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FROM THE NETHERLANDS CANCER INSTITUTE, AMSTERDAM, THE NETHERLANDS.

GENETIC MECHANISMS OF DRUG RESISTANCE

A review

P. BORST

Abstract

An overview of our present understanding of mechanisms of resistance against cytotoxic drugs is presented. Most of this understanding has come from studies on tumor cells made resistant in vitro, but there is reason to think that similar mechanisms are responsible for resistance in patients. After a brief overview of biochemical mechanisms of drug resistance, the types of mutations in tumor cells that can alter drug handling are discussed. Three examples of resistance are analysed in more detail: resistance to the folate analogue methotrexate; the multidrug resistance caused by increased levels of P-glycoprotein, which extrudes drugs from the cell; and resistance to alkylating agents.

Key words: Cancer, chemotherapy, resistance, mechanisms.

Chemotherapy of cancer may fail for various reasons. Among these, drug resistance is the most important one. Resistance may be primary (intrinsic), i.e. the tumor cells do not respond from the start; or it may be secondary (acquired) i.e. the tumor initially responds to therapy, but eventually tumor growth resumes and the patient relapses.

It is likely that the biochemical mechanisms involved in primary and secondary resistance are largely similar, but we know far more of secondary resistance, since it is easier to study in the laboratory. Resistance can be induced in cultured cells and one can then determine the differences between the resistant cells and the parental cells from which they were derived. Usually this allows the investigator to determine which difference is responsible for resistance. In contrast, in primary resistance there is no sensitive cell for comparison that is identical in all properties to the resistant one, but for the resistance.

Acquired drug resistance in cultured cells is nearly always due to a genetic change in these cells, i.e. resistant cells are mutant cells. The ability to clone and sequence genes has led to the identification of the genetic changes underlying several forms of drug resistance. This makes it now possible to delineate the main genetic mechanisms involved in drug resistance.

In this review I shall make no attempt to present a complete overview of the genetic basis of all known biochemical mechanisms of drug resistance. Rather, I shall discuss a few informative examples and argue that the remainder is going to be more of the same.

As resistance genes are more easily studied in established tumor cell lines in tissue culture than in tumors in patients, the information about the latter is still sparse. Wherever it is available, it will be included in this review. More detailed information on general aspects of drug resistance can be found in the general textbooks of cancer biology edited by Farmer & Walker (1), Franks & Teich (2), and Tannock & Hill (3), specialized volumes on cancer drug resistance edited by Fox & Fox (4), and by Woolley & Tew (5), in the (recently published) 3rd edition of the textbook on cancer, edited by DeVita et al. (6) and in the specialized reviews quoted in individual sections of this chapter.

Biochemical mechanisms of acquired drug resistance: an overview

The availability of tumor cell lines, growing in tissue culture, has allowed the experimental biologist to obtain variant cells resistant to virtually any drug. By step-wise increases in the drug dose, one can usually obtain highly resistant cell lines that are suitable for biochemical analysis. In this way a long list of mechanisms of drug resistance has been precisely defined.

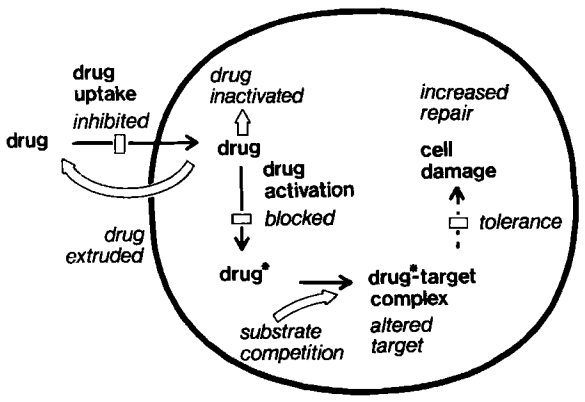

Fig. 1. Simplified scheme illustrating the main biochemical mechanisms of drug resistance. See text for explanation.

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

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.

Increased drug extrusion

Increased synthesis of a plasma membrane protein, *called* P-glycoprotein, that can pump out a wide variety of large hydrophobic drugs, is the cause of the most intensely studied form of multidrug resistance (MDR).

Decreased drug activation

Many cytotoxic drugs exert their effects only after metabolic activation. A decrease in the enzymes doing this job, may lead to resistance. An example is the loss of deoxycytidine kinase, an enzyme required for the conversion of aracytidine (ara-C) into aracytidine-monophosphate (ara-CMP), an essential step in the synthesis of aracytidine-triphosphate (ara-CTP) that is the actual inhibitor of DNA synthesis that kills the tumor cell.

Increased drug inactivation

Cells metabolize drugs and this may not only lead to drug activation, but also to drug inactivation. Increased rates of inactivation could in principle lead to resistance. It has been shown, for instance, that an induced increase in the level of the metal-binding protein metallothionein can result in low-level resistance to cisplatin and some alkylating agents. Resistance to some alkylating agents can also be caused by increased coupling of the drug to glutathione by one of the glutathione-S-transferases. In general, however, this rather obvious line of defense is used less frequently by tumor cells than expected only a few years ago.

Decreased formation of drug-target complexes

This can occur in any of four ways:

a. The target enzyme is altered by an aminoacid substitution, resulting in an enzyme with decreased affinity for the drug.
b. The target enzyme is overproduced making it less easy to get complete inhibition of the enzyme.
c. An increased level of normal substrate competes with the drug for the target enzyme.
d. A decrease in an essential co-substrate decreases the formation of the drug-target complex.

Mechanisms a and b occur in MTX resistance; mechanisms b, c, and d in resistance to 5-fluorouracil (FU).

Increased tolerance of 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 tumor 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.

Increased repair of drug damage

Many carcinostatic 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. However, direct evidence for increased DNA repair in resistant cancer cells is still limited.

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.

Drug resistance in cell lines is usually due to mutation rather than adaptation

Drug resistance can arise by adaptation or by mutation. Adaptation is induced by drug and dependent on the continued presence of the drug; resistance is rapidly lost when the resistant cells are grown without drug. Mutations

resulting in drug resistance can occur in the presence but also in the absence of drug. The mutant sub-population is selected by growth in presence of drug. As mutations are in principle irreversible, the resistance is retained if the cells are grown without drug. Although the distinction is simple in theory, there are sometimes problems in practice. Drugs may induce a change in cellular differentiation that is rather stable, but not associated with changes in DNA. There are also mutations that are fairly unstable; for instance, amplified DNA present as double minute chromosomes (see section on Gene amplification) is often lost rapidly when cells are grown without drug selection. To complicate matters further, not all alterations in DNA are mutations: gene expression may be stably repressed by methylation of DNA by cellular enzymes. Although replacement of a DNA base by its methylated analogue formally changes the DNA sequence, such changes are usually called epigenetic, i.e. changes in differentiation state, rather than mutations, because they cannot be maintained in dividing cells without a maintenance methylase that methylates hemi-methylated DNA after DNA replication.

Inducible drug-metabolizing systems are present in several cell types and one would therefore expect that it should be easy to make cells resistant by growth in sub-inhibitory drug concentrations. In practice, however, this has only rarely been described in cell lines. Resistant cells are nearly always mutant cells. It is possible that this is due to selective reporting, or inadequate testing. One can easily miss adaptation, if cells are only tested in inhibitory drug concentrations.

