

MULTIDRUG RESISTANCE TO CANCER CHEMOTHERAPY: GENES INVOLVED AND BLOCKERS

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في خلال الثلاث عقود الماضية ، بذلت مجهودات بحثية كبيرة لمعرفة الآلية التي تستطيع من خلالها خلايا السرطان التغلب على تأثيرات العديد من أدوية السرطان. وقد ركزت معظم الأبحاث السابقة على مقاومة خلايا السرطان لأدوية السرطان المستخرجة من مصادر طبيعية. إن مقاومة العديد من الأدوية هي مقاومة مشتركة واسعة النطاق للعديد من الأدوية الطبيعية التي تستخدم في علاج السرطان والتي تعتبر السبب الرئيسي لفشل العلاج لعدد كبير من الأدوية مثل قلويدات نبات فينكا وتكسان وإيبودوفيللوتوكسينز وبعض المضادات الحيوية. وتنتج هذه الظاهرة من الزيادة في نسخ أربع جينات مقاومة للعديد من هذه الأدوية وكذلك زيادة نسخ البروتينات المصاحبة لها والتي تعمل كمضخات مستهلكة للطاقة ومرتبطة بجدار الخلية. وتعمل هذه البروتينات على خروج عدد كبير من الأدوية والتي لا تتشابه في الوظيفة أو التركيب ، ولكن جميع هذه الأدوية مستخرج من أصل طبيعي. وقد تكون هذه المقاومة طبيعية أو مكتسبة بعد العلاج الكيماوي. إن وجود المقاومة الطبيعية في خلايا السرطان قبل وبعد العلاج يؤدي إلى سوء في نتائج العلاج وزيادة في انتشار خلايا السرطان في أماكن أخرى. وفي ضوء عدم وجود بديل للأدوية المستخرجة من مصادر طبيعية وفي ضوء المقاومة المتعددة لهذه الأدوية والتي تمثل عقبة رئيسية في نجاح العلاج الكيماوي فقد استهدف هذا البحث إلقاء الضوء على الجينات المسببة للمقاومة المتعددة للأدوية ومثبطاتها وكذلك استخدام العلاج الجيني للتغلب على هذه الظاهرة.

During the last three decades, important and considerable research efforts had been performed to investigate the mechanism through which cancer cells overcome the cytotoxic effects of a variety of chemotherapeutic drugs. Most of the previously published work has been focused on the resistance of tumour cells to those anticancer drugs of natural source. Multidrug resistance (MDR) is a cellular cross-resistance to a broad spectrum of natural products used in cancer chemotherapy and it is believed to be the major cause of the therapeutic failure of drugs belonging to different naturally obtained or semisynthetic groups including vinca alkaloids, taxans, epipodophyllotoxins and certain antibiotics. This phenomenon results from overexpression of four MDR genes and their corresponding proteins that act as membrane-bound ATP-consuming pumps. These proteins mediate the efflux of many structurally and functionally unrelated anticancer drugs of natural source. MDR may be intrinsic or acquired following exposure to chemotherapy. The existence of intrinsically resistant tumour cell clone before and following chemotherapeutic treatment has been associated with a worse final outcome because of the increased incidence of distant metastasis. In view of irreplaceability of natural product anticancer drugs as effective chemotherapeutic agents, and in view of MDR as a major obstacle to successful chemotherapy, this review aimed to highlight the genes involved in MDR, classical MDR blockers and gene therapy approaches to overcome MDR.

Key words: Multidrug resistance, MDR genes, MDR Blockers.

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Introduction

Cancer chemotherapy has been widely and successfully employed in the treatment of various types of cancer. Unfortunately, the usefulness of this important treatment modality is usually limited due to the rapid development of tumour cell resistance (1,2) and organs toxicity (3,4). It is well known that, cellular resistance to chemotherapy is often multifactorial and may be affected by the cell cycle stage, proliferation status, cellular drug transport (influx, efflux and retention) and DNA replication and repair mechanisms (1,5). Multidrug resistance (MDR) is the most widely studied manifestations of tumour cell resistance (6). This type of resistance results from overexpression of MDR genes, which encode for specific proteins that act as membrane-bound ATP-consuming pumps (2,7,8). Genotyping of these MDR genes and identifying haplotypes is an important tool in predicting individual sensibility to chemotherapy (2).

Multidrug Resistance: Historical Background:

The story of multidrug resistance to cancer chemotherapy has been initiated in 1968 and is still continuing. The milestones in its history are highlighted below.

1968 Kessel *et al.* reported that in vivo sensitivity of mouse leukemic cells to daunomycin is positively correlated with their ability to retain it (9).

1970 Biedler and Riehm, have demonstrated that Chinese Hamster Ovary (CHO) cells, selected for resistance to actinomycin D were cross-resistant to mithramycin, vinblastine, vincristine, daunorubicin and mitomycin C. This phenomenon is presently known as MDR (10).

1973 Dano, reported that active drug efflux may play a role in the decreased accumulation of daunorubicin in resistant Ehrlich Ascites Carcinoma (EAC) cells (11).

1976 Juliano and Ling, have detected a 170 KD glycoprotein in the membrane of CHO cells selected for resistance to colchicine, but not in their drug sensitive counterparts. The investigators termed this protein permeability-glycoprotein (P-gp), since it appeared to be unique to mutant cells displaying altered drug permeability (12).

1981 Tsuruo *et al.* found that verapamil inhibits the efflux of vincristine and restores the cytotoxicity of vinca alkaloids in vincristine-resistant murine

leukemic cells both in vivo and in vitro. Since that date, many compounds capable of reversing MDR have been identified (13).

