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REVIEW ARTICLE

Molecular mechanisms of drug resistance

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INTRODUCTION

Man increasingly employs chemicals as insecticides, herbicides and chemotherapeutic agents to treat not only cancer, but also bacterial, viral and parasitic infections. The repeated use of these chemicals often leads ultimately to their becoming ineffective due to the onset of resistance or tolerance by the target cells or organism. This phenomenon is of considerable economic importance and often has grave consequences to health. It also serves as a major challenge to the pharmaceutical industry because the development of resistance ensures that effective drugs become limited in their usefulness. For the purpose of this review, the term drug is used in a general sense to describe all foreign chemicals that are used by man either as chemotherapeutic agents (e.g. antibiotics, antiviral agents, antiparasitic agents and anticancer agents), as herbicides and insecticides or as by-products of the chemical industry. It is the aim of the review to highlight the biochemical events that are responsible for the major resistance mechanisms encountered in Nature, and to discuss how they may have evolved.

Despite the infinite variability in our environment it is remarkable that drug resistance is often achieved by a relatively small number of mechanisms. In the case of a drug which has a unique target site it is perhaps not surprising that resistance to the particular compound occurs through any one of a small number of mechanisms. However, in the case of a drug with a more general mode of action a larger number of resistance mechanisms may operate. Comparisons of resistance mechanisms in bacteria, insects, plants and man reveal several common features, and that certain classes of protein are responsible for conferring resistance to various drugs in a diverse number of organisms.

Over the past two decades understanding the mechanisms of drug resistance has become a central issue as its importance in medicine has assumed ever-increasing significance. During this period there has been an enormous increase in our understanding of the wide variety of mechanisms involved. Whilst the studies of drug resistance in bacteria and the use of bacterial genetics have played a central role in identifying resistance mechanisms, the application of recombinant DNA techniques developed over the past 15 years has made a substantial impact on our understanding of the molecular mechanisms of drug resistance in eukaryotic cells. Without these tools, events that are of fundamental importance, such as gene amplification, may have remained undiscovered. The ability to manipulate genes and to express them in foreign cells has also added a new dimension to these studies. The involvement of a specific protein can only be shown unequivocally if the resistant phenotype can be conferred through the transfection of DNA encoding the protein of interest into a suitable recipient cell. The same technology also allows the consequences of mutations in the gene encoding a target protein to be established. The techniques of molecular biology enable the deletion of specific DNA sequences from the genome and this possibility provides the researcher with an alternative strategy to establish which genes are involved.

Drug resistance can be classified as either intrinsic or acquired. This division may seem somewhat arbitrary. However, from a practical point of view a simple operational distinction can be made by determining whether the organism, or cell, in question was resistant at the time treatment began or whether it was initially drug sensitive and became resistant only after treatment was commenced. The former represents intrinsic resistance (also sometimes called natural or *de novo* resistance), whereas the latter represents acquired resistance. As Table 1 demonstrates, both intrinsic and acquired resistance may exist for a variety of reasons. It is, however, simplest to discuss these two classes of resistance separately.

Table 1. Origins of intrinsic and acquired resistance

For further details see the text.

Type	Duration of resistance	Frequency of resistance within the population
Intrinsic resistance		
1. Absence of target site	Permanent	All cells
2. Species-specific structure of target site	Permanent	All cells
3. High detoxication capacity, arising from:		
(*a*) tissue-specific function	Permanent	All cells
(*b*) ontogenic variations	Variable	All cells
(*c*) sex-specific differences	Permanent	All cells
(*d*) population polymorphisms	Permanent	Variable
(*e*) self defence	Permanent	All cells
(*f*) high repair capacity	Permanent	All cells
4. Low drug delivery	Variable	Variable
5. Cell cycle effects	Variable	Variable
6. Adaptive change	Temporary	All cells
7. Stress response	Temporary	All cells
Acquired resistance		
1. Natural selection	Permanent	Rare
2. Constitutive adaptive change	Permanent	Rare
3. Constitutive stress response	Permanent	Rare
4. Gene transfer	Requires continued selection	Rare
5. Gene amplification	Requires continued selection	Rare

Abbreviations used; MDR, multidrug resistance; GST, glutathione *S*-transferase.

INTRINSIC DRUG RESISTANCE

Intrinsic resistance describes the situation where an organism, or cell, possesses a characteristic 'feature' which allows all normal members of the species to tolerate a particular drug or chemical environment. In this case, the 'feature' responsible for resistance is an inherent, or integral, property of the species that has arisen through the processes of evolution. Life has evolved in a hostile environment in which all organisms have had to protect themselves against numerous forms of insult to ensure their continued existence, and a fundamental characteristic of all cells is their ability to withstand this environmental challenge. All cells will therefore exhibit a degree of intrinsic resistance to the compounds which they normally encounter. The level of exposure and the nature of the chemicals involved will determine the resistance mechanisms which have evolved. It is perhaps not surprising that there is an enormous variation in the sensitivity of cells to chemicals and their level of intrinsic drug resistance presumably reflects the selection pressures endured in the course of evolution.

As many drugs have only been developed relatively recently it is likely that the characteristic which bestows intrinsic drug resistance will have evolved through selection pressures that are entirely independent of the chemical agent against which resistance is observed. On this basis, it is reasonable to assume that the distribution of the trait responsible for resistance within the population will reflect both the length of time since its emergence and the selective advantage it conferred before exposure to the drug. Although the term intrinsic resistance implies that the trait which allows cells, or an organism, to resist a drug is possessed by all members of the species, this is not necessarily true. For example, the polymorphisms associated with certain drug-metabolizing enzymes demonstrate the common occurrence of population variations in proteins that are directly involved with resistance or sensitivity to drugs. Nonetheless, before exposure to the drug, the distribution of the protective 'feature' in most cases of intrinsic resistance will be widespread within the 'wild type' population (i.e. 10–100%) and would be encountered at a much greater frequency than occurs in cases of acquired resistance. In the latter case the protective 'feature', which would arise from a spontaneous mutation within the population, would be a very rare event (10^{-6}) and would not be widely distributed before drug selection.

Mechanisms of intrinsic resistance

This phenomenon can be due to either the presence or the absence of a biochemical 'feature' (Table 2). This may, for example, be the structure of the cell envelope or membrane, the existence of a drug transport protein, the absence of a metabolic pathway, the presence of a drug-metabolizing enzyme, the structure of the drug target site (see below for further details), the expression of specific stress response proteins or high repair capacity. Various experimental approaches have been employed in order to understand these processes. Gene transfer from resistant cells to sensitive cells or gene deletion in the resistant cells can enable the identification of specific proteins involved. The study of cells that have lost the drug-resistant phenotype, or the selection of drug-sensitive mutants (i.e. previously normal cells that have become hypersensitive to a particular chemical), will also facilitate the identification of mechanisms of intrinsic resistance.

Analysis of drug sensitivity in naturally arising mutants or in chemically-induced mutants is valuable. For example, the null mutation in the *Drosophila* Cu/Zn superoxide dismutase gene, which was recovered as an ethylmethanesulphonate-induced recessive lethal mutation (Campbell *et al.*, 1986), provides an illustration of the requirement to produce genetic lesions experimentally if the functions of indispensable proteins in drug resistance are to be assessed. Phillips *et al.* (1989) examined the effects of this null mutation in the superoxide dismutase gene and found that the absence of the enzyme resulted in sensitivity to paraquat as well as infertility and reduced longevity. The classical approach of studying potentially lethal mutations by the selection of temperature-sensitive mutants can also be applied to the investigations into the role of housekeeping genes in drug resistance. However, this approach is only applicable in certain organisms. Studies to determine the cause of intrinsic resistance are unfortunately rare, and the term is often used in an empirical sense to describe a cell that simply does not respond to therapy on initial exposure to a chemotherapeutic agent.

Table 2. Some examples of intrinsic resistance

Example	Organism	Protein or other factor	Type	Drug
Antibiotic producers	*Streptomyces griseus*	Aminoglycoside phosphotransferase	Novel metabolic inactivation	Streptomycin
Antibiotic producers	*Streptomyces erythraeus*	RNA methylation	Modification of target site	Erythromycin
Poor debrisoquine metabolizers	Man	Cytochrome *P*-450	Population polymorphism	Reduced sensitivity to carcinogens
Fast acetylators	Man	*N*-Acetyltransferase	Population polymorphism	Isoniazid, sulphanilamide
Brain tumours	Man	Sanctuary site	Drug delivery	Anticancer drugs
Dihydropteroate synthetase inhibitors	Mammals	Lack of dihydropteroate synthetase	Absence of target site	Sulphonamide drugs
Cell wall inhibitors	Mammals	No cell wall	Absence of target site	β-Lactam antibiotics
DNA synthesis inhibitors	Mammals	Lack of DNA replication	Cell cycle effects	Antipurines, antipyrimidines
Drug priming	Mammals	Exposure to subtherapeutic doses of drug	Adaptive change	Cyclophosphamide, busulphan, X-irradiation, 1-arabinofuranosylcytosine
Chemoprevention	Mammals	Dietary manipulation	Adaptive change	Carcinogen (aflatoxin B_1, benzo[*a*]pyrene)
Heat shock	Chinese hamster fibroblasts	Transient hyperthermia	Stress response	Adriamycin, ethanol

In higher organisms the expression of many proteins involved in protection against chemicals is tissue-specific and may relate to their function. For example, the mammalian lung has to withstand the damage produced by oxygen-induced free radicals. Consequently, this tissue has evolved a variety of antioxidant defence mechanisms including glucose-6-phosphate dehydrogenase, α-tocopherol, glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase (Halliwell & Gutteridge, 1985). Moreover, because the lung is directly exposed to the environment, the bronchiolar epithelium contains moderately high levels of many detoxication enzymes. These characteristics ensure that it has a natural resistance to many drugs that act through the generation of free radicals or act as alkylating agents.