Most tumors arise from a single cell, but by the time they become clinically manifest they consist of 10^9–10^{10} cells and are genetically heterogeneous. This is not surprising. Mutation rates in somatic cells are of the order of 10^{-6}–10^{-8} per DNA base pair per division and if the mutation is not deleterious it will be carried along in the population. Among these mutations some will have consequences for drug sensitivity. The larger the tumor mass, the greater the chance that cells will happen to acquire a mutation that changes drug handling. Drug treatment will select out this resistant cell and its progeny will eventually predominate the recurring tumor. The chance of having two independent mutations in one cell is of course small and this explains the success of the simultaneous administration of drugs affected by different resistance mechanisms. These ideas have predominantly been developed by Skipper et al. (7) and by Goldie & Coldman (8). Although a tumor may not consist just of completely sensitive and completely resistant cells, but of a spectrum of cells with different levels of sensitivity (9), this does not detract from the basic soundness of the mutation-selection theory for understanding tumor cell resistance (see also Goldie & Coldman in Woolley & Tew (10), for a recent discussion of their ideas).

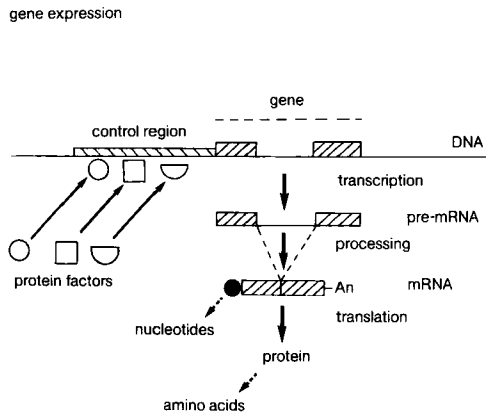

Fig. 2. Simplified scheme illustrating the main steps in protein synthesis and degradation. See text for explanation. The filled black circle represents the cap structure on mRNA; the broken arrows indicate degradation to nucleotides and aminoacids respectively.

How mutations affect the behavior of cells

DNA codes for proteins and for the structural RNAs required for the machinery that makes these proteins. Alterations in the structural RNAs affect the general capacity of cells to synthesize protein. Such alterations are nearly always deleterious and not of interest to oncologists. Mutations that affect cellular behavior in an interesting way nearly always do so by changing the amount or catalytic activity of one or more proteins. It is therefore necessary to consider briefly how cells make and degrade proteins before I go into the mutations that alter these proteins. Fig. 2 gives a simplified scheme of the main steps in protein synthesis and degradation. Synthesis of the messenger RNA (mRNA) that specifies the aminoacid sequence of a protein, starts with the activation of a gene by binding of protein factors to the gene control regions, usually situated in front of the gene. The pre-mRNA is processed to remove non-coding (intron) sequences and is transported to the cytoplasm where the mature mRNA is translated into protein. All subsequent events are encoded in the aminoacid sequence, i.e. folding and modification of the protein, topogenesis (routing to its proper place in the cell) and stability. All these events may therefore be affected by mutations that alter the aminoacid sequence of a protein.

How mutations in DNA affect proteins involved in drug handling

Genetic change affects cellular drug handling by changing one or more proteins, e.g. drug transport proteins or drug metabolizing enzymes. Such changes can be subdivided into three categories on the basis of the biochemical effects of the change:

Decreased production of protein X, or synthesis of an unstable or non-functional form of X

The net result is a lower activity of X in the cell. The most common and drastic alteration is the complete absence of X. From Fig. 2 one can infer how this could happen:

- Part or all of the gene may be deleted; no gene, no protein.
- The gene may be intact but the control regions surrounding the gene may be mutated resulting in a gene that cannot be activated. A special form of inactivation is associated with increased methylation of cytosines in the gene area and a concomitant alteration in chromosome structure. This form of gene inactivation can sometimes be partly reversed by growing cells in azacytidine, a base analogue that is incorporated into DNA. Aza-deoxycytidine in DNA traps the DNA methylating enzyme and thereby prevents DNA methylation.
- There may be alterations in the gene that prevent the proper processing of pre-mRNA. Even a point mutation (a single base pair substitution) may for instance prevent the proper splicing out of an intron, resulting in an unreadable mRNA.
- Aminoacid substitutions in the protein coding sequence may result in a protein that is non-functional, unstable, or susceptible to rapid proteolytic degradation in the cell.
- More rarely, genes may be inactivated in the absence of mutations in or near the gene. This may be caused by an alteration in a gene coding for one of the regulatory proteins that bind to the control region in front of gene X in Fig. 2. This is called inactivation *in trans*, whereas mutations in or near gene X work *in cis*. No clear example of trans-inactivation of proteins involved in drug handling has been reported yet.

Production of a protein with altered affinity for drug

These mutation are nearly always due to aminoacid substitutions caused by point mutations.

Increased cellular levels of a normal protein

This increase can be mediated by alterations that affect any step in gene expression, and it may either be caused by an increase in the production of the mRNA or protein, or by a decrease in its degradation. The most prevalent cause of an increase in protein X is an increased synthesis of mRNA for X. This may either occur by *transcriptional activation*, i.e. an increase in the rate of mRNA X synthesis, or by *gene amplification*, i.e. an increase in the copy number of gene X.

Dominant versus recessive mutations

Human cells are diploid and have two functional copies of most protein-coding genes. If alteration of one of these copies leads to drug resistance, resistance is dominant. If both copies must be altered, resistance is recessive.

Loss-of-function mutations, e.g. the loss of the activation of a pro-drug, are usually recessive, as 50% of the normal enzyme amount is still sufficient to activate the pro-drug. Mutations that make a target-enzyme resistant to drug are usually dominant, it suffices in most cases to keep metabolism going if 50% of the normal enzyme complement is in resistant form. One would expect that recessive forms of drug resistance are rare, since they require two genetic events. In practice, some recessive loss-of-function mutations are frequent, because there are many different mutations that can prevent gene function.

Gene amplification resulting in drug resistance

In 1976, Biedler & Spengler (11) noted the presence of chromosome elongations in cultured hamster cells made resistant to MTX. They called the extra DNA 'homogeneously staining regions' or HSRs. Other names for these chromosome insertions are 'aberrantly staining region' (ABR) or 'extended chromosomal region' (ECR). Schimke and coworkers then showed that the extra DNA contains extra copies of the gene for the enzyme dihydrofolate reductase (DHFR), explaining the increased enzyme levels in the resistant cell (12). The amplified DNA may either be present in chromosomes as HSRs or free as minute chromatin particles usually present in metaphase spreads as paired minutes and therefore usually called double minutes or DMs. HSRs are fairly stable in the absence of drug selection and are lost over months. DMs do not contain a centromere and are therefore not properly retained in the mitotic spindle and distributed over daughter cells during cell division. Hence, the number of DMs per cell tends to be rather variable in the presence of drug selection and they are rapidly lost in dividing cells not exposed to selection (13, 14).

