WHEN CHEMOTHERAPY FAILS

Mechanisms of Drug Resistance and Their Relationship to Carcinogenesis

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INTRODUCTION

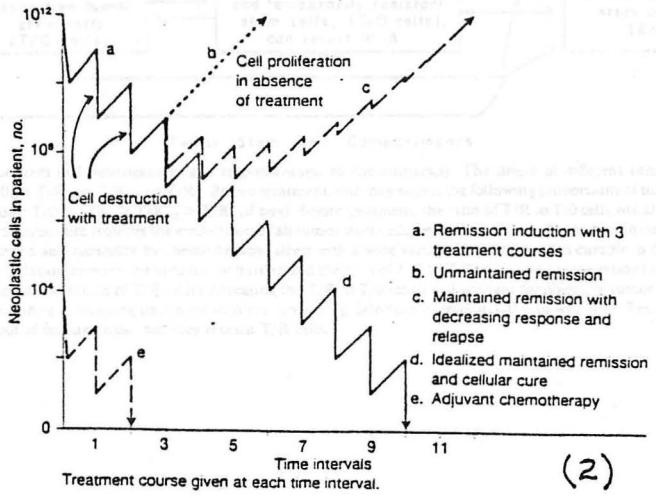
As a general principle the chemotherapy of cancer is the treatment of metastases, since local (or even local-regional) disease can usually be approached for cure by local therapy (ie surgery and/or radiation) (1). Chemotherapy has a firmly established role in the treatment of a variety of disseminated cancers. In at least 13 different cancers (representing approximately 10% of all new cancers each year) the cure of widespread disease is a direct and reasonable therapeutic goal. In another 40% of disseminated cancers of a variety of types significant clinical responses and prolongation of survival has been documented. In spite of these excellent therapeutic responses, relapses occur in both categories of patients and when they do most patients demonstrate that their disease is refractory to further therapy.

The emergence of drug resistance accounts for a remarkable limitation in the efficacy of chemotherapy. As the biology of acquired drug resistance has begun to be characterized, it has become evident that the fundamental mechanisms that develope during therapy are related to de novo ("inherent") resistance to chemotherapy (which seen in approximately 45% of all cancers) and, in addition, are very likely operative in the events of carcinogenesis for at least some tumors. Since acquired drug resistance is generally pleiotropic, the term "multidrug" resistance (MDR) is used to characterize this broad biologic response of "acquired resistance" to a wide variety of natural-product cytotoxic agents. In order to develope rational therapeutic approaches and to prevent or alter the advent of such resistance, extensive recent observations on the operative biochemical and genetic processes have provided important data that now provides an opportunity to integrate this information in clinical practice.

MODELING OF TREATMENT FAILURE

Extensive studies of animal tumor models by Dr. Howard Skipper at the Southern Research Institute over nearly 4 decades have provided much of the basis for our concepts of tumor cell kill and the in vivo kinetics of tumor cell growth. Although recognizing that very significant biologic differences exist between the transplantable animal tumor and de novo tumors in man, a variety of kinetic and biologic parameters had been expressed by Skipper and co-workers (2) to explain the "responsiveness" and "resistance" to chemotherapy.

FIG. 5–3 Idealized time-action response curves for total tumor stem cells.[10] Curve ab: cessation of treatment after three courses. A second response with the same treatment will depend on the T/R to T/0 stem cell ratio at the cessation of treatment and a relapse. Curve ac: a classical illustration of response followed by tumor progression during continuing undiminished treatment with the same drug or combination of drugs. A nadir will be reached when the surviving tumor stem cell burden is composed of about 50% T/R cells (T/R to T/0 ratio = *ca* 1.0). Variations in the initial T/R to T/0 cell ratio in individual tumors will influence the degree and duration of response. Curve ad: This curve would almost never be expected when treating patients with, for example, 10^9 or greater tumor stem cells with single drugs because of the high probability of the presence of T/R cells with specific resistance. Burkitt's lymphoma and choriocarcinoma may be exceptions to this generalization. Curve e: cure of relatively small numbers of metastatic tumor stem cells after local surgery or radiation that left no T/R cells resistant to the drug employed.



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Tumor Stem Cell Heterogeneity and Responsiveness to Chemotherapy

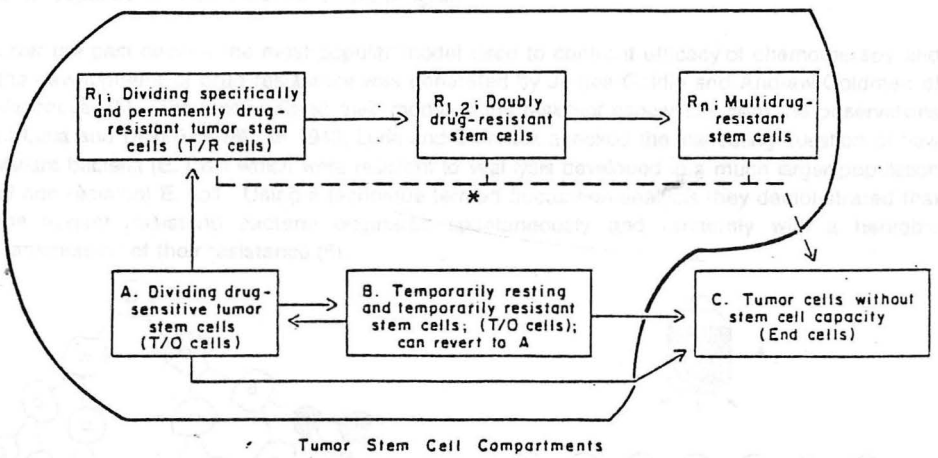


FIG. 5-1 Tumor stem cell heterogeneity and responsiveness to chemotherapy. The origin of different tumor stem cell types is presumed to be T/0 — T/R_1 — $T/R_{1,2}$ — T/R_n. Before treatment, one may expect the following proportions of tumor stem cell types in any type of neoplasm: T/0 > T/R_1 > $T/R_{1,2}$ > T/R_n (if any). Before treatment, the ratio of T/R to T/0 cells will almost never be as high as 1.0. Chemotherapeutic cure requires the eradication of all tumor stem cells, regardless of type. There is an invariable inverse relation between tumor burden and curability by chemotherapy, albeit with a wide variation in total burden curable in different cancers. One may expect a direct relation between the total tumor burden and the ratio of T/R to T/0 cells as well as the ratio of resting to dividing T/0 cells. Chemotherapeutic selection of T/R cells (increasing the T/R to T/0 ratio) will account for objective tumor response followed by tumor progression during continuing treatment with the same drug. Selection of resting T/0 cells will not.* Resistant phenotypes also may go into and out of resting phase, but they remain T/R cells.

(2)

His studies established that resting cells (T/O) had mechanism(s) of resistance that were temporary, non-specific and very different from the changes responsible for the specific, and often permanent resistance of dividing (T/R) cells (2). The studies further characterized the invariable inverse relationship between total tumor cell burden and curability by chemotherapy for any given tumor and provided evidence validating models to explore these (two) different cellular patterns of resistance. The studies of a variety of animal tumor models documented that chemotherapeutic agents kill cancer cells by first-order kinetics (ie a given dose of drug will kill a constant fraction of a population of [tumor] cells, regardless of the size of the total population of tumor cells).

