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Synergistic incorporation of daunorubicin in erythrocytes in the presence of polyene antibiotics. Role of the membrane potential

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The synergistic incorporation into red blood cells of the antitumor compound daunorubicin, in the presence of the polyene antibiotics amphotericin B and vacidin A, depended on the composition of the external medium. Synergism was observed only for concentrations of polyene antibiotics that induce cation permeability. The same synergistic effect was observed with the K^+ selective carrier, valinomycin, but this had a different dependence on the external medium composition. By using the membrane probe 3,3'-dipropylthiadicarbocyanine (diS-C_3-(5)), the synergistic effect was shown to occur under conditions where addition of the ionophores leads to hyperpolarization of the membrane.

Introduction

Daunorubicin is a representative member of the anthracycline glycoside antibiotic family. It is one of the most widely used drugs in the clinical treatment of several cancers. Interaction of daunorubicin with its nuclear target and with membrane has been widely reviewed [1,2], bringing to light several possible mechanisms of action. Unfortunately, cardiotoxicity places a limit on the total dose of daunorubicin that may be given [3]. Interaction of the drug with mitochondrial membrane has been suspected (for a review see Ref. 2) to be responsible for this toxicity. The other limiting factor in the clinical use of daunorubicin is spontaneous or acquired resistance (for a review see Ref. 4).

Membrane transport plays a central role in the antitumor activity of anthracyclines since it determines the rate of drug entry and exit from cells, and therefore the concentration of drug at the intracellular target. The mechanism of transport of these drugs is not yet adequately understood but may involve both carrier-mediated transport and simple diffusion [5].

Drugs that modify membrane properties can also modify membrane transport of daunorubicin. Indeed, amphotericin B, a polyene antibiotic which alters membrane permeability, has been shown to induce sensitivity to anthracycline in human neoplasia [6]: in mice with ovarian cancer simultaneous administration of amphotericin B and adriamycin, another glycoside anthracycline very similar to daunorubicin, resulted in long-term survival in 83% of mice compared to 65% of mice treated with adriamycin only [7]. Potentiation of adriamycin by amphotericin B against AKR leukemia has also been demonstrated [8,9]. The

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Abbreviations: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; diS-C_3-(5), 3,3'-dipropylthiadicarbocyanine.

synergistic effect of amphotericin B is not limited to anthracyclines. It has also been demonstrated for several kinds of antitumor agents. It has particularly been studied for actinomycin D [10] and 1-2-(chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) on the human promyelocytic leukemia cell line HL-60 [11]. The explanation of this synergy at the cellular level and the proposition of a molecular mechanism is therefore of current interest.

In the present work, we studied the synergistic incorporation of daunorubicin into human erythrocytes in the presence of several membrane effectors, polyene antibiotics and valinomycin. The choice of human erythrocytes as a cellular model is supported by the extensive description of the membrane structure and function of these cells. Furthermore, the absence of the nucleus may simplify the interpretation of the results, since interference with DNA-daunorubicin interactions is avoided.

After showing the conditions in which synergism occurs, we will point out the concomitant hyperpolarisation of the membrane by the effectors. We will then be in a position to propose a mechanism for synergism.

Methods

Drugs and chemicals. The polyene antibiotics were generous gifts from Squibb, France (amphotericin B) and Professor Borowski (vacidin A). They were used without further purification. They were dissolved in dimethylsulfoxide aliquot (1 mg/100 μl) and then suspended in aqueous buffer and used on the same day. Their concentrations were determined from their absorption.

Valinomycin was a Sigma product. Concentrated solutions were prepared in ethanol.

Purified daunorubicin was kindly provided by Laboratoires Rhône-Poulenc. Concentrations were determined by diluting aqueous stock solutions to approx. 10^{-5} M and measuring their absorption ($\varepsilon_{480} = 11\,500\ cm^{-1} \cdot M^{-1}$).

Dipropylthiadicarbocyanine (diS-C_3-(5)) has been kindly supplied by Dr. Waggoner.

Sodium azide was from Prolabo.