After the initial demonstration that cells can overcome the MTX inhibition of DHFR by overproducing the enzyme by means of gene amplification, numerous other examples of this escape mechanism have been reported. The ability to amplify segments of DNA under pressure and retain the segment as long as the selection pressure continues, is not limited to mammalian somatic cells, but a general device available to prokaryotes, primitive and higher eukaryotes. In principle, one can expect to select for gene amplification in all cases where the effect of the inhibitor can be overcome by producing more of a protein. Some DNA segments amplify more easily than others, however, for reasons that are still unknown. Moreover, selection for gene amplification is only possible when the gene is active. The low transcriptional activity of

P-glycoprotein gene in many human cell lines may explain why overproduction of P-glycoprotein in these cells is usually due to gene activation alone without amplification. Having more copies of an inactive gene does not help in overcoming resistance.

This review is not the place to present an overview of the entire complex subject of gene amplification. The reader can find the information in recent reviews of Schimke (13, 15), Stark & Wahl (14), Hamlin et al. (16), Stark (17), Stark et al. (18) and Wahl (19). Here I briefly discuss two points that are relevant to this review:

— What is the structure of amplified DNA?
— What induces DNA amplification?

The structure of amplified DNA

To be visible under the light microscope, a minute chromosome must contain 1 Mb (1 megabase pair = 1 000 kb) of DNA. There is considerable variation in the size of DMs in different resistant mutants and in most cases there must be at least 2 Mb or more of DNA per minute chromosome. One minute chromosome contains a single large circular DNA molecule (20, 21). In the one case studied in detail, the amplified segment ('the amplicon') could be interpreted as either being 1 500 kb in size, or alternatively a dimer of a 750 kb segment (21).

Very long amplicons have also been found in the HSRs of some drug-resistant cells. There is now strong circumstantial evidence that the initial amplification step in drug-selected cells, may often involve large expanses of DNA, covering several Mb (22, 23). For instance, the initial amplicon in the series of CHO cell lines of Victor Ling, leading to the much-used CH^RC5 line, is at least 3 Mb and possibly as large as 7 Mb (24, 25). Under continuing stringent drug selection, reamplification of part of the original amplicon occurs and shorter amplicons are selected. The end result at high degrees of amplification is usually a short amplicon (200–300 kb), heterogeneous in size, with variable endpoints relative to the original chromosomal structure (18). The important practical implication of this sequence of events is that amplicons may be quite large under conditions that are most relevant for drug resistance in patients, i.e. low degree of amplification. A 3 Mb segment may contain in the order of 30 genes. Hence, if one finds a gene amplified in a resistant cell, it is not necessarily the gene that confers resistance. It may just be linked to the gene that is being selected for. A case in point is the amplification of the sorcin gene in many MDR cells. This gene is linked to the P-glycoprotein genes and there is no evidence that sorcin contributes to MDR (24).

What induces DNA amplification

The mechanism of gene amplification remains remarkably controversial. At least five principal models have been proposed and none of these can fully explain the experimental results obtained in all cell lines studied. Stark et al. (18) and Wahl (19) present overviews of this complex field.

An important spin-off from studies on the mechanism of DNA amplification was the realization that this process is stimulated by many treatments that block DNA synthesis or damage DNA (14, 15). These treatments include hypoxia, ionizing radiation, carcinogens and cytostatic agents. Although this has not been studied systematically, one may expect that many of the cytotoxic drugs used in the treatment of cancer may promote the DNA amplification that could help the cancer cell to overcome the adverse effects of the drug. As cancer cells have higher rates of gene amplification than normal cells (26, 27), gene amplification could be an important mechanism for drug resistance. In practice, genes coding for drug-metabolizing enzymes have rarely been reported to be amplified in clinical samples. The only exception is the DHFR gene (28). Whether this is due to lack of systematic studies, or the rarity of gene amplification in clinical drug resistance is not clear.

Biochemical and genetic alterations causing methotrexate resistance (29, 30)

Six distinct mechanisms are now known to result in MTX resistance:

1. Decreased uptake of MTX via the high-affinity carrier system for reduced folates

As this transport system has neither been isolated yet nor its gene(s) cloned, the underlying defect is not known. The decrease in transport can be due to a reduction in affinity for MTX and reduced folates (increased K_m), to a decrease in maximal transport rate (decreased V_{max}), or a combination of both. There are two additional routes for cells to meet their folate requirements: a low-affinity folate carrier (29) and a high-affinity folate binding protein that also mediates folate uptake (31). Both alternative routes have a low affinity for MTX, which is only effectively taken up by the high-affinity carrier system for reduced folates.

2. Decreased polyglutamylation of MTX

The polyglutamyl derivatives of MTX formed in the cell have a higher affinity for the target enzyme, DHFR, and are also retained longer in the cell than MTX itself. Hence, decreased polyglutamylation of MTX can lead to low level resistance.

3. Production of an altered DHFR with decreased affinity for MTX

Several different aminoacid subsitutions in DHFR are now known that decrease the enzyme's affinity for MTX

without seriously decreasing catalytic activity. Each of these altered DHFR forms is due to a single point mutation in the gene encoding DHFR.

4. Increased production of normal DHFR

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

5. Decreased level of thymidylate synthase

Thymidylate synthase catalyses the only reaction that converts tetrahydrofolate into dihydrofolates, the substrate for DHFR. In the absence of this enzyme, cells can maintain their tetrahydrofolate pools required for biosynthetic reactions, even if reduction of dihydrofolates to tetrahydrofolates by DHFR is blocked by MTX. In more general terms, the rate of thymidine-monophosphate (TMP) synthesis is an important determinant of MTX cytotoxicity and it has been demonstrated that lower rates of TMP synthesis make cell lines less sensitive to MTX (32, 33).

6. Increased nucleoside salvage

It has been claimed that MTX resistance can arise through increased uptake of thymidine and purine nucleosides. These can be converted into the corresponding nucleotides by salvage pathways circumventing the MTX block in nucleotide biosynthesis. Although this mechanism might contribute to primary MTX resistance, no mutants with increased salvage have been obtained by selection for MTX resistance in cultured cells. There is clinical interest in this potential mechanism for MTX resistance, because cellular nucleoside uptake can be inhibited by dipyridamole. The combination of MTX and dipyridamole is in clinical trial (34).

Table 1 summarizes the main features of the five forms of MTX resistance, studied in cell lines. In the spectrum of MTX resistant mutants one sees the three main types of alterations that can be associated with drug resistance: decrease in functional protein (transport defect, decreased polyglutamylation, decreased thymidylate synthase); synthesis of a protein with altered catalytic properties (DHFR); and increase in normal protein (DHFR). In the latter two cases, the mutations causing the alterations are known; the decrease of function mutations remain to be molecularly defined.

Table 1

Mechanisms of resistance to methotrexate in tumor cell lines

Resistance mechanism	Frequency	Enzyme alteration	Mutation defined?
Decrease in high affinity uptake of MTX	+ + +	Decreased	–
Decreased polyglutamylation of MTX	+	Decreased	–
Altered DHFR with decreased affinity for MTX	+	Altered sequence	+
Overproduction of DHFR	+ + +	Increased	+
Decreased thymidylate synthase	?	Decreased	–

In the cell types studied thus far, MTX resistance is usually due to an uptake defect or to overproduction of DHFR (Table 1). Amplification of the DHFR gene in clinical samples has been reported for chronic myeloid leukemia (35), acute myelocytic leukemia (36) and ovarian cancer (37). As DHFR enzyme or mRNA levels have not been determined in human tumor samples, increased DHFR not associated with DNA amplification may occur as well. Impaired uptake of MTX has not been reported as a cause of resistance in clinical samples.