1984 The first results of clinical trial with MDR-reversal agents have been reported by Rogan *et al.* (14)

1986 The genes encoding human, mouse and hamster MDR protein were isolated and cloned (15,16).

1992 Cole *et al.*, is the first to detect Multidrug Resistance Associated Protein (MRP) in non-small cell lung carcinoma cell line (17).

1995 Scheffer *et al.* is the first to detect Lung Resistance Related Protein (LRP) in lung cancer cell lines (18).

1998 Breast Cancer Resistance Protein (BCRP) was detected in human breast cancer cell line (MCF-7) by Doyle *et al.* (19).

2000 Osman *et al.* detected MDR1/P-gp expression in sensitive EAC cells 6 hours after in vivo exposure to doxorubicin (20).

2002 Kamel *et al.*, reported that the functional assay method is considered the best approach for evaluation of MDR status as it provides information regarding both the real function and the sum of contributions of all genes and gene products involved in MDR phenotype (21).

2003 Nieth *et al.* used small interference RNA (siRNA) technology as strategy to overcome MDR in human tumours (22).

2004 Sorokin, reported that overexpression of cyclooxygenase-2 (COX-2) increases the expression of MDR1 in cancer cells (23).

2005 Zatelli *et al.* found that the selective COX-2 Inhibitors, rofecoxib, reversed MDR phenotype in medullary thyroid Carcinoma cell line by a p-gp mediated mechanism (24).

2007 Kamel *et al.* reported that transduction of MCF-7/DOX cells with COX-2 antisense reversed their resistance to doxorubicin (25).

1- Mechanisms of Multidrug Resistance:

By definition MDR is a term used to describe the phenomenon characterized by the ability of some tumours to exhibit simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents. A number of mechanisms have explained the phenomenon of MDR in mammalian cells. They have been broadly classified into cellular and non-cellular mechanisms (26).

1.1-Non-cellular MDR mechanisms:

The non-cellular drug resistance mechanisms can arise as a consequence of in vivo tumour growth. These phenomena are typically associated with solid tumours which exhibit unique physiological properties compared to circulating tumours such as hematological malignancies (27). Poor tumour vascularization can result in reduced oxygen, nutrients and drug access to regions within solid tumours and thus protect tumour cells from drug cytotoxicity (28). One example of this type of resistance is the increased presence of non-cycling tumour cells in the poorly vascularized sections of solid tumours. These cells are often viable, but non-dividing and consequently are resistant to drugs dependent on cell proliferation (27,28). The acidic environment in tumours, due to lactic acid generation by hypoxic tumour cells, has also been suggested to confer a resistant mechanism for weak bases, where cellular uptake is dependent on pH gradient across membranes (26).

1.2- Cellular MDR mechanisms:

Cellular mechanisms are categorized in terms of alterations in the biochemistry of malignant cells into two major categories namely, non-transport-based MDR (non-classical MDR phenotypes) and transport-based MDR (classical MDR phenotypes).

1.2.1. Non-classical MDR phenotypes:

The term non-classical MDR is used to describe non-transport based mechanisms that affect multiple drug classes. This type of resistance can be caused by altered activity of specific enzyme systems including, glutathione-S-transferase (GST) and topoisomerases, which can decrease the cytotoxic activity of drugs in a manner independent of intracellular drug concentrations, which remain unaltered (29). In addition, changes in the balance of proteins that control apoptosis can also reduce chemosensitivity since most anticancer drugs are believed to exert their cytotoxic effects via apoptotic processes. The decision, whether a cell continues through cell cycle or undergoes apoptosis, is dependent upon a complex interplay of a team of genes and proteins that exert a regulatory role in cellular events (29). Resistance may, therefore, develop with loss of genes required for cell death such as p53 or overexpression of genes that block cell death such as B-cell lymphoma-2 (bcl-2) homodimers (30). Bcl-2 can confer cellular resistance to many natural pro-

duct anticancer drugs including, doxorubicin, taxol, etoposide, mitoxantrone, camptothecin and cisplatin. Unlike, P-gp mediated MDR, overexpression of bcl-2 does not prevent drug influx into tumour cells (31,32). Overexpression of bcl-2 contributes to resistance mechanism. It has been shown that the anticancer drugs promotes cell cycle arrest; however, their effects are cytostatic rather than cytotoxic (31,33). Indeed, a wide variety of human cancers, with a poor clinical response to chemotherapy, exhibit higher levels of bcl-2 expression. The bcl-2 gene inhibits apoptosis induced by a variety of stimuli including growth factor withdrawal, oncogene activation and antitumor drug treatment (30-32).

1.2.2. Classical MDR phenotypes:

The term classical MDR is used to describe a transport-based cross-resistance mechanisms that affect multiple drug classes. This type of resistance is caused by overexpression of MDR transcription genes and MDR proteins with subsequent decrease in intracellular drug accumulation. Since the classical MDR phenotypes constitute the well characterized mechanisms of MDR, it will be discussed in the following sections.