Buffers. Phosphate-buffered saline was made up of 150 mM NaCl buffered with 7 mM Na_2HPO_4 and 3 mM KH_2PO_4 to a pH of 7.4. Sucrose buffer was made up of 250 mM sucrose, 10 mM glucose, 5 mM $MgCl_2$, 5 mM KCl, buffered with 10 mM sodium phosphate to a pH of 7.4.

In the cases specified below, phosphate-buffered saline was supplemented with 2 mM EDTA in the acidic form.

Phosphate and chloride salts were Prolabo products (Normapur grade). Sugars and EDTA were Sigma Grade 1 products.

Preparation of erythrocytes. Human venous blood was collected from normal donors in tubes containing EDTA (1 mg EDTA/ml blood) and immediately centrifuged at $1300 \times g$ for 10 min. The plasma and buffy coat were then removed. Erythrocytes were then washed three times with phosphate-buffered saline and dispersed either in the desired medium for immediate use or in sucrose buffer for later use (not more than 4 days later). Cells were counted in a hematocymeter.

Incubation procedures. Erythrocytes, prewarmed to 37°C, were added to 5 ml of the appropriate buffer supplemented with the effector of membrane transport to be assayed and were incubated for 30 min at 37°C. In all experiments, the final cell concentration was $1.8 \cdot 10^8$ cell per ml. Final concentrations of dimethylsulfoxide or ethanol in which membrane effectors were dissolved were always lower than 1%, v/v. No influence of these substances on the results has been detected. Daunorubicin was then added to a final concentration 10^{-4} M and the solution was further incubated for 1 h at 37°C. The cells were then separated from the buffer by centrifugation at $1300 \times g$ for 10 min and were rinsed three times in 5 ml of phosphate-buffered saline. Cell pellets were lysed in 2 ml of 5 mM hypotonic phosphate buffer (pH 7.4) at 0°C. The membranes were then separated from the hemolysate by centrifugation at $12\,000 \times g$ for 10 min. Pelleted ghosts were resuspended in 2 ml of 5 mM phosphate buffer (pH 7.4).

Determination of the intracellular anthracycline content. Supernatants collected just after incubation, highly concentrated in anthracycline, were analyzed by electronic absorption at 480 nm. Supernatants resulting from cell washings and hemolysis, as well as ghost suspensions with smaller anthracycline contents, were analyzed by measur-

ing fluorescence emission at 595 nm (excitation at 450 nm).

At 480 nm, the effectors of membrane do not absorb and the residual hemoglobin released during incubation does not contribute significantly to the absorption at the concentration present in the medium (Fig. 1). On the other hand, fluorescence measurements of hemolysate and ghost suspensions had to be corrected for inner filter effects due to hemoglobin absorption at 450 and 595 nm.

The correction is given by:

$$F = F_o \text{ antilog}\left(\frac{A_{450} + A_{595}}{2}\right)$$

where A_{450} and A_{595} are the absorbances at the excitation and emission wavelength, respectively, and F and F_o the corrected and observed fluorescence emission intensities, respectively.

Nevertheless, this theoretical correction is not valid for high absorbances; under our conditions, absorbances never exceeded 0.1.

Absorption spectra were recorded with a Cary 219 spectrophotometer and fluorescence spectra with a Jobin-Yvon JY3C spectrofluorimeter.

Membrane potential studies. Membrane potential variations induced by the membrane effectors were followed using the monocationic fluorescent probe diS-C_3-(5).

It has been shown (for a review see Ref. 12) that diS-C_3-(5) fluorescence can be used to record negative membrane potentials of red blood cells. This monovalent cation, which permeates through cell membranes, distributes itself between intra- and extracellular compartments according to the Nernst equation:

$$\frac{[D^+]_o}{[D^+]_i} = e^{V_m F/RT}$$

where $[D^+]_o$ and $[D^+]_i$ are the outside and inside dye concentrations, respectively. The binding of the dye to intracellular contents, that is essentially to hemoglobin in the case of red blood cells, results in a decrease of the fluorescence intensity and in a red shift of the maximum emission wavelength. Since the amplitude of the decrease depends on $[D^+]_i$, it can be correlated to V_m.