The other three resistance mechanisms in Table 1 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 MTX doses. A more clinically relevant selection schedule, i.e. intermittent low-dose selection, may result more often in polyglutamylation defects. Indeed, primary MTX resistance in squamous carcinoma cell lines derived from head and neck tumors was found to be due to decreased polyglutamylation in 2 of 3 cells lines (38). In cell lines selected for very high resistance one may find more than one resistance mechanism operative, e.g. uptake defect and gene amplification, or an amplified gene for an altered DHFR (39).

Whereas overproduction of DHFR will result in cross-resistance to all MTX analogues this does not necessarily hold for the other resistance mechanisms. The more hydrophobic MTX analogue trimetrexate (TMQ), for instance, 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 TMQ (but not MTX) may be part of P-glycoprotein mediated MDR, but may also be caused by another mechanism only affecting hydrophobic antifolates, but not other drugs subject to MDR (40). This latter type of resistance was discovered in human lymphoblastoid cells and is associated with a decreased cellular drug concentration. As the uptake of TMQ 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.

Biochemical and genetic mechanism of P-glycoprotein-mediated multidrug resistance (24, 41–46)

Growth of human tumor 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 (Fig. 3). This lowers the intracellular drug concentration and hence results in drug resistance.

Fig. 4 shows that there are two genes for P-glycoproteins in man, called *mdr*1, and *mdr*3 (or 2). Only the *mdr*1-encoded P-glycoprotein has thus far been shown to contribute to drug resistance. Although the *mdr*3/2-encoded P-glycoprotein closely resembles its *mdr*1-encoded homologue in size, structure and aminoacid sequence (76% identity), it is not known to pump drugs and its physiological function remains unknown.

The specificity of the wild-type version of the human *mdr*1 P-glycoprotein has been studied by introducing and overexpressing the cloned *mdr*1 gene in human cells with low P-glycoprotein levels. P-glycoprotein overproduction in human melanoma cells results in a moderate resistance to anthracyclines, like doxorubicin, and to epipodophyllotoxins, like VP16; high resistance to vinca alkaloids and actinomycin D; and no resistance to a host of other clinically important drugs, like alkylating agents, cisplatin,

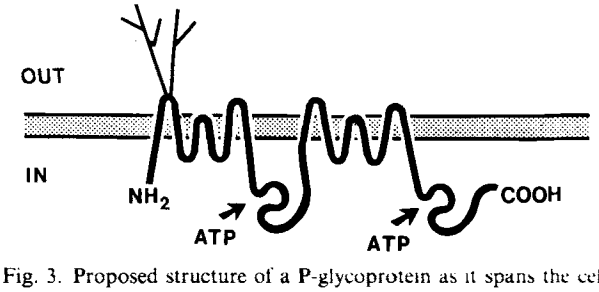

Fig. 3. Proposed structure of a P-glycoprotein as it spans the cell membrane. The nucleotide-binding folds are brought together in this scheme. The potential N-linked glycosylation sites are indicated by the branches sprouting from the polypeptide (141, 142).

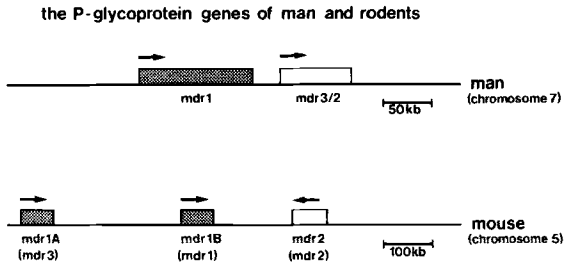

Fig. 4. Schematic maps of the P-glycoprotein genes of man and mouse. The human map is approximately to scale. The size of *mdr*3, the intergenic distance, and the direction of transcription are based on our unpublished results; the size of *mdr*1 is from Chen et al. (143) and its chromosomal location from Callen et al. (144). The schematic map of the mouse genes is adapted from Raymond et al. (145). Note the 2-fold difference in scale with the human map. The intergenic distances shown here are maximal distances based on a large-fragment restriction map. The actual distances could be much smaller. The arrows indicate the direction of transcription.

methotrexate, and purine and pyrimidine analogues (47). A largely similar multidrug resistance phenotype is induced by P-glycoprotein overproduction in other cell types. It has been observed, however, that during the stepwise selection of highly resistant cells, one may select for altered versions of the *mdr*1 gene, that encode a P-glycoprotein with altered drug-transport properties. A single aminoacid substitution was found to result in a 2- to 4-fold increase in the relative resistance to colchicine (48). Whether such mutant versions of P-glycoprotein ever arise in tumors in patients is not known.

How the P-glycoprotein pump works is not known in detail. From the aminoacid sequence of the protein it can be deduced that P-glycoproteins consist of two similar halves, each containing six trans-membrane segments and an ATP binding site (Fig. 3). The trans-membrane 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 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 (49). Substitution of critical aminoacids in one of the ATP-binding sites also abolishes pump function (50). Although the possibility has been raised that P-glycoprotein pumps out a carrier protein to which drugs attach, there is now considerable evidence for a direct binding of drug to P-glycoprotein (51).

Considerable interest was generated by the discovery that P-glycoprotein mediated multidrug resistance can be reversed by the Ca-channel blocker verapamil. Subsequent work has demonstrated that the reversal activity of verapamil is unrelated to its effect on Ca-channels and has led to a large (and still growing) list of compounds that share

the reversal property with verapamil. Although the mechanism of reversal is still controversial, there is considerable evidence that nearly all reversal compounds are in fact substrates of the P-glycoprotein pump and compete with drug for extrusion from the cell (52–54). Reversal compounds have been used with some success to overcome resistance in syngenic tumor models in mice (55) and they could provide the clinician with a tool to combat P-glycoprotein-mediated drug resistance in patients. We return to the clinical trials designed to test this tool below.

The function of P-glycoproteins in normal tissues is still a matter of speculation. The *mdr*1-encoded P-glycoprotein is mainly found in epithelia of excretory organs, like the biliary canalicular surface of hepatocytes, the apical surface of the intestinal epithelium and in the brush border of the proximal tubules of the kidney (56–61). It is also present in other natural barriers, i.e. the blood-brain barrier and in the testis. This would be compatible with a role in the excretion of large hydrophobic toxic compounds or waste products. However, this does not readily explain the high concentration of P-glycoprotein in adrenal cortex and medulla. Progesterone can inhibit drug transport by one of the mouse P-glycoproteins 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 (62). There is even substantial homology between this family and bacterial membrane transport proteins, involved in substrate uptake or toxin excretion. The ongoing analysis of the P-glycoproteins in these simple organisms may yield new ideas about possible natural substrates for the pump in man.

Genetic changes resulting in P-glycoprotein overproduction

Early experiments with rodent cell lines showed that MDR cells arise with a frequency of 10^{-5}–10^{-6} per cell (63). This is compatible with mutations at a single genetic locus. Hybrid cells made by fusing sensitive and resistant cells were resistant, showing that MDR is a dominant trait (64). MDR cells were found to have the karyotypic signs of gene amplification, DMs and/or HSRs (24). Taken together these results suggested that MDR is due to the overproduction of a protein or proteins, encoded by the amplified DNA segment. We now know that these proteins are the P-glycoproteins.

