Perturbation of Glycoprotein Expression and Processing in Multidrug Resistant Cells: Modulation of Drug Transport and Cytotoxicity by Tunicamycin

Donavon Charles Hiss BSc, BSc(Med)(Hons),
MSc(Med), Dip. Freelance Journalism

Thesis Presented for the Degree of Doctor of Philosophy
in the Departments of Pharmacology and Haematology,
Faculty of Medicine, University of Cape Town

Supervisors:
Professor Peter I. Folb
Professor Peter Jacobs

September 1993

To my mother. Dedicated also to the memory of Doreen Hiss.

DECLARATION

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PREFACE

The work described in this thesis was carried out between March 1988 and September 1992 mainly in the laboratories of the Departments of Pharmacology and Haematology, University of Cape Town Medical School. Chapter 1 is divided into three parts. In Part I, the multidrug resistance phenomenon is reviewed - both in historical context of the problem and recent advances of molecular, cellular and pharmacological significance. Part II is an overview of the relevance of glycosylation. In addition, Parts I and II contain special areas of interest which, because of their close relationship with the principal text, are included in shaded boxes to provide adequate background for the research proposition outlined in Part III. The reader might find this presentation valuable to become familiar with the overall objective of the thesis. The research methodology and major experimental data accumulated during the course of the project are presented and discussed in Chapters 2 to 5. The most important results and their implications are summarized in Chapter 6. I have been diligent in keeping apace with the contemporary literature and exercised every effort to compare my findings with related results and information reported by other authors. However, the literature in this field is vast and ever-expanding, and I had to limit the current level of awareness in the thesis to April 1993. The manuscript was subjected to several critical revisions and I have taken care to remove any errors and redundancies. I am, however, accountable for the final version. I hope that this thesis will be of value to its readers and add to their appreciation of the relevance of glycoconjugate research in tumour biology.

ACKNOWLEDGMENTS

It gives me great pleasure to acknowledge the assistance and guidance of my supervisors, Professors Peter Folb and Peter Jacobs. I value the scope and liberty which they have entrusted to me in the development of the project. I learned from them the discipline required to do research at and beyond the Ph.D. level.

A special thanks is due to Gary Gabriels for outstanding assistance in the laboratory.

I am most appreciative of the valuable suggestions of Dr Peter Smith, Department of Pharmacology, University of Cape Town Medical School, for improving the format and content of the thesis.

I am deeply grateful to the staff and students in the Department of Pharmacology for their efforts, help and encouragement.

I want to thank Professors P. Jacobs (Department of Haematology and Leukaemia Centre), E. B. Dowdle (Department of Clinical Science and Immunology), R. E. Kirsch (Liver Research Centre) and M.C. Berman (Department of Chemical Pathology) for making their research facilities available.

This project was funded by grants from The National Cancer Association, The Medical Research Council and the University of Cape Town.

Financial support from The University of Cape Town, The Foundation for Research Development, Beckman Instruments, Adcock-Ingram, The Ackerman Family Educational Trust, the late Mr Solly Yach of The Mauerberger Foundation and Tony Cowell of Electronic Scientific Instrumentation is gratefully acknowledged.

I am greatly indebted to Professor Peter Jacobs, Sr. Lucille Wood and the staff of Haematology-E5, Groote Schuur Hospital, for supplying blood specimens.

I thank Professor Michael M. Gottesman, Drs Stephen J. Currier and Carol O. Cardarelli of the Laboratories of Cell Biology and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA, for generously providing the KB-3-1, KB-8-5-11, NIH-3T3 and NIH-3T3 *mdr*1 transfected cell lines.

I also thank Professor Philippe Gros, Department of Biochemistry, McGill University, Montreal, Canada, for the gift of the LR73 and LR73-1A cell lines.

I am grateful to Dr W.R. Bezwoda and Terry Golombick, Haematology/Oncology, University of the Witwatersrand, for the UWOV2 ovarian carcinoma cell line.

Drs C.A. Wallen and Keiichi Fujiwara, Department of Radiology, Bowman Gray School of Medicine, North Carolina, USA, generously supplied the BG-1 ovarian carcinoma cell line.

The monoclonal antibody MRK16 was kindly provided by Professor Takashi Tsuruo, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan.

A word of appreciation goes to Dot Pink and Desmond Williams, University of Cape Town Purchasing Department, for their courtesy and efficient handling of the many hasty orders for chemicals, books and computer software.

To my friend, Ebrahim Alexander, for his constant encouragement and for sharing a special interest in computers and the art of writing.

Recognition also goes to the maze of human contacts from whom I have learned, and who have contributed directly and indirectly to my personal well-being and development.

Finally, to my family for enduring with me against terrible odds and throughout the difficult times; thanks, thanks and ever thanks.

ABSTRACT

The development of multiple drug resistance (MDR) by tumour cells presents a major impediment to effective cancer chemotherapy. Drug-resistant cells have raised levels of a plasma membrane glycoprotein, designated P-glycoprotein (P-gp), of molecular weight (MW) 170 kilodaltons, relative to their drug-sensitive counterparts. P-glycoprotein functions as an active efflux pump and maintains a reduced level of drug inside the cell, possibly by a mechanism(s) involving decreased drug uptake and/or increased drug efflux. The association of MDR in the failure of tumours to respond favourably to a given regimen of anticancer drugs is the subject of intense research. Approaches for studying the underlying mechanisms of MDR raise hopes for preventing or overcoming the phenomenon. In this study, tunicamycin (TM), an inhibitor of glycoprotein synthesis and processing, has been evaluated for its potential to reverse the MDR phenotype. A correlation was sought between the MDR phenotype (drug resistance and toxicity, drug transport and retention) and the perturbation of cell-surface glycoproteins by TM *in vitro*.

The effects of TM on protein and glycoprotein synthesis were correlated with the responses of drug-sensitive (BG-1-P and KB-3-1) and drug-resistant (BG-1/ADR, KB-8-5-11 and UWOV2) human tumour cell lines, and of the murine fibroblast parental (NIH-3T3-parental) cell line and its drug-resistant transfectant (NIH-3T3-MDR) to various anticancer drugs alone or in combination with TM. The drug-sensitive cell lines (NIH-3T3-parental mouse fibroblasts, LR73 Chinese hamster cells, and KB-3-1 human epidermoid carcinoma) and their corresponding drug-resistant cell lines (NIH-3T3-MDR, LR73-1A and KB-8-5-11, respectively) as well as drug-resistant UWOV2 human ovarian carcinoma cells were also studied for altered vincristine (VCR) transport and retention in response to TM pretreatment. In addition, leukaemia blast cells isolated from peripheral blood of patients with AML, and the human ovarian carcinoma cell line (UWOV2, established from a patient refractory to combination chemotherapy with actinomycin D, doxorubicin, cisplatin and VCR), were characterized for their expression of the MDR-associated 170 kDa P-gp by gel filtration, SDS-PAGE and immunocytochemical analysis using P-gp-specific monoclonal antibodies.

Tunicamycin significantly increased the cytotoxicity of doxorubicin, epidoxorubicin, colchicine, VCR and cisplatin in MDR cells to a greater extent than in the corresponding drug-sensitive (parental) cells. This effect of TM on the

cytotoxicity of the various agents studied suggests a common mechanism of reversal of drug resistance by TM. In NIH-3T3-MDR, KB-8-5-11, and BG-1/ADR cells a drug sensitivity pattern developed in the presence of TM that corresponded to that in the parental line. On removal of TM from the culture media the cells regained their original pattern of drug resistance.

Pretreatment of cells with TM, inhibits protein and glycoprotein synthesis in all the cell lines that were studied. In the NIH-3T3-MDR and KB-8-5-11 cell lines, glycoprotein synthesis was elevated compared with the drug-sensitive NIH-3T3-P and KB-3-1 cell lines, respectively, but similar levels of glycoprotein synthesis were found in drug-resistant LR73-1A cells and the corresponding drug-sensitive LR73 cells. Analysis of several glycosyltransferases in the various parental and MDR cell lines showed that activities of fucosyltransferase (FT) and N-acetylglucosaminyltransferase (NGT) are increased while the activities of sialyltransferase (ST) and galactosyltransferase (GT) are decreased in drug-resistant KB-8-5-11 cells compared with their parental controls. In NIH-3T3-MDR cells, the reactions catalyzed by FT, GT and NGT, but not by ST, occurred at significantly higher rates than those in the NIH-3T3-parental cells. The LR73-1A cells expressed elevated levels of FT whereas the levels of ST, GT and NGT remained constant compared with drug-sensitive LR73 controls. Similar ST, FT and NGT reaction rates were observed in the BG-1/ADR human ovarian carcinoma cell line and its parental control, BG-1-P. Tunicamycin pretreatment of cells produced fluctuations in reaction rates for the various glycosyltransferases in MDR and parental cells and no consistent trend of inhibition of enzyme activity was demonstrated. However, the inhibition by TM of FT-, GT- and NGT-catalyzed reactions was common to NIH-3T3-MDR and LR73-1A cells, although TM did not completely abolish the activities of the glycosyltransferases in these cells. Exposure of KB-8-5-11 cells and UWOV2 cells to TM did not inhibit any of the glycosyltransferases. These results demonstrate that glycoproteins are differentially expressed in various drug-sensitive and drug-resistant cell lines.

Various drug-sensitive cell lines (NIH-3T3-parental, LR73 and KB-3-1) and their corresponding drug-resistant cell lines (NIH-3T3-MDR, LR73-1A and KB-8-5-11, respectively) as well as drug-resistant UWOV2 cells were studied for altered VCR transport in response to TM pretreatment. The uptake of VCR was not affected in the various cell lines following prior exposure and co-incubation with TM. NIH-3T3-MDR cells accumulated less drug than their drug-sensitive parental cells. No differences in the levels of VCR accumulation could be demonstrated between KB-3-1 and KB-8-5-11 cells, as well as between LR73 cells and LR73-1A

cells. Tunicamycin had no effect on the efflux of VCR from NIH-3T3-parental cells. Vincristine efflux was significantly reduced when *mdr*1-transfected NIH-3T3-MDR cells were treated with TM. Tunicamycin increased the retention of VCR in KB-8-5-11 cells, in NIH-3T3-MDR and in UWOV2 cells, but not in NIH-3T3-parental cells. In contrast, KB-3-1 cells expressed a decrease in VCR retention. A consistent association was found between treatment of MDR cell lines with TM and reduced VCR efflux with resultant increase in VCR retention. The results suggest that the enhancement of VCR retention in MDR cell lines by TM may occur as result of inhibition of VCR efflux. Drug-resistant cells appear to be affected to a greater extent by TM pretreatment than their drug-sensitive counterparts, possibly due to raised levels of P-gp in the former. In KB-8-5-11 cells, increasing concentrations of TM resulted in a concentration-related reduction in glycoprotein synthesis which did not correlate with inhibition of VCR efflux. Transient inhibition of both protein and glycoprotein synthesis during TM exposure may contribute to alterations in VCR efflux and retention observed in the MDR cell lines. The binding of [^{14}C]doxorubicin and [^{3}H]azidopine, a photoactive dihydropyridine calcium channel blocker known to bind P-gp, to UWOV2 ovarian carcinoma cells was studied in these cells. Binding of doxorubicin to UWOV2 cells was saturable in the concentration range 10-80 nM, whereas that of azidopine remained linear between 10 and 80 nM. In the presence of equimolar concentrations of doxorubicin, the binding of azidopine to UWOV2 cells was significantly inhibited in the concentration range of 30-80 nM. Tunicamycin increased the binding of both doxorubicin and azidopine to UWOV2 cells. In the presence of TM and equimolar concentrations of doxorubicin, however, the binding of azidopine was unchanged. The binding of both azidopine and doxorubicin to intact UWOV2 cells suggests that P-gp may be present on their cell surfaces.

Leukaemia blast cells isolated from peripheral blood of patients with AML, and the UWOV2 cell line, were characterized for their expression of the MDR-associated 170 kDa P-gp by gel filtration, SDS-PAGE and immunocytochemical analysis using P-gp-specific monoclonal antibodies. Plasma membrane glycoproteins prepared from patients L24 and L100 eluted as distinct peaks in the regions between 450-158 kDa and 158-44 kDa. SDS-PAGE analysis of the 450-158 kDa peak fractions revealed the presence of a 170-kDa band in samples prepared from both patients L24 and L100. SDS-PAGE of L24 and L100 plasma membranes also resolved a 170-kDa protein. In similar experiments, SDS-PAGE analysis of high MW (≥150 kDa) peak fractions (eluted from Sephadex G150, Sephadex G-200 or BioGel A-0.5M columns) and purified plasma membranes, showed a 170-kDa

band in samples prepared from patients L19, L23, L28, L31, L45, L84, L94, L95, and L97, but not from samples prepared from patients L21, L26, L42 and L55. The expression of P-gp in peripheral blood leukaemia blast cells prepared from patients with AML was analysed by the P-glycoCHEK™ C219 immunohistochemical detection kit. Two out of 15 samples prepared from patients with AML stained positively for P-gp. This was expected since the one patient (L24) was resistant to combination chemotherapy with etoposide, cytosine arabinoside and methotrexate, and the other patient (L100) presented with relapsed AML following chemotherapy with cytosine arabinoside, etoposide and daunorubicin. SDS-PAGE analysis of some samples from AML patients, who received either chemotherapy or no prior chemotherapy, showed the presence of a 170-kDa membrane protein, but this could not be confirmed by immunocytochemical staining using the C219 monoclonal antibody reaction. No difference in staining patterns could be discerned between samples prepared from treated and untreated patients in this group. Therefore, no clinical correlations with the presence of the 170-kDa protein could be made because P-gp was not identifiable in samples prepared from this group of patients.

Separation of cell-surface glycoproteins derived from UWOV2 cells metabolically labelled with [^{3}H]glucosamine on a column of BioGel A-0.5M, yielded high MW (240-158 kDa) peak fractions, consistent with the presence of a 170-kDa glycoprotein. This was confirmed by Sephadex G-200 fractionation of [^{3}H]glucosamine-labelled UWOV2 plasma membrane glycoproteins isolated after 72h incubation in the absence (control) and presence (TM-treated) of 5 μg/ml TM. High MW UWOV2 cell-surface glycoproteins eluted as a broad peak in the 200-150 kDa range of the Sephadex G-200 chromatographic profile. This peak was reduced to approximately 53% of control in the presence of TM, indicating inhibition of glycoprotein synthesis. The presence of a 170-kDa glycoprotein in UWOV2 cells and its disappearance as a result of prolonged TM exposure were confirmed by SDS-PAGE analysis of purified plasma membrane samples. When UWOV2 plasma membranes were immunoprecipitated with P-gp-specific monoclonal antibodies MRK16 and C219, the 170-kDa band was again present, although contaminating bands were also noted. The results from this study show that UWOV2 ovarian carcinoma cells express a 170-kDa membrane glycoprotein and that this glycoprotein is synthesized at reduced rates following TM exposure. Thus, TM may be regarded as a resistance modifier in studies related to the mechanisms of MDR.

TABLE OF CONTENTS

CONFERENCE PUBLICATIONS AND PRESENTATIONS

¶Parts of the work presented in this thesis have been presented at conferences and published in abstract form in conference proceedings as follows:

Hiss, D.C., Gabriels, G.A., Jacobs, P. and Folb, P.I. (1988) The Expression of Human Tumour Cell Glycoproteins in Multidrug Resistance with Special Reference to Acute Leukaemia and Ovarian Carcinoma, *23rd Annual Congress of the Pharmacological Society of Southern Africa*, Port Elizabeth, 12-14 October 1988.

Hiss, D.C., Gabriels, G.A., Jacobs, P. and Folb, P.I. (1989) The Expression of Human Tumour Cell Glycoproteins in Multidrug Resistance, *Proceedings of the Tenth International Symposium on Glycoconjugates,* Jerusalem, Israel, 10-15 September 1989.

Hiss, D.C., Gabriels, G.A., Jacobs, P. and Folb, P.I. (1989) Characterization of the Multidrug-Resistance Associated Glycoprotein in Ovarian Carcinoma and Acute Leukaemia, *24th Annual Congress of the South African Pharmacological Society,* Stellenbosch, 11-13 October 1989.

Hiss, D.C., Gabriels, G.A., Folb, P.I. and Jacobs, P. (1990) Drug Resistance Patterns in Ovarian Carcinoma and Acute Leukaemia, *25th Annual Congress of the South African Pharmacological Society*, Johannesburg, 11-12 October 1990.

Hiss, D.C., Gabriels, G.A. Folb, P.I. and Jacobs, P. (1991) Modulation of Multidrug Resistance by Tunicamycin, *26th Annual Congress of the South African Pharmacological Society*, San Lameer, Durban, 7-8 October 1991.

Hiss, D.C., Gabriels, G.A. and Folb, P.I. and Jacobs, P. (1991) Multidrug Resistance in Cancer, *National Cancer Association's Diamond Jubilee Cancer Expo '91*, Good Hope Centre, Cape Town, October 1991.

¶**Submitted:** Tunicamycin Increases Drug Cytotoxicity and Vincristine Retention in Multidrug Resistant KB Carcinoma Cells and in Cells Transfected with the *MDR*1 Gene.

¶**In preparation:** Expression of drug resistance in UWOV2 ovarian cancer cells

LIST OF FIGURES AND PLATES

CHAPTER 1

Part I

Part II

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF TABLES

CHAPTER 4

CHAPTER 5

LIST OF ABBREVIATIONS

↑	Increase(s); increased; increasing
↓	Decrease(s); decreased; decreasing
α-MEM	Alpha minimum essential medium
6-MP	6-Mercaptopurine
6-TG	6-Thioguanine
ActD	Actinomycin D
ADR	Adriamycin
AML	Acute myelogenous leukaemia
AMP	Adenosine monophosphate
APRT	Adenine phosphoribosyltransferase
Ara-C	1-β-D-arabinofuranosyl cytosine
Asn	Asparagine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
at-MDR	Atypical MDR
BLEO	Bleomycin
BSA	Bovine serum albumin
C-terminal	Carboxy terminal
C219	Human P-glycoprotein-specific monoclonal antibody
Ca^{2+}-ATPase	Calcium-dependent ATPase
CHL	Chinese hamster lung

CHO	Chinese hamster ovary
CI	Confidence Interval
CLQ	Chloroquine
CML	Chronic myelogenous leukaemia
COL	Colchicine
CPL	Cisplatin
Cys	Cysteine
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA, copy DNA
cis-DDP	*Cis*-diaminedichloroplatinumII, cisplatin
D_{10}	Dose that will kill 10% of cells
DHAD	Dihydroxyanthracinedione; mitoxanthrone dihydrochloride
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DHFR	Dihydrofolate reductase
DMC(s)	Double minute chromosome(s)
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
DOX	Doxorubicin
Dol-P	Dolichol phosphate
Dol-P-P	Dolichol pyrophosphate

DTN	Dactinomycin
DTT	Dithiothreitol
dCK	Deoxycytidine kinase
dCT	Deoxycytidine
EDTA	Ethylene-diamine tetraacetic acid disodium salt
EGF	Epidermal growth factor
EMS	Ethylmethane sulfonate
EPX	Epidoxorubicin
FT	Fucosyltransferase
Fuc	Fucose
g	Relative centrifugal force
Gal	Galactose
GDP-Man	Guanosine-5'-diphosphate mannose
Glc	Glucose
GlcNAc	N-Acetyl D-glucosamine
GlcNAc-1-P	N-Acetylglucosamine-1-phosphate
Gly	Glycine
GMP	Guanosine monophosphate
GPI	Glycosylphosphatidyl inositol
GRAM	Gramicidin D
GSH	Glutathione
GSTs	Glutathione sulphotransferases
GT	Galactosyltransferase

HIFBS	Heat-inactivated fetal bovine serum
HIFCS	Heat-inactivated fetal calf serum
HPRT	Hypoxanthine phosphoribosyltransferase
$HPRT^+$	Hypoxanthine phosphoribosyltransferase positive
$HPRT^-$	Hypoxanthine phosphoribosyltransferase negative
HSRs	Homogeneously staining regions
h	Hour(s)
IC_{50}	Drug concentration required to reduce the final absorbance to 50% of the control value / drug concentration required to kill 50% of cells
IC_{90}	Concentration of drug required to kill 90% of cells
IL-1	Interleukin-1
IUB-IUPAC	International Union of Biochemistry - and - International Union of Pure and Applied Chemistry
K_{av}	Partition coefficient
kDa	Kilodalton
MAYT	Maytansine
Man	Mannose
MDR	Multidrug resistant, multidrug resistance
MDR^+	MDR positive
MDR^-	MDR negative
MEM	Modified Eagle's medium
MeBlue	Methylene blue
MitoC	Mytomycin C

M_r	Relative molecular weight, apparent molecular weight
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
MW(s)	Molecular weight(s)
m-AMSA	Amsacrine; 4'-[(9-acridnyl)amino]methanesulfon-*m*-anisidide
mAb	Monoclonal antibody
mAbs	Monoclonal antibodies
mRNA	Messenger RNA
mV	Millivolts
N-terminal	Amino terminal
NEM	N-ethylmaleimide
NeuAc	Sialic acid or neuraminic acid
NeuNAc	N-Acetyl neuraminic acid
NGT	N-Acetylglucosaminyltransferase
NTPs	Nucleoside triphosphates
ρ	Density
P-170 or Pgp	P-glycoprotein
Pgp(s)	P-glycoprotein(s)
Pgp-MDR	P-glycoprotein-mediated MDR
Pgp-mAbs	P-glycoprotein-specific monoclonal antibodies
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol

Phe	Phenylalanine
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
POD	Podophyllotoxin
PRPP	5'-Phosphoribosyl-1-pyrophosphate
PURO	Puromycin
pAbs	Polyclonal antibodies
pH	Negative logarithm of the hydrogen ion concentration
pI	Isoelectric point
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
s	Seconds
S.E.M.	Standard error of the mean
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
Ser	Serine
SM	Sulphur mustard
Sorcin or V19	Cytosolic calcium-binding protein overexpressed in some MDR cells
ST	Sialyltransferase
SwN	Swainsonine
TBS	Tris-buffered saline

TFMS	Trifluoromethanesulfonic acid
TFP	Trifluoperazine
Thr	Threonine
TM(s)	Tunicamycin(s)
TM11	Transmembrane domain 11 of P-glycoprotein
TM3	Third transmembrane sequence of P-glycoprotein
TopoII	DNA topoisomerase II
$t_{1/2}$	Half-life
UDP-GlcNAc	Uridine-5'-diphosphate-N-acetyl D-glucosamine
U/ml	Units per millilitre
V_e	Elution volume
V_o	Void volume
V_t	Total volume
Val	Valine
VBL, VLB	Vinblastine
VCR	Vincristine
VDS	Vindesine
VM-26	Teniposide; 4'-demethylepipodophyllotoxin 9-(4,6-*O*-2-phenylidene-β-D-glucopyranoside; VM
VP-16	Etoposide; 4'-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); VP
VPL	Verapamil
v/v	Volume per volume
XR	Cross-resistance, cross-resistant

Factual evidence can never "prove" a hypothesis; it can only fail to disprove it, which is what we generally mean when we say, somewhat inexactly, that the hypothesis is "confirmed" by experience.

Milton Friedman (1912-)
Essays in Positive Economics [1953], pt. I, ch. I
Source: *Bartlett's Familiar Quotations*, 15th and 125th Anniversary Edition, 1980, John Bartlett, Edited by Emily Morrison Beck, Little Brown, Boston.

In formal logic, contradiction is the signal of defeat: but in the evolution of real knowledge it marks the first step in progress toward victory.

Alfred North Whitehead (*Am. J. Med.* **83**:757, 1987)

Credit must be given to observation rather than theories, and to theories only insofar as they are confirmed by the observed facts.

Aristotle

The great tragedy of Science: the slaying of a beautiful hypothesis by an ugly fact.

Thomas Huxley

Although this may seem a paradox, all exact science is dominated by the idea of approximation.

Bertrand Russell

CHAPTER 1

PART I: THE MOLECULAR, CELLULAR, AND PHARMACOLOGICAL BASIS OF MULTIDRUG RESISTANCE

1. HISTORICAL OVERVIEW

For many years the importance of the original observation that a single viable murine leukaemia cell can give rise to a lethal leukaemic population in an appropriate strain of mice (Furth and Kahn, 1938) was overlooked. By the early 1950s it was recognized that transplanted neoplasms (grafts) from any tissue origin will generate tumours in animals (Schabel, 1975). The knowledge that the survival of a malignant population may result in treatment failure led to the *first-order* or *pseudo-first-order* concept (*log-kill hypothesis*) of tumour cell elimination (Skipper *et al.*, 1964). According to this view, a given dose of drug will kill a constant fraction of cells, and not a specific number of cells, provided that they are exposed to similar growth conditions and the ratio of sensitive to resistant targets remains the same.

In the 1940s, Gilman and Philips (1946) noted regression of lymphoma in a patient treated with nitrogen mustard. This historic breakthrough evoked considerable interest in the potential use of cytotoxic agents in the management of neoplasms, and so had begun the modern era of cancer chemotherapy. Later, antitumour activity of the folate antagonists was demonstrated in children with leukaemia (Faber *et al.*, 1948). In 1942 methotrexate was synthesized and proved to be the first antimetabolite capable of curing an advanced malignancy (Hertz *et al.*, 1961). This latter outcome secured a definite place for these agents in chemotherapeutic regimens. The 1950s had seen an exciting period of drug development - the original anthracyclines became available in the 1960s and, by the 1970s, numerous substances were synthesized for chemotherapy (Weiss, 1992). These include alkylating agents, plant alkaloids, nitrosoureas, and hormones (Muss, 1984).

Another landmark observation was that a combination of nitrogen mustard, vincristine, procarbazine and prednisone could cure a majority of patients with advanced Hodgkin's disease (De Vita and Serpick, 1967; De Vita *et al.*, 1970). The importance of this approach was that drugs used together may be more effective than when they are used separately. This is referred to as *collateral sensitivity* (Szybalski and Bryson, 1952; Bech-Hansen *et al.*, 1976). In contrast, it was found that consolidation chemotherapy often resulted in the selection of drug-resistant cells that are also refractory to other regimens. This is defined as *cross-resistance* (Danø, 1972; Hutchison and Schmid, 1973; Cano-Gauci and Riordan, 1991). The two terms rely heavily on the accurate evaluation of sensitivity to different treatment regimens, and can vary for any particular tumour cell-drug system, and should thus not be considered as "*all-or-none*" phenomena (Hill, 1984).

Spontaneous regression of tumours is rare, although there are a number of well-quoted instances (Everson and Cole, 1956; Sumner and Foraker, 1960; Everson, 1964). A complete remission will depend on whether the target cell population is collectively sensitive to the type of treatment. Anything less than a total disappearance of the tumour implies a degree of resistance. Dramatic responses to chemotherapy are frequently followed by relapse due to one or more surviving cancer cells. In this regard, Skipper (1979) considered the research of Luria and Delbrück (1943) to be a significant contribution to oncology. No allusion to the cancer problem is made in their paper, yet it became increasingly apparent that refractory cells of many malignancies continually arise from random mutations - in a manner comparable to phage- and antibiotic-resistant bacteria (Demerec, 1948; Skipper, 1979). Several reports describing similar findings have followed (Burchenal *et al.* 1950; Law and Boyle, 1950; Law, 1952). Drug resistance is now a widely known, yet poorly understood phenomenon.

It is clear that neoplastic transformation and tumour non-responsiveness represent a formidable challenge to generate novel and effective treatment modalities. Over the last few years there has been an explosion of research into multiple drug resistance (MDR). This chapter is intended to explore and present recent advances in understanding the molecular and cellular mechanisms involved in the initiation, progression, regulation and manipulation of this protective response during cytotoxic attack (see Figure 1 for outline). For quick reference, areas of special interest that led to the research undertaken in this thesis are located in shaded text boxes in Parts I & II on pages 8-11, 24, 25, 29, 32, 42, 49 and 50.

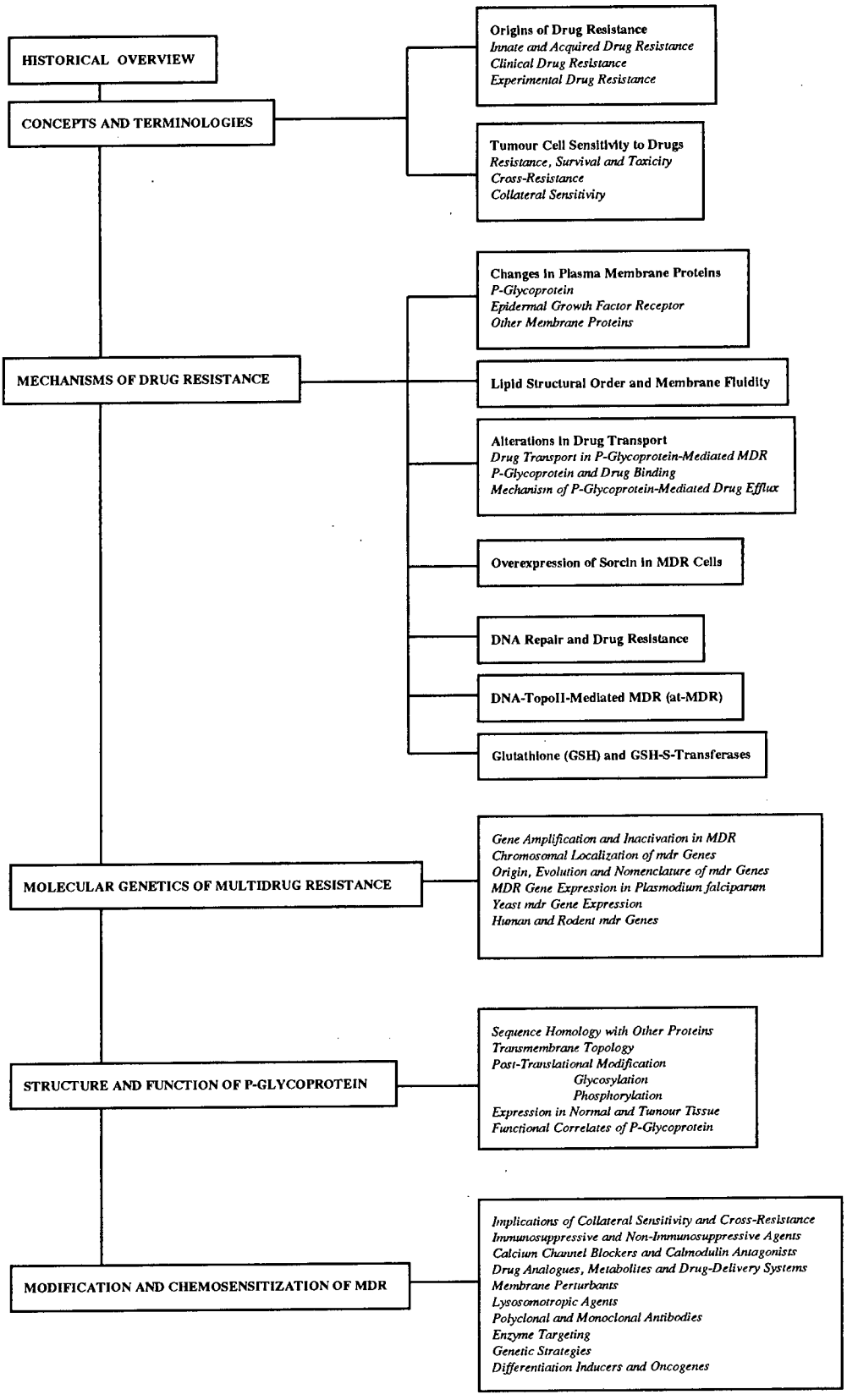

Figure 1. Flow diagram for the molecular, cellular and pharmacological basis of the multidrug resistance phenotype and its modification reviewed in Part I, Chapter 1.

2. CONCEPTS AND TERMINOLOGIES

2.1. Origins of Drug Resistance

Central to the concern of how MDR can be overcome is the question of its aetiology, *i.e.,* the transition of a cell or population of cells from a state of drug sensitivity to one of insensitivity. Resistance is a relative term, with high, intermediate, and low degrees, as defined by the investigator in terms of individual experience (Henderson, 1984). Important also is the notion that the refractory phase is not a property of an individual tumour cell or tissue, but the manifestation of some tumour-drug system.

2.1.1. Innate and Acquired Drug Resistance

The ability of a single tumour cell to survive a local concentration of a cytotoxic drug that would otherwise kill it may be an innate property or may have been acquired by a gradual or rapid adaptive response (Henderson, 1984). If a cell were to survive a given dose of a damaging agent and divide, probably in the face of a loss of many similar cells within the population of which it is part, a new clone of resistant cells would ensue. Subsequent generations may be further selected for reduced sensitivity or actively become transformed by expressing alternative biochemical pathways.

2.1.2. Clinical Drug Resistance

Cancer management over the past few decades has been altered radically by the design of different classes of anticancer drugs. Despite this advancement, few universally successful agents acting solely and preferentially on neoplastic cells have hitherto emerged. Given the right conditions, no tumour cell is completely resistant to the effects of existing chemotherapeutic agents. In clinical practice, any consequence of drug treatment hinges upon its contrasting effects on normal and tumour tissue. An important factor is the degree of drug selectivity for the target cell and its subsequent influence upon it (Zubrod, 1978).

It is the qualitative nature of this specificity that varies from drug to drug when used against different cancers that equates with resistance, *i.e.,* the margins of therapeutic benefit may be narrow in terms the consequences of toxicity to normal tissues. It has been suggested that chemotherapy of tumours may result in the selection of drug-resistant cells in a heterogeneous cell population and the outgrowth of a refractory tumour (Georges *et al.*, 1990b). Some human

malignancies such as multiple myeloma, breast cancer, ovarian carcinoma, childhood neuroblastoma and leukaemia may respond favourably to initial treatment only to be followed by relapse and eventual death of the patient. In contrast, adenocarcinomas of the kidney and colon, non-small-cell lung cancer and glial tumours are at the outset resistant to cytotoxic drugs (Becker *et al.*, 1991; van Kalken *et al.*, 1991; Shin *et al.*, 1992).

The failure of chemotherapy can be ascribed to a variety of factors, including poor drug absorption, unfavourable pharmacokinetics and pharmacodynamics, insufficient tumour vascularization or hypoxia, and low pH. Although clinical drug resistance is not always detected satisfactorily (Ma *et al.*, 1987), recent developments, especially the identification of a molecular marker, P-glycoprotein (Pgp) and its gene (*mdr*), implicated in unsuccessful treatment outcome, are apt to prove effective (Bell *et al.*, 1985; Cazin *et al.*, 1992; Herzog *et al.*, 1992). Tumour cell lines selected *in-vitro* for resistance to anticancer drugs (*Vinca* alkaloids, anthracyclines, epipodophylotoxins, colchicine, actinomycin D) are useful for studying multidrug resistance (MDR) mechanisms. It has yet to be verified, however, whether the experimental resistance pattern observed in such model systems is a reflection of that experienced clinically. Therefore, pretherapeutic detection of MDR in individual patients may be of great value to determine whether the proposed therapy would be beneficial (Verrelle *et al.*, 1991; Mohammad *et al.*, 1992).

P-glycoprotein and its mRNA were found to be frequently present in tumour specimens from some patients (Fojo *et al.*, 1987; Gerlach *et al.*, 1987; Tsuruo *et al.*, 1987), but not in others (Goldstein *et al.*, 1989; Ito *et al.*, 1989; Pieters *et al.*, 1991). The degree of Pgp expression following drug administration is often greater than before treatment was begun (Goldstein *et al.*, 1989) and often correlates with "clinical" MDR (Gekeler *et al.*, 1992; Herzog *et al.*, 1993). Cancer chemotherapy does not appear to enhance the synthesis of this glycoprotein in normal cells (Geromin *et al.*, 1992). Therefore, observations of its expression in both normal and malignant tissues, together with the identification of MDR forms in human cells that do not overproduce Pgp, suggest that clinical assumptions of this nature are likely to be complicated (de Vries and Pinedo, 1991; Kato *et al.*, 1991; Marie *et al.*, 1992a).

Notwithstanding that chemotherapy has been invaluable in the management of many human tumours, effecting long-term survival or cures of haematologic malignancies, ovarian carcinoma and small-cell lung cancers, the overall success

rate against some tumours remains low (Murren and Hait, 1992). The development of newer anticancer agents to overcome this obstacle is a major goal of cancer chemotherapy. The potential of MDR cells in the study and prevention of drug resistance is now widely accepted (Dietel, 1991). Clinical trials emanating from these advances hold great promise for future treatment of refractory tumours (Lum *et al.*, 1992; Philip *et al.*, 1992; Yahanda *et al.*, 1992).

