Jpn. J. Cancer Res. (Gann)
79, 285–296 ; March, 1988

REVIEW

MECHANISMS OF MULTIDRUG RESISTANCE AND IMPLICATIONS FOR THERAPY

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INTRODUCTION

One of the major problems in cancer chemotherapy is the development of drug resistance during treatment. The nature of drug resistance in cancer patients is complex. One reason for the clinical resistance is the metabolic inactivation, or excretion of antitumor agents by the liver, kidney and other organs. In addition, it has been found that tumor cells can acquire resistance to anticancer drugs. It is generally accepted now that drug resistance at the cellular level (cellular resistance) is also an important mechanism of drug resistance in patients.

There are two types of cellular resistance. One is the innate (natural, *de novo*) resistance, and the other is the acquired resistance to antitumor agents. Colon cancer, renal cancer, gastric cancer and other solid tumors are known to respond only marginally to antitumor agents. This type of cellular resistance is classified as innate (natural) drug resistance. During the treatment of tumors with antitumor agents, tumor cells can acquire resistance to the drugs. This type of resistance is classified as acquired drug resistance. Innate and acquired drug resistances are major factors limiting the clinical use of antitumor agents. A common mechanism is now speculated to be involved in these two resistances, as described later. The elucidation of the resistance mechanisms has progressed well recently owing to the application of cellular and molecular techniques in addition to the usual biological and biochemical techniques. In this article, I describe the mechanisms of cellular resistance, especially those of multidrug resistance at the molecular level, and I also discuss possible approaches to overcoming drug resistance.

Multidrug Resistance and Its Biochemical Nature

When tumor cells acquire resistance to naturally occurring antitumor agents such as vinca alkaloids or anthracyclines, they generally show cross resistance to other antitumor agents having different structures and different modes of action. This type of resistance has been widely and generally observed in various experimental tumors, and is called "pleiotropic drug resistance."[1-4] Pleiotropic drug-resistant tumor cells bear similar cytogenetic and biochemical changes which are related to the resistant phenotype as follows.

(1) Cytogenetic alterations including double minute chromosomes and homogeneously staining regions have been reported for drug-resistant tumor cells of rodent origins.[5,6] We also detected double minute chromosomes and homogeneously staining regions in the chromosome spreads of human K562 myelogenous leukemia resistant to adriamycin (K562/ADM).[7] These cytogenetic alterations are considered to be related to the

amplification of drug-resistance genes. Gene amplification related to multidrug resistance has been observed in rodent tumors.[8]

(2) These cells are often also cross-resistant to unrelated drugs. Anthracycline-resistant tumor cells are resistant not only to other DNA-interacting drugs but also to mitotic spindle poisons, protein synthesis inhibitors, and other structurally unrelated compounds.[9-17]

(3) These resistant tumor cells have defects in intracellular drug accumulation and retention. In vincristine- and adriamycin-resistant tumor cell sublines, these agents can be shown to enter the cells but are actively transported to the outside.[12, 15, 18, 19] This results in a relatively low intracellular level of the drugs and thus low cytotoxicity.

(4) Pleiotropic drug-resistant tumor cells possess biochemical changes in the plasma membrane components. An overproduction of a 170 kilodalton (kDa) glycoprotein has been observed widely in rodent tumors resistant to colchicine.[20-22] Human leukemia resistant to vinblastine or adriamycin also expressed this type of protein in the plasma membrane.[23, 24] We observed amplified glycoproteins with molecular weights of 180 and 85 kDa in the plasma membrane of K562/ADM cells, the amounts of which are related to the extent of resistance.[7] Amplification of the genes corresponding to the 170 kDa protein was detected in resistant cells.[25]

(5) Pleiotropic drug-resistant tumor cells possess biochemical changes in cytoplasmic components. Among these changes, the most prominent is the appearance of 19–22 kDa protein in the cytoplasm.[26-28] This protein is a calcium-binding protein and has a homology with calpain. The function of this protein, however, remains to be solved.

(6) Recently, it was found that the calcium content of pleiotropic drug-resistant tumor cells was higher than that of the parent tumor lines. P388 and K562 leukemia cells resistnat to vincristine or adriamycin contained more calcium in the cells and on the cell surface than the parental cells.[29, 30] Similar results were obtained with Chinese hamster ovary cells resistant to colchicine. However, the calcium content of P388 cells resistant to 5-fluorouracil was almost the same as that of the parent P388 cells. The calmodulin contents of pleiotropic drug-resistant tumor lines and a 5-fluorouracil-resistant tumor line were almost the same as those of the corresponding parent lines.[29] The isolated plasma membrane of K562 cells resistant to vincristine contained approximately 1.5-fold more calcium than the parent cells.[29] The higher cellular calcium content might be a characteristic phenotype of pleiotropic resistant tumor lines.

