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Interaction of ^{14}C-labelled amphotericin B derivatives with human erythrocytes: relationship between binding and induced K^+ leak

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Four ^{14}C-labelled amphotericin B (Am B) derivatives with different net electric charges were examined: zwitterionic *N*-fructosyl Am B, positively charged *N*-fructosyl Am B methyl ester, negatively charged *N*-acetyl Am B and neutral *N*-acetyl Am B methyl ester. The binding of these four derivatives to human red cells and their octanol-water partition coefficients were measured. Simple partitioning between red cells and buffer was found for the four compounds, regardless of concentration, within a range of 10^{-8} and 10^{-4} M. This indicates the absence of cooperativity and saturability of binding at least in this concentration range. The constant partition coefficients were found to be three to five times higher for the two methyl ester derivatives than for the two non-esterified compounds. All partition coefficients were proportional to those found for the octanol-water system. Efficiency in inducing K^+ leak from red cells was measured during the binding experiments. Despite the higher partition coefficients of the two methyl ester derivatives, they were found to have much lower ionophoric efficiency than the two non-esterified compounds. These results are discussed in terms of the mechanism of permeability pathway formation by polyene antibiotics.

Introduction

Amphotericin B, an antifungal polyene macrolide, is still the principal drug for the treatment of systemic mycosis despite the numerous side effects it causes, such as nephrotoxicity, anaemia, and electrolyte abnormalities, on account of its low selective toxicity [1].

Amphotericin B interacts with cell membranes, induces permeability to ions and small non-electrolytes, and consequently impairs cell functions. However, the molecular mechanism of action of the drug at membrane level is not understood [1–3].

Numerous amphotericin B derivatives have been synthesized in the search for new, more efficient and less toxic drugs. The relationship between the structure and the toxicity of these derivatives have been systematically studied in order to determine which parts of the antibiotic molecule are essential to induce membrane permeability. The selective toxicity of the compounds examined was determined by comparing the damages such as K^+ leakage and growth inhibition which they induce in yeast cells (representing pathogenic microorganisms) with the damages such as K^+ leakage

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and haemolysis induced in human red cells (representing mammalian host cells) [4]. Further studies are in progress in order to clarify the mechanisms by which the cell toxicity of amphotericin B derivatives can be modified. One of the questions raised in this connection was whether the differences observed in the cell toxicity of these derivatives resulted from their different affinities for the cell or from differences in the toxic effects of bound molecules. The determination of the extent of binding of these compounds to cells is necessary to answer this question.

In spite of the spectroscopic properties of amphotericin B such as absorption and circular dichroism which make it possible to follow this antibiotic interaction with model membranes [5,6] or red cell ghosts [7], it is difficult to use these characteristics when the interaction of amphotericin B with cells is investigated, because they scatter light and induce important spectral artifacts. Furthermore, for red cells, haemoglobin absorption bands completely mask the amphotericin B spectrum.

In this paper we describe the interaction between human erythrocytes and ^{14}C-labelled amphotericin B derivatives in terms of their affinity for red cells and its relation to potassium permeability inducement.

Since, as previously shown [8–10], the nature of the interaction between the polyene macrolide molecule and the membrane largely depends on the electric charge of the molecule, this study was carried out with four derivatives of amphotericin B, rendered zwitterionic, neutral, cationic or anionic by the modification of the ionizable groups of the mother molecule. All four derivatives exhibit different degrees of antifungal activity [4].

Materials and Methods

Four ^{14}C-labelled derivatives of amphotericin B: *N*-fructosyl Am B, *N*-fructosyl Am B methyl ester, *N*-acetyl Am B and *N*-acetyl Am B methyl ester (Fig. 1) were synthesized as previously described [11] using [^{14}C]glucose (uniformly labelled) and [1-^{14}C]acetic anhydride. The specific radioactivity obtained was 12 mCi/mmol for the glycosylated compounds and 20 mCi/mmol for the acetylated compounds.

Their chemical purity after synthesis and their stability during storage in the solid state at $-70\,^{\circ}$C were tested by thin-layer chromatography, autoradiography and absorption spectroscopy.

The biological activity of the four derivatives, expressed as their haemolytic activity in human red cells, was concomitantly measured.

All the above tests showed that the four ^{14}C-labelled compounds remained stable during storage for at least three months.

Human erythrocytes were isolated (by centrifugation at $1500 \times g$ for 5 min) from fresh blood samples from healthy donors, serum and buffy coat were removed, and the erythrocytes were washed three times in isotonic phosphate saline buffer (140 mM NaCl, 30 mM sucrose and 5 mM sodium phosphate, pH 7.4). The red cells were used within 24 h.