2.1.3. Experimental Drug Resistance

Cell lines isolated from tumour-bearing mice following chronic exposure to anticancer drugs mimic to some extent the *in vivo* acquisition of cellular refractoriness under clinical conditions, although the precise relationship between the two modes is not well established. Drug resistance may be due to multiple genetic and biochemical changes, and a single mechanism may not be implicated in all tumours (Masters, 1990; Fairchild and Cowan, 1991; Toffoli *et al.*, 1991). The heterogeneity in spontaneous tumours further complicates the issue and tends to oversimplify current explanations and assumptions (O'Brien and Cordon-Cardo, 1991). Hence, each system has to be evaluated individually. Transfection experiments with cloned cDNA have unequivocally shown that Pgp mediates the MDR phenotype (Ueda *et al.*, 1987; Gros *et al.*, 1988; Schurr *et al.*, 1989). The *in vitro* selection of multidrug-resistant cell lines involves exposure of cells in culture to anticancer drugs. Of the most widely used techniques are:

- continuous exposure of cells to initial subtoxic levels of the drug followed by stepwise dose increments of the same drug,
- brief exposure of the cells to an initial high concentration of the selecting agent and subsequent growth of the cells in the absence of selection pressure,
- chemical mutagenesis which increases the probability of the emergence of a replicating drug-resistant clone.

2.2. Tumour Cell Sensitivity to Drugs

2.2.1. Resistance, Survival and Toxicity

The effectiveness of many anticancer agents is increased by a corresponding escalation of their concentration or accumulation in target sites. As the dose is increased, so normal tissues may become more prone to potential side effects. Therefore, tolerance by the patient is influenced by both the dosage and duration of chemotherapy which, in turn, should be determined by a realistic appraisal of potential therapeutic benefit. Toxicity is a consequence of both tumour resistance to the anticancer drug and its lack of specificity for neoplastic cells. Thus, dosage

must be maximized to the point where tumour cell kill can be achieved without irreversible damage to normal tissues. Quality of life is accordingly regarded as the principal moderator of therapeutic intervention. In the face of full-blown resistance despite rapid tumour proliferation, as for example in terminal malignant melanoma, it is unjustifiable to persist with intensive chemotherapy which may be more deleterious to the patient than to the tumour.

As other specific and selective treatment modalities are made available and drug resistance becomes more accurately predictable, toxicity may possibly be reduced to a minimum and quality of life improved throughout therapy (Benard *et al.*, 1990; Kaye, 1991; Kellen, 1991). Novel molecular and pharmacological approaches such as drug carriers (microspheres and polymers), antibody-directed strategies using pro-drugs and enzymes, genetic manipulation of the immune system, the introduction of suicide genes into tumour cells, or the application of drug-resistance genes to protect normal cells during chemotherapy, are already entering clinical trials and are anticipated to benefit cancer patients.

2.2.2. Cross-Resistance

Some tumours respond favourably to initial chemotherapy only to be complicated subsequently by relapse, which is characterized by decreased sensitivity not only to the primary drug, but often to all secondary regimens (Hutchison and Schmid, 1973; Whitehouse, 1984). This cross-resistance pattern to multiple drugs seems to be largely unaffected by further dose increases and may influence the outcome of treatment (Gerlach *et al.*, 1986b; Benard *et al.*, 1990). The specificity of a given cross-resistance profile is determined by the actual mechanism of resistance, *e.g.*, the altered expression of plasma membrane components and decreased drug accumulation (Bech-Hansen *et al.*, 1976). In addition, cross-resistance may arise from biochemical changes in the tumour cell population which occur independently of the original selection pressure.

2.2.3. Collateral Sensitivity

This term refers to a cell population that is insensitive to some drugs but is more prone than the parent population to the cytotoxic effects of others (Szybalski and Bryson, 1952; Henderson, 1984). Such predisposition may be the consequence of biochemical alterations that accompany the development of resistance. In this regard, Brockman (1970) suggested that the increased sensitivity to deazauridine observed in a cytosine arabinoside (Ara-C)-resistant L1210 cell line could be the

result of their inability to metabolize deoxycytidine (dCT) due to loss of deoxycytidine kinase (dCK) activity. Many tumours that are resistant to Ara-C and dCT, for example, have elevated activities of cytidine deaminase. The relatively inactive compound bromodeoxycytidine was therefore prepared with the idea that its deamination by this enzyme would lead to the formation of bromodeoxyuridine, an antimetabolite with superior potency (Henderson, 1984). Despite insufficient knowledge of its biochemical basis and associated unpredictable clinical response, the collateral sensitivity paradigm has proven useful in the treatment of some tumours, particularly acute leukaemia, but has not improved "disease-free" survival in solid tumours.

3. Mechanisms of Drug Resistance

Resistance to anticancer drugs is associated with multiple genetic, biochemical and pharmacological alterations in tumour cells (Assaraf *et al.*, 1989; Kane *et al.*, 1990; Ban, 1992), some of which are reviewed below.

3.1. Changes in Plasma Membrane Proteins

3.1.1. P-Glycoprotein

Numerous studies have sought a biochemical explanation for antitumour drug resistance in mammalian and other cell lines. Some of the earliest investigations in this area focused exclusively on the cell surface as a possible site of the resistance lesion, and increased synthesis of plasma membrane glycoproteins was found in drug-resistant cells (Bosman, 1971). Specifically, the activity and levels of glycosyl transferases were increased and those of glycosidases were decreased in such cells (Bosman and Kessel, 1970; Kessel and Bosman, 1970; Bosman, 1971). The net effect of such metabolic regulation of enzyme activity would be to increase overall the amount of glycoproteins, but this was found not to be the case since the raised levels appeared to be restricted to specific types of surface glycoproteins.

Drug-resistant cells express on their surfaces a high molecular weight (MW) glycoprotein (designated P-glycoprotein, or P170, or Pgp) which is absent in their drug-sensitive counterparts (Juliano and Ling, 1976; Peterson and Biedler, 1978; Beck *et al.*, 1979). The MW of Pgp, estimated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), has been reported to be 150 kilodalton (kDa) (Peterson and Biedler, 1978), 165 kDa (Juliano and Ling, 1976), and between 170- and 190 kDa (Beck *et al.*, 1979). These discrepancies in MWs would imply that different glycoproteins may be involved in the expression of

resistance in the various cell lines studied. Instead, it has been suggested that such differences may either reflect fluctuations in the extent of glycosylation of similar proteins or may be due to differences in experimental calibration of SDS gels (Beck, 1984).

Distinct protein changes in plasma membranes of MDR cells have been correlated with decreased drug uptake (Garman and Center, 1982; Barrand *et al.*, 1993). Increased expression of a surface membrane glycoprotein of apparent molecular weight (M_r) 170- to 190-kDa has been implicated in the progression of MDR (Beck *et al.*, 1979; Koch *et al.*, 1986; Arsenault *et al.*, 1988). Cells that have acquired resistance to natural-product chemotherapeutic agents such as vincristine (VCR), daunorubicin (DNR), dactinomycin (DTN), or doxorubicin (DOX) and simultaneously exhibit cross-resistance to other drugs in this category are termed multidrug resistant. A large number of these cells express Pgp (Nielsen and Skovsgaard, 1992; Wu *et al.*, 1992).

3.1.2. Epidermal Growth Factor Receptor

Some MDR cells exhibit *in vitro* growth behaviour of non-transformed cells and become less tumourigenic (Biedler *et al.*, 1975). This reverse transformation is thought to be due to altered cellular responses to growth factors and fluctuations in the level and function of their receptors. Furthermore, similarity in the MW and isoelectric point (pI) of Pgp (Peterson *et al.*, 1983) and the EGF receptor (Cohen *et al.*, 1982; Carlin *et al.*, 1983) has prompted investigations into the presence of the latter in MDR cells. Sequence analysis, however, established that these two proteins are distinct (Ullrich *et al.*, 1984; Gros *et al.*, 1986b). Despite observations of increased EGF receptor expression in resistant cells, no quantitative correlation between this increase and the degree of Pgp-mediated resistance could be demonstrated (Meyers *et al.*, 1986; Scotto *et al.*, 1986; Meyers *et al.*, 1988). Although elevated expression of this receptor may be associated with diminished oncogenic potential of MDR cell lines (Meyers *et al.*, 1986), several reports favour the idea that MDR and tumourigenicity are independent events (Hill, B.T. *et al.*, 1988; Slovak *et al.*, 1991).

3.1.3. Other Membrane Proteins

Several researchers have described enhanced synthesis of membrane proteins and glycoproteins not characterized as Pgp. Examples include:

- DOX-resistant HL-60 human leukaemia cells which synthesize glycoproteins of MWs 160- and 110 kDa (Bhalla *et al.*, 1985), 150- and 120 kDa (Marsh *et al.*, 1986) not detectable in sensitive cells (Center, 1987),
- Surface membrane glycoproteins with MWs 130-, 155-, and 180- to 210 kDa have been characterized in vinblastine (VBL)-resistant human leukaemia cells (Danks *et al.*, 1985),
- Membrane proteins of MWs 100- and 160 kDa were found to be expressed in DOX-resistant P388 mouse leukaemia cells (Shanbaky *et al.*, 1986),
- Mouse cells transfected with high MW DNA from DTN-resistant Syrian hamster cells express a 140-150-kDa surface glycoprotein (Suarez *et al.*, 1985),
- DTN-resistant human myeloid leukaemia cells which synthesize three membrane glycoproteins of MWs 65-, 83-, and 180 kDa (Tsuruo *et al.*, 1986).

There are also reports of decreased production of membrane proteins, *e.g.*, loss of a 100-kDa cell-surface protein in Chinese hamster cells (Peterson *et al.*, 1983). These sporadic appearances of other proteins in MDR cells have yet to be phenotype-tested. Nevertheless, the expression and identification of discrete or novel species of MDR-related membrane proteins point to the complexity and diversity of this phenotype (Gros and Shustik, 1991; Deuchars *et al.*, 1992; Krishnamachary and Center, 1992).

3.2. Lipid Structural Order and Membrane Fluidity

Since the transport of molecules across the cell membrane is regulated by the physical state of the lipid bilayer, typically poor accumulation of drugs by MDR cells may be due to alterations in membrane lipids. Compositional analysis of plasma membranes of drug-sensitive and -resistant CHO cells showed no apparent variation in the relative amounts of cholesterol, cholesteryl esters, neutral glycerides, free fatty acids, or total phospholipids (Ling *et al.*, 1977). In contrast, changes in the proportions of phospholipid classes were noted in murine (Ramu *et al.*, 1984) and human (Wright *et al.*, 1985) leukaemia cell lines. The statistical significance of these differences, however, appeared to be marginal. No differences in fatty acid arrangement of plasma membranes between sensitive and resistant cells have been reported. Montaudon *et al.* (1986) postulated that the increase in the level of polyunsaturated fatty acids in drug-resistant glioblastoma cells may appear occasionally in the MDR phenotype. Similarly, Rintoul *et al.* (1984) concluded that alteration of cell membrane lipid structural order is not an essential component of adriamycin (ADR) resistance in Chinese hamster lung (CHL) cells. It is likely, however, that increased fluidity due to elevated levels of polyunsaturated fatty acids

may be able to contribute to the maintenance of reduced intracellular drug levels by preventing diffusion of drugs through the bilayer. In addition, membranes from drug-resistant cells may contain domains of less complex lipids than those found in drug-sensitive cells, thereby contributing to diminished drug permeability in MDR. Of special interest is the observation that the biosynthesis of gangliosides is blocked at the level of haematosides (G_{M3}) in actinomycin D (ActD)-resistant Chinese hamster cells (Peterson *et al.*, 1979). Increased expression of polysialylated gangliosides (particularly the disialoganglioside, GDIa) and decreased expression of the monosialoganglioside G_{M2} may be pivotal during the acquisition of MDR (Mazzoni *et al.*, 1991).

Verapamil, tamoxifen and dipyridamole have been found to induce increased synthesis of phosphatidyl choline in MDR cells. The relative resistance of these cells to permeabilization by digitonin and the observation that verapamil is able to counteract this effect suggests that verapamil may modify the membrane lipid organization in these cells and modulate drug permeability (Ramu *et al.*, 1991). In addition, studies with vesicles of the bile canaliculus membrane (CMV) in which Pgp has been localized to the apical side, have demonstrated that increases in membrane fluidity of CMV inhibited [^{3}H]daunomycin and [^{3}H]vinblastine accumulation (Sinicrope *et al.*, 1992). Therefore, an alteration in the physical state of CMV is likely to be at least one important modulator of Pgp function.

3.3. Alterations in Drug Transport

3.3.1. Drug Transport in P-Glycoprotein-Mediated MDR

The pharmacologic basis for *in vitro* MDR appears to be a decrease in the steady state accumulation of antitumour agents, attributed either to diminished uptake or decreased retention (Skovsgaard, 1978; Fojo *et al.*, 1985; Sirotnak *et al.*, 1986). This may be a direct consequence of reduced drug binding to Pgp and/or active efflux via Pgp (Beck *et al.*, 1983; Bradley *et al.*, 1988; Dordal *et al.*, 1992). There is no consensus in the literature whether the various drug types most frequently involved in the MDR phenotype (*e.g.*, *Vinca* alkaloids, anthracyclines, colchicine and actinomycin D) enter the cells by passive diffusion or carrier-mediated transport mechanisms (Dalmark and Hoffman, 1983; Sirotnak *et al.*, 1986; Inaba *et al.*, 1987). However, the majority of cell lines which overexpress Pgp also exhibit increased drug efflux (Nielsen and Skovsgaard, 1992).

3.3.2. P-Glycoprotein and Drug Binding

The cellular accumulation and retention of drugs may be associated with their binding to transport or carrier proteins. Much insight into this putative mechanism has come from the demonstration that [^{3}H]VBL binds more avidly to plasma membrane vesicles prepared from drug-resistant cells compared to those derived from drug-sensitive cells (Cornwell *et al.*, 1986). In addition, unlabelled VBL, VCR, and DNR competed with the binding of [^{3}H]VBL to crude membranes whereas colchicine (COL) and DTN were comparatively less competitive. It was not evident from these studies that different binding sites and affinities for the various drugs contributed to the observed disparities.

Safa *et al.* (1986) showed that the protein which binds a photoaffinity analogue of VBL could be immunoprecipitated with polyclonal antibody against Pgp, and that approximately 90% of the label could be displaced with a 200-fold excess of VBL. Numerous other reports corroborated these findings and it is now widely accepted that Pgp binds at least to some drugs (Akiyama *et al.*, 1988; Beck *et al.*, 1988; Bruggemann *et al.*, 1992). The binding of [^{3}H]VCR to plasma membranes isolated from ADR-resistant human K562 cells occurs in an adenosine triphosphate (ATP)-dependent manner, and is blocked by non-hydrolyzable ATP analogues and inhibited by excess non-radiolabelled VBL, VCR, ActD, and ADR. These studies support the notion that ATP hydrolysis is a necessary component of drug binding to Pgp, and presumably also for subsequent efflux (Ambudkar *et al.*, 1992; Doige *et al.*, 1992; Shimabuku *et al.*, 1992).

A significant observation is that many of the compounds that participate in the MDR phenotype share properties such as hydrophobicity (lipid solubility) and cationic charge at physiological pH (Zamora *et al.*, 1987; Zamora *et al.*, 1988; Ford *et al.*, 1989). Such common characteristics could contribute to non-specific interactions with hydrophobic proteins such as Pgp that do not require drug-ligand interactions. By contrast, recent evidence has indicated that substrate-specific binding to Pgp does take place (Pearce *et al.*, 1989; Ramu and Ramu, 1989; Spoelstra *et al.*, 1991).

3.3.3. P-Glycoprotein as Active Efflux Pump

Several groups assert that Pgp contains nucleotide binding sites (Chen *et al.*, 1986; Gerlach *et al.*, 1986a; Gros *et al.*, 1986a). P-glycoprotein has been shown to bind photoaffinity analogues of ATP (Cornwell *et al.*, 1987b) and to contain adenosine

triphosphatase (ATPase) activity (Hamada and Tsuruo, 1988). Experiments using Pgp-mAb-Protein A-Sepharose conjugates established that verapamil (VPL) and trifluoperazine (TFP), unlike VCR and DOX, enhanced such activity. Nucleoside triphosphates (NTPs), but not VBL, effectively decrease the affinity of Pgp for the nucleotide, indicating that distinct binding sites for NTPs and drugs may exist on the molecule. Doige *et al.* (1992) confirmed that certain drugs and chemosensitizers, including COL, progesterone, nifedipine, VPL and TFP enhance the catalytic function of the enzyme, and that Mg^{2+} and ATP are required for optimal activity. Doige and Sharom (1992) demonstrated that the uptake of [^{3}H]COL and [^{3}H]VBL into plasma membrane vesicles (PMV) from the MDR CH^RC5 cell line, but not the AuxB1 drug-sensitive parent, was stimulated by the presence of ATP which is also a requirement for the efflux of VBL from PMV isolated from human MDR KB-V1 cells (Horio *et al.*, 1988; Lelong *et al.*, 1992). Furthermore, the observation that expression of the human *MDR*1 gene in cultured insect cells via a baculovirus vector generates a high activity vanadate-sensitive membrane ATPase that is markedly stimulated by compounds known to interact with the Pgp provides further evidence for the existence of an energy-requiring "drug exodus" system in MDR cells (Sarkadi *et al.*, 1992).

3.3.4. Mechanism of P-Glycoprotein-Mediated Drug Efflux

According to the model depicted in Figure 2, lipophilic drugs enter the cells by passive diffusion through the lipid bilayer, bind Pgp on the cytoplasmic side of the plasma membrane and are pumped out of the cell utilizing the energy of ATP hydrolysis. This scenario suffers from a major drawback in that Pgp, to be effective in efflux, should have a higher affinity for the drug than its intracellular target. It is unlikely that the affinity of Pgp for any individual ligand would be very high, since it binds reversibly to a wide variety of structurally dissimilar drugs. Also, anticancer agents are primarily selected on the basis of their high specificity for intracellular targets, *e.g.*, the efficacy of DNR and *Vinca* alkaloids can be attributed to their strong affinity for tubulin.

In an alternative efflux model, drug binding to Pgp is proposed to occur within the membrane phase (interior) instead of in the cytoplasmic domain (Gros *et al.*, 1986a). An example in favour of this view is resistance to gramicidin D (GRAM), a membrane ionophore, which exerts its cytotoxic effect at the cell surface, without entering the cell (Bradley *et al.*, 1989). Mutational inactivation of the nucleotide binding sites of Pgp abolishes resistance to GRAM, suggesting that resistance to this ionophore is also dependent upon ATP (Roninson, 1991a).

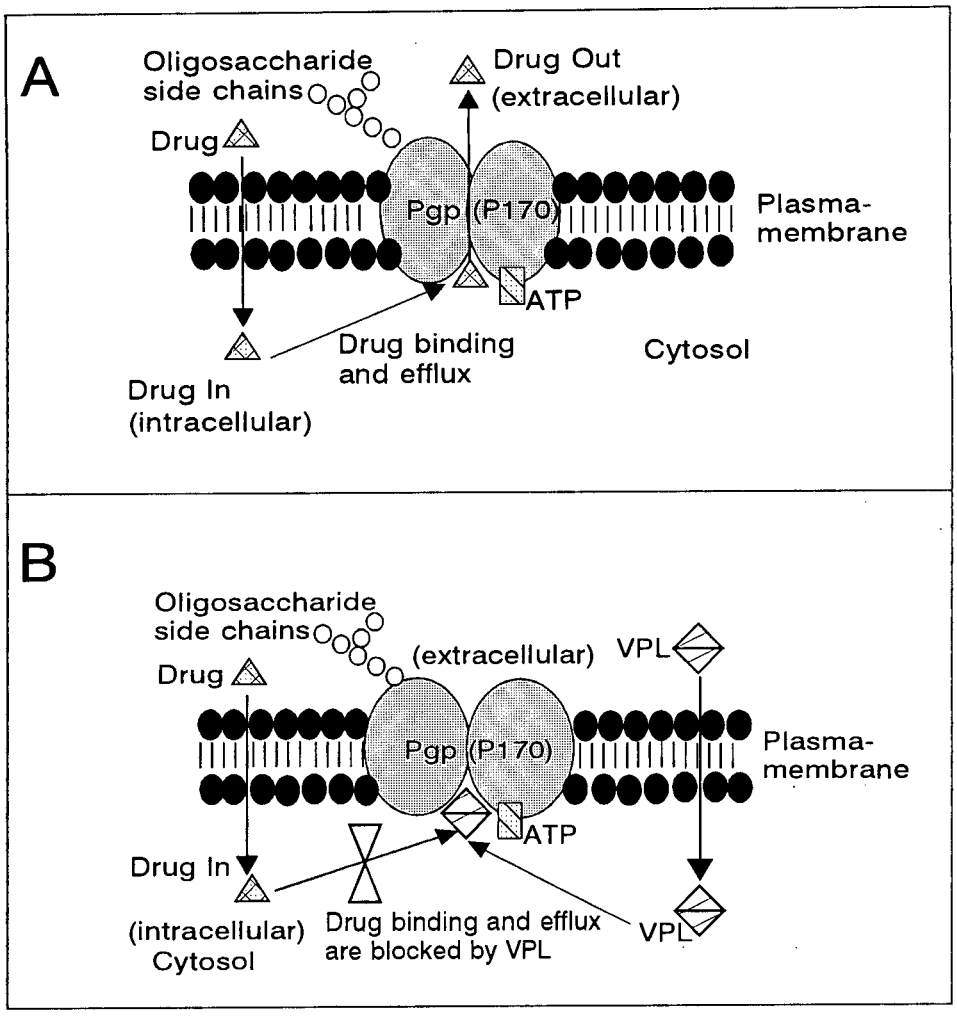

Figure 2. Simplified schematic model of the mechanism of action of P-glycoprotein. A, P-glycoprotein uses ATP as energy source to transport the drug out of the cell; B, Verapamil (VPL) inhibits the active outward transport of the drug by competing for binding site(s) on the P-glycoprotein molecule. The result is a net intracellular accumulation and retention of the drug. (Adapted from Dickson and Gottesman, 1990).

Recently Higgins and Gottesman (1992) proposed that substrates for Pgp first partition into the inner lipid phase of the plasma membrane before being pumped to the extracellular side. The substrate may also be flipped from one face of the membrane to the other (transverse diffusion) by the multidrug transporter, cognate to the rotation of phospholipids by flippases (Devaux, 1991).

Another model, first described by Beck (1987) is based on observations of an increase in the number of vacuoles and apparent localization of anthracyclines to cytoplasmic vesicles in MDR cells. P-glycoprotein is postulated to be anchored in both plasma and intracellular membranes so that drugs entering the cell are pumped into the lumen of intracytoplasmic membranes and subsequently discharged by exocytosis. This hypothesis agrees with immunohistochemical evidence that Pgp is present in both the plasma membrane and cytoplasm (Willingham *et al.*, 1987), and may show primarily intracytoplasmic distribution in cells not selected for drug resistance *in vitro* (Scheper *et al.*, 1988).

3.4. Overexpression of Sorcin in MDR Cells

Sorcin is a 22-kDa cytosolic calcium-binding protein excessively produced in many but not all MDR cells (Meyers and Biedler, 1981). Cells which synthesize this protein also manifest gene amplification (Meyers *et al.*, 1985) which may be a fortuitous or random occurrence and/or the result of its proximity to the *mdr* gene (van der Bliek *et al.*, 1986a; van der Bliek *et al.*, 1986b; Jongsma *et al.*, 1987). Sorcin consists of four "E-F hand" structures typical of calcium binding sites (van der Bliek *et al.*, 1986a) and has striking homology with the light chain of calpain. Two of the calcium binding sites contain putative recognition sites for cAMP-dependent protein kinase. The protein is phosphorylated in a cAMP-dependent manner (Meyers and Biedler, 1982; Biedler *et al.*, 1983) and probably, like calpain, activates protein kinase C (PKC) which would explain the elevated activity of this enzyme in MDR cells (Fine *et al.*, 1986). The abundance of sorcin in some drug-resistant cells may confer increased responsiveness to calcium channel blockers (Cano-Gauci and Riordan, 1987). Furthermore, the use of calcium antagonists in the prevention of drug resistance (see section 6.2.2.) may point to possible interplay of Pgp and sorcin.

3.5. DNA Repair and Drug Resistance

Increased repair of DNA confers resistance to anticancer compounds which exert their cytotoxic effects by intercalating with DNA (Fox and Roberts, 1987) and

causing single- or doublestrand breaks (Jamali *et al.*, 1989; Bungo *et al.*, 1990; Capolongo *et al.*, 1990). These drugs include the alkylating agents: platinum compounds, nitrosoureas, ADR, DNR, bleomycin (BLEO), and related antibiotics. Different cell lines vary in their ability to replace DNA lesions and their repair capacity may be intrinsically related to cellular accumulation and sensitivity to antitumour agents (Jamali *et al.*, 1989). The platinum complexes, *e.g.*, *cis*-DDP (*cis*-dihlorodiaminoplatinum II), represent an important class of cytotoxic drugs generally capable of forming cross-links in DNA critical to their cytotoxicity (Bungo *et al.*, 1990; Chu and Chang, 1990). *Cis*-DDP-resistant cell lines express increased levels of cellular factors that recognize damaged DNA, and concomitantly are more efficient in repair of such lesions. Furthermore, it has been suggested that acquired cellular non-responsiveness to cisplatin may be associated with increased gene-specific DNA repair of interstrand cross-links (Harris and Hochhauser, 1992; Zhen *et al.*, 1992).

It is generally believed that the mechanism of cisplatin refractoriness differs from that of MDR. Although *cis*-DDP-resistant cells also exhibit decreased uptake of platinum compounds (Chao, C.C. *et al.*, 1992), Misawa *et al.* (1992) have clearly established that the reduced accumulation is not due to *mdr*1 gene expression. Cell lines selected for resistance to drugs in the MDR class (*e.g.*, vinblastine, adriamycin, colchicine, vincristine and actinomycin D) usually express cross-resistance to other drugs in the same class (Kimiya *et al.*, 1992), but not to DNA intercalating agents (*e.g.*, methotrexate, cisplatin, nitrosoureas). Similarly, Futscher *et al.* (1992) reported that a DOX-resistant multiple myeloma cell line displayed a sensitivity pattern to the nitrosoureas (streptozotocin, STZ; 1,3-bis[chloroethyl]-1-nitrosourea, BCNU) identical to its drug-sensitive parental cell line. Their studies imply that cells resistant to "MDR-related" drugs are not resistant to nitrosoureas. In general, drugs which modify the MDR phenotype do not show a sensitizing effect to cisplatin (Morikage *et al.*, 1991).

By contrast, Yang *et al.* (1993) have recently indicated that Pgp-mediated MDR and *cis*-DDP refractory phenotypes may coexist in certain tumour types. Nevertheless, insensitivity to platinum-containing drugs and alkylating agents is multifactorial in that patients not only fail to respond to drugs from the same pharmacological classes as used in primary therapy, but they also do not respond to other available drugs or irradiation (Hamilton *et al.*, 1990). Therefore, compounds which will induce DNA strand breaks and inhibit DNA repair could be of particular value in reversing such drug resistance mechanisms (Jamali *et al.*, 1989;

Chen and Zeller, 1990; Zhen *et al.*, 1992). (See also sections 3.6. and 6.3.2. for discussion on DNA damage by anthracyclines and epipodophyllotoxins.)

3.6. DNA-Topoisomerase II-mediated MDR (Atypical MDR)

Atypical MDR (at-MDR) is characterized by decreased DNA topoisomerase II (TopoII) activity (Beck *et al.*, 1987; Danks *et al.*, 1987; Danks *et al.*, 1988), possibly due to reduced binding or hydrolysis of ATP by the enzyme (Danks *et al.*, 1989; Hoban *et al.*, 1992). Cells displaying this form of resistance are cross-resistant to a variety of anticancer agents, except the *Vinca* alkaloids, DNR and ActD, and do not differ from parental sensitive cells in their uptake, accumulation and retention of drugs. They are also unaffected by VPL and chloroquine (CLQ), known modulators (*cf.* section 6) of Pgp-MDR (Beck and Danks, 1991), but may exhibit a form of collateral sensitivity to anti-mitochondrial drugs (de Jong *et al.*, 1992). Some cell lines exhibit features of both Pgp- and at-MDR (Friche *et al.*, 1991; Kamath *et al.*, 1992). However, the large variability in Pgp expression and TopoII activity observed in, for instance, fresh ovarian tumour specimens (van der Zee *et al.*, 1991), implies that clinical correlations of the concomitant expression of Pgp- and at-MDR in cell lines should be made with caution.

3.7. Glutathione and GSH-S-Transferases

The increased expression of Pgp is typical of a vast number of MDR cell lines. There are reports, however, of cell lines that exhibit resistance to anticancer drugs in the absence of Pgp (McGrath and Center, 1988; Nygren *et al.*, 1991; Nakagawa *et al.*, 1992). Alterations in the levels of glutathione (GSH), GSH-S-transferase (GST), GSH reductase, and GSH peroxidase have been observed in DOX-resistant ovarian carcinoma cells (Hamilton *et al.*, 1985; Lewis *et al.*, 1992), multidrug-resistant human colon cancer cells (Chao, C.C. *et al.*, 1992; Peters *et al.*, 1992) and breast tumour cells (Batist *et al.*, 1986; Akman *et al.*, 1990; Whelan *et al.*, 1992), and elevation of non-protein sulfhydryls in myeloma cells (Bellamy *et al.*, 1989). The intrinsic GSH level and GST activity may affect anthracycline toxicity *per se* and not MDR in the human colon cancer cells (Chao, C.C. *et al.*, 1992).

4. Molecular Genetics of Multidrug Resistance

4.1. Gene Amplification and Inactivation in MDR

Drug-resistant cells often display chromosomal aberrations, including homogeneously staining regions (HSRs) and double minute chromosomes (DMCs;

cytogenetic markers of gene amplification) (Chao *et al.*, 1991; Roninson, 1992a; Schoenlein *et al.*, 1992). Molecular characterization of amplified genes in numerous cell lines *in vitro* has contributed to the elucidation of the genetic basis of MDR. Roninson *et al.* (1984) correlated resistance to ADR and DNR, respectively, in Chinese hamster cell lines with amplification of common DNA sequences. It was deduced from this investigation that one or several genes in the region of the amplicon must be activated in MDR. A large DNA segment of the amplified sequence associated with MDR has subsequently been cloned and characterized, the basic transcription unit of the MDR domain being designated *mdr*. Recent evidence suggests that coordinate induction of the *mdr* and *P-450IA* gene families (Thorgeirsson *et al.*, 1991) as well as sequential amplification of the dihydrofolate reductase (*DHFR*) and *mdr* genes (Assaraf *et al.*, 1989) may provide a collateral response to prevent lethal accumulation and effects of xenobiotics.

4.2. Chromosomal Localization of *mdr* Genes

Analysis of human tumour cell lines resistant to the anthracycline DOX, the *Vinca* alkaloid VBL, and the epipodophyllotoxin teniposide VM-26, revealed the involvement of chromosomes 1, 3, 6 and 7 in at least two of the six cell lines (Trent *et al.*, 1987). The latter was the most consistently altered, with breaks along its entire length, but clustered along the mid-distal long arm (*ca.* 57% of all breaks). Despite the finding that breakpoints in several cell lines are cytologically coincident with the loci of the Pgp gene, many are not. If the interruptions along chromosome 7 are aligned with known growth-regulatory sequences, the region corresponds to the EGF receptor gene (Zuckier and Tritton, 1983). It is not clear whether the structural interchanges involving this chromosome are indeed associated with altered expression of the Pgp or EGF receptor genes. However, recent data on SW-1573 cells indicate that the acquisition of non-Pgp-mediated MDR involves a specific deletion or a translocation involving the short arm of chromosome 2, whereas rearrangement of the long arm of chromosome 7 is a critical event in the development of Pgp-mediated MDR (Nieuwint *et al.*, 1992).

4.3. Origin, Evolution, and Nomenclature of *mdr* Genes

The striking sequence homology between the N-terminal and C-terminal domains of Pgp (Chen *et al.*, 1986) warrants consideration that *mdr* arose by internal duplication of its ancestor (Figure 3). Recently, Chen *et al.* (1990) determined the entire intron-exon sequence of the human *MDR*1 gene. On the basis of their results they conceptualized an alternative genealogic scheme. According to this hypothesis,

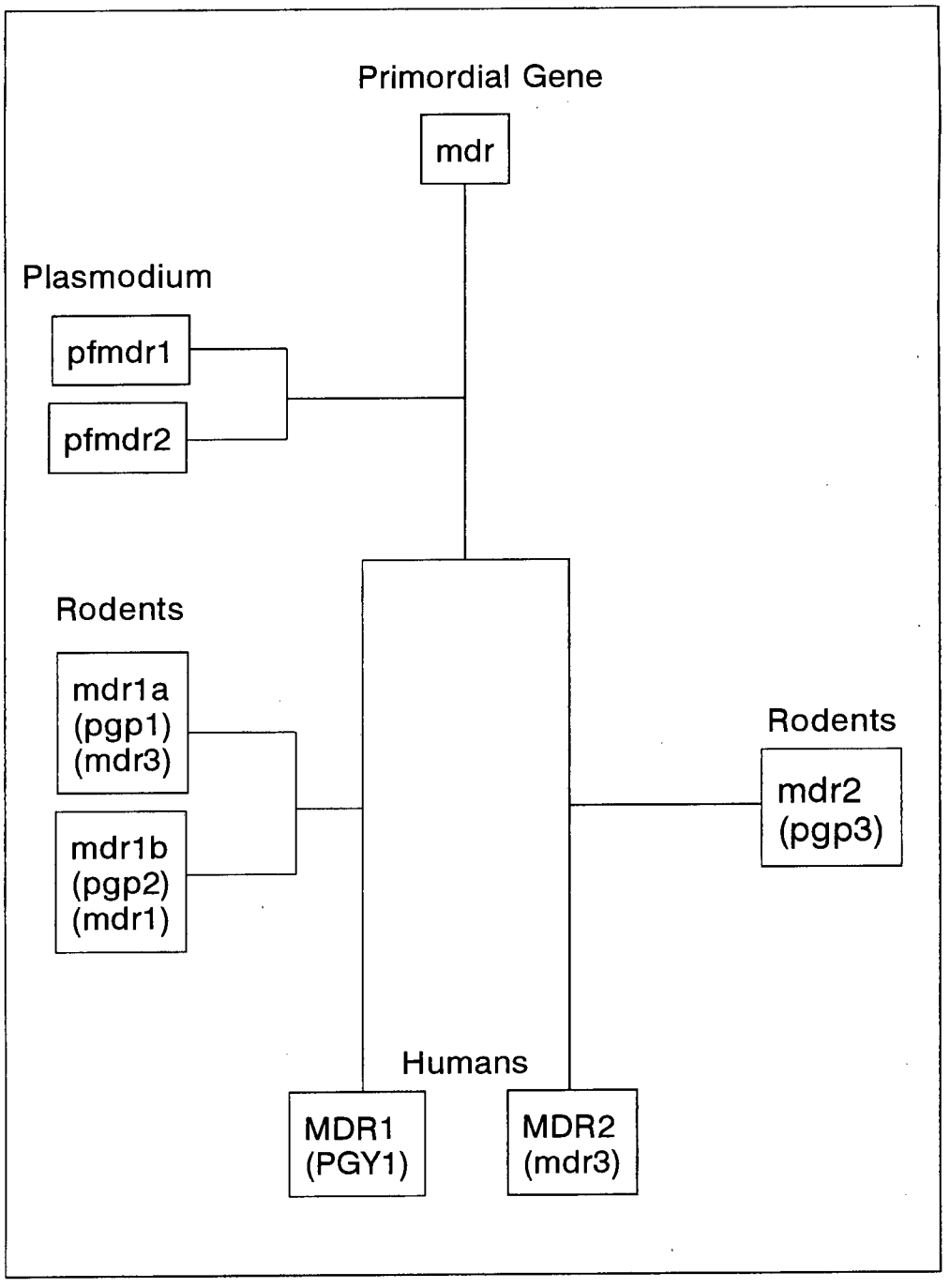

Figure 3. Proposed scheme for the evolutionary relationship between P-glycoproteins. Alternative names for P-glycoprotein are indicated in parenthesis. See section 4.6 for details. (Adapted from Roninson, 1991b).

primordial proteins corresponding to the left and right halves of Pgp were formed separately by fusion of common or related entities coding for the nucleotide-binding domain with genes for different transmembrane proteins. Subsequent convergence of these two independently evolved genes resulted in the formation of Pgp. The availability of *mdr* cDNA clones has facilitated analysis of complete amino acid sequences of the mammalian, protozoan and yeast gene products which share evolutionary links with a diverse group of membrane transport proteins in bacteria and invertebrates (Roninson, 1991b).