(7) Finally, calcium channel blockers and calmodulin inhibitors can inhibit the outward transport of antitumor agents, leading to a high concentration of antitumor agents in resistant cells, and thereby overcoming the resistance.[19, 31-37] The precise mechanisms remain to be solved, but the function of P-glycoprotein seems to be deeply associated with the mechanisms, as described later.

Pleiotropic drug-resistant tumor cells also bear other biochemical changes. For example, adriamycin-resistant tumor cells possessed increased amounts of cellular glutathione and decreased activities of cellular enzymes.[38, 39] Vincristine(VCR)-resistant tumor cells possessed an altered β-tubulin, the target of VCR.[40-42]

These biochemical changes are speculated to be related with each other, but are not fully understood. The observations described in (1)–(7) have led to the assumption that one of the probable mechanisms of pleiotropic drug resistance is a change in membrane characteristics, and the antitumor agents might be transported outside the cells by certain common mechanism(s). Recent progress in the genetic analysis of 170 kDa glycoprotein (P-glycoprotein) revealed that this glycoprotein could be a transporter of various antitumor agents. The mechanism of transport for each drug is not necessarily exactly the same, but possesses similar characteristics.

P-Glycoprotein and the Gene

P-Glycoprotein has been found in plasma membrane of various multidrug-resistant tumor cells, and has long been considered as a candidate transporter protein which pumps out antitumor agents. The cloning of the gene(s) for P-glycoprotein has been accomplished.[25, 43-50] Four methods can be applied for the cloning of the gene(s): (1) screening of a cDNA library by using antibody to P-glycoprotein, (2) isolation of amplified

gene(s) by an in-gel denaturation-renaturation method, (3) transfection of DNA from multidrug-resistant cells to sensitive cells, and (4) differential screening of a cDNA library of multidrug-resistant cells.

Ling's group in Canada has isolated the drug-resistance gene *mdr* from hamster cells by using a monoclonal antibody against P-glycoprotein.[25] The groups of Biedler and Borst isolated *mdr* by differential screening of cDNA of multidrug-resistant cells.[44–46] The groups of Roninson and Pastan isolated amplified genes from adriamycin-resistant Chinese hamster cells and then isolated a drug-resistance gene (*mdr*1) from colchicine-resistant human KB cells.[47–50] The DNA sequence, *mdr*1, is amplified in various multidrug-resistant cell lines.[50] The *mdr*1 was found to encode a 4.5-kb mRNA which is overexpressed in various drug-resistant tumor cells.[50]

Adriamycin-resistant human myelogenous leukemia K562 cells (K562/ADM) established in our laboratory also contained amplified DNA sequences as revealed by in-gel renaturation. As K562/ADM possesses human-specific *Alu* sequences, we have succeeded in isolating a resistance gene by the transfection of DNA from K562/ADM to mouse L-cells.[51] The transfectants showing resistance to ADM or VCR were selected. Secondary transfectants were established and isolated by the same procedures. All transfectants showed cross resistance to ADM and VCR, and possessed a defect in VCR and ADM accumulation, indicating that a putative resistance gene controls both VCR- and ADM-resistance.

From a genomic library of *Eco*RI-digested DNA of a secondary transfectant L-N4-S1, we have successfully isolated *Alu*-positive λ phage plaques. The phages contained 2.6 kb or 1.9 kb *Eco*RI fragments and were designated as λKA2.6 and λKA1.9. The 2.6 kb DNA fragment was amplified in various human resistant tumor lines and hybridized with 4.5 kb mRNA from human resistant sublines.[51]

Complementary DNA clones corresponding to *mdr*1 isolated by Roninson's and Pastan's groups were found to cross-hybridize with a cDNA clone isolated by Ling's group, and threfore *mdr*1 was identified as the human homologue of P-glycoprotein gene.[52] Also cDNA clones reactive to our λKA2.6 were the gene of P-glycoprotein, as the partial DNA sequence was identical to that of an *mdr*1 cDNA clone. These genes were confirmed to be responsible for multidrug resistance by DNA transfection of full-length cDNA.[53–55] As the transfectant showed the typical phenotype of multidrug-resistant cells, it is believed that *mdr*1 gene is sufficient to confer multidrug resistance.[53]