The four compounds were first dissolved in dimethylformamide whose final concentration in the samples never exceeded 0.5% and did not affect the erythrocytes. Before addition to the samples, the antibiotic solution in dimethylformamide was diluted in the buffer to permit the addition of easily measurable volumes.

Binding of amphotericin B derivatives to red cells

For each antibiotic, the same amounts giving final concentrations ranging from 10^{-8} M to 10^{-4} M were simultaneously added to one series of scintillation vials and two series of 5 ml pyrex tubes. One of the series of tubes contained 2 ml of red cell suspension at $2 \cdot 10^9$ cell/ml (haematocrit 20%), and the other, 2 ml of buffer only.

Both series of tubes were incubated for 90 min at $37\,^{\circ}$C with mild shaking and then centrifuged at $1500 \times g$ for 5 min. The radioactivity of the supernatants in the two series of tubes and the scintillation vials was counted, giving three sets of radioactivity counts, m, m' and t, representing, respectively, the amounts of antibiotic in the supernatants in the tubes containing red cells, those containing buffer, and in the scintillation vials.

Since we added the same amount t of antibiotic to one tube containing red cells and the corresponding scintillation vial, we can write:

$$t = m + a + b$$

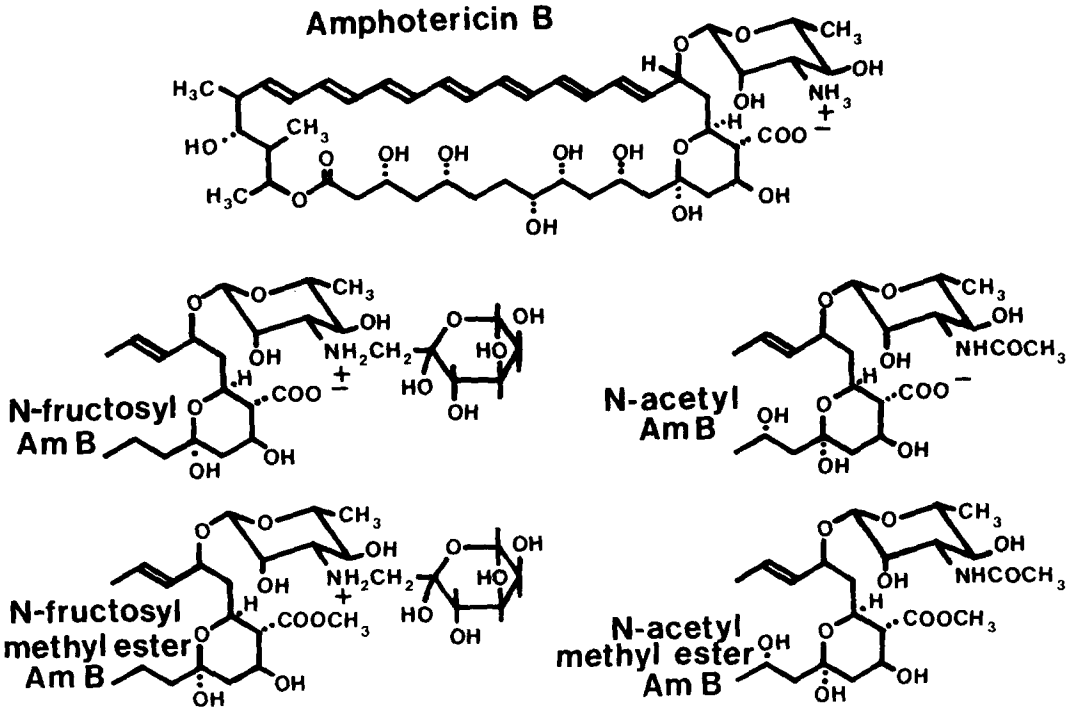

Fig. 1. Structure of amphotericin B and of the four derivatives studied.

where m, a and b are, respectively, the amount of antibiotic in the supernatant, the free antibiotic which aggregates and sediments with the cells, and the antibiotic which binds to the red cells.

Therefore:

$$b = t - (m + a)$$

To estimate the amount, a, of free antibiotic sedimenting as aggregates, a sedimentation curve was drawn plotting the m'/t ratio, representing the fraction of unbound antibiotic which does not sediment, versus (m'), the concentration of non-sedimented antibiotic (Fig. 2).

Partition of antibotic between buffer and octanol

The antibiotic was introduced into the pyrex tube containing buffer only, and octanol was then added. When the tube contained octanol and the antibiotic was added, followed by the buffer, this did not affect the partition coefficient.