Concentrated stock solutions were prepared in ethanol. Fluorescence measurements were made at final concentrations ranging from 50 to 200 nM. Fluorescence emission intensity was monitored at 670 nm as time elapsed with the excitation wavelength set to 622 nm. Red blood cells and then membrane effectors were added to the dye solution once the fluorescence level had reached equilibrium. All recordings were taken at 25°C without stirring. We had previously checked by following the circular dichroism spectrum of amphotericin B that no interaction occurred between the polyene antibiotic and the probe at the concentrations used for fluorescence measurements.

Results

Daunorubicin uptake by erythrocytes: Influence of the polyene antibiotics

(A) Incubation in sucrose buffer. The absorption spectra of the media isolated after incubation and centrifugation were located between 400 and 600 nm (Fig. 1). The amount of daunorubicin contained in these media was determined from the absorbance at 480 nm, where no other species

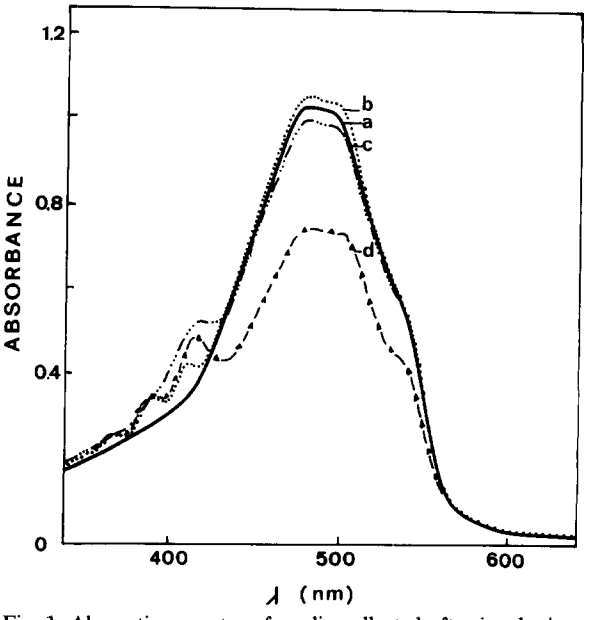

Fig. 1. Absorption spectra of media collected after incubation and centrifugation in sucrose buffer of a (———): 10^{-4} M daunorubicin; b (· · · · · ·): 10^{-4}M daunorubicin + 10^{-6} M amphotericin B; c (· · — · ·): 10^{-4} M daunorubicin + $1.8 \cdot 10^8$ cells per ml; d (▲———▲): 10^{-4} M daunorubicin + 10^{-6} M amphotericin B + $1.8 \cdot 10^8$ cells per ml. Optical pathway: 1 cm.

were absorbant. We had previously checked that the mixture of the drugs, daunorubicin and amphotericin B, did not modify the own absorption spectra of each compound, what was confirmed by fluorescence and circular dichroism. This indicated the absence of any significant interaction between the two drugs. Small peaks around 400 nm were related to the presence of free amphotericin B. Peaks at 416 nm indicated that some hemolysis had occurred. At the concentrations used, the drugs investigated had at most a slight hemolyzing effect when considered separately (less than 3% of the totally hemolyzed cells). However, hemolysis occurred when drugs were combined. For instance, with 10^{-4} M daunorubicin and $2 \cdot 10^{-6}$ M amphotericin B in phosphate-buffered saline, at the end of incubation 10% of the cells had lysed while only 2.5% has lysed in the absence of daunorubicin (for 10^{-6} M amphotericin B there was no difference). It should be noted that the synergistic effects observed in our experiments occurred at concentrations where no lysis was observed.

The amounts of daunorubicin retained by the cells in the absence and in the presence of 10^{-6} M amphotericin B are reported in Table I.

Slight uptake of daunorubicin occurred when erythrocytes were incubated with daunorubicin. In the presence of 10^{-6} M amphotericin B, approx. 5-times more daunorubicin was taken up than without amphotericin B.

