Drug resistance
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INTRODUCTION

Drug resistance is the central problem in cancer therapy today. Whereas surgery and/or radiotherapy can usually remove or destroy the initial tumour and its outgrowths in surrounding tissues, it is the lack of adequate drug treatment for disseminated forms of cancer and failing locoregional control that is responsible for the death of cancer patients.

Resistance to drugs may be primary, where the tumour does not respond to drugs from the start (also called intrinsic, permanent, or natural resistance) or secondary, in which case the tumour initially responds, but becomes resistant during treatment (also called acquired or temporary resistance). This chapter presents a brief overview of known mechanisms of resistance and possible ways to prevent or circumvent resistance, where possible. The emphasis is on clinically relevant forms of resistance, but we also provide an outline of molecular mechanisms of resistance to provide the clinician with a feeling of things to come and for the cunning ways in which cancer cells evade therapy. More detailed information on drug resistance can be found in the general textbooks of cancer biology edited by Farmer and Walker (1985), Franks and Teich (1986), and Tannock and Hill (1987), specialized volumes on cancer drug resistance edited by Fox and Fox (1984), in the EORTC Cancer Chemotherapy Annuals (see Pinedo 1990) and Woolley and Tew (1988), and in the specialized reviews quoted in individual sections of this chapter.
CAUSES OF ‘APPARENT’ AND PRIMARY (INTRINSIC) DRUG RESISTANCE

This topic has been reviewed by McVie (1984). To kill a cancer cell, a drug must reach it in sufficient amounts. This obvious point bears stressing, because one of the reasons for resistance is exactly this failure of the drug to reach the tumour (see Table 1). Resistance may only be apparent, because the drug is given in inadequate dosage, for inadequate times, or via an ineffective route. The oral route may be ineffective, for instance, because of variable absorption or insufficient patient compliance. In the 1970s such inadequate treatment was sometimes interpreted as resistance, an example being the use of oral 5-fluorouracil. Subsequently, clinical pharmacokinetic studies have guided us in the application of more reliable routes of administration. These principles have not, however, been applied to oral 6-mercaptopurine, where pharmacokinetic studies have also shown wide interindividual variation in bioavailability.

‘Humane’ opinionated physicians may decide to lower the drug dose to spare the patient unpleasant side-effects and this may result in imaginary resistance, such as tends to occur in cases of non-Hodgkin’s lymphoma. As cytotoxic drugs are usually most effective at a maximum tolerated drug dosage, suboptimal dosage is nearly always ineffective and may even promote the selection of resistant cells (see below). Little attention has been paid to dose intensity. Unfortunately, clinical protocols usually do not allow for dose intensification and more frequent administration in cases where toxicity is moderate. With certain drugs, dose reduction may result in equal remission rates, but with a higher relapse rate. Inappropriate treatment schedules or impatience are other causes of apparent treatment failure. With slowly growing tumours, several weeks of therapy may be required before the antitumour effects can be assessed. In contrast, overtreatment may occur when sensitive tumours, such as teratomas, respond but the necrotic tissue remains in situ.

Drug interactions represent another possible cause of apparent resistance. In practice this is rarely a problem and only few cases have been reported.

Even if a drug is administered properly, it may still fail to reach the tumour for pharmacological reasons. The drug may not cross the blood–brain barrier and may therefore not reach tumour cells behind this barrier. The clinical relevance of such barriers become apparent following the achievement of long-term remissions in chemosensitive tumours such as acute lymphocytic leukaemia and small-cell anaplastic lung cancer.

Tumours may also be resistant for kinetic reasons. Most cytotoxic agents only kill proliferating cells and tumours with a very small growth fraction may therefore be resistant. Large tumours may also be undervascularized and contain necrotic areas in which drugs penetrate poorly.

Finally, biochemical mechanisms may prevent cell kill even if a drug reaches the proliferating tumour cell. The biochemical mechanisms of primary drug resistance are probably largely the same as those of acquired resistance and these will be discussed below.

BIOCHEMICAL MECHANISMS OF ACQUIRED DRUG RESISTANCE: AN OVERVIEW

The availability of tumour cell lines, growing in tissue culture, has allowed the experimental biologist to obtain variant cells resistant to virtually any drug. By step-wise increases of the drug dose, one can usually obtain highly resistant cell lines that are suitable for biochemical analysis of the molecular basis of drug resistance. In this way a long list of mechanisms of drug resistance has been defined. Which of these possible mechanisms are actually responsible for resistance in patients is more difficult to assess and is often not known. In this section we will first discuss the resistance mechanisms found in cultured cells, with clinical resistance being dealt with in subsequent sections.

Figure 1 presents a simplified cartoon of the major biochemical alterations that may be found in resistant cells.

1. Decreased drug uptake. Several drugs enter cells with the help of a cellular transport system. An example is the high-affinity transport system for reduced folates that allows the folate analogue methotrexate (MTX) to enter cells efficiently. Loss or inactivation of this transport system is one of the causes of MTX resistance.

2. Increased drug extrusion. Increase synthesis of a plasma membrane protein, called P-glycoprotein, that can pump out a wide variety of large hydrophobic drugs, is the cause of classical multidrug resistance (MDR).

3. Decreased drug activation. Many cytotoxic drugs exert their effects only after metabolic activation. A decrease in the levels of
the activating enzymes may lead to resistance. An example is the
loss of deoxythymidine kinase, an enzyme required for the conversion
of deoxythymidine (ara-C) into deoxythymidine-monophosphate (ara-CMP),
an essential step in the synthesis of deoxythymidine-triphosphate
(ara-CTP) that is the actual inhibitor of DNA synthesis that kills
the tumour cell.

4. Increased drug inactivation. Some drugs are inactivated by
acellular enzymes and increased levels of these enzymes may
therefore lead to resistance. An increase of GSTs can, for instance,
lead to resistance to melphalan and related alkylating agents. Thus
far, increased drug inactivation has only been observed in cells
resistant to (some) alkylating agents and to cisplatin.

5. Decreased formation of drug–target complexes. This can
occur in any of four ways:
(1) the target enzyme is altered by an amino acid substitution,
resulting in an enzyme with decreased affinity for the drug;
(2) the target enzyme is overproduced making it less easy to get
complete inhibition of the enzyme;
(3) an increased level of normal substrate competes with the drug
for the target enzyme;
(4) a decrease in essential co-substrates decreases the formation
of the drug–target complex.
Mechanisms (1) and (2) occur in methotrexate resistance;
mechanisms (2), (3), and (4) in 5-fluorouracil resistance.

Increased repair of drug damage
Many cytotoxic drugs eventually kill the cell by DNA damage. It
is known that decreased DNA repair can sensitize cells to DNA
damage and it is therefore logical to expect that increased repair
could protect cells from DNA damage. Direct evidence for increased
DNA repair in resistant cancer cells is, however, still limited. There
are resistant cell lines in which the initial interaction of alkylating
agents or cisplatin with DNA seems unaltered and, by inference,
the repair of damage is probably more effective in these cells.

Increased tolerance of a drug
This is a mixed bag of mechanisms, often poorly defined. All
metabolic alterations that allow a cell to circumvent the block
induced by the drug fall under this heading. An example is the
increased nucleoside uptake postulated to allow cells to circumvent
blocks in de novo pyrimidine and purine synthesis. Another example
is the induction of asparagine synthetase in tumour cells, allowing
them to make their own asparagine. This circumvents the block
in external asparagine supply resulting from the hydrolysis of
asparagine by administration of the enzyme asparaginase.

It is clear from this brief overview that resistance to a single drug
may arise in several different ways. Different drugs are affected
by different mechanisms. Drug resistance is therefore a complex
affair. People speaking about the cause of drug resistance are as
ignorant as those speaking about the cure for cancer.

Genetic alterations resulting in drug resistance
The analysis of drug resistance in tumour cell lines has demonstrated
that drug resistance in cultured cells invariably results from genetic
alterations; that is, resistant cells are mutant cells (see Goldie
and Goldman 1988). The mutant variants arise spontaneously at low
rate in the population, but this rate can be increased by mutagenic
agents, including several clinically used cytotoxic drugs. Goldie and
Goldman (1988) have suggested that drug resistance mutants appear
in solid tumours with a frequency of $10^{-6}$ per cell division. Hence,
a large tumour containing more than $10^{11}$ cells must provide an
excellent breeding ground for resistant mutants. It is therefore not
surprising that treatment of large tumours with single agents usually
fails to cure and only induces short-lasting remissions. Only by
combining different drugs with different modes of action and no
overlapping resistances, can one completely eradicate the mixture
of mutant cells, called a tumour.

