

COMPOUNDS THAT MODULATE MULTIDRUG RESISTANCE IN CANCER CELLS

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The resistance of cancer cells to multiple chemotherapeutic agents remains a major obstacle in cancer therapy [1]. Cancer cell lines developed in vitro have provided significant insights into the mechanisms of multidrug resistance. Cell lines which are highly resistant to a variety of anticancer agents can be obtained by slowly increasing the concentration of cytotoxic agents in the growth medium. The phenomenon of multidrug resistance (MDR) is not restricted to mammalian cells - it occurs from cancer cells to microorganisms. Homologues of mammalian MDR genes have been identified in bacteria, fungi and protozoan parasites [2-4]. Most organisms appear to have developed a lot of strategies to protect them from toxic environmental compounds. A variety of these specific genetic changes have been identified in cancer cells and they are capable of protecting these cells from multiple chemotherapeutic agents.

Mechanisms of drug resistance in cancer

One of the protection mechanisms of cancer cells is an increased efflux of cytotoxic compounds out of the cell due to an increased expression of membrane transport proteins such as P-glycoprotein (MDR1) or multidrug resistance associated protein (MRP) [5]. P-glycoprotein (P-gp) is believed to be one of the key molecules which cause multidrug resistance in cancer. Transmembrane glycoprotein P-gp is an ATP-dependent extrusion pump which confers cross-resistance to a variety of structurally-unrelated cytotoxic agents, such as anthracyclines, taxanes, vinca alkaloids and other drugs which are widely used in cancer treatment. P-gp is a member of the ATP-binding cassette (ABC) superfamily of proteins [6]. A prominent member of the ABC superfamily of transporters is CFTR - cystic fibrosis transmembrane conductance regulator. CFTR is a chloride channel which malfunctions in cystic fibrosis.

P-gp consists of four distinct domains. Two of these are highly hydrophobic, integral membrane domains, each of which spans the membrane six times by alpha-helices. The other two are hydrophilic nucleotide-binding domains (NBDs). Recently, a 2.5 nm resolution structure of P-gp was obtained by electron microscopy and single-particle image analysis [7]. In the P-gp molecule there is a large central pore, ~5 nm in diameter, which is closed at the inner (cytoplasmic) side of the plasma membrane. A gap may be present in the

protein ring; this could allow substrates to access the central pore from the lipid phase. P-glycoprotein is predicted to act as a flippase with drug substrates, gaining access to their binding sites from the inner leaflet of the lipid bilayer [8].

P-glycoprotein is also expressed in normal mammalian tissues, usually at secretory surfaces. It is expressed at high levels in the biliary canaliculi, the proximal tubules of the kidney, intestinal and colonic epithelium and pancreatic ducts. High levels of P-gp have been demonstrated in capillaries found in the central nervous system, suggesting a possible role of P-gp in the blood/brain barrier. The physiological function of P-gp has proved difficult to identify. A role has been proposed both as a protective mechanism against xenobiotics and as a transporter of endogenous substrates [6].

The most puzzling feature of P-glycoprotein is its recognition and transport of a wide variety of substrates. Many, but not all, cytotoxic compounds in the multidrug resistance phenotype are relatively small, lipophilic, and cationic at physiological pH. Apart from many anticancer drugs, there are some fluorescent probes such as Rhodamine 123 and Di(OC)₂ which are substrates of P-gp. They may be used in functional tests to detect chemoresistant cells by the flow cytometry technique [6].

Reversal of drug resistance

The development of pharmacological agents that reverse drug resistance is a very promising way to overcome the difficulties in successful chemotherapy. Resistance to anticancer drugs such as doxorubicin, vinblastine, vincristine or taxol can be reversed, at least *in vitro*, by a variety of resistance modifying agents called MDR reversal modulators or chemosensitizers. Among the many MDR modulators are the following compounds: calcium channel blockers (e.g. verapamil), cyclosporine A, phenothiazines, steroid hormones and non-ionic detergents [9, 10]. MDR modulators often reverse multidrug resistance by competing for the transport system responsible for MDR, but overall the mechanism of MDR modulation is not well understood.