4.4. MDR Expression in *Plasmodium falciparum*

Resistance of *Plasmodium falciparum* to CLQ and successive antimalarial drugs is also associated with increased drug efflux (Krogstad *et al.*, 1987). Interestingly, two genes closely related to mammalian *mdr*, were isolated from *P. falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989). One of these, *pfmdr*1, was amplified in two out of five CLQ-resistant strains (Foote *et al.*, 1989) and in one mefloquine-resistant derivative (Wilson *et al.*, 1989). *Plasmodium falciparum* and mammalian Pgps show a high degree of sequence homology in both halves of the protein, hence the probability that its emergence preceded the divergence of protozoan and metazoan eukaryotes (Roninson, 1991b).

4.5. Yeast *mdr* Expression

Several studies have described an *mdr* gene in the yeast *Saccharomyces cerevisiae* (McGrath and Varshavsky, 1989; Raymond *et al*, 1992) that codes for a protein consisting of 1290 amino acid residues, 57% of which are highly conserved with human *MDR*1-encoded Pgp, and resembles another yeast gene, *STE6*, associated with secretion of *a-factor* pheromone, a modified dodecapeptide that carries a highly hydrophobic farnesyl group attached to its C-terminal cysteine (Cys) residue (Anderegg *et al.*, 1988). The secretion of *a-factor* follows the classical signal sequence-dependent protein secretory pathway, implicating yeast Pgp as a component of a novel protein efflux system. The results of their study exemplify a normal function for Pgp.

Recent evidence indicates that pleiotropic resistance in yeasts mimics MDR in higher eukaryotes (Nevzglyadova *et al.*, 1992). By analogy with *a-factor* secretion, McGrath and Varshavsky (1989) concluded that at least some of the mammalian Pgps may function in transmembrane transport of specific proteins or peptides and that hydrophobic post-translational modifications such as myristylation

may serve to target these proteins to the cell surface and Pgp. Interleukin-1 (IL-1), for example, whose processing and secretion is similar to the biogenesis of the pheromone, has been proposed as a potential substrate for mammalian Pgps.

4.6. Human and Rodent *mdr* Genes

Analyses of hybridization patterns and sequencing of cDNA and genomic clones revealed that human cells contain two and rodent (mouse and hamster) cells three *mdr* genes (Croop *et al.*, 1989; Hsu *et al.*, 1989; Ng *et al.*, 1989). *MDR*1 is directly associated with drug resistance in humans; the function of *MDR*2, also referred to as *mdr*3, remains undetermined. Chin *et al.* (1989) also detected these in green monkey DNA. Two genes appear to be actively involved in drug resistance in rodent cells (Gros *et al.*, 1991a; Gros *et al.*, 1991b). One is designated *pgp*1 in hamsters and *mdr*3 in mice and the other, *pgp*2 in hamsters and *mdr*1 in mice; the sequences of both match closely that of human *MDR*1 (Croop *et al.*, 1989; Ng *et al.*, 1989). The third member, termed *pgp*3 in hamsters and *mdr*2 in mice, is homologous to human *MDR*2 and and also lacks association with MDR.

The alignment of amino acid sequences of mammalian Pgps is almost identical, particularly in the region of the nucleotide binding domains. This reflects a strong selection pressure for the evolution and conservation of these sites (Endicott *et al.*, 1987; van der Bliek *et al.*, 1988; Hsu *et al.*, 1989). The proposed evolutionary relationship between Pgps is illustrated in Figure 3. According to this scheme, *mdr*1 has undergone duplication in the rodent lineage after the divergence of primates and rodents, or before the mammalian branching with subsequent loss of one of the two *mdr*1 genes in the primate lineage. Roninson (1991b) contends that the latter possibility seems less likely, since the rodent *mdr*1a and *mdr*1b products have identical amino acid residues where they differ from the human gene product.

4.7. The P-Glycoprotein Multigene Family

The existence of multiple non-allelic Pgp genes has been postulated by several researchers (Riordan *et al.*, 1985; de Bruijn *et al.*, 1986; Roninson *et al.*, 1986) and adds to the complexity of the MDR phenotype. Information regarding the nomenclature and actual number of these genes is not easily established since newer units are invariably being identified and added to an already indistinct list (Borst and van der Bliek, 1991; Kirschner *et al.*, 1992). The utilization of molecular biology techniques, in combination with mAbs against Pgp (Chen *et al.*, 1986;

Hamada and Tsuruo, 1986a; Gros *et al.*, 1988), has extended our knowledge of the genetics of MDR (Barrand and Twentyman, 1992). Complete cDNA sequences for two human and two murine Pgps (Chen *et al.*, 1986) and partial sequences for Chinese hamster genes (Endicott *et al.*, 1987; Roninson *et al.*, 1984; Gros, *et al.*, 1986b) have been documented.

Sequence analysis of cDNA encoding *mdr* has revealed that the epitope recognized by mAb C219 is present in almost all Pgps, and thus provides a universal probe for its detection. Monoclonal antibody C494, for example, binds preferentially to the *pgp*1 product in human and hamster cells, whereas the mAb C32 is specific for hamster *pgp*1 and *pgp*2 products (Kartner *et al.*, 1985). The requirement for a multigene family is not well established, but can be discerned especially from the perspective of the complexity and diversity in the MDR phenotype. It is possible that Pgp molecules differ in their efficiencies in transporting specific classes of anticancer drugs and, under stringent selection pressure, more efficient molecules may be selected (Endicott and Ling, 1989).

5. Structure and Function of P-Glycoprotein

5.1 Sequence Homology with Other Proteins

The *mdr*1 gene is a member of the ATP binding cassette (ABC) superfamily of active transporters, including the cystic fibrosis transmembrane conductance regulator (*CFTR*), the *hly*B gene of bacteria, the yeast *a-factor* (*STE*6), and the histocompatibility antigen modifier (*HAM*) (Hyde *et al.*, 1990; Berkower and Michaelis, 1991; Bremer *et al.*, 1992). The cystic fibrosis gene product (Fuller and Benos, 1992; Higgins, 1992a; Higgins, 1992b) and Pgp are structurally related proteins and both are probably associated with chloride channel activities (Gill *et al.*, 1992; Gollapudi *et al.*, 1992a; Valverde *et al.*, 1992), and may therefore have a physiological role of regulating epithelial cell volume (Trezise *et al.*, 1992).

In addition, the *mdr*1 efflux pump has been reported to function as an ATP channel (Abraham *et al.*, 1993) which may be specific for ATP transport essential to the regulation of Pgp activity in a manner analogous to the regulation of *CFTR* by phosphorylated channels (Anderson *et al.*, 1992). P-glycoprotein has remarkable homology with other proteins (see also sections 4.3 and 5.1), most notably the nucleotide-binding domains of several bacterial transport proteins (Chen *et al.*, 1986; Gerlach *et al.*, 1986a; Gros *et al.*, 1986a). The best-characterized proteins in this group function as energy-coupling subunits of multicomponent transport

systems, *e.g.*, the uptake of various metabolites from the periplasm into the cytoplasm of bacterial cells (Ames, 1988; Ames, 1992; Ames and Lecar, 1992).

The *white* and *brown* genes of *Drosophila* (Mount, 1987; Dreesen *et al.*, 1988) were the first eukaryotic proteins identified to be homologous to Pgp. These proteins are thought to be involved in the uptake of pteridene precursor into pigment eye cells. This led to speculation that Pgp might be involved in the active uptake of some unknown substances, as well as in the efflux of hydrophobic compounds (Roninson, 1991b). Wu *et al.* (1991) have characterized two *Drosophila* homologues of the mammalian *mdr* gene. These, located in chromosomal regions 49EF and 65A, encode proteins that share over 40% amino acid identity with the human and murine *mdr* products. Studies on disruptions in the homologue in section 49EF implicate this gene in conferring colchicine resistance to the organism.

5.2. Transmembrane Topology

The overall organization of all mammalian Pgps is essentialy identical. The human *MDR*1-encoded glycoprotein, as predicted from its cDNA sequence, consists of 1280 amino acid residues constituting a molecular mass of 141 kDa (Chen *et al.*, 1986). It is possible that post-translational processing such as phosphorylation and glycosylation may alter the sequence of the mature molecule. The apparent MW of 141 kDa agrees with that reported for the unglycosylated protein synthesized by glycosylation-deficient mutants of Chinese hamster cells (Ling *et al.*, 1983). The predicted secondary configuration of the protein, based on analysis of its primary structure, suggests that the protein possesses 12 transmembrane helices and 2 cytoplasmic nucleotide binding sites (Chen *et al.*, 1986; Gerlach *et al.*, 1986a; Gros *et al.*, 1986a). A short mostly hydrophilic N-terminal sequence is followed by a long hydrophobic region and a long relatively hydrophilic C-terminal domain. In this tandem duplication structure, the former and latter halves show homology, especially in the C-terminal region. In addition, the protein has a putative sugar binding site. P-glycoprotein belongs to a class of polytopic or multispanning integral membrane proteins containing signal sequences, which are located internally rather than at the N-terminus (Friedlander and Blobel, 1985). The hydrophobic moment plot (Eisenberg *et al.*, 1984) of the membrane-spanning domains places all of them within or very near the area containing the majority of transmembrane segments from known channel-forming proteins.

Recent evidence strongly suggests that Pgp has a quaternary structure in the membrane and may exist as dimers compared with other active transport systems, such as the sarcoplasmic reticulum Ca^{2+}-ATPase and the H^+,K^+-ATPase which form oligomeric structures in membranes (Boscoboinik *et al.*, 1990). At present, little is known about the direct relationship between the molecular mechanism of the energy-dependent drug efflux pump and its topology in the plasma membrane. Georges *et al.* (1993) have proposed two models based on epitope mapping studies with mAb MRK-16. In the first model, the 12 α-helical transmembrane domains are closely packed in a circle to form a monomeric efflux channel with maximum pore diameter. In the second model, 4 transmembrane helices are packed in proximity to form a pore and more than one Pgp molecule may be included to form multimeric (clusters) pores. It is not evident, however, which of the two (monomer or multimer) is best representative of a functional drug-efflux pump.

5.3. Post-Translational Modification

5.3.1. Glycosylation

P-glycoprotein has been reported to be differentially glycosylated resulting in heterogeneous forms of MDR-associated glycoproteins in different cell lines (Greenberger *et al.*, 1988b; Greenberger *et al.*, 1989; Meyers *et al.*, 1989) which may or may not correlate with drug resistance (Center, 1983; Mukhopadhyay and Kuo, 1989). A VBL-resistant cell line J7.V1-1 and a DNR-resistant cell line J7.C1-100 both express a precursor of 125 kDa that can undergo rapid (half-life, $t_{1/2}\approx20$ min) differential N-glycosylation to a mature 135-140 kDa Pgp (Greenberger *et al.*, 1987). The taxol-selected cell line J7.T1-50 synthesizes two precursors with molecular sizes of 120- and 125 kDa, both products of distinct genes (Greenberger *et al.*, 1988a). In MDR KB cells only a single proform of 140 kDa that undergoes processing to a mature glycoprotein of 170 kDa has been observed. The $t_{1/2}$ of Pgp in KB-V1 cells was estimated to be between 48 and 72 hours (Richert *et al.*, 1988).

The function in MDR of the carbohydrate moieties of Pgp, which account for about 30-40 kDa of its molecular mass has not been evaluated adequately. Early observations that the addition of carbohydrates may not be a necessary component of the MDR phenotype (Beck and Cirtain, 1982; Ling *et al.*, 1983) did not inspire investigations of this nature. It has been reported that anthracycline resistance in HL-60 human promyelocytic leukaemia cells may be due to hypoglycosylation of cell-surface glycoproteins (Gervasoni *et al.*, 1991). P-glycoprotein is, however, not overexpressed in these cells. Several reports of differences between drug-sensitive and drug-resistant cells point to changes in glycosylation patterns of plasma

membrane glycoproteins (Juliano and Stanley, 1975; Beck and Cirtain, 1982; Marsh and Center, 1987). Unglycosylated forms of Pgp have also been detected in some cell lines (Germann *et al.*, 1990). Thus, differential overexpression of distinct species of Pgp may be a possible mechanism of generating diversity in the MDR phenotype (Hsu *et al.*, 1989; Georges *et al.*, 1990a; Miyamoto *et al.*, 1992).

5.3.2. Phosphorylation

The observation that Pgp is phosphorylated *in vivo* (Hamada *et al.*, 1987; Mellado and Horwitz, 1987; Richert *et al.*, 1988) has stimulated interest in the regulatory function which such a process may have in MDR (Sibley *et al.*, 1987; Bates *et al.*, 1992). The addition of phosphate groups is thought to occur by a calcium-dependent mechanism, suggesting the involvement of protein kinase C (PKC) or a calcium-calmodulin-dependent kinase. Calcium channel blockers and calmodulin antagonists (*cf.* section 6.2.2.) enhance the phosphorylation of Pgp (Hamada *et al.*, 1987). Protein kinase C activity is known to be modulated by tumour-promoting agents such as phorbol esters (Castagna *et al.*, 1982). In support of this view, Pgp has been shown to be a substrate for calcium-dependent PKC in the presence of phorbol esters (Hamada *et al.*, 1987).

In addition, Gollapudi *et al.* (1992b) reported that prolonged exposure of P388/ADR cells to phorbol myristate acetate (PMA) down-regulated total PKC activity with an associated correction of daunorubicin accumulation in these cells. Since the level of expression of Pgp in PMA-treated cells was similar to that in untreated cells, their data support the proposed role of PKC in modulating Pgp activity by phosphorylation-dephosphorylation (Chambers *et al.*, 1992; Hait and Aftab, 1992; Blobe *et al.*, 1993). In contrast, protein kinase A (PKA), which is cAMP dependent, has also been shown to mediate the phosphorylation of Pgp (Meyers, 1989) and sorcin (see section 3.4), a calcium-binding protein that is overexpressed concomitantly with Pgp in some MDR cells (Koch *et al.*, 1986; van der Bliek *et al.*, 1986a). These discrepant findings have led to the notion that perhaps any or all of the known protein kinase systems may operate in different aspects of the MDR phenotype (O'Brian *et al.*, 1989; Abraham *et al.*, 1990; Chin *et al.*, 1992a). However, conflicting reports cast doubt on whether the MDR phenomenon is wholly related to the calcium, calmodulin and the cAMP physiologic repertoire (Nair *et al.*, 1986; Cole *et al.*, 1989; Anderson *et al.*, 1991).

5.4. Expression in Normal and Tumour Tissue

P-glycoprotein-specific cDNA probes and monoclonal antibodies (*e.g.*, C219, MRK16, JSB-1, HYB211, HYB612) have provided useful tools for the identification of Pgp in normal and tumour tissues (Kartner *et al.*, 1985; Hamada and Tsuruo, 1986a; Scheper *et al.*, 1988). The glycoprotein has been detected in several normal human tissues (Thiebaut *et al.*, 1987; Hitchins *et al.*, 1988; Pileri *et al.*, 1991). In the liver, mAb MRK16 localizes to the luminal surfaces of the hepatocytes lining the biliary canalicular space and the small biliary ductules. P-glycoprotein is diffusely present on the surfaces of most cells of the adrenal cortex and, in the kidney, it is distributed on the apical surface brush border of the proximal tubular cells (Lieberman *et al.*, 1989). In the gastrointestinal tract, it is prominent on the apical (mucosal) surface of the jejenum and colon, but not on that of the oesophagus or stomach. Highly confined areas of Pgp expression are detectable in the small collecting ducts of the pancreas. Other interesting locations include specialized capillary endothelial cells of the brain and testis (Cordon-Cardo *et al.*, 1989; Thiebaut *et al.*, 1989), placental tissue (Sugiwara *et al.*, 1988), and normal lymphocytes (Coon *et al.*, 1991b).

Significant levels of Pgp may be present in breast cancer before exposure to drugs and may be of prognostic value in patients with locally advanced disease (Wishart *et al.*, 1990; Sanflippo *et al.*, 1991; Verrelle *et al.*, 1991). In metastatic human musculoskeletal tumours and those which did not respond to combination chemotherapy, an increased expression of the *MDR* gene was detected whereas no evidence of appearance of this gene was found in the benign tumours (Nagata *et al.*, 1992). The elevated levels of Pgp in remission phase associated with early and resistant relapse in multiple myeloma is indicative of a significant relationship between failure of chemotherapy in patients and an increase in expression of the *mdr*1 gene (Musto *et al.*, 1991a; Linsenmeyer *et al.*, 1992). The presence of the same gene in human glial tumours suggests that the multidrug transporter may contribute to the clinical non-responsiveness of these tumours to chemotherapy (Becker *et al.*, 1991).

Although the glycoprotein is infrequently detected in untreated lymphomas, it is often well-defined in clinically drug-resistant disease with a close correlation between the level of expression and the clinical course of disease (Dan *et al.*, 1991; Miller *et al.*, 1991; Pileri *et al.*, 1991). The proportion of strongly MDR-positive cells is very common in acute non-lymphoblastic leukaemia (ANLL), albeit higher in relapse than at diagnosis, and failure of standard treatment is associated with a

progressive increase of Pgp levels (Michieli *et al.*, 1992). Numerous other reports have confirmed the notion that the positive identification of Pgp in tumours may be closely associated with clinical drug resistance in some cases but not in others. These include:

- ovarian and gynaecological cancers (Finstad *et al.*, 1990; Rubin *et al.*, 1990),
- leukaemias (Marie *et al.*, 1991; Pirker *et al.*, 1991; Campos *et al.*, 1992),
- tumours of the brain and central nervous system (Chan *et al.*, 1991; Henson *et al.*, 1992; Tishler *et al.*, 1992),
- non-small-cell lung cancer (Scagliotti *et al.*, 1991; Shin *et al.*, 1992),
- colorectal carcinomas (Toffoli *et al.*, 1992), and
- adrenocortical carcinomas (Flynn *et al.*, 1992).

5.5. Functional Correlates of P-Glycoprotein

Observations of evolutionary conserved sequences of Pgp, together with their identification in normal and tumour tissue, have led to an interest in the physiologic functions which this membrane glycoprotein might have in normal embryonic and adult tissues, and the contrasting pathologic functions in neoplastic tissues. The localization of Pgp in normal tissues such as liver, colon, kidney and adrenal gland (Fojo *et al.*, 1987; Cordon-Cardo *et al.*, 1989), as well as in endothelial cells of the blood-brain barrier (Thiebaut *et al.*, 1989; Hegmann *et al.*, 1992; Tatsuta *et al.*, 1992), implies a functional role for the protein in normal physiologic transport processes.

The presence of Pgp on the luminal surfaces of excretory organs exemplifies its postulated function as an energy-dependent drug efflux pump. It is probable that the *mdr* multigene family has evolved to protect animals against hydrophobic xenobiotics and microbial toxins, many of which are present in plants and contaminated food. The liver is known to respond to xenobiotics by expressing a host of cellular oncogenes and detoxifying enzymes. The location of the multidrug transporter in the liver and kidney, therefore, provides excellent pharmacologic defence against the accumulation and retention of potentially harmful substances. Many of the drugs to which MDR cell lines are resistant, including DNR, *Vinca* alkaloids, and DOX, are thought to be excreted by the liver into bile and by the kidney into urine (Bender and Chabner, 1982; Meyers, 1982). Thus, Pgp can be viewed as an energy-transducing transmembrane excretory pump that exudes hydrophobic amphipathic drugs, which have diffused into the proximal tubular cells of the kidney, or the pericanalicular hepatocytes, into the urine (Charuk and Reithmeier, 1992), or the bile (Gottesman *et al.*, 1991). According to

this view, it is possible that drugs that are excreted into bile could be reabsorbed in the jejenum or colon, but such reabsorption is prevented largely by the activity of the glycoprotein on their mucosal surfaces.

The liver is an active modifier of circulating metabolites and also produces bile salts (which are amphipathic, hydrophobic compounds) that may be natural substrates for the active transporter. The widely used immunosuppressive drug, cyclosporine A (CsA), is a substrate for Pgp (Goldberg *et al.*, 1988; Ryffel *et al.*, 1991). Since nephrotoxicity is the major limitation in the use of CsA, the presence of an efflux pump in the apical membrane of the renal proximal tubule may have clinical implications for cyclosporine toxicity. In rat small intestinal brush border membrane vesicles Pgp may be responsible for the transport of substrates into the lumen of the small intestine (Hsing *et al.*, 1992) and perhaps provides protection against potentially damaging exogenous lipohilic cations (Gros *et al.*, 1992). This may contribute to the rarity of small intestinal cancer in humans and animals (Hsing *et al.*, 1992).

The role of Pgp in the adrenal gland is not completely understood, but is speculated to include steroid transport. In support of this view, progesterone has been shown to inhibit photoaffinity labelling of Pgp by [^{3}H]azidopine and to reverse the MDR phenotype (Yang *et al.*, 1989). These studies are corroborated by observations that progesterone can bind the pharmacophore in MDR human leukaemia lymphoblasts (Qian and Beck, 1990) and, under physiological conditions, may play a role in the excretion of the hormone from certain cells. Likewise, cortisol, aldosterone and corticosterone have been postulated as substrates for the protein (Ueda *et al* ., 1992; Wolf *et al.*, 1992). Also, the interaction of organic chemicals with Pgp in the adrenal gland and kidney points to its physiological function of excreting certain classes of carbon compounds (Ichikawa *et al.*, 1991b). On the luminal surface of pancreatic exocrine ductules it is postulated to prevent concentration of toxic metabolites produced in the pancreas or the reabsorption of pancreatic acinar cell secretions.

The localization of Pgp in specialized capillary endothelial cells of the brain and testis has raised the interesting possibility that it might be functional in the blood-brain and -testis barrier. The natural product drugs which are substrates for the MDR transport system are principally excluded from these organs. By analogy, its presence in placental tissue (Sugiwara *et al.*, 1988) and in the human fetus (van Kalken *et al.*, 1992) has been suggested to aid in the protection against harmful substances and might, in addition, serve as an active transporter for nutrients vital

to the fetus. In the mouse, the *mdr* gene is expressed in secretory glands of the gravid uterus, implying an involvement in hormone secretion during pregnancy (Arceci *et al.*, 1988).

In considering the causative role of the glycoconjugate in the development of resistant tumours on the one hand, and its protective role in normal tissues such as the blood-brain barrier, liver, kidney and placenta on the other, future strategies for developing chemotherapeutic regimens, will have to anticipate possible host toxicities that may result from interfering with physiological Pgp-protective functions.

6. Modification and Chemosensitization of MDR

MDR presents a major impediment to effective cancer chemotherapy and has provided the rationale to study cell lines as *in-vitro* model systems for drug resistance, particularly the type mediated by Pgp (Nielsen and Skovsgaard, 1992; Wu *et al.*, 1992). There is considerable optimism that information on and the understanding of the biochemistry and pharmacology of Pgp-associated and other forms of MDR can be exploited and translated into useful clinical applications (Beck, 1991b; Edgington, 1992; Judson *et al.*, 1992).

6.1. Implications of Collateral Sensitivity and Cross-Resistance

An approach to the circumvention of MDR is to take advantage of the sensitivity or hypersensitivity of refractory cells to agents administered simultaneously with the primary drug. Data on the molecular, cellular, biochemical and pharmacological events that lead to collateral sensitivity and cross-resistance are potentially valuable in:

- scheduling antitumour drugs to prevent the appearance of resistance,
- designing optimal drug combinations to exploit synergistic effects and eradicate pleiotropic resistance,
- selecting specific second-line chemotherapeutic regimens in relapsing patients to avoid drugs likely to be ineffective,
- developing antitumour agents which can preferentially overcome resistance or cross-resistance, and
- screening for new agents to which resistant tumours exhibit collateral sensitivity.

Correlations of patterns of cross-resistance and enhanced sensitivity with various tumour types may provide valuable information needed in initial treatment or equally successful secondary therapy of advanced recurrent or untreated disease (Futscher *et al.*, 1992; Shinoda *et al.*, 1992; Warrington, 1992).

6.2. Chemosensitizers and Resistance Modifiers

Much impetus of collateral sensitivity has come from the identification of substances termed chemosensitizers or resistance modifiers that can partially or completely reverse MDR (Georges *et al.*, 1990b; Pearce *et al.*, 1990; Hait and Aftab, 1992). Compounds with such activity include drug analogues, calcium channel blockers, lysosomotropic agents, calmodulin antagonists, cyclosporins, monoclonal antibodies, dihydropyridine analogues, pyrazoloacridines, thioxanthenes, and many other agents. The possible significance of selected agents will be considered in turn.

6.2.1. Immunosuppressive and Non-Immunosuppressive Agents

Cyclosporin A is a potent resistance modifier in a number of human and rodent MDR cells (Foxwell *et al.*, 1989; Gavériaux *et al.*, 1989; Nooter *et al.*, 1990). Although CsA has specific Pgp binding activity but less membrane perturbing effects, inhibition of protein kinase C may also be involved in its sensitizing action. However, this immunosuppressant is toxic to organs, and thus raises concern regarding its clinical use. Nevertheless, a number of its immuno- and non-immunosuppressive analogues are less harmful, possess similar or more potent chemosensitizing activity than the parent molecule (Arceci *et al.*, 1992; Friche *et al.*, 1992; Mizuno *et al.*, 1992), and clinical studies of their MDR modulating effects seem highly relevant. Cyclosporine and some of these agents alone or in combination with other drugs have already been used successfully in clinical trials (Lum *et al.*, 1992; Sonneveld *et al.*, 1992a; Yahanda *et al.*, 1992).

6.2.2. Calcium Channel Blockers and Calmodulin Antagonists

Calcium channel blockers such as VPL and calmodulin inhibitors such as TFP enhance intracellular accumulation of VCR and ADR in cells resistant to these drugs (Tsuruo *et al.*, 1981; Tsuruo *et al.*, 1982). Several reports followed which confirmed the potentiation of cytotoxicity of various drugs by calcium antagonists (Rogan *et al.*, 1984; Bruno and Slate, 1990; Mickisch *et al.*, 1991a), as well as by calmodulin inhibitors (Ganapathi *et al.*, 1991a; Ganapathi *et al.*, 1991b; Philip *et*

al., 1992). The mechanism of action of the calcium channel blockers evidently involves their direct binding to Pgp in competition with other drugs for Pgp-mediated efflux (Cornwell *et al.*, 1987a; Safa *et al.*, 1987; Horio *et al.*, 1991). The inhibition of VCR binding to Pgp by VPL apparently does not require cellular calcium ion fluxes (Naito and Tsuruo, 1989). In contrast, Vasilev *et al.* (1987) postulated that the higher efflux rate of cisplatin in *cis*-DDP-resistant L1210 cells may be due to changes in cellular calcium levels. Verapamil has been demonstrated also to enhance the action of drugs which cause DNA strand breaks or inhibit the repair of damaged DNA (Yalowich and Ross, 1984 ; Harker *et al.*, 1986; Yalowich *et al.*, 1987).

Observations that some MDR cells overproduce cytosolic calcium-binding proteins (sorcin or V19 or CP_{22}) indicate that calcium may have a role to play in MDR and its modulation (Meyers and Biedler, 1981; Meyers *et al.*, 1985; Koch *et al.*, 1986). The mechanism of action of calmodulin inhibitors in the reversal of MDR is less well understood. They may interact with hydrophobic regions of the molecule that are exposed on the surface following conformational changes caused by calcium binding (Zimmer and Hofmann, 1987). By analogy, these agents may bind to the hydrophobic domains on the Pgp molecule and affect the MDR phenotype.

6.2.3. Drug Analogues, Metabolites and -Delivery Systems

The design of drugs, their analogues and metabolites for overcoming MDR is being pursued actively (Ford and Hait, 1990; Pearce *et al.*, 1990; Klopman *et al.*, 1992). Numerous reports have described the synthesis, chemical characterization and use of efficient MDR reversers alone or in combination with other agents (Table 1). Interestingly, several of these compounds (*e.g.*, quinidine, quinine, tamoxifen, TFP and VPL) share structural features such as lipophilic aromatic rings and hydrophilic N-alkyl groups (Pommerenke *et al.*, 1990). In some instances drugs (*e.g.*, ADR) may modulate both the function and expression of Pgp, thus raising the issue of their administration and scheduling to achieve optimal therapeutic effect (Kato *et al.*, 1992). In addition, the ability of liposomes (Thierry *et al.*, 1992; Warren *et al.*, 1992), liposome-encapsulated DOX (Sadasivan *et al.*, 1991; Mickisch *et al.*, 1992c; Rahman *et al.*, 1992) and biodegradable polymers loaded with DOX (Cuvier *et al.*, 1992), to bypass the efflux mechanism responsible for MDR so that accumulation of drugs in resistant cells is restored to levels approximating those in sensitive cells, may provide another effective approach to chemosensitization.

Table 1. Drugs and Related Compounds Used in the Reversal of Multidrug Resistance

REVERSING AGENT	REFERENCES
DOX derivatives	Ripamonti *et al.*, 1992
Quinine and its analogues	Genne *et al.*, 1992 Solary *et al.*, 1992
Quinidines	Wishart *et al.*, 1992
Quinolines	Sato *et al.*, 1991 Wakusawa *et al.*, 1992
Progesterone and related compounds	Fleming *et al.*, 1992
Tamoxifen	Millward *et al.*, 1992 Stuart *et al.*, 1992 Trump *et al.*, 1992
Phenothiazines and N-substituted phenoxazines	Ramu and Ramu, 1992 Thimmaiah *et al.*, 1992
Indole derivatives	Kadam *et al.*, 1992
Toremifene and several of its metabolites	Wiebe *et al.*, 1992
Triazine derivatives	Cros *et al.*, 1992 Dhainaut *et al.*, 1992
Polyether antibiotics	Kawada *et al.*, 1992
Metabolites of niguldipine, nitrendipine and verapamil	Hofmann *et al.*, 1992
Amiloride analogues	Epand *et al.*, 1991

6.2.4. Membrane Perturbants

The plasma membrane has long been regarded as a prime site for chemotherapeutic intervention (Powis *et al.*, 1990; Grunicke, 1991; Daoud, 1992). New strategies for optimization of specific agents that interact with the cell surface and associated signalling pathways, growth factor receptors, and MDR are being developed (D'Incalci *et al.*, 1991; Jandrig and Wunderlich, 1992). These include steroids, steroid hormones, local anaesthetics, surfactants and nonionic detergents (Naito *et al.*, 1989; Coon *et al.*, 1991a; Woodcock *et al.*, 1992).

6.2.5. Lysosomotropic Agents

These agents have recently been reported to reverse MDR in human cancer cells (Shiraishi *et al.*, 1986; Zamora and Beck, 1986). The lysosome-endosome secretory system has been implicated in the enhanced efflux of drugs observed in MDR cells (Beck, 1987; Sehested *et al.*, 1987). It has further been speculated that Pgp may function in the trans-Golgi network vesicles rather than in the lysosomal compartment or at the level of the plasma membrane (Klohs and Steinkampf, 1988).

P-glycoprotein presumably pumps drugs into secretory vesicles derived from the Golgi apparatus where they become entrapped and extruded from the cell by fusion of the vesicle with the plasma membrane.

Klohs and Steinkampf (1988) presented evidence that lysosomotropic agents (Triton WR-1339 and CLQ), and secretory inhibitors (monensin, cytochalasin B, and VBL) are effective in limiting DNR and DOX efflux from MDR P388 leukaemia cells and potentiating their cytotoxicity. They exert their effects by alkalinizing the normally acidic intralysosomal compartment (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981). Neither methylamine nor ammonium chloride, both of which raise endosomal and lysosomal pH, could be demonstrated to inhibit drug efflux from MDR cells (Zamora *et al.*, 1987; Klohs and Steinkampf, 1988).

These discrepancies make the acidic vesicles of the trans-Golgi network, which are destined for secretion, prime candidates for the energy-dependent removal of drugs from MDR cells. Such a mechanism implies that after drugs have entered the cells they can be sequestered in the Golgi vesicles either by Pgp, which would bind and actively transport them into these vesicles, or by passive diffusion. Once inside the vesicular compartment, the drugs become protonated and entrapped in the acidic environment. This process of vesicular drug accumulation is analogous the concentration of catecholamines in storage granules of the adrenal medulla and might even explain the elevated expression of Pgp in this tissue (Fojo *et al.*, 1987). P-glycoprotein may be related to the monamine transporter of the chromaffin granules. After drug concentration, these vesicles migrate to and fuse with the plasma membrane to shed their contents by exocytosis (Klohs and Steinkampf, 1988).

6.2.6. Polyclonal and Monoclonal Antibodies

A vast number of polyclonal- (pAbs) and monoclonal antibodies (mAbs) have been used appropriately in defining the biology of tumour cells and in various aspects of cancer management (Schlom, 1986; Oettgen, 1990). Antibodies are being used as targeting vehicles for toxins, chemotherapeutic agents or their activating enzymes, cell-surface receptors, as well as inflammatory activators at the site of their interaction with tumour cell-surface antigens (Richardson *et al.*, 1989; Ding *et al.*, 1990). Their potential application in the circumvention of MDR is now widely recognized. Several mAbs have been raised against Pgp (Danks *et al.*, 1985; Kartner *et al.*, 1985; Scheper *et al.*, 1988) and are useful for detecting the

glycoprotein in human tumour specimens (Finstad *et al.*, 1990; Efferth *et al.*, 1992; Mattern and Volm, 1992).

Recombinant fragments (Marquardt *et al.*, 1990; Tanaka *et al.*, 1990) should also be useful to raise antibodies to Pgp in order to define the function of the various domains in MDR. Georges *et al.* (1991) have demonstrated that mAbs C219, C494, and C32 directed against short linear regions of the Pgp molecule inhibit its ATP binding capacity *in vitro*. They also presented evidence that both predicted domains bind ATP, that co-operativity exists between the two sites and that antibodies differentially inhibit the binding capacity of Pgp to the calcium channel blocker, azidopine. These observations couple specific perturbations of the molecule with ATP and drug binding. Monoclonal antibody-drug conjugates have been used successfully to augment the cytotoxicity of the conjugated drug (Pimm *et al.*, 1990).

Studies with mAbs MRK16 and MRK17, suggest that Pgp is involved in drug transport and proliferation of MDR cells (Tsuruo *et al.*, 1989; Hamada and Tsuruo, 1991). Both mAbs exhibit growth-inhibitory effects on MDR cells and may be clinically useful to circumvent multidrug resistance (Beck, 1991a; Pearson *et al.*, 1991; Heike *et al.*, 1992). However, the obstacle of undesirable antibody-induced toxic effects, arising either from Pgp expression in normal tissues or from host-immune responses, first needs to be addressed. An important advance to this end has been the selective elimination of MDR positive (MDR^+) cells *in vitro* with MRK16 conjugated to the ribosome-inactivating protein saporin 6 (Dinota et al., 1990). This has also been performed on MDR^+ and MDR negative (MDR^-) cell lines, normal human bone marrow-derived haemopoietic precursors, and MDR^+ cells mixed with normal bone marrow to simulate a therapeutic bone marrow purging. The results implied that it may be possible to eradicate MDR^+ cells without affecting the *in vitro* proliferation of haemopoietic precursors (Dinota et al., 1990).

Similarly, a combination of VP-16 and CsA was shown to be effective in purging MDR leukaemia cells from transplanted bone marrow in a murine model (Kuhl *et al.*, 1992). Oher reports describe the successful purging of MDR cells (Aihara *et al.*, 1991; Chao, N.J. *et al.*, 1992) and the ability of MRK16 coupled to *Pseudomonas* exotoxin to effectively kill bone marrow cells in MDR-transgenic mice (Mickisch *et al.*, 1992b; Mickisch *et al.*, 1993). Correspondingly, a mouse-human chimeric mAb, MH162, against Pgp was found to be more effective than MRK16 in lysis of MDR tumour cells by blood mononuclear cells, suggesting that

such mAbs may also be beneficial for *in vivo* destruction of MDR tumours (Ariyoshi *et al.*, 1992; Nishioka *et al.*, 1992).

6.3. Enzyme Targeting

Various enzymic mechanisms that confer cellular protection against anticancer drugs have been reported (Giai *et al.*, 1991), some of these will be described briefly according to their proposed function in MDR.