Genetic change affects cellular drug handling by changing a
protein (or proteins), such as drug transport proteins or drug
metabolizing enzymes. Such changes can be one of three types:

1. Decreased production of a protein, or synthesis of an unstable
or non-functional protein. The most common and drastic alteration
is the complete loss of functional protein. This can happen by gross
alterations in DNA (deletions, translocations) that inactivate the
gene, by mutations that prevent synthesis of the complete protein,
or by hypermethylation inactivating the gene without mutation. A
drastic decrease in the amount of functional protein can also occur
by mutations that result in amino acid substitutions in the protein
incompatible with function or stability. Loss of functional protein
by these mutations are usually recessive; that is, they require the
inactivation of both gene copies present in a diploid cell (the
exception being X-chromosomal genes).

2. Production of a protein with altered affinity for a drug. These
mutations are nearly always due to amino acid substitutions
caused by point mutations. Such mutations usually behave in a
dominant fashion.

3. Increased production of a normal protein. This increase can
be mediated by alterations that affect any step in gene expression.
The most prevalent are transcriptional activation (an increase in the
rate of mRNA synthesis) and gene amplification (an increase in the
copy number of the protein-coding gene). The genomic instability
of most cancer cells may promote such DNA rearrangements.

BIOCHEMICAL AND GENETIC ALTERATIONS CAUSING
METHOTREXATE RESISTANCE
This topic has been reviewed by Bertino et al. (1987) and Sirotmak
(1987). Six distinct mechanisms are now known to result in
methotrexate resistance.

1. Decreased uptake of methotrexate via the high-affinity carrier
system for reduced folates. This transport system has not been
isolated yet, nor have its gene(s) been cloned, thus the underlying
defect is not known. The decrease in transport could be due to a
decrease in affinity for methotrexate and reduced folates (increased
$K_m$), to a decrease in maximal transport rate (decreased $V_{max}$), or
to a combination of both. There are two alternative routes for cells
to meet their folate requirements: a low-affinity folate carrier
(Sirotmak et al. 1987) and a high-affinity folate-binding protein that
also mediates folate uptake (Jansen et al. 1989). Both alternative
routes have a low affinity for methotrexate, which is only effectively
taken up by the high-affinity carrier system for reduced folates.
2. Decreased polyglutamylation of methotrexate. The poly-
 glutamyl derivatives of methotrexate formed in the cell have a higher affinity for the target enzyme, dihydrofolate reductase and are also retained longer in the cell than methotrexate itself. Hence, decreased polyglutamylation of methotrexate can lead to resistance.

3. Production of an altered dihydrofolate reductase with decreased affinity for methotrexate. Several different amino acid substitutions in dihydrofolate reductase are now known that decrease the enzyme’s affinity for methotrexate without seriously decreasing catalytic activity. Each of these altered forms of dihydrofolate reductase is due to a single point mutation in the gene encoding the enzyme.

4. Increased production of normal dihydrofolate reductase. Although methotrexate has a high affinity for dihydrofolate reductase, the drug does not bind irreversibly to this enzyme. Hence, it is difficult to obtain more than 95 per cent enzyme inhibition with the drug concentrations that can be reached in patients. Five per cent of the normal enzyme level is insufficient for cell survival, but if the amount of enzyme is increased 10-fold in the resistant cell, 95 per cent inhibition will leave 50 per cent of the normal enzyme complement in active form and this is sufficient for normal cell growth. Increased enzyme levels in resistant cells are nearly always the result of gene amplification.

5. Decreased level of thymidylate synthase. This enzyme catalyses the only reaction that converts tetrahydrofolate into dihydrofolates, the substrate for dihydrofolate reductase. In the absence of this enzyme, cells can maintain their tetrahydrofolate pools required for biosynthetic reactions, even if the reduction of dihydrofolates to tetrahydrofolates by dihydrofolate reductase is blocked by methotrexate. In general, the rate of thymidine monophosphate synthesis is an important determinant of methotrexate cytotoxicity and it has been demonstrated that lower rates of thymidine monophosphate synthesis make cell lines less sensitive to methotrexate (Moran et al. 1973; Ayasawa et al. 1981).

6. Increased nucleoside salvage. It has been claimed that methotrexate resistance can arise through increased uptake of thymidine and purine nucleosides. These can be converted into the corresponding nucleotides by salvage pathways circumventing the methotrexate block in nucleotide biosynthesis. Although this mechanism might contribute to primary methotrexate resistance, no mutants with increased salvage have been obtained by selection for methotrexate resistance in cultured cells. There is clinical interest in this potential mechanism for methotrexate resistance, because cellular nucleoside uptake can be inhibited by dipyridamole. The combination of methotrexate and dipyridamole is undergoing clinical trials (Wilson et al. 1989).

Table 2 Mechanisms of resistance to methotrexate in tumour cell lines

<table>
<thead>
<tr>
<th>Resistance mechanism</th>
<th>Frequency</th>
<th>Circumvention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Decrease in high-affinity uptake of methotrexate</td>
<td>+++</td>
<td>(a) Lipid-soluble analogues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(trimetrexate)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) High-dose methotrexate</td>
</tr>
<tr>
<td>2. Decreased polyglutamylation of methotrexate</td>
<td>+</td>
<td>(a) Lipid-soluble analogues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(trimetrexate)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) High-dose methotrexate</td>
</tr>
<tr>
<td>3. Altered dihydrofolate reductase with decreased affinity for methotrexate</td>
<td>+</td>
<td>High-dose methotrexate2</td>
</tr>
<tr>
<td>4. Overproduction of dihydrofolate reductase</td>
<td>+++</td>
<td>High-dose methotrexate2</td>
</tr>
<tr>
<td>5. Decreased thymidylate synthase</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

The other four resistance mechanisms occur much less frequently in cell lines and have also not yet been observed in clinical samples. It has been suggested, however, that the low frequency of polyglutamylation defects in resistant cell lines may be due to the use of continuous selection with high methotrexate doses. A more clinically relevant selection schedule, intermittent low-dose selection, may result more often in the detection of polyglutamylation defects (Pizzorno et al. 1988). Primary methotrexate resistance in squamous carcinoma cell lines derived from head and neck tumours was also found to be due to decreased polyglutamylation in two or three cell lines (Pizzorno et al. 1989).

In cell lines selected for very high resistance, one may find more than one resistance mechanism operative, for example an uptake defect and gene amplification or an amplified gene for altered dihydrofolate reductase (Srimatkandada et al. 1989).

Whereas overproduction of dihydrofolate reductase will result in cross-resistance to all methotrexate analogues, this does not necessarily hold for the other resistance mechanisms. For example, the more hydrophobic methotrexate analogue, trimetrexate, enters the cell by diffusion and is not glutamylated; it is therefore not affected by alterations in the folate uptake system or in polyglutamylation.

Resistance to trimetrexate (but not methotrexate) may be caused by P-glycoprotein-mediated multidrug resistance (Arkin et al. 1989; Assaraf et al. 1989), but also by another mechanism affecting hydrophobic analogues but not drugs subject to multidrug resistance (Fry and Besserer 1988). This latter type of resistance was discovered in human lymphoblastoid cells and is associated with a decreased cellular drug concentration. As the uptake of trimetrexate appears not to be carrier-mediated, the decreased cellular concentration must be due to decreased binding or increased extrusion of the drug. The nature of the alteration in the mutant remains undefined.

THE MECHANISM OF RESISTANCE TO BASE AND NUCLEOSIDE ANALOGUES

This topic is reviewed by Allegra et al. (1990). Base and nucleoside analogues have to be converted into the corresponding nucleotide analogues by cellular enzymes before they can inhibit their target, namely one of the enzymes required for the normal biosynthesis of nucleotides. According to Fig. 1 one may therefore expect resistance to arise by a decrease in analogue uptake or in its conversion into the active inhibitor, by an alteration in the target enzyme, or by more complex alterations leading to more effective
production of normal nucleotides. In practice, each of these resistance mechanisms has been observed in resistant cell lines. The best characterized mechanism of resistance to purine analogues, such as 6-mercaptopurine or 6-thioguanine, is a decreased conversion by hypoxanthine-guanine phosphoribosyl transferase (HGPTase) into the corresponding nucleoside monophosphate (Brennard and Caskey 1984) by a loss of HGPTase activity. Other reported mechanisms of resistance include decreased uptake, increased capacity of de novo purine synthesis, or increased degradation of the purine analogue or the purine analogue nucleotide. The genetic basis of these alterations has not been studied yet, and reconstruction of resistance by transfection of cloned genes has not been done.

