Drug Resistance

GIUSEPPE GIACCONE, HERBERT M. PINEDO

Free University Hospital, Department of Oncology, Amsterdam, The Netherlands

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ABSTRACT

Chemotherapy fails to cure most cancer patients with advanced disease, particularly patients with the most common forms of solid tumors. The presence or development of resistance to anticancer agents is the major cause of this failure. Several of the mechanisms underlying drug resistance to cytotoxic drugs have been elucidated in the last two decades, largely by employing in vitro drug-selected cancer cell lines. In unselected cell lines and probably also in human cancer, multiple mechanisms are redundantly present to defend the organism from the insults of drugs. Mechanisms have been unraveled by which cross-resistance ensues to multiple drugs (multidrug resistance), similar to what is commonly seen in patients. More recently, the identification of downstream genes, intimately involved in cell-cycle checkpoints, appears also to directly contribute to determining the sensitivity to cytotoxic drugs by regulating the response of the cell to the drug damage. The identification of mechanisms of drug resistance has provided ways of attempting to revert the drug resistance. Although, so far, attempts to revert P-glycoprotein-mediated multidrug resistance have only sorted out limited efficacy, new drugs and new strategies are being devised and implemented, such as high-dose chemotherapy and gene transfer. *The Oncologist 1996;1:82-87*

INTRODUCTION

The presence or development of resistance to anticancer drugs is the main cause of failure of chemotherapy in the majority of the most common forms of cancer (e.g., lung, colon, breast). Resistance to chemotherapeutic drugs can be already present at diagnosis or it can develop after treatment with chemotherapy. These two forms of drug resistance are respectively called intrinsic and acquired. It is unknown whether the underlying mechanisms of drug resistance are the same in these two forms of drug resistance.

Many causes of drug resistance are well recognized, such as those due to administration of inadequate doses or scheduling of the drug, or to altered pharmacokinetics, or to limited penetration of the drug into the tumor. Limited penetration may be caused by poor vascularization, or extensive necrosis of parts of the tumor. It can also be due to localization of the tumor in areas of the body which are difficult to reach (sanctuary sites) because of the presence of a tissue-blood barrier (e.g., blood-brain, blood-testis, placenta).

A well-known reason for poor sensitivity to chemotherapy is the slow growth kinetics of the tumor; in fact, most known anticancer agents exquisitively act on proliferating cells.

Research on drug resistance in the last two decades has focused primarily on the study of cellular mechanisms of drug resistance. Whether these are more important than physical mechanisms or drug-related mechanisms is still rather unclear, and numerous examples of both cases are present in the literature.

CELLULAR DRUG RESISTANCE

The establishment of cancer cell lines which are in vitro-selected resistant by exposure to increasing concentrations of anticancer agents has made possible the identification of a number of mechanisms of cellular drug resistance. The cell clones that survive in culture in the presence of escalating concentrations of drug can be analyzed, and alterations at biochemical and/or genetic levels identified.

Several biochemical causes of drug resistance have been described for exposure to several drug types and are summarized in Table 1. Some drugs, such as methotrexate, are transported into the cell

Table 1. General mechanisms leading to cellular drug resistance

- Decreased drug uptake
- Increased drug extrusion
- Decreased drug activation
- Increased drug inactivation
- Decreased formation of drug-target complexes
- Increased repair of drug damage

Correspondence: G. Giaccone, M.D., Ph.D., Department of Medical Oncology, Free University Hospital, 1117 de Boelelaan, HV 1081 Amsterdam, The Netherlands. Telephone: 31-20-4444336; Fax: 31-20-4444355. *Accepted for publication January 4, 1996.*

through a high-affinity transport system; a loss of, or reduced activity of, this system may induce resistance.

There are now at least two well-known cell membrane proteins which can extrude a number of drugs from the cell and cause multidrug resistance (MDR); this mechanism is extensively described later in the text. Obviously, a reduced uptake or an increased efflux will lower the intracellular concentration of the drug, thereby diminishing its activity.