6.3.1. Glutathione S-Transferases

The cytosolic glutathione S-transferases (GSTs) constitute a family of dimeric xenobiotic-detoxifying isozymes which are associated with resistance to anticancer drugs, *e.g.*, alkylating agents (Hall *et al.*, 1989; Miyazaki *et al.*, 1990; Puchalski and Fahl, 1990). The mechanisms by which GSTs confer resistance to alkylating agents have not been elucidated, but it is hoped that combination chemotherapy with specific inhibitors of GST may improve the therapeutic effectiveness of alkylating agents (Hall *et al.*, 1989).

6.3.2. DNA Topoisomerases

The regulation of DNA topology is obligatory for replication, transcription, recombination and repair. Topoisomerases are nuclear enzymes which catalyze the isomerization of DNA by breaking and rejoining DNA strands transiently (Wang, 1985; Glisson and Ross, 1987; Preston and White, 1990). The cytotoxicity and DNA damage induced by anthracyclines (*e.g.*, DOX and DNR), DNA intercalating agents and epipodophyllotoxins (*e.g.*, etoposide and teniposide) appear to be mediated by TopoII (Utsugi *et al.*, 1990; Utsumi *et al.*, 1990; Sullivan and Ross, 1991). Recent studies have implicated alterations in activity and function of this enzyme as an alternative mechanism of MDR (Deffie *et al.*, 1989; De Isabella *et al.*, 1991; Zwelling *et al.*, 1991).

Although TopoII is a principal determinant of cell sensitivity to DNA poisons, other changes involved in the regulation of enzyme function and/or in the cellular processing of drug-induced DNA damage may be critical in determining disparate cell responses to antitumour agents (Beck, 1990; Lefevre *et al.*, 1991; Baguley *et al.*, 1992). The precise nature of these alterations in MDR has yet to be established. However, several studies have demonstrated that the design of well-defined DNA intercalators and drugs is both feasible and potentially useful to

overcome at-MDR (Baguely *et al.*, 1990; Morrow and Cowan, 1990; Zwelling *et al.*, 1991).

6.3.3. Protein Kinases

The relationship between protein kinases and MDR is an area of intense interest. The phosphorylation of Pgp is thought to involve multiple kinases, including, cAMP-dependent PKA, calcium-sensitive PKC, and calcium-calmodulin-dependent kinases (Hamada *et al.*, 1987; Chambers *et al.*, 1990; Hait and Aftab, 1992). These kinases may have an effect on Pgp-mediated drug efflux, *e.g.*, substances which stimulate the activity of PKC decrease the cellular accumulation of anticancer drugs such as *Vinca* alkaloids (Ido *et al.*, 1986) whereas inhibitors of the enzyme enhance drug accumulation and reverse resistance (O'Brian *et al.*, 1989; Sato *et al.*, 1990; Gollapudi *et al.*, 1992b). In addition, chemotherapeutic agents commonly producing drug resistance have been shown to inhibit PKC (Palyoor *et al.*, 1987). However, it has been reported that the kinase can be decreased and increased in certain MDR cells and in other cells may be unrelated to MDR (Schwartz *et al.*, 1991). This may therefore complicate its targeting in strategies to overcome MDR. Observations of the modulation of basal MDR by PKA suggest that such intervention, too, has potential therapeutic implications (Abraham *et al.*, 1990).

6.4. Genetic Strategies

Genetic approaches to the treatment of cancer promise to revolutinize patient care. There has been significant advances in recent years concerning the regulation of the cell cycle, the role of oncogenes in the maintenance of genomic stability, induction of terminal differentiation, signal transduction and growth regulation as well as the mechanisms by which cytotoxic drugs kill cells. Some of these advances together with molecular analysis of the structure-function relationship of Pgp in MDR should reveal new targets for cancer chemotherapy. Novel appoaches, based on gene therapy of cancer by manipulating the immune system, introducing suicide genes into tumours, or using drug-resistant genes to protect normal cells during chemotherapy have already entered clinical trials. Several genetic strategies have been explored in reversing MDR, some of which are detailed below.

6.4.1. Site-Directed Mutagenesis

P-glycoprotein, encoded by the *mdr* multigene family, consists of 12 transmembrane domains and at least two nucleotide binding sites. Azzaria *et al.*

(1989) have demonstrated that the introduction of direct mutations into the predicted nucleotide binding sites of the *mdr*1 gene abolishes its ability to confer resistance. Simlar efforts to inactivate either or both of the nucleotide binding domains by site-directed mutagenesis have confirmed that these sites are essential for the Pgp-multidrug transporter in human epidermoid KB carcinoma cells (Kioka *et al.*, 1989; Dickson and Gottesman, 1990). In addition, a mutational substitution of a Val residue for a Gly residue at amino acid position 185 (in the first cytoplasmic loop, near the third transmembrane sequence, TM3) alters the specificity of the pump, apparently by affecting drug transport but not initial binding.

Major functional differences between *MDR*1 and *MDR*2 Pgps reside in a small segment of the first intracytoplasmic loop; the *MDR*2 gene product does not appear to be a functional drug efflux pump (Buschman and Gros, 1991; Currier *et al.*, 1992). Analysis of the effect of replacing Asn183 of *MDR*1 with Ser which occurs in *MDR*2 as well as substitution of Ser at position 183 in combination with Val at position 185 in Pgp resulted in a relative increase in resistance to ActD, VBL and DOX in transfected NIH-3T3 cells. This is indicative of the importance of the first intracytoplasmic loop in determining function and relative drug specificity (Currier *et al.*, 1992).

These findings are in accordance with observations that single amino acid substitutions, Ser→Phe in transmembrane domain 11 (TM11) of *mdr*1 (amino acid position 941) and *mdr*3 (amino acid position 939), markedly alter the activity and substrate specificity of the mouse *mdr*1 and *mdr*3 drug efflux pumps (Gros *et al.*, 1991a). The latter study has identified TM11 as a critical structural and functional determinant of the *mdr*1 and *mdr*3 gene products. The requirement for both TM3 and TM11 in MDR is further in agreement with the notion that the activity of a transacting positive regulatory factor which may exist constitutively in various tissues might be modulated by anticancer drugs (Kohno *et al.*, 1989). It also seems likely that the *mdr* gene is differentially expressed in response to the degree of cytotoxic exposure. Recently, Devine *et al.* (1992) have implicated the sixth transmembrane domain of Pgp in the mechanism of drug recognition and efflux. Moreover, their results revealed a close functional homology between Pgp and the cystic fibrosis transmembrane regulator in which the sixth transmembrane domain has likewise been shown to influence substrate specificity.

6.4.2. Antisense Oligonucleotides

Another genetic approach, utilizing the ability of nuclease-resistant antisense oligonucleotides to suppress Pgp gene expression preferentially, has been described by Vasanthakumar and Ahmed (1989). These authors showed that antisense oligonucleoside methylphosphonates elicited sequence-specific, concentration-related inhibition of Pgp synthesis and also increased the toxicity of DNR in human K562 erythroleukaemia cells. Since the entire length of the *mdr*1 is delineated, a complementary base sequence would be advantageous in selectively targeting a desired mRNA segment and in arresting the synthesis of Pgp. Such studies may have implications for the genetic control of MDR (Rivoltini *et al.*, 1990).

6.4.3. Transgenic Mice

This biotechnology is becoming increasingly sophisticated so that genes can be targeted for replacement by a mutated version of the same gene (McGrane *et al.*, 1992; Mickisch *et al.* 1991d). The system is aimed at evaluating the efficacy, potency and toxicity associated with chemotherapy and chemosensitization of MDR cells in animals (Mickisch *et al.* 1991b; Mickisch *et al.* 1991c; Pastan *et al.*, 1991). The transplantation of bone marrow cells from transgenic mice expressing the human *MDR*1 gene confers long-term protection against the myelosuppressive consequences of chemotherapy in mice (Mickisch *et al.* 1992a). This selective advantage conferred by expression of the *MDR*1 cDNA provides a strategy for the use of *MDR*1 gene in cancer chemotherapy and for the introduction of unselected genes into sensitive tissues such as normal bone marrow.

6.5. Differentiation Inducers and Oncogenes

Valuable insight into the maturation-dependent response of certain forms of cancer, including MDR tumours, to antineoplastic agents has been gained by the use of differentiation inducers (Bates *et al.*, 1989; Mickley *et al.*, 1989; Michaeli, *et al.*, 1990). The potential therapeutic advantage of differentiation inducers is based on their ability to convert unresponsive non-differentiated neoplastic cells to their non-proliferating (*i.e.*, terminally differentiated) mature counterparts (Shwartz *et al.*, 1983; Estervig *et al.*, 1989; Hassan and Rees, 1990).

Certain human MDR cell lines are unaltered in their state of differentiation compared with parental cells (Bhalla *et al.* 1985; Beck *et al.*, 1986; Beran and Anderson, 1987) and they grow equally well as their drug-sensitive parental lines

in nude mice (Mirski *et al.*, 1987; Hill *et al.*, 1988a; Hill *et al.*, 1988b). However, Ades *et al.* (1992) have demonstrated that the differentiation agent hexamethylene bisacetamide and transforming growth factor beta-1 (TGF-β1) acted synergistically on a multidrug-resistant human hepatocellular carcinoma cell line and resulted in a cell death rate of 80%. Their data support the notion of programmed cell death and suggest that drug-resistant tumour cells may be susceptible to a combination of cytokines and differentiation inducers. Okabe-Kado *et al.* (1991) have shown that the MDR phenotype can be reversed partially by treatment of K562/VCR cells with erythroid differentiation factor (EDF). It is presumed that EDF causes down-regulation of Pgp in K562/VCR cells. Thus, differentiation-induction therapy may prove beneficial in patients who are unresponsive to conventional cytotoxic chemotherapy.

Cellular differentiation is a multistep process (Chen *et al.*, 1982), involving PKC, proto-oncogenes (including *c-myc*, *c-myb*, *c-fos*, and *p53*), and expression of genes for differentiated phenotypes (Marks *et al.*, 1987; Richon *et al.*, 1989; Bhushan *et al.*, 1992). The promoter of the human *MDR*1 gene is a target for both the *c-Ha-ras-1* oncogene and the *p53* tumour suppressor gene products (Chin *et al.*, 1992b). This implies that the *MDR*1 gene may be developmentally regulated and might also be activated during tumour progression associated with mutations in the *ras* and *p53* oncogenes (Fearon and Vogelstein, 1990; Chin *et al.*, 1992b). Recent data indicate that increased expression of *c-fos* and *c-erb* proteins is a constant feature of several MDR cell lines and may play a role in the development of MDR (Efferth and Volm, 1992). These oncogenes may be explored as molecular targets for MDR reversal.

PART II: GLYCOSYLATION OF MEMBRANE PROTEINS

1. INTRODUCTION

The term *glycoconjugate* is used to describe molecules in which carbohydrate moieties (mono-, oligo-, or polysaccharides) are covalently linked to proteins or lipids. Glycolipids are lipids (*e.g.*, diacylglycerol, sphingosine, ceramide or dolichol) to which oligosaccharides are attached. Oligosaccharides are coupled via N- and/or O-glycosidic bonds to polypeptides and may represent up to 70% of their overall molecular weight (Beeley, 1985). Cell membranes of eukaryotic cells usually have a carbohydrate content of between 2 and 10% contributed by the sugar residues of their constituent glycolipids and glycoproteins. The oligosaccharide moieties of glycoproteins are discrete, stereo-specific and highly conserved structures. Four different monosaccharides can be combined to form over 36,000 different tetrasaccharides. In addition, glycoproteins differ from other glycoconjugates, such as proteoglycans, glycosaminoglycans, mucoproteins and peptidoglycans, in having multiple branched, relatively short oligosaccharide chains with a greater aggregation of monosaccharides and without repeating disaccharide units (Shreeve, 1974).

The core protein of glycoproteins is generally more prominent than it is, for example, in proteoglycans where amino acids are linked to carbohydrate chains composed of variable branches of repeating structural units (Abbreviated terminology of oligosaccharide chains, IUB-IUPAC recommendations, 1982). Furthermore, an outer sequence, sialic acid (neuraminic acid, NeuAc; or N-Acetyl neuraminic acid, NeuNAc) or fucose (Fuc)→galactose (Gal)→N-Acetyl D-glucosamine (GlcNAc), linked to an inner core of ~3 mannose (Man) and 2 GlcNAc residues, is common to many glycoproteins (Shreeve, 1974). Another important class of glycoconjugate is glycosylphosphatidyl inositol (GPI) lipid anchors (Low *et al.*, 1986; Low, 1987; Ferguson *et al.*, 1988) which link cell surface glycoproteins to membrane lipids through an oligosaccharide bridge. Thus, proteins may be glycosylated with N- and O-linked oligosaccharides (N-glycans and O-glycans, respectively), as well as a GPI membrane anchor (Abeijon and Hirschberg, 1992). Glycoconjugates have a ubiquitous distribution in almost all

organisms ranging from bacteria to mammals (Ginsburg and Neufeld, 1969). This universality, together with the structural diversity of the carbohydrate moieties of glycoconjugates, has stimulated much interest in their biochemical and cellular functions (Rademacher *et al.*, 1988). Indeed, glycoproteins have been implicated in a broad spectrum of processes (Table 1).

Table 1. Functional Significance of Glycoconjugates in Biological Systems

FUNCTIONS	REFERENCES
Embryonic development	Loveless *et al.*, 1990; Varki *et al.*, 1991
Cell adhesion and ligand-receptor interaction	Brandley *et al.*, 1990; Springer, 1990; Roche, 1991
Biosynthetic disorders	Kornfeld, 1986; Fukuda *et al.*, 1987
Lymphocyte homing and migration	Yednock *et al.*, 1987; Stoolman, 1989
Parasite-host binding	Friedman *et al.*, 1985; Lev *et al.*, 1986
Oncogenesis	Leathem *et al.*, 1987; Paietta *et al.*, 1987; Santer, *et al.*, 1989
Tumour metastasis	Humphries *et al.*, 1986; Dennis *et al.*, 1987; Benedetto *et al.*, 1989
Resistance to proteolytic degradation	Loh and Gainer, 1978
Natural cytotoxicity	Mueller *et al.*, 1990
Multiple drug resistance	Endicott and Ling, 1989

2. N-GLYCOSYLATION OF PROTEINS

Glycosylation of proteins is a prevalent post-translational modification process detected in all eukaryotes (Abeijon and Hirschberg, 1992; Haltiwanger *et al.*, 1992). Glycoproteins are classified into two broad categories: N-glycosylated proteins and O-glycosylated proteins. The bond in the former is via the amide nitrogen of an asparagine (Asn) residue of the protein, whereas that of the latter occurs via the hydroxyl of serine (Ser), threonine (Thr), hydroxylysine, or hydroxyproline. The structures of several hundreds of N-linked oligosaccharides associated with many glycoproteins share a number of characteristics, including the pentasaccharide core, $Man_3GlcNAc_2$, which forms the attachment point between the oligosaccharide and the Asn residue of the protein. This core then undergoes modification into diverse branches, *viz.*, high/oligomannose-type, complex-type and

hybrid-type structures (Hirschberg and Snider, 1987; Roth, 1987). The biosynthetic pathway of N-linked glycoproteins is schematically illustrated in Figures 1 and 2.

3. Inhibitors of N-Linked Oligosaccharide Processing

Since all types of Asn-linked oligosaccharides arise from different branches of a common biosynthetic pathway, perturbation of that pathway can inhibit or alter the type and extent of sugars transferred to protein. The structural and functional importance of the carbohydrate moieties of glycoproteins can thus be explored by arrest of N-glycosylation with compounds that selectively block one or more steps in the pathway. Inhibitors of oligosaccharide processing have proved useful, for example, in establishing the transport and assembly of enveloped viruses (Datema *et al.*, 1982; Elbein, 1987), and could be important in the development of anti-HIV drugs (Fleet *et al.*, 1988; Taylor *et al.*, 1991).

Treatment of metastatic B16 melanoma cells with tunicamycin (TM), a glycosylation inhibitor, alters their morphology and adhesive properties with concomitant loss of metastatic potential *in vivo* (Nicolson, 1984). Of special interest is the observation that TM-induced modifications correlate with the loss of a specific class of B16 melanoma cell-surface sialoglycoproteins that may be essential for metastasis (Irimura *et al.*, 1981). Similarly, Desantis *et al.* (1987) have demonstrated that swainsonine (SwN) can block the expression of the transformed phenotype of 3T3 cells transfected with human tumour DNA. The *in vivo* and *in vitro* effects of inhibitors of glycoprotein synthesis appear to be both concentration- and cell-dependent (Elbein, 1987).

Numerous other compounds which interfere with glycosidases and glycosyltransferases have been advanced. The properties of some natural and synthetic processing inhibitors are summarized in Table 2 and their sites of action indicated in Figures 1 and 2. Current applications of these compounds include AIDS and cancer research.

4. Application of Tunicamycin in Glycobiology

The chemistry of the tunicamycins (TMs) has been reviewed by Elbein (1981). The TMs are a group of nucleoside antibiotics produced by *Streptomyces lysosuperificus*. Tunicamycin (produced as a mixture of at least 10 homologous

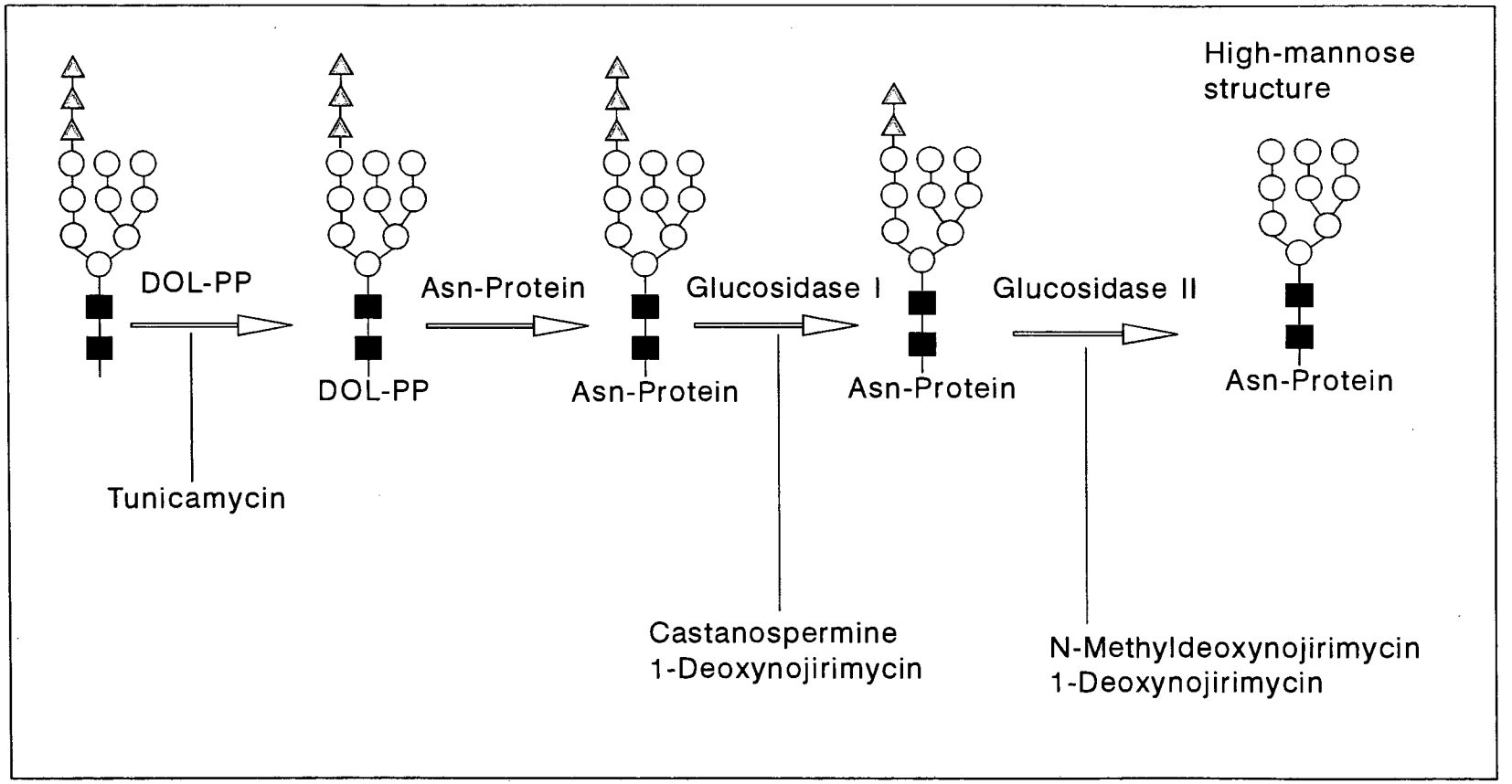

Figure 1. Initial steps in the assembly of N-linked oligosaccharide chains: transfer to protein and trimming to high-mannose structures. Arrows represent enzyme-catalyzed reactions. Lines perpendicular to arrows indicate inhibition sites of glycosylation inhibitors. Dol-PP, dolichol pyrophosphate; Asn, asparagine. Modified from Kornfeld and Kornfeld (1985) and *Glycosylation Inhibitors*, Boehringer Mannheim *Biochemica* (1987), Boehringer Mannheim GmbH, Germany.

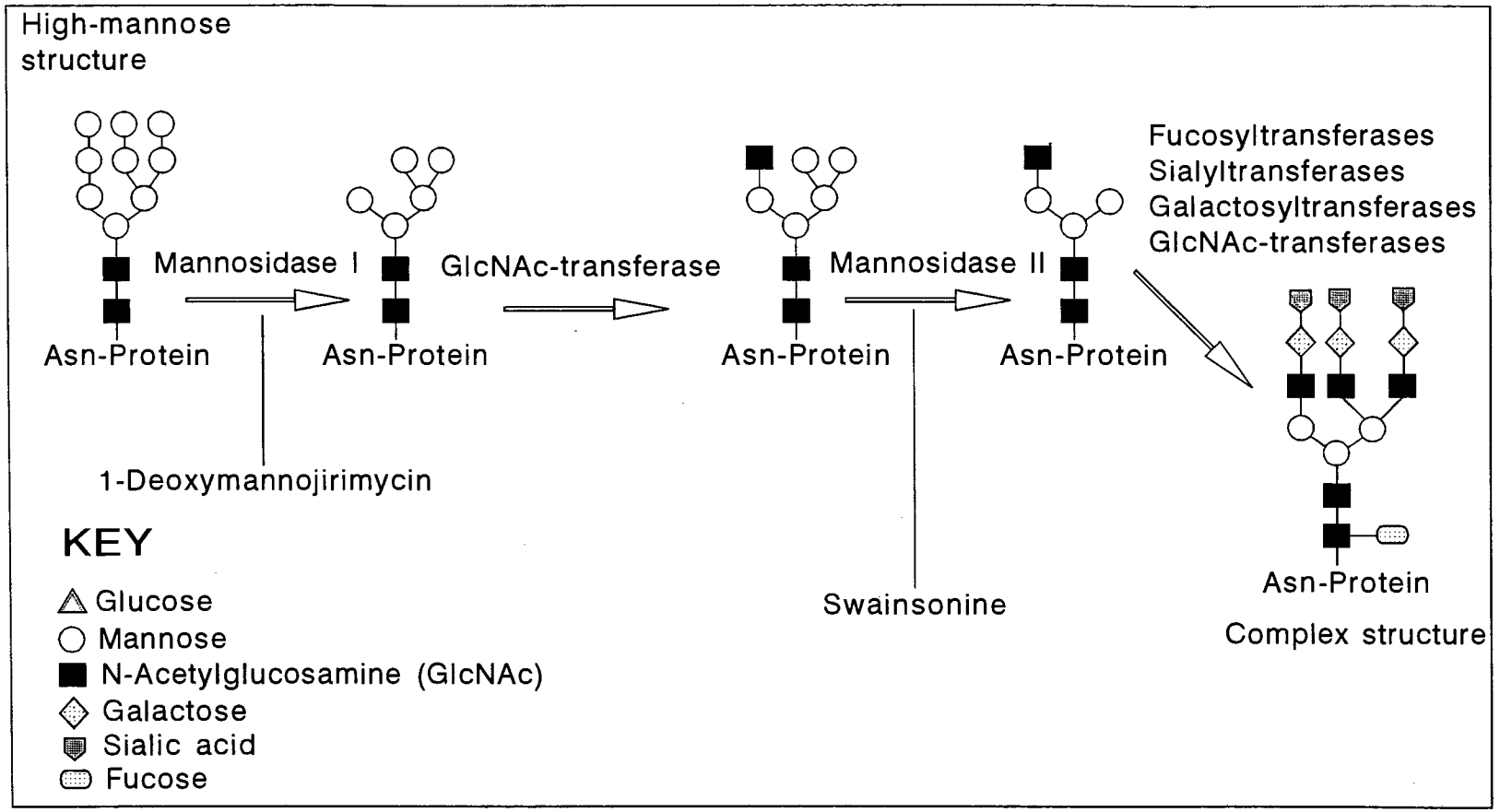

Figure 2. Processing of high-mannose structures to complex structures. Arrows represent enzyme-catalyzed reactions. Lines perpendicular to arrows indicate inhibition sites of glycosylation inhibitors. Asn, asparagine. Modified from Kornfeld and Kornfeld (1985) and *Glycosylation Inhibitors*, Boehringer Mannheim *Biochemica* (1987), Boehringer Mannheim GmbH, Germany.

Table 2. Properties of Inhibitors of Glycosylation and Trimming of Asparagine-Linked Oligosaccharides

Inhibitor	Reactions Inhibited	Comments on Use	References
ANTIBIOTICS			
Tunicamycin (TM)	First step in synthesis of lipid-linked oligosaccharide *i.e.*, forma-tion of GlcNAc-P-P-Dol from UDP-GlcNAc and Dol-P. In presence of TM, newly synthesized proteins carry no Asn-linked oligosaccharides.	Used with intact cells at 0.1-10 μg/ml) or in cell-free systems at (0.5-5 μg/ml).	Schwarz and Datema, 1982; Elbein, 1987
Bacitracin	Forms complex with Dol-P-P and prevents its dephosphorylation, *i.e.*, formation of Dol-P from Dol-P-P.	Inhibitor probably does not cross cell membrane.	Wedgewood and Strominger, 1980; Elbein, 1987
Amphomycin	Formation of Dol-linked monosaccharides	Cell-free systems, 500 μg/ml	Schwarz and Datema, 1982; Elbein, 1987
Showdomycin	Formation of Glc-P-Dol	Cell-free system, 5-10 μg/ml	Schwarz and Datema, 1982; Elbein, 1987
Diumycin	Formation of $GlcNAc_2$-P-P-Dol, of Man-P-Dol and of GlcNAc-P-P-Dol	Cell-free system, 50 μg/ml	Schwarz and Datema, 1982

(Continued)

Table 2. (Continued)

Inhibitor	Reactions Inhibited	Comments on Use	References
INHIBITORS OF PROCESSING ENZYMES			
1-Deoxynojirimycin (dNM)	Release by glucosidase I and II of Glc residues from $Glc_3Man_9GlcNAc_2$; also inhibits synthesis of $Glc_3Man_9GlcNAc_2$-P-P-Dol; cells incubated in presence of dNM synthesize ↓ amounts of complex oligosaccharides and ↑ amounts of high mannose structures; Subsequent transport and secretion of glycoproteins have been shown to be impaired in some cases.	Used with intact cells at 1-5 mM and as glucosidase inhibitor (100-500 μM).	Elbein, 1987
1-Deoxymannojirimycin (dMAN)	Potent inhibitor of Golgi α-mannosidase I which normally removes several Man residues from the precursor. The principal product formed in the presence of dMAN is $Man_9GlcNAc_2Asn$, a high mannose structure; dMAN blocks formation of complex and hybrid oligosaccharide structures.	dMAN at 50 μM is sufficient to inhibit rat liver Golgi α-mannosidase I *in vitro* without significantly affecting other α-mannosidases; Used at 1-5 mM in culture media, to prevent conversion of high mannose oligosaccharides to complex structures.	Gross *et al.*, 1983; Bischoff and Kornfeld, 1984; Elbein *et al.*, 1984; Elbein, 1987

(Continued)

Table 2. (Continued)

Inhibitor	Reactions Inhibited	Comments on Use	References
INHIBITORS OF PROCESSING ENZYMES			
Castanospermine (CsP)	Inhibits only glucosidase I which releases terminal Glc from $Glc_3Man_9GlcNAc_2$; the inhibition causes accumulation of Glc-containing oligosaccharides of secreted and membrane glycoproteins; has no apparent effect on protein synthesis; Recent studies have shown that CsP interferes with HIV infectivity.	CsP at 100 μg/ml completely blocks mammalian glucosidase I; In intact cells 10-50 μg/ml is used.	Pan *et al.*, 1983; Elbein, 1987; Gruters *et al.*, 1987; Fleet *et al.*, 1988; Taylor *et al.*, 1991
Swainsonine (SwN)	Inhibits Golgi α-mannosidase II, *i.e.* formation of $GlcNAc_2Man_5$ is blocked. Processing to hybrid-type oligosaccharide structures containing Fuc and NeuNAc can occur as well as high mannose and complex-type structures.	Intact cells, 0.1-2 μg/ml; Cell-free systems, 0.1-10 μM	Gross *et al.*, 1983; Elbein *et al.*, 1983; Schwarz and Datema, 1984; DeSantis *et al.*, 1987; Elbein, 1987
Bromoconduritol (BrC)	Inhibits mammalian α-glucosidase II; Does not affect mammalian α-glucosidase I, Golgi α-mannosidase I or formation of Dol-P-P-oligosaccharide or its transfer to proteins.	For inhibition of glycoprotein processing, 1-5 mM used *in vivo* and *in vitro*. As a glucosidase inhibitor, 0.2-2 mM is recommended.	Datema *et al.*, 1982; Schwarz and Datema, 1984; Elbein, 1987

(Continued)

Table 2 (Continued)

Inhibitor	Reactions Inhibited	Comments on Use	References
SUGAR ANALOGUES*			
2-Deoxy-D-glucose (dGlc)	Formation of Dol-linked monosaccharides by trapping Dol-P as dGlc-P-P-Dol; Also inhibits the initial assembly of lipid-linked oligosaccharides by incorporation of dGlc instead of Glc or Man	Intact cells 1-50 μM or 1-120 μM GDP-dGlc or UDP-dGlc in cell-free systems	Schwarz and Datema, 1982; Elbein, 1987
2-Deoxy-2-fluoro-D-glucose	Formation of Man-P-Dol and Glc-P-Dol is blocked; ↓UDP-Glc pool size.	Intact cells 0.2-0.3 mM	Schwarz and Datema, 1982; Elbein, 1987
2-Deoxy-2-fluoro-D-mannose	Synthesis of lipid-linked oligosaccharides	Intact cells 0.2-10 mM	Schwarz and Datema, 1982; Elbein, 1987
D-Glucosamine	Synthesis of lipid-linked oligosaccharides	Intact cells 4-40 mM, inactive in cell-free systems.	Schwarz and Datema, 1982; Elbein, 1987

See **List of Abbreviations** for abbreviations and nomenclature used. Adapted and condensed from: Beeley, 1985; *Tools for Glycobiology*, Oxford Glycosystems Catalogue, 1992, Oxford Glycosystems Ltd., UK; *Reagents for Glycoconjugate Research, Inhibitors of Glycoprotein Synthesis, Glycohydrolases*, Boehringer Mannheim Biochemica Information, 1987, Boehringer Mannheim GmbH, Germany; *Inhibitors of Glycoprotein Processing*, Genzyme Corp., Boston, USA. *See also Reutter and Bauer (1985) for additional sugar analogues; ↑, increase(d); ↓, decrease(d).

antibiotics, the main components being TMs V, VII and X, also referred to as A, B, C and D, respectively; The Merck Index, 1989), is composed of uracil, an 11-carbon aminodialdose called *tunicamine*, GlcNAc that is attached to one of the anomeric carbons of tunicamine, and a fatty acid coupled to the amino group of *tunicamine* via an amide bond. The homologues vary in their fatty acid composition, saturation, chain length and branching. The molecular weights of the TMs range from about 814 to 872. Tunicamycin is easily soluble in dimethylsulphoxide (DMSO), dimethylformamide (DMF), and pyridine (>10 mg/ml). Solutions in DMSO and DMF are stable for 3-4 weeks if stored at -20°C. Tunicamycin is readily soluble in aqueous solutions at alkaline pH. Solutions are stable at neutral and alkaline pH, but not at acid pH (The Merck Index, 1989). TM is a higly toxic substance with an LD_{50} of 6.5 mg/kg (oral) and 1.8 mg/kg (i.p. or i.v.) in mice.

In prokaryotic cells, TM inhibits peptidoglycan synthesis by blocking the formation of lipid intermediates. In eukaryotic cells, *in vitro,* it blocks the initial step in oligosaccharide biosynthesis, *viz.*, the transfer of GlcNAc-1-P from UDP-GlcNAc to the lipid carrier, Dol-P. Thus, in the presence of this antibiotic, the lipid-linked oligosaccharide is not formed and the protein cannot be glycosylated. Tunicamycin has also been demonstrated to inhibit the incorporation of [^{3}H]mannose and [^{3}H]glucosamine by mammalian cells in culture (Irimura *et al.*, 1981). The degree of inhibition obtained varies with the cell type and is also dependent upon the concentration of TM (range from 0.1-10 μg/ml, or higher). Tunicamycin is used as a tool for elucidating the role of the carbohydrate moieties of glycoproteins in a wide variety of biologic phenomena such as protein secretion and turnover (Olden *et al.*, 1978), membrane transport (Olden *et al.*, 1979a), assembly of enveloped viruses (Schultz and Oroszlan, 1979) and changes in cell-surface properties during transformation (Duksin and Bornstein, 1977). In the majority of systems studied, somewhat conflicting and confusing results have been obtained which make it difficult to present a unifying hypothesis regarding the action of this antibiotic. Thus, any effect of TM has to be studied and interpreted with caution since glycosylation is cell-type specific. Nevertheless, the TMs have proved useful in studying the potential roles of carbohydrate in glycoprotein-mediated processes.

5. BIOCHEMICAL RELEVANCE OF GLYCOSYLATION

Transformed phenotypes are characterized by quantitative alterations in surface properties that enable tumour cells to invade, disseminate, implant, survive and multiply in secondary sites (Nicolson, 1984). Several lines of evidence support the role that cell surfaces play in the regulation of phenotypic diversity. The selective removal of metastatic cell-surface components with proteolytic enzymes and TM has been shown to modify invasive properties and subsequent metastasis without affecting tumour cell viability (Irimura *et al.*, 1981). In addition, multiple differences in the expression of carbohydrates on cell-surface glycoproteins have been found between low and high metastatic cell variants (Koyama *et al.*, 1979; Takasaki *et al.*, 1980; Dennis *et al.*, 1984). Many transformed cells show alterations in their terminal saccharide residues, most prominently increased expression of NeuNAc-Gal-GlcNAc sequences, although this observation may not be generally applicable (Irimura *et al.*, 1981; Steck and Nicolson, 1983). It is important to note that highly metastatic cells have higher rates of spontaneous mutation than their normal counterparts and that alterations in the tumour cell peripheral components that typify these cells are consistent with the mutational-regulatory gene hypothesis (Hart and Fidler, 1981). However, other mechanisms such as gene amplification, differential gene activation, transcriptional and translational controls, microenvironmental signals, changes in extracellular milieu, cell-cell communication and certain inducers may be equally important for the expression and regulation of altered phenotypes.

Many aspects of cellular reponses are influenced by the composition of cell-surface glycoproteins. The putative functional significance of the carbohydrate moieties in such phenomena has been recognized for many years (Olden *et al.*, 1982; Smets and Van Beek, 1984; Jentoft, 1990). Recent advances in glycobiology have highlighted the profound importance of glycoconjugates in several disease states (Dennis and Laferte, 1987; Stanley, 1987; Rademacher, 1988). Dramatic changes in the branching patterns of cell-surface glycans occur during normal and pathological processes (Hakomori, 1985; Warren *et al.*, 1979). Cancerous tissues and transformed cells express aberrant carbohydrates and blood group antigens (Marshall and Neuberger, 1968; Holgersson *et al.*, 1989). Highly branched sialic acid- and fucose-containing Asn-linked (N-glycans) oligosaccharides show one of the most reproducible correlations with transformed and malignant cells (Yogeeswaran and Salk, 1981). Moreover, abnormal expression of sugars on human cells may trigger autoimmune processes (Rademacher *et al.*, 1988).

