The Anticancer Drug Cisplatin Interacts with the Human Erythrocyte Membrane

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Drugs which exert their effects by interacting with DNA cause structural and functional membrane alterations which may be essential for growth inhibition by these agents. This paper describes the interaction of cisplatin with the human erythrocyte membrane and models constituted by bilayers of dimyristoylphosphatidylethanolamine (DMPE) and diacylphosphatidylserine (DAPS), representative of phospholipid classes located in the inner monolayer of the erythrocyte membrane, and of dimyristoylphosphatidylcholine (DMPC), a class present in its outer monolayer. Cisplatin ability to perturb DMPE, DAPS and DMPC bilayer structures was determined by X-ray diffraction and fluorescence spectroscopy. Electron microscopy disclosed that human erythrocytes incubated with 35 μM cisplatin, which is its therapeutical concentration in serum, developed cup-shaped forms (stomatocytes). According to the bilayer couple hypothesis, this means that the drug is inserted into the inner monolayer of the erythrocyte membrane, a conclusion supported by the studies on model systems.

Introduction

Cisplatin is one of the largest selling cancer chemotherapeutic agents (Scanlon *et al.,* 1989). It has been successfully used in the treatment of testicular, ovarian, esophagean, bladder, small cell lung (Speelmans *et al.*, 1996) cancers as well as those of the head and neck (Suzuki and Kaga, 1997). It has been well established that it forms covalent cross-linked adducts with DNA (Huang *et al.*, 1995; Yang *et al.*, 1995); thus, its anticancer activity has been directly related to its ability to bind to DNA in the cell. Although there is good correlation between DNA cross-linking and growth inhibition, clear-cut evidence that this is the only essential event leading to blockade of cell proliferation is still lacking. It is known that growth factors regulate the proliferation of cells by binding to specific high-affinity receptors on the cell surface. Receptor occupancy triggers a cascade of biochemical and physiological changes which ultimately leads to stimulation of DNA synthesis and cell division. Among the consequences of growth factor-receptor interaction are the activation of a protein tyrosine kinase activity, the phospholipase-C-mediated breakdown of inositol phospholipids generating several signal molecules, and the activation of various ion transport systems in the plasma membrane and changes in intracellular ionic composition (Moolenar, 1988). Thus, the plasma membrane as a possible target of antitumor drugs has gained increasing attention (Luxo *et al.,* 1996; Speelmans *et al.*, 1996; Marutaka *et al.*, 1994), where they may act as growth factor antagonists, growth factor receptor blockers, interfere with mitogenic signal transduction or exert direct cytotoxic effects (Grunicke and Hoffmann, 1992). Furthermore, drugs which exert their antiproliferative effect by interacting with DNA cause structural and functional membrane alterations which may be essential for growth inhibition by these agents (Grunicke *et al.*, 1985). In fact, at membrane level, cisplatin may inhibit amino acid transport, calcium ion uptake, and modulate protein kinase C activity (Scanlon *et al.*, 1989). As doses of cisplatin have

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DAPS, diacylphosphatidylserine; LUV, large unilamellar vesicles; DPH, 1,6-diphenyl-1,3,5-hexatriene; laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; r, fluorescence anisotropy; GP, general polarization; SEM, scanning electron microscopy.

escalated in recent years, neurotoxicity has emerged as an undesirable side effect (Holmes *et al.*, 1998). The symptoms of neurotoxicity may include peripheral sensory neuropathy (Mollman, 1990), decreased sensory nerve conduction velocity (Hamers *et al.*, 1991) and preferential loss of large myelinated fibers (Mollman, 1990).

The complexity of living cells is often a serious problem when trying to sort out the various effects caused by a drug. It is therefore useful to study simpler cells such as the erythrocytes which, lacking DNA and RNA, preclude drug interaction with nucleic acids. Furthermore, the red cell membrane, and in particular that of man, is the most particularly characterized (Roelofsen, 1991). This paper describes the interaction of cisplatin with the human erythrocyte membrane and models constituted by phospholipid multilayers and large unilamellar vesicles (LUV). These systems have been used in our laboratories to determine the interaction and perturbing effects on membranes by other anticancer drugs such as tamoxifen (Suwalsky *et al.*, 1998), adriamycin (Suwalsky *et al.*, 1999) and chlorambucil (Suwalsky *et al.*, 1999). The interaction of cisplatin with human erythrocytes was examined by scanning electron microscopy (SEM) to detect shape changes induced by the drug. The multilayers consisted of the phospholipids dimyristoylphosphatidylethanolamine (DMPE) and diacylphosphatidylserine (DAPS), representative of phospholipid classes located in the inner monolayer of the erythrocyte membrane, and of dimyristoylphosphatidylcholine (DMPC), a class present in its outer monolayer (Devaux and Zachowsky, 1994). Cisplatin perturbation of DMPE, DAPS and DMPC multilayers structures was determined by X-ray diffraction. The effect of the drug on the physical properties of DMPC LUV was studied evaluating DPH steady state fluorescence anisotropy and laurdan fluorescence spectral shifts.