Some anticancer agents require metabolic activation in the body, such as the alkylating agent cyclophosphamide; an alteration of the metabolic conversion (e.g., loss of deoxycytidine kinase activity for Ara-C activation) can lead to reduced activity of the drug. A mechanism of this sort has also been recently described for the new camptothecin analog Irinotecan (CPT-11), a DNA topoisomerase I inhibitor, which requires conversion to the active metabolite SN-38 in order to exert antitumor efficacy. This conversion is catalyzed by carboxyestherase, an enzyme present in several cell types. Reduced levels of this enzyme have been observed in cell lines resistant to CPT-11 as a possible cause of drug resistance. Of course, this mechanism is difficult to extrapolate to the in vivo situation, where, in fact, the conversion will occur very rapidly after intravenous administration and the tumor will be exposed directly to SN-38, bypassing the need for activation of CPT-11 in the tumor cells.

Drug inactivation is also a well-described mechanism of drug resistance, such as the one due to increased glutathione-S-transferase activity in resistance to alkylating agents.

Decreased formation of drug-target complexes can occur in one of several different ways: 1) an amino acid substitution in the target enzyme determines a decreased affinity of the enzyme for the drug; 2) overproduction of the target enzyme, making impossible a complete inhibition; 3) an increased level of normal substrate competes with the drug for the target enzyme; and 4) a decrease of essential cosubstrates reduces the formation of the complex. More than one of these mechanisms are implicated in resistance to the antimetabolites methotrexate and 5-fluorouracil. Finally, an increased repair of damage produced by the drug can also lead to resistance (e.g., increased repair of DNA damage induced by alkylating agents or topoisomerase inhibitors).

Genetic analysis of cell lines selected for resistance to anticancer drugs has shown that various genetic alterations can ensue in these cells [1]. The mutation rate is usually low in a normal cell population, but it can increase by exposure to mutagenic agents. Drug resistant mutants have been hypothesized to appear with a frequency of 10^{-6} cell divisions. Large tumors have a greater chance of developing mutants which are drug resistant because more duplications will have occurred. This may be an additional reason why, in general, large tumor masses are less sensitive to eradication by chemotherapy. Several different types of biochemical changes leading to drug resistance are associated with gene alterations, such as mutations or amplifications of genes involved in resistance to specific drugs. Mutations in a gene can lead to decreased production of a protein, synthesis of an unstable or nonfunctional protein, production of a protein with altered drug affinity (often caused by point mutations), or increased production of a normal protein. This last alteration is, however, caused mainly by transcriptional activation or gene amplification in the absence of mutations.

Multiple pathways and different mechanisms of action have been described for a number of drugs, but in particular for antimetabolites. It is therefore not surprising that tumor cells can present many mechanisms of drug resistance to a single drug. As an example, resistance to the antimetabolite methotrexate can occur through decreased activity of the membrane transport system, decreased polyglutamation of methotrexate, alteration or increased production of dihydrofolate reductase, decreased levels of thymidilate synthase, or increased nucleoside salvage. Although one specific mechanism is usually overwhelming, multiple mechanisms can coexist in the same cell line.

All of the above-mentioned mechanisms have been primarily described in in vitro-selected cell lines, whereas much less is known about the mechanisms of resistance and differential sensitivity to drugs of unselected cell lines. In general, it would appear that the process of selection drives the cells, in a rather extreme fashion, to develop mechanisms to escape death; it is dubious whether these same mechanisms are in place or expressed at comparable levels in unselected cell lines as in in vitro-selected resistant cell lines. This is, of course, a matter of great concern, as drug exposure in patients is rather different from that obtained in vitro. Whether in vitro findings at all reflect the basis of drug resistance at the cellular level in the patient remains to be elucidated.

Multidrug Resistance

Although in patients, resistance to one drug does not necessarily always imply resistance to other drugs, this is quite often the case. This may partly be due to the present use of combined chemotherapy programs for the treatment of most forms of cancer, but also likely to the presence of broad drug resistance to multiple anticancer drugs with no common mechanism of drug action.

By exposing cells in vitro to one drug, clones may be selected which display resistance mechanisms to the single drug or class of drugs, but cells can also simultaneously become resistant to several anticancer drugs not belonging to the same type. This phenomenon is called cross-resistance and mirrors the clinical setting where patients who have become resistant to the first chemotherapy are very

frequently also resistant to the second chemotherapy, regardless of whether the drugs employed are different by mechanism of action or chemical class.

The best characterized of this type of drug resistance has been called multidrug resistance. MDR is the in vitro resistance induced by exposure to a drug, which leads to cross-resistance to a number of other drugs which are not related by chemical structure or mechanism of action. MDR is due to overexpression of P-glycoprotein, a cell membrane protein of 170 kDa molecular weight (P-170) [2]. The anticancer drugs involved in this phenotype are natural products such as anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, actinomycin D and taxanes.