Therefore, the spatial structures of the glycan chains of glycoproteins play important roles in biological functions, and an evaluation of the control points in both N- and O-linked assembly may yield valuable information on how certain diseases progress and might be controlled (Göttlinger *et al.*, 1988; Holzmann *et al.*, 1988). Receptor-based strategies involving glycoconjugates may define, in conformational terms, the structural features on both participants (*i.e.*, ligand and receptor) essential for the control of several diseases (Carver, 1989; Davidson, 1989; Feizi, 1989). The use of lectins with their multiplicity of sugar specificities and binding capacities offers enormous possibilities as probes of various disease states (Smith and Walborg, 1977; Smets and Van Beek, 1984; Lis and Sharon, 1986). Moreover, the clinical utility of monoclonal antibodies to glycoconjugates may be increased by the very many carbohydrate structures and specificities (Schlom, 1986; Lloyd and Lloyd, 1989). Thus, the identification of the carbohydrate recognition elements in cellular function will be pivotal for drug development and the safe and effective treatment of a number of human diseases.

PART III: RESEARCH PROPOSAL

Many of the difficulties encountered in the chemotherapy of human tumours can be attributed to the manifestations of drug resistance. P-glycoprotein is present in most drug-resistant cells and functions as an energy-dependent efflux pump that maintains subtoxic intracellular levels of a broad spectrum of chemotherapeutic agents. It is generally accepted that MDR cells accumulate less drug than their corresponding parental or drug-sensitive counterparts. Therefore, the extent of penetration into and accumulation and retention within tumour cells of certain anticancer compounds appears to be an important determinant of their cytotoxicity. Considering the relationship between its structure and function in the MDR phenotype, it may be that modification of Pgp can be applied usefully for chemosensitization purposes. P-glycoprotein is processed post-translationally by glycosylation, which may regulate its activity and result in disparities in cell responses to cytotoxic drugs. [Refer to shaded text boxes in Parts I and II, pages 8-11, 24, 29, 32, 42, 49 and 50, for selected areas of interest that led to the research undertaken in this thesis.]

In this study, tunicamycin, a specific inhibitor of N-glycosylation, will be evaluated for its potential to reverse the MDR phenotype in various cell lines. No attempt will be made to characterize the specific oligosaccharide alterations induced by antibiotic treatment in a single class of membrane glycoproteins, *i.e.*, P-glycoprotein. Rather, the inhibition of glycoprotein biosynthesis in general will be correlated with resistance and cytotoxicity, drug uptake, efflux and retention *in vitro* in cells transfected with the *mdr* gene. In addition, the partial characterization of Pgp expression in a continuous human ovarian carcinoma cell line (UWOV2) and peripheral blood leukaemia blasts obtained from patients will be undertaken. The UWOV2 cell line was isolated from an ovarian cystadenocarcinoma in a patient who had not responded to combined chemotherapy with actinomycin D, doxorubicin, vincristine and cisplatin. This cell line has not been characterized previously with regard to its expression of Pgp, and will be studied in association with the *mdr*1-transfected cell lines.

CHAPTER 2

EFFECTS OF TUNICAMYCIN ON GLYCOPROTEIN BIOSYNTHESIS IN VARIOUS DRUG-SENSITIVE AND DRUG-RESISTANT CELL LINES

1. INTRODUCTION

A working hypothesis for acquired drug resistance at the biochemical level has been postulated, *viz.*, enhanced glycoprotein synthesis and/or increased glycosyltransferase activity and decreased glycosidase activity (Bosman, 1971). Several reports have confirmed that cells selected *in vitro* for resistance to any of the natural product drugs (*e.g.*, adriamycin, daunorubicin, actinomycin D, vincristine and vinblastine) display cross-resistance to the others, altered rates of glycoprotein synthesis, reduced glycoprotein degradation and a higher level of glycosyltransferase activity than in the parent or drug-sensitive counterparts (Biedler and Riehm, 1970; Bosman and Kessel, 1970). Exposure of certain drug-sensitive cells to adriamycin modified their pattern of glycoprotein synthesis by enhancing the incorporation of radioactive fucose into plasma membrane glycoproteins (Kessel, 1979). However, the hyperglycosylation induced by adriamycin was not accompanied by a net gain in protein synthesis and this presumably involved amplified glycosylation of existing glycoproteins. Drug-resistant cells displayed a significantly greater degree of fucosylation during the labelling period contrary to drug-sensitive cells, even in the absence of drug treatment.

Studies with human multidrug-resistant KB carcinoma cells have shown that P-glycoprotein (Pgp) is synthesized as a 140-kDa precursor that is converted to a 170-kDa glycoprotein. Tunicamycin, an inhibitor of N-glycosylation, prevents the conversion of the precursor to the mature form (Richert *et al.*, 1988). By contrast, Yoshimura *et al.* (1989) have presented evidence that human multidrug-resistant KB-C2 cells synthesize a 125-kDa Pgp that is modified to a 140-150-kDa protein, and that treatment with tunicamycin produces a 120-kDa species that is not further processed. These data, together with observations of molecular mass variations of

Pgps in normal human tissues such as liver, kidney, adrenal gland, capillary endothelium of the brain and testis, indicate that Asn-linked glycosylation represents a major modification of Pgp and that a variety of N-glycans may be present in normal tissue and tumour Pgps (Ichikawa *et al.*, 1991a). Futhermore, Gervasoni *et al.* (1991) have established that in drug-sensitive human HL-60 leukaemia cells a type of drug resistance, in the absence of Pgp expression, is partially inducible by inhibition of Asn-linked glycosylation of cell-surface proteins.

Notwithstanding these observations, relatively few studies have directly evaluated the role of glycosylation in the expression of MDR, which makes the characterization of the glycoprotein synthesis in MDR a challenging task. This chapter explores the effects of tunicamycin, an inhibitor of the earliest step of N-linked glycosylation, on total protein and glycoprotein synthesis in various drug-sensitive and drug-resistant cell lines. Tunicamycin was also tested for possible inhibition of the glycosyltransferase activities in these cells. The studies described in this chapter were necessary before further investigation of the possible consequences of TM treatment on the expression of the MDR phenotype in the cell lines described could be undertaken.

2. Materials and Methods

2.1. Radioisotopes and Biochemicals

Trypsin 1:250 (Difco Laboratories, Detroit, MI, USA), PBS Dulbecco 'A' [phosphate-buffered saline] (Oxoid, UK), tissue culture media (Gibco, UK), tunicamycin (Boehringer Mannheim, Germany), EDTA [ethylene-diamine tetraacetic acid Na_2 salt] (Merck Chemicals, Germany), asialofetuin, ovalbumin and colchicine (Sigma Chemical Co., St. Louis, MO., USA), insulin monotard®(Novo Pharmaceuticals, South Africa), gentamicin sulphate (cidomycin®, Roussel Laboratories, South Africa), doxorubicin (adriamycin, generously supplied by Farmitalia Carlo Erba, Italy), radioactive precursor isotopes (Amersham, U.K.), and Bio-Rad™ protein assay dye reagent concentrate (Bio-Rad Laboratories Ltd., U.K.) were used in this study. All other reagents were of analytical grade and were obtained from either Merck Chemicals or Sigma Chemical Co.

2.2. Cell Lines and Culture Conditions

The UWOV2 ovarian carcinoma cell line was provided by Professor W.R. Bezwoda (University of the Witwatersrand, South Africa). This cell line was derived from an ovarian cystadenocarcinoma in a patient who had not responded to combined chemotherapy with actinomycin D, vincristine, cisplatin and doxorubicin (Golombick *et al.*, 1990), and therefore represents a cell line with *in vivo*-acquired drug resistance. The characterization of this cell line is descibed in Chapter 5. The BG-1 ovarian carcinoma cell line (Geisinger *et al.*, 1989) was obtained from Dr C.A. Wallen (Bowman Gray School of Medicine, North Carolina, USA). The BG-1/ADR cell line was selected for resistance to doxorubicin by stepwise exposure of BG-1 cells to the drug as described in Chapter 3. The KB-3-1 human epidermoid carcinoma cell line, and its MDR derivative, KB-8-5-11, which contains the amplified *mdr*1 gene encoding Pgp (Richert *et al.*, 1985), were provided by Professor M.M. Gottesman (National Cancer Institute, Bethesda, MD, USA). The isolation and properties of the KB carcinoma cell lines have been described (Akiyama *et al.*, 1985; Fojo *et al.*, 1985). The NIH-3T3 murine fibroblasts and NIH-3T3-MDR cells, obtained by transfection of the NIH-3T3 cell line with the retroviral expression vector, pHaMDR1, containing a full-length complementary DNA from the human *mdr1* gene, (Shen *et al.*, 1986a) were also provided by Professor M.M. Gottesman. The LR73 drug-sensitive and LR73-1A drug-resistant Chinese hamster cells were a gift from Professor P. Gros (McGill University, Montreal, Canada). LR73-1A cells were obtained by transfection of drug-sensitive LR73 cells with expression constructs containing the *mdr*1 cDNA and selection of drug-resistant clones in adriamycin or colchicine (Schurr *et al.*, 1989; Gros *et al.*, 1991b).

Cell lines were maintained and propagated under the following conditions: UWOV2, in RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum (HIFCS), penicillin G (100 U/ml) and streptomycin sulphate (100 μg/ml) or gentamicin sulphate (50 μg/ml); BG-1, in McCoy's 5A medium supplemented with 10% HIFCS, 100 U/ml insulin, 200 mM L-glutamine and antibiotics; KB-3-1 and NIH-3T3 parental cells, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% HIFCS and antibiotics as above; KB-8-5-11 and NIH-3T3-MDR cells, in DMEM as for parental cells with addition of 1 μg/ml colchicine. LR73 and LR73-1A, in alpha minimum essential medium (α-MEM) containing ribo- and deoxyribonucleotide precursors, 10% HIFCS, 2mM L-glutamine and antibiotics. LR73-1A were grown in the presence of adriamycin or colchicine at

100 ng/ml. Incubator settings were calibrated to 37°C, 5% CO_2:air and 85% relative humidity. Cells were routinely subcultured with trypsin-EDTA (0.25%-0.02%, w/v) in Ca^{+2}- and Mg^{+2}-free PBS and maintained in the logarithmic phase of growth. Cell lines were periodically tested according to the method described by Chen (1977) and found to be free of mycoplasma contamination.

2.3. Assay of Glycosyltransferases

Glycosyltransferase activities in cell-free extracts prepared from various drug-sensitive and -resistant cell lines were assessed follwing a 16h preincubation without (Control) or with (TM-treated) 5 μg/ml tunicamycin by modification of the procedure previously reported by Liu *et al.* (1981).

2.3.1. Preparation of Cell-Free Extracts

Cell pellets were suspended at 10^7 cells/ml in cold lysis medium composed of Triton X-100/DMEM (0.1% v/v) and 1 mM glutaraldehyde to stabilize Golgi enzymes (particularly glycosyltransferases, Warley and Cook, 1976). The cell suspensions were frozen-thawed twice and cell-free extracts obtained by lysis for 10 min. on ice. No intact cells remained after this treatment as judged by phase-contrast microscopy. However, the lysates were centrifuged at 10 000g for 30s in a Beckman microfuge and the supernatants used as the crude enzyme preparation. Total protein in cell-free extracts was determined by the automated Bio-Rad™ dye-binding assay described by Bradford (1976).

2.3.2. Sialyltransferase Assay

Sialyltransferase activity was measured by the transfer of [^{14}C]sialic acid from CMP-[^{14}C]sialic acid to an exogenous acceptor (asialofetuin) catalyzed by cell-free extracts. Assay mixtures in 1.5-ml microfuge tubes consisted of 10 μl of 0.25 M sodium phosphate buffer, pH 7.0, containing 50 mM $MgCl_2$; 10 μl of asialofetuin (50 mg/ml in the above buffer); 20 μl of CMP-[^{14}C]sialic acid (262 mCi/mmol, final specific activity 11.5 dpm/pmol); 50 μl of the crude enzyme preparation (*i.e.*, cell-free extract) and 28 μl serum-free DMEM. Assay mixtures were incubated for 2h at 37°C in a shaking waterbath.

2.3.3. Galactosyltransferase Assay

Galactosyltransferase activity was assessed by measuring the transfer of [^{3}H]galactose from UDP-[^{3}H]galactose to exogenous desialylated fetuin. Reaction mixtures in 1.5-ml microfuge tubes consisted of 10 μl 0.75 M sodium cacodylate buffer, pH 7.0, containing 0.20 M $MnCl_2$; 10 μl fetuin free of sialic acid and galactose (50 mg/ml in the above buffer); 20 μl of UDP-[^{3}H]galactose (20 Ci/mmol; final specific activity 11.5 dpm/pmol); 50 μl of cell-free extract and 28 μl serum-free DMEM. Reaction mixtures were incubated for 2h as described for the sialyltransferase assay.

2.3.4. Fucosyltransferase Assay

The activity of fucosyltransferase was determined by measuring the transfer of [^{14}C]fucose from GDP-[^{14}C]fucose to exogenous asialofetuin (free of sialic acid and galactose) in cell-free extracts. Assay mixtures in 1.5-ml microfuge tubes consisted of 10 μl of 0.375 M Tris-HCl buffer, pH 7.0, containing 18.75 mM EDTA; 10 μl asialofetuin (50 mg/ml in the above buffer); 20 μl of GDP-[^{14}C]fucose (292 mCi/mmol, final specific activity 11.5 dpm/pmol); 50 μl of cell-free extract and 28 μl serum-free DMEM. Assay mixtures were incubated for 2h as described for the sialyltransferase assay.

2.3.5. N-Acetylglucosaminyltransferase Assay

N-Acetylglucosaminyltransferase activity was measured by the transfer of [6-^{3}H]GlcNAc from UDP-[6-^{3}H]GlcNAc to an exogenous acceptor, *viz.*, ovalbumin, in cell-free extracts. Reaction mixtures in 1.5-ml microfuge tubes consisted of 10 μl of 0.6 M sodium cacodylate buffer, pH 7.0, containing 0.12 M $MnCl_2$ and 1.2 mM ATP; 10 μl ovalbumin (50 mg/ml in the above buffer); 20 μl of UDP-[6-^{3}H]GlcNAc (60 mCi/mmol, final specific activity 11.5 dpm/pmol); 50 μl cell-free extract and 28 μl serum-free DMEM. Assay mixtures were incubated as described for the sialyltransferase assay.

2.3.6. Harvesting of Assay Mixtures

Glycosyltransferase assays were terminated by the addition of 1 ml ice-cold 10% trichloroacetic acid (TCA) to the reaction vessels and incubation for a further 10 min. on ice. The precipitate containing the glycosylated acceptor protein was

poured onto glass microfibre filters (Ø25mm, Schleicher & Schüll or Millipore HAWP02500). The filters were washed three times with 1 ml ice-cold 10% TCA and once with 96% ethanol. The washed filters were air-dried, transferred to 10 ml Beckman Ready Protein™ scintillation cocktail in 20-ml glass vials and counted by liquid scintillation spectrometry. The radioactivity obtained in DPM was converted to pmoles and enzyme activity expressed as the number of pmoles of radiolabelled sugar transferred from nucleoside diphosphate sugar carrier to exogenous acceptor protein per hour.

2.4. Precursor-Incorporation-Inhibition Studies

Inhibition of protein and glycoprotein synthesis was studied by minor modification of the procedure described by Irimura *et al.* (1981). The effects of prior exposure to TM on the incorporation of labelled precursors [^{14}C]leucine (50-60 mCi/mmol) or [^{35}S]methionine (>1000Ci/mmol) and [^{3}H]mannose (30-60 Ci/mmol) or [^{3}H]glucosamine (20-40 Ci/mmol) into trichloroacetic acid-insoluble macromolecules (proteins and glycoproteins, respectively) were evaluated in the various cell lines as described in the results. Following an initial 16h exposure to TM (5 μg/ml), cells in the logarithmic phase of growth (usually $4x10^5$ cells/ml/well) in 24-well plates were incubated in the absence and presence of 5 μg/ml or various concentrations of TM (0.005 to 5 μg/ml) and 5-10 μCi/ml of [^{3}H]mannose or [^{3}H]glucosamine, 2.5 μCi/ml [^{14}C]leucine or 10 μCi/ml [^{35}S]methionine in medium devoid of leucine or methionine (DMEM or RPMI selectamine kit, Gibco) for various time intervals.

After each incubation period, medium containing radioactive precursor was aspirated and the cells washed three times in ice-cold PBS and solubilized with 0.5ml of 1% SDS/0.3M NaOH. One aliquot (0.1ml) was subjected to protein determination. To the other aliquot (0.4ml) was added 1 ml of ice-cold 10% trichloroacetic acid (TCA) and the mixture left on an ice bath for 20-30 min. TCA-insoluble material (proteins or glycoproteins) was collected onto glass-fibre filters (Ø25mm, Schleicher & Schüll). Filters were washed twice with 2ml of ice-cold 10% TCA, twice with 3ml 96% ethanol and once with chloroform:methanol:water (10:10:3, v/v) to remove glycolipids (Kuo and Lampen, 1976). Filters dried in air were placed in 20-ml scintillation vials to which 10ml scintillation fluid (Beckman Ready-Protein™) was added for radioactive counting. Counting efficiencies were 33% for ^{3}H and 66% for ^{14}C. All samples were corrected for background radioactivity.

2.5. Protein Determination

The total protein content in solubilized cells and cell-free extracts was estimated by utilizing the Bio-Rad™ protein dye-binding assay kit based on the method described by Bradford (1976) with bovine serum albumin as standard.

2.6. Statistical Method

Differences between control and TM-treated cells as well as between drug-sensitive and drug-resistant cells were evaluated using the GraphPad™ INSTAT© Student's *t*-test statistical program. The level of significance was set at $p \leq 0.05$. Actual p values are presented.

3. Results

3.1. Protein and Glycoprotein Synthesis

The incorporation of [^{3}H]mannose or [^{3}H]glucosamine into glycoprotein and of [^{14}C]leucine or [^{35}S]methionine into protein was analysed in various drug-resistant (NIH-3T3-MDR, KB-8-5-11, LR73-1A and UWOV2) and drug-sensitive (NIH-3T3-parental, KB-3-1 and LR73) cell lines in the absence (control) or presence of TM (TM-treated) at different time intervals following an initial 16-h preincubation with the antibiotic. Synthesis was standarized and expressed as the amount of DPM incorporated into trichloroacetic acid-insoluble fractions per total cell protein content.

3.1.1. NIH-3T3-Parental and NIH-3T3-MDR Murine Fibroblasts

The incorporation of glycoprotein precursor isotopes by both NIH-3T3-parental and NIH-3T3-MDR was significantly inhibited by TM at the time intervals indicated (Figure 1). The NIH-3T3-MDR cells showed a consistently greater rate of glycoprotein synthesis than their drug-sensitive counterparts (Figure 2). Similarly, total protein synthesis was significantly greater (except at the 4-h time interval, $p=0.3752$) in the NIH-3T3-MDR cell line compared with its parental cell line (Figure 3). Tunicamycin also inhibited protein synthesis in NIH-3T3-MDR cells, except at 4 hours of incubation (Figure 3).

The antibiotic exerted varying time-dependent effects on NIH-3T3-parental cells, with inhibition of protein synthesis achieved at incubation times of 4h, 16h

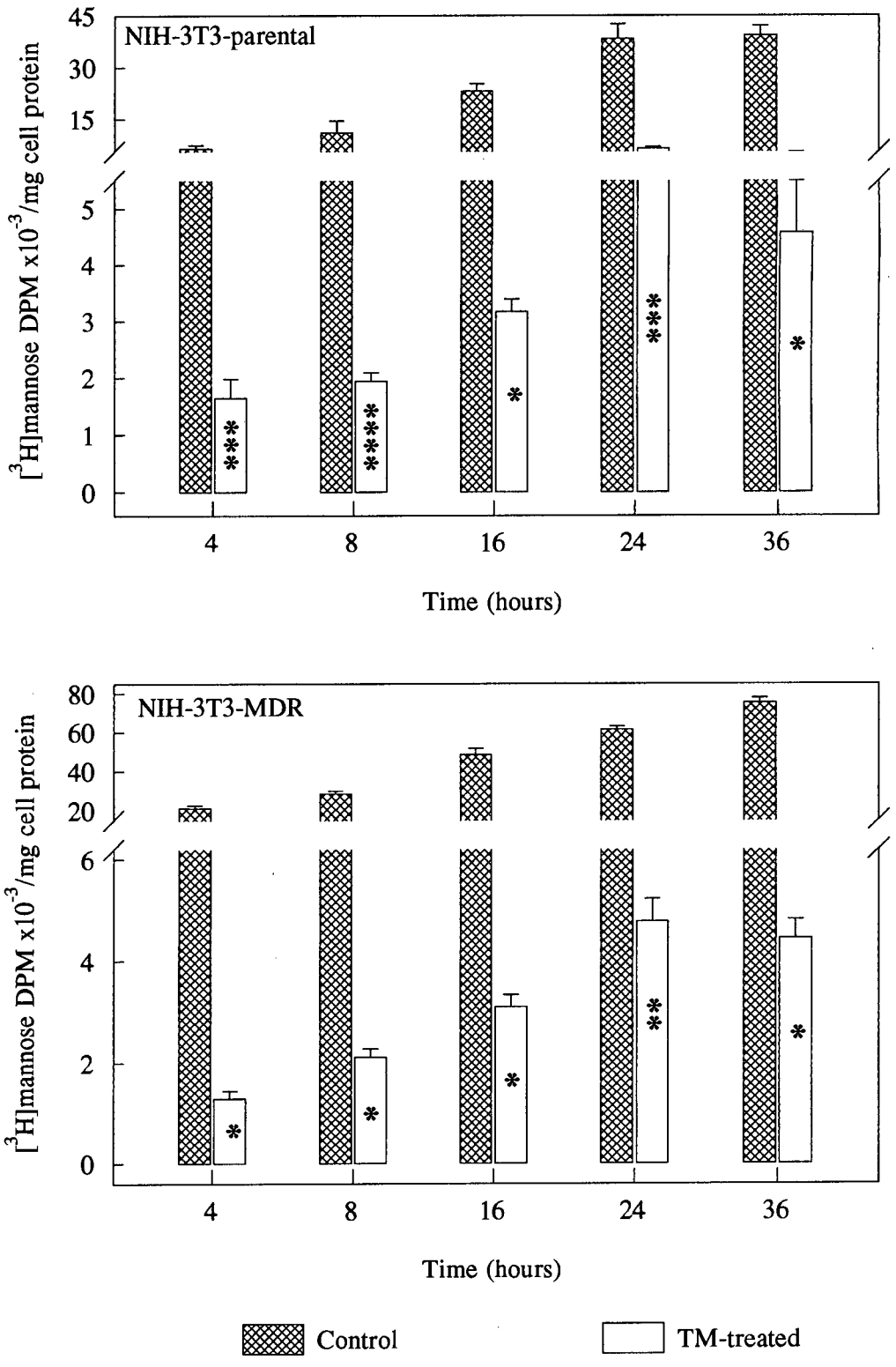

Figure 1. Time-course of the effect of tunicamycin (5 μg/ml) on the incorporation of radiolabelled mannose by NIH-3T3-parental and NIH-3T3-MDR cells in culture. Results are expressed as means $\pm$ S.E.M. of quadruplicate cultures. Asterisks indicate two-tailed p values for the differences between control and TM-treated cells as follows: *$p < 0.0001$; **$p = 0.0003$; ***$p = 0.003$; ****$p = 0.038$.

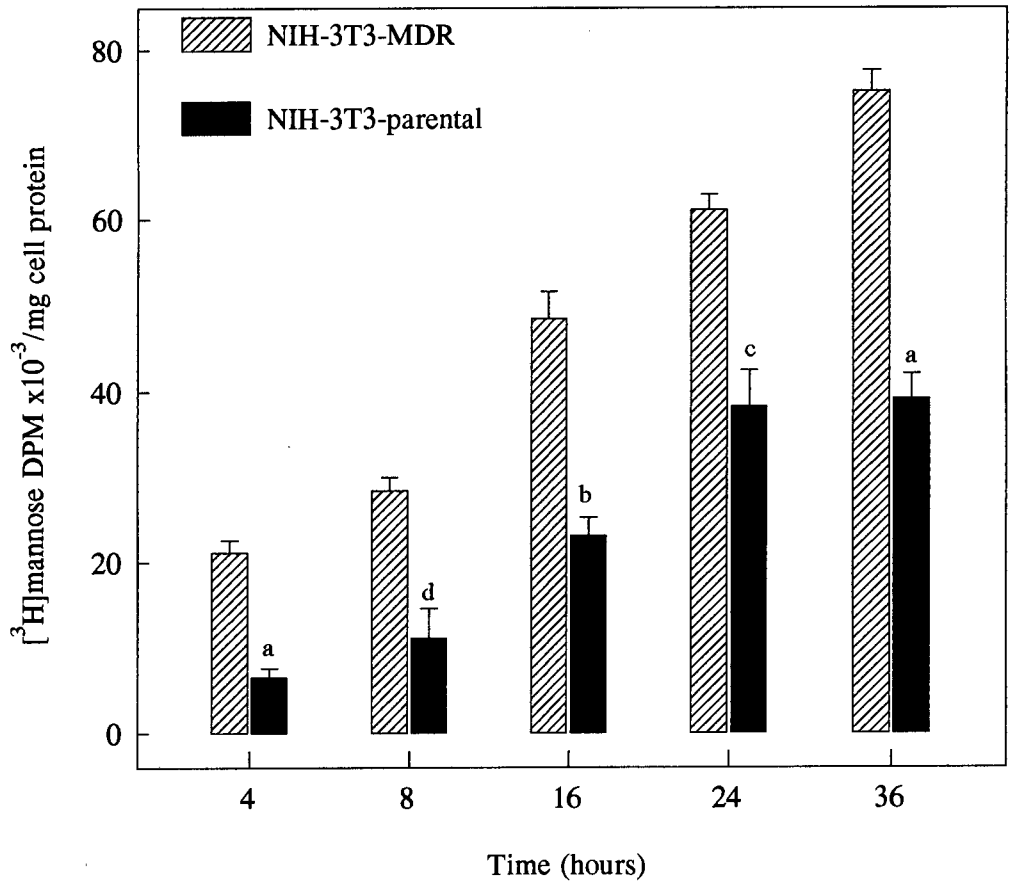

Figure 2. Time-course of total glycoprotein synthesis in NIH-3T3-MDR and NIH-3T3-parental cells in culture. Data were replotted from Figure 1 and are expressed as means ± S.E.M. of quadruplicate cultures. Lowercase letters above error bars indicate two-tailed p values for the difference in glycoprotein synthesis between NIH-3T3-MDR and NIH-3T3-parental cells as follows: $^{a}p \leq 0.0001$; $^{b}p = 0.006$; $^{c}p = 0.0023$; $^{d}p = 0.0038$.

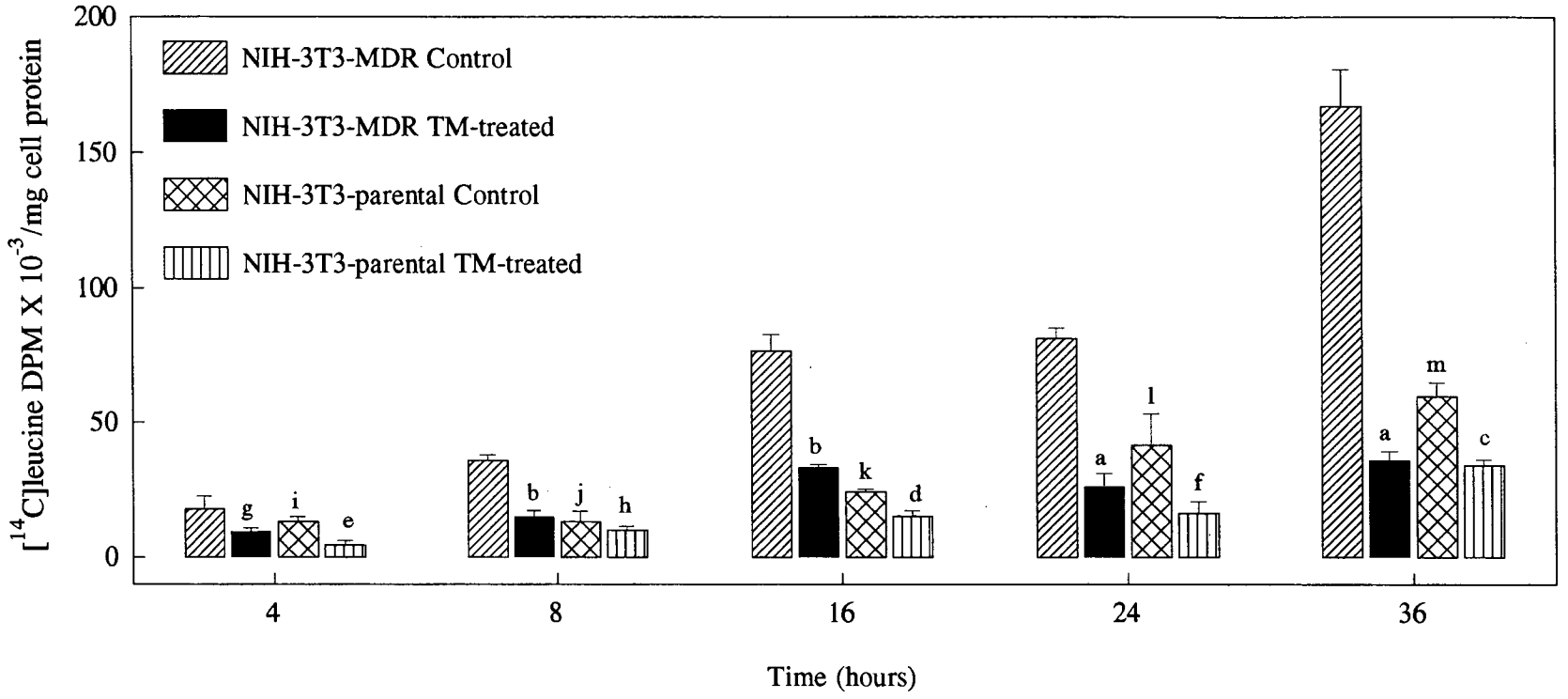

Figure 3. Comparison of total protein synthesis in NIH-3T3-MDR and NIH-3T3-parental cells in the absence (control) or presence (TM-treated) of tunicamycin (5 μg/ml). The effect of TM on total protein synthesis was measured by determining the incorporation of [^{14}C]leucine into cellular protein. Data represent means $\pm$ S.E.M. (n=4). $^{a\text{-}h}$Indicate two-tailed p values for the difference between control and TM-treated cells as follows: $^{a}p \leq 0.0001$; $^{b}p=0.0004$; $^{c}p=0.0036$; $^{d}p=0.0067$; $^{e}p=0.0074$; $^{f}p=0.0827$; $^{g}p=0.128$; $^{h}p=0.4677$. $^{i\text{-}m}$Depict two-tailed p values for the difference in protein synthesis between MDR and parental cells as follows: $^{i}p=0.3752$; $^{j}p=0.0019$; $^{k}p=0.0002$; $^{l}p=0.0170$; $^{m}p=0.0003$.

and 36h, but not at 8h and 16h (Figure 3). NIH-3T3-parental and NIH-3T3-MDR cells differed markedly in their susceptibility to suppression of protein synthesis by TM at 8h, 16h and 36h, but not at 4h and 24h (Figure 4A). The percentage inhibition of mannosylglycoprotein synthesis induced by TM in both the NIH-3T3-parental and NIH-3T3-MDR cell lines consistently exceeded 75-80% of the controls (Figure 4B). However, no differences in the degree of glycoprotein synthesis inhibition by TM could be demonstrated between NIH-3T3-parental cells and their MDR transfectants (Figure 4B).

3.1.2. KB-3-1 and KB-8-5-11 Human Epidermoid Carcinoma Cells

Multidrug-resistant KB-8-5-11 cells expressed higher rates of glycoprotein synthesis compared with parent KB-3-1 cells (Figure 5). In the absence of TM, at the end of selected time intervals, the level of radiolabelled mannose associated with trichloroacetic acid-insoluble material was significantly higher in KB-8-5-11 drug-resistant cells than in KB-3-1 drug-sensitive cells. At all incubation times studied, exposure of both cell lines to TM resulted in a considerable decrease in the quantity of cell-associated [^{3}H]mannose-labelled macromolecules (Figure 5).

3.1.3. LR73 and LR73-1A Hamster Cells

A time-course of the synthesis of mannosylglycoproteins in LR73 and LR73-1A cells in medium containing or lacking TM is shown in Figure 6. In the absence of TM no differences in either the level or rate of glycoprotein synthesis were noted between these two cell lines. In both the drug-sensitive and drug-resistant cell lines TM significantly reduced glycoprotein synthesis compared with untreated controls.

3.1.4. UWOV2 Human Ovarian Carcinoma Cells

In the presence of TM, a marked inhibition of both glycoprotein synthesis (Figures 7 and 8B) and protein synthesis (Figure 8A) was observed in UWOV2 cells at every time period at which they were studied. The incorporation of [^{35}S]methionine into cellular protein was greatly diminished at all incubation times, except at 4h. The concentration-dependent effect of TM on [^{3}H]glucosamine incorporation by UWOV2 cells is shown in Figure 8. At concentrations less than 0.1μg/ml, TM had no inhibitory activity, but at higher concentrations (0.5-50 μg/ml) the antibiotic significantly reduced glycoprotein synthesis compared with the control.

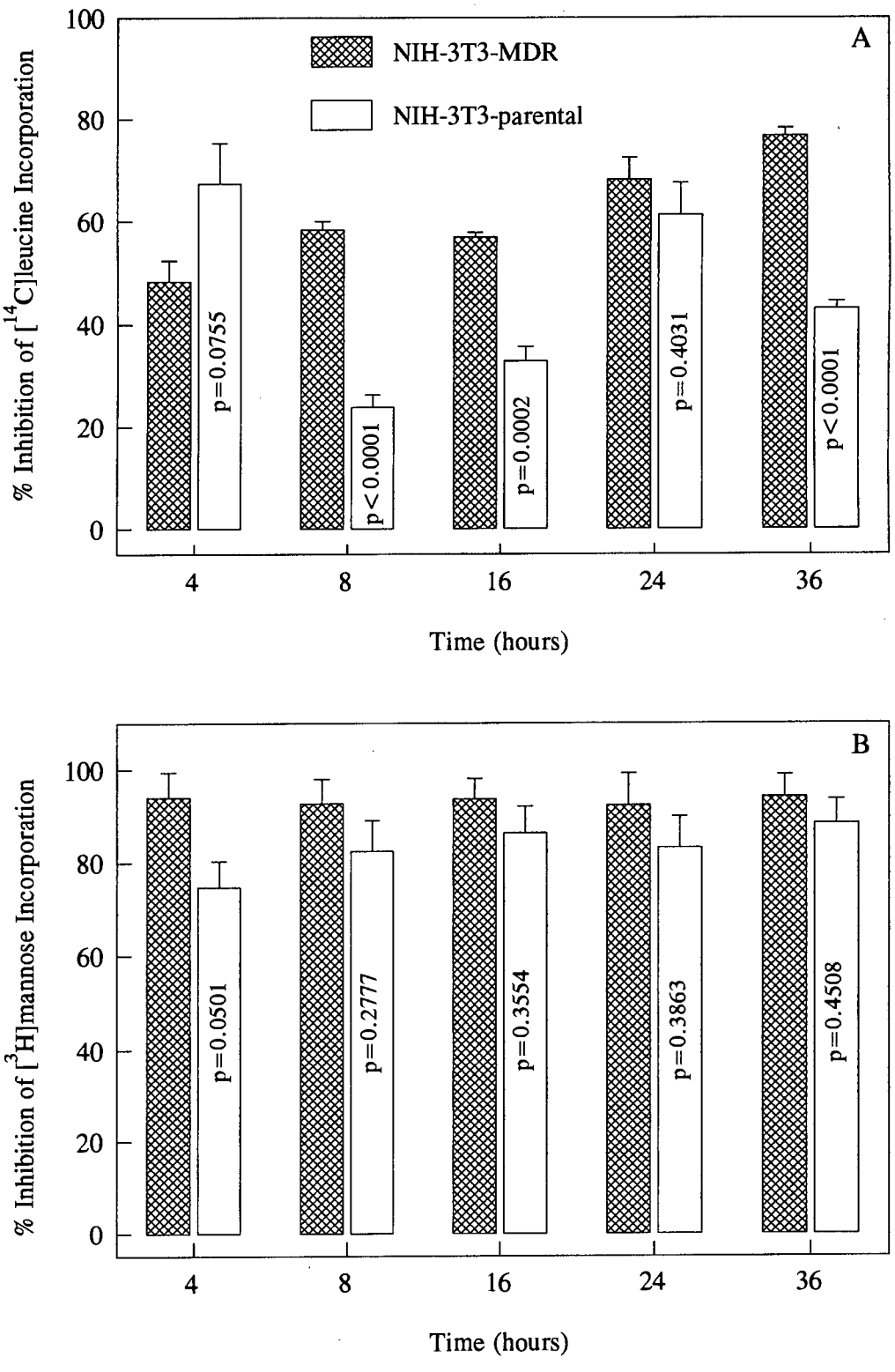

Figure 4. Comparison of the inhibitory effects of tunicamycin (5 μg/ml) on protein synthesis (A) and glycoprotein synthesis (B) in NIH-3T3-parental and NIH-3T3-MDR cells. Values are means $\pm$ S.E.M. of quadruplicate cultures. Two-tailed p values for the difference between parental and MDR cells are presented within bars.