Materials and Methods

Phase contrast and scanning electron microscopy (SEM) studies on human erythrocytes

Blood samples taken from clinically healthy male adult donors by puncture of the ear lobe disinfected with 70% ethanol were incubated with cisplatin. Two drops of blood were collected in a plastic tube containing 1 ml of saline (0.9% NaCl) at room temperature. In order to wash and dilute the erythrocytes this blood suspension was twice transferred into syringes, each time with 10 ml of saline. These suspensions were used to prepare the following samples in tuberculin syringes: a) control, by mixing 0.1 ml of blood stock with 0.9 ml of saline, b) 35 μM, 50 μM, 0.1 mM and 6 mM cisplatin by mixing 0.1 ml of blood stock with 0.9 ml of saline cisplatin in adequate concentrations. The samples were incubated at 37 °C for 1 h and then drops of each sample were examined in a phase contrast microscope. They were then fixed overnight at 5 °C by adding one drop of each sample to a plastic tube containing 1 ml of 2.5% glutaraldehyde. The fixed samples were washed with distilled water and left for 2 h to allow sedimentation; the supernatant was discarded and drops of each remnant were placed on siliconized Al stubs, air dried at 37 °C for 1 h and gold coated for 3 min at 10^{-1} Torr in a sputter device (Edwards S150, Sussex, England). Resulting specimens were examined in an Etec Autoscan SEM (Etec Corp., Hayward, CA, USA).

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), DAPS (from bovine brain, lot 69F8371) and cisplatin (lot 55H3471, MW 300.0) from Sigma (St. Louis, MO, USA) were used without further purification. About 3 mg of each phospholipid were mixed in 1.5 mm dia glass capillaries (Glas Technik & Konstruktion, Berlin, Germany) with aqueous solutions of cisplatin in a range of concentration from 35 μM up to 6 mM. They were diffracted at least 2 days after preparation in flat-plate cameras built by us with 0.25 mm dia glass collimators provided with rotating devices. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuKα radiation from a Philips PW1140 X-ray generator (The Netherlands) was used. The relative reflection intensities on films were measured by peak-integration in a Bio-Rad GS-700 microdensitometer (Hercules, CA, USA) using the Bio-Rad Molecular Analyst/PC image software; no correction factors were applied. The experiments were performed at 17 ± 2 °C. Higher

temperatures would have induced transitions to more fluid phases making it harder to detect the structural changes produced by cisplatin.

Fluorescence measurements on large unilamellar vesicles (LUV)

DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspension (final lipid concentration 0.5 mM) through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp., Cambridge, MA, USA) under nitrogen pressure at 10 °C over the lipid transition temperature. DPH and Laurdan were incorporated into LUV by addition of small aliquots of concentrated solutions of the probe in tetrahydrofurane and ethanol respectively to LUV suspensions and gently shaken for about 30 min. Fluorescence spectra and anisotropy measurements were respectively performed in a Spex Fluorolog (Spex Industries Inc., Edison, NJ, USA) and in a phase shift and modulation Greg-200 steady-state and time-resolved spectrofluorometer (I. S. S. Inc., Champaign, IL, USA), both interfaced to computers. Software from I. S. S. was used for data collection and analysis. Measurements of LUV suspensions were made at 18 °C in 10 mm path-length square quartz cuvettes. Sample temperature was controlled by an external bath circulator (Cole Parmer, Chicago, IL, USA) and measured prior and after each measurement using a digital thermometer (Omega Engineering Inc., Stanford, CT, USA). Anisotropy measurements were made in the "L" configuration using prism polarizers (Glan Thompson, I. S. S.) in both exciting and emitting beams. The emission was measured across a high pass filter (Schott WG-420, Mainz, Germany) with negligible fluorescence. Laurdan fluorescence spectral shifts were quantified through the General Polarization (GP) concept which was evaluated by GP=(Ib-Ir)/(Ib+Ir), where Ib and Ir are the 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). Cisplatin was incorporated in LUV suspensions by addition of small aliquots of a concentrated solution and incubated at 40 °C for ca. 15 min. Samples with probes but without cisplatin showed no variation in the measured parameters during periods longer than those employed in the experiments. Blank subtraction was performed in all measurements using unlabelled samples without probes.