In mammalian cells the rate of cell division is also an important determinant in intrinsic resistance to particular drugs. This is illustrated by the fact that the major dose-limiting determinant in cancer chemotherapy is toxicity to the normal rapidly dividing cells such as bone marrow and cells of the gastrointestinal tract. Many of the drugs used in cancer chemotherapy are most effective against rapidly proliferating malignancies (Tidd, 1984). Solid tumours which exhibit slow growth are frequently drug-resistant, which could be attributed to the finding that most of the cells are in the G_0 resting state (Tidd, 1984).

Self-protection mechanisms associated with intrinsic drug resistance

Many organisms survive in the environment through their ability to produce chemicals which are toxic or distasteful to their predators or their competitors. As a consequence, they require their own defence against the noxious chemicals they produce. Studies on the antibiotic-producing micro-organisms such as the various species of *Streptomyces* provide good examples of this form of intrinsic drug resistance. In a review by Cundliffe (1984), the mechanisms used by organisms to protect themselves against their own antibiotic products were divided into two types, firstly, resistance involving inactivation of antibiotics such as streptomycin and neomycin by the phosphotransferases and acetyltransferases and secondly, resistance resulting from modification of potential target sites within the organism. For example, the ribosomal RNA is protected by methylation in the erythromycin producer *Streptomyces erythraeus*.

Chemically-induced adaptive change and intrinsic resistance

Drugs and a wide variety of toxic agents (e.g. radiation, osmotic shock and heat shock) provoke many biochemical changes in cells that allow them to overcome the toxic effects of either the same or other compounds. In some circumstances this ability to resist chemical insult arises immediately following administration of the drug or, alternatively, there may be a significant time lag following exposure to the drug before the adaptive process is manifest. The potential number of phenotypic changes that may be produced includes alterations in membrane structure, in enzyme and DNA structure, in cofactor availability or in metabolic capacity. The interest in adaptive changes, particularly as it relates to chemical-induced damage, has focused largely on enzyme induction, DNA repair and detoxication capacity. The phenomenon of enzyme induction is exemplified by the increase in chromosomally encoded penicillinases (type C β-lactamases) in certain bacteria exposed to β-lactam antibiotics. Similarly, in the case of bacterial resistance to trimethoprim, production of the plasmid-encoded type-IV dihydrofolate reductase is induced in response to challenge by the drug (Young & Amyes, 1986); other prokaryotic dihydrofolate reductases described to date are not inducible (Amyes, 1989). Only in very few instances are the molecular details of the events responsible for enzyme induction understood.

An important difference exists between intrinsic resistance produced by adaptive change and other examples of intrinsic resistance. Adaptive change is relatively short-lived and is normally reversed when the toxic agent is removed (Table 1). The 'priming' effects of certain cytotoxic drugs serve to demonstrate the temporal nature of resistance produced by adaptive change in mammals. The term 'priming' has been used to describe the phenomenon where exposure to a low dose of cytotoxic compound provides protection, for only a limited period of time, against the subsequent administration of a normally lethal dose of the same compound (Miller *et al.*, 1975, Kimball *et al.*, 1976). This phenomenon is exploited clinically, during cancer chemotherapy, as it allows protection of the normal host cells, but hopefully not tumour cells, from the chemotherapeutic agent. Many toxins have been shown to initiate 'priming' effects, and often one cytoxic agent will confer resistance against other structurally unrelated toxic compounds (Adams *et al.*, 1985; Wolf *et al.*, 1987*b*). Administration of the 'priming' dose does not confer resistance immediately, but rather resistance develops several days later.

Physiological stress response and intrinsic resistance

Environmental factors, other than drugs, can, through the ability to stress cells, elicit an adaptive response that confers resistance against chemicals. Phenomena such as heat, anoxia, viral infection, trauma, u.v. irradiation, pH, osmotic shock and oxidative stress stimulate a genetic reflex in all cells that is 'designed' to confer tolerance against subsequent exposure to the same physiological insult. Intriguingly, not only does a response provoked by one of these types of insult often provide protection against a different physiological stress, but it may also protect against drugs. Prokaryotes have at least four major regulons which are induced by stress, namely, the SOS response (Walker, 1985), the adaptive response to alkylating agents (Samson & Cairns, 1977; Demple *et al.*, 1985), the oxy R network (Christman *et al.*, 1985; Storz *et al.*, 1990) and the heat-shock response (Lindquist, 1986; Carper *et al.*, 1987).

The study of cellular adaption has shown that possibly 30–40 proteins can be implicated in the stress response. Each insult may induce a specific sub-set of stress response proteins, but many of the adaptive responses appear to be inter-related. For example, in *Escherichia coli* the *gro*EL and *dna*K heat-shock proteins are not only induced by hyperthermia but are also induced by u.v. irradiation or nalidixic acid, both of which effect the SOS response (Krueger & Walker, 1984). Similarly, in *Salmonella typhimurium* the ability of a cell to adapt to H_2O_2 induced oxidative stress also confers resistance to heat killing (Christman *et al.*, 1985).

The most rigorously studied physiological insult is transient hyperthermia, otherwise known as heat shock. Therefore, for historical reasons, the proteins involved in adaption to physiological stress have been called 'heat-shock' proteins (hsps). During heat shock there is a transient arrest of cell growth and a block in the synthesis of DNA, RNA and protein is observed. Although there is a marked cessation in the general biosynthesis of macromolecules, the heat-shock response is characterized by the marked increase in transcription of a small number of genes (HSPs). Three families of heat-shock genes, HSP90, HSP70 and HSP20, have been identified and these encode proteins of about 110–80 kDa, 60–78 kDa and 20–30 kDa, respectively. The proteins are defined by their molecular mass and, for example, the major protein in most eukaryotic cells is hsp70, a protein with an M_r of 70000. The heat-shock genes in eukaryotes are activatable because they contain the sequence C--GAA--TTC--G in the gene

promoter region (Pelham, 1982, 1984). The change in the genetic programme produced by heat-shock confers a transient resistance upon cells against a subsequent potentially lethal heat challenge. The basis for the protection is unclear, as the functions of the majority of the hsps are unknown. However, it has been reported that the expression of hsp70 will confer heat resistance. Amongst the HSP90 family, hsp89 binds to the glucocorticoid receptor and other hormone receptors. It also interacts with the glucocorticoid-like 'dioxin' (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) receptor which mediates the induction of several drug-metabolizing enzymes (Denis *et al.*, 1988). This involvement of hsps with xenobiotics is not restricted to the HSP90 family. Certain members of the HSP70 family, hsp70 and hsp72, bind peroxisome proliferators, such as clofibrate, and, although the physiological significance of this interaction is, as yet, unclear. Alvares *et al.* (1990) have suggested that the binding of clofibrate may inhibit the activity of hsp70 and hsp72. The HSP70 family is involved in protein–protein interactions, and not only interacts with the tumour suppressor protein p53 but may be involved in actin microfilament reassembly as well as the recovery of nucleolar morphology and the association of proteins with the endoplasmic reticulum or the mitochondria (Pelham, 1984; Burdon, 1986; Chirico *et al.*, 1988; Deshaies *et al.*, 1988). Little is also known of the function of the small heat-shock proteins, but in *Drosophila* hsp27, 26, 23 and 22 share substantial homology with mammalian α-crystallin (Ingolia & Craig, 1982; Southgate *et al.*, 1983).

There appears to be a fundamental association between adaption to physiological stress and resistance to drugs. Though the basis for this association is not known, the implication is that heat-shock proteins protect against both physiological and chemical stress. The observation by Li & Hahn (1978) that ethanol can induce resistance to both heat and adriamycin was of great importance in establishing the link between thermotolerance and drug tolerance. Subsequently, Li *et al.* (1982) reported that a variety of heat-shock protein inducers, such as ethanol, hypoxia, arsenite and cadmium, all confer resistance to adriamycin. These workers have extended this study and have found that thermotolerant cells also display resistance to the anticancer drugs actinomycin D, bleomycin and the epipodophyllotoxin, VM-26 (Li, 1987). Another interesting example of the association between the response to heat-shock and drug resistance is provided by a permanently heat-resistant CHO cell line that overexpresses hsp70 at normal growth temperature, and which shows appreciable resistance to both adriamycin and VM-26 (Wallner & Li, 1986).

Despite the fact that the function of the heat-shock proteins is the subject of conjecture, it is interesting that chemicals themselves can induce hsp synthesis. The ability of ethanol and amino acid analogues to elicit the heat-shock response is well known (Burdon, 1986). However, adriamycin and VM-26, against which thermotolerant cells show cross-resistance, both induce hsp70 transcription in Chinese hamster fibroblasts and *Drosophila* cells (Rowe *et al.*, 1986). Carcinogens can also induce heat-shock proteins (Carr *et al.*, 1986).

Intrinsic drug resistance and selective toxicity

Evidence for the widespread existence of intrinsic resistance is provided by the study of species-specific selective toxicity exhibited by agents such as insecticides, herbicides and antibiotics. Many agents have been developed that are toxic towards weeds, insects, fungi and bacteria but are not harmful to mammals. Just as the analysis of drug-sensitive mutants provides an insight into the mechanisms of intrinsic resistance, so the study of the mode of action of selectively toxic compounds can shed light on resistance mechanisms. Three categories of selectivity can be defined which exploit differences in drug accumulation, intermediary metabolism or structure of drug target sites (Albert, 1985).