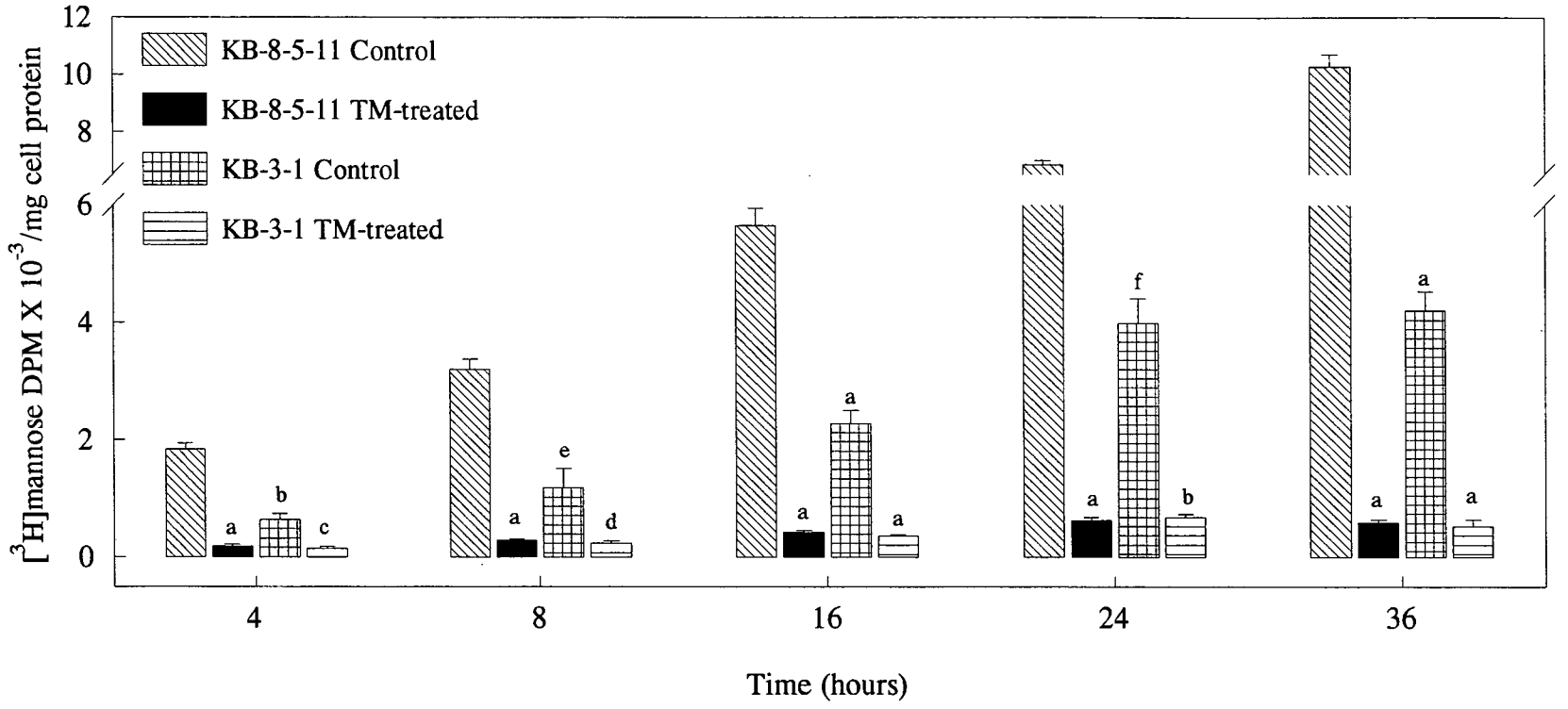

Figure 5. Effect of tunicamycin (5 μg/ml) on the synthesis of mannosylglycoproteins in KB8-5-11 and KB-3-1 cells measured by the incorporation of [^{3}H]mannose into trichloroacetic acid-precipitated cellular protein. Data represent means $\pm$ S.E.M. (n=4). Lowercase letters above bars indicate two-tailed p values for the difference between control and TM-treated cells, and between KB-8-5-11 control and KB-3-1 control as follows: $^a p \leq 0.0001$; $^b p = 0.0002$; $^c p = 0.0035$; $^d p = 0.0294$; $^e p = 0.0016$; $^f p = 0.0007$.

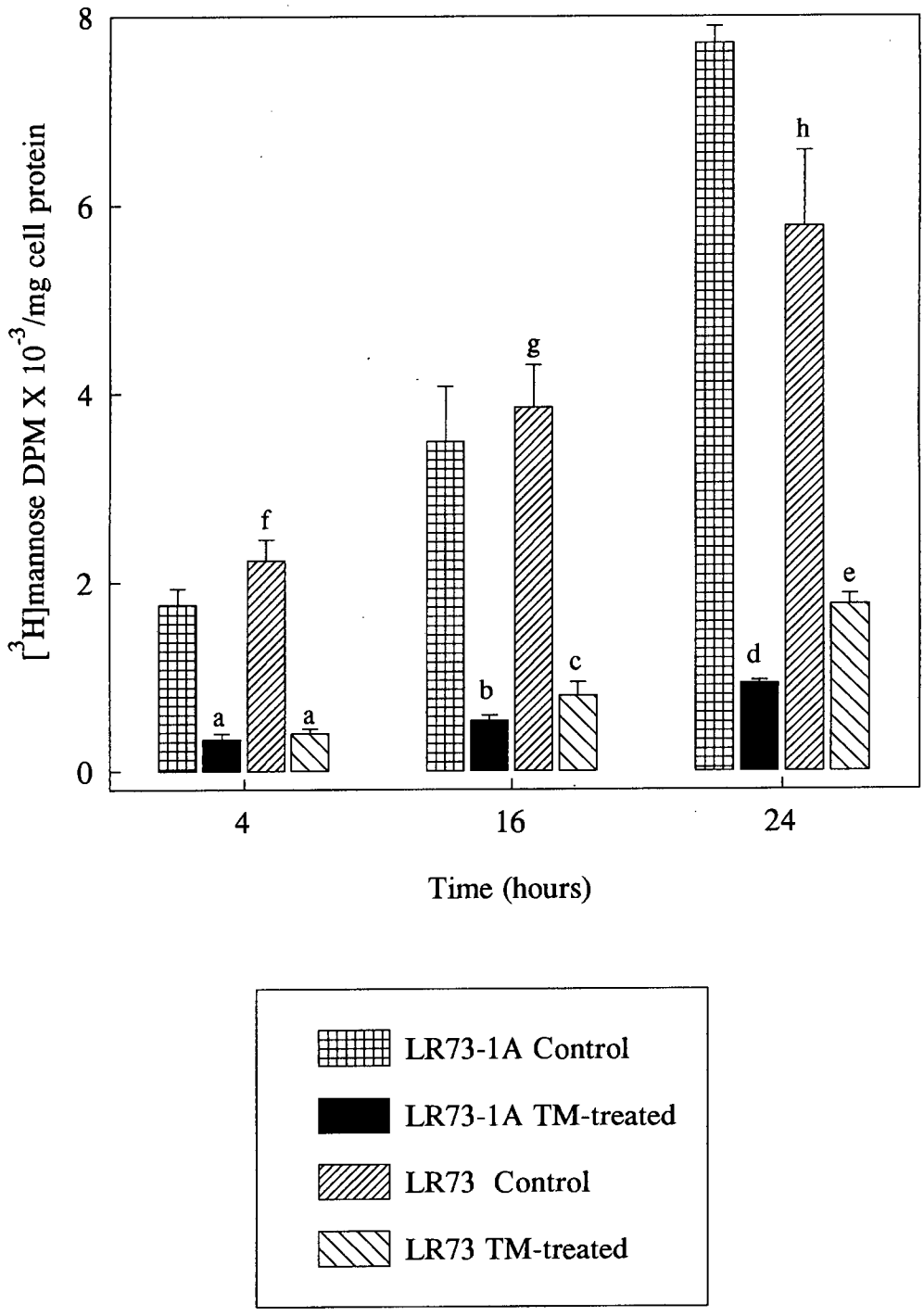

Figure 6. Effect of tunicamycin (5 μg/ml) on the incorporation of radiolabelled mannose by LR73-1A and LR73 cells in culture. Results are expressed as means ± S.E.M. of quadruplicate cultures. [a-e]Indicate two-tailed p values for the difference between control and TM-treated cells as follows: [a]$p=0.0002$; [b]$p=0.0024$; [c]$p=0.0006$; [d]$p<0.0001$; [e]$p=0.0025$. [f-h]Represent two-tailed p values for the difference between LR73-1A control and LR73 control as follows: [f]$p=0.1497$; [g]$p=0.6325$; [h]$p=0.0547$.

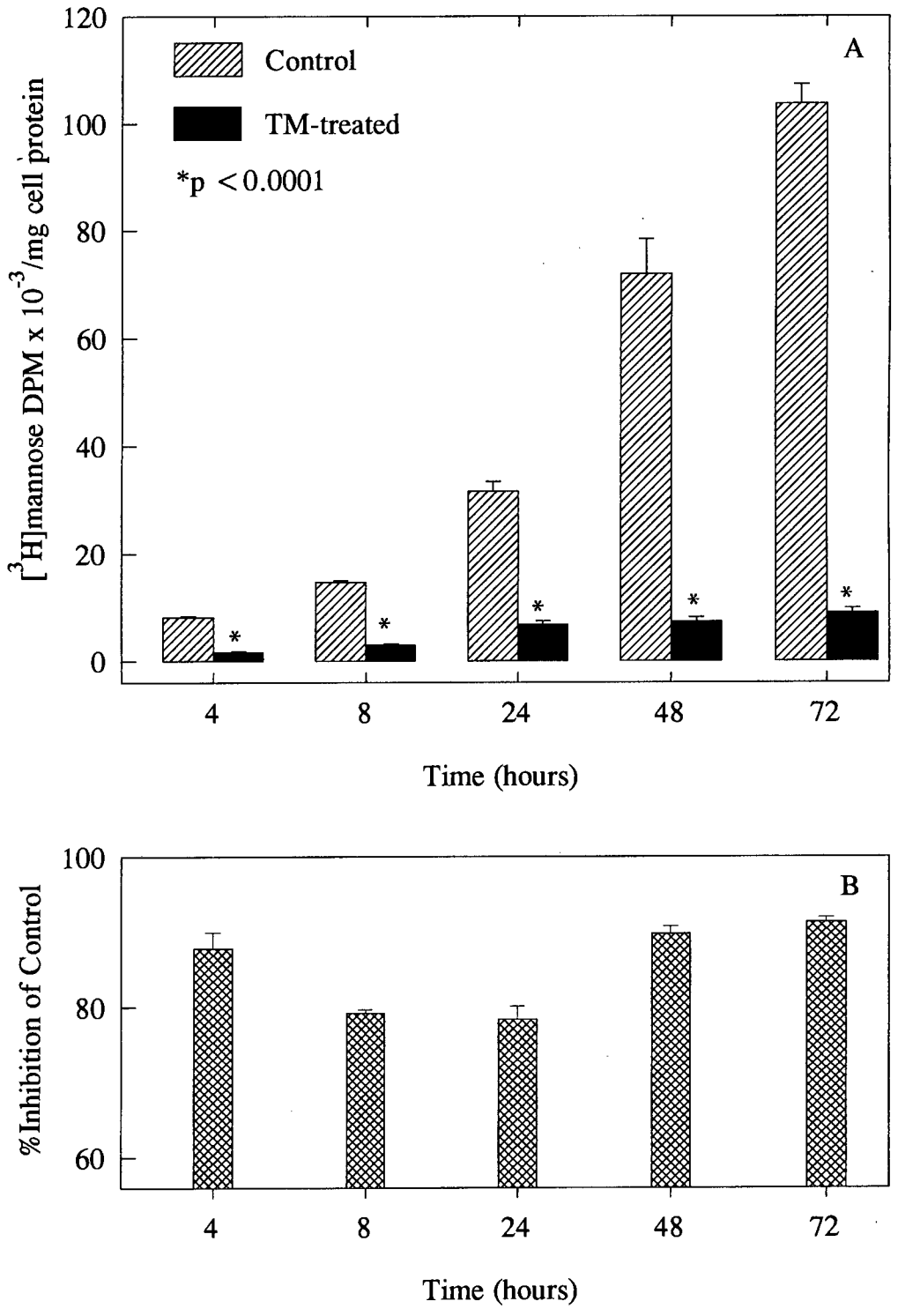

Figure 7. Effect of tunicamycin (5 μg/ml) on the incorporation of [^{3}H]mannose by UWOV2 ovarian carcinoma cells in culture (A) and the corresponding level of inhibition of mannosylglycoproteins synthesis (B). Values are means $\pm$ S.E.M. of quadruplicate experiments.

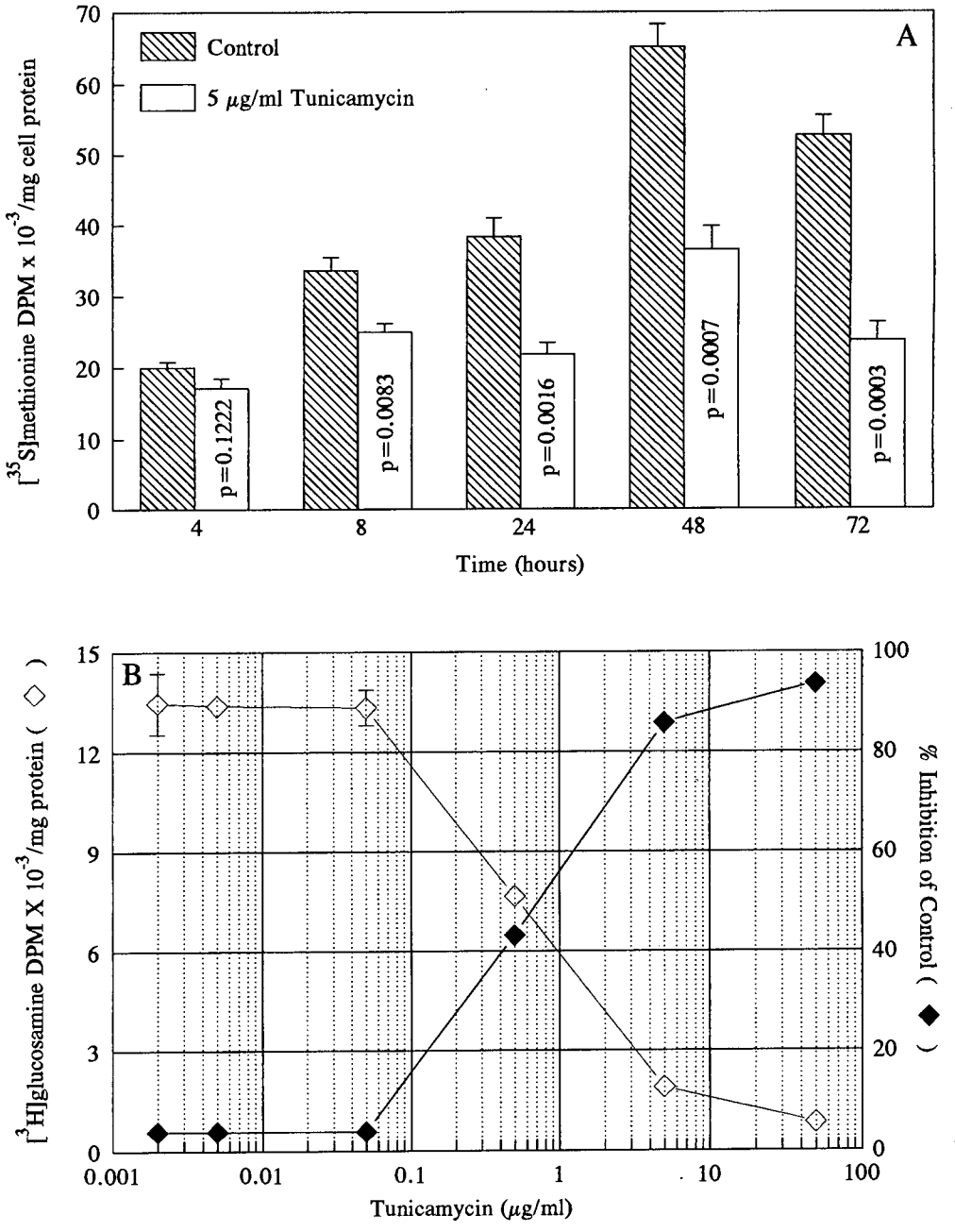

Figure 8. Effects of tunicamycin (TM) on protein synthesis (A) and glycoprotein synthesis (B) in UWOV2 ovarian carcinoma cells in culture. Protein synthesis was monitored by measuring the incorporation of [^{35}S]methionine into cellular protein at various time intervals in the absence (control) or presence (TM-treated) of the antibiotic. Glycoprotein synthesis as a function of TM concentration was evaluated by determining the amount of [^{3}H]glucosamine incorporated into cellular protein after 16h of exposure to the antibiotic. Data represent means ± S.E.M. (n=4). Two-tailed p values for the difference between control and TM treated cells are presented within bars.

3.2. Glycosyltransferase Activities

Glycosyltransferase activities measured in cell-free extracts prepared from various drug-sensitive and drug-resistant cell lines following a 16-h pre-incubation with (TM-treated) or without TM (control) are summarized in Table 1.

3.2.1. KB-3-1 and KB-8-5-11 Human Epidermoid Carcinoma Cells

Cell-free extracts from control multidrug-resistant KB-8-5-11 cells contained higher fucosyltransferase (FT) and N-acetylglucosaminyltransferase (NGT) activities, but considerably less sialyltransferase (ST) and galactosyltransferase (GT) activities than those from the corresponding untreated parental KB-3-1 cells. Tunicamycin significantly inhibited the activity of ST, but not that of FT, GT and NGT in KB-3-1 cells. In KB-8-5-11 cells, TM pretreatment failed to inhibit the catalytic activity of any of the glycosyltransferases.

3.2.2. NIH-3T3-Parental and NIH-3T3-MDR Murine Fibroblasts

When the levels of glycosyltransferases in cell-free extracts prepared from untreated NIH-3T3-parental and NIH-3T3-MDR cells were compared, the activities of FT, GT and NGT in NIH-3T3-MDR cells were increased approximately 1.5-fold above that of their parental cells. No differences in ST activities were detected between controls of the NIH-3T3-MDR cell line and its parental cell line. In both the parental and MDR cell lines, TM pretreatment significantly reduced the activities of all the glycosyltransferase enzymes investigated.

3.2.3. LR73 and LR73-1A Hamster Cells

Control LR73-1A cells contained equivalent levels of ST, GT and NGT activities, but a lower level of FT activity than their parental cells. In LR73 cells, TM did not alter the activities of ST, FT, and NGT, but markedly reduced the activity of GT in these cells. By comparison, the levels of FT, GT and NGT in LR73-1A cells were lowered by pretreatment with the antibiotic, whereas that of ST was not affected.

3.2.4. BG-1-P and BG-1/ADR Human Ovarian Carcinoma Cells

Analysis of glycosyltransferases in cell-free preparations from control BG-1-P and BG-1/ADR cells yielded similar ST, FT, and NGT activities. By contrast,

Table 1. Comparison of Glycosyltransferase Activities in Various Drug-Sensitive and Drug-Resistant Cell Lines Following Exposure to Tunicamycin

	Glycosyltransferase Activity (pmol·μg protein^{-1}·h^{-1})							
	Sialyltransferase		Fucosyltransferase		Galactosyltransferase		N-Acetylglucosaminyltransferase	
Cell Line	Control	TM treated	Control	TM treated	Control	TM treated	Control	TM treated
KB-3-1	0.89 ± 0.02	0.67 ± 0.02^{a}	0.14 ± 0.004	0.14 ± 0.01^{b}	37.4 ± 6.62	27.0 ± 1.22^{b}	0.13 ± 0.004	0.14 ± 0.009^{b}
KB8-5-11	0.74 ± 0.05^{n}	0.57 ± 0.05^{c}	0.71 ± 0.08^{o}	0.50 ± 0.10^{b}	2.28 ± 0.26^{p}	2.52 ± 0.20^{b}	0.58 ± 0.05^{q}	0.63 ± 0.04^{b}
NIH-3T3-P	4.48 ± 0.57	2.71 ± 0.11^{d}	1.38 ± 0.01	1.23 ± 0.03^{e}	4.75 ± 0.28	2.66 ± 0.12^{f}	27.1 ± 2.28	18.0 ± 0.72^{g}
NIH-3T3-MDR	6.08 ± 0.64^{b}	3.55 ± 0.04^{h}	2.06 ± 0.02^{i}	1.46 ± 0.02^{i}	7.08 ± 0.05^{a}	3.96 ± 0.13^{i}	44.6 ± 0.04^{m}	35.8 ± 0.16^{i}
LR73	5.32 ± 0.88	3.96 ± 2.27^{b}	1.34 ± 0.18	2.40 ± 1.16^{b}	1.42 ± 0.02	1.15 ± 0.04^{j}	30.1 ± 3.93	23.7 ± 2.28^{b}
LR731A	6.22 ± 4.20^{b}	3.25 ± 0.22^{b}	2.03 ± 0.10^{r}	1.67 ± 0.08^{k}	1.59 ± 0.16^{b}	0.98 ± 0.04^{l}	26.5 ± 1.31^{b}	16.4 ± 1.04^{j}
BG-1-P	0.44 ± 0.05	0.59 ± 0.05^{b}	0.86 ± 0.06	0.76 ± 0.05^{b}	6.69 ± 0.21	3.79 ± 0.33^{m}	0.59 ± 0.05	0.68 ± 0.05^{b}
BG-1/ADR	0.62 ± 0.11^{b}	0.55 ± 0.04^{b}	0.64 ± 0.08^{b}	0.77 ± 0.05^{b}	9.02 ± 0.01^{i}	5.12 ± 0.03^{i}	0.54 ± 0.05^{b}	0.60 ± 0.08^{b}
UWOV2	0.29 ± 0.06	0.26 ± 0.02^{b}	0.24 ± 0.04	0.35 ± 0.03^{b}	1.15 ± 0.10	0.99 ± 0.03^{b}	0.16 ± 0.01	0.19 ± 0.01^{b}

Values are means±S.E.M. (n=4). Superscripts represent Student's two-tailed p values for the differences between control and TM treated cells, and between parental and MDR cells as determined with the GraphPad INSTAT statistical program: $^{a}p=0.0002$; ^{b}not significant; $^{c}p=0.053$; $^{d}p=0.022$; $^{e}p=0.0032$; $^{f}p=0.0005$; ^{g}p0.0089; $^{h}p=0.0076$; $^{i}p<0.0001$; $^{j}p=0.0009$; $^{k}p=0.0307$; $^{l}p=0.010$; $^{m}p=0.0003$; $^{n}p=0.0318$; $^{o}p=0.0004$; $^{p}p=0.0018$; $^{q}p=0.0001$; $^{r}p=0.0154$.

untreated BG-1/ADR cells expressed an increased GT activity relative to their parental cells. In both BG-1-P and BG-1/ADR cells, the activities of ST, FT and NGT were unaffected by TM pretreatment, whereas GT was significantly reduced.

3.2.5. UWOV2 Human Ovarian Carcinoma Cells

In this cell line for which no corresponding drug-sensitive cell line exists, the activities of all the glycosyltransferases remained unchanged under the conditions of TM exposure.

4. Discussion

Numerous studies with tumour and other cell types have shown that the development of resistance to multiple chemotherapeutic drugs (MDR) correlates with amplified glycoprotein synthesis. In particular, the overexpression of a 170-kDa membrane glycoprotein (Pgp) has consistently been associated with MDR (Endicott and Ling, 1989; Schinkel and Borst, 1991). Although there is ample evidence to implicate Pgp as the causative molecule in MDR, other reports point out that it may not be the only membrane protein or glycoprotein that is increased in MDR (Gros and Shustik, 1991; Krishnamachary and Center, 1992; Scheper *et al.*, 1992). By contrast, reduced synthesis of specific surface proteins has also been observed in some MDR cells (Peterson *et al.*, 1983). This adds further to the diversity of the MDR phenotype.

The data indicate that glycoprotein synthesis in NIH-3T3-MDR and KB-8-5-11 cells was increased above that of their parental cells. On the other hand, the LR73-1A drug-resistant cell line expressed equivalent levels of glycoprotein synthesis relative to its drug-sensitive control. Tunicamycin inhibited the synthesis of proteins and glycoproteins in the various cell lines studied. When glycosyltransferases in drug-resistant cells were compared with those in drug-sensitive cells, no constant pattern of altered enzyme activity was observed. Fucosyltransferase (FT) activity in KB-8-5-11, NIH-3T3-MDR and LR73-1A cells was consistently higher than in the corresponding drug-sensitive cells. Increased activity of N-acetylglucosaminyltransferase (NGT) was measured in the KB-8-5-11 and NIH-3T3-MDR cell lines compared with the parental cell lines, whereas GT activity in NIH-3T3-MDR and BG-1/ADR cells exceeded that in their parental cells. Sialyltransferase (ST) acitivity remained constant in both the drug-sensitive and drug-resistant NIH-3T3 and the LR73 cell lines. In KB-8-5-11 cells, the

activities of ST and GT were reduced in relation to the parental cells. The BG-1/ADR cells did not express altered ST, FT or NGT activities. In addition, the results presented in Table 1 point to differential inhibition of glycosyltransferase function by TM in the various cell lines. The lack of inhibition of glycosyltransferases by tunicamycin observed in KB-8-5-11 cells supports the claim that interference of N-linked oligosaccharide synthesis may not affect the surface expression of certain integral membrane proteins (Burke *et al.*, 1984). This, together with the finding that TM reduced the activity of all the glycosyltransferases in both NIH-3T3-parental and NIH-3T3-MDR cells, is further in agreement with reports of differences between drug-sensitive and drug-resistant cells due to changes in glycosylation patterns of plasma membrane glycoproteins (Beck and Cirtain, 1982; Marsh and Center, 1987; Ichikawa *et al.*, 1991a).

Differential glycosylation of Pgp has been shown to result in heterogeneous forms of MDR-associated glycoproteins in different cell lines (Greenberger *et al.*, 1988b; Greenberger *et al.*, 1989; Meyers *et al.*, 1989). It is not clear whether post-translational processing of Pgp correlates with drug resistance (Center, 1983; Mukhopadhyay and Kuo, 1989). P-glycoprotein is expressed in VBL-resistant J7.V1-1 and a DNR-resistant J7.C1-100 cells as a 125-kDa nascent polypeptide that undergoes rapid (half-life, $t_{1/2}$ ≈20 min) N-glycosylation to a 135- or 140-kDa species (Greenberger *et al.*, 1987). The J7.T1-50 cell line, selected for resistance to taxol, synthesizes two Pgp precursors of molecular sizes 120- and 125 kDa, both products of distinct genes (Greenberger *et al.*, 1988a). In MDR KB cells (Richert *et al.*, 1988) a single 140-kDa protein is modified to a mature 170-kDa glycoform.

The function of the carbohydrate moieties of Pgp, which account for about 18-24% of its molecular mass, has not been adequately evaluated in MDR. Previous suggestions that glycosylation may not be an obligatory component of the MDR phenotype (Beck and Cirtain, 1982; Ling *et al.*, 1983) were not followed up by further research. Gervasoni *et al.* (1991) postulated that anthracycline resistance in HL-60 human promyelocytic leukaemia cells may be the result of hypoglycosylation of cell-surface glycoproteins. P-glycoprotein is, however, not overexpressed in these cells. It has also been shown to exist in an unglycosylated form in certain MDR cell lines (Germann *et al.*, 1990).

Structural analysis of the oligosaccharides produced by different cell lines in the absence or presence of TM was not the purpose of this study. Nevertheless, the results suggest that the degree of inhibition by TM of glycosyltransferases and of protein and glycoprotein synthesis in drug-sensitive and drug-resistant cells,

respectively, may depend on their specific complement of oligosaccharide structures. From these considerations it may be concluded that the effects of TM on glycoprotein synthesis in drug-sensitive and -resistant cells may be relevant in evaluating the role of Asn-linked glycosylation in multiple drug resistance.

5. Summary and Conclusions

Tunicamycin, an antibiotic which prevents N-glycosylation of proteins, has been evaluated for its effects on protein and glycoprotein synthesis in several drug-sensitive (parental) and drug-resistant cell lines. Pretreatment of cells with the antibiotic, inhibits protein and glycoprotein synthesis in all the cell lines that have been studied. In the multidrug-resistant NIH-3T3-MDR murine fibroblast and KB-8-5-11 human epidermoid carcinoma cell lines, glycoprotein synthesis was elevated compared with drug-sensitive (NIH-3T3-P and KB-3-1, respectively) controls, but no such elevation was found in drug-resistant Chinese hamster LR73-1A cells and their corresponding drug-sensitive LR73 cells.

Analysis of several glycosyltransferases in the various parental and MDR cell lines showed that activities of fucosyltransferase (FT) and N-acetylglucosaminyltransferase (NGT) are increased while the activities of sialyltransferase (ST) and galactosyltransferase (GT) are decreased in drug-resistant KB-8-5-11 cells compared with their parental controls. In NIH-3T3-MDR cells, the reactions catalyzed by FT, GT and NGT, but not by ST, occurred at significantly higher rates than those in the NIH-3T3-parental cells. The LR73-1A cells expressed raised levels of FT whereas the levels of ST, GT and NGT remained constant relative to drug-sensitive LR73 controls. Similar ST, FT and NGT reaction rates were observed in the BG-1/ADR human ovarian carcinoma cell line and its parental control (BG-1-P).

Tunicamycin pretreatment of cells produced fluctuations in reaction rates for the various glycosyltransferases in MDR and parental cells and no consistent trend of inhibition of enzyme activity was detected. However, the suppression by TM of FT-, GT- and NGT-catalyzed reactions was common to NIH-3T3-MDR and LR73-1A cells, although TM did not completely abolish the activities of the glycosyltransferases in these cells. Exposure of KB-8-5-11 cells and UWOV2 human ovarian carcinoma cells to tunicamycin did not alter any of the glycosyltransferases. These results demonstrate that glycoproteins are differentially expressed in various drug-sensitive and drug-resistant cell lines.

CHAPTER 3

THE EFFECTS OF TUNICAMYCIN ON THE *IN VITRO* CYTOTOXICITY OF VARIOUS ANTICANCER DRUGS IN DRUG-SENSITIVE AND DRUG-RESISTANT CELL LINES

1. INTRODUCTION

The tumour cell surface has long been regarded as a target for chemotherapeutic intervention (D'Incalci *et al.*, 1991; Daoud, 1992; Jandrig and Wunderlich, 1992). In particular, cell-surface glycoproteins are thought to mediate a number of normal and tumour-related processes such as tissue invasion and metastatic spread (Nicolson, 1984; el-Battari *et al.*, 1986), cell attachment, growth and differentiation (Iwakura, 1987; Engstrom and Larsson, 1989; Kabakoff *et al.*, 1990), and multidrug resistance (Peterson and Biedler, 1978; Beck *et al.*, 1979; Gervasoni *et al.*, 1991).

As with other glycoproteins, the multidrug resistance-associated P-glycoprotein is subject to post-translational modification, such as N-glycosylation (Greenberger *et al.*, 1989; Yoshimura *et al.*, 1989; Ichikawa *et al.*, 1991a). It is possible to interfere with different steps in the biosynthesis of N-linked glycoproteins by the use of specific inhibitors (Elbein, 1987; McDowell and Schwarz, 1988). The biological consequences of the alterations caused by glycosylation inhibitors are numerous, including enhanced susceptibility of proteins to proteases, improper protein processing and misfolding of polypeptide chains, loss of biological activity (McDowell and Schwarz, 1988) and sensitization of cells to cancerostatic protein toxins (Sandvig *et al.*, 1986). Thus, sensitizing agents may prove valuable in enhancing the actions of immunotoxins and anticancer agents. In this chapter we explored the effects of TM, an antibiotic which prevents protein N-glycosylation and synthesis of glycoproteins (Elbein, 1987), on the reponses of several drug-sensitive and drug-resistant cell lines to various cytotoxic drugs.

2. Materials and Methods

2.1. Drugs and Chemicals

Doxorubicin (Adriamycin) and epidoxorubicin (Farmitalia Carlo Erba, Milan, Italy), cisplatin (Lennon), vincristine sulphate, colchicine, methylene blue, sarkosyl [sodium N-lauroyl sarcosine] and MTT 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO, USA), trypsin 1:250 (Difco Laboratories, Detroit, MI, USA), PBS Dulbecco 'A' [phosphate-buffered saline] (Oxoid, UK), tissue culture media (Gibco , UK), tunicamycin (Boehringer Mannheim, Germany), EDTA [ethylene-diamine tetraacetic acid Na_2 salt] and DMSO [dimethylsulphoxide] (Merck Chemicals, Germany) were used in this study. All reagents were of analytical grade.

2.2. Cell Lines and Culture Conditions

The UWOV2 ovarian carcinoma cell line was provided by Professor W.R. Bezwoda (University of the Witwatersrand, South Africa). This cell line was derived from an ovarian cystadenocarcinoma in a patient who had not responded to combined chemotherapy with actinomycin D (ActD), vincristine, cisplatin and doxorubicin (Golombick *et al.,* 1990). The BG-1 ovarian carcinoma cell line (Geisinger *et al.,* 1989) was received from Dr C.A. Wallen (Bowman Gray School of Medicine, North Carolina, USA). The KB-3-1, a human epidermoid carcinoma cell line, and its MDR derivative, KB-8-5-11, which contains the amplified *mdr*1 gene (Richert *et al.,* 1985), were donated by Professor M.M. Gottesman (National Cancer Institute, Bethesda, MD, USA). The isolation and properties of the KB carcinoma cell lines have been described (Akiyama *et al.,* 1985; Fojo *et al.,* 1985). The NIH-3T3 murine fibroblasts and NIH-3T3-MDR cells, obtained by transfection of the NIH-3T3 cell line with the retroviral expression vector, pHaMDR1, containing a full-length complementary DNA from the human *mdr*1 gene, (Shen *et al.,* 1986a; Shen *et al.,* 1986b; Shen *et al.,* 1986c) were supplied by Professor M.M. Gottesman.

Cell lines were maintained and propagated under the following conditions: UWOV2, in RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum (HIFCS), penicillin G (100 U/ml) and streptomycin sulphate (100 μg/ml) or gentamicin sulphate (50 μg/ml); BG-1, in McCoy's 5A medium containing 10% HIFCS, 100 U/ml insulin, 200 mM L-glutamine and antibiotics; KB-3-1 and NIH-3T3-parental cells, in Dulbecco's modified Eagle's medium (DMEM) composed of

10% HIFCS and antibiotics as above; KB-8-5-11 and NIH-3T3-MDR cells, in DMEM as for parental cells with addition of 1 μg/ml colchicine. Incubator settings were calibrated to 37°C, 5% CO_2:air and 85% relative humidity. Cells were routinely subcultured with trypsin-EDTA (0.25%-0.02%, w/v) in Ca^{+2}- and Mg^{+2}-free PBS and maintained in the logarithmic phase of growth. Cell lines were periodically tested according to the method of Chen (1977) and found to be free of mycoplasma contamination.