These studies were used as an important basis for the introduction of multi-agent chemotherapy in medical oncology. Indeed, as recently as 1977, Norton and Simon (3,4) exploited these observations to emphasize a rationale for "cross-over intensification" therapy using (presumably) non-cross-resistant drugs for therapy in order to achieve maximum cell kill following the induction of a response with an initial treatment program.

Over the past decade the most popular model used to confront efficacy of chemotherapy and the development of drug resistance was generated by James Goldie and Andrew Coldman of Vancouver (5). They extrapolated their model of the resistant cancer cell from the observations of Luria and Delbrüick (6). In 1943, Luria and Delbrück attacked the interesting question of how variant bacteria (E. Coli) which were resistant to viral lysis developed in a much larger population of non-resistant E. coli. Using a technique termed fluctuation analysis they demonstrated that the variant (resistant) bacteria originated spontaneously and randomly with a heritable transmission of their resistance (6).

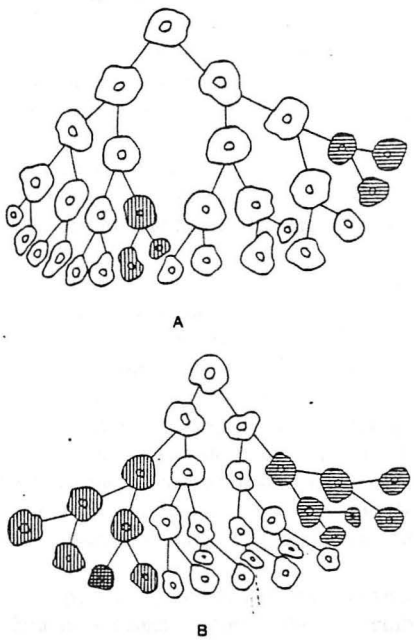


Fig 2. Schematic representation of the emergence of singly and doubly resistant tumor cells by the process of clonal expansion and spontaneous mutation. (A) Two independent classes of resistant cells are postulated, one represented by vertical lines, the other by horizontal. No doubly resistant cells emerge because of the relative small size of the singly resistant compartments. In B, higher mutation rates are postulated giving rise to larger populations of singly resistant cells with the consequent appearance of one doubly resistant cell (cross hatched) rendering the tumor cell population incurable. (9)

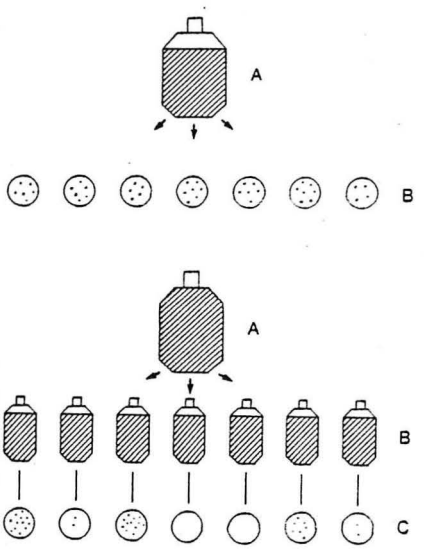


Fig 1. The technique of the fluctuation analysis. The first part of the test is depicted in the upper part of the diagram. Cells are distributed from a large stock culture (A) into dishes containing selecting agents (B). The number of colonies per dish is counted and there is found to be little variation in numbers from one dish to the other. In the lower part of the diagram the second stage of the test is performed. Beginning with stock culture A a series of parallel subclonal culture are grown up, B (either from small inocula or ideally from single cells). These are then transferred to the dishes containing the selecting agent (C). There is now observed substantial variation in numbers of colonies per dish. (9)

Goldie and Coldman proposed that these observations in bacterial genetics were applicable to the development of cancer cell resistance in man (5). They accepted the spontaneous mutation rates of 10^{-6} (as seen in E-coli) as a fundamental proposition. Since most cancers in man are identified when larger than 1 cm^3 (ie 10^9 cells), they proposed that at least one resistance cell line is likely to be present in most human tumors. Incurability could be considered with the development of doubly resistent lines; again these could be expected at very minimal tumor masses. This concept of somatic mutation was used as the explanation for observed effectiveness of intensive early chemotherapy and has slowly become the eponymic designation (eg Goldie-Coldman model) applied for intensive multiagent chemotherapy with alternating cycles of non-cross resistant cytotoxic drugs (5,7,8,9,10).

The Goldie-Coldman model has commonly been termed a mathematical one; it is in fact an extrapolative hypothesis from bacterial genetics. Although this model is attractive and very popular, it's specifics have been recently been examined from several aspects. Thus, Kendal and Frost (11) have brilliantly reviewed the problems of translating the bacterial genetic observations, defined by Luria-Delbrück with fluctuation analysis, to somatic cells. They conclude that, at best, this approach may apply to somatic cell genetics in only the qualitative demonstration of the consequences of variation (11). Other related concerns focus on our understanding of tumor cell progression (12), heterogeneity (13) and most importantly that our current knowledge of drug resistance mechanisms in tumor cells shows these to be complex and pathophysiologically different then that of the projected Luria-Delbrück model (14).

In the clinical setting it has been difficult to prove the somatic mutation concept posed by the mathematical model of Goldie and Coldman. The best therapeutic best results using this model of alternating multi-agent combination chemotherapy to date were the initial reports by Bonnadonna and colleagues in the treatment of Hodgkins Disease with alternating courses of MOPP and ABVD (these are combinations of non-cross reactive drugs) (15). Subsequent reports by others have failed to confirm the advantage of this approach (16), sensible though this such a theraputic program appears to be (17). Goldie and Coldman (18) have recently reviewed the clinical trials and concur that the application of their conceptual model has not resulted in any durable data in its support, although they stress that serious examination of the reports of "test(s)" of the theory (ie clinical trials) identifies significant design flaws which can be used to explain the failures (18,19). It must be stressed that the intensive and alternating non-cross-resistant combination chemotherapy approach can and does have many other rationales and will have a significant future in clinical therapy design.

MULTIDRUG (PLEIOTROPIC) DRUG RESISTANCE

Studies over the past decade have now clearly elucidated a variety of biochemical and genetic alterations when chemotherapeutic agents interact with cells. Some of these changes result in resistance to the drug(s) involved. The studies show that resistance to drugs can involve virtually

every biologic and biochemical step at which a drug interacts with a cell and its contents. Extensive effort has been expended to identify and delineate a series of classes of mechanisms that are specific for individual drugs (20). These mechanisms of resistance are highly specific for certain specific individual agents and will be considered later.