TABLE I

AMOUNTS OF DAUNORUBICIN RETAINED IN THE ABSENCE AND IN THE PRESENCE OF AMPHOTERICIN B

(A) Concentration of daunorubicin determined in the supernatant after incubation and centrifugation of samples containing (a) 10^{-4} M daunorubicin, (c) $1.8 \cdot 10^{8}$ cells/ml + 10^{-4} M daunorubicin, (d) $1.8 \cdot 10^{8}$ cells/ml + 10^{-6} M amphotericin B for 30 min. + 10^{-4} M daunorubicin for 1 h. (B) Daunorubicin (in mg) retained by 10^{10} cells under the same conditions as in (A).

Samples	Daunorubicin [(A)] (10^{-4} M)	Daunorubicin [(B)] (mg)
a	1.02 ± 0.01	–
c	0.96 ± 0.01	0.17 ± 0.05
d	0.76 ± 0.01	0.82 ± 0.05

Fig. 2 shows a plot of the ratio, $R([X])$, of the amount of daunorubicin taken up by erythrocytes at a given concentration [X] of amphotericin B or vacidin to the amount of daunorubicin taken up under the same conditions but without the polyene antibiotic as a function of the concentration of amphotericin B or vacidin.

The strong increase in the daunorubicin retention occurred when amphotericin B concentration was higher that $4 \cdot 10^{-7}$ M. This increase was constant up to $2 \cdot 10^{-6}$ M. At higher concentration, hemolysis occurred to such an extent that significant results could not be obtained. Similar effects were observed with vacidin for which a comparable dose-response curve was obtained, except that threshold for the facilitation of daunorubicin incorporation was shifted to a lower polyene antibiotic concentration ($5 \cdot 10^{-8}$ M) (see Fig. 2).

The daunorubicin contents of the supernatants of washings with phosphate-buffered saline and of hemolysis of the pellets, as well as those of the erythrocyte membranes, were estimated by fluorescence as described in Methods.

The corresponding results are given in Table II. After three washings, significant amounts of daunorubicin were still found in the supernatant; this could not be attributed to dilution of the small volumes of supernatant not removed in the previ-

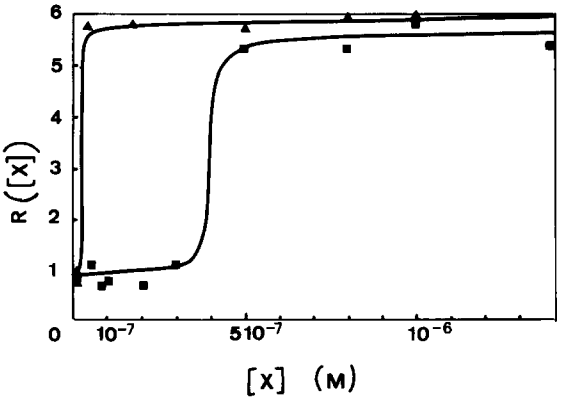

Fig. 2. Ratio of the amount of daunorubicin incorporated in the presence of vacidin (▲) or amphotericin B (■) to the amount incorporated in the absence of antibiotic, as a function of [X], the concentration of antibiotic present in the medium. Results obtained in sucrose buffer (pH 7.4), 10^{-4} M daunorubicin and $1.8 \cdot 10^{8}$ cells per ml.

TABLE II

AMOUNTS OF DAUNORUBICIN DETERMINED IN THE ABSENCE AND IN THE PRESENCE OF AMPHOTERICIN B AT EACH STEP OF THE EXPERIMENT

A, B and c, d same as in Table I.

Samples	Daunorubicin [A] (10^{-5} M)		Daunorubicin [B] (mg)
1st washing	(c)	0.025	0.07
	(d)	0.65	0.18
2nd washing	(c)	0.12	0.03
	(d)	0.38	0.11
3rd washing	(c)	0.06	0.02
	(d)	0.42	0.12
Hemolysate	(c)	0.25	0.03
	(d)	1.61	0.18
Membrane suspensions	(c)	0.12	0.013
	(d)	1.05	0.12

ous washings. If the washings were performed in sucrose buffer instead of phosphate-buffered saline, twice as much daunorubicin was found in the supernatants. We considered therefore, that the binding equilibrium of the drug to erythrocytes may have been perturbed by the washings. Thus, the data obtained from the supernatants of hemolysis and the erythrocyte membranes did not correspond to the initial situation just after incubation. However, they indicated that daunorubicin was, in part, bound to the membranes.