Differences in drug accumulation as the basis for selective toxicity are typified by certain parasites which, in comparison with their mammalian hosts, preferentially accumulate drugs. For example, trypanosomes readily concentrate organic arsenicals and helminths rapidly concentrate phenothiazine. In the latter case, if phenothiazine is administered intravenously to an infected sheep, it is equally toxic to host and worm. However, when given orally, only the helminth is affected as phenothiazine is poorly absorbed by the epithelial cells of the gut.

Species differences in the pathways of intermediary metabolism are exemplified by the sulphonamide drugs. These antibacterial and antimalarial agents, which include sulphanilamide and sulphadiazine, are relatively harmless to mammals; they inhibit the enzyme dihydropteroate synthetase and prevent the formation of dihydropteroic acid, which is later converted to the nucleotide precursor dihydrofolic acid. Most pathogenic bacteria, with the exception of *Streptococcus faecalis*, are obliged to synthesize their own dihydrofolic acid as they lack the folate permease. As mammalian cells do not possess the target enzyme dihydropteroate synthetase, but rather use preformed folates in the diet to synthesize dihydrofolic acid, they are resistant to the toxic effects of sulphonamides.

The lack of toxicity of the β-lactam antibiotics to mammalian cells represent an example of selective toxicity that is due to differences in cell structure (i.e. bacteria possess a cell wall whereas mammalian cells lack this structure). Another example of cytological differences resulting in drug selectivity is the differential inhibition by various drugs of protein synthesis in prokaryotes and eukaryotes. The bacterial 70 S ribosome comprises a 30 S and 50 S subunit. Aminoglycoside antibiotics, such as streptomycin and gentamicin, inhibit protein synthesis by binding to the 30 S subunit whereas chloramphenicol and erythromycin bind to the 50 S subunit. None of these antibiotics binds to the eukaryotic ribosome. Conversely, cycloheximide and emetine, which have a high affinity for eukaryotic ribosomes and selectively inhibit protein synthesis in mammalian cells, do not prevent protein synthesis in prokaryotes.

ACQUIRED DRUG RESISTANCE

The term acquired resistance is used to describe the case where a resistant strain, or cell line, emerges from a population that was previously drug-sensitive. In addition to resistance towards the selective agent, drug resistance may also be observed towards other chemicals. The biological 'feature' responsible for resistance is either absent from the population or is not expressed in the major portion of the population before drug exposure. This form of resistance can arise by several different mechanisms (Table 1). However, mutation and selection for protective genes are central to this process. Three major types of genetic change can be envisaged: (1) mutations and amplifications of specific genes directly involved in a protective pathway; (2) mutations in genes which regulate stress-response processes and lead to the altered expression of large numbers of proteins; (3) gene transfer. These types of change are of course not mutually exclusive, and examination of the multiple changes that are frequently seen in resistant tumour cell lines suggests that several mechanisms can operate simultaneously.

Natural selection and acquired resistance

The distinction between acquired resistance through natural selection and intrinsic drug resistance lies in the frequency with which the mutated gene is observed in the 'wild type' population.

Table 3. Examples of acquired drug resistance

Example	Organism	Resistance to:	Procedure	Type of resistance
Bacterial drug resistance	*Escherichia coli*	Chloramphenicol, ampicillin	Exposure to drug	Gene transfer (+natural selection)
Bacterial drug resistance	*Serratia marcescens*	Fosfomycin	Exposure to drug	Gene transfer (+natural selection)
Preneoplastic hepatocyte nodules	Rat	Toxins, carcinogens	Carcinogen exposure	Carcinogen-induced stress response
Persistant hepatocyte nodules	Rat	Toxins, carcinogens	Carcinogen exposure	Natural selection: altered expression of drug-metabolizing enzymes
Oxy R1 network (adaptive response to oxidative stress)	*Salmonella typhimurium*	Peroxides, ethanol	*In vitro* selection of cell line	Constitutive overexpression of a stress response
*amp*C, R and D genes (adaptive response to cephalosporins)	*Citrobacter freundii*	Cefuroxime, cefotaxime, cetazidime	*In vitro* selection of cell line	Constitutive overexpression of an adaptive response
Ada gene (adaptive response to alkylating agents)	*Escherichia coli*	*N*-Methyl-*N*-nitrosourea, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine	*In vitro* selection of cell line	Constitutive overexpression of an adaptive response
Multidrug resistance	Tumour cell lines	Adriamycin, vincristine, actinomycin D	Stepwise exposure to increasing concentrations of cytotoxic drug	Amplification of *P*-glycoprotein genes
Alkylating agent resistance	Tumour cell lines	Alkylating agents	Stepwise exposure to increasing concentrations of cytotoxic drug	Overexpression of drug-metabolizing enzymes
DNA gyrase mutants	*Escherichia coli*	Nalidixic acid	*In vitro* exposure to drug	Natural selection
Penicillin binding protein mutants	*Escherichia coli*	Penicillin	Exposure to drug	Natural selection
Acetylcholinesterase mutants	Houseflies	Organophosphorus	Exposure to drug	Natural selection

These changes arise independently of exposure to the drug and are part of biological variation. The presence of resistant cells within the population long before the presence of any selection pressure has been shown convincingly by the demonstration that antibiotic resistance existed in bacterial strains which were freeze-dried and stored at a time before antibiotic drugs were developed commercially. In many instances of acquired drug resistance natural selection, with its requirement for mutation and biological variation, is the most likely process to explain the resistant phenotype (Table 3). The essence of this mechanism is the selection of individuals that can withstand the chemical insult and hence outgrow their susceptible counterparts. This scheme, which is a restatement of the principles of natural selection outlined by Darwin, predicts that if, for example, the site of action of a drug is a particular receptor then, as a consequence of random mutation, a few individuals within a large population will produce a structurally abnormal target site that will fail to interact with the drug and prevent it from producing its deleterious effects. This hypothesis is supported by the substantial increase in the frequency of generating resistant cells observed when the cells are pre-treated before selection with mutagens such as ethylmethanesulphonate.

There are many examples of acquired drug resistance through natural selection. In *E. coli*, structural changes in penicillin-binding proteins can result in resistance to the antibiotics mecillinam or cepholosporin (Spratt, 1978, 1983), changes in the structure of the β-subunit of RNA polymerase can confer resistance to rifampicin (Rabussay & Zillig, 1969), and changes in the structure of DNA gyrase can confer resistance to nalidixic acid or novobiocin (Reynolds, 1984). In houseflies, changes in the structure of acetylcholinesterase can result in resistance to Rabon (Tripathi & O'Brien, 1973).

Drug-mediated genetic changes and acquired resistance

Herbicides, insecticides or antimicrobials are not mutagenic. However, many drugs used in cancer chemotherapy are mutagens and therefore treatment with anticancer drugs, in addition to providing the selection pressure for resistance, can significantly increase the frequency of mutations that will produce resistant cells. This is probably greatly potentiated by the inherent genetic instability of cancer cells. Such effects are exemplified by the significant increase in the frequency of DNA amplification following the exposure of tumour cells to mutagens such as monofunctional and bifunctional alkylating agents and u.v. irradiation (Connors, 1984; Stark, 1986).

It is technically difficult to demonstrate whether resistant cells in tumours arise from drug-mediated mutations or were present before chemotherapy was initiated. Indeed, in tumour biology the relative importance of natural selection and adaptive change is still hotly debated. In any event, acquired resistance is a common phenomenon and a major limiting factor in cancer patients receiving chemotherapy and, in hindsight, is a predictable consequence of chronic treatments with mutagenic agents.

Inter-relationship between acquired resistance through the mechanisms of natural selection and adaptive change

Whilst subdividing acquired resistance into different classes according to the type of cellular change that is responsible for resistance is helpful, the distinction should not be regarded as rigid. In particular, the acquired resistance which arises from natural selection and the intrinsic resistance that is the product of adaptive changes to chemical or physiological stress are not mutually exclusive. Clearly, mutations in the DNA sequences that are involved in producing an adaptive response (e.g. in genes encoding transcription factors or in *cis*-acting elements) may result in the constitutive over-expression of stress-response proteins. Phenotypically, such mutants would give constitutive expression of at least some of the genes involved in the adaptive response.

In *E. coli*, mutations have been described in the Lex A repressor protein which prevent it from binding to and regulating genes involved in the SOS response (Little & Mount, 1982;

Walker, 1984). Such mutants express SOS response proteins constitutively and are resistant to DNA damage by u.v. and mitomycin C.

In the H_2O_2 resistant *Salmonella typhimurium* Oxy R1 mutants isolated by Christman *et al.* (1985), nine stress-responsive proteins were found to be constitutively overexpressed. The nine proteins include three heat-shock proteins as well as glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase (Morgan *et al.*, 1986). Besides being resistant to oxidative stress, the Oxy R1 cells were found to be more resistant to killing by heat. The mutation responsible for the increased expression of these nine proteins resided in a single regulatory element (Christman *et al.*, 1989). Other prokaryotic cell-lines that possess mutations in stress-responsive regulons have also been described. In *E. coli*, mutants that constitutively express proteins associated with the adaptive response to DNA-methylating agents have been isolated (Sedgwick & Robins, 1980). These mutants possess high levels of the DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase, which catalyses the removal of alkyl groups from O^6-alkylguanine to a specific cysteine residue within the enzyme itself; this reaction is suicidal as the modified transferase is inactive. The alkyltransferase is encoded by the *ada* gene and in normal cells its expression is increased following exposure to methylating agents; the *ada* gene comprises part of a small operon including the *alk*A and *aid*B genes and is co-induced with the *alk*B genes. Interestingly, the *ada* operon is regulated by the methylated O^6-methyltransferase protein which is formed during the repair process.