The role of the lipid bilayer in chemosensitization

P-gp probably interacts with substrates in the inner leaflet of the lipid bilayer of the plasma membrane, and translocates them in flip-flop fashion to the outer leaflet of the lipid bilayer (the "vacuum cleaner" hypothesis). The binding of drug substrates to P-glycoprotein may thus be modulated by the properties of lipid bilayer [11]. It was shown for various P-gp substrates that the nature of the lipid acyl chains, kind of lipid headgroup and state of the lipid phase modulate drug-protein binding [12]. The perturbation of the lipid phase of the cell membrane by modulators seems to be important for anticancer drug cytotoxicity potentiation. Beyond the interaction of modulators with MDR proteins, there is their interaction with the lipid bilayers of the plasma membrane, which may be

essential. So, membrane lipids can be regarded as one of the targets for MDR reversing agents. Most of the MDR reversing compounds are preferentially soluble in lipids and they may also exert an influence on the physical properties of lipid bilayers. A lot of them can alter membrane fluidity and increase membrane permeability [13]. Alterations in the physical state of plasma membrane lipids can influence a number of important carrier-mediated processes and they appear to be important factors modulating efflux pump systems [14].

Interactions of MDR modulators with phospholipids

The aim of our research is to reveal the putative role of the interaction of some chemosensitizers with the lipid phase in the mechanism of drug resistance reversal caused by these compounds. The interactions of MDR modulators with phospholipid liposomes and multilamellar lipid structures were studied using fluorescence spectroscopy, absorption spectroscopy and differential scanning microcalorimetry. The modulators represented two groups of compounds: phenothiazines and isoflavones.

Phenothiazines as MDR modulators

Phenothiazines, tricyclic antidepressant drugs, are among the compounds known to modify multidrug resistance mediated by P-gp in different kinds of cancer cell lines [15].

Artificial lipid membranes of different composition were used to study the ability of phenothiazines to interact with phospholipid membranes. The interactions of chemosensitizers with lipid model membranes were studied via fluorescence spectroscopy with the application of some fluorescence probes: N-phenyl-1-naphthylamine (NPN), 1,6-diphenyl-1,3,5-hexatriene (DPH) and Laurdan. The incubation of NPN-labelled liposomes with given antidepressant tricyclic compounds resulted in strong changes in the emission spectra of membrane-bound NPN. The alterations in NPN spectra were strongly dependent on the chemical structure of the chemosensitizers (e.g. on the phenothiazine ring substituents) and on the phospholipids used to produce liposomes. Compounds with substituents: CF₃, Cl, SCH₃, SCH₂CH₃ and OCH₃ in position 2 of their phenothiazine ring were more effective quenchers of the fluorescence of membrane-bound NPN than promethazine (-H).

It was shown in different cancer cell lines that certain substitutions in position 2 of the phenothiazine ring: CF₃, Cl, SCH₃, SCH₂CH₃, OCH₃ improve MDR reversal activity. The order showed by the substituents is: CF₃ > Cl > SCH₃ > H [16]. The distance between the amino group in the side chain and the tricyclic ring nucleus is also important for antagonism of MDR. Piperazinyl amines are more potent than noncyclic groups.

Comparing our results with data from the literature on structure-MDR reversal activity relationships for phenothiazines, we may conclude that the quenching

efficiency of the membrane-bound NPN emission caused by these compounds correlates well with their MDR reversal activity. The correlation between lipid bilayer perturbation and the reversal activity of the studied compounds indicates that modulator–lipid interaction is probably an important factor in the mechanism of drug resistance reversal.