(B) Sodium azide (NaN_3)-treated erythrocytes in sucrose buffer. The same experiments as reported in (A) were performed with erythrocytes treated, 1 h before the beginning of the incubation, with 10^{-2} M NaN_3. This salt is known to be a strong nonspecific inhibitor of enzyme activity. Measurements of daunorubicin absorbance in the incubation supernatants showed that the inhibitor did not significantly alter daunorubicin uptake by the erythrocytes, nor did it modify the uptake enhancement induced by amphotericin B.

(C) Incubation in phosphate-buffered saline. As already observed [13], the hemolysis induced by polyene antibiotics occurred for much lower concentrations in phosphate-buffered saline than in sucrose buffer. In order to perform the experiments in phosphate-buffered saline in the same concentration range as in sucrose buffer, 2 mM EDTA, which protects cells against hemolysis, was added to phosphate-buffered saline. Prior to these determinations, we checked that in sucrose buffer, EDTA did not affect the characteristics of daunorubicin incorporation described above.

Under these conditions, the uptake of daunorubicin by erythrocytes was found to be twice as high as in sucrose buffer. This retention was not modified by the presence of amphotericin B over the concentration range extending from 10^{-8} to 10^{-6} M, that is in a range where the synergistic effect was clearly displayed in sucrose buffer.

Thus, amphotericin B did not affect daunorubicin incorporation in erythrocytes in phosphate-buffered saline.

Daunorubicin uptake by erythrocytes: influence of valinomycin

The uptake of daunorubicin by erythrocytes suspended in phosphate-buffered saline was followed in the presence of the peptide ionophore valinomycin at concentrations of 10^{-6} and 10^{-5} M. This treatment, at either concentration, led to an enhancement of the amount of drug retained by the cells (Table III). The enhancement was similar to that obtained with the polyene antibiotic treatment of erythrocytes suspended in sucrose.

TABLE III

AMOUNTS OF DAUNORUBICIN DETERMINED IN THE ABSENCE AND IN THE PRESENCE OF VALINOMYCIN

A and B as in Table I. Samples: (a) $5 \cdot 10^{-5}$ M daunorubicin, (b) $3.6 \cdot 10^{8}$ cells per ml + $5 \cdot 10^{-5}$ M daunorubicin, (c) $3.6 \cdot 10^{8}$ cells per ml + 10^{-6} M valinomycin + $5 \cdot 10^{-5}$ M daunorubicin, (d) $3.6 \cdot 10^{8}$ cells per ml + 10^{-5} M valinomycin + $5 \cdot 10^{-5}$ M daunorubicin.

Samples	Daunorubicin [A] (10^{-5} M)	Daunorubicin [B] (mg)
a	5.01 ± 0.1	–
b	4.50 ± 0.1	0.07 ± 0.05
c	3.25 ± 0.1	0.24 ± 0.05
d	3.4 ± 0.1	0.22 ± 0.05

Membrane potential and membrane effectors

Membrane potential changes induced by valinomycin. It is well documented that the peptide valinomycin creates in red blood cells a specific and dominating conductance to K^+ setting membranes at a potential close to the K^+ equilibrium (Eqn. 1):

$$V_m = -\frac{RT}{F}\log\frac{[K^+]_i}{[K^+]_o} \quad (1)$$

Fig. 3 shows the fluorescence tracings obtained when adding diS-C_3-(5), then cells and then valinomycin to isotonic buffered mixtures of NaCl/KCl of different ratios. As expected at physiological external K^+ concentration valinomycin induced a significant decrease of DiS-C_3-(5) fluorescence together with a red shift of the maximum emission. Indeed, in those conditions valinomycin-induced K^+ leakage led to a hyperpolarisation of the membrane. The subsequent entry of the dye into the cells is at the origin of the fluorescence decrease. From these results, a calibration curve connecting fluorescence level after valinomycin addition with membrane potential calculated from the preceding equation was obtained (Fig. 4). That was then used to estimate potential changes induced by polyene antibiotics in buffer conditions in which daunorubicin uptake experiments had been performed.