P-170 is a transmembrane protein present in tumor and normal cells and is responsible for the active transport of anticancer agents and possibly several other physiological substances from the cytosol of the cell to the extracellular space. By active drug extrusion, the drug concentration at the intracellular target site is insufficient, thereby resistance occurs. P-170 is encoded by the *MDR*-1 gene. From the amino acid sequence of the protein it is hypothesized that P-170 consists of two similar halves, each containing six transmembrane domains and an ATP binding site; the transmembrane segments probably form a channel through which the drug can be extruded [3].

P-170 expression occurs in cells of several normal tissues, such as adrenal cortical cells, apical surface of intestinal epithelium, a small percentage of biliary canaliculi, some $CD34^+$ cells, brush border of proximal tubules of the kidney, pancreas excretory ducts, and endothelial cells of brain, testicles and placenta. The latter localizations provide evidence for the presence of the blood-tissue barrier in these organs, which is also likely responsible for the difficult penetration of most of the cytotoxic drugs into those organs. The other localizations in normal tissue are consistent with a functional extrusion of toxins from the organism, steroid transport in the case of adrenals, and defense mechanism in $CD34^+$ bone marrow cells [4].

A number of tumors overexpress the *MDR*-1 gene; among these are: neuroblastoma, rhabdomyosarcoma, myeloma, non-Hodgkin's lymphomas, colon carcinoma, breast carcinoma and renal cell cancer. Several tumor types with high *MDR*-1 expression actually derive from tissues which have a high expression of the gene (e.g., colonic epithelium). Expression of the *MDR*-1 gene has been shown to increase after exposure to chemotherapy on several occasions [5], but whether this increase is responsible for enhanced resistance to anticancer agents or is simply a feature of a more malignant phenotype is still a matter of discussion [5-8].

A number of cell lines have been described with cross-resistance patterns which are very similar to those of P-170-mediated MDR, but in which *MDR*-1 is not overexpressed. These other MDR phenotypes have been generically identified as non-P-glycoprotein-mediated MDR. Recently, another drug transport protein has been identified, called MRP, or multidrug resistance-associated protein, which, like P-glycoprotein, is localized mainly on cell membranes [9]. The *MRP* gene is overexpressed and amplified in a number of cell lines which were selected in vitro for resistance to doxorubicin. These cell lines lack expression of the *MDR*-1 gene. The patterns of cross resistance are very similar, although paclitaxel does not seem to be a substrate of MRP, whereas it is for P-170 [10].

Both *MRP* and *MDR*-1 genes belong to the ATP-binding cassette superfamily of transporter proteins which can transport a number of different substrates from drugs to peptides to ions [11]. Actually, the *MRP* gene sequence is much closer to the cystic fibrosis and other genes encoding for transport proteins, than to the *MDR*-1 and *MDR*-3 genes. As with the *MDR*-1 gene, transfection experiments can induce MDR in cells transfected with the *MRP* gene. Recently, the *MRP* gene product has been shown to be a primary-active ATP-dependent export pump for conjugates of lipophilic compounds with glutathione and several other anionic residues [12], suggesting that *MRP* overexpression can cause MDR by promoting the export of drug modification products from cells [13]. Whether overexpression of the *MRP* gene is of clinical importance is a matter of current investigation.

More recently, another gene has been cloned and identified which might be responsible for yet other forms of non-P-glycoprotein-mediated MDR. The gene product is targeted by a monoclonal antibody called LRP56 (Lung Resistant Protein), because it was identified in a lung cancer cell line. Overexpression of LRP56 has been observed in in vitro-selected resistant lung cancer cell lines, mainly after exposure to anthracyclines [14]. Interestingly, there is no analogy between the *LRP* gene and the ATP-binding cassette superfamily of transporter proteins, and the gene appears to be the human major vault protein [15]. This 110 kDa protein is one of a complex of four proteins and RNA which is found in the nucleopore complex and in cytoplasmic organelles [15]. This localization points to a possible role of *LRP* in intracytoplasmatic trafficking and trapping of cytotoxic drugs (Fig. 1).

The overexpression of this protein is more frequently present in tumors which are intrinsically resistant to chemotherapy than in tumors that can be cured by chemotherapy [16]. Furthermore, ovarian cancer patients whose tumors had higher *LRP* expression had poorer response to cisplatin-containing chemotherapy and shorter progression-free survival and overall survival [17]. Additional clinical studies investigating the expression of the *LRP* gene are in progress and results are awaited.

