), where $\Delta F$ corresponds to the difference $F_0 - F$. LUV were incubated for 1 h with the different drugs. Untrapped drug was separated from LUV on a 2 ml Sephadex G-50 column and anthracycline fluorescence quenching by iodide was measured. Values of $f_a$ are shown in Tables II to IV.

TABLE II

*Amount of drug entrapped into LUV containing no CL and exhibiting a membrane potential*

Anthracyclines (0.2 mM) were incubated with LUV (2 mM lipids) for 1 h. For details on the determination of the accessibility of anthracyclines, see Fig. 8 and Materials and Methods.

| Drug | Total amount of drug associated to LUV after 1 h (nmol drug/ $\mu$mol phospholipid) | Fraction of drug inaccessible to $I^-$ (%) | Amount of drug entrapped into LUV (nmol drug/ $\mu$mol/phospholipid) |
|---|---|---|---|
| ADM | 48.5 ± 1.1 | 65.5 | 31.8 ± 0.7 |
| 4'-EpiADM | 40.6 ± 2.9 | 66.5 | 27 ± 1.9 |
| 4'-DeoxyADM | 47 ± 4.2 | 72.5 | 34 ± 3 |
| 4'-Deoxy-4'-iodoADM | 11.2 ± 0.8 | 55.4 | 6.2 ± 0.4 |
| DNR | 49.5 ± 4.3 | 76 | 37.6 ± 3.3 |
| 4-DemethoxyDNR | 50.9 ± 0.5 | 81 | 41.2 ± 0.4 |

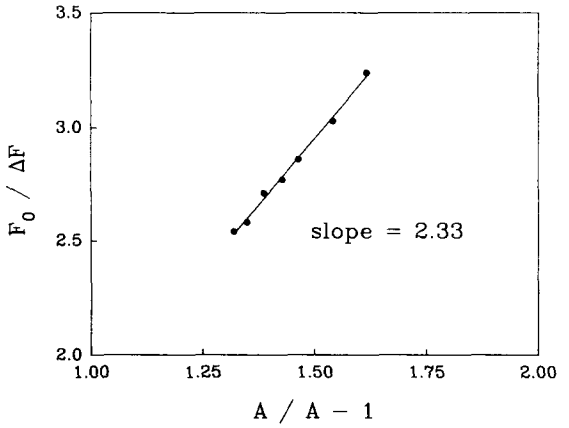


Fig. 8. Modified Stern-Volmer plot used to determine graphically the accessibility of iodide to ADM associated to PC/PE/CL LUV exhibiting a membrane potential. LUV (2 mM lipid) were incubated during 1 h with ADM (0.2 mM). After elimination of the free drug, 50 μl of the LUV suspension were added to 500 μl of a solution containing KI and KCl in various proportions. The total ion concentration (0.165 M) corresponds to an osmolarity of 300 mosM, preventing the lysis of the LUV. The ADM fluorescence was measured at different $I^-$ concentrations. For further experimental details, see Materials and Methods.

From the third column of Table II it appears that 4-demethoxyDNR and DNR penetrate more easily than the other drugs into LUV of class I (without CL) exhibiting a membrane potential, whereas 4'-deoxy-4'-iodoADM concentrates poorly into the LUV. In each case, a significant amount of drug adsorbed at the surface of the vesicles is accessible to iodide (between 19% for 4-demethoxyNR and 45% for 4'-deoxy-4'-iodoADM). With LUV of class II (containing CL) displaying a membrane potential, a situation which mimics the conditions existing near the inner mitochondrial membrane, a large amount of antimitotic, likely bound to CL, is accessible to $I^-$. The fraction of antimitotic accessible to $I^-$ is higher than in the absence of CL (between 32% for 4-demethoxyDNR and 59% for 4'-deoxy-4'-iodoADM) (Table III). Again, 4-demethoxyDNR and DNR accumulate more efficiently into the LUV than the other compounds. For each drug, accumulation of anthracyclines inside the vesicles is much weaker in the absence of membrane potential (Table IV).

