

The Anticancer Drug Cytarabine Does not Interact with the Human Erythrocyte Membrane

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Cytarabine, an analog of deoxycytidine, is an important agent in the treatment of ovarian carcinoma, acute myeloid and lymphoblastic leukemia. Its mechanism of action has been attributed to an interference with DNA replication. The plasma membrane has received increasing attention as a possible target of antitumor drugs, where the drugs may act as growth factor antagonists and receptor blockers, interfere with mitogenic signal transduction or exert direct cytotoxic effects. Furthermore, it has been reported that drugs that exert their antiproliferative effect by interacting with DNA generally cause structural and functional membrane alterations which may be essential for growth inhibition by these agents. This paper describes the studies undertaken to determine the structural effects induced by cytarabine to cell membranes. The results showed that cytarabine, at a concentration about one thousand times higher than that found in plasma when it is therapeutically administered, did not induce significant structural perturbation in any of these systems. Therefore, it can be unambiguously concluded that this widely used anticancer drug does not interact at all with erythrocyte membranes.

Key words: Cytarabine, Erythrocyte Membrane, Phospholipid Bilayer

Introduction

Arabinosyl nucleosides are among the most potent nucleoside analogs available for the treatment of viral infections and cancer (Gmeiner *et al.*, 1998). Cytarabine (1- β -D-arabinofuranosylcytosine; Fig. 1), an analog of deoxycytidine, is an important agent in the treatment of ovarian carcinoma (Jamieson *et al.*, 1989), acute myeloid and lymphoblastic leukemia (Gati *et al.*, 1998; Bishop *et al.*, 1998; Feldman, 2000). Its mechanism of action has been attributed to an interference with DNA replication. After entering the cells, cytarabine is converted to the active metabolite 5'triphosphate ester, which incorporates into nascent DNA strands slowing and eventually terminating strand elonga-

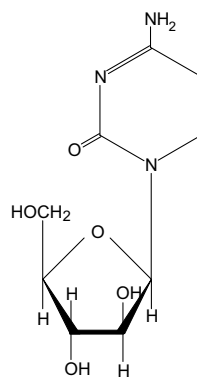


Fig. 1. Schematic formula of cytarabine.

tion (Higashigawa *et al.*, 1991; Gati *et al.*, 1997). Its adverse effects include leucopenia, thrombocytopenia, anemia, fever, anorexia, nausea, vomit, acute cerebellar syndrome and aseptic meningitis (Yeshurun and Marsot, 2001; Kantar *et al.*, 1999; Takeuchi, 2001; Van der Berg *et al.*, 2001). The plasma membrane has received increasing attention as a possible target of antitumor drugs, where the drugs may act as growth factor antagonists and

Abbreviations: SEM, scanning electron microscopy; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; IUM, unsealed human erythrocyte membrane; LUV, large unilamellar vesicles; *r*, anisotropy; *GP*, general polarization.

receptor blockers, interfere with mitogenic signal transduction or exert direct cytotoxic effects (Luxo *et al.*, 1996; Speelmans *et al.*, 1997; Marutaka *et al.*, 1994; Grunicke and Hoffmann, 1992). Furthermore, it has been reported that drugs that exert their antiproliferative effect by interacting with DNA generally cause structural and functional membrane alterations which may be essential for growth inhibition by these agents (Grunicke and Hoffmann, 1992). However, information about the interaction of cytarabine with cell membranes is very scanty.

The complex structure of cell membranes is a serious problem when trying to sort out the various effects caused by a drug. It is therefore useful to study those of simpler cells such as the erythrocyte membrane, in particular that of humans, which is the best characterized membrane (Røelofsen, 1991). Although less specialized than those of many other cells, the erythrocyte membrane does carry on enough common functions to be considered representative of the plasma membrane in general. Furthermore, lacking DNA and RNA, this cell precludes drug interactions with nucleic acids. Therefore, we have studied the structural effects induced by cytarabine to the human erythrocyte membrane and molecular models. With these aims we used

a) human erythrocytes, which were observed by phase contrast and scanning electron microscopy (SEM);

b) isolated unsealed human erythrocyte membrane (IUM), examined by fluorescence spectroscopy to evaluate DPH steady state fluorescence anisotropy and laurdan fluorescence spectral shifts;

c) multibilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the erythrocyte membrane, respectively (Devaux and Zachowsky, 1994; Boon and Smith, 2002), which were analyzed by X-ray diffraction, and

d) large unilamellar vesicles (LUV) of DMPC, examined by fluorescence spectroscopy.