Resistance to the two major pyrimidine analogues, 5-fluorouracil and cytarabine, has been the subject of extensive studies and these will be summarized in separate sections.

RESISTANCE TO 5-FLUOROURACIL

5-Fluorouracil and its nucleoside analogue are converted into 5-fluoro-deoxyuridine monophosphate by cellular enzymes and this inhibits thymidylate synthase, the key enzyme in the biosynthesis of deoxythymidine monophosphate (Fig. 2), essential for DNA synthesis (see Chapter 4.8). Although the affinity of 5-fluoro-deoxyuridine monophosphate for thymidylate synthase is approximately 1000-fold higher than that of the natural substrate, deoxyuridine monophosphate, effective inhibition requires formation of the ternary complex of thymidylate synthase, 5-fluoro-deoxyuridine monophosphate, and the folate co-factor that transfers its methyl group to deoxyuracil as monophosphate (see Fig. 2). Hence, cell killing by 5-fluorouracil only occurs when the cell contains adequate levels of leucovorin (methylene-tetrahydrofolate), presumably in the polyglutamate form. 5-Fluorouracil is also converted into 5-fluorouridine triphosphate and 5-fluoro-deoxyuridine triphosphate and incorporated to a low extent into RNA and DNA. There is no evidence that this contributes to the cytotoxic effect of 5-fluorouracil. Also in clinical samples, there is a good correlation between the degree of thymidylate synthase inhibition and the tumour response to 5-fluorouracil (Spears et al. 1988; Swain et al. 1989; Peters et al. 1990).

In cultured cells, resistance can arise by one of the following mechanisms (see Pinedo and Peters 1988; Peters et al. 1990):

1. uptake defect which has been observed for 5-fluorodeoxyuridine, but not for 5-fluorouracil;
2. decreased conversion of 5-fluorouracil into 5-fluoro-deoxyuridine monophosphate;
3. increased hydrolysis of 5-fluoro-deoxyuridine monophosphate to 5-fluoro-deoxyuridine by nucleotidases;
4. increased concentrations of uridine competing with 5-fluoro-deoxyuridine for conversion into the corresponding nucleotide;
5. lowered levels of the tetrahydrofolate co-factor required for thymidylate synthase function;
6. increased levels of thymidylate synthase.

Among these six mechanisms, the last two seem most important clinically and these will therefore be discussed in more detail.

Resistance in cell lines, associated with elevated thymidylate synthase levels, can be due to amplification of the thymidylate synthase gene (Berger et al. 1985). Amplification of this gene has also been found in a colon cancer biopsy of a single patient with acquired resistance to 5-fluorouracil (Clark et al. 1987). It has also been noted that repeated 5-fluorouracil administration may result in an increase of thymidylate synthase levels, which can be prevented by α-interferon in experimental systems (Keyomarsi and Moran 1989). The effect of the combination of 5-fluorouracil and α-interferon is now being tested in patients with colorectal cancer.

Cellular depletion of folates is an important potential source of clinical resistance (Spears et al. 1989) and, indeed, leucovorin can enhance the cytotoxicity of 5-fluorouracil in cell lines of various histological origins. The effect is schedule-dependent and appears most pronounced for leukemic cell lines (Peters et al. 1990). The co-administration of leucovorin and 5-fluorouracil is being tested in clinical trials of colon cancer and may occasionally reverse even acquired clinical resistance to 5-fluorouracil (Hines et al. 1988; Swain et al. 1989).

RESISTANCE TO CYTOSINE ARABINOSIDE (CYTARABINE)

In cells cytarabine is converted into cytidine triphosphate arabinoside, which acts as a substrate for cellular DNA polymerases in competition with the normal substrate, deoxycytidine triphosphate (Fig. 3). The incorporated cytidine monophosphate arabinoside moiety prevents further DNA chain elongation; this block in DNA synthesis eventually kills the cell (see Chapter 4.8). Resistance to cytarabine can arise by one of the following mechanisms (see Fig. 3).

1. Cellular uptake of nucleosides, like cytarabine, can occur by two different transport systems (see Belt and Noel 1988). The rate of transport into leukemic cells appears to correlate with the sensitivity of these cells to cytarabine (Wiley et al. 1982), but this only applies at conventional cytarabine dosage resulting in plasma concentrations below 1 μM. At the higher concentrations achieved by the high-dose regimens, passive diffusion of cytarabine plays a more important role than active uptake. No mutant cell lines with acquired resistance to cytarabine have been described in which an uptake defect was the sole reason for resistance. This may be due to the presence of two uptake systems in many tumour cell lines.

2. Decreased conversion of cytarabine into cytidine monophosphate arabinoside by deoxycytidine kinase, an enzyme deficient in some cytarabine-resistant mutants. In one resistant cell line, a low expression of the inactive kinase gene could be induced by 5-azacytidine, an inhibitor of DNA methylation (Antonsson et al. 1987). This indicates that the defect in the resistant cell is not a real mutation, but a reversible shut-off of the gene by methylation. As reversal by 5-azacytidine is poor, we see no realistic basis for the combination of this drug and cytarabine in clinical practice.

3. Decreased ability to retain intracellular cytidine triphosphate arabinoside following elimination of the extracellular drug, resulting from an increased deamination of cytarabine and cytidine monophosphate arabinoside.

4. Increased cellular pools of deoxycytidine triphosphate competing with cytidine triphosphate arabinoside for DNA polymerases. High deoxycytidine triphosphate also inhibits deoxycytidine kinase, leading to a decrease in the conversion of cytarabine into cytidine monophosphate arabinoside.

Alterations in the target enzyme, DNA polymerase, have not been found in cytarabine-resistant cells. Apparently, a DNA
Fig. 2. Simplified scheme showing the conversion of 5-fluorouracil (5FU) into the 5-fluoro-deoxyuridine monophosphate (FdUMP) that inhibits the conversion of deoxyuridine monophosphate (dUMP) into thymidine monophosphate (TMP) by thymidylate synthase (TS). The insert shows the ternary complex of folate co-factor, FdUMP, and enzyme.

Fig. 3. Simplified scheme showing the fate of cytarabine (ara-C) in the cell. Shaded blocks indicate (a) decreased uptake (b) deficiency of deoxycytidine kinase; bold arrows indicate high enzymatic activity leading to enhanced drug degradation. The blocks of cytidine triphosphate arabinoside (ara-CTP) and ara-C-DNA indicate that the presence of these forms is essential for activity of the drug.

polymerase that discriminates effectively between cytidine triphosphate arabinoside and deoxycytidine triphosphate is not easily obtained by mutation.

As cytarabine is used in the treatment of leukaemia, the mechanism of resistance has been studied extensively in clinical specimens that can be obtained with relative ease. The results of these studies are disappointing. Although there are indications that resistant cells may be low in cytarabine transport (see above), in most cases no clear biochemical explanation has been found for acquired resistance in clinical samples.

BIOCHEMICAL AND GENETIC MECHANISMS OF P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE

(These mechanisms have been reviewed by Bradley et al. (1988), Gottesman and Pastan (1988, 1989), Tsuroo (1988), Endicott and Ling (1989), Kartner and Ling (1989), and Van der Bliek and Burst (1989).) Growth of human tumour cells in the presence of inhibitory concentrations of large hydrophobic drugs, like doxorubicin or vincristine, may lead to the selection of stable variants that overproduce a large cell membrane glycoprotein, discovered by Victor Ling and his co-workers and called the P-glycoprotein (P for permeability). This P-glycoprotein acts as a molecular pump that can extrude a wide variety of drugs. This lowers the intracellular drug concentration at the target (Schuurhuis et al. 1989) and, hence, results in drug resistance.

There are two genes for P-glycoproteins in man, called mdr1 and mdr3 (or 2). At the present time, only the mdr1-encoded P-glycoprotein has been shown to contribute to drug resistance. Although the mdr2/3-encoded P-glycoprotein closely resembles its mdr1-encoded homologue in size, structure, and amino acid sequence (76 per cent identity), it is not known to pump drugs and its physiological function remains unknown.