After 15 min incubation at 37°C under shaking, the partition equilibrium was reached. Centrifugation at 1500 × g for 5 min permitted a clear phase separation and the amount of radioactivity in each phase was determined.

Potassium determination

For each binding experiment, the K^+ leakage from red cells induced by bound radiolabelled antibiotic was measured on an aliquot using a K^+-selective electrode (F2312K Radiometer Copenhagen). 100% potassium release was measured after complete haemolysis by osmotic shock.

Preparation of red cell resealed ghosts

The resealed human erythorcyte ghosts were prepared by the method of Steck [12] and the amount of ghosts in the suspension was determined as the amount of membrane phospholipids, using ammonium ferrothiocyanate colorimetry [7,13].

Binding to red cell resealed ghosts

(A) By radioactivity counting. The experiments were performed as for red cells, except that centrifugations were carried out at 16000 × g for 20 min.

(B) By circular dichroism. Binding was determined by monitoring the strong excitonic doublet of free antibiotic and its disappearance when the antibiotic bound to membranes, as described in detail previously [7].

Results

Adsorption of amphotericin B derivatives on tube walls

In the preliminary experiments, the possible adsorption of the radiolabelled amphotericin B derivatives was tested on different materials. For this purpose, 1 ml of the derivative solution in buffer at concentrations ranging from 10^{-8} to 10^{-4} M was incubated for 5 min in 5 ml tubes of plastic, ordinary glass and pyrex. The counts per minute found in the solution after incubation (m_c) were compared to the counts denoted t, obtained by direct addition of the same amount of antibiotic to the scintillation vial as the amount added to the tubes. It was found that for antibiotic concentrations up to 10^{-6} M, the m_c/t ratios were as low as about 0.7 for solutions in plastic and ordinary glass tubes, indicating that about 30% of the total amount of derivative was taken up by the tube walls.

For solutions in the pyrex tubes, the m_c/t ratios obtained from four independent experiments were on the contrary, found to be 1 ± 0.05, 1 ± 0.1, 1 ± 0.05 and 0.9 ± 0.05 for *N*-fructosyl Am B, *N*-fructosyl Am B methyl ester, *N*-acetyl Am B and *N*-acetyl Am B methyl ester, respectively, indicating that for concentrations of 10^{-8} to 10^{-6} M, pyrex only adsorbed the last derivative significantly (about 10%).

Pyrex tubes were therefore used in subsequent experiments.

Antibiotic sedimentation during centrifugation

It is well known that amphotericin B and most of its various derivatives are poorly water-soluble. At concentrations between 10^{-7} and 10^{-6} M, depending on the compound, they generally form aggregates whose amount increases with the total antibiotic concentration, as shown by circular dichroism spectroscopy [5,14]. Furthermore, at higher antibiotic concentrations, these aggregates tend to sediment, even at low centrifugation rates. To test the extent of this sedimentation during the centrifugation used to sediment the red cells in binding experiments, the four derivatives were incubated in phosphate saline buffer at concentrations between 10^{-8} and 10^{-4} M and then centrifuged.

The resulting sedimentation curve (Fig. 2) shows that the m'/t decreases progressively above 10^{-6} M for *N*-fructosyl Am B methyl ester, *N*-acetyl Am B and *N*-acetyl Am B methyl ester and above 10^{-5} M for the *N*-fructosyl derivative, indicating an increasing antibiotic sedimentation.

This sedimentation was taken into account when measuring antibiotic binding to red cells (see Materials and Methods).

Binding to red blood cells

Preliminary experiments showed that the binding became independent of time after 60 min. Consequently, an incubation period of 90 min was chosen for the binding experiments.

The results are given in Figs. 3A and B in which bound/free ratios are plotted versus the initial antibiotic concentration in the sample.

For the four amphotericin B derivatives, these ratios were constant and independent of the concentration. No cooperativity or saturability were

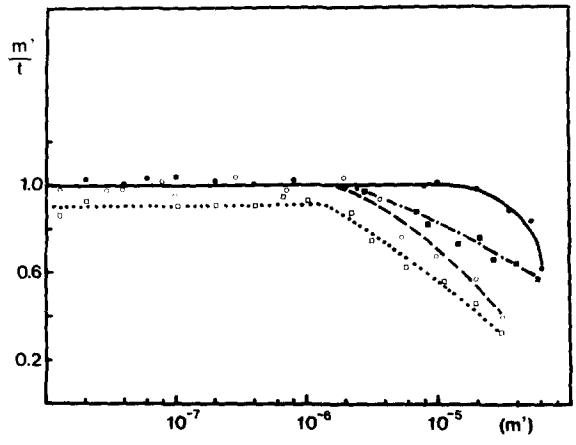

Fig. 2. Sedimentation curve of the four ^{14}C-labelled amphotericin B derivatives, plotted as the ratio of the amount of antibiotic in the supernatant m', to the total amount t, versus the antibiotic concentration in the supernatant (m'), after 90 min incubation in buffer at 37 °C. *N*-Fructosyl Am B (——), *N*-fructosyl Am B methyl ester (- - - - -), *N*-acetyl Am B (·—·—·) and *N*-acetyl Am B methyl ester (· · · · · ·).