Recently the DNA sequence, and the amino acid sequence of P-glycoprotein were determined.[56, 57] According to these findings, human P-glycoprotein possesses the following properties. (1) The total number of amino acids is 1280 and the protein has a tandem duplication structure, the former and the latter halves of which show homology, especially in each C-terminal region. (2) The protein possesses 12 transmembrane domains. (3) Each half molecule of the duplex structure contains a nucleotide binding site. (4) The protein has a putative sugar-binding site. (5) By means of sequence homology analysis, P-glycoprotein was discovered to possess striking homology to Hly B protein, His P protein, Pst B protein, Mal K protein and Opp D protein. These proteins are bacterial membrane-associated proteins related to active transport systems.[56, 57] From these observations, P-glycoprotein, with its internal duplex structure is speculated to be involved in the transport of antitumor agents through plasma membrane. Recently, Cornwell actually observed that 150 to 170 kDa protein (presumably P-glycoprotein) has an affinity for analogs of vinblastine.[58] Calcium channel blockers varapamil, diltiazem and nitrendipine can bind to the plasma membrane from resistant KB cells, and these agents can inhibit the binding of vinblastine to P-glycoprotein.[59] Recently, we have succeeded in purifying P-glycoprotein by means of a one-step immunoaffinity chromatography using a monoclonal antibody against P-glycoprotein, and found that the purified P-glycoprotein has ATPase activity.[60] Based on these observations and speculations, a hypothetical schematic model for the membrane orientation and localization of the P-glycoprotein is presented (Fig. 1). The molecular mechanisms of the action of P-glycoprotein, however, remain

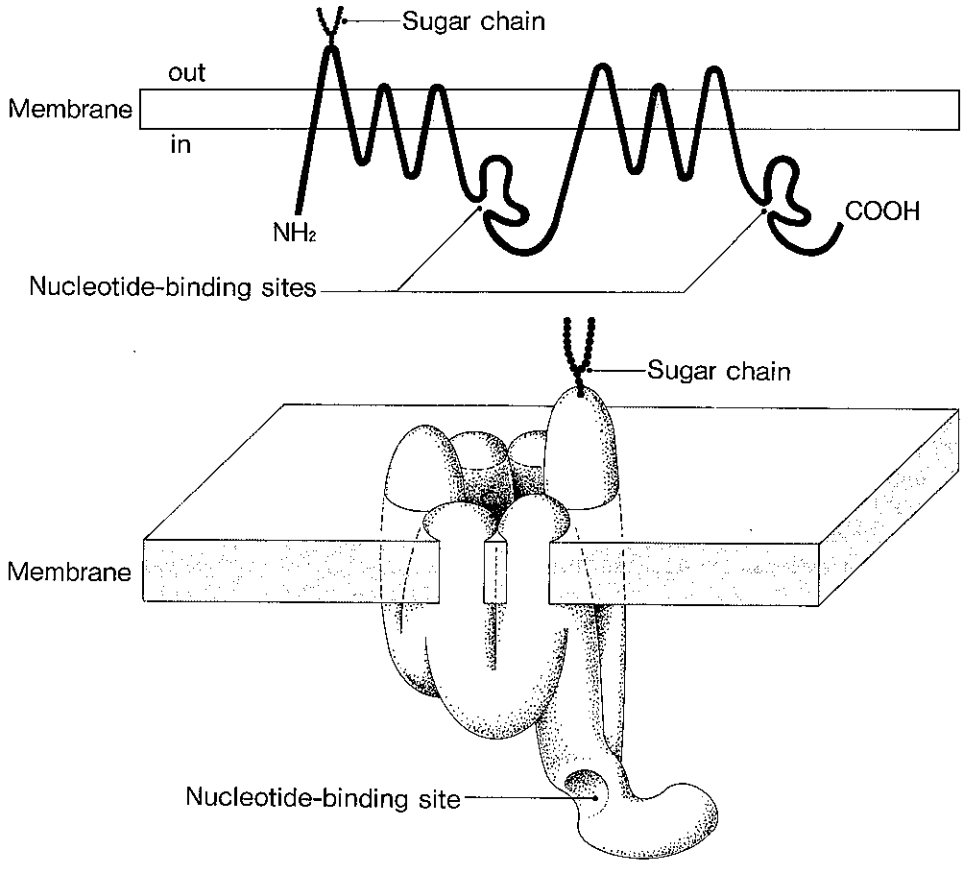

Fig. 1. Speculative model for transmembrane localization of P-glycoprotein based on the data of references 56 and 57.

to be confirmed by using biochemical techniques in the future. Also the mechanism of the expression of *mdr* gene and its regulation remain to be solved.[61]

Monoclonal Antibodies against P-Glycoprotein

Because of the important role of P-glycoprotein in the mechanisms of multidrug resistance, establishment of monoclonal antibodies against this protein should be beneficial not only for studies of drug resistance but also for the diagnosis and therapy of drug resistance in patients.