2- Genes Involved In Multidrug Resistance:

Genes which are responsible for MDR phenomenon and their protein products are broadly classified into four families namely:

- 1- Multidrug Resistance (MDR)
- 2- Multidrug Resistance-associated Protein (MRP)
- 3- Lung Resistance Related Protein (LRP)
- 4- Breast Cancer Resistance Protein (BCRP)

2.1 Multidrug Resistance Genes (MDR genes):

In human, three different MDR genes have been identified (15,16). These genes, MDR1, MDR2 and MDR3, have a higher degree of homology and the same chromosomal (7q21) location (34). Among these genes, only overexpression of MDR1 in cell lines has been correlated to and responsible for the development of MDR to cancer chemotherapy (7,35). In murine, there are three MDR genes (mdr1, mdr2, and mdr3) out of which only two genes (mdr1 and mdr3) are involved in MDR. In mice, two genes encoding drug-transporting proteins have been identified (36), mdr1a (mdr3) and mdr1b (mdr1). Their products (mdr1a and mdr1b P-gp) are highly homologous to each other and to the single human

MDR1 P-gp (37). A full description of human MDR1 gene and its products are highlighted below (38):

2.1.1. Human MDR1 Gene:

Name MDR1 (multiple drug resistance 1)

Chromosome 7q21.

DNA Spans on a 120 kb genomic fragment and separated from MDR3 gene (which is transcribed in the same direction) by only 34 kb of intergenic DNA.

RNA 5 kb mRNA.

PROTEIN Size 1280 amino acids.

Name P-glycoprotein 1 (P-gp1), ATP-Binding Cassette, subfamily B, member 1 (ABCB1).

Description 170 kDa transmembrane glycoprotein; the N-terminal half of the molecule contains six transmembrane domains and large cytoplasmic domain with ATP binding site, and then a second section with 6 transmembrane domain and an ATP binding site.

Expression Overexpressed in many MDR cell lines and in tumours resistant to chemotherapy. Also expressed at secretory surface of a number of normal tissues, including biliary canaliculi in liver, proximal tubules of the kidney, intestinal and colonic epithelium and hematopoietic stem cells.

Localization Integral membrane protein.

Function The P-gp is an energy-dependent efflux pump involved in extrusion of many structurally unrelated anticancer drugs resulting in a decrease in intracellular drug concentration. In normal tissues, it acts as a protective mechanism against noxious xenobiotic and carcinogens.

Homology Closely related gene to MDR3 (also called P-gp3), located at the same chromosomal site but not involved in MDR. There are 3 murine homolog genes (*mdr1*, *mdr2* and *mdr3*) out of which only 2 genes (*mdr1* and *mdr3*) are involved in MDR.

Cytogenetics The genomic amplification of MDR1 appears as extrachromosomal 'double minute chromosomes (DM) or intrachromosomal "homogeneous staining regions" (HSR).

Oncogenesis Amplification.

2.1.1.1. Expression of MDR1 gene in normal tissues:

Human MDR1 mRNA is expressed in normal tissues including colon, small intestine, kidney and liver, and its protein product P-gp is located on the apical surface of these cells facing the lumen (39,40). This strategic localization suggests that

multidrug transporters may have normal physiological role in transporting cytotoxic compounds and also confirm the excretion function of this protein (38). In the liver, expression of MDR1 gene is increased after both partial hepatectomy and exposure to carcinogens. The combination of both partial hepatectomy and carcinogen exposure is synergistic resulting in over a 100 fold increase in MDR protein expression (39). Based on this information, it has been postulated that this protein is part of an integrated system for protecting cells against xenobiotics (38).

2.1.2. Human MDR2 and MDR3 Genes:

The human MDR2 and MDR3 genes and their products are completely homologues to each other and are not involved in MDR (40). The human MDR3 gene and its product p-gp3 and the murine *mdr2* gene and its product P-gp are phospholipid translocator involved in phospholipid excretion and predominantly expressed in the canalicular membrane of hepatocytes which is essential for the formation of phospholipid vesicles and mixed lipid/bile salt micelles (41,42). Accordingly, patients with complete defect in MDR3 gene underlies a Progressive Familial Intrahepatic Cholestasis, a progressive liver disease of childhood, in which cholestasis is of hepatocellular origin and often occurs in the first year of life and leads to death due to liver failure (43). Similarly, disruption of the murine *mdr2* gene as in case of *mdr2* knockout mice (*mdr2/p-gp (-/-)*), leads to complete absence of biliary phospholipids (44). Histological and biochemical features in knockout mice and in patients with genetic defect in MDR3 are similar including, periportal inflammation, extensive bile duct proliferation, feather degeneration of hepatocytes and liver malignancy (43). All these histological pictures result from the cytotoxicity of bile salts are antagonized by phospholipids. Increased levels of *mdr2* mRNA in the liver of mice treated with fibrates had been recently reported (44). The authors (44) concluded that the pharmacological modulation of biliary lipid secretion mediated by fibrates was related to overexpression of a specific liver gene product, *mdr2* P-gp. Using *mdr2/P-gp (-/-)* deficiency mice, Voshol *et al.* (45) reported that excess dietary phospholipids and cholesterol does not normalize low HDL associated with *mdr2/p-gp* deficiency. The use of *mdr2* knockout mice as an experimental model, highlights the possible mecha-

nisms for the pathogenesis of cholestatic liver disease and may be helpful in establishing therapeutic approaches in this complex disease and other diseases.

2.1.3. P-glycoprotein (p-gp):

Using CHO cells, Juliano and Ling (12) initially described P-gp and subsequent investigations have confirmed that the 170 Kd P-gp is responsible for drug efflux and resistance to several unrelated natural products used in cancer chemotherapy. P-glycoprotein is the most studied MDR protein and it belongs to the ATP-Binding Cassette protein, subfamily B (ABCB) which is related to the superfamily ABC. Information regarding this peptide is highlighted below (38):

Names P-glycoprotein 1 (P-gp1).

Multiple Drug Resistance 1 (MDR1).

ATP-Binding Cassette, subfamily B, member 1 (ABCB1).

Amino Acid 1280.

Function Energy-dependent efflux pump.

Subcellular

Localization Integral membrane protein.