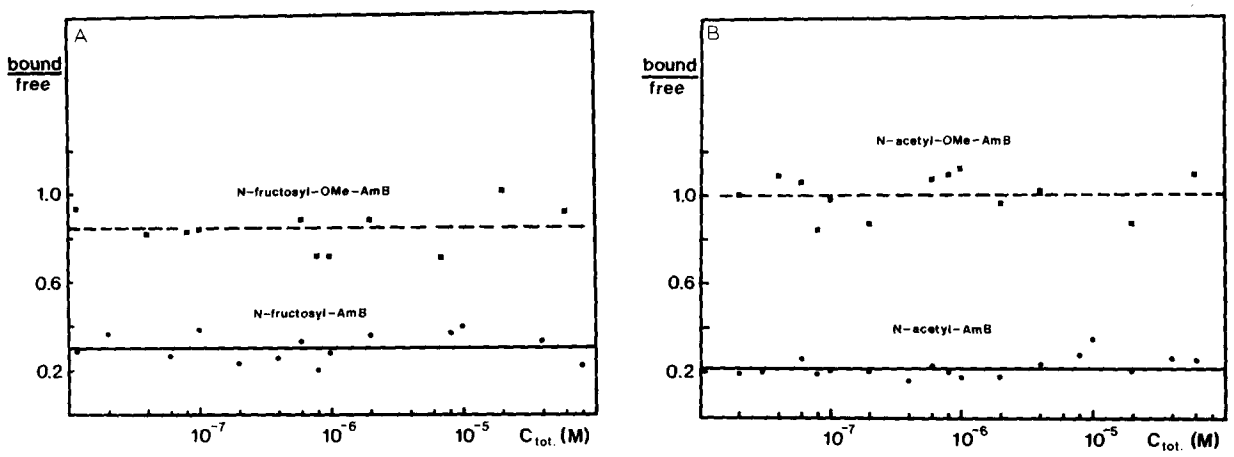

Fig. 3. Binding of the four ^{14}C-labelled amphotericin B derivatives to human red cells. Red cells ($2 \cdot 10^9$ cell/ml) were incubated with antibiotics for 90 min at 37 °C. Bound/free antibiotic ratios are plotted versus the initial antibiotic concentration C_{tot} in the suspension. (A) *N*-Fructosyl Am B (———) and *N*-fructosyl Am B methyl ester (- - - - -). (B) *N*-acetyl Am B (———) and *N*-acetyl Am B methyl ester (- - - - -).

observed. For the entire concentration range (10^{-8} to 10^{-4} M) this behaviour is characteristic of a simple antibiotic partitioning between red cells and external medium.

The effect of the red cell concentration in the suspension was also tested by measuring the binding of the *N*-fructosyl derivative to red cells in a suspension containing $2 \cdot 10^8$ cells/ml. Bound/free ratios were found to be constant, independent of the antibiotic concentration and about ten times lower than with $2 \cdot 10^9$ cells/ml (Table II).

The determination of antibiotics binding in the concentration range from 10^{-6} to 10^{-4} M, where sedimentation of aggregated antibiotics occurs, is based on the assumption that the red cells are without influence on monomer-aggregate equilibrium of free antibiotic i.e. without influence on the monomer/aggregate ratio.

In order to check this assumption the binding of *N*-fructosyl amphotericin B to red cell ghosts was determined in the concentration range from 10^{-6} to 10^{-4} M, by radioactivity counting and by circular dichroism. While the latter method monitors the free aggregated antibiotic and its subsequent disappearance when binding occurs, the former monitors the free monomeric form. Consequently, if the red cell ghosts had shifted the monomer/aggregate ratio, the results obtained by these two method would have been different. The last column of Table II shows that the results are not significantly different.

Therefore it can be concluded that the membranes does not shift the monomer/aggregate ratio and that both methods of binding measurement are valid.

Another fact which also confirms this validity is the absence of any deviation in binding curves for the antibiotic concentrations corresponding to the beginning of the aggregation ($\approx 10^{-6}$ M).