The specificity of the wild-type version of the human mdr1 P-glycoprotein has been studied by introducing and overexpressing the cloned mdr1 gene in human cells with low P-glycoprotein levels. P-glycoprotein overproduction in human melanoma cells results in a moderate resistance to anthracyclines, such as doxorubicin and epipodophyllotoxins, such as VP16, high resistance to vinca alkaloids and actinomycin D, and no resistance to a host of other clinically important drugs, such as alkylating agents, cisplatin, methotrexate, and purine and pyrimidine analogues (Lincke et al. 1990). A largely similar multidrug-resistance phenotype is induced by P-glycoprotein overproduction in other cell types. It has been observed, however, that during the step-wise selection of highly resistant cells, one may select for altered versions of the mdr1 gene, which encode a P-glycoprotein with altered drug-transport properties. A single amino acid substitution was found to result in a 2–4-fold increase in the relative resistance to colchicine (Choi et al. 1988). Whether such mutant versions of P-glycoprotein ever arise in tumours in patients is not known.

How the P-glycoprotein pump works is not known in detail. From the amino acid sequence of the protein it can be deduced that P-glycoproteins consist of two similar halves, each containing six transmembrane segments and an adenosine triphosphate (ATP) binding site (Fig. 4). The transmembrane segments are thought to form a channel through which the drug is extruded. The ATP-binding site is thought to be involved in the ATP hydrolysis required to pump out the drug against a concentration gradient. This is in agreement with experiments on multidrug resistance showing that
a decrease in cellular ATP reverses drug resistance and that high P-glycoprotein pump activity increases cellular ATP utilization (Broxterman et al. 1988). Substitution of critical amino acids in one of the ATP-binding sites by reverse genetics also abolishes pump function (Azzaria et al. 1989). Although the possibility has been raised that the P-glycoprotein pumps out a carrier protein to which drugs attach, there is now considerable evidence for a direct binding of drugs to P-glycoprotein (see Horio et al. 1988).

The function of P-glycoproteins in normal tissues is still a matter of speculation. The mdr1-encoded P-glycoprotein is mainly found in the epithelia of excretory organs, such as the biliary canalicul surface of hepatocytes, the apical surface of the intestinal epithelium, and the brush border of the proximal tubules of the kidney. It is also present in other natural barriers, such as the blood–brain barrier and in the testes. This would be compatible with a role in the excretion of large hydrophobic toxic compounds or waste products. This does not, however, readily explain the high concentration of P-glycoprotein in the adrenal cortex and medulla. One of the mouse P-glycoproteins can bind progesterone and it is therefore possible that P-glycoprotein is involved in steroid transport.

More information may come from the study of organisms more amenable to genetic manipulation than man. The P-glycoprotein genes are part of a large gene family, with representatives in fruit flies, nematode worms, protozoa, and baker’s yeast. There is even substantial homology between this family and bacterial membrane transport proteins, involved in substrate uptake or (offensive) polypeptide toxin excretion. The ongoing study of the P-glycoproteins in these simple organisms may yield new ideas about possible natural substrates for the pump in man.

**REVERSAL OF P-GLYCOPEPTIDE-MEDIATED MULTIDRUG RESISTANCE IN CULTURED CELLS**

Considerable interest was generated by the discovery that P-glycoprotein-mediated multidrug resistance can be reversed by the calcium-channel blocker verapamil. Subsequent work has led to a large (and still growing) list of compounds that share this property with verapamil. A selection from this long list is presented in Table 3. Although the mechanism of reversal is still controversial, it is now clear that reversal has nothing to do with calcium channels.

**Table 3** Some chemical compounds known to reverse P-glycoprotein-mediated multidrug resistance in cultured cells (Kessel 1986; Gottesman and Pastan 1989)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Function</th>
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<tbody>
<tr>
<td>Ca(^2+)-channel blockers and (inactive) analogues</td>
<td>Verapamil (1 and 1)</td>
</tr>
<tr>
<td>Calmodulin inhibitors and (inactive) analogues</td>
<td>Phenothiazines</td>
</tr>
<tr>
<td>Anti-oestrogens and (inactive) analogues</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>Miscellaneous large basic hydrophobic drugs</td>
<td>Reserpine</td>
</tr>
<tr>
<td></td>
<td>Quinidine</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A (analogues)</td>
</tr>
<tr>
<td>Non-cytotoxic analogues of vinca alkaloids and anthracyclines</td>
<td>A-Acetyl-daunomycin</td>
</tr>
<tr>
<td>Other compounds with high affinity for P-glycoprotein</td>
<td>Amiodarone</td>
</tr>
</tbody>
</table>

There is considerable evidence that nearly all reversal compounds are, in fact, substrates of the P-glycoprotein pump and compete with the drugs for extrusion from the cell (see Safa 1988; Naito and Tsuru 1989; Yusa and Tsuru 1989). Hence, reversal agents can also be used to study the requirements for interaction with the mdr1 P-glycoprotein. Although effective agents appear to be lipophilic and weakly basic and to contain planar polycyclic systems, none of these features are shared by all P-glycoprotein substrates (DeGregorio et al. 1989; Gosland et al. 1989; Nogae et al. 1989; Pearce et al. 1989; Kiue et al. 1990).

Reversal compounds have been used with some success to overcome resistance in syngeneic tumour models in mice (see Shinoda et al. 1989) and they could provide the clinician with a tool to counteract P-glycoprotein-mediated drug resistance in patients. We return to the clinical trials designed to test this tool below.

**MULTIDRUG RESISTANCE NOT INVOLVING P-GLYCOPEPTIDE**

Two types of multidrug resistance not involving P-glycoprotein have been induced in cultured cells.

1. ‘Atypical’ multidrug resistance. This type of resistance is not really a multidrug resistance, because it only involves drugs that hit a common cellular target, topoisomerase II (Danks et al. 1988). This will be discussed in the next section.

2. Non-P-glycoprotein-mediated multidrug resistance. This type of resistance is negatively defined: it is not associated with raised P-glycoprotein and it also includes drugs that do not act on topoisomerase II, such as vincristine, colchicine, and the membrane pore-forming drug, gramicidin D. It was first observed in a small cell lung cancer line by Mirski et al. (1987), but it has subsequently been induced in leukaeinoma (Marsh and Center 1987; Haber et al. 1989), fibrosarcoma (Slovak et al. 1988), squamous cell lung (Keizer et al. 1989; Baas et al. 1990), and rat brain (Webber et al. 1989) cell lines. The drug-resistance profiles of these cell lines differ and the mechanism of resistance is not known for any of them. For the squamous cell lung line of Keizer et al. (1989), several independent resistant lines were obtained with the same phenotype, suggesting that this phenotype is due to a single mutation. Table 4 contrasts the drug-resistance profile of this mutant with that seen in P-glycoprotein-mediated multidrug resistance and topoisomerase II mutants. Non-P-glycoprotein-mediated multidrug resistance could
Table 4  A comparison of three forms of resistance to doxorubicin/adriamycin in cultured cells

<table>
<thead>
<tr>
<th></th>
<th>P-glycoprotein-mediated multidrug resistance</th>
<th>Topoisomerase II mutants</th>
<th>Non-P-glycoprotein-mediated multidrug resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Acridines (m-AMSA)</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Eppipodophyllotoxins (VP16)</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Vinca alkaloids</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ratio Doxor/Vinca</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Ratio VP16/Gram. D</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

*This group is not homogeneous and results differ for different members of the group. Here we present the phenotype of the resistant squamous cell lung line of Keizer et al. (1989) and Ras et al. (1989), which contains decreased levels of topoisomerase II in addition to non-P-glycoprotein-mediated multidrug resistance.

be clinically important and methods to study it in clinical samples are eagerly awaited.

RESISTANCE DUE TO ALTERED TOPOISOMERASE II

This is reviewed by Orlic and Franco (1988), Wang (1987) and Liu (1989). Selection of cells resistant to anthracyclines may not only lead to mutant cells overproducing P-glycoprotein or displaying a non-P-glycoprotein-mediated form of multidrug resistance, but also to mutants cross-resistant to elliptoines, acridines, actinomycin D, mitoxantrone, and eppipodophyllotoxins, but not to vinca alkaloids, colchicine, or gramicidin D. As eppipodophyllotoxins were initially not known to bind weakly to DNA and as anthracyclines such as doxorubicin were initially thought to kill cells by generating O2 radicals, it took time before it was discovered that all these drugs have topoisomerase II as their common target and that the resistant mutants are in fact altered in this enzyme. At least two types of mutants exist.