Most enterobacteria, with the exception of *Salmonella typhimurium*, possess chromosomally-encoded class C-type β-lactamases that are distinct from the plasmid-mediated TEM β-lactamases [for definitions of nomenclature, see Ambler (1980) and Coulson (1985)], In some of these bacteria, such as *Citrobacter freundii* and *Enterobacter cloacae*, the expression of the chromosomal class C β-lactamase can be increased as much as 100-fold by the addition of inducing agents to the growth medium. These agents include β-lactam-containing drugs and other unrelated compounds (Gootz & Saunders, 1983; Cullmann *et al.*, 1984). *E. coli* and *Shigella sonnei* also possess β-lactamase, but it is not inducible by β-lactams and is produced at low constitutive levels. Induction of the chromosomal β-lactamase by drugs is an example of an adaptive response and it can have a profound effect on the antimicrobial activity of the cephalosporins cefoperazone and cefsulodin (Saunders & Saunders, 1983; Eliopoulos, 1988). However, besides induction, the expression of chromosomal β-lactamase can also be increased greatly in bacteria by mutation in the genes that control its expression. These mutants constitutively express high levels of the β-lactamase. Such mutations occur at a high frequency (i.e. 10^{-7}–10^{-6}) in organisms with inducible β-lactamases, whereas in bacteria with non-inducible enzymes, they represent a much rarer event (i.e. 10^{-18}–10^{-9}).

Lindberg *et al.* (1986) have studied the genetic basis for the constitutive over-expression of the inducible chromosomal β-lactamase and reported that mutations in two genes, *amp*R and *amp*D, affect the synthesis of the enzyme (encoded by *amp*C). These workers found that the presence of the *amp*R gene is essential for inducibility. It encodes a polypeptide of M_r 31000, and its absence results in the failure of β-lactams to induce β-lactamase; *E. coli* and *S. sonnei* lack the *amp*R gene. The *amp*D gene product is thought to be part of the cellular machinery which recognises the β-lactam inducer, and interacts with the AmpR protein to cause induction of β-lactamase. Evidence suggests that mutations in *amp*D result in the constitutive over-expression of the chromosomal β-lactamase and in resistance to cephalosporins. Drug resistance in bacteria which results from this type of mechanism appears to be of central importance in the protection of mammals from toxic chemicals, in that many of the proteins involved in the metabolism and detoxification of drugs in mammals are induced by their substrates themselves (Talalay *et al.*, 1988).

Adaptive drug resistance in pre-neoplastic cells

An increasing number of parallels can be drawn between the genotypic changes which result in adaptive drug resistance in bacteria to those observed in mammalian cells. In mammals, drug resistance in certain cases appears to arise from mutation or the modification of an adaptive response. Perhaps this is best exemplified by the study of hepatocarcinogenesis. The development of cancer is a stepwise process that involves, at least in part, the selection of new cell populations that exhibit a drug-resistant phenotype (Farber, 1984*a*,*b*). These steps include initiation, promotion and progression. One of the earliest events in chemical hepatocarcinogenesis in rodents is the production of pre-neoplastic hepatocyte nodules. The paradoxical facts that many carcinogens are cytotoxic, but nodules arise from a carcinogen-containing environment, suggested to Solt & Farber (1976) that either the nodules or their precursors would, of necessity, be drug-resistant. Consistent with this hypothesis, major alterations are observed in the levels of drug-metabolizing enzymes in these hepatocyte nodules. The changes in expression are highly complex and include reduced expression of the cytochrome *P*-450 and sulphotransferases and over-expression of the glutathione *S*-transferases, the UDP-glucuronyltransferases, epoxide hydrolase, DT-diaphorase and γ-glutamyltransferase. In addition, the drug efflux pump, P-glycoprotein, is over-expressed in persistent hepatocyte nodules and the intracellular concentration of glutathione is increased (Farber, 1984*a*,*b*). Carr (1987) has shown that cells derived from these nodules display resistance to a variety of compounds.

The remarkable diversity of these changes and their consistency in a variety of liver carcinogenesis protocols suggests the existence of a global control mechanism that is responsible for co-ordinating the expression of drug-metabolizing enzymes and the other proteins involved in handling drugs, as well as providing an elevation in some of the enzyme cofactors like glutathione (Beer & Pitot, 1987; Fairchild *et al.*, 1987; Thorgeirsson *et al.*, 1987; Wolf *et al.*, 1987*a*; Hayes & Wolf, 1988; Hayes *et al.*, 1990*b*). This protection mechanism presumably evolved to enable cells to overcome the genotoxic and cytotoxic insults exerted by a wide spectrum of toxic and mutagenic compounds. The existence of such a non-specific defence process is supported by the fact that, in any particular instance, many of the changes that typically occur in the nodules are apparently not involved in combating the toxic actions of the actual carcinogen employed. Two basic mechanisms could account for the gross disturbance in the expression of drug-metabolizing enzymes in the rat hepatic nodules; it could be due to changes in the level of a transcription factor that mediates the expression of many genes, or an initial mutation event (for example leading to the activation of oncogenes) that could initiate a cascade of events which result in the over-expression of drug-resistance proteins. In view of the gross disturbance in the expression of drug-metabolizing enzymes in rat hepatic nodules, it is likely that the putative mutation involves a gene which regulates the stress-response process. The latter possibility is the most attractive as it will give rise to constitutive expression of stress-response proteins and would be compatible with models identified in bacteria. Also the fact that these multiple changes are consistently observed after a single dose of chemical carcinogen indicates that a single mutation event is responsible for the changes observed. It is interesting that most pre-neoplastic nodules disappear and only a few (1–3%) go on

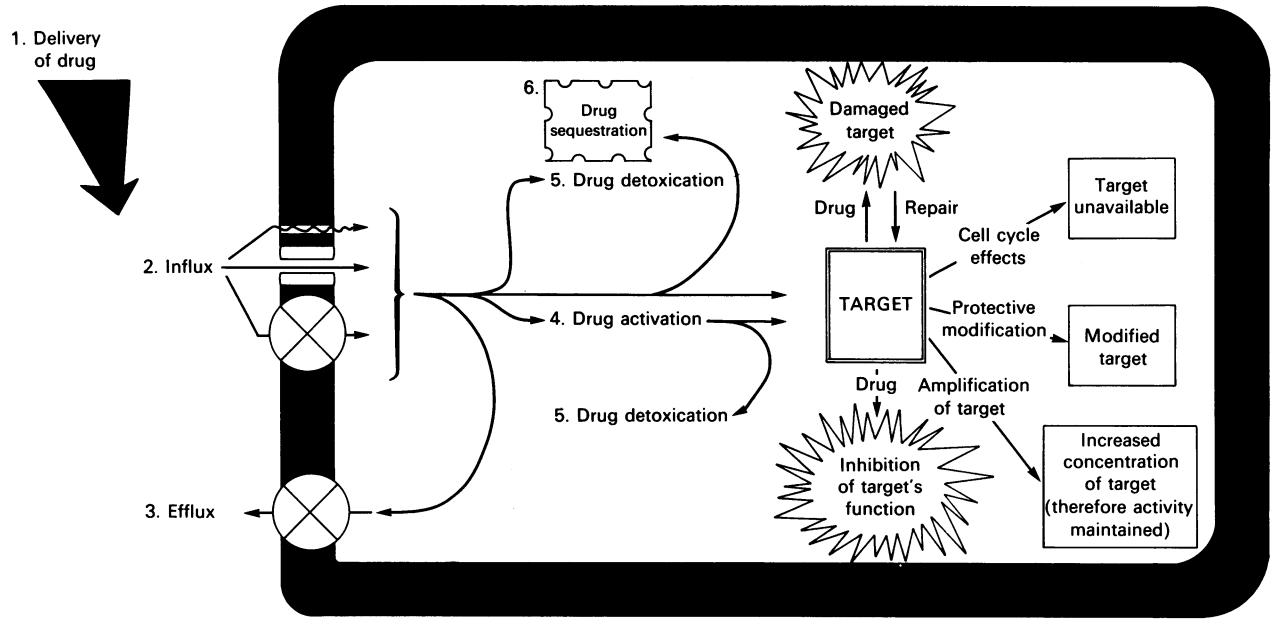

Fig. 1. Biochemical mechanisms of drug resistance

For details see the text.

to become tumours (Tatematsu *et al.*, 1983). This may be because further specific changes or mutations are required within the nodules for their progression. The arguments for a cascade of events leading to the pre-neoplastic phenotype are further supported by the finding that, in rat liver epithelial cells or rat hepatocytes, transformation with v-H-*ras* or V-*raf* results in the multi-drug resistant phenotype as well as the over-expression of pi-class glutathione *S*-transferase (Power *et al.*, 1987; Burt *et al.*, 1988).

BIOCHEMICAL MECHANISMS OF DRUG RESISTANCE

Drug resistance can arise as a consequence of various biochemical mechanisms (see Fig. 1); these include: (*a*) reduced drug delivery; (*b*) decreased drug uptake; (*c*) increased drug efflux; (*d*) reduced metabolic activation of the drug; (*e*) increased deactivation of the drug; (*f*) sequestration of the drug to prevent interaction with target site; (*g*) increase in intracellular concentration of target sites; (*h*) structural alterations in the target site; (*i*) duplication of the functions of the target site; and (*j*) increased repair of damaged target site.

It is important to realise that resistance to a particular drug can be achieved by more than one mechanism. On occasions, particularly in the case of changes in either drug transportation or drug detoxication, protection against more than one chemical is invariably observed. This can be manifest as cross-resistance to structurally-related compounds or structurally unrelated chemicals. This latter phenomenon has been referred to as multidrug, or pleotropic drug, resistance.