More detailed analysis of rodent cell lines has shown that in most cases the increased P-glycoprotein production is due to gene amplification without gene activation, i.e. the increase in P-glycoprotein is proportional to the increase in gene copy number (24). There is an exceptional rodent cell line, however, in which the increase in P-glycoprotein level far exceeds the increase in copy number. In this case the DNA rearrangements that accompany DNA amplification have probably also resulted in P-glycoprotein gene activation, but this remains to be verified. Conversely, one revertant cell line has been described in which the amplified DNA had not been lost, but the P-glycoprotein genes in the amplicons had been switched off (65). The molecular basis for this switch-off is unknown.

Whereas gene amplification is the main mechanism for P-glycoprotein overproduction in rodent cells, transcriptional activation is the main mechanism in human cells. In the human MDR cell lines analysed, the increase in P-glycoprotein mRNA levels always exceeds the increase in gene copy number (66–68). In clinical samples with raised P-glycoprotein (mRNA), gene amplification was never observed (69, 70). How this transcriptional activation occurs is not known. Notwithstanding considerable effort (F. Baas, pers. comm.) it has even been impossible to decide whether activation is in *cis* or in *trans*.

It is not known why selection for resistance leads to gene amplification in rodent cells and to transcriptional activation of the P-glycoprotein gene in human cells. It may be related to the normal level of P-glycoprotein in sensitive cells (67). This level is substantial in the hamster and rodent cells used for drug resistance studies and low or even undetectable in most human lines. If P-glycoprotein already contributes to the resistance level of the parental cell, a doubling of the P-glycoprotein gene copy number may have a significant effect on resistance. If gene activity is zero, a doubling of the copy number will not help; only activation of the gene will result in resistance.

Induction of P-glycoprotein synthesis; a form of adaptive resistance?

Although I have stressed in a previous section that resistance to cytotoxic drugs is rarely inducible, there are now a few reports indicating induction of P-glycoprotein synthesis. This was first observed as part of a more general defense response of liver to carcinogenic agents (71–73). In view of the range of inducible detoxification mechanisms that liver cells have at their disposal, it is not surprising that P-glycoprotein overproduction is included in this range of metabolic defenses. Up to 20-fold increases in *mdr*1 levels have also been observed in regenerating rat liver after partial hepatectomy. This is due to mRNA stabilization, rather than increased transcription of the *mdr*1 gene (74).

More recently induction of P-glycoprotein has been observed in human cancer cell lines derived from other tissues than liver. Fojo's group studied the effect of cellular differentiating agents on neuroblastoma and colon carcinoma cell lines. Retinoic acid induced an up to a 20-fold increase in *mrd*1 mRNA and P-glycoprotein in neuroblastoma cell lines, but without detectable decrease in drug uptake (75). Similar results were obtained with sodium

butyrate in one of the colon lines. This line also did not become detectably resistant even though the additional P-glycoprotein was at the surface and could be labeled with [^{3}H]-azidopine. In another cell line the additional P-glycoprotein did result in an MDR phenotype. The authors suggest that the activity of P-glycoprotein is not a simple function of its concentration, but that it may be present in an inactive (possibly phosphorylated) form. Such discrepancies between P-glycoprotein concentration and P-glycoprotein activity have not been noted in MDR mutants or transfectants and recent work by Fojo's group (T. Fojo, pers. comm.) which indicates that butyrate may inhibit P-glycoprotein transport activity, explaining the inactivity of the induced P-glycoprotein in butyrate-grown cells.

Chin et al. (76) noted the presence of heat shock consensus elements in the promoter area of the *mdr*1 gene and showed that heat shock or sodium arsenite treatment led to a 7-fold increase in the level of *mdr*1 mRNA and a corresponding increase in P-glycoprotein in a renal adenocarcinoma cell line. The increase correlated with a (transient) increase in drug resistance.

Although these results establish that the *mdr*1 gene is inducible, it remains to be shown whether this ever occurs under clinically relevant conditions, i.e. by moderate drug concentrations. The in vitro treatments used thusfar are rather exotic and only work with some cell lines and not with others. This holds even for the heat shock induction (76). We have never observed induction by cytotoxic drugs, even in cell lines, and to my knowledge this has not been observed in other laboratories either.

The possible contribution of elevated P-glycoprotein levels to clinical drug resistance (77, 78)

P-glycoprotein-mediated MDR can be considered a paradigm for the ongoing attempts to define mechanisms of clinical drug resistance. How P-glycoprotein renders cells resistant to drug is known in outline. 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 tumor samples and specific DNA probes to detect overexpression or amplification of the gene. Several sets of cell lines with increasing levels of resistance are available to relate roughly the P-glycoprotein (mRNA) level to the degree of resistance. Hence, it is possible to assess approximately 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 tumor marker and be correlated with clinical outcome. This will provide a reliable basis for rational treatment modification, e.g. avoidance of drugs affected by P-glycoprotein in the treatment of tumors with high levels of this protein, or attempts to modify resistance with reversal agents.

An extensive study of *mdr*1 mRNA in more than 400 human tumors was carried out by the NCI groups (56, 69). High levels in untreated tumors were generally found in tumors derived from tissues known to have a substantial *mdr*1 expression, such as colon, kidney, liver, adrenals, pancreas. Low amounts of *mdr*1 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 tumors analysed (69). This overall distribution of P-glycoprotein in human tumors inferred from mRNA levels, has been confirmed in a more limited series of tumors with P-glycoprotein-specific antibodies (79). Although the tumors that are high in P-glycoprotein are known to be intrinsically resistant to antineoplastic drugs, it should be noted that these tumors 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 tumors with high mRNA. It might be possible to test further the role of P-glycoprotein in these tumors by combining drugs with reversal agents (see below).

In general P-glycoprotein (mRNA) levels tend to be higher in tumors after treatment relapse, but the number of tumors analysed is small (69). Indications for a clear increase of P-glycoprotein levels during treatment have been obtained in ovarian cancer (80, 81), ANLL (82), CML in blast crisis (69, 83), ALL (70) and neuroblastoma (69, 84).

Recent work by Dalton and co-workers (pers. comm.) 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 VCR + DOX, 42% had elevated P-glycoprotein against 4% in the untreated controls. In the treated non-Hodgkin's patients 70% were positive, compared with 2% in controls. This strongly suggests that intensive treatment selects for cells with elevated P-glycoprotein levels.

Some correlation has also been seen between the level of *mdr*1 mRNA and resistance of renal carcinoma samples to vinblastine in a clonogenic assay (85). A clearer correlation was observed in studies with myeloma, non-Hodgkin lymphoma and breast cancer between P-glycoprotein expression and in vitro resistance to doxorubicin (86).

The most remarkable correlation between raised P-glycoprotein and poor response to chemotherapy has thus far been observed in childhood sarcoma patients (87, 88). Gerlach et al. (87) found 6 out of 25 sarcomas positive for P-glycoprotein with a relatively insensitive immuno-blotting assay that did not detect P-glycoprotein in other tumors, including 7 colorectal and 4 renal tumors. In a subsequent series of 30 children with rhabdomyosarcoma or undifferentiated sarcoma, a more sensitive immunocytochemical detection of P-glycoprotein was used (89). P-glycoprotein in these patients was an almost 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 5 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.