The fact that bound/free ratios were independent of the antibiotic concentration and proportional to the red cell concentration is characteristic of a simple equilibrium partitioning between phases.

The bound/free ratios obtained from three independent experiments with $2 \cdot 10^9$ red cell/ml fall into two groups: first, those for the methyl ester derivatives, which were 1 ± 0.02 and 0.84 ± 0.03 for *N*-acetyl Am B methyl ester and *N*-fructosyl Am B methyl ester, respectively, and second, those for the non-esterified compounds, which were 0.30 ± 0.02 and 0.22 ± 0.01 for *N*-fructosyl and *N*-acetyl derivatives, respectively.

The methyl-esterification of the carboxyl group increased binding to red cells, which rose 5-fold for *N*-acetyl derivatives and 3-fold for *N*-fructosyl derivatives compared to the corresponding non-esterified compounds.

As the bound/free ratios were found to be constant i.e. concentration-independent, it may be assumed that derivatives are simply partitioned between membrane lipids and the external aqueous medium. Bound/free ratios can therefore be computed as partition coefficients P_{RBC}, between two phases:

$$P_{RBC} = (\text{bound/free}) \cdot (V_{buffer}/V_{membrane})$$

assuming that the lipid volume V of the membrane is $5 \cdot 10^{-13}$ ml/red cell [15].

The above results were compared to partition coefficients P_{OCT} measured in the octanol-buffer system, for antibiotic concentrations between 10^{-7} and 10^{-6} M.

The P_{RBC} and P_{OCT} for the four derivatives studied are listed in Table I.

As this table shows, P_{OCT} and P_{RBC} fall into two groups corresponding to the methyl ester derivatives and the non-esterified compounds. The last column of the table shows that the P_{RBC}/P_{OCT} ratios are not significantly different for three of the four compounds, but that the *N*-fructosyl derivative ratio is apparently higher than the others.

This result is in agreement with the hypothesis that binding of amphotericin B derivatives on red cells, depends on their lipid solubility.

On the basis of the present antibiotic binding data, it was also possible to calculate the corresponding molar ratios of membrane lipids/bound antibiotic, assuming $4.8 \cdot 10^8$ lipid molecules per red cell [16]. At the highest antibiotic concentration studied (10^{-4} M) these molar ratios are: 40, 44, 86 and 111 for the *N*-acetyl methyl ester, the *N*-fructosyl methyl ester, the *N*-fructosyl and the *N*-acetyl derivative, respectively.

TABLE I

COMPARISON, FOR ^{14}C-LABELLED AMPHOTERICIN B DERIVATIVES, OF P_{RBC} WITH P_{OCT}

P_{RBC} is the coefficient for partition between red cells and buffer; P_{OCT} is the coefficient for partition between octanol and buffer.

Amphotericin B derivatives	P_{RBC} ($\times 10^{-2}$)	P_{OCT}	P_{RBC}/P_{OCT} ($\times 10^{-2}$)
N-Fructosyl	2.4±0.2	0.20±0.01	12.0
N-Acetyl	1.8±0.1	0.19±0.01	9.5
N-Fructosyl methyl ester	6.7±0.2	0.99±0.05	6.8
N-Acetyl methyl ester	8.0±0.2	0.93±0.05	8.9

Binding to resealed red cell ghosts

Recently, the binding of amphotericin B to resealed red cell ghosts was studied using circular dichroism spectroscopy [7]. This method is based on the determination of the amount of free amphotericin B by monitoring the strong excitonic doublet of free aggregated antibiotic, and its subsequent disappearance when amphotericin B binds to membranes. This method allows determination of amphotericin B binding in whole membrane suspensions, without centrifugation. However, its application is restricted to antibiotic concentrations greater than 10^{-6} M, in which significant amounts of aggregates are detectable, and to relatively diluted suspensions of red cell ghosts, which exhibit sufficiently small light scattering and absorption. Using this method, amphotericin B binding to ghosts in a suspension of $2 \cdot 10^8$ ghosts/ml was earlier found to be cooperative [7].

To establish whether the difference between these results and the present data was due either to the difference between red cells and ghosts, between amphotericin B and its derivatives or between the methods used, binding of *N*-fructosyl amphotericin B to red cell ghosts was measured by radioactivity counting (at $2 \cdot 10^8$ and $2 \cdot 10^9$ ghosts/ml) and by circular dichroism (at $2 \cdot 10^8$ ghosts/ml). These data were compared with the binding to red cells at $2 \cdot 10^8$ and $2 \cdot 10^9$ red cells/ml for the same antibiotic concentration range (10^{-6}–10^{-4} M) (Table II).