Results

Phase contrast and scanning electron microscopy (SEM) studies on human erythrocytes

Phase contrast microscopy and SEM of red blood cells incubated with 35 μM, 50 μM, 0.1 mM and 6 mM cisplatin revealed abnormalities in their shapes. In contrast to the normal discoid erythrocyte profile a number of cisplatin treated cells underwent stomatocytic shape changes, i.e., evagination of one surface and invagination of the opposite face. The lowest concentration is that present in serum when the drug is administered to patients (Scanlon *et al.*, 1989).

X-ray diffraction analysis of phospholipid multilayers

The molecular interactions of cisplatin with multilayers of the phospholipids DMPC, DMPE and DAPS were investigated in an aqueous media. Fig. 1A shows a comparison of the diffraction pattern of DMPC alone and that incubated with 6 mM cisplatin. Results indicated that DMPC bilayer structure was not affected by the drug as its X-ray pattern remained essentially the same. Similar results were obtained with DMPE, although a slight decrease of high angle reflection intensities induced by 6 mM cisplatin was observed (Fig. 1B). Nevertheless, a structural perturbation was observed in DAPS (Fig. 1C). In fact, DAPS presents very poor X-ray diagrams (Atkinson *et al.*, 1974) characterized by a diffuse and central halo in the low angle region and a sharp 4.2 Å reflection. The latter is usually associated with the average separation of the fully extended phospholipid acyl chains organized with rotational disorder in a hexagonal lattice. 35 μM cisplatin reduced by about a 25% the intensity of the 4.2 Å reflection and enhanced the intensity of the central halo, effects that increased with higher concentrations of the drug. Thus, it can be concluded that cisplatin, at

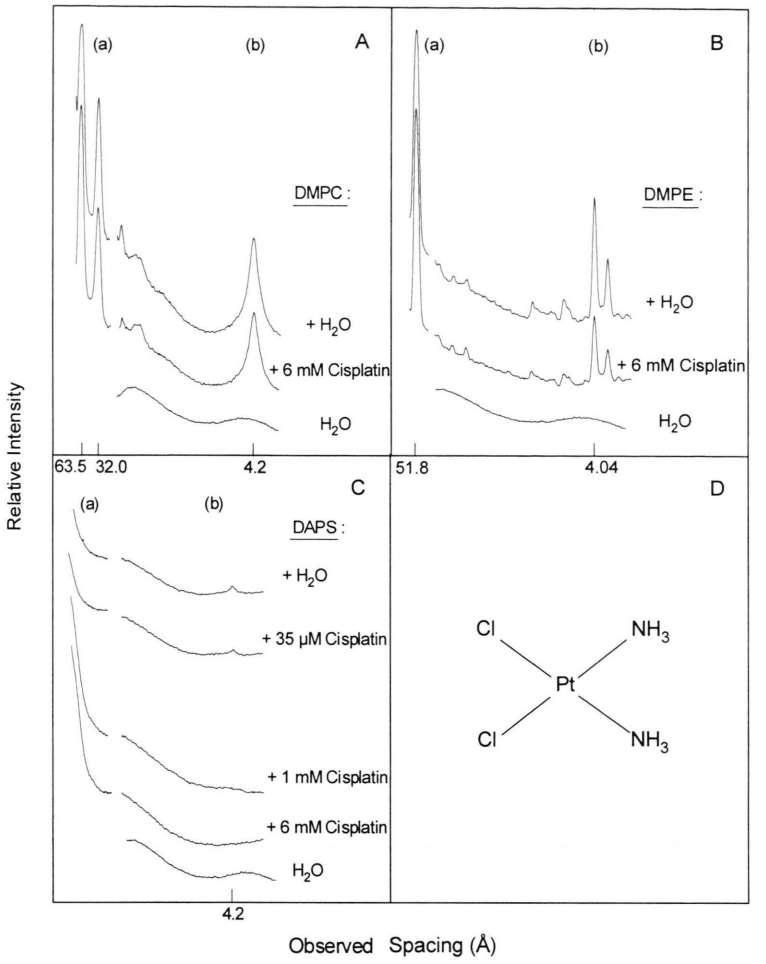

Fig. 1. Microdensitograms from X-ray diagrams of (A) dimyristoylphosphatidylcholine (DMPC), (B) dimyristoylphosphatidylethanolamine (DMPE) and (C) diacylphosphatidylserine (DAPS) with water and aqueous solutions of cisplatin; flat-plate cameras; specimen-to-film distances: (a) 14 cm, (b) 8 cm. (D) Structural formula of cisplatin (*cis*-diamminedichloroplatinum (II)).

the same concentration as that found in serum when it is therapeutically administered, interacts with and perturbs the bilayer structure of DAPS.