1. Mutants with lowered topoisomerase II content. Topoisomerase II makes duplex breaks in DNA, catalyses strand passage through the break without letting go of the ends, and then rescals the duplex break. All the cytotoxic drugs acting on topoisomerase II block the enzyme before rescaling, resulting in a duplex break in DNA when the enzyme is removed. A decrease in the topoisomerase II concentration therefore decreases the number of potential drug targets. As topoisomerase II is indispensable for cell division, there are limits to its decrease and, therefore, to the degree of resistance generated in this way.

2. Mutants with an altered topoisomerase II. These mutants produce an enzyme that is less sensitive to inhibition by drugs. Presumably these mutant enzymes contain amino acid substitutions, but this has not been verified.

One would expect that substantial resistance cannot be obtained by alterations in one allele of the topoisomerase II gene, in other words, that sensitivity will be dominant. Indeed, recent studies on murine mutants show alterations in both alleles, one being rearranged, the other underexpressed, possibly due to DNA hypermethylation (Delfin et al. 1989; Tan et al. 1989). It is possible that additional proteins interacting with topoisomerase II affect the fate of the drug–topoisomerase II complex and that alterations in genes other than that coding for topoisomerase II may also result in resistance to drugs affecting this enzyme (see Liu 1989). This remains to be verified.

How cells containing duplex DNA breaks held together by drug-associated topoisomerase II are eventually killed is still not completely clear. Experiments with temperature-sensitive topoisomerase II mutants in yeast have shown that the enzyme is required to disentangle segregating chromosomes during mitosis (Holm et al. 1989). One would expect therefore that the inactivated topoisomerase II would lead to chromosome breaks and non-disjunction during mitosis, and recent experiments bear this out. The activity of topoisomerase II in normal cells is strongly dependent on the growth phase. Proliferating cells may contain up to 100-fold more enzyme than resting cells and the enzyme level in tissue samples is therefore highly dependent on the growth fraction of the tissue. There are indications that the topoisomerase II level is less tightly controlled in tumours than in normal cells (see Liu 1989). Hence, the mean topoisomerase II concentration in tumours may be higher than in normal tissues and this may explain the success as antitumour agents of drugs inhibiting topoisomerase II.

Whether alterations in topoisomerase II contribute to drug resistance in cancer patients is not yet known and not easily studied. The large changes in the level of this enzyme with changes in the growth phase of the cell make it difficult to decide whether the level found in a tumour is lowered or not. It should be possible, however, to test for point mutations in topoisomerase II in clinical specimens, once the mutations that give rise to resistance are known.

THE MECHANISM OF RESISTANCE TO ALKYLATING AGENTS AND CISPLATIN

This topic has been reviewed by Goldenberg and Begleiter (1984) and De Graeff et al. (1988). Whereas it is relatively easy to select mutant cells highly resistant (more than 1000-fold) to methotrexate or one of the drugs extruded by P-glycoprotein, resistance to alkylating agents and cisplatin is difficult to obtain and resistance is no more than 10–2 fold. This has complicated the analysis of resistance mechanisms. A summary of the documented mechanisms is presented in Table 5. Only in the case of nitrogen mustard and melphalan are well-defined transport proteins known which may be less active in resistant cells. How tumour cells manage to reduce the uptake of other drugs is not known. In fact, it is still not unambiguously proven that a drug such as cisplatin enters cells with
Table 5  Documented mechanisms of resistance to alkylating agents and cisplatin in tumour cell lines (modified after De Graeff et al. (1988), Frei et al. (1988), Kelley et al. (1988))

<table>
<thead>
<tr>
<th>Drug</th>
<th>Resistance mechanism</th>
<th>GST increase</th>
<th>Glutathione increase</th>
<th>Methyl transferase increase</th>
<th>Increased DNA repair</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen mustard</td>
<td>+</td>
<td>+?</td>
<td></td>
<td>+?</td>
<td>+?</td>
<td></td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>+</td>
<td>+?</td>
<td></td>
<td>+?</td>
<td>+?</td>
<td></td>
</tr>
<tr>
<td>Melphalan</td>
<td>+</td>
<td>+?</td>
<td></td>
<td>+?</td>
<td>+?</td>
<td></td>
</tr>
<tr>
<td>Nitrosoureas</td>
<td>+?</td>
<td>+?</td>
<td></td>
<td>+?</td>
<td>+?</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>+?</td>
<td>+?</td>
<td></td>
<td>+?</td>
<td>+?</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>+?</td>
<td>+?</td>
<td></td>
<td>+?</td>
<td>+?</td>
<td></td>
</tr>
</tbody>
</table>

*Increased methyl transferase removing $\text{O}^{3}$-methyl group from guanine.

*Increase in liver aldehyde dehydrogenase.

the help of a carrier protein, as the influx of labelled cisplatin is non-saturable and cannot be prevented by cold cisplatin.

The possible involvement of increased repair is also inferred from indirect evidence, for example increased DNA repair synthesis after short-term drug exposure (Lai et al. 1988). As the first enzymes involved in excision repair of human DNA have only recently been characterized, it may take some time before this important area is fully accessible to biochemical analysis (see Fox and Roberts 1987). The only repair system in mammalian cells that is fully characterized is the $\text{O}^{3}$-methyl transferase system, which can remove the methyl group from the $\text{O}^{3}$-position of guanine. $\text{O}^{3}$-Methylation of guanine appears to be the major lesion induced in tumours by methylating cytotoxic drugs such as dacarbazine (DTIC), streptozotocin, and the nitrosoureas. It has been shown that overproduction of methyl transferase can lead to resistance to nitrosoureas in cultured cell lines (Harris 1983) and that expression of a transfected bacterial alkyltransferase in mammalian cells induces resistance to carbamustine ($\text{bis}$-chloroethyl-nitrosourea; BCNU) (Dumenco et al. 1989).

The role of glutathione and GST in resistance is still not defined precisely. Although there are elevated in a variety of resistant cell lines (Pickett and Lu 1989), this elevation appears to be a rather general and non-specific response to any selection pressure, not necessarily implying a role of GST in resistance. In fact, transfection experiments with plasmid GST class have shown only marginal effects of the overproduction of this enzyme, both on resistance to alkylating agents and to doxorubicin (Moskow et al. 1989). The most compelling evidence for a contribution of GST to drug resistance has come from the analysis of a chlorambucil-resistant Chinese hamster ovary line studied by Robson et al. (1986, 1987) and Lewis et al. (1988). This mutant line was 20-fold resistant, cross-resistant to mechlorethamine and melphalan, but not to other agents, such as mitomycin C or cisplatin. Resistance was associated with a 4–8-fold amplification of a DNA segment containing a gene for an alpha (basic) type GST. The corresponding 50-fold elevation of a GST coupling of melphalan (and chlorambucil) to glutathione, would seem to account for the resistance of this mutant cell line.

The ability of buthionine sulfoximine to lower glutathione in melphalan-resistant ovarian cancer cells and increase their sensitivity to melphalan, is the basis for a clinical trial of buthionine sulfoximine in drug-resistant ovarian cancer (Fojo et al. 1987). The drastic decrease in glutathione induced by buthionine sulfoximine may make glutathione rate-limiting in cell survival under drug stress, even if resistance is due to another mechanism. Even though it is now clear that the resistance of the doxorubicin-resistant MCF-7 cells is predominantly due to raised levels of P-glycoprotein (Morrow and Cowan 1990), possibly increased by a limited alteration in topoisomerase II, Kramer et al. (1988) and Dusre et al. (1989) have found that glutathione depletion of resistant cells by pre-treatment with buthionine sulfoximine leads to a partial reversal of resistance (dose-modifying factor 4–7 at 75–90 per cent glutathione depletion). This clearly illustrates that modification of resistance by alterations of cellular glutathione does not necessarily mean that resistance involves glutathione and cellular detoxification of oxygen radicals.

In contrast, overproduction of metallothionein, a metal-binding protein, has been shown, in direct gene transfection experiments, to result in low-level resistance to alkylating agents and cisplatin (Kelley et al. 1988). Whether overproduction of this protein is ever responsible for resistance in cell lines selected for resistance, however, is unclear.

Several reports have appeared linking cisplatin resistance to increased levels of dihydrofolate reductase and/or thymidylate synthase (see Scanlon and Kashani-Sabet 1988). The biochemical basis for this link is not obvious and it will be necessary to demonstrate that cisplatin resistance can be induced by overexpression of transfected dihydrofolate reductase or thymidylate synthase genes to prove that the association is causal. Nevertheless, the collateral methotrexate resistance, regularly seen in head and neck carcinoma cells selected for cisplatin resistance, may be of clinical relevance (Rosowsky et al. 1987).