It should be stressed that in several major tumors, increased levels of P-glycoprotein do not seem to contribute to clinical resistance. These include tumors of breast (90) and lung (91). Hence, increased P-glycoprotein levels appear to make only a modest contribution to drug resistance in patients.

Multidrug resistance not involving P-glycoprotein

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

'Atypical' multidrug resistance

This type of resistance is not really a multidrug resistance, since it only involves drugs that hit a common cellular target, topoisomerase II (topo II) (92). This will be discussed in the next section.

Non-pgp mediated multidrug resistance

The latter type of resistance is negatively defined: it is not associated with raised P-glycoprotein and it includes drugs that do not act on topo II, such as vincristine, colchicine or the membrane-pore forming drug gramicidin D. It was first observed in a small cell lung cancer line by Mirski et al. (93), but it has subsequently been induced in leukemia (94, 95), fibrosarcoma (96), squamous cell lung (68, 97) and rat brain cell lines (98). The drug resistance profiles of these cell lines differ and for none of them the mechanisms of resistance is known. Only for the squamous cell lung line of Keizer et al. (97), several independent resistant lines were obtained with the same phenotype, suggesting that this phenotype is due to a single mutation (68). Table 2 contrasts the drug resistance profile of this mutant with that seen in P-glycoprotein-mediated MDR and topo II mutants. Potentially non-Pgp-mediated MDR could be clinically important and methods to study it in clinical samples are eagerly awaited.

Table 2

A comparison of three forms of resistance to doxorubicin/adriamycin in cultured cells

	P-glycoprotein-mediated MDR	Topo II mutants	Non-Pgp-mediated MDR*
Doxorubicin	+ +	+ +	+ +
Acridines (m-AMSA)	+ +	+ +	ND
Epipodophyllotoxins (VP16)	+ +	+ +	+ + +
Vinca alkaloids	+ + +	−	+
Actinomycin D	+ + +	+ +	+.
Gramicidin D	+ + +	−	+
Ratio Doxo/Vinca	Low		High
Ratio VP16/Gram D	Low		High

*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. (97) and Baas et al. (68).

Resistance due to altered topo II; biochemical background (99–101)

Selection of cells resistant to anthracyclines may not only lead to mutant cells overproducing P-glycoprotein or displaying a non-pgp-mediated form of multidrug resistance, but also to mutants cross-resistant to ellipticines, acridines, actinomycin D, mitoxantrone, and epipodophyllotoxins. These mutants differ from truly MDR cells in their lack of cross-resistance to vinca alkaloids, colchicine or gramicidin D. As epipodophyllotoxins were initially not known to (weakly) bind to DNA and as anthracyclines like doxorubicin were initially thought to kill cells by generating O_2 radicals, it took time before it was discovered that all drugs affected in these mutants have topo II as their common target and that the resistant mutants are in fact altered in topo II. At least two types of mutants exist:

1. Mutants with lowered topo II content.
   Topo II makes duplex breaks in DNA, catalyses strand passage through the break without letting go of the ends, and then reseals the duplex break. All the carcinostatic drugs acting on topo II block the enzyme before resealing, resulting in a duplex break in DNA when the enzyme is removed. A decrease in the topo II concentration therefore decreases the number of potential drug targets. As topo II is indispensable for cell division (102), there are limits to the decrease of topo II and therefore to the degree of resistance generated in this way.
2. Mutants with an altered topo II, that is less sensitive to inhibition by drugs. Presumably these mutant enzymes contain aminoacid substitutions, but this has not been verified.

It is possible that additional proteins interacting with topo II affect the fate of the drug-topo II complex and that alterations in other genes than that coding for topo II may also result in resistance to drugs affecting topo II (101). This remains to be confirmed.

For the drugs that interact with topo II, there is a good correlation between cytotoxicity and the number of duplex

breaks induced in DNA (after removal of topo II). This has recently been confirmed in a series of anthracycline analogues (103). Moreover, CHO cell mutants defective in the repair of duplex DNA breaks are hypersensitive to all classes of cytotoxic drugs that interact with topo II (104). There is little doubt therefore that the lethal event is the formation of the 'cleavable complex', the duplex break held together by drug-associated topo II. How cells containing this complex are eventually killed is still not completely clear. One would expect that the cleavable complex would lead to chromosome breaks and non-disjunction during mitosis and recent experiments bear this out.

The activity of topo II in normal cells is strongly growth phase dependent. 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 topo II level is less tightly controlled in tumors than in normal cells (101). Hence, the average topo II concentration in tumors may be higher than in normal tissues and this may explain the success of drugs inhibiting topo II as anti-tumor agents.

Whether alterations in topo II contribute to drug resistance in cancer patients is not yet known and not easily studied. The large changes in topo II level with changes in the growth phase of the cell make it difficult to decide whether the level found in a tumor is reduced or not. Point mutations in topo II are not simple to determine in clinical specimens.

The genetic basis of alterations in topo II

As explained in the preceding section, drug resistance may either be due to a decrease in (normal) topo II, or to the replacement of normal topo II by an altered enzyme, less sensitive to drug. The altered enzyme is presumably altered in aminoacid sequence, but this alteration remains to be determined. One would expect that resistance due to an altered enzyme should be genetically recessive. Normal cells are diploid for topo II (105) and synthesis of half the amount of normal enzyme should result in half the number of duplex breaks in DNA; this should still be sufficient for killing. Indeed, in the resistant mutants studied thusfar, there is less than half the normal enzyme complement present (106–110).

Thus, when resistance is due to a decrease in (normal) enzyme, both alleles should be affected. This could either be due to (recessive) alterations in both alleles, or to an alteration in a gene for a repressor. Mutations in such a 'trans-acting' gene could still be dominant. Dominance can be tested in cell-fusion experiments. The mutants for which this experiment was done were all recessive (111–113).

A more detailed analysis of the mutations in two mouse cell lines with altered topo II was recently published. Tan et al. (114) describe an amsacrine-resistant subline of the P388 leukemia with an approximately 2-fold decrease in topo II activity and mRNA. This decrease is smaller than in any of the other mutants studied, but as the resistant line was created in mice and only tested in mice, it is possible that resistance in these cell lines is also low. Tan et al. (114) report that one of the topo II alleles in this mutant is rearranged, as judged from alterations in restriction digests. The nature of this rearrangement remains undefined, but it has presumably led to a block in the expression of the affected allele. In addition, Tan et al. (114) present data that they interpret to mean that the topo II genes in the resistant mutant are hypermethylated. Whether this only affects the rearranged allele or both alleles has not been determined.

Two other P388-derived cell lines, made resistant to doxorubicin, were studied by Deffie et al. (115). These lines were 5- to 10-fold resistant and were found to contain a strongly decreased amount of immunoreactive topo II protein and 7- to 8-fold less of the normal 6.6-kb topo II mRNA than the sensitive parental cells. In addition, both mutant cells contained a novel 5.5-kb RNA hybridizing to the topo II cDNA probe. Also in these mutants, restriction digests show one altered allele of the topo II gene and this may be the one giving rise to the truncated mRNA, but this remains to be verified. Clearly the expression of the unrearranged allele in these mutants must also be partially suppressed to explain the more than 2-fold decrease in normal mRNA. Both alleles are therefore altered, as one would expect for a recessive mutation.

Although the characterization of the genetic alterations in the P388 mutants of Tan et al. (114) and Deffie et al. (115) is still rather preliminary, it is clear that the tools are now at hand to characterize such mutants in detail and to elucidate how recessive mutations arise at high frequency.