## Discussion

The accumulation of various drugs (dibucaine, safranine, ADM) into LUV in response to a membrane potential has been described [40–44]. Conditions were, however, quite different from those chosen in this work. We examined the ability of ADM and derivatives

TABLE III

*Amount of drug entrapped into LUV containing CL and exhibiting a membrane potential*

Conditions are those described in legend to Table II.

| Drug | Total amount of drug associated to LUV after 1 h (nmol drug/ μmol phospholipid) | Fraction of drug inaccessible to $I^-$ (%) | Amount of drug entrapped into LUV (nmol drug/ μmol phospholipid) |
|---|---|---|---|
| ADM | 67.5 ± 2.5 | 57.1 | 38.5 ± 1.4 |
| 4'-EpiADM | 65.4 ± 2.8 | 49 | 32.8 ± 1.4 |
| 4'-DeoxyADM | 59.3 ± 2.7 | 58.5 | 34.6 ± 1.6 |
| 4'-Deoxy-4'-iodoADM | 40 ± 2.7 | 41.2 | 16.5 ± 1.1 |
| DNR | 71.2 ± 3.5 | 64.4 | 45.8 ± 2.2 |
| 4-DemethoxyDNR | 76.3 ± 2.5 | 68.1 | 52 ± 1.7 |

TABLE IV

*Amount of drug entrapped into LUV containing CL and exhibiting no membrane potential*

Conditions are those described in legend to Table II.

| Drug | Total amount of drug associated to LUV after 1 h (nmol drug/ μmol phospholipid) | Fraction of drug inaccessible to $I^-$ (%) | Amount of drug entrapped into LUV (nmol drug/ mmol phospholipid) |
|---|---|---|---|
| ADM | 46.3 ± 4.6 | 4.5 | 2.1 ± 0.2 |
| 4'-EpiADM | 47.4 ± 6 | 4.5 | 2.1 ± 0.3 |
| 4'-DeoxyADM | 47.7 ± 5 | 7 | 3.3 ± 0.4 |
| 4'-Deoxy-4'-iodoADM | 39.6 ± 4 | 3 | 1.2 ± 0.1 |
| DNR | 51.2 ± 6 | 11 | 5.6 ± 0.7 |
| 4-DemethoxyDNR | 56.6 ± 4 | 15.5 | 8.8 ± 0.6 |

to accumulate into LUV in conditions comparable to those prevailing in the vicinity of the inner mitochondrial membrane. LUV displayed a transmembrane potential (inside negative) and a $\Delta$pH (outside acidic) mimicking those existing across the inner membrane of respiring mitochondria. The initial transmembrane potential was higher with LUV containing CL (class II) than with LUV containing no negative phospholipid (class I) and decayed more rapidly with class II LUV. The presence of 20% CL in the LUV bilayer reduced the stability of the membrane potential.

Our data provide evidence that the potential favours the association of anthracyclines to the vesicles. Experiments carried out with LUV of class I (containing no CL), displaying a membrane potential, showed that this association can reach 50 nmol anthracycline/$\mu$mol phospholipid after 1 h in the case of 4-demethoxyDNR (Fig. 3A and Table II, column 1), whereas it is almost practically undetectable in the absence of potential (Fig. 3B). The association of anthracyclines to LUV of class II (containing CL), displaying a membrane potential, is higher (76 nmol anthracycline/$\mu$mol phospholipid after 1 h incubation in the case of 4-demethoxyDNR) (Fig. 4 and Table III, column 1). This higher association of anthracyclines can be explained in terms of the higher initial potential associated to class II LUV as compared with class I LUV. After this initial phase however, the uptake decreases slowly for most of the drugs and could correspond partially to the decay of the membrane potential. Another reason for the high association of drugs to class II LUV is the binding of a substantial fraction of the antimitotics to CL on their outer side, since significant drug association is still observed with LUV of the same composition but displaying no membrane potential. An anthracycline fluorescence quencher ($I^-$) has been used to discriminate between drug adsorbed at the surface of the LUV and drug accumulated inside the vesicles. Quenching measurements were carried out after elimination of the unbound drug on 2 ml Sephadex G-50 minicolumns. After filtration, one can reasonably expect that the drug concentration in the LUV membrane is too low to promote fluorescence self-quenching through chromophore stacking, which could interfere in the iodide quenching measurements [45]. LUV containing no CL (class I) and displaying a membrane potential showed that between 55% and 81% of anthracycline is inaccessible to iodide depending on the derivative used. For each compound, a significant amount of drug is adsorbed at the surface of the vesicles and accessible to the quencher. Several studies provided evidence of an interaction between anthracyclines and vesicles made of uncharged lipids [46,47]. Antimitotics are located either at the surface of liposomes either more deeply into the lipidic bilayer and inaccessible to the quencher. However, in our conditions, association of anthracyclines to LUV of class I displaying no transmembrane potential is negligible (Fig. 3B). As a consequence, it appears that the potential not only promotes the uptake of an important amount of drug into the liposomes, but also the binding of a fraction of the anthracyclines on external sites of these neutral LUV. Uptake proceeds in two steps: binding of drugs at the surface of the LUV, in much greater amount than in the absence of potential, followed by their penetration into the vesicles.