For red cells and ghosts, the bound/free ratio were independent on antibiotic concentration. The corresponding partition coefficient P_{GH} between ghosts and buffer at $2 \cdot 10^8$ ghosts/ml were not significantly different for the two methods used. This fact shows that the binding difference between amphotericin B and its *N*-fructosyl derivative is attributable to their different chemical structure.

The results given in Table II also show that P_{GH} decreases when ghosts concentration increases and that at $2 \cdot 10^8$ cells or ghosts/ml P_{GH} is about seven times higher than P_{RBC}.

TABLE II

P_{RBC} AND P_{GH} FOR *N*-FRUCTOSYL AMPHOTERICIN B FOR TWO MEMBRANE CONCENTRATIONS IN SUSPENSION

P_{RBC} is the coefficient for partition between red cells and buffer; P_{GH} is the coefficient for partition between resealed red cell ghosts and buffer. All values were obtained by radioactivity counting as described in Materials and Methods. For ghosts at $2 \cdot 10^8$ ghosts/ml, binding was also monitored by circular dichroism.

Cell or ghost concentration (*n* per ml)	P_{RBC} ($\times 10^{-2}$) from radioactivity measurement	P_{GH} ($\times 10^{-2}$)	
		from radioactivity measurement	from circular dichroism
$2 \cdot 10^8$	2.5 ± 0.5	16.0 ± 3	18.0 ± 2
$2 \cdot 10^9$	2.4 ± 0.2	4.6 ± 0.9	–

These results could be ascribed, respectively, to the ghost's folding surface [18] allowing contact between membranes at high ghost concentrations and to the modification of phospholipid packing of ghosts comparing to intact erythrocytes, due to the perturbation of membrane skeleton during haemolysis [20].

Nevertheless the higher partition coefficient P_{GH} of ghosts, $16 \cdot 10^2$, compared with the partition coefficient P_{RBC} on red cells, $2.5 \cdot 10^2$ would rule out the contribution of the red cell content (especially haemoglobin) to the antibiotic binding. This is in agreement with the widely admitted but not proved assumption that polyene antibiotic-cell interaction is limited to the cell plasma membrane.

Permeability inducement

The biological activity of the four amphotericin B derivatives presently studied was previously determined [4] on the basis of dose-response curves yielding 50% hemolysis (H_{50}) and 50% potassium loss (K_{50}), for a currently used suspension containing $2 \cdot 10^7$ cells/ml. For direct comparison of binding and permeability inducement, we measured, for each binding experiment, the K^+ leak of red cells at $2 \cdot 10^9$ cells/ml induced by each of the four radiolabelled derivatives during 90 min incubation at 37 °C.

The dose-response curves obtained (Fig. 4) clearly show that the non-esterified derivatives, i.e.

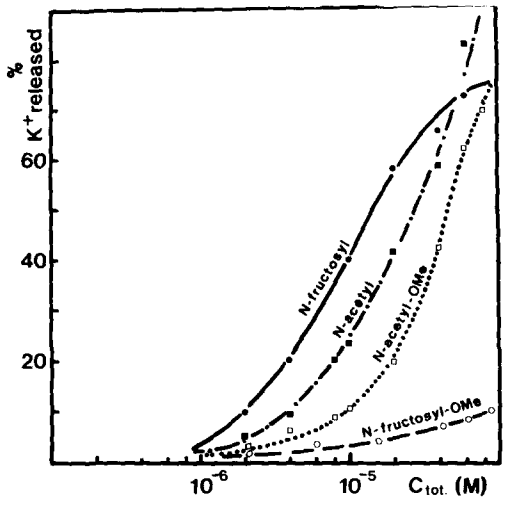

Fig. 4. Dose-response curve of K^+ release from red cells induced by ^{14}C-labelled amphotericin B derivatives during the binding experiments (90 min incubation at 37 °C). Experimental conditions as in Fig. 3. K^+ leak, expressed as per cent of total cellular K^+, is plotted versus the initial antibiotic concentration C_{tot} in the suspension. *N*-Fructosyl Am B (———), *N*-fructosyl Am B methyl ester (- - - - -), *N*-acetyl Am B (—·—·—) and *N*-acetyl Am B methyl ester (· · · · · ·).

N-fructosyl Am B and *N*-acetyl Am B whose binding to red cells was 3–5-fold smaller than that of their esterified homologues, exhibited a much higher ionophoric activity.