One of the most useful practical generalizations to come from these studies on resistant cell lines is the limited degree of cross-resistance between the different classes of alkylating agents. This is not unexpected. Each type of alkylating drug appears to enter cells by a different route, usually by an uptake system for a structurally related amino acid. Nitrogen mustard uses the transport carrier for choline, melphalan uses two transport systems for larger amino acids. Other drugs, such as nitrosoureas and chlorambucil appear to enter by passive diffusion. The lack of cross-resistance is therefore not surprising if resistance is due to decreased uptake.

**RESISTANCE TO OTHER DRUGS**

Resistance to glucocorticoids is invariably due to loss of the glucocorticoid receptor protein. Resistance to oestrogen and androgen antagonists is always associated with the loss of hormone dependence of growth of the resistant cell. How cells escape from hormone dependence is still not known in detail. It is clear that several genes that require a hormone for their activation become constitutionally active, examples are genes for growth factors and growth factor receptors, but the underlying genetic changes remain unknown.
Asparaginase acts by hydrolysing blood asparagine and thereby depriving the tumour (lymphocytic leukaemia cells) of an essential amino acid. Resistance is caused by an increased production of asparagine synthase in tumour cells; the genetic alteration responsible for this enzyme induction is not yet known.

Resistance to other clinically important drugs is mainly accounted for by the mechanisms discussed in previous sections. For instance, substantial resistance to vinca alkaloids is invariably associated with multidrug resistance. Resistance associated with alterations in the target protein, tubulin, has been described (Cabral and Barlow 1989), but resistance in these mutants is only 2–3-fold and the growth of the mutants is often slower than that of the parental cells in the absence of the drug. It is doubtful whether such mutations could contribute to clinical resistance.

PITFALLS IN THE STUDY OF CULTURED CELLS SELECTED FOR HIGH LEVELS OF RESISTANCE IN VITRO

It cells selected for high levels of resistance contain an increased level of enzyme A, the usual inference is that increased A is the cause of resistance. This inference may be incorrect for several reasons.

1. If the level of enzyme A is increased because of the amplification of gene A, it should be realized that DNA amplicons are large and may contain many genes. Selection may be for gene B and enzyme A is only amplified because it happens to reside next to B. An example is the overproduction of the Ca²⁺-binding protein sorcin in many (but not all) multidrug resistant cells. The gene for this protein is close to the P-glycoprotein gene (Van der Bliek and Borst 1989) and it is now likely that the sorcin overproduction in multidrug-resistant cells has no effect on drug resistance (although this remains to be rigorously proven).

2. If the activity of gene A is controlled by a regulatory protein that also controls genes B and C, changes in this regulatory protein may co-ordinately affect the expression of genes A, B, and C, even if these genes are on different chromosomes. An example is the simultaneous induction of P-glycoprotein and pi class GST and the repression of P450 synthesis in the liver by carcinogens (Gottesman 1988, Ivy et al. 1988). There is circumstantial evidence that the genes for these proteins are also controlled by a common regulatory circuit in other cells. Hence, one may find an increased level of pi-class GST in multidrug-resistant cells even though the enzyme makes no contribution to resistance.

3. Cells selected for high levels of resistance have gone through several rounds of stringent selection (99.9 per cent cell kill), often preceded by treatment with mutagens to increase the frequency of resistant mutants. Such cells may require the simultaneous expression of more than one mechanism for resistance to cope with the high drug loads. Documented examples are methotrexate-resistant cells with decreased methotrexate transport and increased dihydrofolate reductase levels and multidrug-resistant cells with raised levels of P-glycoprotein and an alteration in topoisomerase II. Obviously such stringently selected cells can be expected to contain a lot of other irrelevant mutations.

In view of these pitfalls it is essential that putative resistance mechanisms be reconstructed by demonstrating that the introduction of a purified gene into the parental cells can faithfully reproduce the entire resistance spectrum. A good example is the recent analysis of a doxorubicin-resistant MCF-7 breast cancer line. This line was shown to contain multiple alterations relative to its parent, increased P-glycoprotein, a small decrease in topoisomerase II, and a large increase in pi class GST and in P450, to mention only the most relevant. By the appropriate transfection experiments of cloned genes, Moscow et al. (1989) have shown that resistance is largely due to P-glycoprotein overproduction and that the pi class GST makes no significant contribution.

When resistance is thought to be due to recessive changes (enzyme loss), reconstruction of the genotype with cloned DNA is more complicated, even though techniques to specifically knock out cellular genes by homologous recombination with an added defective gene have improved enough to make such experiments feasible. As mutations leading to loss of gene function are fairly frequent, however, it is usually possible to demonstrate that several independent resistant mutants all contain defects in the same genes.

The multiple alterations often found in highly resistant cells have led to the notion that resistance in general is usually multifactorial. There are good reasons to expect that this notion will turn out to be incorrect for clinical resistance. The very steep dose–response curve and the narrow therapeutic window for most cytotoxic drugs predicts that a 2–3-fold increase in the IC₅₀ of a tumour cell would usually be sufficient for complete resistance. Such a modest increase could easily be obtained by a single biochemical change. As resistant cells are mutant cells and mutations occur at a low frequency, the vast majority of mutants will contain a single mutation altering a rate-limiting step in drug metabolism. Even though the total range of resistance mechanisms is bewilderingly complex, we expect that in each individual patient a single mechanism will usually be responsible for resistance against each of the drugs used. Obviously more than one resistance mechanism may arise simultaneously in different cells of the same tumour and with multiple drugs one can select for multiple resistance mechanisms in the same tumour cells. The elementary considerations presented here strongly predict, however, that the average tumour of the average patient treated with doxorubicin will only display a single acquired resistance mechanism.

GENERAL PROBLEMS IN ASSESSING DRUG RESISTANCE IN PATIENTS

Most of our knowledge of drug efficacy in cancer is empirical. We know that cisplatin is effective in testicular and ovarian carcinoma and not in colon carcinoma, but we cannot give a molecular explanation why. This is because the cellular pharmacodynamics of most cytotoxic drugs are not known in sufficient detail. Ideally one would like to know how every drug is handled in every specialized tissue of the body, what the molecular basis is for the dose-limiting toxicity, and why it kills some tumour cells and not others. Without this knowledge we cannot hope to fully understand resistance.

Even if a resistance mechanism is precisely defined by studies of established tumour cell lines, made resistant in vitro, it may still be very difficult to extrapolate the results to tumours in patients. Cells in tissue culture lack stromal interaction, they are usually much less heterogeneous than a real tumour, they cannot be rescued by metabolic cooperation, and one cannot expect to get a homogeneous drug exposure of cells in a real tumour. A case in point is the recent work on P-glycoprotein levels in clinical tumour samples (see below). By sensitive immunocytochemistry one can determine the level of
P-glycoprotein in individual cells, but it is difficult to translate these levels into the response of the tumour as a whole. The only rational approach is to treat P-glycoprotein levels as just another tumour marker and determine in controlled prospective studies whether the presence of this marker correlates with response to therapy. As more potential resistance mechanisms are unravelled, the number of such markers for resistance may increase and gradually improve the ability of the clinician to predict tumour response.

In the following sections we shall present the empirical data on primary and secondary resistance of the main tumour types and the major antineoplastic drugs and, where this information is available, we shall give the probable mechanism of resistance. In the last section we shall discuss briefly how knowledge of resistance mechanisms can help to overcome or circumvent resistance.

RESISTANCE MECHANISMS IN TUMOUR SAMPLES FROM PATIENTS: AN OVERVIEW

Resistance mechanisms in clinical samples have either been studied directly or on cultured cells. Direct tests are limited to analyses on surviving, non-replicating cells, such as drug uptake, drug binding to target, inhibition of metabolic pathways, and acute cellular defence reactions (such as induced DNA repair). Such direct tests are hampered by problems in the preparation of representative intact cell suspensions from fresh solid tumours and by the presence of variable amounts of normal tissue and stromal elements in the suspension. Analysis of cultured cells is complicated by difficulties in growing a representative fraction of the tumour cells and the potential loss of resistance during growth in vitro.

Table 6 gives an overview of the resistance mechanisms observed. Most of these have already been discussed in previous sections, together with resistance mechanisms in cell lines. Since 1986, considerable effort has been invested in defining the possible contribution of mdr1-encoded P-glycoprotein to clinical resistance and these studies will be summarized in the following paragraphs.