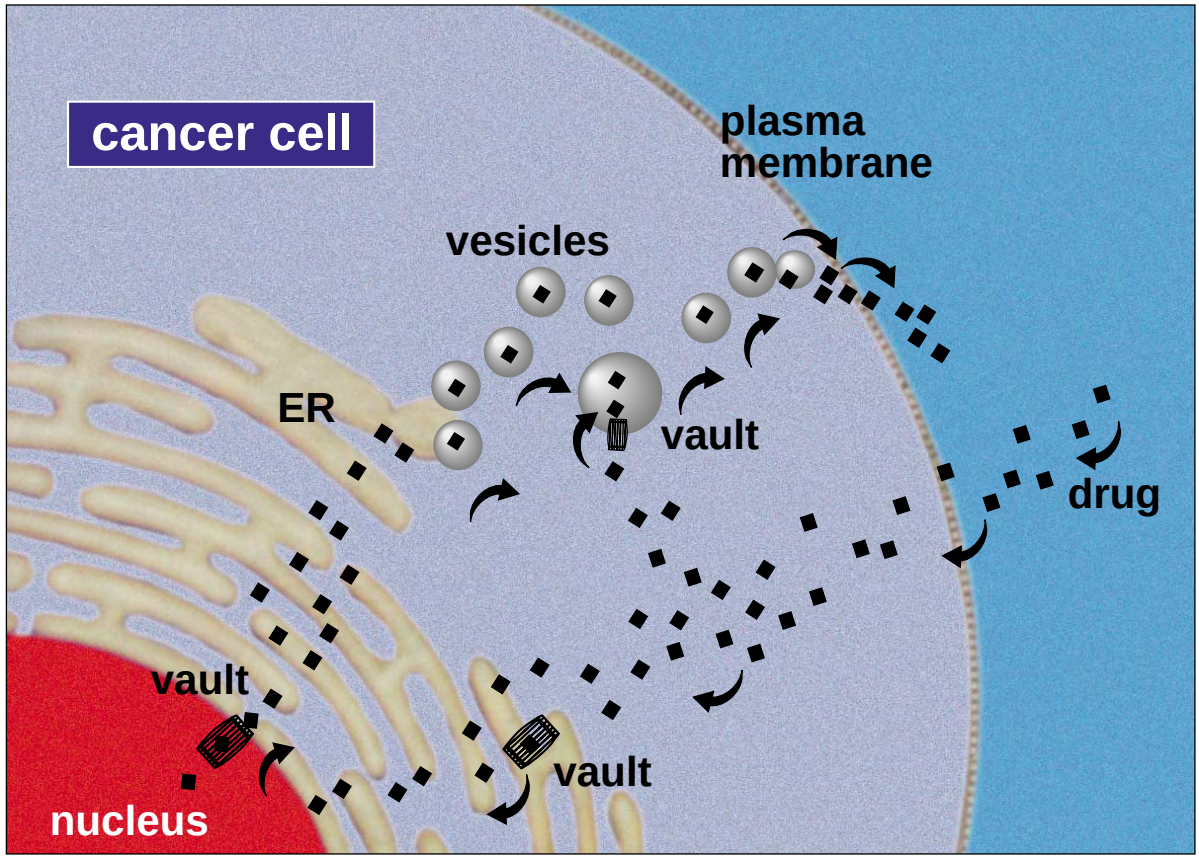

Figure 1. Schematic representation of the mode of action of the* LRP *gene product.

Finally, another type of non-P-glycoprotein-mediated MDR, also called atypical MDR, is due to alterations of the function of the enzyme DNA topoisomerase II and concerns cross-resistance between drugs which are targeted to the enzyme. DNA topoisomerase II is an essential nuclear enzyme which catalyzes conformational changes of DNA, important for several steps of DNA metabolism such as transcription, DNA synthesis, chromosome segregation, and recombination. The conformational modifications are rendered possible by formation of a transient double DNA break, followed by a DNA strand passage and eventually by relegation. Inhibitors of topoisomerase II prevent the relegation process from occuring by freezing the so-called "cleavable complex" formed between the enzyme and DNA. DNA topoisomerase II-targeted drugs are anthracyclines, anthracenediones, epipodophyllotoxins, actinomycin D and amsacrines. The drugs involved in MDR due to alteration of topoisomerase II are essentially the same drugs involved in the P-170-mediated MDR, with the exception of *Vinca* alkaloids and taxanes [18]. The reduced activity of the enzyme and/or reduced expression of the gene have been identified as responsible for the decreased chemosensitivity to topoisomerase II inhibitors in cell lines which had been selected by exposure to several topoisomerase II-targeted drugs. Nevertheless, a good correlation between activity/expression and antitumor efficacy could also be found in unselected cell lines of various types, indicating that topoisomerase II-mediated MDR might be responsible for a more generalized form of drug resistance than the transport-mediated MDR types [19].