In accordance with the usual mode of expression, the abscissa of the dose-response curve in

TABLE III

AMOUNTS OF RADIOLABELLED DERIVATIVES NECESSARY TO INDUCE A 10% LEAK OF RED CELL INTRACELLULAR POTASSIUM

Incubation time 90 min, red cell suspension at $2 \cdot 10^9$ cell/ml, at 37 °C.

Amphotericin B derivatives	C_{tot} (μM)	*N* number of molecules bound per cell	*R*, lipid/bound Am B derivative (molar ratio)
N-Fructosyl	2	$1.1 \cdot 10^5$	4364
N-Acetyl	4	$1.7 \cdot 10^5$	2824
N-Acetyl methyl ester	10	$1.2 \cdot 10^6$	399
N-Fructosyl methyl ester	100	$1.1 \cdot 10^7$	44

Fig. 4 is the total initial concentration of antibiotic in the red cell suspension. The concentrations necessary to induce a 10% K^+ leak are given in the first column of Table III. However, this 10% leak was induced by the antibiotic actually present in the membrane, whose concentration can be calculated from the known partition coefficients P_{RBC} and expressed either as the number N of bound antibiotic molecules per red cell, or as the molar ratio R of membrane lipids/bound antibiotic (second and third columns of Table III).

Comparison of these sets of values shows that the difference in the permeabilizing activity of esterified and non-esterified derivatives is larger when estimated from the amount of antibiotic bound than from its total initial concentration.

These data definitely establish that, in spite of the greater binding of the methyl esterified compounds, they exhibit markedly lower permeabilizing activity calculated per bound molecule than the homologous non-esterified compounds.

Discussion

Many difficulties are encountered when studying the binding of polyene macrolides to membranes. The presence of hydrophilic and hydrophobic fragments in the macrocyclic ring is responsible for the ionophoric properties of these compounds and for their particular physicochemical properties.

One of the latter properties is surface adsorption, which means that the laboratory vessel must be carefully chosen as regards the material of which it is made and the total surface area of the suspension's contact with its walls. Another property is the self-association of molecules in aqueous media resulting from their low water solubility. When dissolved in buffer, the monomers form aggregates, even at concentrations lower than 10^{-6} M (Ref. 14 and circular dichroism data (not shown)) well before the aggregates reach a size which makes them easily sedimentable in low centrifugational fields.

It should therefore be stressed that under the present experimental conditions, 'soluble' simply means 'not sedimentable'. From this point of view, the fact that the circular dichroism, which monitors free aggregates, and radiolabelling, which monitors 'soluble' forms both yield coherent data confirms the validity of the two methods of binding measurement used here, even though this measurement became more and more complex as the polyene concentration and aggregation increased.

Another important result obtained in this work shows that the binding of the four amphotericin B derivatives studied behaves like a simple partitioning between red cells and buffer. The constant partition coefficients measured paralleled the octanol-buffer partition coefficients indicating that they are determined by liposolubility.

These results raised the question of the polyene-cell interaction and its relationship with ionophoric activity. While it is indisputable that the membrane sterols play a crucial role in the amphotericin B-model membrane interaction and in the resulting permeability inducement, it is also clearly proved that the phospholipid composition of the membrane has a substantial influence on the antibiotic-membrane interaction [19]. Consequently, it is risky to extrapolate the results observed for interaction of amphotericin B with model membranes to the interaction of this antibiotic with erythrocyte membranes whose composition and molecular organisation are more complex. This assumption is well confirmed by recent results [20] which clearly demonstrate that the red cell membrane skeleton strongly influences the interaction between another polyene antibiotic, filipin, and the red cell membrane.

So, the finding that the binding observed here for amphotericin B derivatives displayed no detectable cooperativity or saturability might reflect the particular characteristics of the red cell membrane. In fact this undetectable saturability simply implies that if some specific binding sites exist they are either already saturated at polyene concentrations as low as 10^{-8} M or not yet saturated at 10^{-4} M. The former possibility, unfortunately cannot be tested using radiolabelling. It would demand a specific activity too high to be endured by the fragile polyene materials. On the other hand such sites would be uncorrelated to permeability which is induced at antibiotic concentrations beyond 10^{-6} M. The second possibility that sites are not yet saturated at 10^{-4} M must be thoroughly considered, since at this polyene concentration the lipid/bound polyene molar ratio is

only about 40–100, depending on the derivative. Unfortunately it is impossible to test this possibility experimentally, since beyond 10^{-4} M the antibiotics induce extensive haemolysis of red cells indicative of membrane damage. Let's consider then the partition coefficients found here as expressing the overall affinity of amphotericin B derivatives for red cells.