It is interesting to note that close fluorescence levels were obtained after addition of untreated cells in phosphate-buffered saline and in sucrose buffer indicating potential values around −30 mV (negative-inside). Cells in phosphate-buffered saline exhibited only slightly more negative value than cells in sucrose buffer. On the other hand, when cells were placed in media of increasing KCl proportion, potential evolved to less negative values. For higher KCl concentrations fluorescence level indicated potential values around −10 mV. Generally, electrical potential of red blood cells at thermodynamical equilibrium under standard conditions is admitted to be given by chloride ratio according to constant field equation and assuming that membrane permeability to Na^+ and K^+ are negligible compared to permeability to Cl^-. It

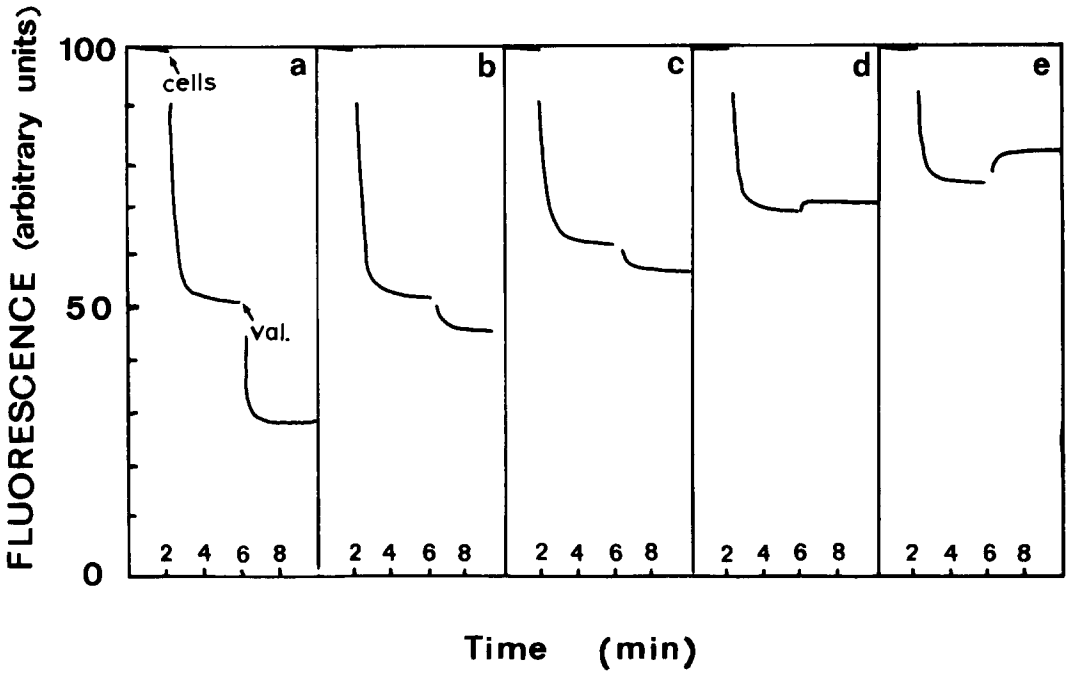

Fig. 3. Fluorescence tracings from 66 nM diS-C_3-(5) on addition of first cells at final hematocrit 0.1% and then valinomycin at final concentration 1 μM in isotonic mixtures * of KCl and NaCl buffered with 5 mM phosphate. Excitation and emission wavelength were set to 622 and 670 nm, respectively. * (a: 150 mM NaCl; b: 15 mM KCl+135 mM NaCl; c: 30 mM KCl+120 mM NaCl; d: 60 mM KCl+90 mM NaCl; e: 90 mM KCl+60 mM NaCl.)