In recent years, some genes involved in response to cell damage (particularly DNA damage) have also been investigated as possibly responsible for drug resistance. *p53* and *bcl*-2 are important genes that control programmed cell death (apoptosis). Tumor cells which have mutant *p53* are less sensitive to a large spectrum of drugs including doxorubicin, cisplatin and 5-fluorouracil [20]. Given the fact that *p53* appears to be the gene that most frequently is altered in human malignancy, these considerations may be of extreme importance. This is a field in very rapid development, and it now appears clear that downstream genes may play an important role. This new understanding of the tumor biology opens up new therapeutic possibilities, including gene therapy.

With the advances in understanding the mechanisms of drug resistance, it appears more and more clear that within a given tumor there may well be multiple mechanisms in action.

Table 2. Drugs used in clinical trials to revert P-glycoprotein-mediated MDR and examples of each

Calcium channel blockers	Calmodulin antagonists	Steroidal agents	Immunosuppressive drugs	Miscellaneous compounds
verapamil	trifluoperazine	progesterone	cyclosporin A	dipyridamole
nifedipine	prochlorperazine	tamoxifen	PSC-833	quinidine
bepridil		megestrol acetate		amiodarone

Overcoming Drug Resistance

A large number of trials attempting to revert P-glycoprotein-mediated MDR have been performed. Many substances with drug-resistance-modifying capabilities are known. They belong to different chemical classes, ranging from beta-blockers to anti-arrhythmics, to hormones, to immunosuppressives (Table 2). Although the mechanism of MDR reversal can be complex, nearly all reversal compounds are substrates of P-glycoprotein and compete with the cytotoxic drug for extrusion from the cell. The first of these substances to be tested was verapamil, which unfortunately was found to be extremely cardiotoxic at doses which give patients plasma concentrations necessary to revert drug resistance in vitro. More recently, cyclosporin-A and analogs like PSC-833, which is devoid of cardiac, renal and immunosuppressive properties, have been developed and are under clinical investigation.

A large number of phase I and dose-finding studies have been performed with drug resistance reversal agents and cytotoxic drugs; however, randomized trials are few. Despite occasional responses in patients with hematological malignancies, and a small positive randomized study of verapamil added to chemotherapy in untreated non-small cell lung cancer [21], three large randomized trials failed to show any benefit of reverters in influencing response to chemotherapy or survival in refractory patients with multiple myeloma [22], untreated small cell lung cancer [23] and breast cancer [24].

Some reverters, such as verapamil and cyclosporin A, including the analog PSC-833, substantially influence the pharmacokinetics of the anticancer drug possibly by decreasing their elimination and increasing their plasma concentration over time by 40%-60%, thereby increasing their toxicity. Reverters which are active in vitro in reverting P-glycoprotein-mediated MDR appear to have significantly less activity in reverting MDR which is due to *MRP* overexpression. Other substances that appear to more efficiently block the drug efflux due to overexpression of *MRP* rather than of *MDR*-1 are being investigated [25].

Another approach for reversing or preventing the development of drug resistance to anticancer agents is the use of very high doses of chemotherapy. Many anticancer agents, in fact, possess a steep dose-response relationship, and higher doses of a drug have a much higher therapeutic activity in several tumor types. The recent introduction of colony-stimulating factors and peripheral stem cell infusions into routine use has replaced, at least in solid tumors, the use of bone marrow transplantation, which is complicated by much higher morbidity and mortality rates. These improvements have made the administration of high-dose chemotherapy more feasible by substantially reducing the intensity and length of myelosuppression. Randomized studies comparing traditional chemotherapy doses with high-dose chemotherapy are ongoing in several tumor types.

Another way of allowing higher doses of chemotherapy to be administered is to transfect peripheral stem cells with the *MDR*-1 gene in order to make them more resistant to chemotherapy. Clinical studies employing this approach are also in progress.

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