Although the results discussed thusfar paint a fairly plausible picture of drug resistance involving topo II alterations, a major complication has recently turned up. It had been known for some time, that some cell lines contain two topo II activities, the conventional 170 kDa topo IIα, and a 180 kDa topo IIβ (108). A recent paper by Chung et al. (116) demonstrates that topo II α and β are related enzymes specified by co-migrating 6.5-kb mRNAs, but that these are encoded by different genes. This has obvious consequences for resistance to topo II inhibitors, especially since the topo II α and β levels are differentially regulated (116). Alterations in the gene for one of the enzymes can only lead to resistance, if the other enzyme makes a small contribution to cleavable complex formation in the cells under study. More work is required to determine the consequences of this multiplicity of topo II genes for drug resistance.

The mechanism of resistance to alkylating agents and cisplatin (117, 118)

Whereas it is relatively easy to select mutant cells highly resistant to MTX or one of the drugs extruded by P-glyco-

Table 3

Documented mechanisms of resistance to alkylating agents and cisplatin in tumor cell lines (118, 132, 140)

Drug	Resistance mechanism					
	Reduced uptake	GST increase	GSH increase	MT increase	Increased DNA repair	Other
Nitrogen mustard	+	+?			+?	
Chlorambucil	+	+?		+	+?	
Melphalan	+		+?	+	+?	
Nitrosoureas		+?			+ (a)	
Cyclophosphamide						+ (b)
Cisplatin	+	+?		+		

(a) Increased methyl transferase removing O^6-methyl group from guanine.
(b) Increase in liver aldehyde dehydrogenase.

protein (even in excess of 1 000-fold resistant), resistance to alkylating agents and cisplatin is difficult to obtain and in vitro resistance does not exceed 10- to 20-fold. This has complicated the analysis of resistance mechanisms. A summary of the mechanisms documented is presented in Table 3. Only in the case of nitrogen mustard and melphalan are well-defined transport proteins known that may be less active in resistant cells. How tumor cells manage to reduce uptake of other drugs is not known. In fact, it is still not unambiguously proven that a drug like cisplatin enters cells with the help of a carrier protein, as the influx of labeled cisplatin is non-saturable and cannot be prevented by cold cisplatin.

The possible involvement of increased repair is also inferred from indirect evidence, such as increased DNA repair synthesis after short-term drug exposure (119). 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 (120). The only repair system in mammalian cells that is fully characterized, is the O^6 methyl transferase which can remove the methyl group from the O^6 position of guanine. O^6 methylation of guanine appears to be the major lesion induced in tumors by methylating cytotoxic drugs, like decarbazine (DTIC), streptozotocin, and the nitrosoureas. It has been shown that overproduction of the methyl transferase can lead to resistance to nitrosoureas in cultured cell lines (121) and that expression of a transfected bacterial alkyltransferase in mammalian cells induces resistance to bischloroethyl nitrosourea (BCNU) (122).

The role of glutathione and glutathione-S transferase (GST) in resistance is still not precisely defined. Although glutathione and GST are elevated in a variety of resistant cell lines (123), this elevation appears to be a rather general and non-specific response to any selection pressure, not necessarily implying an effect of GST on resistance. In fact, transfection experiments with GST-pi have shown only marginal effects of overproduction of this enzyme, both on resistance to alkylating agents and to doxorubicin (124). 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. (125, 126) and Lewis et al. (127). This mutant line was 20-fold resistant, cross-resistant to mechlorethamine and melphalan, but not to other agents like mitomycin C or cisplatin. Resistance was associated with a 4- to 8-fold amplification of a DNA segment containing a gene for a alpha (basic) type GST. The corresponding 50-fold elevation of a GST coupling melphalan (chlorambucil) to GSH, would seem to account for the resistance of this mutant cell line. This is supported by the recent finding that indomethacin, another substrate of alpha-type GST, potentiates chlorambucil toxicity 5-fold in the resistant line, presumably by competing for GST (128).

An argument for the potential importance of GST in resistance comes from the observation that the toxicity of many cytotoxic agents can be increased by lowering cellular GSH with BSO (buthionine sulfoximine). It should be realized, however, that the drastic decrease in GSH induced by BSO may make GSH 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 (124), possibly increased by a limited alteration in topo II, Kramer et al. (129) and Dusre et al. (130) have found that GSH depletion of resistant cells by pretreatment with BSO leads to a partial reversal of resistance (dose-modifying factor 4–7 at 75–90% GSH depletion). This clearly illustrates that modification of resistance by alterations of cellular GSH does

not necessarily mean that resistance involves GSH and cellular detoxification of oxygen radicals.

The risk of equating increased GST in resistant cells with a function of the enzyme in the resistance observed is further illustrated by recent work of Wang et al. (131). They selected cell lines resistant for one of 3 alkylating agents or cisplatin from a melanoma cell line. Most of these lines were only resistant to the drug used for selection, but nevertheless they all had elevated levels of GST-pi. Wang et al. (131) conclude that 'the lack of cross-resistance among cell lines selected for resistance to different alkylating agents, all of which have elevated GST-pi levels, indicates that increased levels of GST-pi cannot be the predominate mechanism for resistance to the tested drugs and these cell lines".

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 (132). Whether overproduction of this protein is ever responsible for resistance in cell lines selected for resistance, remains to be proven.

Several reports have appeared linking cisplatin resistance to increased levels of DHFR and/or thymidylate synthase (TS) (133). 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 DHFR or TS genes to prove that the association is causal. Nevertheless, the collateral MTX resistance, regularly seen in head and neck carcinoma cells selected for cisplatin resistance, may be of clinical relevance (134).

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 an uptake system for a structurally related aminoacid. Nitrogen mustard uses the transport carrier for choline, melphalan uses two transport systems for larger aminoacids. Other drugs, like nitrosoureas and chlorambucil appear to enter by passive diffusion. The lack of cross-resistance is not surprising if resistance is often due to decreased uptake.

Using drug resistance genes to increase the drug resistance of normal tissues

The cloning of drug resistance genes has opened the theoretical possibility to introduce these genes into host cells to render them more resistant to subsequent drug treatment. When the dose-limiting toxicity of a drug affects a tissue that is potentially accessible to gene therapy, it is conceivable that the normal tissues might be rescued in this way obviating the need to replace them after destruction by tissue transplantation. This concept has been tested recently in animals for two different genes:

— Activated DHFR has been introduced into the germ line of mice. This results in an increased expression of the enzyme in tissues and an increased resistance (at least 2-fold) to high-dose MTX (135). Activated DHFR has also been introduced in murine and human hematopoietic stem cells. As with all gene transfers into such cells, gene expression is initially high but tends to be shut off in the course of weeks. Nevertheless, it is conceivable that such a temporary expression would be sufficient to allow protection of the patients' transfected bone marrow cells during high-dose chemotherapy.

  It has been shown that tumors transplanted into mice transgenic for the DHFR gene are more easily destroyed by high-dose MTX, demonstrating that the concept of a widened therapeutic window actually works in model systems.

— Mice transgenic for the activated human *mdr*1 gene display an increased resistance of their bone marrow against anthracyclines (136). Retroviral constructs containing the *mdr*1 gene have been made, but these have not been systematically tested in murine stem cells for their ability to confer resistance on reconstituted bone marrow.