With LUV of class II, iodide quenching experiments reveal a large amount of drug adsorbed on the external face of the liposomes whether a membrane potential is present or not. The potential is still required for the penetration of the drugs into the vesicles since in its absence, the anthracycline fluorescence is almost entirely quenched by iodide (Table IV). This suggests that self-quenched drug molecules, inaccessible to further quenching by iodide, are not associated to the LUV membranes. However, a significant amount of DNR and 4-demethoxyDNR remains inaccessible to iodide, indicating that these compounds, more lipophilic than ADM, penetrate into the core of the bilayer.

The antimitotics can be classified according to their ability to concentrate into liposomes mimicking the inner mitochondrial membrane (lipid composition and membrane potential): 4-demethoxyDNR > DNR > ADM > 4′-deoxyADM > 4′-epiADM > 4′-deoxy-4′-iodoADM.

This sequence shows that despite structural analogies, the anthracyclines do not penetrate to the same extent into the vesicles. This property is not related to a different affinity of the drugs for CL, since we recently provided evidence that the association constants with this phospholipid were identical for all the drugs, within the limits of the experimental error (our unpublished data).

Steric hindrance plays a crucial role as illustrated for 4′-deoxy-4′-iodoADM which contains a voluminous iodine atom. This drug, whatever the conditions, accumulates weakly into the vesicles. The $pK_a$ of its amine moiety is 6.4 and 8 for ADM [48,49]. The iodine atom at position 4′ of the aminosugar reduces the basicity of the amine in 3′ and increases the lipophilicity of the molecule [49]. At pH 6.2 (the pH outside the LUV), most of the ADM molecules are ionized, whereas only about 50% of the 4′-deoxy-4′-iodoADM molecules are in the ionized form. Hasmann provided evidence that a membrane potential facilitates the uptake of positively charged molecules, and that this effect is increased for highly lipophilic compounds [29]. Obviously the 4′-deoxy-4′-iodoADM lipophilicity does not counterbalance the effects associated to the basicity of the amine residue and the volume of the iodine atom. Therefore, this anthracycline penetrates poorly into the vesicles.

On the contrary, 4-demethoxyDNR and DNR which are more lipophilic than the other compounds [48] penetrate more easily into the LUV.

Although the inner mitochondrial membrane is classically described as impermeable to most biological solutes, the passage of ADM through this membrane is a plausible hypothesis. Cheneval et al. showed on intact mitochondria and intact mitoplasts extracted from rat liver and rat heart that ADM binds CL located on the matrix face of the inner membrane [36]. Moreover, administration of ADM to rats slows down the synthesis of circular non-supercoiled cardiac mitochondrial DNA in the heart and causes its breakage in linear fragments [50]. This suggests a direct interaction of ADM with the mitochondrial cardiac DNA in vivo. The possibility for ADM to concentrate itself in the matrix space was also proposed by Kashfi [51]. The ability of anthracyclines to cross the bilayer of LUV mimicking the inner mitochondrial membrane can partly explain the differences of cardiotoxicities associated to compounds closely related from a structural point of view. 4'-deoxy-4-iodoADM is structurally very similar to ADM but is much less cardiotoxic [52]. Our data suggest that this molecule could penetrate poorly into the mitochondrial matrix limiting its access to free radicals production sites facing this mitochondrial compartment. Such a correlation is confirmed for 4'-deoxyADM and 4'-epiADM, for which the uptake into vesicles is lower than for ADM. The correlation is not verified with 4-demethoxyDNR, which penetrates more efficiently than ADM into the vesicles but is less cardiotoxic [48,52–55]. Obviously, the ability to enter into the mitochondrial matrix is not the only parameter involved in the modulation of the cardiac mitochondrial toxicity of anthracyclines. This toxicity depends also on the capacity of a given compound to mediate efficiently the formation of free radicals. Efforts are being made in our laboratory to demonstrate the relationship between the free radical formation and the mitochondrial damage observed in vivo and in vitro (unpublished data).

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