These systems have been used in our laboratories to study similar effects induced by other anticancer drugs such as tamoxifen (Suwalsky *et al.*, 1998); adriamycin (Suwalsky *et al.*, 1999a); chlor-

ambucyl (Suwalsky *et al.*, 1999b) and cisplatin (Suwalsky *et al.*, 2000).

Materials and Methods

Scanning electron microscope (SEM) studies on human erythrocytes

The interaction of cytarabine with human erythrocytes was achieved by incubating blood samples from clinically healthy male adult donors not being subject to any drug treatment. Blood samples were obtained by puncture of the ear lobule disinfected with 70% ethanol. Two drops of blood were collected by aspiration into plastic tuberculin syringe containing 50 units/ml heparin in 1 ml of saline solution (0.9% NaCl) at 5 °C. Red blood cells were centrifuged, washed twice in saline, resuspended in saline containing cytarabine at a final 10 mM concentration. Controls were erythrocytes resuspended in saline solution without cytarabine. These samples were incubated at 37 °C for 1 h in an oven. Red blood cells were then fixed with glutaraldehyde adding one drop of each sample to a tube containing 1 ml of 2.5% glutaraldehyde in saline solution, reaching a final fixation fraction of about 2.4%. After overnight repose at 5 °C the fixed samples were directly placed on Al stubs, air dried in an oven at 37 °C for half to one hour and gold coated for 3 min at 10⁻¹ Torr in a sputter device (Edwards S150). The observations and photographic records were performed in an Etec Autoscanner SEM.

Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)

The influence of cytarabine on the physical properties of IUM and DMPC LUV was examined by fluorescence spectroscopy using DPH and laurdan (Molecular Probe, Eugene, OR, USA) fluorescent probes. DPH is widely used as a probe for the hydrophobic regions of the phospholipid bilayers because of its favorable spectral properties. Its steady-state fluorescence anisotropy measurements were used to investigate the structural properties of IUM and DMPC LUV as it provides a measure of the rotational diffusion of the fluorophore, restricted within a certain region such as a cone, due to the lipid acyl chain packing order.

Laurdan, an amphiphilic probe, has a high sensitivity of excitation and emission spectra to the physical state of membranes. With the fluorescent moiety within a shallow position in the bilayer, laurdan provides information of the dynamic properties at the level of the phospholipid glycerol backbone. The quantification of the laurdan fluorescence shift was effected using the general polarization (*GP*) concept (Parasassi and Gratton, 1995), which is related to the lipid polar head organization in the zone of the erythrocyte membrane and DMPC LUV.

Erythrocytes were separated from heparinized venous blood samples obtained from normal casual donors by centrifugation and washing procedures. IUM were prepared by lysis according to Dodge *et al.* (1963). DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspensions (final lipid concentration 0.3 mM) through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp., MA, USA) under nitrogen pressure at 10 °C above the lipid phase transition temperature. DPH and laurdan were incorporated into IUM and LUV by addition of small aliquots of concentrated solutions of the probe in dimethylformamide and ethanol, respectively, and incubated at 37 °C for 45 min. Fluorescence spectra and anisotropy measurements were performed on a Spex Fluorolog (Spex Industries Inc., Edison, N. J., USA) and in a phase shift and modulation Gregg-200 steady state and time-resolved spectrofluorometer (ISS Inc., Champaign, IL, USA) respectively, both interfaced to computers. Software from ISS was used for data collection and analysis.

Measurements of IUM were made at 37 °C and measurements of LUV suspensions were made at 18 °C using 10 mm path-length square quartz cuvettes. Sample temperature was monitored by an external bath circulator (Cole-Parmer, Chicago, IL, USA) and controlled before and after each measurement using an Omega digital thermometer (Omega Engineering Inc., Stanford, CT, USA). Anisotropy measurements were made in the \perp configuration using Glan Thompson prism polarizers (I. S. S.) in both exciting and emitting beams. The emission was measured using a WG-420 Schott high-pass filter (Schott WG-420, Mainz, Germany) with negligible fluorescence. Laurdan

fluorescence spectral shifts were quantitatively evaluated using the *GP* concept (see above) which is defined by the expression $GP = (I_b - I_r) / (I_b + I_r)$, where I_b and I_r are the emission intensities at the blue and red edges of the emission spectrum, respectively. These intensities have been measured at the emission wavelengths of 440 and 490 nm, which correspond to the emission maxima of laurdan in the gel and liquid crystalline phases, respectively (Parasassi and Gratton, 1995). DPH fluorescence anisotropy (r) was calculated according to the definition: $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, where I_{\parallel} and I_{\perp} are the corresponding parallel and perpendicular emission fluorescence intensities with respect to the vertically polarized excitation light. Cytarabine was incorporated in IUM and LUV suspensions by addition of small aliquots of a concentrated solution and incubated at 18 °C or 37 °C, depending on the work temperature, for *ca.* 15 min. Blank subtraction was performed in all measurements using labeled samples without probes.

