Combination of the anti-tumour cell ether lipid edelfosine with sterols abolishes haemolytic side effects of the drug

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Abstract Edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) is an anti-tumour cell ether lipid with surface-active properties. Pure edelfosine can be dispersed in aqueous media in the form of micelles. One important, negative side effect of edelfosine is that it is highly haemolytic. In this paper, we show that edelfosine can be co-dispersed in water with certain lipids (particularly cholesterol, campesterol or β -sitosterol) so that it gives rise to liposomes. Surface pressure measurements demonstrate that edelfosine is slowly released from these liposomes. In liposomal form, edelfosine remains apoptogenic for a variety of leukemia cell lines, while its haemolytic effect is abolished. The phenomenon is explained on the basis of the complementarity of the molecular geometries of sterols and edelfosine.

Keywords Edelfosine · Ether lipid · Sterols · Antitumour drugs · Lipid molecular geometry · Surface pressure

Introduction

The ether lipid edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) is the best-known member of a family of synthetic ether lipids that show a selective anti-tumour cell activity [1, 2]. At variance with most conventional chemotherapeutic drugs, edelfosine does not target DNA but is rather taken up by malignant cells, triggering apoptosis in a large variety of human tumour cell lines, while normal cells are spared [3–5]. The apoptotic activity of edelfosine involves membrane rafts and the Fas/CD95 death receptor [4–6], and the drug displays various dose-dependent effects on PKC α and PKC ϵ [7].

Studies involving different physical techniques have shown that edelfosine is a surface-active molecule, with a high affinity for model and cell membranes but weak detergent activity [8]. It displays critical micellar concentrations at 3.5 and 19 μ M in water [8]. In the presence of a lipid monolayer at the air–water interface, edelfosine becomes inserted in the phospholipid monolayer, thus increasing surface pressure. This increase is dose-dependent and reaches a plateau at approximately 2 μ M edelfosine bulk concentration [8]. The ether lipid can become incorporated in phospholipid monolayers with initial surface pressures of up to 33 mN/m, which ensures its capacity to become inserted into cell membranes [8]. Only at very high concentrations of this lipid (>40 mol% in the bilayer) are the membrane bilayers destabilized, probably because of the formation of micelles or small vesicles [9].

One serious drawback in the use of edelfosine is its high haemolytic activity that would prevent its parenteral use [10]. Various attempts have been carried out to develop pharmaceutical preparations with a low haemolytic potential. In this report, we describe some simple binary mixtures of edelfosine with other lipids, particularly sterols, that combine

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high anti-tumour cell activity and low haemolytic effects. Furthermore, we show that these binary mixtures give rise to vesicles and that the corresponding bilayers act as edelfosine reservoirs, from which it is slowly released. The behaviour of these non-haemolytic edelfosine preparations can be explained on the basis of the Israelachvili's lipid shape hypothesis [11].

Materials and methods

Reagents

Edelfosine was obtained from INKEYSA (Barcelona, Spain). Campesterol, cholesterol, ergosterol, β -sitosterol, 1,2-dimyristoyl-L- α -phosphatidylcholine (C14:0) (DMPC), 1,2-dioleoyl-L- α -phosphatidylcholine (C18:1, [cis]-9) (DOPC) and 1,2-dioleoyl-L- α -phosphatidylethanolamine (C18:1, [cis]-9) (DOPE) were provided by Sigma (St. Louis, MO, USA). L- α -phosphatidylcholine, hydrogenated (egg, chicken; ePC) was supplied by Avanti Polar Lipids (Alabaster, AL, USA). All assays were carried out in 10 mM 4-2-hydroxyethyl-1-piperazineethanesulphonic acid (HEPES), 150 mM NaCl, pH 7.4 buffer. All lipids were used without further purification. All other reagents were of analytical grade.