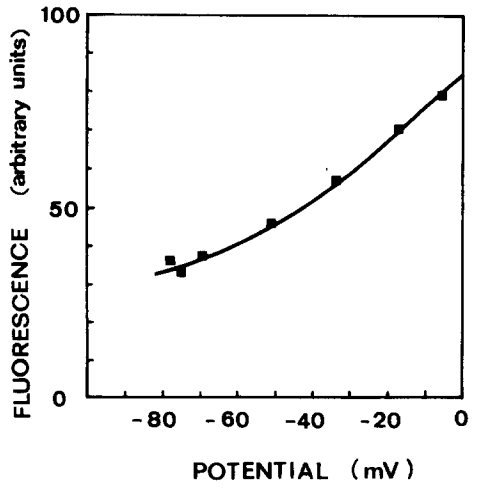

Fig. 4. Calibration curve giving the relationship between the fluorescence level of suspensions containing red blood cells at final hematocrit 0.1% in media with various concentrations of KCl and 10^{-6} M valinomycin, and the membrane potential calculated from K^+ equilibrium (internal K^+ concentration was taken equal to 110 mM). Same excitation and emission wavelength as in Fig. 3.

seemed that we cannot, under the experimental conditions used here, neglect membrane permeability to K^+ and that the K^+ concentration gradient induced a slight leakage of K^+ leading to more negative values of potential in NaCl buffer than in KCl buffer. Nevertheless, what is significant for calibration and the following experiments presented here is that membrane permeability to K^+ is largely predominant in the presence of valinomycin and that red blood cell membranes reached comparable values in phosphate-buffered saline and in sucrose buffer.

Membrane potential changes induced by amphotericin B. Experiments similar to those performed with valinomycin were done in sucrose buffer and in phosphate-buffered saline using amphotericin B as membrane effector. The final concentration of amphotericin B was $5 \cdot 10^{-7}$ M and cells were diluted to a hematocrit equal to 0.1%. Fluorescence tracings, presented in Fig. 5, show the decrease of diS-C_3-(5) fluorescence intensity for amphotericin B-treated cells suspended in sucrose buffer. Amphotericin B-treated cells in phosphate-buffered saline followed a different pathway. In the latter case, the addition of amphotericin B was followed by a slight increase in fluorescence intensity, indicating that in phosphate-buffered saline amphotericin B induced a very slight depolarisation, whereas in sucrose buffer it induced a strong hyperpolarisation comparable to that observed with valinomycin. The kinetics of hyperpolarisation in sucrose buffer are slower with amphotericin B than with valinomycin.

Discussion

We observed that the amount of daunorubicin taken up by erythrocytes is about 5-times greater: (1) in the presence of the polyene antibiotics

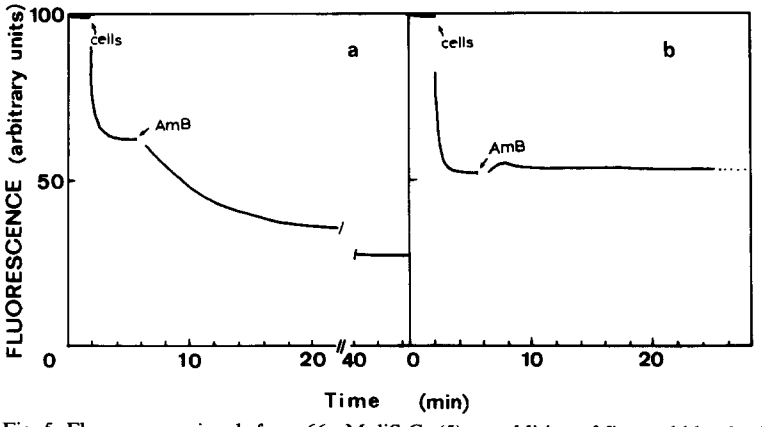

Fig. 5. Fluorescence signals from 66 nM diS-C_3-(5) on addition of first red blood cells at final hematocrit 0.1% and then amphotericin B at final concentration of $5 \cdot 10^{-7}$ M in (a) sucrose buffer and (b) phosphate-buffered saline. Excitation and emission wavelength as in Fig. 3.

amphotericin B or vacidin, in sucrose buffer and (2) in the presence of valinomycin in either phosphate-buffered saline or in sucrose buffer. The absence of effect of the enzyme inhibitor NaN_3 on the synergism permits us to rule out the hypothesis of any involvement of enzymatic process in this phenomenon.