Tumour tissue

specificity Tumours displaying MDR to natural product anticancer drugs.

Normal Tissue

Specificity Liver, Kidney, colon and small intestine.

2.1.3.1. Expression of MDR1/p-gp and its Clinical Correlation:

The human p-gp1, the product of human MDR1 gene, is responsible for the efflux and resistance to several structurally and functionally unrelated natural product drugs used in cancer chemotherapy. P-glycoprotein substrates or agonists (drugs which are being pumped out the cell by P-gp) are listed below (7).

- | | |
|--------------------|------------------------|
| 1- Anthracyclines | 2- Anthracenes |
| Doxorubicin | Mitoxantrone |
| Daunorubicin | Bisantrene |
| Epirubicin | |
| Idarubicin | |
| 3- Vinca Alkaloids | 4- Epipodophyllotoxins |
| Vinblastine | Etoposide (VP-16) |
| Vincristine | Teniposide (VM-26) |
| Vindesine | |
| Vinorelbine | |

- | | |
|---------------------------|---------------------------|
| 5- Taxanes | 6- Antitumour antibiotics |
| Paclitaxel (Taxol) | Actinomycin |
| Docetaxel (Taxoter) | Bleomycin |
| | Mitomycin C |
| 7-Topoisomerase Inhibitor | 8-HIV-Protease Inhibitors |
| Topotecane | Ritonavir |
| Irenocane | Indinavir |
| Camptothecin | Saquinavir |

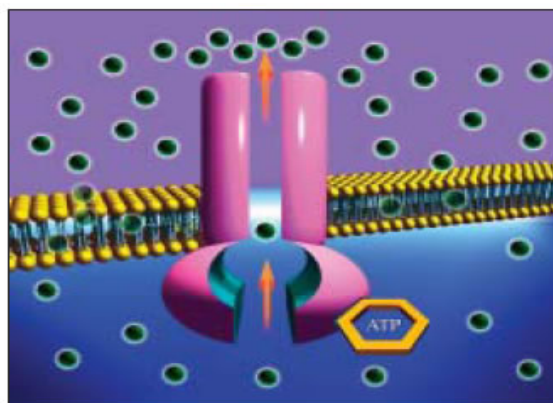


Fig 1. — Mechanism of action of P-glycoprotein (P-gp) inhibitors, showing normal P-gp function in the plasma membrane of a cancer cell during chemotherapy. Activation of the efflux pump by the hydrolysis of a bound ATP molecule drives the cytotoxic drug molecules out of the cell. (Cited from Ref. 25)

Several mechanisms have been put forward to explain the transport function of P-gp. Earlier studies have demonstrated that, P-gp possesses multiple drug-binding sites (7). Using P-gp preparation from CHO cells, Shapiro and Ling, (46) demonstrated that Rhodamine 123 and Hoechst 33342 each stimulated the P-gp-mediated transport of the other compound, suggesting that each molecule bound to a separate and distinct binding site within P-gp. A minimum of four drug binding sites on P-gp have been recognized (47). Figure 1 postulates that P-gp encounters anticancer drugs in the inner leaflet of the plasma membrane and flips these agents to the out leaflet, where they diffuse to the extracellular region (29) P-gp has also been postulated to increase intracellular pH (48), depolarizing plasma membrane electrical potential of the cell by acting as a proton pump, or a chloride channel, thus reducing intracellular accumulation of weak bases or reducing pH-dependent binding of agents to their intracellular targets (49). Overexpression of P-gp has been reported in refractory tumors such as leukemias, breast and ovarian cancers and sarcomas (34). Ferrao *et al.* (50) reported that in leukemic cell lines and 50

Acute Myeloid Leukaemia (AML) patient samples, P-gp mRNA levels correlated better with drug efflux than with cell surface P-gp protein content. In an earlier study, Kanerva *et al.*, reported that 53% of samples from ALL patients had increased P-gp expression, however there was no difference in event free survival or overall survival between patients with lower or higher P-gp expression (51). Wuchter *et al.* have reported that in 121 AML adult patients and 102 pediatric ALL patients, P-gp expression as detected by MRK16 and 4E3 antibodies did not differ between the AML and ALL patients (52). Several studies have analyzed MDR1 mRNA levels by Northern blotting and P-gp content by immunohistochemistry (51-54). Lu *et al.* have described that in pancreatic cancer samples there was a small increase in the amount of MDR1 mRNA content and patients with high MDR1/P-gp expression had a shorter postoperative survival time compared with those patients with weak to moderate expression (53). However, survival of patients with high P-gp expression was not significantly different from those patients with nil P-gp expressing tumours. Jiang *et al.*, reported an inverse relationship between MDR1 gene expression and achievement of complete remission in Acute Leukaemia patients (54). Schneider *et al.*, reported that MDR1 mRNA expression in tumour tissues from breast cancer patients was associated with the presence of invaded axillary nodes in 18/22 cases (81.8 %) as compared with 13/24 (54.2 %) in the group with undetectable MDR1 expression (55). Guanghan *et al.*, studied MDR1/p-gp expression in 34 cases with acute leukaemia. The authors reported that MDR1 expression is not related to age, sex, peripheral white blood count and the percentage of immature cells and directly related to the status of an illness, drawing up and adjusting the chemotherapy plan and reasonably judging the prognosis (56). In renal cell carcinoma patients, Mignogna *et al.*, reported a strong association between MDR1 and worse outcome and concluded that monitoring MDR1 expression is useful in predicting cancer evolution and choosing the appropriate treatment (57).