One important conclusion can be drawn from the present data as referred the relationship between the binding and the biological activity of the four amphotericin B derivatives studied. The difference in their efficiencies in inducing ionic permeability in red cells was not due to the extent of their overall binding to red cells, since the compounds with an esterified carboxyl group bound three to five times as much as their non-esterified homologues, and nevertheless induced markedly lower ionic permeability.

According to the current hypothesis concerning the mechanism of ionic permeabilization by polyene macrolides, the efficiency of this permeabilization depends upon several parameters: they include the extent of binding, the ability to form permeability pathways in the membrane and the structure of these pathways which determines their stability and intrinsic permeability. The present work deals with the first of these parameters. Since for the methyl ester derivatives studied here, permeabilization was small despite considerable binding, it is clear that permeabilization does not simply result from the extent of binding. This absence of a simple relationship between the binding and biological activity of amphotericin B and other polyene macrolides has already been reported [17]. It implies that different permeabilizing efficiencies result, not from the binding, but from the subsequent steps leading to membrane permeabilization.

From ^{31}P-NMR data obtained in lipid vesicles containing cholesterol, the smaller permeabilizing efficiency of polyene antibiotics with an esterified carboxyl group seems to be due to the lower stability of the pores formed by the antibiotic in the presence of membrane cholesterol [9,10].

From the present data it may be concluded that this lower stability is not due to smaller overall affinity of the antibiotic for the membrane, but rather to its reduced ability to form a permeability pathway.

The same reasoning might apply to the red cell membrane. However, the presence of membrane proteins in erythrocytes offers to the antibiotic other possibilities of interaction [7] and therefore the mechanism of permeability induction may be more complex than in the case of lipid vesicles.

The spectroscopic study of bound species of amphotericin B derivatives, as well as the kinetic study of their binding and dissociation in relation to the development of permeability, are all in progress in our laboratory, and will provide more precise information about the mechanism by which polyene macrolides induce the formation of permeability pathways in the membrane.

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References

1 Schaffner, C.P. (1984) in Macrolide Antibiotics Chemistry, Biology and Practice (Omura, S., ed.), pp. 457–507, Academic Press, New York.
2 Thomas, A.H. (1986) J. Antimicrob. Chemiother. 17, 269–279.
3 Bolard, J. (1986) Biochim. Biophys. Acta 864, 257–304.
4 Cheron, M., Cybulska, B., Mazerski, J., Grzybowska, J., Czerwinski, A. and Borowski, E. (1988) Biochem. Pharmacol., in press.
5 Bolard, J., Seigneuret, M. and Boudet, G. (1980) Biochim. Biophys. Acta 599, 280–293.
6 Vertut-Croquin, A., Bolard, J., Chabbert, M. and Gary-Bobo, C.M. (1983) Biochemistry 22, 2939–2944.
7 Szponarski, W. and Bolard, J. (1987) Biochim. Biophys. Acta 897, 229–237.
8 Cybulska, B., Ziminski, T., Borowski, E. and Gary-Bobo, C.M. (1983) Mol. Pharmacol. 24, 270–276.
9 Hervé, M., Cybulska, B. and Gary-Bobo, C.M. (1985) Eur. Biophys. J. 12, 121–128.
10 Cybulska, B., Hervé, M., Borowski, E. and Gary-Bobo, C.M. (1986) Mol. Pharmacol. 29, 293–298.
11 Grzybowska, J., Czerwinski, A. and Borowski, E. (1987) Drugs Exp. Clin. Res., in press.
12 Steck, T.L. (1974) Methods Membr. Biol. 2, 245–281.

13 Marshall Steward, J.H. (1980) Anal. Biochem. 104, 10–14.
14 Mazerski, J., Bolard, J. and Borowski, E. (1982) Biochim. Biophys. Acta 719, 11–17.
15 Van Deenen, L.L.M. and De Gier, J. (1974) in the Red Blood Cell (Surgenor, D.M., ed.), 2nd Edn., Vol. 2, pp. 147–211.
16 Rosenberg, S.A. and Guidotti, G. (1969) in Red Cell Membrane, Structure and Function (Jameson, G.A. and Greenwald, eds.), pp. 93–109, Lippincott, Philadelphia.
17 Witzke, N.M. and Bittman, R. (1984) Biochemistry 23 1668–1674
18 Ponder, E. (1961) in The Cell (Bracket, J. and Mirsky, A. eds.), Vol. 2, pp. 1–84.
19 Clejan, S. and Bittman, R. (1985) J. Biol. Chem. 260 2884–2889.
20 Clark, M.R., Mel, S., Lupu, F. and Friend, D.S. (1987) Exp Cell. Res. 171, 321–330.