Amphotericin B and vacidin can increase quite specifically the permeability of the human erythrocyte membrane to monovalent cations [14–16]. The dose-response curves obtained from K^+ leakage [13] show the same sharp increase over the same range of antibiotic concentration as do the dose-response curves obtained when daunorubicin uptake is followed (our work). The first point is then that the synergistic effect of the membrane effectors amphotericin B, vacidin and valinomycin seems to be related to their ionophore properties.

The second point is linked to the activity of these effectors on membrane potential. We have shown that hyperpolarisation of the membrane occurs with amphotericin B or vacidin treatment in sucrose buffer and with valinomycin in both sucrose and phosphate saline buffer. This may be understood by taking into account the ionophore properties of each effector and the ionic composition of the buffers. Since vacidin and amphotericin B have poor intercationic selectivity and since the only monovalent cation present was K^+, we expected to observe in sucrose buffer the same hyperpolarisation with vacidin as with amphotericin B. In contrast, in phosphate-buffered saline, vacidin and amphotericin B transported Na^+ of the buffer as well as K^+ of the erythrocytes and no hyperpolarisation occurred, while valinomycin transported only K^+ which resulted in the same hyperpolarisation as in sucrose buffer.

The transmembrane potential of erythrocytes in sucrose buffer after addition of amphotericin B has been calculated by other authors [17]. A sharp drop from 30 to -20 mV was calculated, confirming our observation. Indeed, we have observed synergistic incorporation only under conditions where amphotericin B induced membrane hyperpolarisation. More, at pH 7.4 a large number of the daunorubicin molecules are positively charged on the NH_2 group [18] and since it has been proposed that daunorubicin enters the cell in part by simple diffusion [7], we can ascertain that daunorubicin could be partitioned between intra- and extracellular compartments according to the Nernst relation, i.e., following membrane potential.

A similar dependence of cellular uptake of positively charged drug molecules on the electric membrane potential has recently been demonstrated with 2-*N*-methylellipticinium and L-1210 cells in the presence of valinomycin [19].

The synergistic erythrocyte entrapment of daunorubicin has already been demonstrated to occur in the presence of amphotericin B [20], but no remark has been given about the importance of the medium on the phenomenon and about the extension of the synergism to other antibiotics. Moreover, the hypothesis of membrane lesion responsible for the enhancement of the uptake seems quite unlikely.

At the concentrations used, the polyene antibiotics create channels of which the diameter has been estimated to be 8 Å [21]. That means that only ions and very small molecules, not larger than glycerol, may permeate through these channels. It must be also pointed out that the studies devoted to amphotericin B, these last 20 years have led to the common assent that polyene antibiotics attacked the sterol domain of the cell membranes [22]. On the other hand, it has been shown that daunorubicin did not permeate through lipid vesicles [23], making very likely the hypothesis of a passive but protein-related transport of daunorubicin in the cells. So the perturbation caused by amphotericin B to the lipid matrix is certainly not adequate to facilitate the daunorubicin uptake in the absence of a driving force identified in this paper as an electrical driving force.

The synergistic incorporation of daunorubicin in erythrocytes that we have observed is unlikely to occur in conditions closer to the biological situation that is in serum. Indeed, it has been demonstrated [24] that erythrocytes are protected from the amphotericin B action because the drug is bound to lipoproteins and serum albumin. However, such a protection is much lower with other cells which allows the possible occurrence of the synergistic uptake of daunorubicin. We are currently studying this opportunity.

Generally considering, anthracycline therapy

poses problems such as resistance and cardiotoxicity, or more precisely the specific accumulation in, respectively, resistant cells and mitochondria. There is some support for the hypothesis that membrane potential could be a determining factor in how anthracyclines affect their targets. For instance, resistance to anthracyclines can be reversed by verapamil [25] which is a Ca^{2+} channel blocker. Ca^{2+} follows a complicated and not well understood transport pathway. Transport is probably electrogenic as it is for other cations, and its blockage would not be without effect on membrane potential. On the other hand, it should be considered that mitochondria, which seem to be preferential sites for anthracycline action, are cellular organelles where potentials change at high frequency and reach high absolute values.

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