Gad El Mawla *et al.* monitored the expression of MDR1/P-gp among Non-Hodgkin Lymphoma (NHL) patients, using immunohistochemistry technique, and concluded that 46 % from previously untreated aggressive NHL were positive for MDR1-gene (58). Using JSB-1 monoclonal antibody, El-Sayed, (59) monitored the MDR1/P-gp expression in

de novo cases of NHL by immuno-histochemistry. The author reported that 38 % of NHL patients were positive for MDR1/p-gp. The significant correlation between MDR1/p-gp expression and the response showed that P-gp expression was valuable in predicting the two years-overall survival rates. Osman *et al.* (20) have detected MDR1/P-gp expression in sensitive EAC cells after 6 hours exposure to Doxorubicin *in vivo*. In blast cells from 43 patients with de novo Acute Leukaemia, Zekri *et al.* (60), have investigated the expression of MDR1 gene using RT-PCR. Results from this study demonstrated high frequency of MDR1 gene expression; 17 out of 32 with de novo ALL and 3 of 11 with de novo Acute Non-Lymphatic Leukaemia (ANLL), were MDR1 mRNA positive. The authors concluded that MDR1 gene expression in patients with Acute Leukaemia can be used as a prognostic factor and may be helpful in determining chemotherapeutic protocols for those patients. Zekri *et al.* (61) have examined the expression of MDR1 mRNA and five Mismatch Repair (MMR) genes in 23 hepatocellular carcinoma patients using RT-PCR technique. The authors reported that 14 out of 23 (61 %) cases were MDR1 mRNA positive and the expression of MDR1 gene was higher in cirrhotic than non cirrhotic cases indicating that hepatocellular carcinoma developing on top of cirrhosis is expected to be more resistant to chemotherapy. Defective expression of MMR genes reported in this study along with increased expression of MDR 1 gene confirm the roles of these genes in MDR phenotype and in design of chemotherapy protocols for treatment of hepatocellular carcinoma patients. Kamel *et al.* (21) studied MDR1 /p-gp expression in 114 patients with acute leukaemia by flow cytometry using UIC2 and 4EC monoclonal antibodies compared with RD 123 efflux as a functional assay method. In ANLL group, RD 123 was positive in 35/41 (85 %), while MDR1 expression was positive only in 45 %. Discrepancies were encountered in 16 cases with 14 showing positive and negative functional assay. In ALL, function assay was positive in 51/72 (71 %) while MDR1 expression was positive only in 9/60 (15 %). In 14/46 positive function assay was associated with negative MDR1 expression while 6 of the 9 cases expressing MDR1 showed negative function assay. The authors concluded that the discrepancies between high positive function assay and negative MDR1 expression in ALL cases might indicate that other MDR proteins including MRP and LRP are

responsible for the positive function assay and that MDR1 expression might be more important in determining resistance probably through its antiapoptotic effect.

2.2. Multidrug Resistance Associated Protein (MRP):

MRP, is a gene involved in MDR, discovered in MDR P-glycoprotein-negative, non-small cell lung carcinoma cell line (17). Information regarding this gene and its protein products are highlighted below (62).

Name Multidrug Resistance Associated Protein (MRP).

Chromosome 16p13.

DNA Spans at least 200 kb and contains 31 exons.

RNA 7 kb mRNA transcript.

PROTEIN

Size 1531 amino acids.

Name Multidrug Resistance Associated Protein (MRP).

Description 190 KD contains 3 transmembrane-spanning helices and two ATP binding domains.

Expression Overexpressed in many MDR cell lines and in tumours resistant to chemotherapy, including solid and hematological tumours. Also expressed at basal level in a wide variety of normal tissues, epithelial cells and all and hematopoietic cell types.

Localization Mainly at cell membrane of tumour cells and in cytoplasm of normal cells.

Function Plasma membrane, an energy-dependent efflux pump; overexpression confers MDR phenotype to a wide variety of many structurally unrelated anticancer drugs.

Several investigators have reported the presence of drug resistance in the absence of P-gp, suggesting that P-gp is not the sole protein responsible for MDR and several other proteins might contribute to resistance by controlling drug transport and intracellular drug distribution. Cole et al. (17) described MRP as being belongs to ATP-Binding Cassette, subfamily C (ABCC) which is related to ATP-Binding Cassette superfamily (ABC). Seven isoforms of MRP subgroup have been identified in human and share many features contributing to functional and mechanistic similarities among this subgroup of proteins transporter(7). Among the seven isomers of MRP, only MRP1 is responsible for MDR (7,63). Recently, Hasegawa et al., reported that MRP4 plays an important role in luminal efflux of many diuretics

from proximal tubular epithelial cells (64). Information regarding the seven human MRP, including number of amino acids and their homology (% amino acids) to MRP1, are highlighted below (7).

Name	Symbol	Amino acids	Homology to MRP1
MRP1	ABCC1	1531	100 %
MRP2	ABCC2	1545	49 %
MRP3	ABCC3	1523	58 %
MRP4	ABCC4	1325	39 %
MRP5	ABCC5	1437	34 %
MRP6	ABCC6	1503	45 %
MRP7	ABCC10	1513	44 %

2.2.1. Expression of MRP and its clinical correlation:

In blast cells from AML patients, Ross et al. (65) reported that 18 out of 24 patients had MRP mRNA expression and daunorubicin accumulation equal to or less than that of HL-60/W cells, thus suggesting a functional defect in drug transport of cells with high MRP expression. Young et al. (66), reported that MRP1 expression in lung tumor cell lines was correlated with drug response and mRNA levels. Wright et al. (67), used a monoclonal antibody (QCRL- I, highly specific for a defined linear epitope in a relatively poorly conserved region of the human MRP) for monitoring the expression of MRP in archival and snap frozen samples of normal lung and untreated small cell and non-small cell lung cancer. Immunohistochemical studies showed that nearly all histological subtypes of non small cell lung cancer had detectable levels of the expressed MRP. On the other hand, MRP expression in untreated small cell lung cancer was significantly lower. Studies on retinoblastoma by Chan et al. (68) concluded that MRP might result in failure of chemotherapy despite the elimination of P-gp expressing clones by cyclosporine. In human gliomas, Abe et al. reported that MRP and P-gp expression may be involved in acquired or intrinsic drug resistance (69).