THE POSSIBLE CONTRIBUTION OF ELEVATED P-GLYCOPROTEIN LEVELS TO CLINICAL DRUG RESISTANCE

This has been reviewed by Kaye (1988) and Ling (1989). P-glycoprotein-mediated multidrug resistance can be considered a paradigm for the ongoing attempts to define mechanisms of clinical drug resistance. How P-glycoprotein renders cells resistant to a drug is known in essence. Resistance is associated with a single gene product, encoded by a single gene. There are sensitive and specific monoclonal antibodies available to detect the protein in individual cells of tumour samples and specific DNA probes to detect overexpression or amplification of the gene. Several sets of cells line with increasing levels of resistance are available which make it possible to relate the amount of P-glycoprotein (mRNA) to the degree of resistance. Hence, it is possible to assess roughly whether the levels of P-glycoprotein (mRNA) observed might contribute to resistance. In the final analysis, P-glycoprotein (mRNA) levels can be used like any other tumour marker and be correlated with clinical outcome. This will provide a reliable basis for rational treatment modification, for example the avoidance of drugs affected by P-glycoprotein in the treatment of tumours with high levels of this protein or attempts to modify resistance with reversal agents.

An extensive study of mdr1 mRNA in more than 400 human tumours was carried out by the National Cancer Institute group (Fojo et al. 1987; Goldstein et al. 1989). High levels were generally found in untreated tumours from tissues known to have a substantial mdr1 expression, such as colon, kidney, liver, adrenal, and pancreas (Table 7). Low amounts of mdr1 mRNA were also found in a fraction of the breast cancer, non-small-cell lung cancer, and bladder cancer samples and the mRNA was not detected in all other tumours analysed (Goldstein et al. 1989). This overall distribution of P-glycoprotein in human tumours inferred from mRNA levels, has been confirmed in a more limited series of tumours with P-glycoprotein-specific antibodies (Van der Valk et al. 1990). Although the tumours that are high in P-glycoprotein are known to be intrinsically resistant to antineoplastic drugs, it should be noted that these tumours also fail to respond to drugs not affected by P-glycoprotein and that the level of P-glycoprotein mRNA varies considerably, even in the group of tumours with high mRNA. It might be possible to further test the role of P-glycoprotein in these tumours by combining drugs with reversal agents (see below).

In general, P-glycoprotein (mRNA) levels tend to be higher in tumours after treatment relapse, but the number of tumours analysed is small (Goldstein et al. 1989). Indications for a clear increase of P-glycoprotein levels during treatment have been obtained in ovarian cancer (Bell et al. 1985; Bourhis et al. 1989a), acute non-lymphocytic leukaemia (Ma et al. 1987), chronic myelogenous leukaemia in blast crisis (Tsuruo et al. 1987; Goldstein et al. 1989), acute lymphoblastic leukaemia (Rothenberg et al. 1989), and neuroblastoma (Bourhis et al. 1989b; Goldstein et al. 1989).

Recent work by Dalton and co-workers (personal communication) has further strengthened the association between P-glycoprotein elevation and acquired drug resistance in myeloma and non-Hodgkin's patients. In 31 myeloma patients treated with regimens including vincristine+doxorubicin, 42 per cent had elevated P-glycoprotein, against 7 per cent in the untreated controls. In the treated non-Hodgkin's patients 70 per cent were positive, compared with 2 per cent in controls. This suggests strongly that intensive treatment selects for cells with elevated P-glycoprotein levels.

Some correlation has also been seen between the level of mdr1 mRNA and resistance of renal carcinoma samples to vinblastine in a clonogenic assay (Kakchi et al. 1988). A clearer correlation was observed in studies with myeloma, non-Hodgkin's lymphoma, and breast cancer between P-glycoprotein expression and in vitro resistance to doxorubicin (Salmon et al. 1989).

The most remarkable correlation so far between raised P-glycoprotein and a poor response to chemotherapy has been observed in childhood sarcoma patients (Gerlach et al. 1987; Chan et al. 1990). Gerlach et al. (1987) found six out of 25 sarcomas positive for P-glycoprotein with a relatively insensitive immunoblotting assay that did not detect P-glycoprotein in other tumours, including seven colorectal and four renal tumours. In a subsequent series of 30 children with rhabdomyosarcoma or undifferentiated sarcoma, a more sensitive immunocytochemical detection of P-glycoprotein was used (Chan et al. 1988). P-glycoprotein in these patients was a nearly absolute predictor of the response to chemotherapy. In the nine patients in which P-glycoprotein was detected, four were already positive before treatment; the other five became positive at first or subsequent relapses during standard chemotherapy. Increasing resistance to therapy was associated with an increasing fraction of positive cells and higher levels of staining.
Table 6  Documented mechanisms of acquired drug resistance in human tumour specimens

<table>
<thead>
<tr>
<th>Drug</th>
<th>Tumour</th>
<th>Mechanism of resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>Leukaemia</td>
<td>Increased DHFR (gene amplification)</td>
<td>Sobrero and Bertino (1986)</td>
</tr>
<tr>
<td></td>
<td>Small cell lung cancer</td>
<td>Increased DHFR (gene amplification)</td>
<td>Sobrero and Bertino (1986)</td>
</tr>
<tr>
<td></td>
<td>Leukaemia</td>
<td>Altered DHFR</td>
<td>Sobrero and Bertino (1986)</td>
</tr>
<tr>
<td></td>
<td>Leukaemia</td>
<td>Increased cytidine deaminase</td>
<td>Sobrero and Bertino (1986)</td>
</tr>
<tr>
<td></td>
<td>Leukaemia</td>
<td>Impaired transport</td>
<td>Sobrero and Bertino (1986)</td>
</tr>
<tr>
<td></td>
<td>Leukaemia</td>
<td>Decreased HGPRT</td>
<td>Sobrero and Bertino (1986)</td>
</tr>
<tr>
<td></td>
<td>Leukaemia</td>
<td>Increased alkaline phosphatease or increased 5-nucleotidase</td>
<td>Sobrero and Bertino (1986)</td>
</tr>
<tr>
<td></td>
<td>Breast cancer</td>
<td>Decreased inhibition of TS</td>
<td>Swain et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Acute lymphocytic leukaemia</td>
<td>Increased level of asparagine synthetase</td>
<td>Kashani et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>Colon cancer</td>
<td>Increased TS-mRNA</td>
<td>Kashani et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>Ovarian cancer</td>
<td>Increased TS-mRNA</td>
<td>Tidjef et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>Acute myeloid leukaemia</td>
<td>Decreased accumulation</td>
<td>Nooter et al. (1989)</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>Ovarian cancer</td>
<td>Increased P-gp expression</td>
<td>Bell et al. (1985)</td>
</tr>
<tr>
<td>Asparaginase</td>
<td>Chronic myeloid leukaemia</td>
<td>Increased P-gp expression</td>
<td>Ma et al. (1987)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Leukaemia (blast crisis)</td>
<td>Increased P-gp expression</td>
<td>Tsuruo et al. (1987)</td>
</tr>
<tr>
<td></td>
<td>Childhood sarcoma</td>
<td>Increased mdrl-RNA expression</td>
<td>Goldstein et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Ovarian cancer</td>
<td>Increased P-gp expression</td>
<td>Chan et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>Acute lymphocytic leukaemia</td>
<td>Increased mdrl-mRNA expression</td>
<td>Bourhis et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Myeloma</td>
<td>Increased mdrl-RNA expression</td>
<td>Rothenberg et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Lymphoma</td>
<td>Increased mdrl-RNA expression</td>
<td>Dalton et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Acute myeloid leukaemia</td>
<td>Increased mdrl-RNA expression</td>
<td>Goldstein et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Neuroblastoma</td>
<td>Increased mdrl-RNA expression</td>
<td>Miller et al. (1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Holmes et al. (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bourhis et al. (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Goldstein et al. (1989)</td>
</tr>
</tbody>
</table>

Abbreviations: DHFR, dihydrofolate reductase; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; TS, thymidylate synthase; mRNA, messenger ribonucleic acid; P-gp, P-glycoprotein.