X-ray diffraction analysis of phospholipid multilayers

Synthetic DMPC (lot 80H-8371 A grade MW 677.9), DMPE (lot 13H-83681 A grade MW 635.9) and cytarabine (lot 34H7814 MW 243.22) from Sigma were used without further purification. About 3 mg of each phospholipid were mixed with 200 μ l of 10 mM cytarabine into special glass capillaries 1.5 mm diameter (Glastechnik & Konstruktion, Berlin, Germany). X-ray diffractograms were recorded 2 days after preparation in flat-plate cameras with 0.25 mm dia glass collimators provided with rotating devices. Specimen-to-film distances in the flat-plate cameras were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered $\text{CuK}\alpha$ radiation from a Philips PW1140 X-ray generator was used. The relative reflection intensities were obtained from films by peak integration in a Bio Rad GS-700 (Hercules, CA, USA) microdensitometer using the Bio-Rad Molecular Analyst image software. No correction factors were applied. The experiments were performed at 17 ± 2 °C, which is below the main transition temperature of both DMPC and DMPE.

Results

Scanning electron microscopy (SEM) studies on human erythrocytes

The SEM examination of red cells incubated with cytarabine did not reveal abnormalities in their shapes. The normal discoid erythrocyte pro-

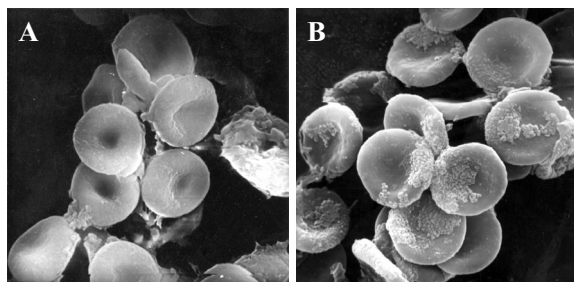


Fig. 2. Scanning electron microscope (SEM) images of human erythrocytes. (A) Control (3250 \times); (B) incubated with 10 mM cytarabine (2500 \times).

file (Fig. 2A) was not affected by 10 mM cytarabine (Fig. 2B). This was observed in all the assayed samples. These results clearly indicated that cytarabine neither penetrated the membrane structure nor perturbed its molecular arrangement.

Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)

The influence of cytarabine upon the bilayers of IUM and DMPC LUV was evaluated at the phospholipid acyl chain hydrophobic core and at the hydrophilic/hydrophobic interface, *i.e.*, the phospholipid polar head level. This was respectively achieved evaluating DPH steady state fluorescence anisotropy (r) and laurdan fluorescence

spectral shifts which were quantified through the general polarization (GP) parameter. The presence of increasing concentrations of cytarabine did not produce significant changes in DPH fluorescence anisotropy and laurdan GP as shown in Table I. These results imply that cytarabine did not interact with the lipid acyl chains nor with the polar headgroups of IUM and DMPC LUV.

X-ray diffraction studies on phospholipid multilayers

Given the solubility of cytarabine, its molecular interaction with the phospholipids DMPC and DMPE was assayed in aqueous media. Fig. 3A shows a comparison of the diffraction pattern of DMPC and of its mixture with 10 mM cytarabine. The fact that the interplanar spacings and intensities of the phospholipid reflections were practically the same when immersed alone in water as well as in the presence of the drug clearly indicated that cytarabine neither interacted with DMPC nor perturbed the phospholipid bilayer structure. Fig. 3B shows the results obtained after DMPE was incubated with 10 mM cytarabine. As it can be observed, cytarabine did not affect the X-ray pattern of DMPE. In fact, both its reflection intensities and interplanar spacings remained practically unchanged. These results indicated that cytarabine was unable to interact with DMPE bilayers or to perturb its molecular structure.