2.3. Lung Resistance Related Protein (LRP):

Lung Resistance Related Protein (LRP), is a drug resistance protein that originally detected in lung carcinoma cell line (18). Information regarding this gene and its protein product are highlighted below (70).

Name Lung Resistance Related Protein (LRP).

Other Name Major vault protein (MVP).

Chromosome 16p11.

DNA/RNA Coding sequence, corresponding to full mRNA is 2840 Transcription bp long.

PROTEIN

Name Lung resistance related protein (LRP).

Size 896 amino acids.

Description 110 KDa, major cytoplasmic vault protein; vaults are nucleoprotein.

complex composed of the MVP associated with two high molecular weight proteins p240 and p193 which surround a small 140 nucleotides RNA species.

Expression Overexpressed in many MDR human tumours, including primary solid tumours, colon carcinoma, ovarian carcinoma and many hematological tumours. Also expressed at basal level in a wide variety of normal tissues, with particularly high levels in epithelial cells of the digestive tract.

Localization Mainly at cell membrane of tumour cells and in cytoplasm, with a little fraction (approximately 5 %) located in the nuclear membrane and nuclear pores.

Function Vaults are cytoplasmic organelles which mediate bidirectional nucleocytoplasmic transport of a wide range of substrates, including many structurally unrelated anticancer drugs.

Lung resistance related protein was first detected by Scheffer *et al.* (18), in the drug resistant lung cancer cell line SW1573/2R120. LRP is a 110 KD protein, and the gene is located on chromosome 16 near the loci for MRP. However, these genes are activated separately and contribute to resistance independently (71). LRP distribution is similar to that of vault proteins, which are believed to transport cellular substances through the nuclear pores. Several studies have concluded that LRP as a vault protein must act in cooperation with the other vaults in order to confer resistance and regulate transport both between the nucleus and the cytoplasm and into and out of the cell itself (71,72).

2.3.1. Expression of LRP and its clinical correlation:

In drug resistant cells negative for P-gp expression, the anti LRP monoclonal antibody (LRP-56) recognizes LRP overexpression. LRP expression has been reported in both normal and tumor tissues (2). Izquierdo *et al.* noted distinct patterns of LRP expression in normal and malignant cells (73). High levels of expression were found in normal

tissues of the bronchus, digestive tract, renal proximal tubules, macrophages, and adrenal cortex. Likewise, Scheffer *et al.* (18) reported higher LRP levels in normal tissues involved in secretion and excretion or those normally exposed to natural drugs and toxins. LRP expression levels varied among different types of cancer and low levels of LRP-positive cells were found in testicular cancer, neuroblastoma and AML. High levels were found in colon, renal, and pancreatic carcinomas.

2.4. Breast Cancer Resistance Protein (BCRP):

In 1998 Doyle *et al.*, reported that the human breast cancer cell line (MCF-7) selected for resistance to doxorubicin and vinblastine, did not overexpress any of the known MDR transporter proteins, suggesting the presence of a new MDR transporter protein (19). Using the RNA finger print technique, Doyle *et al.* identified overexpression of a 2.4 kb mRNA which encoded breast cancer resistance protein (BCRP), a new member of the ABC superfamily of transporters. Enforced expression of BCRP resulted in resistance to mitoxantrone, doxorubicin, daunorubicin and efflux of Rhodamine 123 in the transfected cells. Based on phenotype analysis of a panel of drug resistant cell lines, Several studies suggested that drug resistance due to BCRP is overlapping with but distinct from that of the p-gp (7,74). Litman *et al.* (74) reported that cells with BCRP overexpression were more resistant to mitoxantrone than cells with P-gp-mediated drug resistance. Accordingly, these authors called BCRP gene as mitoxantrone Resistance gene (MXR). Information regarding this gene and its protein product are highlighted below (7,74).

Name BCRP (Breast Cancer resistance Protein).

Mitoxantrone Resistance (MXR).

Other ABCP (ATP-Binding Cassette transporter, Placenta-specific.

Chromosome 4p22.

RNA 2.4 kb mRNA.

PROTEIN

Size 663 amino acids.

Name Breast cancer resistance protein (BCRP).

Description a member of ATP-binding cassette (ABC) superfamily of transporters. Highly expressed in placenta. A xenobiotic transporter that appears to play a major role in the MDR phenotype of a specific human breast cancer.