Table 7  Generally high MDRI RNA levels in untreated cancer (from Goldstein et al. 1989)

<table>
<thead>
<tr>
<th>Cancer type/ cell line</th>
<th>Total number of cancers</th>
<th>Number of positive (≥ 30 U)</th>
<th>Number of low positive (2-29 U)</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon carcinoma</td>
<td>41</td>
<td>10</td>
<td>25</td>
<td>85.5</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>50</td>
<td>35</td>
<td>5</td>
<td>80.0</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>12</td>
<td>7</td>
<td>5</td>
<td>100.0</td>
</tr>
<tr>
<td>Adrenocortical cancer</td>
<td>9</td>
<td>6</td>
<td>1</td>
<td>77.8</td>
</tr>
<tr>
<td>Phaeochromocytoma</td>
<td>20</td>
<td>11</td>
<td>4</td>
<td>75.0</td>
</tr>
<tr>
<td>Melanotic tumour of pancreas</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>50.0</td>
</tr>
<tr>
<td>(ML blast crisis)</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>Gastrointestinal tumour</td>
<td>9</td>
<td>2</td>
<td>5</td>
<td>77.8</td>
</tr>
<tr>
<td>SCLC</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>83.3</td>
</tr>
</tbody>
</table>

MDRI RNA levels were measured by RNA slot blot analysis and are expressed relative to level in the drug-resistant KB-S-5 cell line, which has been assigned a value of 30 U. All values refer to RNA level in 10 μg of total RNA.

CLINICAL TRIALS ON REVERSAL OF P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE

Miller et al. (1988) used the calmodulin-inhibitor and antipsychotic phenothiazine, trifluoperazine, in a diverse group of cancers. No response was seen in 15 patients with primary doxorubicin resistance, but six partial remissions and one complete remission were obtained in 21 patients with acquired resistance (two non-Hodgkin's lymphomas, one small-cell carcinoma of the bronchus, one sarcoma, two breast cancers, one ovarian cancer). No attempt was made to verify whether resistance was associated with elevated P-glycoprotein levels.

This was done in a more recent study by Dalton et al. (1989) on eight patients with acquired resistance, six of whom had significant tumour P-glycoprotein levels. Five of these had multiple myeloma, one a drug-refractory non-Hodgkin's lymphoma. Addition of a continuous infusion of verapamil to the VAD (vincristine, doxorubicin, dexamethasone) regimen led to a brief partial or complete remission (maximum 6 months) in three patients. The myeloma cells isolated from two of these patients showed a 40–50 per cent increase in doxorubicin or vincristine uptake in the presence of verapamil. The cardiac toxicity of verapamil was substantial and was dose-limiting in this trial. As pointed out by Gottesman and Pastan (1989), there is no formal proof that the verapamil effect in this trial was due to a reversal of P-glycoprotein drug pumping. Verapamil might have sensitized the myeloma and non-Hodgkin's lymphoma cells to the VAD regimen in another fashion. This would

It should be stressed that in several major tumours, increased levels of P-glycoprotein do not seem to contribute to clinical resistance. These include tumours of the breast (Merkel et al. 1989) and lung (Lai et al. 1989). Hence, increased P-glycoprotein levels appear to make only a modest contribution to drug resistance in patients.
explain the lack of any response to the verapamil–doxorubicin combination in the ovarian cancer patients studied by Ozols et al. (1987). To interpret the results of additional trials, it will be important to determine tissue levels of the reversal agent, in addition to P-glycoprotein levels.

The potential of these reversal agents will depend on the ability to sensitize tumour cells more to the cytotoxic agent than some of the normal tissues that contain high P-glycoprotein levels. For this reason further delineation of the physiological function of P-glycoproteins is important. Horton et al. (1989) have recently shown that verapamil enhances vincristine toxicity in mice so profoundly that this reversal actually decreases the cytotoxic efficacy of vincristine. It is known, however, that rodents have substantially higher P-glycoprotein levels in normal tissues than man and therefore these unfavourable results with rodents cannot be simply extrapolated to man.

KNOWLEDGE-BASED CIRCUMVENTION OF DRUG RESISTANCE

Although knowledge of the biochemical mechanisms of drug resistance has had little impact on the treatment of cancer patients as yet, this situation should and will change. It is possible to envisage the application of new knowledge at several levels.

1. A further increase in the use of tumour cell suspensions from fresh biopsies, clonogenic assays, and xenografts to assess drug response. These tests will increasingly incorporate more sophisticated measurements, for example the rates of drug uptake and extrusion, intracellular distribution of the drug, and the effect of reversal agents such as verapamil.

2. A rapid increase in the use of markers that predict drug response, allowing the clinician to avoid ineffective drug treatment. An example is provided by the rapidly improving tests for the cytochemical detection of P-glycoprotein in standard pathological specimens. As details of other common mechanisms of drug resistance are unravelled, more tests based on the recognition of (altered) proteins involved in resistance should become available. Such tests are more easily incorporated into routine clinical practice than tests requiring culture of tumour samples or xenografts and, moreover, they do not suffer from the risk that the tumour sample cultured is not representative of the tumour.

3. An understanding of resistance mechanisms may help to direct the development of analogues that can circumvent resistance. An example is provided by the hydrophobic antibiotics that can kill those cells not taking up methotrexate. The development of topoisomerase II inhibitors that are not extruded from the cell by P-glycoprotein would be an obvious challenge for the future. The anticycline derivatives now available with high activity against multidrug-resistant cells, all seem to be rather hydrophobic (see Coley et al. 1989). They may therefore enter the cell so rapidly that the P-glycoprotein pump cannot cope with the influx. Whether such derivatives will be too toxic for normal tissues with high P-glycoprotein content, remains to be seen.

4. Once the biochemical basis for resistance is known and recognizable in clinical samples, it may become possible to devise drug regimens that overcome resistance. Examples include the agents able to reverse P-glycoprotein-mediated multidrug resistance in tissue culture, which are now being tested in clinical trials.

5. Resistance mechanisms may result in changes in metabolism that could be exploited for therapy. An example is the overproduction of P-glycoprotein, which might be exploited using antibodies against the extracellular part of this protein. Fitzgerald et al. (1987) have shown, for instance, that such a monoclonal antibody coupled to Pseudomonas toxin will specifically kill highly multidrug-resistant cells in culture.

6. For responsive tumours, the empirical demonstration that drug combinations are generally more effective than each drug given separately in sequence is now attributed to the presence of resistant mutant cells in the tumour populations, which are more likely to be resistant to one drug than to a cocktail of drugs (see Dexter and Leith 1986). However, it is a serious mistake, often made, to assume that the dose reduction applied in the combination will not affect the antitumour effect of the particular drug. Similarly, dose reduction in adjuvant chemotherapy is a further problem. Dosages in standard treatment protocols used for advanced disease have been reduced in order to 'protect' the patient receiving adjuvant chemotherapy. Such reductions lack any scientific basis.

To optimize such combinations, it is essential to explore the full range of the dose–response curve and to control the dose intensity properly. Occasional responses observed in resistant patients receiving megadose chemotherapy followed by autologous bone-marrow transplantation, indicate that some types of clinical resistance may be overcome with this approach, which has not to date been adequately explored in solid tumours. Perhaps more importantly, extensive studies should be initiated to investigate the role of high-dose chemotherapy with autologous bone-marrow transplantation as the initial treatment. According to the Goldie–Coldman theory (1988), such an approach would reduce the chance of the development of resistant cell clones, as the start of chemotherapy after operation means quite a significant delay in the treatment of micrometastases.

Besides 'conventional' high-dose chemotherapy with autologous bone-marrow transplantation, several approaches are being explored in unresponsive tumours. The availability of haemopoietic growth factors, such as granulocyte–macrophage colony-stimulating factor, granulocyte-stimulating factor, interleukin-3, and perhaps interleukin-1, may make it possible to increase the doses of existing drugs even more. As the dose–response curves for the most effective drugs are steep, this may make some of the unresponsive tumours even more accessible to drug treatment.

The co-administration of growth factors which increase the growth fraction of a tumour should be tested together with the subsequent administration of cycle-specific cytotoxic drugs, the aim of this approach being to increase the number of tumour cells in the sensitive phase of the cell cycle, thus optimizing cell kill. New drugs should therefore be tested in non-pretreated patients over the dose–response curve before 'falsely' rejecting a new agent. While such studies are being undertaken in patients with leukaemia, they are also needed in patients with solid tumours.

7. In general, it is obvious of great importance to know which resistance mechanism is most likely to interfere with the action of a certain drug in the tumours against which it is being used. This will allow the construction of more rational drug combinations, which are at present being deduced empirically from clinical trials.

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4. THE SCIENTIFIC BASIS OF CANCER TREATMENT