We have developed monoclonal antibodies against adriamycin-resistant human myelogenous leukemia K562 (K562/ADM).[62] Two monoclonal antibodies designated MRK16 and MRK17, are specifically reactive against K562/ADM and a human ovarian cancer cell line resistant to adriamycin (2780^{AD}). Both antibodies recognized the P-glycoprotein. MRK16 modulated vincristine and actinomycin D transport in the resistant cells, while MRK17 specifically inhibited the growth of the resistant cells. These data indicate that the P-glycoprotein is involved in the drug transport mechanisms and the proliferation of multidrug-resistant tumor cell lines.[63]

Other monoclonal antibodies against drug-resistant cells have been reported. Danks *et al.* developed monoclonal antibodies that specifically recognize resistance-associated proteins of human origin, and Kartner *et al.* described monoclonal antibodies that are specific for P-glycoprotein in different multidrug-resistant mammalian cells.[64, 65] Functional

modifications of resistant tumor cells by these monoclonal antibodies have not been reported. Since our monoclonal antibodies MRK16 and MRK17 are reactive to viable tumor cells and exhibit unique effects on the cellular function of multidrug-resistant cells, they might be useful in the study of the mechanism of multidrug resistance as well as in the diagnosis of refractory stages of malignancies.

Cellular Function of P-Glycoprotein

P-Glycoprotein has been speculated to be deeply associated with the drug transport function of resistant cells. As the gene of P-glycoprotein was isolated from drugsensitive normal cells, the protein is speculated to be present in normal cells.[53] According to the observation of Pastan's group, the *mdr*1 gene is expressed at high levels in human adrenal gland and kidney, and at intermediate levels in lung, liver, jejunum, colon and rectum.[66] Several human tumors derived from these tissues also express *mdr*1 gene. The increased expression of *mdr*1 gene in some normal organs implies that P-glycoprotein has a normal function in normal organs. Recently it was reported that P-glycoprotein is also expressed partly in carcinogen-initiated hepatocytes and regenerating liver cells.[67] These findings are of considerable interest in understanding the cellular function of P-glycoprotein. From a localization study of P-glycoprotein with monoclonal antibody MRK16, it was found that the protein was detected in a highly polarized fashion on the apical surface of the epithelial cells of ductules of various organs.[68] These findings suggest that the P-glycoprotein has a role in secretion of metabolites and some other chemicals, including toxic environmental compounds and chemotherapeutic agents, outside the cells.

We have recently found that: (1) P-glycoprotein is phosphorylated in the basal state; (2) verapamil and trifluoperazine, which inhibit active drug efflux and restore drug sensitivity in resistant cells, caused an increase in the phosphorylation of the P-glycoprotein; (3) 4β-phorbol 12β-myristate 13α-acetate (PMA) and 1-oleoyl 2-acetylglycerol enhanced phosphorylation of the protein; (4) the protein was phosphorylated at serine residues; (5) tryptic phosphopeptide mapping of the P-glycoprotein showed that PMA treatment induced an increase in phosphorylation at different sites of the protein from those induced by verapamil or trifluoperazine treatment, suggesting that the protein is phosphorylated by an array of complexly regulated mechanisms including C-kinase.[69] Phorbol diester-induced regulation of P-glycoprotein might play a role in the regulation of processes affecting cellular proliferation or other functions in multidrug-resistant tumor cells.[63, 69] So far, there have been only a few reports concerning the biological effects of phorbol diester on multidrug-resistant cells, and nothing has been reported on the relevance of phorbol diester-induced phosphorylation of P-glycoprotein to the function of P-glycoprotein.[70, 71] Further experiments along this line would be rewarding.

Clinical Relevance of Multidrug Resistance Studies

The drug-resistance genes and the monoclonal antibodies against P-glycoprotein could be used in diagnosis and therapy of patients at the refractory stage, as well as in studies of drug resistance mechanisms.