Discussion

The cell membrane is a diffusion barrier that protects the cell interior. Therefore, its structure and functions are susceptible to alterations as a consequence of interactions with foreign molecules, particularly anticancer drugs that must cross

Table I. Effect of cytarabine on the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the general polarization (GP) of Laurdan embedded in isolated unsealed human erythrocyte membrane (IUM) and large unilamellar vesicles (LUV) of dimyristoylphosphatidylcholine (DMPC) (probe: lipid ratio 1:600).

Cytarabine [mM]	r DPH		GP Laurdan	
	IUM*	DMPC LUV**	IUM*	DMPC LUV**
0	0.223	0.293	0.345	0.482
1	0.223	0.295	0.344	0.487
5	0.228	0.293	0.356	0.493
10	0.231	0.292	0.363	0.487

* 37 $^{\circ}\text{C}$; ** 18 $^{\circ}\text{C}$.

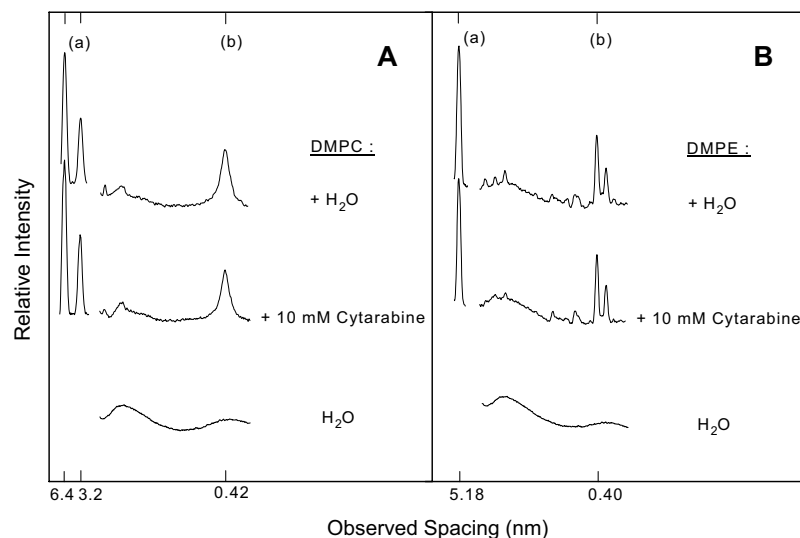


Fig. 3. Microdensitograms from X-ray diffraction diagrams of DMPC (A) and DMPE (B) in water and 10 mM cytarabine; (a) low-angle and (b) high-angle reflections.

it in order to interact with DNA. Our previous studies have shown that tamoxifen, adriamycin, chlorambucil and cisplatin interacted with and perturbed the structure of the human erythrocyte membrane (Suwalsky *et al.*, 1998; 1999a; 1999b; 2000). In fact, human erythrocytes incubated with these drugs in concentrations equivalent to those found in plasma when they are therapeutically used, underwent shape changes. Accordingly to the bilayer couple hypothesis (Sheetz and Singer, 1974) such changes are due to the differential expansion of the outer or the inner membrane monolayer as a consequence of the drug insertion in the corresponding monolayer. Additional experiments performed in membrane models constituted by phospholipid bilayers showed that indeed these drugs interacted with classes of lipids located in either the outer or inner human erythrocyte membrane.

In the case of cytarabine, its concentration in plasma after 72 h continuous infusion of high doses ranges between $3.6 \mu\text{M}$ and $22.6 \mu\text{M}$ (Donenhower *et al.*, 1986). However, cytarabine concentration as high as 10 mM did not induce any significant perturbation in the human erythrocyte shape. In order to further test this lack of interaction cy-

tarabine in similar concentration was incubated with bilayers built-up of phospholipids that are preferentially located either in the outer or inner monolayers of the erythrocyte membrane. Analysis performed by X-ray diffraction on DMPC and DMPE multilayers and by fluorescence spectroscopy on DMPC large unilamellar vesicles and in isolated unsealed human erythrocyte membranes also showed that cytarabine did not induce significant structural perturbation in any of these systems. It can be, therefore, unambiguously concluded that this widely used anticancer drug does not interact at all with erythrocyte membranes. This property can be of therapeutic interest since its cytotoxicity is determined by its permeation across the plasma membrane of mammalian cells mediated by nucleoside-specific transporters and accumulation in leukemia cells (Belt *et al.*, 1993; Gatti *et al.*, 1997).

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