Liposome preparation

For liposome preparation, the proper amount of lipid was dissolved in chloroform/methanol (2:1, v/v) solution, and the solvent evaporated exhaustively. Multilamellar vesicles (MLVs) were then prepared by hydrating the lipid film with buffer by vigorous vortexing. The samples were finally incubated at 50 °C for 40 min, vortexed again and left to equilibrate at room temperature prior to use. Large unilamellar vesicles (LUVs) were prepared by the extrusion method, passing the MLV suspension ten times through polycarbonate filters 0.1 μ m in pore diameter. Average size of the vesicles was 90–110 nm as measured by quasi-elastic light scattering (QELS) in a Malvern Zeta-Sizer 4 spectrometer. Final lipid concentration of glycerophospholipids was measured in terms of lipid phosphorus. Thin layer chromatography (TLC) analysis showed that the initial 40:60 edelfosine/additional lipid mol ratio was maintained.

Surface activity measurements

Edelfosine monomolecular layer formation at the buffer–air interface was measured at 22 °C using a μ Trough-S equipment (Kibron, Helsinki, Finland) consisting of a small 2 cm rounded multi-well plate that allowed for 1 ml subphase measurements. Edelfosine (“control”) or edelfo-

sine/lipid (40:60 mol ratio) mixtures were added into the subphase through an adjusted hole, and edelfosine migration to the interface was followed as changes in surface pressure.

Cell culture

Human cell lines HL-60, Raji, Molt-4, Peer, Jurkat and U937 were grown in RPMI-1640 culture medium containing 10% (v/v) heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in humidified 95% air and 5% CO₂.

Apoptosis

Quantitation of apoptotic cells was calculated by flow cytometry as the percentage of cells in the sub-G₁ region (hypodiploidy) in cell cycle analysis as previously described [12] in a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA, USA).

Haemolysis

Erythrocytes were pelleted by centrifugation of human peripheral blood at 3,000 rpm for 7 min, subsequently washed twice with phosphate-buffered saline (PBS), and a final 4% erythrocyte suspension in PBS was used for the assays. Each lipidic sample (0.3 ml), previously diluted with HEPES/NaCl buffer (10 mM HEPES, 150 mM NaCl, pH 7.4), was incubated with 0.3 ml of 4% erythrocyte suspension at 37 °C for 20 h with

Table 1 Apoptotic (HL-60 cells) and haemolytic activities of edelfosine, free and in mixtures with other lipids

	% Apoptosis (HL-60)	% Haemolysis
Control (buffer)	–	0.1 $\pm$ 0.1
Control (growth medium)	6.3 $\pm$ 0.8	–
Edelfosine (in buffer)	49.9 $\pm$ 1.5	121.0 $\pm$ 5.4
Edelfosine (in growth medium)	50.5 $\pm$ 3.5	124.8 $\pm$ 7.8
Edelfosine + DMPC	59.6 $\pm$ 4.6	111.4 $\pm$ 4.1
Edelfosine + DOPC	52.3 $\pm$ 4.6	42.9 $\pm$ 10.1
Edelfosine + DOPE	42.0 $\pm$ 3.9	8.6 $\pm$ 0.7
Edelfosine + cholesterol	43.4 $\pm$ 5.6	0.9 $\pm$ 0.2
Edelfosine + campesterol	41.8 $\pm$ 3.1	1.0 $\pm$ 0.2
Edelfosine + β -sitosterol	46.5 $\pm$ 4.0	1.7 $\pm$ 0.2
Edelfosine + ergosterol	46.5 $\pm$ 3.4	32.6 $\pm$ 3.9

Total edelfosine concentration was 10 μ M in the apoptosis assays and 50 μ M in the haemolysis assays. Data are shown as mean values $\pm$ SE ($n=4-6$). HL-60 leukemia cells (2.5×10^6 cells per millilitre) were incubated at 37 °C for 7 h in culture medium in the absence and in the presence of the agents indicated above, and the proportion of cells in each phase of the cell cycle was quantitated by flow cytometry. Cells in the sub-G₁ region represent apoptotic cells. Per cent haemolysis was determined as shown in the “Materials and methods” section.