In the clinical setting, an increased mRNA expression occurs without gene amplification.[72] Thus mRNA detection by using an *mdr* gene probe might be useful for the detection of resistance in patients. More suitably, *in situ* detection of mRNA is applicable for the diagnosis. The possibility of using the *mdr*1 gene for diagnosis is now being studied.

The use of monoclonal antibodies for various clinical purposes can be considered. Monoclonal antibodies can be used for diagnosis.[73] Our monoclonal antibody MRK16 was found to be reactive to some hematological tumors rather refractory to therapy.[74] The percentage of the patients with multidrug resistance who show increased P-glycoprotein is difficult to estimate at the present time. According to our preliminary study, approximately 10% of samples from patients with hematological malignancies who had received chemotherapy was found to be positive for expression of P-glycoprotein as measured by the monoclonal antibody MRK16 (unpublished results). It is too early to reach any conclusion regarding the expression of P-glycoprotein in patients, and further studies

are needed. As MRK16 is reactive to living cells expressing P-glycoprotein, the sorting of such cells is possible both in clinical and experimental studies. For therapeutic purposes, monoclonal antibodies can be used for the destruction of tumor cells with complement, or it is possible to expect a cytostatic effect of the monoclonal antibody itself. For example, MRK17 inhibits the growth of multidrug-resistant human cells.[62,63] The development of conjugated drugs composed of monoclonal antibody and cytotoxic substances could also provide a means to destroy tumor cells. For this purpose, conjugates of *Pseudomonas* toxin with MRK16 have been prepared.[75] These conjugates specifically killed multidrug-resistant human cells and seem to be useful in cancer therapy. Finally, the monoclonal antibodies could be used for the elimination of contaminating drug-resistant human cells during the course of bone marrow transplantation.

Approaches for Overcoming of Drug Resistance

For the purpose of overcoming of drug resistance, the development of new antitumor agents effective against multidrug-resistant tumor cells is most important. In this regard, inhibitors of DNA topoisomerases are of considerable interest.[76] The approach of targeting the biochemical changes of multidrug-resistant tumor cells is also beneficial for overcoming of drug resistance. For this purpose, P-glycoprotein, the key protein in the mechanism of multidrug resistance, could be the most important target. P-Glycoprotein possesses an ATP-binding site, ATPase activity,[60] a binding site for vincristine and adriamycin,[58] a binding site for calcium channel blockers,[59] and phosphorylation sites which might be involved in the regulation of the function of P-glycoprotein.[69] These sites could theoretically be targets for therapeutic purposes. The monoclonal antibodies for P-glycoprotein could also be useful for directly overcoming drug resistance as described above.

P-Glycoprotein is supposed to possess drug efflux function. If we could find inhibitors which inhibit the drug efflux function of P-glycoprotein, such compounds would be of great interest for overcoming drug resistance. In 1981 and 1982, we found that calcium channel blockers and calmodulin inhibitors could inhibit the efflux of antitumor agents, thus overcoming drug resistance.[19,31] Since then various compounds including quinidine, tamoxifen, cyclosporin A and so on have been reported to overcome drug resistance[33–37] (see ref. 37 for review). It is now believed that these agents can interact with P-glycoprotein and thus inhibit the drug efflux function of the protein. Actually some calcium channel blockers can bind to the P-glycoprotein, and the binding of some antitumor agents can be inhibited competitively by calcium channel blockers and other potentiating agents.[58,59] The molecular mechanisms of action of calcium channel blockers, as well as that of P-glycoprotein remain to be solved.

The use of calcium channel blockers and related compounds for the overcoming of drug resistance is a practical approach.[37] Combination chemotherapy with calcium channel blockers is potentially useful against refractory acute lymphocytic leukemia of children, various advanced solid tumors, small cell lung cancer and brain tumors.[77–80] Further studies are needed on pharmacokinetics, optimal schedules, and toxicology to determine the clinical effectiveness. Calcium channel blockers should be more effective clinically when combined with vinca alkaloid class antitumor agents than with anthracycline antibiotics. The combination therapy induces side effect such as reversible hypotension and arrhythmia. Most importantly, more effective agents (calcium channel blockers and structurally related compounds) with fewer side effects than the present calcium channel blockers are needed. The calcium influx blocking action of the calcium channel blockers, which is closely related to hypotension, is not involved in the mechanism of potentiation of antitumor agents. Thus, it should be possible to find potentiating agents without calcium influx blocking action. Research along this line, as well as the search for more effective drugs with less side effects is now in progress.[37]