2.3. Selection of BG-1/ADR Cells

The BG-1/ADR cells were selected by a modification of the procedure described by Jamali *et al.* (1989). BG-1-P cells were grown in increasing concentrations of doxorubicin over a period of 6 months until resistance to 0.2 μg/ml was achieved. The cells were subsequently cultured in growth medium containing doxorubicin and reselected every 2-3 months in order to achieve maximum resistance. The resultant BG-1/ADR cells expressed a 59-fold increase in resistance to doxorubicin compared with the parental line. The cells also showed relative cross-resistance to colchicine and vincristine (1.4- and 10-fold, respectively). However, efforts in our laboratory to phenotype test BG-1/ADR cell line and to further characterize it with regard to Pgp expression and its lack of cross-resistance to colchicine were met with several obstacles, principal of which were the failure to propagate the cells to critical density from cryopreserved stocks and inability of the cells to attach to the culture substratum due to cytostasis.

2.4. Pretreatment of Cells with Tunicamycin

Tunicamycin stock solutions were prepared by dissolving the contents of a 10-mg vial in 25 mM NaOH and then diluting to 0.8 mg/ml TM and 10 mM NaOH with pyrogen-free distilled-deionized water. The solution was sterilized by passing through a 0.22-μm disposable filter (Millipore, Millex-GV) and was stored at -20°C for a maximum period of 3 weeks. Immediately before each experiment, the TM solution was diluted to a final concentration of 5 μg/ml in culture medium. This concentration was chosen because it had little effect on cell viability, which remained greater than 95% between 4h and 72h exposure, as assessed by the trypan blue dye exclusion method (Shier, 1985; Shier, 1988). Cell viability in the presence of TM at this concentration was also found by more rigorous cytotoxicity assays (Figure 1) to exceed 90% for all the cell types studied. Tunicamycin was tested for its ability to enhance the cytotoxicity of doxorubicin (DOX), epidoxorubicin (EPX), vincristine (VCR), colchicine (COL) and cisplatin (CPL).

Cells were pretreated with TM for 16h prior to their exposure to the different drugs. To control cultures were added an equal volume of 10 mM NaOH (vehicle), the final concentration (62.5 μM) of which was confirmed not to affect the pH of the culture medium.

2.5. *In Vitro* Cytotoxicity Assays

To determine the effect of TM on the cytotoxicity of DOX, EPX, COL, VCR and CPL, preconfluent cells from stock cultures were detached with trypsin-EDTA (0.25%-0.02%, w/v) in PBS, washed twice with PBS and resuspended in complete culture medium to obtain single-cell suspensions. Cells were counted in an electronic particle counter (Coulter, Hialeah, FL, USA). Standardization of cell numbers in individual wells of a 96-well microtitre plate was confirmed by a linear correlation ($r=0.97$) between cell number and absorbance up to a maximum density of 3.5-4.5 x 10^4 cells/well (Twentyman, 1988). Cells were seeded at a density of 3 x 10^3 cells/well in a total volume of 200 μl as follows: After trypsinization, cells were rinsed twice with PBS, resuspended in 10 ml complete culture medium and repeatedly pipetted to ensure a homogeneous mixture during dispensing of 100-μl aliquot/well. The cells were then allowed to attach and grow for 48-72h.

Cytotoxic drugs were dissolved in PBS and sterilized through 0.22-μm disposable filters. The drugs were diluted in culture medium free of phenol red (Modified Eagle's medium- or RPMI-1640-selectamine kit) to avoid interference with spectrophotometric assays (Denizot and Lang, 1986). After the addition of drugs at various concentrations to octuplicate wells, cells were incubated in the presence or absence of TM for a further 72h, unless stated otherwise in the text. The cytotoxicity of drug in the TM-treated and TM-free cultures was determined by spectrophotometry. For the methylene blue (MeBlue) microassay, the cells were fixed and stained with 0.5% MeBlue in 50% ethanol for 30 min at room temperature. Excess stain was removed by blotting inverted plates onto adsorbent paper and immersing them in distilled water (3-4 sequential washes). Plates were blotted as described above and air-dried. Stained cells were solubilized overnight in 1% sarkosyl in PBS containing 0.001% sodium azide, 100 μl/well.

Absorbances were read by a microplate reader (Titertek Multiskan model MCC/340) at a sample wavelength of 620 nm and a reference wavelength of 405 nm (Finlay *et al.*, 1984). For the MTT (3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) assay (Mosman, 1983), 20 μl of MTT (5 mg/ml in

sterile PBS) were added to each well and the plates were incubated for 5h at 37°C. Plates were then centrifuged at 400 x g for 5 min to pellet any floating cell aggregates. The supernatant was aspirated and the formazan crystals dissolved in DMSO (200 μl/well). The plates were then gently agitated for 10 min and the absorbance read at a sample wavelength of 540 nm and a reference wavelength of 630 nm on a microplate reader as described above for the MeBlue assay.

For both assays the IC_{50} of the dose-response curve was defined as the drug concentration required to reduce the final absorbance to 50% of the control value. Dose-response curves were corrected for the decrease in absorbance caused by TM *per se*, which was always less than 10% of the control value. The potency ratio for each drug in the presence of TM was determined from the following relationship (Twentyman *et al.*, 1990):

$$\text{Potency Ratio} = \frac{IC_{50} \text{ of drug in absence of TM}}{IC_{50} \text{ of drug in presence of TM}}$$

2.6. Data Analysis

Dose-response curves were evaluated by the method of Litchfield and Wilcoxon (1948) using a computer-assisted program (PHARM/PCS) to determine IC_{50} concentrations (Tallarida and Murray, 1987). The values are representative of the means $\pm$ S.E.M. of 3 experiments (n=8 for each experiment). Statistical analysis was performed on the variables in this study using the Student's two-tailed t-test. The level of significance was set at $p \leq 0.05$. The significance of the potency ratio was obtained by computing either the 95% or 99% confidence interval using Fieller's ratio-of-means test (Fieller, 1944), as simplified by Bliss (1956) or by estimating confidence limits of the potency ratios using the method of Litchfield and Wilcoxon (1948), using the PHARM/PCS computer program as described by Tallarida and Murray (1987).

3. RESULTS

The effects of TM on the viability of various drug-sensitive and drug-resistant cell lines following 72h continuous exposure to the antibiotic were compared (Figure 1). In all the cell types, TM did not affect cell viability in the concentration range 0.001-10 μg/ml and survival was consistently greater than 90% or similar to control (*i.e.*, cells not treated with TM). Higher concentrations of the antibiotic (30-100 μg/ml) were necessary to achieve greater than 20% reduction in cell survival as measured by the MTT cytotoxicity assay. This decrease in cell survival was clearly concentration dependent. The consequence of TM pretreatment of cells for 16-72h was not toxic and completely reversible with time after its removal from the culture medium. Similar results were obtained with the MeBlue cytotoxicity assay (not shown). Accordingly, a TM concentration of 5 μg/ml and conditions under which cell viability remained unaffected were chosen to test the outcome of antibiotic treatment on the sensitivity of drug-sensitive and drug-resistant cells to various anticancer drugs.

The effect of TM at 24h, 48h and 72h, respectively, on the dose-response relation for DOX in NIH-3T3-MDR cells is shown in Figure 2. The survival of these cells at any particular concentration of DOX was significantly decreased in the presence of TM, except at DOX concentrations of 5 μg/ml at 48h and 72h exposure (in the latter there is an inversion of this effect at doses of DOX in excess of 5 μg/ml). An enhancement of DOX cytotoxicity by TM was also observed for BG-1/ADR cells after 24h, 48h and 72h (Figure 3). The sensitivity of BG-1/ADR cells to VCR was unaffected by TM after 24h, but was increased consistently after 48h and 72h with doses greater than 0.05 μg/ml. Vincristine alone had little effect on the survival of BG-1/ADR cells as judged by the survival curves for 24h, 48h and 72h. The effects of TM were transient since the cells consistently regained their original state of resistance to DOX and VCR within 24h of removal of TM from the culture medium (data not shown).

For both NIH-3T3-MDR and BG-1/ADR cells, TM markedly lowered the IC_{50} for DOX at each of the indicated times when determined by the MTT assay (Table 1). When assessed by the MeBlue cytotoxicity assay, a similar reduction in IC_{50} was observed only after 24h and 48h in the case of BG-1/ADR but not for NIH-3T3-MDR after 48h and 72h (Table 1). This discrepancy between the MTT and MeBlue assays can probably be ascribed to differences in slopes of the dose-response curves. This was not investigated further.

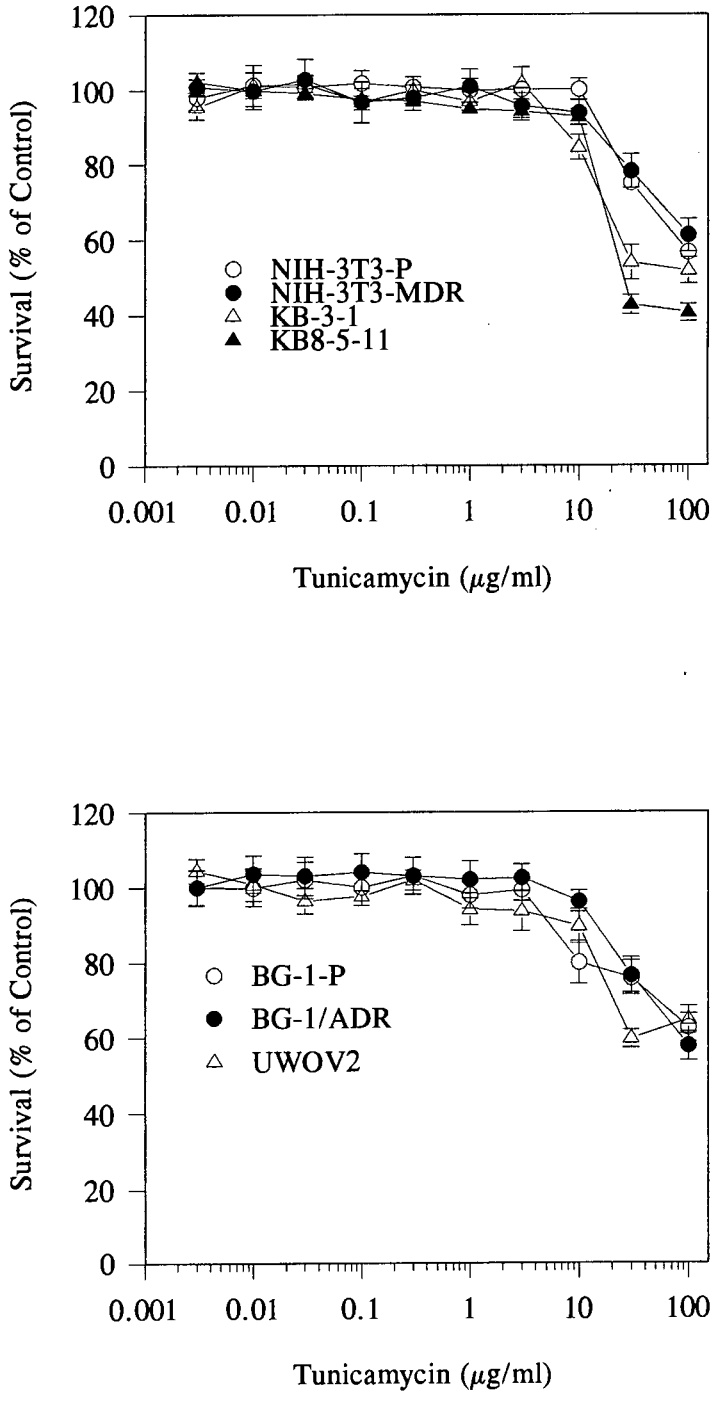

Figure 1. The effects of tunicamycin on the viability of various cell lines following 72h continuous exposure to the antibiotic. Cells were treated with increasing concentrations of tunicamycin and viability measured by the MTT cytotoxicity assay as described in "Materials and Methods". Values are means ± S.E.M. of octuplicate cultures.

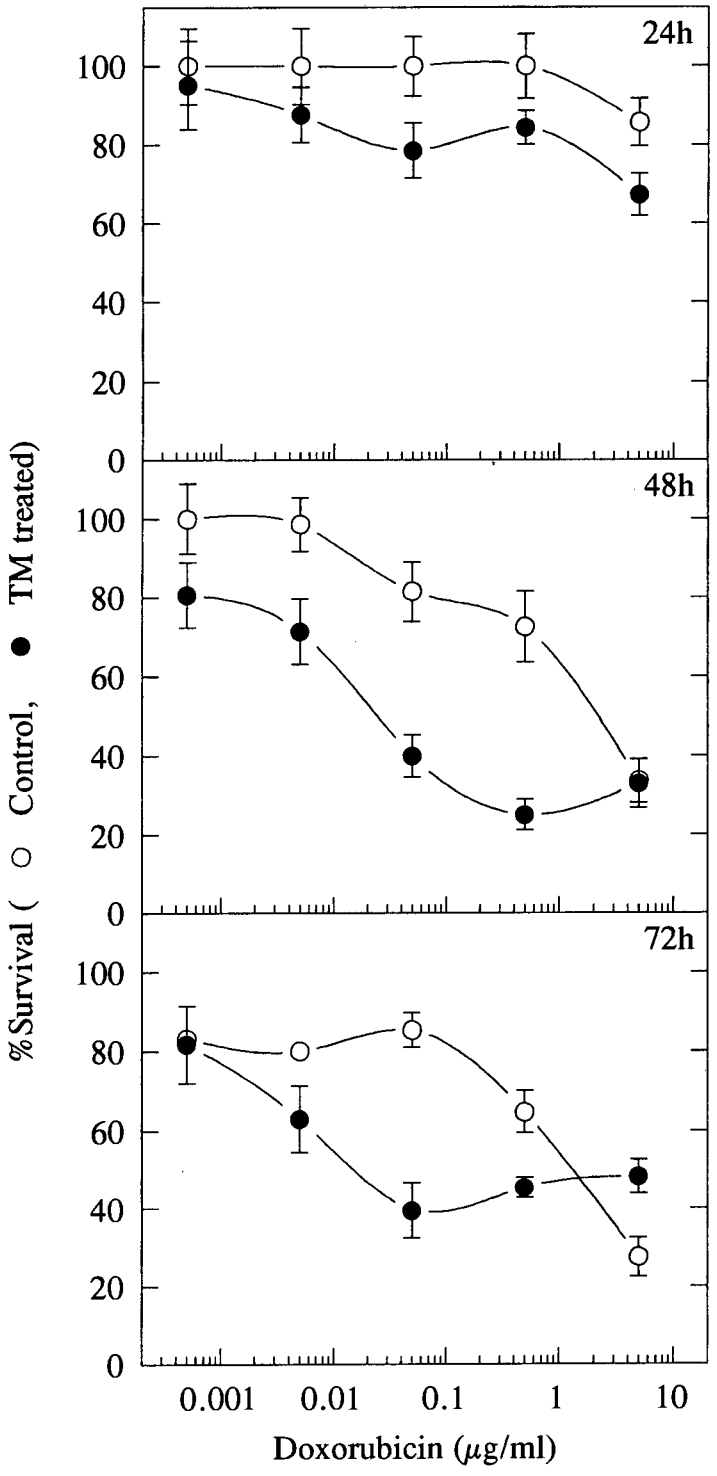

Figure 2. The effect of TM on the dose-response relation for DOX in NIH-3T3-MDR cells. Cells were exposed for the indicated times to DOX alone or to DOX in the presence of TM after preincubation with the antibiotic. Cell viability was determined by the MTT cytotoxicity assay. Values are means ± S.E.M. for 3 experiments (n=8 for each experiment).

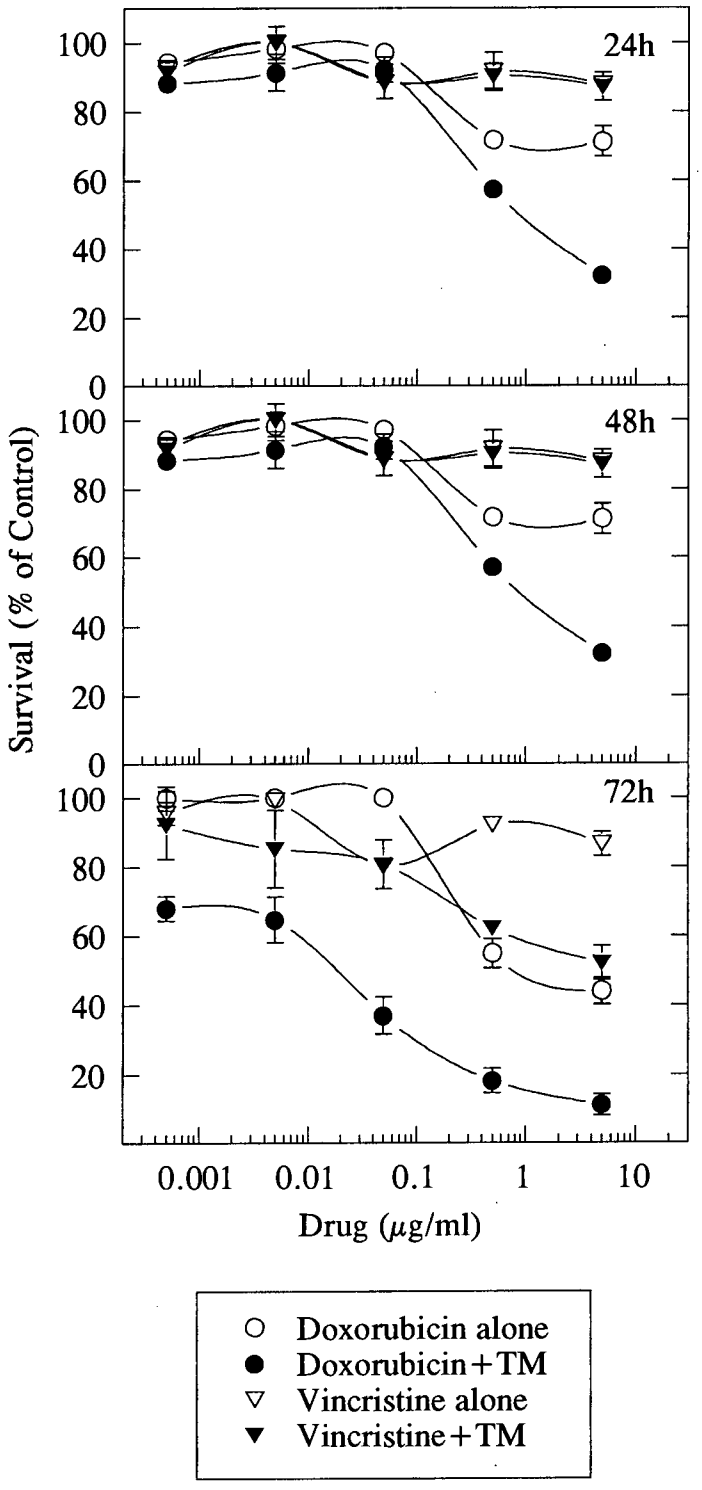

Figure 3. The effect of TM on drug cytotoxicity in BG-1/ADR ovarian carcinoma cells. Cells were exposed for the indicated times to drug alone or to drug in the presence of TM after preincubation with the antibiotic. Cell viability was determined by the MTT cytotoxicity assay. Values are means ± S.E.M. for 3 experiments (n=8 for each experiment).

Table 1. Comparison of the MTT and MeBlue Cytotoxicity Assays for Evaluating the Effects of TM on the Sensitivity of BG-1/ADR and NIH-3T3-MDR Cells to DOX *In Vitro*

		Mean IC_{50} ± S.E.M.(-TM)		Mean IC_{50} ± S.E.M.(+TM)	
Time(h)	Cell Line	MTT	MeBlue	MTT	MeBlue
24	BG-1/ADR	16.0±3.20	11.4±1.50	4.80±1.44[c]	0.70±0.142[a]
	NIH-3T3-MDR	2.72±0.56	ND	0.25±0.06[a]	ND
48	BG-1/ADR	0.98±0.26	6.15±1.02	0.03±0.01[b]	0.52±0.22[a]
	NIH-3T3-MDR	2.51±0.60	1.67±0.80	0.20±0.003[b]	0.03±0.015[d]
72	BG-1/ADR	0.59±0.10	2.07±1.03	0.04±0.005[a]	0.01±0.006[d]
	NIH-3T3-MDR	1.62±0.05	1.05±0.49	0.20±0.09[a]	0.02±0.01[d]

Mean IC_{50} values in μg/ml; S.E.M. standard error of the mean for 3 experiments (n=8 for each experiment); _ND not determined; _[a-c]significantly different from the value obtained for cells treated with DOX alone: _[a]$p<0.001$; _[b]$p<0.005$; _[c]$p<0.01$; _[d]not significantly different from control (DOX alone).

Survival curves for UWOV2 cells treated with different drugs in the presence or absence of TM are presented in Figure 4. Tunicamycin potentiated the cytotoxicity of DOX, VCR, COL, CPL, and EPX. Pretreatment of cells with TM effectively decreased the IC_{50} for DOX, VCR, COL and CPL, but not for EPX (Figure 5).

Table 2 summarizes data on the time dependence of the effects of TM on the sensitivity of NIH-3T3-P and NIH-3T3-MDR cells to COL. Tunicamycin did not cause any change in the cytotoxicity of COL in both cell lines after 24 and 48h, but significantly augmented the cytotoxicity of the drug in NIH-3T3-MDR cells after 72h. The IC_{50} values for DOX separately or in combination with TM for each of the cell lines studied are presented in Table 3. Tunicamycin lowered the IC_{50} for DOX in NIH-3T3-parental and -MDR cells at 24h, 48h and 72h. In the presence of TM, the response of NIH-3T3-MDR cells to DOX after 48h and 72h approximated closely that of its parental line treated with the drug alone. This was also noted in the BG-1/ADR cells after 48h and 72h. In contrast, the KB-8-5-11 cells retained a DOX-sensitivity pattern that was significantly less than its parental line.

The potency ratios for DOX were consistently higher in drug-resistant cells than in the corresponding parental cells (Table 4). This could not be tested in the case of UWOV2 cells for which no equivalent parental cell line exists. The effects of TM on the sensitivity of the same cell lines to other anticancer drugs are presented in Table 5. Sensitization of NIH-3T3-MDR cells to COL, VCR, EPX and CPL by TM was substantially greater compared with their parental cells. Tunicamycin failed to increase the sensitivity of NIH-3T3-parental cells to these drugs. Similarly, responsiveness was greater to COL and VCR in BG-1/ADR relative to its parental line, and to COL in KB-8-5-11 cells as opposed to their parental cells. Tunicamycin did not enhance the sensitivity of KB-3-1 cells to COL. The enhancing effect of TM on the toxicity of CPL, a drug that is thought not to belong to the same pharmacological class of MDR-related drugs (see section 3.4, Chapter1), in UWOV2 and NIH-3T3-MDR cells implies a mechanism which does not at all involve Pgp. This was not resolved in this study.

4. Discussion

Chemosensitizers may represent an important advance in overcoming MDR in cancer chemotherapy (Georges *et al.*, 1990b; Pearce *et al.*, 1990; Hait and Aftab, 1992). However, the application of such agents in clinical medicine is limited by their lack of specificity, their potential side effects and insufficient understanding of

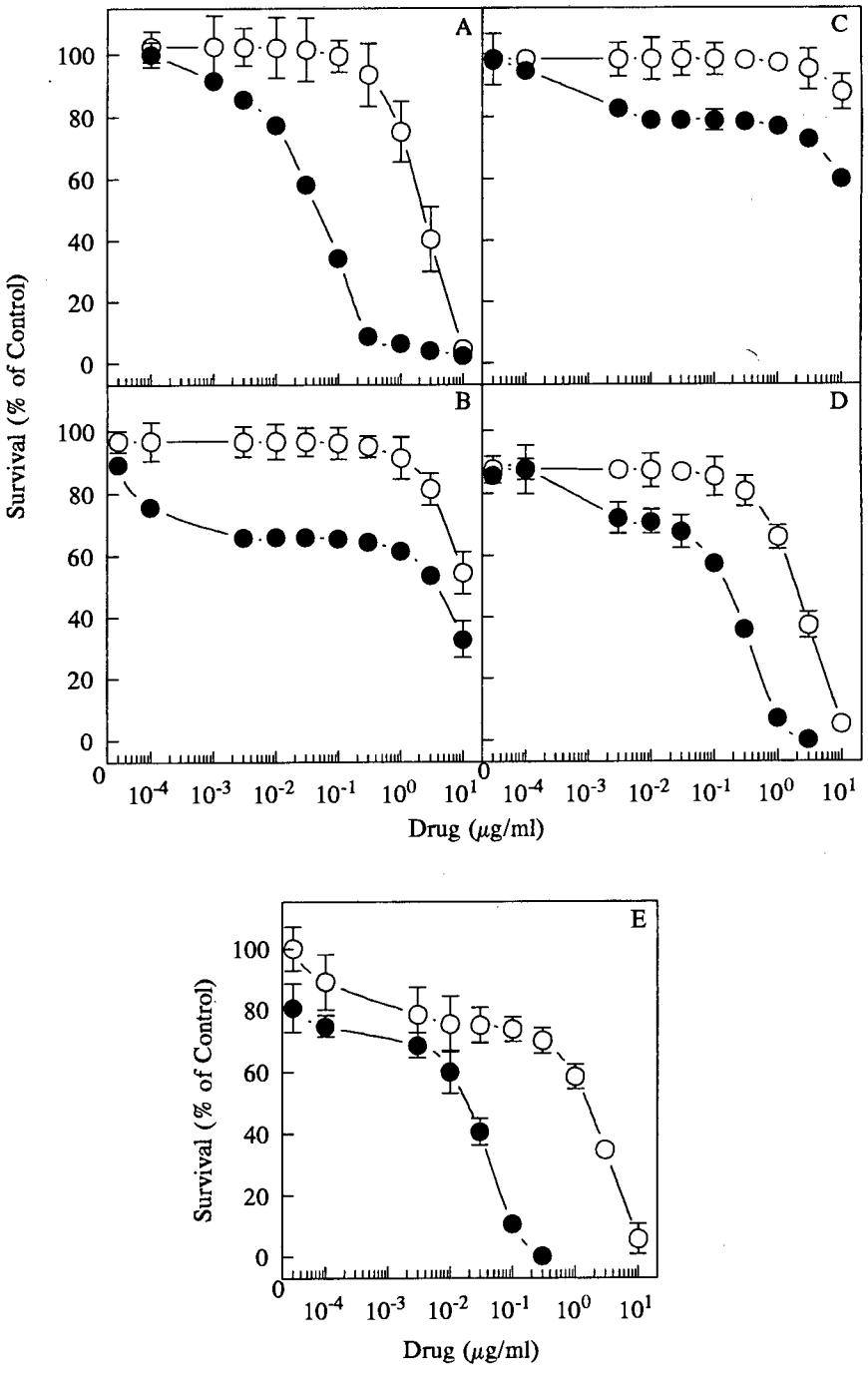

Figure 4. The effects of TM on drug cytotoxicity in UWOV2 ovarian carcinoma cells. Cells were exposed to drug alone (open circles) or to drug in the presence of TM (solid circles). A, DOX; B, VCR; C, COL; D, CPL; E, EPX. Cell viability was determined by the MTT cytotoxicity assay. Values are means ± S.E.M. for 3 experiments (n=8 for each experiment).

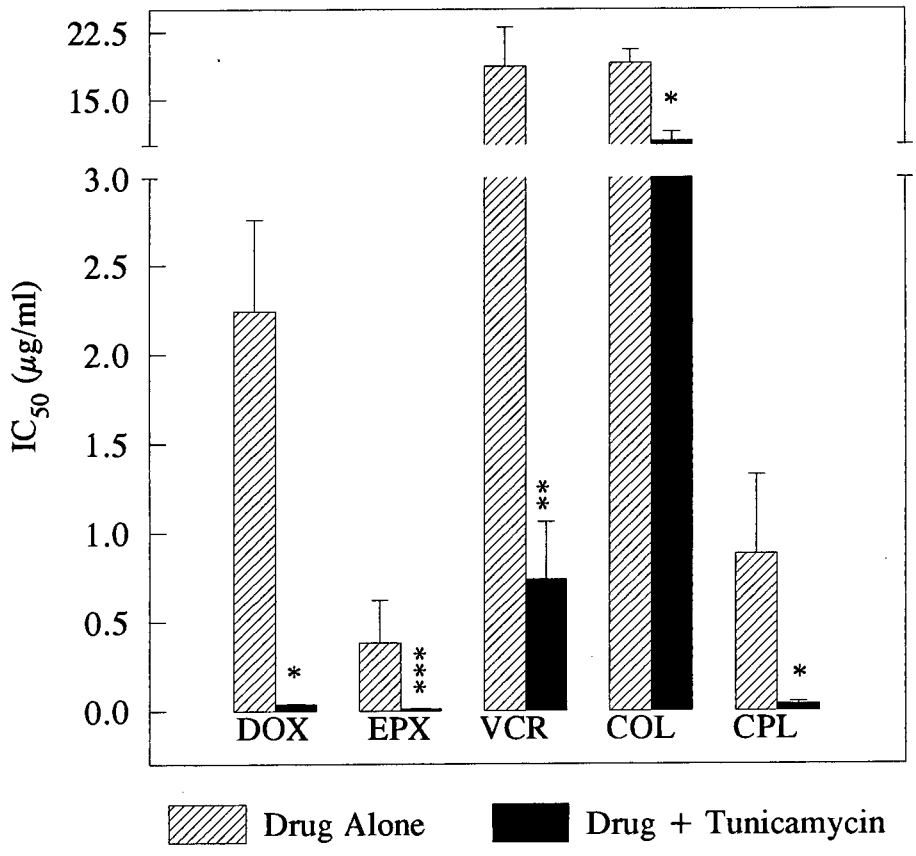

Figure 5. The effects of TM pretreatment on the IC_{50} of various drugs in UWOV2 ovarian carcinoma cells after 72h of exposure to the drug alone or in combination with the antibiotic. DOX, doxorubicin; EPX, epidoxorubicin; VCR, vincristine; COL, colchicine; CPL, cisplatin. Data represent the means ± S.E.M. for 3 experiments (n=8 for each experiment). *$p < 0.001$; **$p < 0.005$; ***not significant.

Table 2. Time-Dependence of the Effects of TM on the Sensitivity of NIH-3T3-Parental Cells and NIH-3T3-MDR Cells to COL Measured by the MTT Cytotoxicity Assay

		Mean IC_{50}[1] (95% Confidence Interval[2])		
Time (h)	Cell Line	Control	TM-treated	Potency Ratio (95% Confidence Interval)
24	Parental	11.00 (7.06 to 17.16)	3.05 (0.46 to 20.33)	3.60 (0.51 to 25.26)[a]
	MDR	32.12 (2.51 to 410.33)	6.22 (0.21 to 183.03)	5.16 (0.08 to 356)[a]
48	Parental	10.07 (1.49 to 68.07)	1.20 (0.16 to 9.03)	8.37 (0.52 to 134.6)[a]
	MDR	26.53 (1.21 to 583.56)	1.85 (0.12 to 29.67)	14.35 (1.23 to 914)[a]
72	Parental	3.39 (2.14 to 5.37)	1.20 (0.09 to 16.38)	2.82 (0.19 to 40.1)[a]
	MDR	31.81 (16.16 to 62.63)	0.51 (0.05 to 4.83)	62.4 (5.96 to 652)[b]

[1]Mean IC_{50} values in μg/ml; [2]95% CI values are presented in accordance with the statistical guidelines of the Br. Med. J.(Altman *et al.*, 1990); [a]not significant; [b]$p < 0.05$.

Table 3. The Effects of Tunicamycin on the Sensitivity* of Various Parental and Drug-Resistant Cell Lines to DOX

	Mean IC_{50} ± S.E.M.(μg/ml)					
	24h		48h		72h	
Cell line	Control	TM-treated	Control	TM-treated	Control	TM-treated
NIH-3T3-P	1.20±0.30	0.33±0.09[d]	0.31±0.05	0.13±0.01[b]	0.33±0.05	0.12±0.02[b]
NIH-3T3-MDR	2.72±0.56	0.25±0.06[a]	2.51±0.60	0.20±0.003[b]	1.63±0.05	0.20±0.09[a]
KB-3-1	ND	ND	ND	ND	0.12±0.01	0.29±0.02[f]
KB-8-5-11	ND	ND	ND	ND	5.58±0.22	0.92±0.05[a]
BG-1-P	0.99±0.16	0.42±0.17[e]	0.22±0.02	0.04±0.005[d]	0.01±0.003	0.02±0.004[f]
BG-1/ADR	16.0±3.20	4.76±1.44[c]	0.98±0.26	0.03±0.01[b]	0.59±0.10	0.04±0.005[a]
UWOV2	ND	ND	ND	ND	2.24±0.52	0.03±0.004[a]

P, parental; MDR, multidrug-resistant; S.E.M., standard error of the mean for 3 experiments (n=8 for each experiment; ND, not determined; [a-e]significantly different from control: [a]$p<0.001$; [b]$p<0.005$; [c]$p<0.01$; [d]$p<0.02$; [e]$p<0.05$; [f]not significant. *assessed by the MTT assay.

Table 4. Tunicamycin-Induced Potentiation of Doxorubicin Cytotoxicity* in Parental and Drug-Resistant Cells

	Potency Ratio (± Confidence Interval)		
Cell Line	24h	48h	72h
NIH-3T3-P	3.63±2.51[a,f]	2.42±2.67[b,d]	2.78±4.03[b,d]
NIH-3T3-MDR	10.96±2.39[a,c]	12.9±2.05[a,d]	8.08±13.8[b,c]
KB-3-1	ND	ND	0.42±0.13[b,h]
KB-8-5-11	ND	ND	6.08±2.72[b,c]
BG-1-P	2.33±6.33[b,g]	6.13±9.57[a,f]	0.50±0.49[b,h]
BG-1/ADR	3.36±4.89[a,e]	32.7±1.78[a,d]	16.4±2.95[a,c]
UWOV2	ND	ND	67.9±3.83[b,c]

Potency ratios were calculated from the data presented in Table 3._*assessed by the MTT assay; _ND, not determined; _[a]99% CI; _[b]95% CI; _[c]$p<0.001$; _[d]$p<0.005$; _[e]$p<0.01$; _[f]$p<0.02$; _[g]$p<0.05$; _[h]not significant.

Table 5. Tunicamycin-Induced Potentiation of Anticancer Drug Cytotoxicity* in Various Parental and Drug-Resistant Cells *In Vitro*

	Potency Ratio (95% Confidence Interval)			
Cell Line	Colchicine	Vincristine	Epidoxorubicin	Cisplatin
NIH-3T3-P	2.82 (0.19 to 40.1)[d]	1.48 (1.43 to 5.07)[d]	0.81 (0.16 to 4.20)[d]	1.07 (0.35 to 3.25)[d]
NIH-3T3-MDR	62.4 (5.96 to 652)[c]	95.2 (17.04 to 532)[c]	10.0 (2.80 to 36.0)[c]	107 (10.5 to 1088)[c]
KB-3-1	0.44 (0.36 to 0.52)[d]	ND	ND	ND
KB-8-5-11	366 (358 to 374)[a]	ND	ND	ND
BG-1-P	0.96 (0.63 to 1.29)[d]	16.4 (12.19 to 20.61)[a]	ND	ND
BG-1/ADR	149 (145.4 to 152.6)[a]	541 (537 to 545)[a]	ND	ND
UWOV2	1.82 (1.24 to 2.40)[a]	25.0 (22.7 to 27.3)[c]	41.1 (40.05 to 42.15)[d]	25.2 (23.9 to 26.5)[a]

*Assessed by the MTT assay; ND, not determined; [a-c]significantly different from cells treated with the drug alone: [a]$p < 0.001$; [b]$p < 0.01$; [c]$p < 0.05$; [d]not significant.

their mechanisms of action and metabolic effects. The MDR phenotype characteristically manifests itself by overexpression of Pgp in a manner analogous to the expression of aberrant glycoproteins or glycolipids in transformed cells (Hakomori, 1985; Dennis and Laferte, 1987; Holzmann *et al.*, 1988). Biochemical characterization of the role of N-glycosylation of Pgp should be useful in delineating the resistance mechanisms of MDR cell lines.

The effects of TM have been studied in various biological systems, primarily with the aim of elucidating the role of the carbohydrate moieties in the cellular function of glycoproteins. It has been suggested that TM may be selectively more toxic to transformed cells than to non-transformed cells, and thus therapeutically useful as an antitumour agent effective against certain malignant cells while sparing normal cells (Olden *et al.*, 1979b). In agreement with this view, Eren and Duksin (1985) have shown that a purified homologue of TM (B2-tunicamycin) caused greater inhibition of sugar incorporation into glycoproteins in transformed cells than in wild-type cells. However, Brysk *et al.* (1986) observed a contrary pattern with two mouse epidermal cell lines; the malignant cells were more resistant to TM than their non-malignant counterparts, as measured by growth and viability. Such enhanced cytotoxicity may be due to differences in penetration of TM into cells, inhibition by TM of the synthesis of glycoproteins, of their insertion into the plasma membrane or their secretion, or of metabolic processes which may be essential for the altered phenotype (Duksin and Bornstein, 1977; Eren and Duksin 1985).