Drug delivery

Of the mechanisms listed above, only the first is a consequence of factors which are essentially independent of the target cell. Clearly, drug delivery in a single cell organism is less complicated than in complex organisms. In mammals, blood circulation is a factor of crucial importance in the delivery of the drug to the target tissue. The brain, for example, is difficult to target in cancer chemotherapy because of the blood-brain barrier, and it is therefore referred to as a sanctuary site for tumour cells. Further, the vascularization of tumours is highly variable and tumours that are poorly vascularized are difficult to target. The delivery of drugs to target cells is also dependent on their half-life in plasma. This is itself dependent on a wide range of factors, particularly on their rate of metabolism, invariably at sites separate from the target cells. The activity of drug-metabolizing enzymes is influenced by a host of factors such as hormonal status, bacterial and viral infection etc. As the majority of detoxication enzymes are inducible by xenobiotics, previous drug therapy or exposure to other inducing agents can exert a profound effect on drug availability. Other factors can also be of importance for drug delivery; for example, in insects, behaviour is an important factor in the effectiveness of insecticides as certain houseflies deliberately avoid insecticide-contaminated materials, presumably due to an acquired hypersensitivity to them.

Drug uptake

The defective transport of drugs is a general mechanism of resistance, the importance of which varies considerably depending on both the lipophilicity of the drug and the structure of the cell membrane (Table 4). It should be recognized that the uptake of a particular drug may occur by several distinct mechanisms (for a review, see Goldenberg & Begleiter, 1984).

This resistance mechanism can involve drugs which are absorbed passively and those that are actively transported into cells. Passive uptake, or permeability, is dependent on the physiochemical properties of the membrane. For example, many hydrophobic antibiotics are more effective against Gram-positive bacteria than Gram-negative bacteria. This difference is due to a reduced drug diffusion rate across the outer membrane. The low permeability of the outer membrane to hydrophobic drugs is attributed to the asymmetric structure of its lipid bilayer, which comprises only lipopolysaccharides in the outer leaflet and glycerophospholipids in the inner leaflet (Nikaido, 1988). The lipopolysaccharide layer is much less fluid than other membranes and the rigidity is thought to contribute to the ability of the outer membrane to exclude many hydrophobic antibiotics. In Gram-negative bacteria the transport of hydrophilic compounds across the outer membrane is accomplished through water-filled dif-

Table 4. Examples of decreased drug accumulation by cells

Organism or cell	Type of resistance	Drug	Cause	Reference
DECREASED UPTAKE				
Staphylococcus aureus	Acquired	Fusidic acid	Alteration in membrane phospholipids	Chopra (1976)
Escherichia coli	Acquired	Chloramphenicol	Change in cytoplasmic membrane?	Gaffney *et al.* (1981)
Pseudomonas aeruginosa	Intrinsic	Cephacetrile	Porin structure	Yoshimura & Nikaido (1982)
Escherichia coli	Acquired	Ampicillin, cephaloridine	Lack of outer membrane pore proteins b and c	van Alphen *et al.* (1978)
Escherichia coli	Acquired	Tetracycline	Reduction in many membrane proteins including protein b	Rossouw & Rowbury (1984)
Enterobacter cloacae	Acquired	Cephalosporin	Plasmid-determined suppression of porin expression	Sawai *et al.* (1982)
Staphylococcus aureus	Acquired	Gentamicin	Loss of energy-dependent transport system	Miller *et al.* (1980)
Staphylococcus aureus	Acquired	Tetracycline	Loss of energy-dependent transport system	Sompolinsky *et al.* (1970)
Trypanosoma brucei	Acquired	α-Difluoromethylornithine	Reduced drug uptake	Phillips & Wang (1987)
Leishmania donovani	Acquired	Methotrexate	Deficient folate transporter	Kaur *et al.* (1988)
Mouse L5178Y leukaemia cells	Acquired	Methotrexate	Reduced drug uptake	Hill *et al.* (1979)
Mouse L5178Y leukaemia cells	Acquired	Nitrogen mustard	Reduced drug uptake (choline transporter	Goldenberg *et al.* (1970)
Human L1210 leukaemia cells	Acquired	Melphalan	Reduced drug uptake (L-amino acid carrier)	Redwood & Colvin (1980)
INCREASED EFFLUX				
Escherichia coli	Acquired	Tetracycline	Membrane-located proteins encoded by Tet A, B, C and pA 124 involved in the active efflux of the antibiotic	Chopra (1984)
Plasmodium falciparum	Acquired	Chloroquine	Possession of an efflux pump that is inhibited by desipramine	Bitonti *et al.* (1988)
Chinese hamster ovary cells	Acquired	Colchicine, vinblastine, actinomycin D etc.	Overexpression of P-glycoprotein	Ling & Thompson (1974)
Chinese hamster lung cells	Acquired	Actinomycin D, vincristine, daunorubicin etc.	Overexpression of P-glycoprotein	Gerlach *et al.* (1986)

fusion channels which are formed by the porin proteins. Compounds which are subject to porin-mediated transport include nutrients, products of metabolism, and drugs such as cephalosporin. The loss of porins (e.g. Omp F and Omp C) can confer resistance to cephalosporin.

In addition to the passive mechanisms described above, the importance of active transport mechanisms in resistance to several anticancer drugs has been described. For example, the uptake of nitrogen mustard is via the choline transport system (Goldenberg *et al.*, 1971), the uptake of melphalan is via the L-amino acid carrier (Vistica *et al.*, 1978; Goldenberg & Begleiter, 1984), the uptake of 5-fluorouracil is via the purine and pyrimidine uptake system (Wohlhueter *et al.*, 1980), and the transport of methotrexate is via the folate transport carrier (Bertino, 1980; Sirotnak, 1985). Mutations affecting the activity of these transport processes have been implicated in resistance to these anticancer drugs (Table 4).

Drug efflux

Proteins involved in drug efflux play an important role in resistance to certain compounds. In Enterobacteriaceae, a number of plasmid-encoded proteins (Tet A, B, C and pAB 124) which act as membrane-located, energy-dependent efflux pumps, can confer resistance to tetracycline (Chopra, 1984, 1986). Similarly, in human tumours the presence of a trans-membrane, energy-dependent efflux pump, P-glycoprotein, can confer resistance to a number of anticancer drugs (see below).

Drug metabolism

Drug-metabolizing enzymes can also play an important role in reducing the intracellular concentration of drug. Interestingly, certain drugs require to be metabolized by these enzymes before they exert their chemotherapeutic effects. The expression of drug-metabolizing enzymes can therefore either potentiate or reduce the toxicity of chemicals, so changes in both the activation and de-activation pathways are important variables that can lead to drug resistance. Examples of both the reduced expression of activating enzymes or the over-expression of detoxication enzymes have been described. In model systems it appears that both oxidation (phase I) and conjugation (phase II) enzymes play critical roles in protecting cells against many drugs.

The ability of cytochrome *P*-450, a phase I enzyme, to catalyse the formation of the ultimate toxic and carcinogenic metabolites of a host of compounds is well established. This enzyme system is implicated in the activation of the chemotherapeutic agent cyclophosphamide; reduced expression of the *P*-450 responsible for this reaction is a potential mechanism of resistance. Altered levels of expression, or inhibition, of cytochrome *P*-450 can have profound effects on the sensitivity of the target cell to toxic compounds. Many quinone-containing drugs, such as adriamycin and mitomycin C, can generate superoxide and hydroxyl radicals by redox cycling. This one-electron-reduction activation pathway is catalysed by enzymes such as cytochrome *P*-450 reductase and changes in *P*-450 reductase activity have been associated with resistance to these compounds. The DNA interstrand cross-linking agent 5-(aziridin-1-yl)-4-hydroxylamino-2-nitroben-

Table 5. Examples where changes in the metabolism of drugs are implicated in the mechanism of resistance

Organism or cell	Type of resistance	Drug	Cause	Reference
INCREASED DEACTIVATION OF DRUGS BY CHROMOSOME-ENCODED ENZYMES				
House-fly	Acquired	Methylcarbamates	Induction of cytochrome *P*-450	Tsukamoto & Casida (1967)
Anopheles stephensi	Acquired	Malathion, phenthoate	Overexpression of carboxylesterase	WHO Expert Committee Report (1986)
Anopheles gambiae	Acquired	DDT	DDT-dehydrochlorinase (a GST)	WHO Expert Committee Report (1986)
House-fly (Third Yumenoshima)	Acquired	Lindane, malathion	Glutathione *S*-transferase	Tanaka *et al.* (1981)
Maize (GT112 RfRf)	Intrinsic	Atrazine	Presence of a polymorphic GST	Shimabukuro *et al.* (1971)
Chinese hamster ovary cells	Acquired	Chlorambucil, mechlorethamine	Overexpression of a GST	Lewis *et al.* (1988)
Providencia stuartii	Acquired	Gentamycin, neomycin	Production of gentamycin acetyltransferase II	Chevereau *et al.* (1974)
Escherichia coli	Acquired	Cephalosporins	Inducible β-lactamase (class C)	Medeiros (1984)
Streptomyces vinaceus	Intrinsic	Viomycin	Viomycin phosphotransferase	Skinner & Cundliffe (1980)
Streptomyces capreolus	Intrinsic	Viomycin, capreomycin	Viomycin and capreomycin phosphotransferase	Skinner & Cundliffe (1980)
Streptomyces griseus	Intrinsic	Streptomycin	Inducible aminoglycoside 6-phosphotransferase	Cundliffe (1984)
Mouse	Intrinsic	Aflatoxin B_1	Glutathione *S*-transferase	O'Brien *et al.* (1983)
INCREASED DEACTIVATION OF DRUGS BY ENZYMES ENCODED BY RESISTANCE PLASMIDS AND TRANSPOSONS				
Escherichia coli, *Staphylococcus aureus*	Acquired	Chloramphenicol	Chloramphenicol acetyltransferase	Shaw (1984)
Escherichia coli, *Staphylococcus aureus*	Acquired	Penicillin	β-Lactamases (class A)	Medeiros (1984)
Pseudomonas aeruginosa	Acquired	Streptomycin	Phosphotransferase [ADPH(3)″]	Phillips & Shannon (1984)
Klebsiella pneumonia	Acquired	Gentamicin, tobramycin, kanamycin	Adenyltransferase	Benveniste & Davies (1971)
Escherichia coli	Acquired	Gentamicin, tobramycin	Acetyltransferase [AAC(3)II]	Le Goffic *et al.* (1974)
Escherichia coli	Acquired	Fosfomycin	Glutathione *S*-transferase	Arca *et al.* (1988)

zamide is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by the nitroreductase activity of the drug-metabolizing enzyme DT-diaphorase [NAD(P)H:(quinone-acceptor) oxidoreductase]. Knox *et al.* (1988) have shown that the resistance of Chinese hamster V79 cells to CB1954, and the exceptional sensitivity of Walker tumour cells to this compound, is due to differences in the levels of DT-diaphorase.