The most vexing clinical problem is the recognition that the cancer cells in patients who relapse or recur following successful therapy are usually resistant to the drugs previously effective. In addition and somewhat suprising is that they are often cross-resistant to agents to which they have not been exposed. Our current understanding of this important mechanism of resistance began with a study of Joan Biedler et al (21) on P388 leukemia cells and chinese hamster lung cells that were exposed to the chemotherapeutic agent Actinomycin D. Cells resistant to the Actinomycin grew, and when these selected (resistant) cells were tested they demonstrated cross resistance to several other agents (ie daunomycin, mitomycin, puromycin, vinblastine and vincristine) to which the cells had no previous exposure. In addition, the agents and drugs that showed cross resistance had no evident relationships to one another in terms of structure or function. The development of resistance to drugs with no obvious structural features or cellular targets in common had no biochemical precedent (22).

CHARACTERIZATION OF THE MULTIDRUG RESISTANCE PHENOTYPE:

The term multidrug resistance (MDR) defines the simultaneous expression of cellular resistance to a wide range of structurally and functionally different moieties (23). This "resistance" is variable and relative. A feature, to date unexplained, is increased sensitivity (termed collateral sensitivity) to certain hydrophobic compounds.

Table 1. Relative drug resistance[a]

	Cell line	
Drug	*CH^RC5 (CHO)*	*CEM/VLB_{100} (human)*
Colchicine	180	45
Colcemid	16	NA
Vinblastine	30	420
Vincristine	NA	>600
Doxorubicin	25	110
Daunorubicin	76	120
Gramicidin D	~5000	NA
Melphalan	4–15	NA
Methotrexate	NA	3
1–dehydrotestosterone	0.1	NA
Triton X–100	0.3	NA
Lidocaine	0.1	NA

[a]Relative resistance was calculated as the concentration of drug required to inhibit growth (50% reduction in a 48 h growth assay) in the drug resistant cell line, divided by that required for the same inhibition in the drug sensitive, parental cell line. Cross resistance is denoted by values greater than 1 and collateral sensitivity by values less than 1. NA = not assayed. CH^RC5 is a clonal CHO cell line selected in three discrete steps for resistance to colchicine (Ling and Thompson, 1974). CEM/VLB_{100} is a human leukaemia cell line selected by continuous culture in increasing vinblastine concentration (Beck *et al*, 1979). Data for this table were compiled from Bech-Hansen *et al* (1976), Ling *et al* (1983) and Beck (1984a) (27)

This (relative) resistance has now been shown to a wide range of cytotoxic agents in a large number of mammalian cell lines and transplantable tumors. Commonly, the cell lines show the greatest degree of resistance to the "selecting" agent or analogues of that agent and lesser degrees of resistance to other compounds.

CHEMOTHERAPEUTIC AGENTS TO WHICH CELLS RESISTANT TO MULTIPLE DRUGS ARE RESISTANT OR SENSITIVE (22)

Resistant	Sensitive
Dactinomycin (Actinomycin D)	BCNU (Carmustine)
Daunomycin	Bleomycin
Doxorubicin (Adriamycin)	Cyclophosphamide
Plicamycin (Mithramycin)	Cytarabine (ARA-C)
VP 16 (Etoposide)	Dexamethasone
VM 26 (Teniposide)	
Vinblastine	Methotrexate
Vincristine	Thioguanine

BIOLOGICAL CHANGES IN MULTIDRUG RESISTANT CELLS:

The possible mechanisms whereby "resistance" is conferred on cells was suggested when decreased drug accumulation was shown in these drug resistant cells (24,25,26). The event of decreased drug accumulation in the MDR cell is so consistent that this finding is now considered part of the phenotypic characterization of these cells. Although most of the data suggests that the decreased drug accumulation is the result of an increased capacity to "pump out" of the cell the toxic agents, definitive proof is lacking. Ling, a major pioneer in these studies, has carefully analyzed the physiologic studies relative to this question and concludes that we do not have a simple model to account for this observed reduced drug accumulation in MDR cells (23).

As he has extensively reviewed reduced drug influx, increased drug efflux and altered intracellular drug binding have all been shown under different circumstances (27). He notes that it is exceedingly difficult to visualize a single molecule capable of mediating all of these functions for structurally diverse drugs already identified for the MDR phenotype (27). Recent studies from Ira Pastans laboratory (28) do provide convincing evidence that an energy-dependent drug efflux pump is an (? perhaps the) important basis for the decreased accumulation. These studies show that when multidrug-resistent cell lines are grown in the presence of metabolic inhibitors that deplete intracellular ATP, the drug-resistent cells accumulate the cytotoxic agents to levels approaching the parental drug-sensitive line. Then, when the inhibitors of ATP generation are removed there is a rapid efflux of the cytotoxic agent out of the cell (28).

A second feature common to the multidrug resistant cells are cytogenetic alterations (21). These are usually paired extrachromosomal elements, called double minutes (DM). Their presence provide structural evidence of a gene amplified state, generally considered to be unstable (29). A second alteration, which appears related to the double minutes are chromosomes expanded in length with homogeneously staining regions (HSR) the HSR may be the complement DM they also represent evidence of gene amplification, albeit it more commonly a stable amplification (29).

The third feature of the multi-drug resistant cell is the presence of a cellular membrane alteration with the recognition of a high molecular weight (170k Da) glycoprotein, commonly termed P-glycoprotein, a name generated by it's association with the pleiotropic MDR phenotype (23, 25, 27). Although a great number of other membrane changes are seen in neoplastic cells, the most outstanding common denominator in MDR cells is the increased expression of this cell surface glycoprotein. Some variation in the size of this glycoprotein has been observed (range 140-220 k Da) depending upon the system used for size analysis.

MOLECULAR BIOLOGY AND FUNCTIONAL ASPECTS OF P-GLYCOPROTEIN

Comparative analysis of the MDR-associated glycoproteins identified by a variety of investigators from several multidrug resistant cell lines have shown them to be conserved in molecular size and antigenic structure. Thus, homologous P-glycoproteins are expressed in MDR cells of a variety of different origins (23).

Table 2. Positive identification of P-glycoprotein overexpression in multidrug resistant cell lines[a]

Cell type		*Selecting drug*	*References*
Chinese hamster:			
CHO	(fibroblastoid)	Colchicine*	Ling and Thompson (1974) Kartner *et al* (1985)
CHO	(fibroblastoid)	Daunorubicin*	Kartner *et al* (1983a,b, 1985)
CHO	(fibroblastoid)	Auromomycin	Rauscher *et al* (1984) Rauscher and Kartner (unpublished)
CHEF	(fibroblastoid)	Vincristine	Kuo *et al* (1982) Kartner *et al* (1985)
DC-3F	(fibroblastoid)	Actinomycin D	Biedler and Riehm (1970) Gerlach and Ling (unpublished)
V79	(fibroblastoid)	Doxorubicin*	Howell *et al* (1984) Gerlach and Ling (unpublished)
Syrian hamster:			
Cl_2TSV_5	(SV40-transformed)	Actinomycin D	Langelier *et al* (1974) Kartner *et al* (1985)
Mouse:			
L cell	(fibroblastoid)	Colchicine*	Debenham *et al* (1982) Kartner *et al* (1985)
S180	(sarcoma)	Doxorubicin*	Siegfried *et al* (1983) Kartner *et al* (1985)
B16	(melanoma)	Doxorubicin	Ganapathi (personal communication) Bell and Ling (unpublished)
UV-2237	(fibrosarcoma)	Doxorubicin	Giavazzi *et al* (1983, 1984) Kartner *et al* (1985)
P388	(leukaemia)	Doxorubicin	Shoemaker *et al* (1985)
P388	(leukaemia)	Vinblastine	Shoemaker *et al* (1985)
P338	(leukaemia)	Actinomycin D	Shoemaker *et al* (1985)
Human:			
CCRF-CEM	(leukaemia)	Vinblastine*	Beck *et al* (1979) Kartner *et al* (1985)
RPMI 8226	(myeloma)	Doxorubicin	Dalton *et al* (1985) Gerlach and Ling (unpublished)
MES-SA	(sarcoma)	Doxorubicin	Harker and Sikic (1985) Gerlach and Ling (unpublished)
HeLa/KB	(carcinoma)	Colchicine	Akiyama *et al* (1985) Gerlach and Ling (unpublished)