Perturbations in the biophysical properties of the plasma membrane, both increases and decreases in membrane fluidity, may result in increased cell accumulation of chemotherapeutic agents. To test the membrane fluidising or rigidifying effects of the chemosensitizers, DPH fluorescence polarisation measurements were performed. The increase in the degree of polarization of DPH fluorescence in PS liposomes in the presence of phenothiazines was observed. The effect was strongly dependent on substitutions in the phenothiazine ring

The influence of trifluoperazine on phospholipid bilayers

Trifluoperazine (TFP) is usually more effective than chlorpromazine (CPZ) in reversing drug resistance mediated by P-gp activity [16]. The influence of TFP on bilayers composed of either zwitterionic or charged phospholipids was studied using differential scanning microcalorimetry (DSC). Upon an increase in TFP concentration, asymmetrical broadening and shifting of chain melting transition peaks to lower temperatures were observed. In multilamellar structures formed of phosphatidylcholines, for higher TFP:lipid mole ratios (0.04 and 0.06 for DPPC and DMPC, respectively), the deconvolution of chain melting transition profiles into two separate Gauss components was possible [17]. The presence of more than one Gauss component in the phase transition profile is usually attributed to the existence of phase separation in the studied system.

In the thermograms obtained for the charged phospholipid, dimyristoyl-phosphatidylglycerol (DMPG), in the presence of TFP, broadening of the transition peaks was also observed but, contrary to the results for phosphatidylcholines, the DMPG transition profiles remained symmetric and their deconvolution into multiple Gauss components was not possible. Perturbation of lipid bilayer structure induced by trifluoperazine seems to be dependent on the lipid chain length, as may be concluded from similar transition enthalpy changes in DMPG and DMPC in the presence of TFP [17]. DSC experiments show that trifluoperazine, a known potent modulator of drug resistance in mammalian cancer cells and bacteria, alters lipid phase transition properties and is able to produce phase separation in TFP:phosphatidylcholine systems.

Phase separation and membrane fluidisation induced by TFP in phosphatidylcholines might be responsible for the permeability increase observed by other authors.

A new group of MDR modulators - isoflavones

Isoflavones are widely distributed in the plant kingdom. Genistein and daidzein are the two major soy isoflavones. The interest in the mechanism of genistein action originated from the discovery that the compound, like other isoflavones, displays a remarkable estrogenic activity. Genistein is also a specific and potent inhibitor of tyrosine kinases and it interferes with many biochemical pathways [18]. Among the various cell targets of genistein, ABC transporters were also identified. Genistein was found to be a modulator of non-P-gp mediated multidrug resistance, not affecting Pgp MDR cells [19]. Other isoflavone and flavone compounds have been found to be active against P-gp [20]. The number and position of hydroxyl groups are determining factors for isoflavone and flavone activity. The influence of OH substitution on MDR modulating activity appeared to be highly dependent on the position of substitution [20]. For example, the presence of an unsubstituted OH group at position 5 appears to be preferable in the case of resistance mediated by P-gp. This behaviour could be related to the presence of an intramolecular hydrogen bond between this phenol and the adjacent ketone group. This creates a structure which brings an additional lipophilic contribution to the flavonoid nucleus. Lipophilicity by itself does not determine MDR modulating activity, but may be regarded as a favorable parameter within a homologous series.

The interaction of isoflavones with the lipid phase of the membrane could represent an additional contribution to the other effects of these compounds on cellular functions. In our experiments, the interaction of a set of isoflavones extracted from various plants with phospholipid membranes was examined. Some of the isoflavones induced aggregation of liposomes, dependent on the substitutions in the isoflavone molecule, kind of phospholipid and cholesterol content. DSC experiments revealed strong perturbation of the thermal phase behaviour of DPPC multilamellar structures by the isoflavones. In many cases isoflavones decreased both the main transition temperature T_m and the enthalpy of phase transition.

REFERENCES

1. Simon, S.M. and Schindler, M. Cell biological mechanism of multidrug resistance in tumors. **Proc. Natl. Acad. Sci. USA** 91 (1994) 3497–3504.
2. Holland, I.B. and Blight, M.A. ABC-ATPases, Adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. **J. Mol. Biol.** 293 (1999) 381–399.
3. Kołaczkowski, M., van der Rest M., Cybularz-Kołaczkowska A., Soumillion J.-P., Konings W. N. and Goffeau A. Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. **J. Biol. Chem.** 271 (1996) 31543–31548.