Fig. 1 Edelfosine-induced haemolysis is abolished by campesterol. Haemolysis was assayed (as detailed under “Materials and methods”) in the presence of pure edelfosine micelles (*–camp*) or of edelfosine/campesterol (40:60, mol ratio) liposomes (*+camp*). Average values $\pm$ SE ($n=3$)

constant gentle shaking (65 rpm). Mixtures of 0.3 ml 4% erythrocyte suspension + 0.3 ml distilled water and 0.3 ml 4% erythrocyte suspension + 0.3 ml HEPES/NaCl buffer were used as positive and negative control samples, respectively. Then, tubes were centrifuged at 3,000 rpm for 7 min, and haemolysis was quantified by determining the absorbance at 550 nm in the

Table 2 Apoptotic activities of edelfosine, added in the presence or absence of campesterol, on various leukemia cell lines

Cell line	Treatment	% Apoptosis	
		7 h	24 h
HL-60	Control	7.2 $\pm$ 0.8	8.2 $\pm$ 1.0
HL-60	Edelfosine	35.8 $\pm$ 1.9	65.2 $\pm$ 3.1
HL-60	Edelfosine+campesterol	32.8 $\pm$ 0.2	59.9 $\pm$ 3.9
Raji	Control	5.3 $\pm$ 0.9	8.0 $\pm$ 1.3
Raji	Edelfosine	39.3 $\pm$ 2.1	66.4 $\pm$ 3.4
Raji	Edelfosine+campesterol	24.9 $\pm$ 3.1	42.6 $\pm$ 5.2
Molt-4	Control	9.2 $\pm$ 2.0	8.4 $\pm$ 1.4
Molt-4	Edelfosine	42.0 $\pm$ 1.4	79.2 $\pm$ 1.9
Molt-4	Edelfosine+campesterol	27.9 $\pm$ 4.0	49.4 $\pm$ 3.0
U937	Control	11.1 $\pm$ 2.6	13.2 $\pm$ 2.0
U937	Edelfosine	42.4 $\pm$ 1.5	59.5 $\pm$ 5.0
U937	Edelfosine+campesterol	25.6 $\pm$ 1.0	36.4 $\pm$ 3.3
Peer	Control	9.2 $\pm$ 0.3	10.5 $\pm$ 1.8
Peer	Edelfosine	28.9 $\pm$ 2.1	70.5 $\pm$ 3.9
Peer	Edelfosine+campesterol	27.0 $\pm$ 2.6	65.6 $\pm$ 2.0
Jurkat	Control	3.9 $\pm$ 0.5	6.9 $\pm$ 2.5
Jurkat	Edelfosine	18.9 $\pm$ 2.5	52.9 $\pm$ 1.6
Jurkat	Edelfosine+campesterol	14.3 $\pm$ 2.0	39.8 $\pm$ 3.7

Total edelfosine concentration was 10 μ M. Data are shown as mean values $\pm$ SE. ($n=3$). Leukemia cells (7×10^5 cells/ml) were incubated at 37 $^{\circ}$ C for 7 h or 24 h in culture medium in the absence and in the presence of the agents indicated above, and the proportion of cells in each phase of the cell cycle was quantitated by flow cytometry. Cells in the sub-G₁ region represent apoptotic cells.

supernatant in a Ultrospec Plus 4054 UV/Visible LKB-Pharmacia spectrophotometer. Data are expressed as percent haemolysis as follows:

$$\% \text{ haemolysis} = \frac{([A550_{\text{sample}}] - [A550_{\text{control-}}])}{([A550_{\text{control+}}] - [A550_{\text{control-}}])} \times 100$$

Results

The apoptotic activity of edelfosine on human acute myeloid leukemia HL-60 cells is shown in Table 1. Edelfosine induces apoptosis after 7 h treatment in $\approx 50\%$ of the cells, measured as number of cells in the sub-G₁ zone after fluorescence-activated cell sorting, vs. $\approx 7\%$ of the cells in the

Fig. 2 Surface activity of edelfosine is decreased by other lipids. **a** Time-course of the increase in surface pressure in a Langmuir balance after injection of edelfosine (*CONTROL*) or edelfosine/lipid mixtures into the aqueous subphase. Representative traces. **b** The change in surface pressure after 60 min injection of edelfosine or edelfosine/lipid mixtures, measured from experiments as shown in **a**. Average values $\pm$ SE ($n=3$)

untreated control experiment. However, edelfosine caused full haemolysis in a red blood cell preparation (Table 1, right-hand column).