Plasmid-encoded enzymes that detoxify antibiotics and thereby confer resistance to their bacterial hosts have been recognized for about 25 years. An increasing number of proteins have been implicated in this mechanism of resistance. For example β-lactamases, acetyltransferases, phosphotransferases and glutathione *S*-transferases (Table 5) detoxify penicillin, chloramphenicol, streptomycin, kanamycin, gentamicin and fosfomycin (Falkow, 1975; Arca *et al.*, 1988). Increase in detoxication capacity as a mechanism of drug resistance is not restricted to bacteria. In mammalian cells, resistance to 1-arabinofuranosylcytosine ('araC') has been attributed to increased deactivation by specific deaminases (Ho & Frei, 1971), and resistance to alkylating agents can involve the overexpression of glutathione *S*-transferases or aldehyde dehydrogenase (Clapper *et al.*, 1987; Wolf *et al.*, 1987*b*; Colvin *et al.* 1988; Hayes & Wolf, 1988; Hayes *et al.*, 1990*b*). Expression of human pi-class and alpha-class glutathione *S*-trasferase cDNAs in yeast cells confers 7–12-fold resistance to chlorambucil (Black *et al.*, 1990).

Resistance to drugs which interfere with nucleic acid metabolism is often associated with decreases in the activation reaction responsible for their conversion into compounds which are able to interfere with biosynthesis of macromolecules. Unlike other examples described in this section, the activation of antimetabolites involves normal housekeeping enzymes and not detoxication enzymes (Table 5). For example, the purine antimetabolites, 6-mercaptopurine and 6-thioguanine, are activated by hypoxanthine-guanine phosphoribosyltransferase and decreased expression of this enzyme represents a major resistance mechanism to these drugs (Brockman, 1960, 1963). Similarly, the loss of enzymes responsible for converting 5-fluorouracil to inhibitory nucleosides is responsible for resistance to this drug (Laskin *et al.*, 1979; Ardalan *et al.*, 1980).

Drug sequestration

A reduction in active drug concentration can also be achieved by drug sequestration due to increased intracellular drug binding. The increased expression of metallothionein, a low-M_r cysteine-rich protein, has been implicated in this mechanism (Kelley *et al.*, 1988). In man there may be as many as 10 metallothionein genes which can be divided into two major groups, MT-I and MT-II. Expression of the human metallothionein II_A cDNA in human carcinoma cells results in a 4-fold increase in resistance to the anti-tumour agents *cis*-diamminedichloroplatinum, melphalan and chlorambucil (Kelley *et al.*, 1988). Metallothionein can scavenge oxygen free radicals and the ability of metallothionein II_A to confer a limited level of resistance to adriamycin may be due to this activity rather than drug sequestration.

Alterations in target site

Structural changes. There are a variety of mechanisms of drug resistance which do not involve either drug accumulation or the rate of drug metabolism. In certain instances the cellular target becomes structurally altered in a manner which reduces the affinity of the toxin for the target enzyme or protein (Table 6). This type of drug resistance is the result of point mutations in the structural gene(s) encoding the target and is usually associated with drugs whose target is well-defined. For example, mutations

Table 6. Structural changes in target site which lead to drug resistance

Organism or cell	Type of resistance	Drug	Event	Reference
Escherichia coli	Acquired	Mecillinam	Reduced affinity of PBP2 for mecillinam	Spratt (1978)
Escherichia coli	Acquired	Cephalosporin	Reduced affinity of PBP3 for cephalosporin	Spratt (1983)
Clostridium perfringens	Acquired	Benzylpenicillin	Reduced affinity of PBP1 for benzylpenicillin	Williamson (1983)
Streptococcus pneumoniae	Intrinsic	Penicillin	Low affinity of PBP1 and 2 for penicillin	Hakenbeck *et al.* (1980)
Escherichia coli	Acquired	Nalidixic acid	Changes in the structure of the A subunit of DNA gyrase	Reynolds (1984)
Escherichia coli	Acquired	Novobiocin	Changes in the structure of the B subunit of DNA gyrase	Reynolds (1984)
Escherichia coli	Acquired	Rifampicin	Alteration in a subunit of RNA polymerase	Rabussay & Zillig (1969)
Escherichia coli	Acquired	Streptomycin	Changes in protein S12 in the 30 S ribosome subunit	Funatsu & Wittmann (1972)
Streptomyces aureus	Intrinsic	Thiostrepton	Methylation of 23 S RNA in the 50 S ribosome subunit	Thompson *et al.* (1982)
Staphylococcus aureus	Acquired	Erythromycin	Altered methylation of ribosomal RNA	Lai & Weisblum (1971)
Chinese hamster ovary cells	Acquired	Etoposide	Mutant type II DNA topoisomerase	Glisson *et al.* (1987)
Plasmodium falciparum	Acquired	Pyrimethamine	Mutation in dihydrofolate reductase	Peterson *et al.* (1988)
Mouse 3T6 subline	Acquired	Methotrexate	Mutation in dihydrofolate reductase	Simonsen & Levinson (1983)
Human rhabdomyosarcoma cells	Acquired	Vincristine	Structural changes in β tubulins	Houghton *et al.* (1985)
Chinese hamster ovary cells	Acquired	Vinca alkaloids	Changes in microtubule-associated proteins	Gupta & Gupta (1984)
House-fly	Acquired	Rabon	Mutant acetylcholinesterase	Tripathi & O'Brien (1973)
Herpes simplex virus	Acquired	Arabinofuranosyl-adenine	Mutation in the gene encoding viral DNA polymerase	Coen *et al.* (1982)
Influenza A virus	Acquired	Amantadine	Mutation in the M2 protein	Hay *et al.* (1986)

in dihydrofolate reductase can result in resistance to methotrexate (Simonsen & Levinson, 1983), mutations in thymidylate synthetase can result in resistance to 5-fluorouracil, mutated topoisomerase I can result in resistance to camptothecin (Kjeldsen *et al.*, 1988), mutations in topoisomerase II may be responsible for resistance to VP-16 (Glisson *et al.*, 1987) and changes in the tubulins or microtubule-associated proteins may represent a resistance mechanism towards vinca alkaloids (Cabral *et al.*, 1980; Keates *et al.*, 1981; Gupta & Gupta, 1984).

In addition to mutations in the genes encoding target proteins it is conceivable that mutations or changes in other genes, for example those involved in post-translational modification, can contribute towards this type of resistance. Phosphorylation represents such a post-translational change that can substantially influence the activity of proteins. Fine *et al.* (1988) have shown that activation of protein kinase C in breast tumour cells, using phorbol esters, results in the potentiation of the multidrug resistant (MDR) phenotype which is paralleled by an increase in protein kinase C and the phosphorylation of a 20 kDa paticulate protein. The increasing evidence that phosphorylation plays a central role in regulating protein function, for example, in the case of hormone receptors and hsp70 and gene transcription, indicates that changes in phosphorylation pathways may play an extremely important role in the acquisition of drug resistance.

Changes in target concentration. Resistance can also be achieved by increasing the intracellular concentration of the target protein. In this case the structure of the target site is unchanged and although its activity is inhibited, i.e. the turnover number of the enzyme is reduced, the overall activity is maintained through an increase in target site abundance. The best-characterized example is the increase in dihydrofolate reductase concentration often observed in methotrexate-resistant cell lines (Alt *et al.*, 1978; Nunberg *et al.*, 1978). However, there are many other examples. A methionine suphoximine-resistant Chinese hamster ovary cell line has been described that over-produces the target enzyme glutamine synthetase (Sanders & Wilson, 1984) and a histidinol-resistant Chinese hamster ovary cell line has been reported that overproduces histidyl-tRNA synthetase (Tsui *et al.*, 1985). Resistance to *N*-phosphonoacetyl-L-aspartate has been associated with the overproduction of the target protein aspartate transcarbamoylase (Kempe *et al.*, 1976; Wahl *et al.*, 1979). This type of resistance is often associated with an increase in copy number of the gene encoding the target protein. This gene amplification has been demonstrated for all the examples of drug resistance described above. It should be emphasized that gene amplification is not the exclusive mechanism of protein overexpression. For example, derepression of the gene encoding arginosuccinate synthetase, the target protein for canavanine, is believed to be responsible for the over-production of arginosuccinate synthetase in a canavanine-resistant human cell line (Su *et al.*, 1981). There are many other examples where increased protein expression resulting in drug resistance is not a consequence of amplification of the target gene.