Fluorescence measurements on large unilamellar vesicles (LUV)

The structural effects of cisplatin on DMPC LUV were determined at the acyl chain deep hydrophobic core and at the hydrophilic/hydrophobic interface regions of the phospholipid bilayer by evaluation of DPH steady state fluorescence anisotropy (r) and laurdan general polarization (GP), respectively. Table I shows that increasing concentrations of cisplatin up to 10 mM did not significantly change the r and GP values. The DPH steady state anisotropy is primarily related to the rotational motion restriction due to the hydrocarbon chain packing order. Therefore, the maintenance of this parameter indicated that no interaction occurred between cisplatin and DMPC hydrophobic region. On the other hand, the stability of laurdan GP indicated that the dynamics of the dipolar relaxation was not affected and/or there was no water penetration into the polar head group region. These results lead to the conclusion that cisplatin did not interact with the hydrophilic region of DMPC LUV. These conclusions agreed with those obtained from the X-ray diffraction experiments.

Discussion

Our experimental results indicate that human erythrocytes incubated with a dose of cisplatin close to its therapeutical concentration in serum

Table I. Effect of cisplatin on the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the general polarization (GP) of laurdan embedded in large unilamellar dimyristoylphosphatidylcholine (DMPC) vesicles (probe:lipid ratio 1.600).

Cisplatin conc. [mM]	r DPH	GP laurdan
0.00	0.316	0.338
0.01	0.315	0.340
0.10	0.316	0.343
1.00	0.317	0.344
10.00	0.317	0.344

Each result represents the average of data in duplicate samples; between 6 and 12 determinations were performed in each sample. Mean standad deviation ± 0.003.

developed cup shaped forms (stomatocytes). Accordingly to the bilayer couple hypothesis (Sheetz and Singer, 1974), the shape changes induced by foreign molecules in erythrocytes are due to differential expansion of the two monolayers. Thus, stomatocytes are produced when the added compound locates into the inner leaflet whereas spiculated-shaped equinocytes arise when it inserts into the outer moiety (Lange *et al.*, 1982). It can be concluded that cisplatin inserted into the inner monolayer of the erythrocyte membrane. This conclusion is supported by the results obtained from the incubation of cisplatin with bilayers composed of the phospholipids DMPC, DMPE and DAPS. While DMPC represent a class of lipids preferentially located in the outer monolayer of the erythrocyte membrane, the other two are mostly found in its inner leaflet. X-ray diffraction and fluorescence spectroscopy analysis of DMPC under the respective forms of multilayers and large unilamellar liposomes showed that cisplatin in a concentration as high as 10 mM did not significantly perturbed the structure of any of these forms A similar result was found in DMPE multilayers by X-ray diffraction. It is possible, therefore, to conclude that cisplatin does not interact with zwitterionic phospholipids. This conclusion agrees with binding studies reported in the literature (Speelmans *et al.*, 1996). However, X-ray diffraction results indicated that 35 μM cisplatin perturbed the bilayer structure of DAPS, which was practically destroyed when the concentration of cisplatin was increased to 1 and 6 mM. This result is not surprising as it has already been reported that cisplatin forms a stable coordination complex with phosphatidylserine in model membrane systems (Speelmans *et al.*, 1997). Although this complex seemed to be absent when cisplatin was incubated with freshly isolated human erythrocytes, the drug was efficiently taken up by the red cells (Burger *et al.*, 1999). Our results clearly indicate that cisplatin *in vitro* locates into the inner monolayer of the intact erythrocyte membrane. Although its interaction with a protein located in the inner leaflet cannot be disregarded, the fact that cisplatin had a strong affinity for DAPS tends to support the hypothesis that its location in the cytosolic moiety of the erythrocyte membrane is due to its affinity with phosphatidylserines. This lipid plays an important role in cellular processes such as signal transduction, cell proliferation and apoptosis, the blood clotting cascade, biogenesis of mitochondria, and is necessary for the activity of membrane enzymes such as $(Na^+ + K^+)$-ATPase and protein kinase C (Burger *et al.*, 1999). Therefore, the interactivity of cisplatin with the cell membrane might affect its structure and vital functions and explain its cytotoxic effects.

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