[a]Plasma membrane isolation (Riordan and Ling, 1979) followed by SDS-PAGE and Western blot analysis (Debenham *et al*, 1982; Kartner *et al*, 1983b) using P-glycoprotein specific monoclonal antibody (Kartner *et al*, 1985) was used to identify P-glycoprotein in the cell lines listed. Positive identification required both distinct labelling with the monoclonal antibody and a position corresponding to a Mr of ca 170 000

*A series of sequentially selected cell lines with increasing drug resistance was tested. The relative amount of P-glycoprotein correlated with the degree of drug resistance in each case.

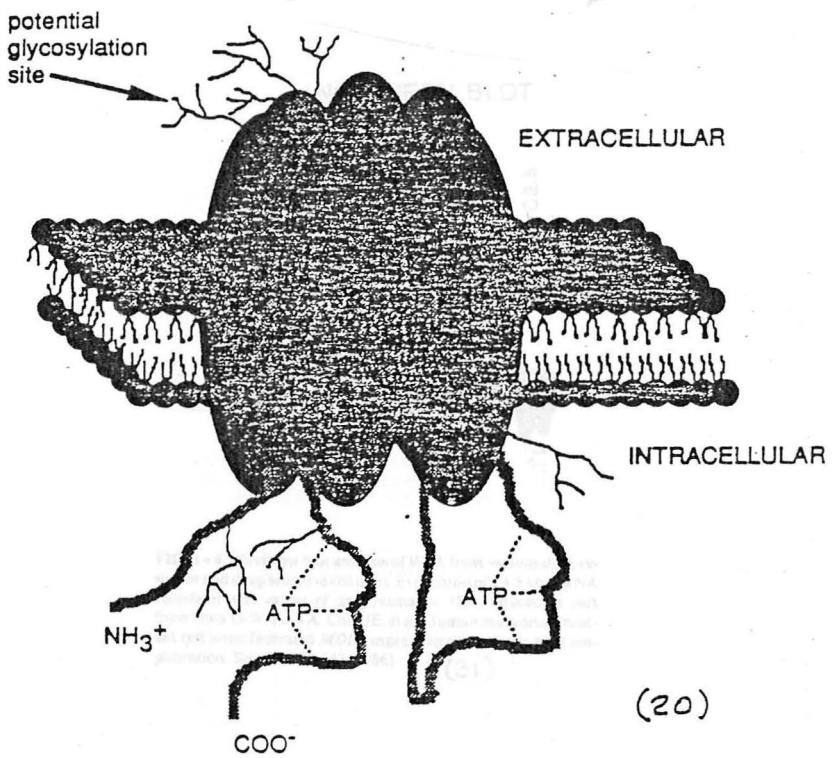
(23)

A variety of well established features now help define the P-glycoprotein:

1.] The level of P-glycoprotein expression is always elevated over drug sensitive parental levels by an amount consistent with the degree of drug resistance.

2.] It behaves as an intrinsic membrane protein.

Schematic Representation of P-glycoprotein



(20)

3.] It has drug binding ability.

4.] Calcium channel blocking agents can bind to the P-glycoprotein and can compete with cytotoxic agents for binding to the P-glycoprotein (30).

5.] A cDNA clone has been identified that contains an insert of approximately 600 base pairs that code for a portion of the (in the CHO cell) P-glycoprotein polypeptide. The insert was shown to code for three different epitopes of P-glycoprotein firmly establishing its identity. Northern blot analysis using the c DNA insert revealed a 4.7 kb m RNA specific to multidrug resistant cells. The size of this m RNA was consistent with the molecular size of the P-glycoprotein polypeptide, and the amount of m RNA expressed correlated with the level of P-glycoprotein expression in all cell lines of different degrees of multidrug resistance (23,27,30,31).

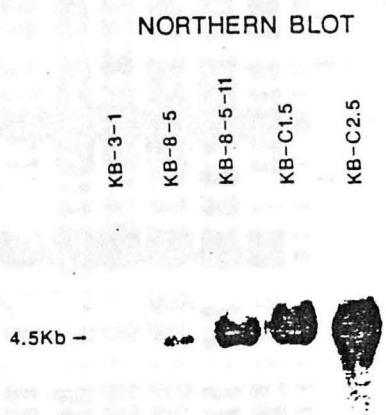


FIG. 1-4 Northern blot analysis of RNA from various drug-resistant and drug-sensitive cell lines. Expression of a 4.5 kb mRNA correlates with extent of drug resistance. (Reproduced in part from Shen D-W, Fojo A, Chin JE, et al: Human multidrug-resistant cell lines: Increased *MDR1* expression can precede gene amplification. Science 232:643, 1986). (31)

FIG. 1–3 In-gel renaturation analysis used to detect amplified genes in human KB multidrug-resistant cells. Bands in the autoradiograms *(left side)* represent amplified sequences in various KB cell lines selected in colchicine (C4), adriamycin (Ad), or vinblastine (Vb) and in parental (3-1) and revertant (R) lines. The schematic representation *(right side)* shows the novel bands amplified in multidrug-resistant cells and the bands amplified in common (Com) in the three different multidrug-resistant cell lines examined. (Fojo AT, Whang-Peng J, Gottesman MM, Pastan I: Amplification of DNA sequences in human multidrug-resistant KB carcinoma cells. Proc Natl Acad Sci USA 82:7661, 1985) (31)

6.] When the P-glycoprotein specific cDNA was used to probe Southern blots of genomic DNA from drug sensitive cell lines, amplification of the genomic DNA sequences coding for the P-glycoprotein was seen.

7.] Of interest is that the number of bands seen in Southern blots are greater than expected (in light of the small size of the cDNA probe). This has suggested that there are a number of different genes that code for the P-glycoprotein. That is, P-glycoprotein appears to be coded for by a multigene family (23,32,33).

8.] Transfer of genetic material encoding the P-glycoprotein by transfection (and a variety of other methods) has shown that the MDR phenotype can be transferred to otherwise normal cells (30, 34, 35, 36). This ability of a defined sequence of DNA to confer resistance on otherwise normal cells is strong evidence that the gene(s) responsible for multidrug resistance has been identified (37); this is now commonly termed the mdr 1 gene.