Duplication of the function of the target site

Trimethoprim resistance in bacteria provides a good example of the by-pass of a metabolic block through the production of a novel protein. In this instance the new protein which has the same function as the target protein no longer interacts with the drug. Trimethoprim inhibits bacterial dihydrofolate reductase of M_r 21000. However, the resistance plasmid R388 encodes a distinct enzyme with an M_r of 35000 which is unaffected by this compound (Smith & Amyes, 1984; Amyes, 1989).

Repair of drug-induced damage

Increased rates of repair of cellular damage represents an important mechanism of resistance to alkylating agents and particularly radiation. While DNA repair has been extensively studied, the replacement of proteins and the repair of membranes has been essentially ignored. A number of DNA repair enzymes have been described (Fox & Roberts, 1987) and in the context of drug resistance O^6-alkylguanine–DNA-alkyltransferase has been shown to be responsible for resistance to methylating agents

Table 7. Patterns of multidrug resistance in some mammalian lines

The cell lines were selected using: DC-3F/ADIV, actinomycin D (Biedler & Riehm, 1970); C5, colchicine (Ling & Thompson, 1974); CH^RC5, colchicine (Ling *et al.*, 1983); DNR^R51, daunorubicin (Ling *et al.*, 1983); KB-C4, colchicine (Choi *et al.*, 1988); Adr^RMCF-7, adriamycin (Batist *et al.*, 1986); ARN2, doxorubicin (Schisselbauer *et al.*, 1989); CHO-$Ch1^R$, chlorambucil (Robson *et al.*, 1986).

Drug	Mode of action	Cross-resistance patterns of cell lines (fold resistance compared to wild type)							
		DC-3F/ADIV	C5	CH^RC5	DNR^R51	KB-C4	Adr^RMCF-7	ARN2	CHO-$Ch1^R$
Colchicine	Inhibition of tubulin assembly	–	300	180	25	1750	65	–	–
Vinblastine	Inhibition of tubulin assembly	239	42	30	22	159	274	8	–
Vincristine	Inhibition of tubulin assembly	189	–	–	–	–	> 170	13	–
Emetine	Inhibition of protein synthesis	–	–	29	11	–	–	–	–
Puromycin	Inhibition of protein synthesis	84	–	100	38	–	–	–	–
Chlorambucil	Alkylating agent	–	–	–	–	–	–	–	24
Mechlorethamine	Alkylating agent	–	–	–	–	–	–	–	34
Melphalan	Alkylating agent	–	–	4–15	–	–	–	1.8	14
Actinomycin D	Inhibition of DNA and RNA synthesis	376	725	–	–	–	357	–	–
Doxorubicin	Free radical generation, inhibition of topoisomerase II, membrane effects	–	–	25	30	254	192	13	–
Daunorubicin		–	–	76	41	–	–	–	–
Taxol	?	–	–	20	5	–	–	–	–
VP-16 (etoposide)	Topoisomerase II inhibitor	–	–	–	–	–	175	100	–

such as *N*-methyl-*N*-nitrosourea, methylmethane sulphonate and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (Demple *et al.*, 1985; Sekiguchi & Nakabeppu, 1987). The adaptive response system in prokaryotes allows bacteria to overcome DNA damage produced by some alkylating drugs and the SOS system allows bacteria to combat DNA damaging agents such as u.v. and mitomycin C (Walker, 1985). DNA repair has also been implicated in resistance to chemical carcinogens and resistance to a wide range of anticancer drugs (see Kessel, 1986).

GENETIC MECHANISMS THAT PRODUCE RESISTANCE

At a molecular level, the biochemical changes described in the previous section can result from gene amplification, gene transfer, gene deletion, point mutations, the loss of *cis*-acting regulatory elements, the loss or dysfunction of *trans*-acting factors, transcriptional activation, hypo- or hyper-methylation, or the stress-induced production of 'alarmones'. All these effects could be on genes directly involved in combating the cytotoxic compounds and/or could be in genes involved in their regulation or processing. In theory, the number of ways cells could become drug resistant seems almost limitless. An example where many of the above types of changes occur is provided by the studies on P-glycoprotein.

ROLES OF P-GLYCOPROTEIN IN DRUG RESISTANCE

Multi-drug resistance

Since the early 1980s considerable attention in cancer research has focussed on multidrug resistance (MDR), where tumour cells selected for resistance to a single agent, such as vinblastine or actinomycin D, demonstrates resistance to a broad range of structurally diverse drugs, namely anthracyclines, vinca alkaloids and podophyllotoxin derivatives. This type of resistance was not restricted to any particular cell line and, indeed, an extremely broad range of cell types have been shown to display this phenotype (Table 7). It is evident that the relative resistance of the cell lines that exhibit MDR for particular drugs (e.g. vinblastine) varies substantially; cells usually exhibit greatest resistance towards the compound used in the selection process. It is also important to note that MDR does not extend to alkylating agents nor to *cis*-platinum. Accompanying the marked increase in resistance to the drugs which characterize MDR is a modest decrease in resistance (collateral sensitivity) to a small number of other agents, such as Triton X-100, 1-dehydrotestosterone and lidocaine. The reason for this sensitization is unclear.

P-glycoprotein and multidrug resistance

The most probable explanation for the MDR phenotype was considered to involve alterations in drug-transport systems. In early studies using CHO cells, Juliano & Ling (1976) showed that the level of resistance to colchicine correlated with the level of a 170 kDa protein, now termed P-glycoprotein. The finding of an inverse relationship between the expression of P-glycoprotein and the accumulation of certain anthracyclines and vinca alkaloids suggested that this membrane protein may be involved in drug transport. These data, together with the observation that the MDR phenotype is energy-dependent and is reversed by respiratory inhibitors such as KCN, 2,4-dinitrophenol and sodium azide suggested that P-glycoprotein serves as an energy-dependent drug efflux pump. Biochemical analysis of P-glycoprotein is consistent with this proposed physiological role. The use of photoactivatable derivatives of vinblastine has demonstrated that P-glycoprotein is able to bind drugs (Cornwell *et al.*, 1986; Safa *et al.*, 1986). Moreover, ATP binds specifically to P-glycoprotein (Cornwell *et al.*, 1987) and the protein has been found to exhibit ATPase activity (Hamada & Tsuruo, 1988).

The primary sequence analysis of P-glycoprotein shows that it shares substantial homology with several bacterial membrane-associated transport proteins (Gerlach *et al.*, 1986; Gros *et al.*, 1986*a*,*b*) which strongly supports its putative role in membrane transport. P-glycoprotein was found to share the greatest homology with haemolysin B, which is responsible for secreting α-haemolysin. It is intriguing that there is also significant homology between P-glycoprotein and the *Salmonella typhimurium* His P protein, which is a component of the histidine permease system, and the *Escherichia coli* Mal K protein, which is part of the maltose and maltodextrin transport system (Gerlach *et al.*, 1986).

The amino acid sequences deduced from the mammalian *mdr*1 genes (Chen *et al.*, 1986; Gerlach *et al.*, 1986; Gros *et al.*, 1986*a*,*b*) suggest that these proteins each contain a total of 12

membrane-spanning helices and two nucleotide (ATP)-binding folds. The possession of this number of transmembrane segments represents a structural motif which is a characteristic feature of pore-forming proteins (Henderson & Maiden, 1987). In the case of P-glycoprotein, it is envisaged that these segments would form a channel through which drugs could be extruded. The presence of two highly conserved ATP-binding regions is consistent with a role for P-glycoprotein as an energy-dependent drug efflux pump. It is clear that certain structural properties associated with this class of membrane transport system have been highly conserved through evolution. In mammals, several genes have these conserved motifs in common including the cystic fibrosis transmembrane conductance regulator (Riordan *et al.*, 1989), the nicotinic acetylcholine receptor (Stroud & Finer-Moore, 1985) and the Gaba receptor (Schofield *et al.*, 1987).

P-glycoprotein: a multigene family with multiple functions

Mammalian P-glycoproteins are encoded by a multigene family where the number of members varies between species; two human genes, three hamster genes, three murine genes, three bovine genes and five porcine genes (Gottesman & Pastan, 1988; Endicott & Ling, 1989; van der Bliek & Borst, 1989). On the basis of sequence homologies the P-glycoprotein genes in mammals have been divided into two major classes, called *mdr*1 and *mdr*2; the *mdr*1 family can be subdivided into *mdr*1a and *mdr*1b (Kane & Gottesman, 1989). Surprisingly, not all these genes have the ability to produce the multidrug resistant phenotype. Whilst the P-glycoproteins encoded by the *mdr*1a and *mdr*1b class genes can confer drug resistance it is now recognized that the *mdr*2 class genes encode a protein that probably does not serve as a drug efflux pump (at least not for vincristine and adriamycin). The physiological function of *mdr*2 is not yet known. There are now several reports demonstrating that transfection of *mdr*1a and *mdr*1b cDNAs into cells produces the MDR phenotype (Gros *et al.*, 1986*b*; Veda *et al.*, 1987; Croop *et al.*, 1987; Pastan *et al.*, 1988). The ability of P-glycoprotein to confer resistance to cytotoxic drugs has also been established *in vivo* using transgenic mice carrying the human *mdr*1 cDNA; in this model the human gene was under the control of the β-actin promoter. The transgenic mice, expressing the human P-glycoprotein at high levels in the bone marrow, were able to withstand the myelosuppressive effects of daunomycin (Galski *et al.*, 1989).