4. Köhler, T., Pèchere, J.-C. and Plésiat, P. Bacterial antibiotic efflux systems of medical importance. **Cell. Mol. Life Sci.** 56 (1999) 771–778.
5. Gottesman, M.M. and Pastan, I. Biochemistry of multidrugresistance mediated by the multidrug transporter. **Annu. Rev. Biochem.** 62 (1993) 385–427.
6. Bosch, I. and Croop, J. P-glycoprotein multidrug resistance and cancer **Biochim. Biophys. Acta** 1288 (1996) F37-F54.
7. Rosenberg, M.F., Callaghan, R., Ford, R. C. and Higgins, C. F. Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. **J. Biol. Chem.** 272 (1997) 10685-10694.
8. Higgins, C.F. and Gottesman, M.M. Is the multidrug transporter a flippase? **Trends Biochem. Sci.** 17 (1992) 18-21.
9. Ford, J.M. Experimental reversal of P-glycoprotein – mediated multidrug resistance by pharmacological chemosensitisers. **Eur. J. Cancer** 32(A) (1996) 991–1001.
10. Woodcock, D.M., Linsenmeyer, M.E., Chojnowski, G., Kriegler, A.B., Nink, V., Webster, L.K. and Sawyer, W.H. Reversal of multidrug resistance by surfactants. **Brit. J. Cancer** 66 (1992) 62–68.
11. Callaghan, R., Stafford, A. and Epan, R.M. Increased accumulation of drugs in a multidrug resistant cell line by alteration of membrane biophysical properties. **Biochim. Biophys. Acta** 1175 (1993) 277–282.
12. Romsicki, Y. and Sharom, F.J. The membrane lipid environment modulates drug interactions with the p-glycoprotein multidrug transporter. **Biochemistry** 38 (1999) 6887–6896.
13. Drori, S., Eytan, G.D. and Assaraf, Y.G. Potentiation of anticancer–drug cytotoxicity by multidrug–resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability. **Eur. J. Biochem.** 228 (1995) 1020–1029.
14. Pajeva, K., Wiese, M., Cordes, H.P., Seydel, J.K. Membrane interactions of some catamphilic drugs and relation to their multidug–resistance–reversing ability. **J. Cancer Res. Clin. Oncol.** 122 (1996) 27–40.
15. Ford, J.M., Prozialeck, W.C. and Hait, W.W. Structural features determining activity of phenothiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance. **Molecular Pharmacology** 35 (1989) 105–115.
16. Ramu, A. and Ramu, N. Reversal of multidrug resistance by phenothiazines and structurally related compounds. **Cancer Chemother. Pharmacol.** 30 (1992) 165–173.
17. Hendrich, A.B., Wesolowska, O., Michalak, K. Trifluoperazine induces domain formation in zwitterionic phosphatidylcholine but not in charged phosphatidylglycerol bilayers. **Biochim. Biophys. Acta** 1510 (2001) 414-425.

18. Polkowski, K. and Mazurek, A.P. Biological properties of genistein. A review of in vitro and in vivo data. **Acta Pol. Pharm-Drug Res.** 57 (2000) 135-155.
19. Versantvoort, C.H.M., Broxterman, H.J., Lankelma, J., Feller, N. and Pinedo, H.M. Competitive inhibition by genistein and ATP dependence of daunorubicin transport in intact MRP overexpressing human small cell lung cancer cells. **Biochem. Pharmacol.** 48 (1994) 1129-1136.
20. Ferte, J., Kühnel, J.-M., Chapuis, G., Rolland, Y., Lewin, G. and Schwaller, M.A. Flavonoid-related modulators of multidrug resistance: synthesis, pharmacological activity and structure-activity relationships. **J. Med. Chem.** 42 (1999) 478-489.