Other Factors in Multidrug Resistance

As described in the previous chapter, clinical studies to date have indicated that P-glycoprotein is not always found in refractory tumors; rather, it is found in a limited number

of tumors. Further studies are needed to evaluate the relevance of P-glycoprotein in refractory patients, but these findings indicate that other mechanisms are also important in multidrug resistance in patients. For example, adriamycin-resistant human breast cancer cells (MCF-7) showed phenotypic changes associated with increases in glutathione transferase.[81] The increase in transferase activity, however, was not associated with any change in the cellular level of glutathione.[81] In ovarian cancer cell lines resistant to adriamycin, melphalan, and cisplatin, analysis of total cellular glutathione revealed moderate to marked elevations (50 to 700%) when compared to sensitive lines,[82, 83] but the level of glutathione transferase did not change. In these cell lines, reversal of resistance was accomplished by glutathione depletion. Treatment of the cells with buthionine sulfoximine, an amino acid analog which irreversibly binds and inactivates γ-glutamyl-cysteine synthetase, resulted in an increase (2–10 fold) in the cytotoxicity of these drugs.[83]

In our studies no increase in the activity of glutathione S-transferase was detected in myelogenous leukemia K562 resistant to adriamycin, ovarian carcinoma A2780 cells resistant to adriamycin and acute lymphoblastic leukemia CCRF-CEM resistant to vinblastine as compared to the drug-sensitive parent lines.[84] Human breast cancer cell lines Hattori and MCF-7, used by Batist *et al.*,[81] had a 12–63-fold lower level of glutathione S-transferase activity than K562, A2780, CCRF-CEM and their drug-resistant sublines. Induction of colchicine resistance in MCF-7 resulted in a 70-fold increase in the activity of glutathione transferase, thus reaching the level of K562, A2780, CCRF-CEM and their drug-resistant sublines. Our findings indicate that increased cellular glutathione transferase activity is not associated with the development of multidrug resistance.[84] Glutathione level and glutathione transferase activity in multidrug-resistant tumor cells seem to be interesting and important factors in resistance mechanisms, but additional studies are needed to clarify the situation.

Another factor in multidrug-resistant tumors which might have some relevance to the resistance mechanisms is related to the cytochrome P-450 linked microsomal mixed-function oxidase system, which is usually involved in the metabolism of foreign compounds. Adriamycin-resistant P388 leukemia showed a decreased content of cytochrome P-450 and other mixed-function oxidase components as compared to the parent line.[39] These types of enzyme seem to be deeply associated with the metabolism of antitumor agents, but again, many problems remain to be solved.

Epilogue

Since the finding of P-glycoprotein in multidrug-resistant tumor cells and the discovery of calcium channel blockers as agents for overcoming of drug resistance in 1981, studies on multidrug resistance have progressed enormously at the cellular as well as the molecular level. Calcium channel blockers are now considered to bind to P-glycoprotein. P-Glycoprotein is interesting because of its function in the drug resistance mechanisms, and also because of its action in normal cellular function.

In the field of cancer chemotherapy, it is notable that the phenomenon of multidrug resistance is being elucidated from the cellular level to the molecular (DNA and protein) level. As described above, other mechanisms of multidrug resistance, not related to P-glycoprotein, can be considered. The importance of the majority of them still remains unclear, but such mechanisms also need to be elucidated at the molecular level in the near future. Theoretical approaches, by targeting the mechanisms of multidrug resistance, may eventually solve the problems of multidrug resistance, which is crucial in the treatment of cancer patients.

ACKNOWLEDGMENTS

I thank the staff of the Cancer Chemotherapy Center, Drs. H. Hamada, Y. Sugimoto, K. Yusa, M. Naito and T. Yamori for their collaboration in these studies. I also thank H. Iida-Saito, T. Oh-hara, H. Kawabata, E. Ohkouchi, R. Takamori, M. Hashizume and S. Tsukahara for their help in the work. I am grateful to Drs. H. Sugano and Y. Sakurai for their encouragement. These experiments were supported by grants from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare, Japan, and the Uehara Memorial Foundation. Finally, I thank Ms. M. Shimizu and M. Hashizume for their expert secretarial assistance and Drs. H. Hamada, Y. Sugimoto and T. Yamori for their critical reading of the manuscript.

(Received October 23, 1987/Accepted January 9, 1988)

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