9.] Using a human gene probe (pMDR1) it has been possible to show gene amplification was directly related to levels of drug resistance.

Table 2. Expression and Amplification of the *mdr*1 Gene in Cell Lines with Increasing Levels of Drug Resistance.

Cell Line*	Relative Resistance to			Relative Amplification of *mdr*1 Gene	Relative Level of *mdr*1 RNA Expression
	colchicine	doxorubicin	vinblastine		
KB-3-1	1	1	1	1	1
KB-8	2.1	1.1	1.2	1	14
KB-8-5	4	3	6	1	42
KB-8-5-11	40	23	51	7	1,100
KB-C1	260	160	96	10	3,800
KB-C6	2100	320	370	80	11,000
KB-C1-R1†	6	3	4	1	14

*Cell lines were selected by culturing them in increasing concentrations of colchicine. The amount of *mdr*1 DNA and *mdr*1 RNA was determined with an *mdr*1-specific gene probe as described in the text. Data are from Shen et al.[23] and Fojo et al.[26]

†KB-C1-R1 is a revertant obtained by culturing KB-C1 in medium without colchicine.

10.] Current data is consistent with the suggestion that mdr-1 gene encodes the P-glycoprotein (37,38). The expressed human polypeptide contains 1280 amino acids with a calculated molecular weight of approximately 140 kDa. These studies have led to further delineation of the putative structure.

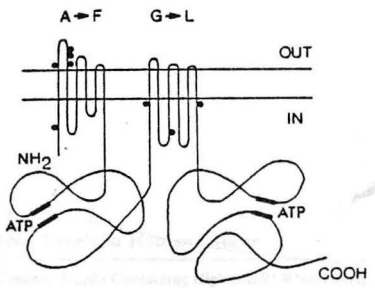


Figure 3. Schematic Representation of the Putative Structure of the *mdr* Protein and Its Orientation in the Cell Membrane

Putative N-linked glycosylation sites (closed circles), putative transmembrane domains, ATP-binding folds and amino and carboxyl termini are indicated.

(38)

11.] A sensitive way to examine the expression of mdr 1 gene is to extract RNA from the given tissue and measure the level of mdr1-RNA (using a ^{32} P-labeled cDNA from the mdr 1 gene).

These have been done in normal tissues.

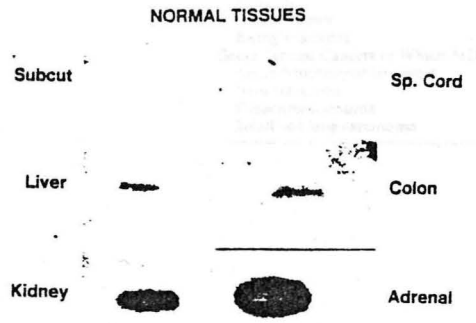


Table 1–4
Levels of *MDR1* RNA in Normal Tissues (31)

High	Low
Liver	Bone marrow
Colon	Spleen
Small intestine	Ovary
Kidney	Skeletal muscle
Adrenal cortex	Heart muscle
Adrenal medulla	Prostate
Lung (2/7)	Stomach
	Nervous tissue
	Lung (5/7)

and in a variety of human tumors:

Table 1–5
***MDR1* RNA Levels in Human Cancer**

Untreated Cancers Usually Containing High *MDR1* RNA Levels
Colon adenocarcinoma
Kidney adenocarcinoma
Adrenal cortical carcinoma
Some pheochromocytomas
Untreated Cancers Usually Containing Low *MDR1* RNA Levels
Acute lymphocytic leukemia
Acute nonlymphocytic leukemia
Chronic myelogenous leukemia
Hodgkin's lymphoma
Nodular lymphoma
Burkitt's lymphoma
Ovarian cancer
Prostate cancer
Bladder cancer
Breast cancer
Squamous cell lung carcinoma
Small cell lung carcinoma
Neuroblastoma
Head and neck cancer
Melanoma
Wilms' tumor
Ewing's sarcoma
Some Treated Cancers in Which *MDR1* RNA Levels May Rise
Acute lymphocytic leukemia
Neuroblastoma
Pheochromocytoma
Small cell lung carcinoma

(31)

PHYSIOLOGIC ROLE FOR P-GLYCOPROTEIN:

The exact physiologic role for P-glycoprotein is not known. Its biologic importance is certainly suggested from its strong sequence homology with several well-characterized bacterial proteins that function in the transport of molecules through the bacterial cell membrane (38,39,40).

Bacterial Transport Proteins with Homology to P-glycoprotein

Protein	Transport System	Organism
oppD	oligopeptides	S. *typhimurium*
mal K	maltose	E. *coli*
his P	histidine	S. *typhimurium*
pst B	phosphate	E. *coli*
hly B	hemolysin	E. *coli*

(20)

Evidence supports its role as cellular export pump (20,23,37,41,42), although how a single molecule could effectively handle the large number of structurally diverse drugs has been difficult to delineate. The nucleotide sequence of the P-glycoprotein gene indicates that the protein has eight potential N-linked glycosylation sites and one possibility is that functional heterogeneity is related to the glycosylation of this protein (20). Alternatively, the recognition of a multigene family of P-glycoproteins suggests different forms may interact with different agents (43,44).

An interesting biochemical alteration also suggests an important physiological role and, in addition, has focused current interest in approaches to modify the "malfunction"defined as the development of drug resistance during therapeutic intervention. P-glycoprotein is rapidly phosphorylated and dephosphorylated in the cell membrane (45,46). Of particular interest is that phosphorylation appears to be a calcium dependent process and the dephosphorylation cycle can be blocked by metabolic inhibitors which are now known to also abolish the MDR phenotype (23,45,47,48).

Croop, et al (37) have emphasized that the high degree of evolutionary conservation of genes in the mdr-1 (P-glycoprotein) gene family between rodents and primates suggests that the function of this gene family has been under strong selective pressure during evolution. Foja et al (49) have analyzed total mdr-1 RNA in slot blots from a variety of human tumors using a probe from mdr cDNA.

Table 3. Expression of *mdr*1 RNA in Normal Tissues and Tumors.*

Normal Tissue	Untreated Cancer	Cancers That Become Resistant to Drugs†
	HIGH EXPRESSION OF *mdr*1	
Adrenal	Pheochromocytoma‡	Pheochromocytoma‡
Colon	Adrenal cortex	Acute lymphocytic leukemia
Kidney	Colon cancer	Neuroblastoma
Liver	Kidney cancer	
Lung (2/7)‡		
	LOW EXPRESSION OF *mdr*1	
Bone marrow, spleen	Leukemias	
Skin, subcutaneous tissue	Breast cancer	
Lung (5/7)‡	Ovarian cancer	
Skeletal and heart muscle	Thyroid cancer	
Prostate	Neuroblastoma‡	
Ovary	Pheochromocytoma‡	
Stomach	Adrenal cortex	

*The level of *mdr*1 RNA was determined by hybridization with an *mdr*1-specific gene probe. Data are from Fojo et al.[28]

†Doxorubicin, vinblastine, or dactinomycin.

‡Some untreated cancers of the same histologic type and different normal lung samples fell into both the high and low groups.