Binary mixtures of edelfosine with other lipids were prepared and dispersed in water, as described under “Materials and methods”. The second lipid was either DMPC, DOPC, DOPE, cholesterol, β -sitosterol or ergosterol. In all cases, edelfosine molar fraction was $X=0.4$. After dispersion in aqueous buffer, the suspension presented the opalescent aspect that is typical of MLV preparations. In samples of MLV or LUV, chemical analysis showed that edelfosine concentration in the bilayer remained at $X=0.40\pm0.05$. When LUV was formed by the extrusion method using $0.1\ \mu\text{m}$ polycarbonate filters, quasi-elastic light scattering measurements revealed highly monodisperse suspensions of particles 90–110 nm in diameter, irrespective of the nature of the lipid other than edelfosine. This is interpreted as the lipids giving rise to regular bilayers, organized in the form of liposomes. QELS did not reveal the presence of smaller particles that could be interpreted as pure or mixed micelles. This, together with the fact that LUV keeps the 40:60 edelfosine/additional lipid mol ratio for all lipid mixtures, confirms that the lipid couples are being dispersed in the form of bilayers and that both kinds of lipids are incorporated more or less equally to the bilayers.

When edelfosine was added in binary mixtures (in the form of MLV) to HL-60 cells, it was found to be as active as the pure drug in causing apoptosis (Table 1, left-hand column). However with most lipids, with the exception of DMPC, its haemolytic activity was greatly reduced, becoming virtually zero for the mixtures containing cholesterol, campesterol or β -sitosterol. Of the diacyl

phospholipids used, DOPE was most effective in reducing haemolysis (Table 1, right-hand column). The various sterols and diacyl phospholipids, in the absence of edelfosine, had neither apoptotic nor haemolytic properties (data not shown). The antihaemolytic properties of campesterol are shown in detail in Fig. 1.

The apoptotic effects of edelfosine were not limited to HL-60 cells but were equally seen on B lymphoid leukemia Raji cell line, T lymphoid leukemia Molt-4, Peer and Jurkat cell lines and histiocytic lymphoma U937 cell line (Table 2). In all these cell types, adding edelfosine/campesterol (40:60 mol ratio) MLV had almost similar apoptotic effects than adding edelfosine alone (Table 2), the number of apoptotic cells in the presence of campesterol being about two thirds of those in its absence. Peer cells were exceptional in that they were equally sensitive to edelfosine in the presence and absence of campesterol. Note that this sterol was one of the molecules that virtually abolished edelfosine-induced haemolysis (Fig. 1).

In order to understand the mechanism by which the molecules in Table 1 decrease or abolish haemolysis caused by edelfosine, a series of surface tension measurements were carried out in a Langmuir trough. As shown in our previous paper [8], when injected into the aqueous subphase, edelfosine alone causes a rapid increase in surface pressure due to its migration to the air–water interface, where it orients with the hydrophobic moiety facing the air. This effect can be seen in Fig. 2a, “control” trace. However, when edelfosine is added to the subphase in the form of multilamellar vesicles in binary mixtures with other lipids, then the increase in surface pressure is much slower, actually undetectable for the sterols even after 60 min, when edelfosine alone reaches a maximum after