P-glycoproteins are widely distributed in Nature. In addition to those mammalian species mentioned above, others, such as the malaria parasite *Plasmodium falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989), the yeast *Saccharomyces cerevisiae* (McGrath & Varshavsky, 1989) and the kinetoplastid protozoan flagellate *Leishmania tarentolae* (Ouelette *et al.*, 1990) also possess genes that are homologous to *mdr*1. In the case of *P. falciparum* the *mdr*-like genes (Pf *mdr*1 and Pf *mdr*2) are associated with resistance to the anti-malarial drugs chloroquine and mefloquine, and their protein products are thought to function in an analogous fashion to the mammalian P-glycoproteins. Interestingly, the yeast P-glycoprotein-like protein, encoded by the STE6 gene, may be involved in the secretion of the mating factor, a-factor pheromone (McGrath & Varshavsky, 1989). However, the STE6 gene does not confer resistance to adriamycin and, although there is no evidence that the STE6-encoded P-glycoprotein can promote the efflux of drugs, the structure of a-factor pheromone (Anderegg *et al.*, 1988) suggests that the protein may be involved in the secretion of interleukin-1 or, more speculatively, xenobiotic–peptide conjugates. In the case of *L. tarentolae*, Ouellette *et al.* (1990) have shown that the P-glycoprotein gene (Lt *pgpA*) is located on the extrachromosomal H circles of duplex DNA. The amplification of H circles has been associated with methotrexate resistance (White *et al.*, 1988) but Ouellette *et al.* (1990) were unable to demonstrate that Lt *pgp*A conferred resistance to either methotrexate, adriamycin or vincristine.

P-glycoprotein: control of gene expression and regulation of activity

It is evident that increases in either the expression of P-glycoprotein or changes that affect its function as an efficient efflux pump are of central importance in producing MDR. Whilst a large body of literature testifies to the involvement of P-glycoprotein in drug resistance, less is known about the molecular events that are responsible for its overexpression. Remarkably little is known about the biological control of P-glycoprotein or the post-translational events which may control its activity, although there is evidence that both are important in modulating resistance of cells to chemical insult. The early observation that, in some cases, P-glycoprotein overexpression was associated with gene amplification has been to the detriment of studies into other mechanisms of control of this protein. The MDR phenotype was originally defined in mammalian cell lines that were selected for resistance to cytotoxic natural products. The highly resistant sublines which were obtained by the selection procedures invariably possessed abnormal chromosomes; karyotypic analyses usually revealed the presence of multiple double minute chromosomes as well as homogeneously staining regions of chromosomes. These cytogenetic changes are the hallmarks of gene amplification (Stark, 1986) and hence the link between MDR and gene amplification was established in the early descriptions of this phenotype. Following cloning of *mdr* genes, examination of the highly resistant cells by Southern blotting revealed a marked increase in the copy number of *mdr* genes and in many of these cell lines the amount of P-glycoprotein (and level of resistance) and the extent of gene amplification showed good correlation. Recently, Choi *et al.* (1988) have shown that point mutations in the human *mdr*1 gene can produce a P-glycoprotein that is significantly more efficient at conferring resistance to colchicine than is the normal protein. Hence, the processes of selection for MDR may involve both point mutations and gene amplification; the cross-resistance displayed by the KB-C4 cell line that expressed the mutant P-glycoprotein is shown in Table 7. Interestingly, the mutation identified by Choi *et al.* (1988) was a glycine-to-valine change at position 185 which is situated in the first putative transmembrane segment. To date, all the evidence for the importance of gene amplification in MDR has been obtained from cell culture methods and the physiological significance of the highly resistant cell lines produced by these techniques is unclear. The identification of transcription factors which regulate P-glycoprotein expression will be of central importance in helping establish how this protein functions.

Gene amplification is not the only mechanism which can result in the overexpression of P-glycoprotein. Increased P-glycoprotein mRNA levels have been observed in cell lines displaying MDR without amplification of the gene (Shen *et al.*, 1986). These data suggest that factors besides gene copy number influence the amount of P-glycoprotein message. Elevation of mRNA encoding P-glycoprotein has been noted in the livers of rodents that have been treated with carcinogens or in regenerating livers of rodents that have undergone partial hepatectomy (Thorgeirsson *et al.*, 1987; Fairchild *et al.*, 1987). The increase in P-glycoprotein mRNA in regenerating liver occurs without transcriptional activation, possibly by message stabilization (Gottesman, 1988). The transient induction of P-glycoprotein by drugs and carcinogens is also intriguing and it will be important to establish the regions within the P-glycoprotein genes which regulate this response. Burt & Thorgeirsson (1988) have suggested that the induction of P-glycoprotein by the xenobiotics *N*-hydroxy-2-

(acetylamino)fluorene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is mediated via the cytoplasmic aromatic hydrocarbon (Ah) receptor. Interestingly, these workers also showed that aflatoxin B_1 is able to induce P-glycoprotein in the mouse despite the fact that the mouse, through the possession of a glutathione *S*-transferase (GST Ya_3Ya_3) with high activity for aflatoxin B_1-8,9-epoxide, is resistant to the hepatocarcinogenic effects of this mycotoxin (Hayes *et al.*, 1990*a*). Clearly, in this case at least, the induction of P-glycoprotein is separate from the process of carcinogenesis. It appears likely that the transient induction of P-glycoprotein by xenobiotics is co-ordinately regulated with that of certain drug-metabolizing enzymes.

In addition to the effects of xenobiotics on P-glycoprotein, recent work has shown that the expression of this protein can be modulated by other forms of environmental stress. In the renal adenocarcinoma cell line HTB-46, P-glycoprotein expression can be induced by heat shock as well as exposure to either sodium arsenite or cadium chloride (Chin *et al.*, 1990). It was found that in these cells the increase in P-glycoprotein expression was paralleled by an increase in resistance to vinblastine. Consistent with this observation, Chin *et al.* (1990) also reported that the promoter region of the human *mdr*1 gene contains two heat shock consensus sequences. Whilst these workers have shown that P-glycoprotein is induced by heat shock and heavy metal toxicity its expression is not, however, increased by other physiological stresses such as glucose deprivation and anoxia.

P-glycoprotein was originally identified as a phosphorylated glycoprotein. Mellado & Horwitz (1987) have shown that this phosphorylation is partly cyclicAMP-dependent *in vitro*. The level of phosphorylation can be increased by phorbol esters and by agents which reverse the MDR phenotype. Although the effect of phosphorylation on the activity of P-glycoprotein is uncertain its potential importance should not be overlooked. For example, Center (1983, 1985) has reported that the phosphorylation of P-glycoprotein modulates the exodus of anthracyclines from Chinese hamster lung cells. Whether the function of P-glycoprotein can be regulated through protein kinases has not been thoroughly investigated. However, Fine *et al.* (1988) have shown that drug-resistant MCF7 cells have a 7-fold greater protein kinase C activity than the drug-sensitive parental cell line and have suggested this as a mechanism of P-glycoprotein regulation. The involvement of protein kinase C in MDR regulation was supported by the observation that in the MCF7 cells both drug resistance and phosphorylation were increased by phorbol esters (Fine *et al.*, 1988). In this context, it is interesting to note that a number of the agents that can reverse MDR (see below) could operate via protein kinase C or protein kinase A.

P-glycoprotein is subject to tissue-specific expression (van der Bliek & Borst, 1989; Georges *et al.*, 1990). It is often commented that it is expressed in highest levels in tissues that have an excretory or transport function; such tissues include kidney, liver, stomach, intestine, colon and spleen. Immunohistochemistry has revealed intense localization of P-glycoprotein in the epithelial cells in the villi of stomach, small intestine and colon and the surface of bile ducts as well as kidney tubules. Many of the organs described above play a role in chemical detoxication, but it is interesting to note that P-glycoprotein is also expressed in the human adrenal gland where Sugawara *et al.* (1988) have shown it to be located in the cortex, which is involved in biosynthesis of steroids.

Reversal of multidrug resistance

MDR can be reversed by a variety of pharmacological agents which promote drug accumulation. These include calcium-channel blockers and calcium-calmodulin antagonists such as verapamil, forskolin, nifedipine and its analogues, perhexiline maleate, trifluoperazine and chlorpromazine as well as the antiarrythmic agent, quinidine (Tsuruo *et al.*, 1982; Kessel, 1986; Schuurhuis *et al.*, 1987; Wadler & Wiernik, 1988). Other agents, like the anti-oestrogen tamoxifen and the antibiotic cyclosporin A, are also effective at reversing MDR (van der Bliek & Borst, 1989). The mode of action of these compounds is at present unclear and whilst competitive binding for the transport site(s) on P-glycoprotein has been proposed, the agents verapamil and tamoxifen appear to exert their effects at different sites or through different processes (Kessel, 1986). Monoclonal antibodies provide a potentially valuable alternative means of reversing MDR. Tsuruo (1988) has described a monoclonal antibody which recognises an extracellular epitope on P-glycoprotein and is able to inhibit the function of P-glycoprotein; this monoclonal may be of therapeutic, as well as of diagnostic, use.

Much of the information on the mode of regulation of P-glycoprotein is equivocal and it is clear that there are still many central issues to be resolved in the study of this protein.

CONCLUSIONS

Drug resistance is of increasing concern in modern medicine. Its consequences in terms of human misery are grave and the financial implications of its increasing incidence are substantial. In this review we have attempted to demonstrate the diverse nature of drug resistance, both in its origin and the number of potential biochemical mechanisms involved. Because of this complexity, resistance may not always be attributable to changes in single genes. Moreover, different examples of resistance to a specific drug will not necessarily involve the same mechanism. At the molecular level it is evident that much remains to be learnt about the control of expression of drug-resistance genes. However, such information will be invaluable as it holds the promise of enabling the development of new, urgently required, therapeutic strategies that will help circumvent drug resistance.

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