(22, 48)

Although mdr expression was shown to vary from sample to sample, significant tissue differences are evident.

An interesting feature relative to function is the immunocytochemical localization of mdr-P-glycoprotein to the luminal surface of tissues involved in secretary function. Nevertheless, to date the normal substrates for function of the P-glycoprotein are unknown.

Finally, it should be emphasized that in circumstances of gene amplification linked genes which may be otherwise irrelevant to MDT can be co-amplified in the DM's and HSR's of the multidrug resistant cells (23). Thus, its still somewhat difficult to be certain which changes in the MDR cells are secondary and which change is primary (50,51).

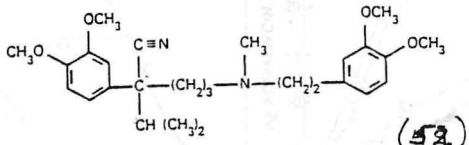
PHARMACOLOGIC MANIPULATION OF P-GLYCOPROTEIN

It is now clear that multidrug resistance can be overcome in experimental systems by a variety of agents that are not cytotoxic (31).

Table 1-2
Agents that Overcome Multidrug Resistance

Verapamil	Trifluoperazine
Desmethoxyverapamil	Chlorpromazine
Diltiazem	Chloroquine
Quinidine	SDB-ethylenediamine
Thioridazine	Reserpine (31)

As is evident these agents do not common structural relationships, although they do have a complex ring structure and hydrophobic properties. An early attractive hypotheses was generated by the evidence that verapamil (47,48) and other calcium channel blocking agents were effective at reversing the MDR phenotype.



(52)

FIG. 6–3 Structure of verapamil.

Table 6-6
Effect of Verapamil on Drug Cytotoxicity in Human Tumor Cell Lines

Cell Line	Drugs Studied*	Dose-Modifying Factor† with Verapamil	Reference
Leukemia Cell Lines			
K562 myelogenous leukemia	VCR, ADR	VCR-4; ADR-2	200
K562/VCR	VCR, ADR	VCR-100; ADR-5	200
CEM/VLB lymphoblasts	VLB, VCR, ADR	VLB-87; VCR-156; ADR-4	201
GM3639 T-cell ALL	VCR	VCR-2	202
Solid Tumor Cell Lines			
Ovarian cancer: *in vitro* acquired resistance			
1847^{AD}	ADR	ADR-6	26
2780^{AD}	ADR	ADR (3–6 depending on dose of verapamil)	26
Cell lines from refractory patients			
OVCAR-2	ADR	ADR-2.4 (requires high concentration of verapamil—3000 ng/ml)	26
OVCAR-3	ADR	ADR-1.5	26
OVCAR-4	ADR	ADR-4.3	26
OC cell line	ADR	No effect	203
Breast cancer			
MCF-7 pleiotropic resistance induced with colchicine	ADR	No effect	204
Sarcoma			
MES-SA	ADR	No effect	205
MES-SA Dx_{5-0}	ADR	ADR-5	205

(52)

* VCR, vincristine; ADR, adriamycin; VLB, vinblastine.

† Dose-modifying factor is the ratio of drug concentration producing 50% survival/concentration of drug producing 50% survival in the presence of verapamil.

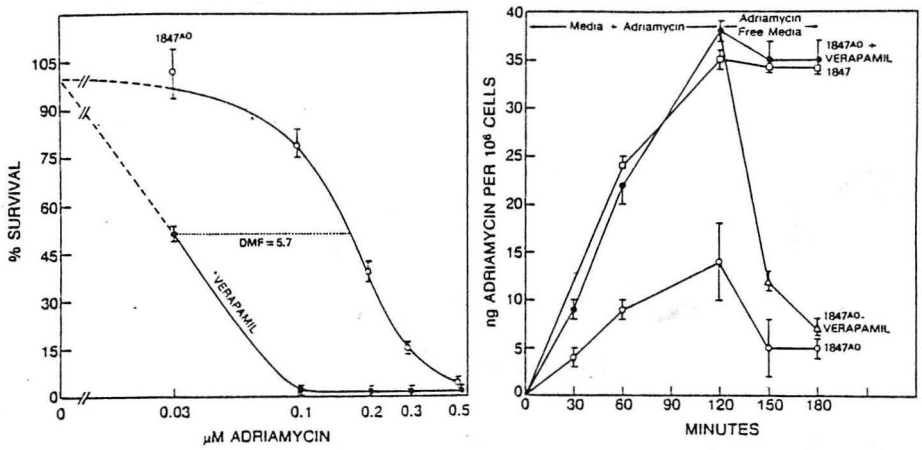


FIG. 6–4 (*Left*) Dose-response curves for Adriamycin-resistant cell line 1847^{AD} with and without verapamil. The dose modifying factor (*DMF*) with verapamil was 5.7 and completely restored sensitivity to Adriamycin. (*Right*) Accumulation and efflux of Adriamycin. The 1847 Adriamycin-sensitive cell line (– □ –) accumulated three times more Adriamycin than did the resistant variant 1847^{AD} (– ○ –). In the presence of verapamil (– ● –), 1847^{AD} accumulated the same amount of Adriamycin as did 1847. When the cells were placed in drug-free media, there was rapid efflux of Adriamycin from 1847^{AD} (– △ –); however, the efflux was markedly reduced when verapamil was present in the Adriamycin-free efflux media (– ● –).

(52)

Although studies such as these led to the thesis that drug resistance could be suppressed by inhibition of the high voltage calcium channels, it is now clear that no relationship exists between a drugs ability to suppress the MDR phenotype and its calcium channel effect (31, 52). However, all agents capable of this change do produce an increase in intracellular drug content (22,31).

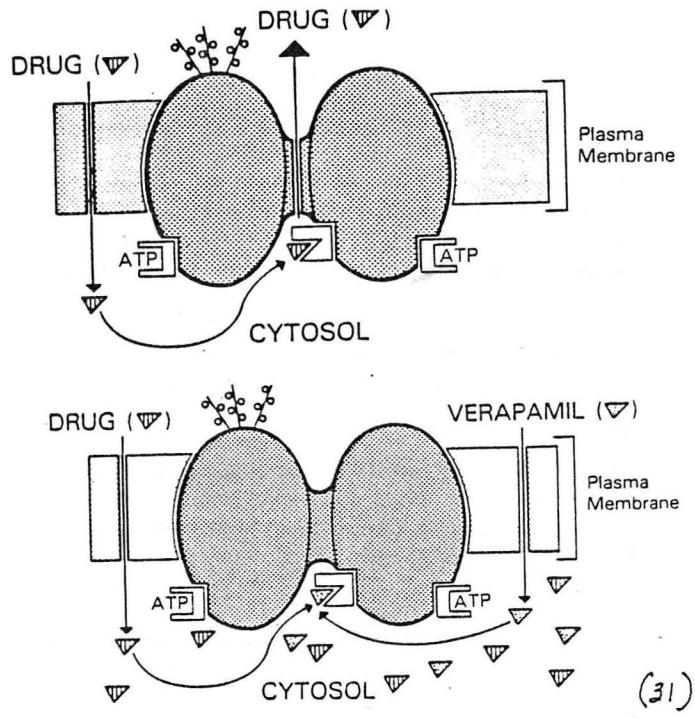


FIG. 1-6 Model of how P-glycoprotein might be involved in transporting cytotoxic drugs, such as vinblastine, out of cells *(A)* and how drugs such as verapamil that reverse multidrug-resistance complete with vinblastine to block the pump *(B)*.