Ross *et al.* (76) screened 21 Acute Leukemia patients and described that BCRP RNA expression varied more than 1000-fold with low expression in 50% of the samples. 33% of the samples had high BCRP expression, which did not correlate strongly with high P-glycoprotein expression, thus indicating that BCRP may be responsible for resistance to drugs in P-gp-negative patients. Scheffer *et al.* developed a monoclonal antibody (BXP-34) to BCRP and reported that the transporter was mainly located in the plasma membrane of the drug resistant cell lines (77). Maliapaard *et al.* (2001) compared the expression of the two antibodies against BCRP in cell lines and normal tissues (78). In contrast to BXP-34, BXP-21 was able to detect BCRP expression in immunoblots and was reactive with BCRP in formalin-fixed paraffin embedded tissues. BCRP expression was seen in placental trophoblasts, in small intestine and colon epithelium, in liver canalicular membranes and in ducts and lobules of the breast. Positive expression was seen in venous and capillary but not in the arterial endothelial cells. In epithelium of the small intestine and colon, BCRP expression was seen in the apical areas suggesting a possible role of this resistance protein in regulation and absorption of orally administered drugs. Besides the use of anti-BCRP monoclonal antibody for its detection, Robey *et al.* used a flow cytometric functional assay to monitor drug retention and efflux in BCRP positive cells (79). They reported that Fumitremogin C (FTC) inhibited mitoxantrone and prazosin efflux and data provided by this functional assay correlated well with BCRP levels as determined by Northern blotting. Reversal of MDR using BCRP inhibitors has been reported (80).

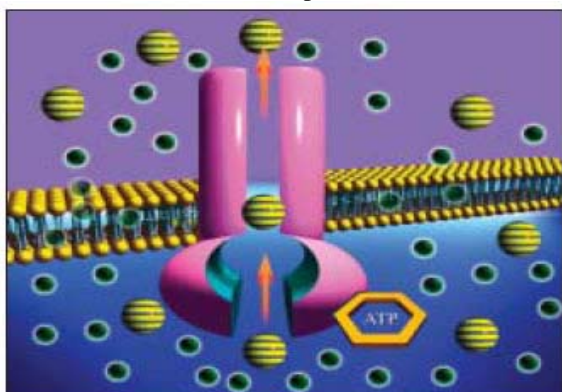


Fig 2. — Competitive inhibition of the P-glycoprotein transporter. First- and second-generation modulators compete as a substrate with the cytotoxic agent for transport by the pump. This limits the efflux of the cytotoxic agent, increasing its intracellular concentration. (Cited from Ref. 25)

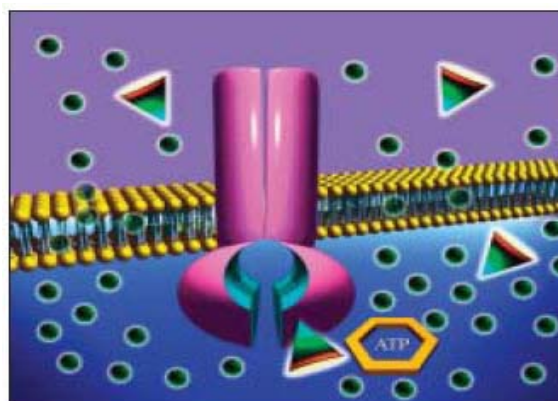


Fig 3. — Noncompetitive inhibition of the P-glycoprotein transporter. Third-generation inhibitors of P-gp, such as tariquidar, bind with high affinity to the pump but are not themselves substrates. This induces a conformational change in the protein, thereby preventing ATP hydrolysis and transport of the cytotoxic agent out of the cell, resulting in an increased intracellular concentration. (Cited from Ref. 25)

3- Blockers of Multidrug Resistance:

Blockers or antagonists of MDR phenotype are drugs that inhibits the efflux of anticancer drugs either by a competitive (competes with the drug binding site on P-gp, fig. 2) and/or non-competitive (inhibits the ATPase activity of P-gp, fig. 3) mechanisms (7). In order to control and/or prevent MDR function, a number of pharmacological agents, not antitumour drugs in their own right, are known to modulate the MDR phenotype to increase cellular sensitivity to anticancer drugs (8,81). This process of chemosensitization involves the co-administration of a P-gp inhibitor (MDR Blocker) with those anticancer drugs, known to be substrates for one or more than one of the MDR proteins, in order to enhance intracellular anticancer drug accumulation via impairing the P-gp function. Numerous Pharmaceutical compounds with different pharmacological actions have been shown to inhibit the drug efflux function of P-gp (82). The most commonly used P-gp antagonists are:

1- Calcium channel blockers

Verapamil
Diltiazem
Nifedipine

2- Immunosuppressants

cyclosporin A
PSC 833

3- Antiestrogen

Tamoxifen

4- Calmodulin inhibitors

Trifluoperazine

Chlorpromazine

Prochlorperazine .

5- Antimalarial drugs

Quinine

Mefloquine

6- Antiarrhythmic drugs

Quinidine

Amiodarone

Classification of MDR blockers:

3.1. First Generation MDR Blockers:

Tsuruo and co-workers (13,83) were the first to demonstrate the ability of the calcium channel blocker, verapamil, to reverse MDR with the consequent increase of intracellular accumulation of many anticancer drugs, including doxorubicin in numerous cell lines (48). Subsequent studies revealed that this MDR reversing property is shared by many other calcium channel blockers. Clinically available calcium antagonists known to reverse MDR in vitro include, felodipine, nifedipine and diltiazem; (83,84). However, these agents modulated MDR at very high concentrations, ranging from 5 to 50 μM . At these high concentrations, enhanced cytotoxicity was observed in normal cells such as cardiomyocytes (85). The efficacy of verapamil as MDR blocker has been confirmed in clinical study by Naito et al. which have demonstrated that intravesical instillation of doxorubicin plus verapamil was safe and more effective than instillation of doxorubicin alone in preventing early intravesical recurrence after transurethral resection of superficial bladder cancer (86). Cyclosporin A, a commonly used immunosuppressant for organ transplantation, remains to be one of the most effective first generation MDR modulators studied. In conjunction with several anticancer agents, Cyclosporin A effectively reverses MDR in many cell lines, such as P388, CHO, and L1210 (87,88). Moreover, Nooter et al. reported that cyclosporin A increased the uptake of daunorubicin by leukaemia cells from patients with MDR1 mRNA positive (89). Calmodulin antagonists such as trifluorperazine, chlorpromazine and prochlorperazine reverse MDR significantly at concentrations ranging from 1 to 10 μM (90-92). Using the P-gp associated MDR human SCLC cells, Shrivastava et al (8). reported that the quinoline derivative, MS-209, completely reversed the resistance against doxorubicin and vincristine. More-over, MS-209 in combination with verapamil and cyclosporin A, synergistically potentiated the anti-cancer activity of doxorubicin and vincristine in