Pitfalls in the study of cultured cells selected for high levels of resistance in vitro

If 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. The selection may be for enzyme B and gene A may only be amplified, because it happens to reside next to gene B.
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 in trans, even if these genes are on different chromosomes. A simple scheme illustrating this principle is presented in Fig. 5. A practical example is the simultaneous induction of P-glycoprotein and GST-pi and the repression of P450 synthesis in liver by carcinogens (137, 138). 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 GST-pi 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% cell kill), often preceded by treatment with mutagens to increase the frequency of resistant mutants. Such cells

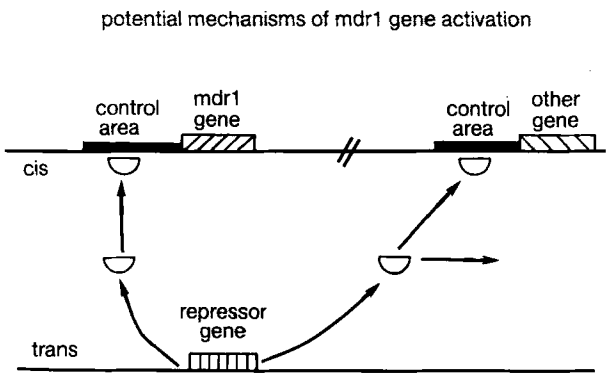

Fig. 5. Simplified (and theoretical) scheme showing how a single repressor might control the expression of several genes. Inactivation of this repressor gene leads to simultaneous *trans*-activation of all genes controlled by the repressor.

may require the simultaneous expression of more than one mechanism for resistance to cope with the high drug loads. Documented examples are MTX resistant cells with decreased MTX transport *and* increased DHFR levels; and MDR cells with raised levels of P-glycoprotein *and* an alteration in topo II. Obviously such mutagenized and 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.

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

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 narrow therapeutic window for most carcinostatic drugs predicts that a 2- to 5-fold increase in the IC_{50} of a tumor 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 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, I 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 tumor and with multiple drugs one can select for multiple resistance mechanisms in the same tumor cells. The elementary considerations presented here strongly predict, however, that the average tumor of the average patient treated with doxorubicin will only display a single acquired resistance mechanism.

Why are tumor cells (sometimes) sensitive to cytoxic drugs?

Oncologists think about resistant cells as abnormal cells, the odd-balls that spoil their wonderful treatment. This is also the picture painted here: resistant cells are mutant cells; sensitivity is the norm. How reasonable is that picture? It is not, as Harris (121) has persuasively argued. The mouse tumors that are so spectacularly cured by chemotherapy are usually immunogenic and only kill the host, because they outgrow the host's immunological defense. They row against the stream and as soon as we stop them (g) rowing, they are washed away by host defense. Likewise, some of the human tumors most sensitive to chemotherapy, like choriocarcinoma or Wilms' tumor, consist of highly abnormal cells that are either immunogenic or easily driven into terminal differentiation. Harris stresses that most normal tissues are quite resistant to cytotoxic agents. It is therefore not surprising that many tumors derived from these tissues are equally resistant. After all, it takes only the activation of 3–7 oncogenes to turn a normal cell into a cancer cell. As the human genome contains some 100 000 genes, the average cancer cell can be over 99.99% normal in genetic terms, at least initially.

The logical consequence of this line of reasoning is that tumor cells can be expected to be as sensitive as normal cells to chemotherapy (i.e. primary resistant) unless altered by mutation. Sensitive tumor cells are mutant cells, hypersensitive mutants, and acquired resistance may either arise by reversion to the normal state or by the secondary changes discussed in this review.

There are insufficient experimental results available yet to judge whether the provocative hypothesis of Harris is correct. There is no clear-cut example yet where acquired resistance is clearly attributable to a reversion to the normal state, rather than an abnormal state (like gene amplification or gene deletion). As we do not know the molecular basis for most forms of acquired resistance yet, the Harris hypothesis remains an interesting frame of reference for thinking about drug resistance.

Outlook

Drug resistance is the central problem in cancer therapy today. Whereas surgery and/or radiotherapy can usually

remove or destroy the initial tumor and its outgrowth in surrounding tissues, it is the lack of adequate drug treatment for disseminated forms of cancer and failing loco-regional control that is responsible for the death of cancer patients. Nevertheless, we still know little of the causes of drug resistance in patients. Although this is unfortunate, it is neither unexpected nor embarrassing. It is becoming clear that many resistance mechanisms affect drug transport through membranes or DNA metabolism, two areas of biochemistry that developed rather late. P-glycoproteins and topoisomerases were discovered only recently, and without that knowledge it is difficult to even start understanding anthracycline resistance. Even now, large areas of cellular biochemistry remain to be charted. The first human genes required for excision repair of DNA were only recently cloned and there are still many at large. Without a complete inventory of cellular biochemistry, it will be difficult to get a full description of the cellular pharmacodynamics of carcinostatic drugs and its alteration in resistance. Ideally one would like to know of every drug how it is handled in every specialized tissue of the body, what the molecular basis is for the dose-limiting toxicity and why it kills some tumor 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 tumor cell lines, made resistant in vitro, it may still be very difficult to extrapolate the results to tumors in patients. Cells in tissue culture lack stromal interaction, they usually are much less heterogeneous than in a real tumor, they cannot be rescued by metabolic co-operation, and one cannot expect to get a homogeneous drug exposure of cells in a real tumor.

Nevertheless, the examples presented in this review show that progress is being made. We understand in principle how genetic change alters proteins and how alteration of proteins can lead to resistance. The power of modern biochemistry and genetics makes it possible to define resistance mechanisms in exquisite detail, once the mechanism is stably in operation in a cell line that can be grown and analysed. The human genome is large, but finite and in 10–15 years we shall know all human genes and hopefully also how they could affect drug handling. As this knowledge accrues, it will be applied at several levels:

1. A rapid increase in the use of markers that predict drug response allowing the clinician to avoid non-effective 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 being unravelled, more tests based on 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 tumor samples or xenografts and moreover, they do not suffer from the risk that the tumor sample cultured is not representative of the tumor.
2. An understanding of resistance mechanisms may help to direct the development of analogues circumventing resistance. An example is provided by the hydrophobic anti-folates that can kill cells that do not take up MTX. The development of topo II inhibitors that are not extruded from the cell by P-glycoprotein would be an obvious challenge for the future.
3. Once the biological basis for resistance is known and recognizable in clinical samples, it may become possible to devise drug regimens that overcome resistance. An example are the reversal agents able to reverse P-glycoprotein-mediated MDR in tissue culture and now being tested in clinical trials.
4. Resistance mechanisms may result in changes in metabolism that could be exploited for therapy. An example is the overproduction of P-glycoprotein that might be exploited with antibodies against the extracellular part of P-glycoprotein. Fitzgerald et al. (139) have shown for instance that such a monoclonal antibody coupled to *Pseudomonas* toxin will specifically kill highly multidrug resistant cells in culture.
5. At a more sophisticated level one may hope that a full understanding of the biochemical differences between cancer cells and their normal counterparts may lead to the development of rationally designed drugs that exploit these differences more effectively than the empirical drug therapy now available.

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Corresponding author: Dr P. Borst, The Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands.

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