For instance, recent studies on the modulation of protein kinase C activity have shown that drug transport and accumulation in breast cancer cells was reduced with stimulation of protein kinase C; that is, MDR was induced by an increase in enzyme activity (53).

CLINICAL IMPLICATIONS AND THERAPEUTIC STRATEGIES FOR THE MULTIDRUG RESISTANCE PHENOTYPE

The exact clinical implications of the MDR phenotype are not certain. Although all of the clinical studies to date suggest that it is the primary issue in the so called intreatable relapse; that is generally defined as disease transformed by therapy and then resistant to it. The following comments summarize the important issues:

1.] Although mechanisms other (see below) than the development of multidrug resistance certainly are factors in some circumstances of chemotherapy failure, at the experimental and clinical level MDR appears to be the most important mechanism in drug resistance that is acquired (20,31,52,54,55).

2.] From the measurements of mdr-1 gene expression in de novo tumors (and tissues), it appears that those human cancers that are inherently resistant to chemotherapy (ie resistant defined as occuring before exposure to any agents and, in general, non responsive to therapy) are those with relatively high activity. This certainly suggests that their primary resistance to therapy may be the result of existent MDR phenotype (31,49).

3.] Several authors have proposed a variety of therapeutic strategies, in the past few years (56-66). However, only a few studies have actually be done:

a.] Some studies purporting to focus on the MDR status, have employed drugs and drug sequences known to be irrelevant in terms of this mechanism of resistance (67,68,69). Some begin with unbridled enthusiasm and then provide "satisfactory responses" (91).

b.] In a few studies clear failure of the strategy appears evident (70). The best published failure (reported as a prospective study in abstract form and then as a full report) from the N.C.I. examined ovarian cancer responsiveness and utilized verapamil as an approach to circumvent MDR development. Unfortunately, they failed to measure the mdr-1-RNA levels in the tumors prior to therapy; when this was subsequently done the tumors were all low levels of activity. In addition, the levels of verapamil (dictated by the data on its cardiovascular toxicity) were too low to even expect a response (72). A Japanese study used diltiazem in children with drug refractory acute lymphocytic leukemia and demonstrated cytoreduction drug response to vincristine (a drug well known to be affected by the MDR phenotype) in 4 of the 5 patients (73). A recent study by Alexanian (74) used an antibody to the cytoplasmic domains of the P-170 protein to measure activity in 22 patients with multiple myeloma. In the previously treated and presently drug resistant patients the "MDR cell proportion" showed good correlation with the clinical presence of drug resistance. In addition, 2 patients who had never been treated demonstrated high levels of MDR; they failed on primary induction chemotherapy.

c.] Clear characterization of dose response for the agents with probable efficacy in affecting MDR development has not been done. For instance, most of the in vitro murine leukemia studies have used verapamil concentrations of 1 to 3/ug/ml which us significantly higher then the peak plasma levels (400-500 ng/ml) that were measured by David Hillis et al (75) in cardiac patients. Although the maximal tolerated dose of verapamil in non-cardiac patients has not been established, the required levels appear to pose risks of cardiovascular toxicity, especially arrhythmias.

Important recent data suggests that quinidine may be more likely to provide a response within dose ranges that are relatively safe (31).

Thus, from the data now available it does appear that MDR is a very important mechanism in cancer chemotherapy treatment failure and that the true test of strategies to circumvent or alter this failure have not yet been done. Pastan has recently reviewed other potential routes to reverse or alter mulitdrug resistance with monoclonal antibodies since the P-glycoprotein is located on the plasma membrane and is at least partially exposed (31). Other pharmacologic approaches also have shown possible roles in limiting the development of multidrug resistant tumor cells. These include agents that perturb the lipid structure or permeability of the plasma membrane (eg non-ionic detergents, anesthetics, amphotericin B), inhibitors of energy metabolism, non-toxic drug analogues of cytotoxic agents, and a variety of calcium channel blockers and calmodulin antagonists (23).

In addition, it must be stressed that the important observations and our knowledge relative to multidrug resistance span but a few years. It clearly represents a family of genes and our understanding of the multiple forms is just beginning. Certainly it will not be the only form of such multiagent resistance and the current data should be looked at as part of a new chapter in our understanding of drug delivery. Such strategies will require prospective and serial measurement of mdr-1 activity, since considerable tumor to tumor variation is well known (49). The strategy may invoke complex pharmacologic interactions, since it appears that a multigene complex is involved. Thus, each member of the complex may have unique functions (37,76), and in fact may even express increased (so called collateral) sensitivity. Nevertheless, clinical models can now be constructed and approached with rational measurements and different drug sequence for the first time.

OTHER MECHANISMS OF ACQUIRED DRUG RESISTANCE

In this current era of multiagent chemotherapy, clearly multidrug resistance appears to have the greatest clinical relevance. Nevertheless, extensive studies of the pharmacologic factors involved in drug resistance have demonstrated that certain drugs are associate with the development of resistance for that specific individual drug. Examination of biochemical and

cellular mechanisms for such specific drug resistance have provided great insight in clinical oncology (52). It should be emphasized that this form of acquired drug resistance is one specific to a single agent without evidence of cross-resistance or cross-resistance or collateral sensitivity.

Mechanisms of Drug Resistance

Mechanism	Drug
Alterations in drug uptake (carrier mediated)	MTX Melphalan Methchlorethamine
Alterations in intracellular drug accumulation	Doxorubicin Vinca alkaloids Dactinomycin
Decreased drug activation	Cytarabine 5-fluorouracil 6-mercaptopurine 6-thioguanine MTX
Increased metabolic inactivation	6-mercaptopurine 6-thioguanine Cytarabine Alkylators Cisplatin
Alterations in target proteins	MTX Steroid hormones Vinca alkaloids FUDR
Alterations in cellular metabolism	Cytarabine 5-fluorouracil MTX 6-mercaptopurine
Alterations in cofactor levels	6-thioguanine FUDR
Alterations in cellular repair mechanisms	Alkylators Nitrosoureas
Increased levels of target proteins	MTX Phosphonoacetyl-l-Aspartate FUDR Deoxycoformycin

(52)

Pharmacologic Factors Involved in Relative "Drug Resistance" (Ineffective Drug Concentrations at Site of Tumor)

1. Variations in drug bioavailability
2. Variations in drug metabolism
3. Variations in drug elimination
4. Presence of tumor in inaccessible sanctuary sites
5. Excessive host toxicity
6. Limited drug diffusion
7. Differences in cell kinetics
8. Variation in salvage factors

(52)

For many agents, gene amplification is now clearly identified as the basic mechanism of acquired drug resistance (29).