MDR expressing human SCLC. Indole alkaloids including, the anti-malarial quinine and the anti-arrhythmic quinidine, have also been shown to reverse MDR in vitro in experimental cell lines in conjunction with doxorubicin (93,94).

3.2. Second Generation MDR Blockers:

First generation MDR blockers are therapeutic agents and typically reverse MDR at concentrations much higher than those required for their individual therapeutic activity. The search for non-toxic second generation MDR blockers resulted in newer analogs of the first generation agents, which were more potent and considerably less toxic. Structural analogs of verapamil including dexverapamil (less cardiotoxic), emopamil, and gallopamil, reverse MDR in vitro to a degree equivalent to VRP, but with marginal toxicity in many animal models (95,96). The non-immunosuppressive analog of cyclosporin A, PSC 833, has demonstrated superior MDR reversal efficacy in many experimental cell lines in vitro (97). PSC 833, has been shown to reverse MDR in conjunction with daunorubicin, doxorubicin, vincristine, vinblastine, taxol, and mitoxantrone in MDR expressing P388 leukemic cell line at in vitro concentrations of 0.5-2 μM (97,98). Dorr et al. have recently reported on the use of PSC 833 in AML patients and correlated clinical response with measurement of P-gp and LRP expression (99).

3.3. Third Generation MDR Blockers:

Several MDR blockers have recently been developed using structure-activity relationships as an approach targeted against specific MDR mechanisms. Specific P-gp blockers such as the cyclopropylidibenzosuberane LY 335979 (100), the acridone-carboxamide GF 120918 (101), the diketopiperazine XR9051 (102), and the diarylimidazole OC144-093 (103) exhibit effective reversing concentrations in the nanomolar range (20-100 nM), thus lower doses are required to achieve effective reversing concentrations in vivo.

3.4. Gene therapy approaches to overcome MDR:

Gene therapy represents an alternative, less toxic and more efficient strategy to overcome MDR including RNA interference technology and antisense strategy.

3.4.1. RNA interference (RNAi) technology:

Alternative procedure to overcome P-gp mediated MDR in cancer cells is to prevent the biosyn-

ntesis of P-gp by selective blocking of the expression of P-gp specific MDR1 mRNA. This approach increases the efficiency and specificity of chemosensitization of multidrug resistant cancer cells leading to reduction in the cytotoxic drug dose to minimize toxicity and undesirable side effects (104). Nieth *et al.* used siRNA duplexes to treat human pancreatic carcinoma (EPP85-181RDB) and gastric carcinoma (EPG85-257RDB) cells (22). In both cellular systems, siRNA inhibited MDR1 expression at the mRNA and protein levels. Another study was conducted by Hao *et al.* using MCF7/AdrR, MCF7/BC-19 and human ovarian carcinoma cell lines A2780 and A2780DX5 and demonstrated that siRNA can be used to modulate MDR (105). Moreover, siRNA increased the intracellular accumulation of paclitaxel and doxorubicin, two P-gp substrates, but had no effect on non-P-gp substrates like hydroxyurea. This suggests that silencing of gene expression using siRNA is specific. However, there was lack of complete inhibition of p-gp (only 65%) and incomplete restoration of drug sensitivity in MCF7/ADR and A2780DX5. Also, similar to previous studies, silencing was observed to be short lived (106). Such limitations of synthetic siRNA can be overcome by the use of a DNA vector-based siRNA expression system (107-109). In the clinical situations, application of P-gp inhibiting siRNAs should be restricted to P-gp-expressing cancer cells .

3.4.2. Cyclooxygenase-2 antisense strategy:

In tumour specimens from breast cancer patients and in a series of resistant cell lines, Ratnasinghe *et al.* found a strong correlation between expression of COX-2 and MDR1/p-gp and concluded that pretreatment with selective COX-2 inhibitors may be useful in the prevention of MDR in response to cancer chemotherapy (110). These authors hypothesized that increased prostaglandines production by COX-2 induces the expression of the anti-apoptotic protein, Protein Kinase B (PKB), as well as the transcriptional factor c-jun, which in turn induces the expression of MDR1/p-gp. Overexpression of COX-2 is related to tumorigenesis by mechanisms of chronic inflammation, apoptosis resistance and angiogenesis (111,112). Selective COX-2 inhibitors have also received an increasing attention for their potential usage as chemopreventive and therapeutic agents in cancer treatment (113). Another role of the contribution of COX-2 to tumor growth was found to be the up-regulation of MDR1 gene suggesting

the potential role of COX-2 in regulation of active drug efflux and maintenance of multidrug resistance phenotype (23). Also, Zatelli *et al.* found that the COX-2 Inhibitors, rofeoxib, reversed chemoresistance phenotype in medullary thyroid Carcinoma cell line by a p-gp mediated mechanism (24). More recently, Kamel *et al.* reported that transduction of MCF-7/DOX cells with COX-2 antisense reversed their resistance to doxorubicin by decreasing the expression of MDR1, Cycline D1 and bcl2 genes (25).

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