Fig. 3 The relationship between lipid molecular geometries and structure of the aggregates. See details on lipid molecular geometries in [11]. *Inset:* The formation of bilayer structures combining lipids with opposite (complementary) non-bilayer geometries

approximately 20 min. The change in surface pressure caused by edelfosine alone, or in a series of binary mixtures, after 60 min, is shown in Fig. 2b. Essentially similar results (not shown) were obtained when edelfosine mixtures were added in the form of LUV instead of MLV. Comparison of data in Fig. 2b and Table 1 (right-hand column) show an excellent agreement. Some mixtures, e.g. those containing campesterol, cholesterol or β -sitosterol, release edelfosine very slowly, as judged from the very slow increase in surface pressure, and they are virtually non-haemolytic while retaining their apoptotic power. Thus edelfosine-induced apoptosis appears to rely on the slow, sustained release of drug, while haemolysis requires an instantaneous high concentration. Note that the data in Fig. 2b do not correspond to equilibrium values (in some cases equilibrium was attained only after many hours), yet they are useful in allowing a comparison of the rates of change in surface pressure after a given time, 60 min in our case.

Discussion

The main result in this contribution is that, in mixtures with certain other lipids, edelfosine retains its apoptotic properties while reducing its haemolytic side effect. It has also been shown that, in those binary mixtures that abolish haemolysis, edelfosine is slowly released from the lipid mixture to the aqueous environment. In fact, all our binary mixtures are seen to form liposomes when dispersed in water. Liposomes have long been recognized as devices for the slow, sustained release of drugs [13, 14]. The fact that the slow release of edelfosine reduces or abolishes haemolysis while keeping almost intact its apoptotic effect suggests that both phenomena occur through different mechanisms. Several aspects of the molecular mechanisms of edelfosine-induced cell death are known [2, 7]. However, the mechanism of haemolysis caused by edelfosine is not known at present, the data in our previous paper [8] showing that the ether lipid is scarcely able, even at high concentrations (250 μ M in [8] vs. 50 μ M in Table 1) to break down the bilayer permeability barrier. In any case, comparison of the data in Table 1 and Fig. 2 indicate clearly that haemolysis requires a higher instantaneous concentration of edelfosine than apoptosis.

The reason why edelfosine forms bilayers (liposomes) in the presence of certain other lipids requires some comment. Some of the added lipids, e.g. DMPC, DOPC, spontaneously form bilayers, and it was shown previously [8] that these bilayers can accommodate rather large proportions of edelfosine (up to 40 mol%) while remaining stable. DOPE, however, does not form bilayers spontaneously under many conditions [15], and sterols are never found to form bilayers spontaneously. Israelachvili et al. [11] proposed that the molecular geometry of the lipid molecules dictates the

structure of the lipid aggregates. Phosphatidylcholine (PC) molecules would have a “cylinder shape” (Fig. 3), i.e. the cross-section of the polar headgroup and of the fatty acyl chains would be approximately the same, thus PC would spontaneously organize into bilayers; phosphatidylethanolamine or sterols would have a “cone shape” (cross-section of the polar headgroup smaller than that of the nonpolar part) and give rise to hexagonal phase aggregates. Finally, single-chain lipids, such as lysophospholipids, or sodium dodecylsulphate, or edelfosine, would have an “inverted cone shape” and disperse in water in the form of micelles (Fig. 3). These suggestions have received ample experimental support, particularly from the X-ray measurements of the lipid spontaneous curvature by Rand and co-workers [16, 17]. In our case, we suggest that sterols and edelfosine combine their conical and inverted conical shapes to give rise to a bilayer (Fig. 3, inset). Previous examples are known of this sort of geometry compensation [18, 19]. Indeed, the bilayers arising from the combination of conical and inverted conical molecules (e.g. sterols + edelfosine) appear to be more stable than those containing cylindrical + inverted conical lipids (e.g. PC + edelfosine); thus, edelfosine release is slower in the former case. Interestingly, edelfosine does not form sterol-rich domains in fluid model membranes containing phospholipid and cholesterol [20], the sterol-edelfosine combination occurring only in the absence of other bilayer-forming lipids.

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