Gene Amplification and Drug Resistance

Selection	Protein Overproduced	Gene Amplification Demonstrated
MTX	DHFR	+
PALA	CAD protein	+
FUDR	Thymidylate synthase	+
Deoxycoformycin	Adenosine deaminase	+
Hydroxyurea	Ribonucleotide reductase	
Dimethylfluoro-ornithine	Ornithine decarboxylase	+
Cadmium	Metallothionein	+
Vinca alkaloids	170K glycoprotein	
Dactinomycin	150K glycoprotein 19K protein	
Compactin	Hydroxymethylglutaryl-CoA reductase	+
Methionine sulfoximine	Glutamine synthetase	+
Pyrazofurin, azauridine	UMP synthase	+

(52)

INHERENT DRUG RESISTANCE

Unifying theories of disease are always attractive! Although it is quite clear that we are only beginning to understand the mdr family of genes, measurements of gene expression in untreated neoplasms in man have provided some provocative data (49). The evidence is clear that in normal tissues the mdr 1 gene is expressed at very high levels in adrenal and kidney and at high levels in lung, liver, jejunum, colon and rectum. This tissue pattern distribution has led many to consider this as evidence that the mdr gene complex is a mechanism for handling a wide variety of "toxic exposures". This rationale has been emphasized by the immunocytochemical localization of the mdr P-glycoprotein to sites where contact with potential toxins is realistic (eg on biliary front of hepatocytes, the apical surface of epithelial cells in the biliary ducts, in the proximal tubules of the kidney, and on the luminal surfaces in colon and small intestinal epithelium.

New untreated cancers were examined and the elevated levels in colon and kidney led Pastan and coworkers (49) to propose that increased expression of the mdr-1 gene (and possibly other members of that family) might be involved in the known intrinsic resistance of these tumors to chemotherapeutic agents. Other studies in untreated tumors over the past year have further supported view.

ANALOGY BETWEEN CHEMICAL CARCINOGENESIS AND MULTI DRUG RESISTANCE

Cowen and coworkers (20,30,77) have developed an interesting hypotheses that relates multi-drug resistance to chemical carcinogenesis. The Solt-Farber model (78) of carcinogenesis is a two step process with an irreversible initiation event followed by promotion this model is one in which a rat is exposed to a chemical carcinogen (and at least 40 different chemicals been now been characterized) for a two week period, and then liver cell proliferation is stimulated by partial pepatectomy. Within a few weeks hyperplastic nodules develop, some of which progress to hepatocellular carcinoma. Farber (79) had noted the relationship of many of the carcinogens to cytotoxic drugs and had predicted that the hyperplastic nodular cells had been selected for resistance, a phenomena now clearly documented. In addition, the hyperplastic nodules show cross-resistance to a wide range of hepatotoxic agents. These agents lack structural or functional similarity. In addition, the biochemical basis and sequence of this carcinogen-induced resistance has now been defined (77).

Biochemical Changes Associated with the Solt-Farber Model

Altered accumulation of a range of toxic compounds
Phase 1 — enzymes of activation
↓ Cytochromes P450 and b_5
↓ Expression and induction of four mixed-function oxidases, including aryl hydrocarbon hydroxylase
Phase 2 — enzymes of conjugation
↑ UDP-glucuronyl transferase 1
↑ Anionic glutathione transferase
↑ Glutathione
↑ γ-Glutamyltransferase
↓ Sulfotransferase
Other changes
↑ Epoxide hydrolase
↑ DT-Diaphorase
↓ Ferritin and hemosiderin
↑ Pentose phosphate activity (77)

This complex biochemical change has been carefully reviewed by Cowan et al (77). As he has shown this transformation does not appear to be the product of a specific adaptation (or sequence of mutations) to a single selecting toxin. The phenotype that develops does so within two weeks of exposure and it can result from exposure to a wide variety of unrelated agents. Of particular interest is that the phenotype is reversible, since more than 90% of the nodules regress if no further injury occurs. This phenomenon can be considered a programmed, well-coordinated cellular response to a broad range of toxins. One can even consider the response to such exogenous toxins as a mechanism that may provide survival advantage, since each of the biochemical changes noted appear to have potentially significant functions in protecting cells from foreign toxins.

Cowan, et al (77,80,81) explored the relationships common to the Solt-Farber model and a multidrug resistant human breast cancer cell line (MCF-7) that initially developed against Adriamycin. Remarkably parallel changes can be seen in the properties in these two experimental systems.

Parallel Between Changes Observed in Drug Resistance and Chemical Carcinogenesis

	Carcinogen Resistance	MCF-7 Multidrug-Resistant Cell Line
↓ Toxin uptake	Yes	Yes
↑ P170	Not done	Yes
↑ Role for protein kinase C activity	Yes	Yes
↑ P20	~~Not done~~ Yes	Yes
↓ P450 isozymes	Yes	Yes, aryl hydrocarbon hydroxylase
↑ Anionic glutathione transferase	Yes	Yes
↑ Pentose phosphate pathway activity	Yes	Yes
↑ UDP-glucuronyl transferase 1	Yes	Yes
↑ Glutathione	Yes	No change
↑ γ-Glutamyltransferase	Yes	No change
↓ Sulfotransferase	Yes	No, ↑ twofold
↑ Epoxide hydrolase	Yes	Not done
↑ DT diaphorase (quinone reductase)	Yes	No, ↓ 50%
↓ Ferritin	Yes	Not done (77)

Again the principle changes in both systems are that exposure to a single toxin confers resistance to that agent and cross-resistance to a variety of other agents. Resistance is associated with a decreased intracellular accumulation of the toxin. An overexpression (3 to 12 fold) of the P-glycoprotein gene has now been shown in the hyperplastic nodules of the rat (20) just as had previously been seen in multi-drug resistant cells. Of course, this analogy between chemical carcinogenesis and MDR raises the possibility that during chemical carcinogenesis the resultant tumor cells may be "programed" to be multidrug resistant. Perhaps then, these findings further define a biochemical basis for the intrinsic chemotherapy resistance of some of the carcinomas in man (eg lung, colon, kidney) that may be induced by chemicals.

CONCLUSIONS

There has been a significant decrease in the development of new cancer chemotherapeutic agents during the past decade. The present data suggests that the absence of a great new enlarged arsenal of drugs may not be as serious a deficit as previously projected. Instead, it appears that the most serious deterrent to cancer chemotherapy efficacy and improved cure rates of cancer is the presence or development of drug resistance. It seems very appropriate that current research has intensively focused upon the occurrence of drug resistance seen during the chemotherapy of cancer. Such resistance has been frustrating to the clinician because of what often appears to be a splendid early response to therapy deteriorates into an evident losing battle. The evidence of drug resistance to a variety of agents the tumor has "not yet seen" typifies multi-drug resistance in the clinical setting.

As we've reviewed, this type of resistance is complex. The current evidence suggests that multiple genes are involved, thereby promising new information as well as correlative observations. Nevertheless, the present data has provided tools to allow the exploration of a variety of new treatment strategies. The translation of this data to patient care is already underway. The extrapolation of even these initial observations to inherent chemotherapy drug resistance and chemical carcinogenesis promises exciting clinical opportunities.

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