Recently, Engstrom and Larsson (1988) found that the TM-induced inhibitory effect on *in vitro* cell proliferation of transformed cell lines was cell cycle-specific since only cells in G1 phase were blocked in their cell-cycle progression. Moreover, they found that TM suppressed the activity of 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA), which constitutes the rate-limiting step in the biosynthesis of cholesterol and isoprenoid derivatives, by catalysing the reduction of HMG-CoA to mevalonate, and it has been suggested that it plays a role in the control of cell proliferation and in tumour transformation (Larsson and Engstrom, 1989). This raises the possibility that TM exerts its inhibitory effects on cell proliferation via the isoprene-synthetic pathway along with its effects on asparginé-linked glycosylation (Kabakoff *et al.*, 1990; Langan and Slater, 1991). These pathways may have clinical ramifications for the expression of drug resistance which can occur during tumour development. We did not correlate the enhancing effects of TM on the cytotoxicity of various drugs with

cell-cycle changes, but applied the criterion most often used in cytotoxicity assays, *viz.*, the investigation of cells in exponential phase of the growth cycle.

The results of this study indicate that TM augments the susceptibility to various anticancer agents in drug-resistant cell lines to a greater extent than in their respective parental cells. The extent of the potentiation of anticancer drug action by TM differed in the various cell lines that have been studied. We did not explore the nature of this differential sensitization, but the consistent pattern of increased drug cytotoxicity that was observed in all drug-resistant cell lines suggests a common mechanism of reversal of drug resistance. The differences between the cell lines can perhaps be attributed to variation in cross-resistance to the anticancer drugs which, in turn, is determined by dissimilarities in genetic regulation and biochemical mechanisms involved in the protection of cells against cytotoxic agents (Felsted *et al.,* 1985; Capranico *et al.,* 1989; Lehrman and Zeng, 1989). It is interesting also that resistance to TM in Chinese ovary cells is associated with gene amplification (Waldman *et al.*, 1987; Scocca *et al.*, 1988; Zhu *et al.*, 1992) comparable to that occurring MDR cells (Chao *et al.*, 1991; Roninson, 1992a; Stahl *et al.*, 1992), which may be considered in studies related to TM.

An important concern was whether any cumulative cytotoxicity of TM might have influenced the results described in this chapter. However, cytotoxicity solely attributable to TM was taken into account, and the dose-reponse curves were corrected accordingly. Tunicamycin at a concentration of 5 μg/ml did not significantly affect cell viability in the various cell lines. Protein and glycoprotein synthesis are perturbed to varying degrees by TM in different cell lines (Irimura *et al.,* 1981; Elbein, 1987; Barathan *et al.,* 1990). We found that TM, at a concentration of 5μg/ml, simultaneously impaired glycoprotein and protein synthesis in the cell lines studied, as assessed respectively by [^{3}H]glucosamine or [^{3}H]mannose and [^{35}S]methionine or [^{14}C]leucine incorporation (data not presented, see Chapter 2 for results).

We did not compare the TM-induced increase in susceptibility of MDR cells to cytotoxic drugs with the effect of TM on the precise glycosylation of Pgp. We have assumed that the TM effect is based on the known action of the antibiotic on Pgp (Greenberger *et al.,* 1987; Richert *et al.*, 1988; Yoshimura *et al.*, 1989). Tunicamycin has been shown to inhibit N-glycosylation of Pgp in drug-resistant cells, but this inhibition did not abolish resistance in these cells (Beck and Cirtain, 1982). Similarly, a glycosylation mutant of drug-resistant CH^{R}C5 cells has been reported to retain its drug-resistance attributes (Ling *et al.,* 1983), suggesting that

the oligosaccharide chains of Pgp are probably not involved in the expression of drug resistance. These findings do not necessarily contradict our own results since we found that the effect of TM is reversible. Such discrepancies may also be explained by differences in the methods used. The fact that not all glycoproteins become unstable or non-functional in the unglycosylated form (Elbein, 1984; Elbein, 1987) suggests that Pgp may be synthesized at normal rates, although deficient in carbohydrate, but still sustain its biological activity in MDR (Chou and Kessel, 1981). Differential glycosylation of the *mdr*1 gene product (Greenberger *et al.*, 1989) and the biosynthesis of heterogeneous forms of MDR-associated glycoproteins in various cell lines (Greenberger *et al.*, 1988a; Greenberger *et al.*, 1988b; Meyers *et al.*, 1989) indicate that complex genetic regulation may determine cell sensitivity to cytotoxic drugs.

The principal finding reported in this chapter is that TM augments the cytotoxicity of different anticancer drugs in MDR cells. This enhancement of cytotoxicity may be due to a mechanism which probably involves impairment of (glyco)protein function. Tunicamycin treatment of NIH-3T3-MDR cells and UWOV2 cells resulted in a significant increase in the sensitivity of these cells to CPL. It is commonly held that CPL does not belong to drugs in the MDR class (*e.g.*, VBL, DOX, COL, VCR and ActD) and that the mechanism of resistance to CPL differs from that of MDR (Futscher *et al.*, 1992; Kimiya *et al.*, 1992; Misawa *et al.*, 1992). However, recent observations by Yang *et al.* (1993) that Pgp-mediated MDR and CPL resistance phenotypes may coexist in cells with primary resistance to CPL complicate the issue. Therefore, an understanding of the precise relationship between the potentiating effect of TM on CPL toxicity must await clarification of the action TM on Pgp-mediated drug resistance. Our findings do not directly demonstrate that the modulating effects of TM on drug cytotoxicity are mediated by inhibition of Pgp, but they do establish that TM is a biological response modifier in MDR. Further research based on the knowledge that has been acquired in this study should allow the development of strategies to overcome MDR.

5. Summary and Conclusions

The responses of the drug-sensitive (BG-1-P and KB-3-1) and drug-resistant (UWOV2, BG-1/ADR, KB-8-5-11) human tumour cell lines, and of the murine fibroblast parental (NIH-3T3-parental) cell line and its drug-resistant transfectant (NIH-3T3-MDR) to various anticancer drugs in the presence or absence of tunicamycin (TM) have been investigated. Tunicamycin alone at a concentration of

5 μg/ml did not exert any toxic effects on the different cell lines as measured by the MTT viability assay. However, pretreatment of cells with 5 μg/ml TM for 16h and its combination with doxorubicin, epidoxorubicin, colchicine, vincristine and cisplatin significantly increased the cytotoxicity of these drugs in a time-dependent manner in drug-resistant cells to a greater extent than in the respective drug-sensitive (parental) cells. This effect of TM on the cytotoxicity of the various agents studied suggests a common mechanism of reversal of drug resistance by this agent. In the NIH-3T3-MDR, KB-8-5-11, and BG-1/ADR cell lines drug sensitivity patterns developed in the presence of TM that corresponded to those in the parental cell lines. The effect of TM was reversible upon its removal from the culture media as the cells regained their original degree of drug resistance within 24h. Tunicamycin inhibited both protein and glycoprotein synthesis in the cell lines studied. The effect of the antibiotic on drug-resistant cells, in general, may be explained by this action.

CHAPTER 4

THE EFFECTS OF TUNICAMYCIN ON THE UPTAKE, EFFLUX AND RETENTION OF VINCRISTINE IN VARIOUS MULTIDRUG-RESISTANT CELL LINES

1. INTRODUCTION

Human tumour cell lines that have developed pleiotropic drug resistance often express increased levels of P-glycoprotein, a product of the *mdr*1 gene, compared with their drug-sensitive or related normal cells (Gerlach *et al.*, 1986b; Endicott and Ling, 1989; Schinkel and Borst, 1991). P-glycoprotein functions as an energy (ATP)-dependent drug efflux pump (Gerlach *et al.*, 1986a; Hamada and Tsuruo, 1988; Sarkadi *et al.*, 1992) which protects cells against a broad spectrum of cytotoxic agents. The extent of penetration into and accumulation and retention within tumour cells of anticancer drugs is an important determinant of their cytotoxicity (Jamali *et al.*, 1989; Nooter *et al.*, 1990; Nakagawa *et al.*, 1992), although this may not always be the case (Kato *et al.*, 1991). Multidrug-resistant (MDR) cells accumulate less anticancer drug than their parental counterparts (Fojo *et al.*, 1985; Lemontt *et al.*, 1988; Keizer *et al.*, 1989), presumably as a result of active efflux mediated by P-glycoprotein (Inaba *et al.*, 1987; Spoelstra *et al.*, 1991; Dordal *et al.*, 1992).

Major advances have been made in understanding the role of the plasma membrane in multidrug resistance, drug transport, development and disease (D'Incalci *et al.*, 1991; Daoud, 1992; Jandrig and Wunderlich, 1992). P-glycoprotein may be strategically positioned at the cell surface to mediate a complex array of interactions between the cell and its external and internal environments. P-glycoprotein is modified post-translationally by N-glycosylation (Ichikawa *et al.*, 1991a). Oligosaccharides form an integral structural component of glycoproteins, and as such may affect many of their properties, such as cell-cell and cell-molecule recognition events (Varki, 1993). Moreover, proteolytic succeptibility, specific bioactivity, immunogenicity, half-life and physical

properties in general can all be influenced by glycosylation (Yamaguchi *et al.*, 1991; Dennis, 1992; Rudd, 1993). Glycoprotein processing inhibitors may therefore be valuable in establishing whether or not the actions of P-glycoprotein are regulated by glycosylation and if such modification can be applied usefully to overcome MDR.

The aim of this study was to determine whether suppression of glycoprotein synthesis with tunicamycin, a specific inhibitor of protein N-glycosylation (Elbein, 1987), would alter the capacity of various drug-sensitive and drug-resistant cells to transport and retain radiolabelled VCR. The effects of TM on doxorubicin and azidopine binding to intact UWOV2 cells was also investigated in an attempt to explain drug-resistance in these cells. In addition, azidopine, a photoactivated dihydropyridine calcium channel blocker known to interact with Pgp (Kamiwatari *et al.*, 1989; Tamai and Safa, 1991; Safa, 1992), was assayed for its ability to compete for doxorubicin binding to UWOV2 cells.

2. Materials and Methods

2.1. Drugs and Chemicals

Colchicine (Sigma Chemical Co., St. Louis, MO, USA), trypsin 1:250 (Difco Laboratories, Detroit, MI, USA), phosphate-buffered saline [PBS] (Oxoid, UK), tissue culture media and antibiotics (Gibco, UK), tunicamycin [TM] (Boehringer Mannheim, Germany), ethylene-diamine tetra-acetic acid disodium salt [EDTA], trichloroacetic acid (Merck Chemicals, Germany), radiochemicals (Amersham, UK), were obtatained from the sources indicated in parentheses. All other chemicals were of analytical grade.

2.2. Cell lines and Culture Conditions

Multidrug-resistant KB-8-5-11 cells and drug-sensitive KB-3-1 human epidermoid carcinoma cells were obtained from Professor M.M. Gottesman, National Cancer Institute, Bethesda, MD, USA. The KB-8-5-11 cell line was selected as a colchicine-resistant subclone from the parent KB-3-1 cell line, and it contains the amplified *mdr*1 gene encoding Pgp (Akiyama, *et al.*, 1985; Fojo, *et al.*, 1985; Shen *et al.*, 1986a; Shen *et al.*, 1986b; Shen *et al.*, 1986c; Choi, *et al.*, 1988).

NIH-3T3 murine fibroblasts and NIH-3T3-MDR cells (derived from NIH-3T3-parental cells transfected with the retroviral expression vector, pHaMDR1, containing a full-length cDNA from the human *mdr*1 gene (Shen *et al.*, 1986c;

Ueda, *et al.*, 1987) were also provided by Professor M.M. Gottesman. The drug-sensitive LR73 Chinese hamster cell line and its *mdr*1-transfectant, LR73-1A, were a gift from P. Gros (McGill University, Montreal, Canada).

The human ovarian carcinoma cell line, UWOV2, was derived from an ovarian cystadenocarcinoma in a patient who had not responded to combined chemotherapy with actinomycin D, vincristine, cisplatin and doxorubicin (Golombick *et al.*, 1990), and thus represents a cell line with drug resistance acquired *in vivo*. The UWOV2 cells were obtained from Professor W.R. Bezwoda (University of the Witwatersrand, South Africa).

All cell lines were grown as adherent monolayers at 37°C in 5% CO_2:air, relative humidity close to saturation under the following conditions: UWOV2 cells, in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum (HIFBS), penicillin (100 units/ml), streptomycin (100 μg/ml) and/or gentamicin (50 μg/ml); KB-3-1 and NIH-3T3-parental cells, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% HIFBS and antibiotics as above (complete DMEM); KB-5-8-11 and NIH-3T3-MDR cells, in complete DMEM with addition of 1 μg/ml colchicine; LR73 cells in α-MEM containing ribo- and deoxyribonucleotides, 10% HIFBS, 2 mM L-glutamine and antibiotics and LR73-1A medium as for LR73, but supplemented with 100 ng/ml of adriamycin or colchicine. The cells were subcultured by brief exposure to trypsin-EDTA (0.25-0.02%) in Ca^{2+}- and Mg^{2+}-free PBS and maintained in the exponential phase of growth. Cell lines were regularly tested using the method described by Chen (1977) and found to be free of mycoplasma contamination.

2.3. Treatment of Cells with Tunicamycin

Tunicamycin (TM) was solubilized in 10 mM NaOH at a concentration of 2 mg/ml, sterilized by passing through a 0.22-μm disposable filter (Millipore, Millex-GV) and either used immediately or stored at -20°C for not more than 2 weeks. Before each experiment, the TM solution was diluted in culture medium. Cells were pre-incubated at 37°C for 16h in medium containing TM (5 μg/ml, unless stated otherwise). Untreated cells (controls) received an equivalent amount of TM-vehicle, *viz.*, NaOH (final concentration 62.5 μM, which did not affect the pH of the medium). TM was tested by the trypan blue dye-exclusion procedure described previously (Shier, 1985; Shier, 1988) and confirmed not to affect cell viability (>95%) between 4h and 72h exposure.

2.4. Assay of Vincristine Uptake and Efflux

To assay vincristine (VCR) uptake and efflux, cells were seeded at a density of $5x10^4$ cells/ml in 24-well plates and allowed to grow for 48h under standard conditions. Cells were pretreated with 5 μg/ml TM as described in section 3.3 above. Parallel controls were set up. Total cellular accumulation of VCR was determined by exposing cells in quadruplicate wells to [G-^{3}H]VCR sulphate (final specific activity 9.48 cpm/pmol in the case of UWOV2 cells, NIH-3T3-parental and NIH-3T3-MDR cells; and 7.74 cpm/fmol in the case of KB-3-1 and KB-8-5-11 cells) in the continued absence or presence of TM in a final volume of 0.5 ml for various incubation times.

At the end of each incubation period, cells were washed three times with 1 ml ice-cold PBS and solubilized in 0.5 ml of 1% sodium dodecyl sulphate (SDS)/0.3M NaOH. One aliquot (0.4 ml) was neutralized by the addition of 0.2 ml of 2M acetic acid and mixed with 10 ml scintillation fluid (Beckman Ready-Solv EP™) and counted in a Beckman scintillation spectrometer. Intracellular drug at each time point was determined by subtracting the value for non-specific/surface-bound drug obtained by incubation with 100 μM unlabelled VCR for 10s at 0-4°C from the value for total drug. The other aliquot (0.1 ml) was assayed for total cellular protein.

Vincristine efflux was measured by loading control and TM-pretreated cells with [^{3}H]VCR for 60 min (0-time value for efflux) followed by washing preloaded cells three times with ice-cold PBS and subsequently incubating at 37°C in serum- and antibiotic-free medium (2ml) for various time intervals. The absence or presence of TM was maintained throughout the post-incubation periods. Cells were harvested as described for uptake studies. To measure VCR efflux rate, KB-8-5-11 cells were seeded at a density of $2x10^6$ cells/ml in quadruplicate 35-mm dishes. Cells were pre-incubated at 37°C for 16h in the absence or presence of TM (concentration range 0.005 - 5 μg/ml) in serum- and antibiotic-free medium. The cells were then washed free of medium and pre-exposed to VCR (final specific activity 667 dpm/pmol) in fresh serum- and antibiotic-free medium with or without TM for 60-min.

The cells were then washed three times in cold PBS and exposed to drug-free medium with or without TM. At 5-min intervals after the 60-min pre-exposure period, cells were washed three times with ice-cold PBS and solubilized in 1 ml 1% SDS/0.3M NaOH. One aliquot (0.5ml) was mixed with 10 ml scintillation fluid

and counted. Another aliquot (0.1ml) was used for protein determination. A large volume ratio (*i.e.*, preloading volume/postincubation volume of 4) was maintained during efflux measurements to minimize reutilization of extruded drug (Sirotnak *et al.*, 1986).

2.5. Binding of [^{3}H]Azidopine and [^{14}C]Doxorubicin to UWOV2 Cells

The binding of [^{3}H]azidopine to UWOV2 ovarian carcinoma cells was measured by a modification of the procedure described by Tamai and Safa (1991). UWOV2 cells were seeded at a density of 5 X 10^4/ml in 24-well plates and allowed to reach confluence. The cell monolayers were cooled to 4°C (by placing the plates on ice for 10 min) and washed four times with 1 ml cold phosphate-buffered saline (PBS), pH 7.4 (to remove serum glycoproteins) and maintained for 60 min at 4°C with binding buffer (10 mM glucose, 3 mM ATP and 5 mM $MgCl_2$ in 10 mM Tris-HCl, pH 7.4) containing various concentrations of [^{3}H]azidopine or [^{14}C]doxorubicin, or both. Following this incubation period, the cells were washed five times with cold PBS to remove unbound [^{3}H]azidopine and [^{14}C]doxorubicin. Cells were solubilized with 0.1 M NaOH and samples were counted to determine the amount of azidopine or doxorubicin bound to the cells, and aliquots were removed for protein determination. Specific binding was distinguished from non-specific binding to the cells and plastic wells by dilution with a large excess (100 μM) of unlabelled drug. To examine whether doxorubicin competes for azidopine binding sites, UWOV2 cells were exposed to equimolar concentrations of both compounds and the specific binding of azidopine determined as described above.

2.6. Protein Estimation

Total cellular protein was determined by the automated Bio-Rad™ dye-binding microassay system described by Bradford (1976) using bovine serum albumin as a standard.

2.6. Data Analysis

The p-values for the differences in the variables obtained in this study as well as for the comparison of VCR retention in the presence or absence of TM were calculated using the two-sided Student's t-test computer program in GraphPad™ INSTAT©. The increase in VCR retention induced by TM and the associated 95% confidence interval (95% CI) were determined by Fieller's ratio-of-means test (Fieller, 1944) as modified by Bliss (1956), using a computer-assisted program

(Tallarida and Murray, 1987). The rates of VCR efflux from KB-8-5-11 cells were determined from the slopes of the linear portion of efflux curves over the first 20 minutes.

3. RESULTS

Uptake and efflux curves for the human epidermoid carcinoma KB-3-1 parental cell line and its multidrug-resistant derivative, KB-8-5-11, are shown in Figure 1. Uptake of VCR by KB-3-1 cells was unaffected by the presence of TM (Figure 1A), but efflux was increased for the 60- 80- and 100-min post-incubation periods (Figure 1C). Tunicamycin failed to alter the uptake of VCR by KB-8-5-11 cells, except at 120 min when a significant decrease ($p=0.0143$) in intracellular VCR was noted in TM-treated cells (Figure 1B). The rapid efflux of VCR was significantly impaired in KB-8-5-11 cells following pretreatment and co-incubation with TM (Figure 1D).

The effects of TM on the uptake and efflux of VCR in cultures of NIH-3T3-parental and NIH-3T3-MDR cells are shown in Figure 2. Pretreatment of NIH-3T3-parental cells with TM did not significantly modify their ability to accumulate VCR (Figure 2A). In NIH-3T3-MDR cells, TM treatment had no effect on the level of VCR accumulation (Figure 2B). NIH-3T3-MDR cells also accumulated less drug than their parental or drug-sensitve counterparts. In both cell lines, uptake displays a consistent biphasic course with a rapid initial component and a subsequent linear component (the drug-cell system reaches a steady state within 20-30 min). Efflux curves for NIH-3T3-parental and NIH-3T3-MDR cells are depicted in Figure 2C and Figure 2D, respectively. The efflux of VCR from NIH-3T3-parental cells was not affected by tunicamycin (Figure 2C). By contrast, VCR efflux was significantly reduced ($p<0.05$ at all post-incubation times) when *mdr*1-transfected NIH-3T3 cells (NIH-3T3-MDR) were treated with TM (Figure 2D). It is noteworthy that the NIH-3T3-MDR cell line contained significantly less VCR after an initial 1-hour pre-exposure period when compared to its parental cell line (Figure 2C and Figure 2D). This observation further supports the idea that MDR, as expressed in NIH-3T3-MDR cells, is associated with decreased intracellular accumulation of drug.

Exposure of drug-sensitive LR73 cells to TM significantly enhanced the accumulation of VCR at incubation periods of 15, 30, 75 and 90 min, but not at 45 and 60 min (Figure 3A). In drug-resistant LR73-1A cells, TM augmented the intracellular accumulation of VCR at all the times that are indicated (Figure 3B).

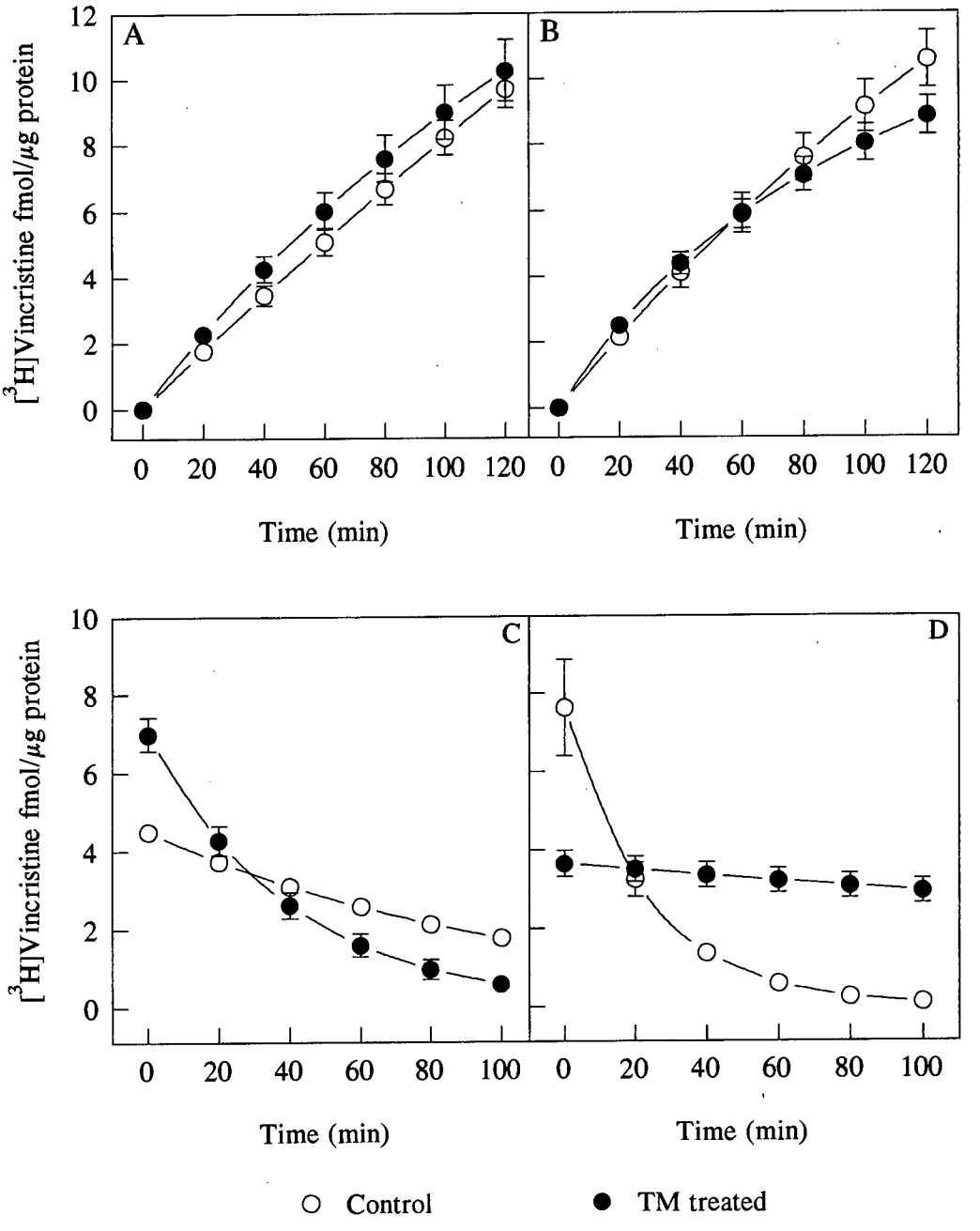

Figure 1. Time course of vincristine uptake (A, B) and efflux (C, D) in drug-sensitive KB-3-1 (A, C) and multidrug-resistant KB-8-5-11 cells (B, D) cultured in the absence or presence of tunicamycin following prior exposure to the antibiotic for 16h. Values are means ± S.E.M.(n=4). Where error bars do not overlap, Student's two-tailed p values are less than 0.05 (not shown).

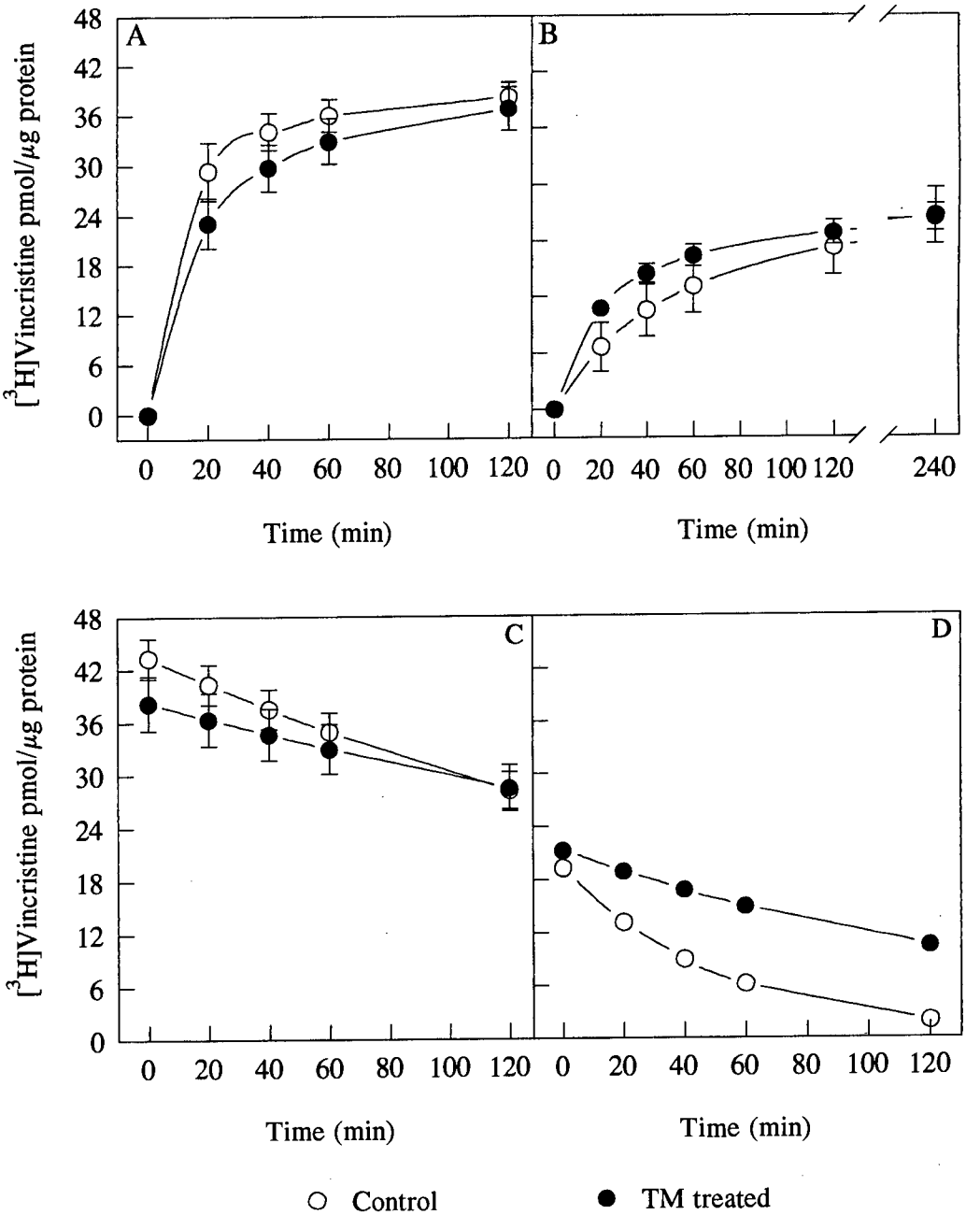

Figure 2. Time course of vincristine uptake (A, B) and efflux (C, D) in NIH-3T3-parental (A, C) and NIH-3T3-MDR cells (B, D) cultured in the absence or presence of tunicamycin following a 16h TM pretreatment. Details are described in the legend to Figure 1. Error bars are shown on the figure when larger than the plot symbols.

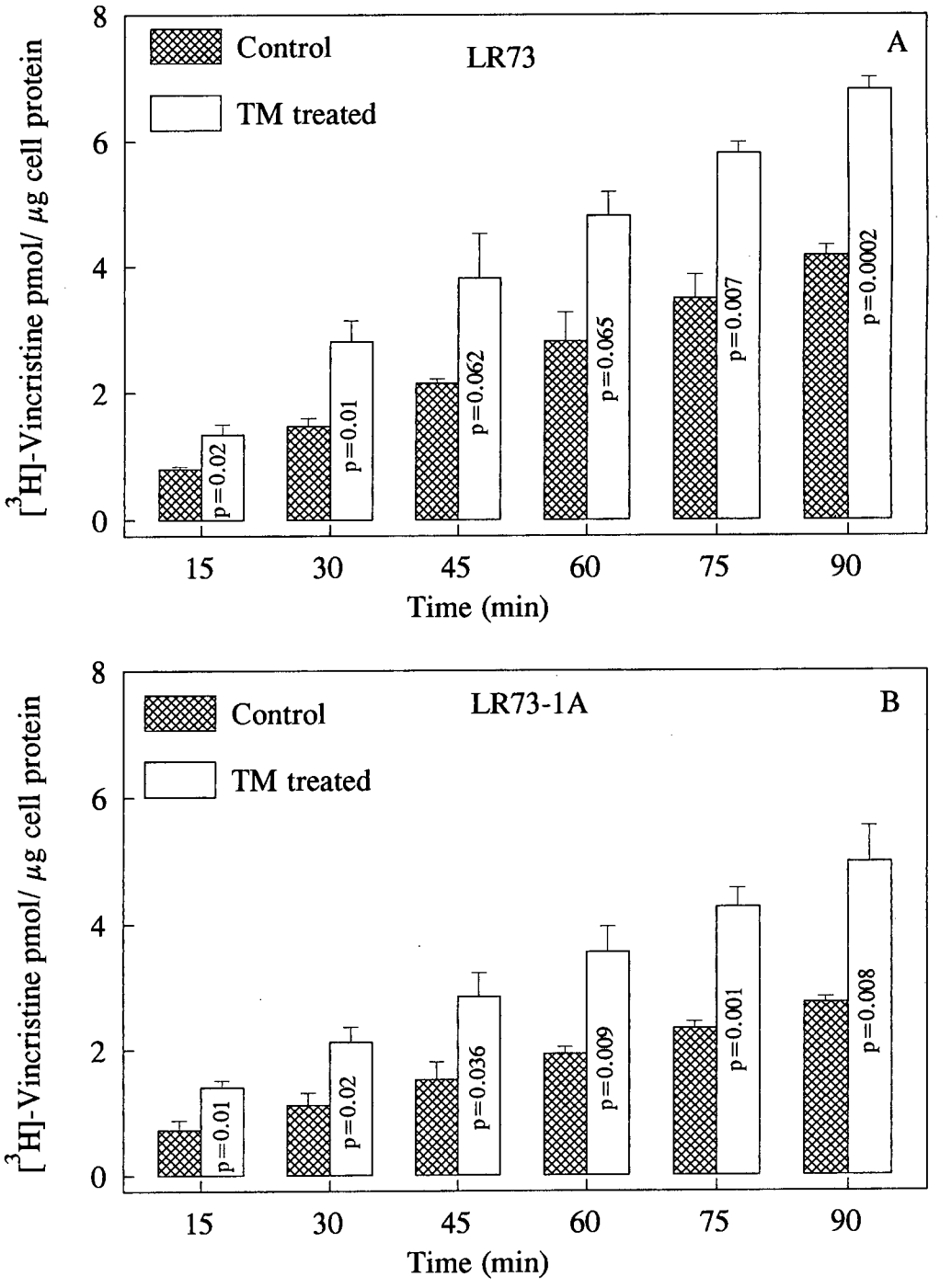

Figure 3. Effects of tunicamycin on vincristine accumulation in drug-sensitive LR73 (A) and drug-resistant LR73-1A (B) cells at various time intervals following an initial 16-h pretreatment with the antibiotic. Data represent the means ± S.E.M.(n=4). Student's two-tailed p values are indicated within bars.

As in the case of NIH-3T3-MDR cells, the LR73-1A cells accumulated significantly less VCR than the parental LR73 cells, but only at 75 and 90 min (Figure 4A). Following pretreatment with TM, LR73-1A cells accumulated VCR in amounts approximating those in untreated LR73 cells (Figures 4A and 4B). Tunicamycin had no effect on the uptake of VCR by UWOV2 ovarian carcinoma cells (Figures 5A), but VCR efflux from these cells was significantly inhibited ($p < 0.05$) at post-incubation times 60-180 min (Figures 5B).

Figures 6 and 7 summarize data (obtained in separate experiments similar to those described for the efflux studies) on the retention of VCR in various drug-sensitive and drug-resistant cell lines in response to TM treatment. TM increased the retention of VCR in KB-8-5-11 cells, in NIH-3T3-MDR cells (at post-drug-loading periods of 40, 60 and 80 min), in LR73-1A cells at post-drug-loading periods of 40, 80 and 100 min (marginally significant), but not so at 20 and 60 min (Figure 6), and in UWOV2 cells at post-incubation times 60, 80 and 100 min (Figure 7), but not in NIH-3T3-parental and LR73 (Figure 6) cells. By contrast, KB-3-1 cells expressed a decreased VCR retention at 40-, 60- and 80-min post-incubation periods (Figure 6). Tunicamycin induced the greatest increase in VCR retention at all the times studied in KB-8-5-11 cells (2.78- to 4.29-fold) compared with NIH-3T3-MDR and LR73-1A cells (Table 1).

In order to determine whether the increased VCR retention induced by TM is associated with altered VCR efflux and inhibition of glycoprotein synthesis, KB-8-5-11 cells were exposed to various concentrations of TM and the changes were measured. The effects of TM concentrations over the range 0.005 - 5 μg/ml on glycoprotein synthesis as reflected by [^{3}H]glucosamine incorporation as a percentage of the control and on the rate of VCR efflux from KB-8-5-11 cells are shown in Table 2. When these cells were treated with TM, a considerably reduced efflux rate was induced at all concentrations of TM compared with the controls. This decreased VCR efflux did not correlate with the inhibition of glycoprotein synthesis as measured by the incorporation of [^{3}H]glucosamine. Tunicamycin at concentrations of 0.005 and 0.05 μg/ml did not affect glycoprotein synthesis, but markedly inhibited VCR efflux. Although the inhibition of glycoprotein synthesis was increased to 43% and 81% at TM concentrations of 0.5 and 5 μg/ml, respectively, the inhibition of VCR efflux remained constant at 70% for these TM concentrations.

The MDR status of UWOV2 ovarian carcinoma cells was confirmed, in part, by measuring the specific binding of [^{3}H]azidopine, a photoactive

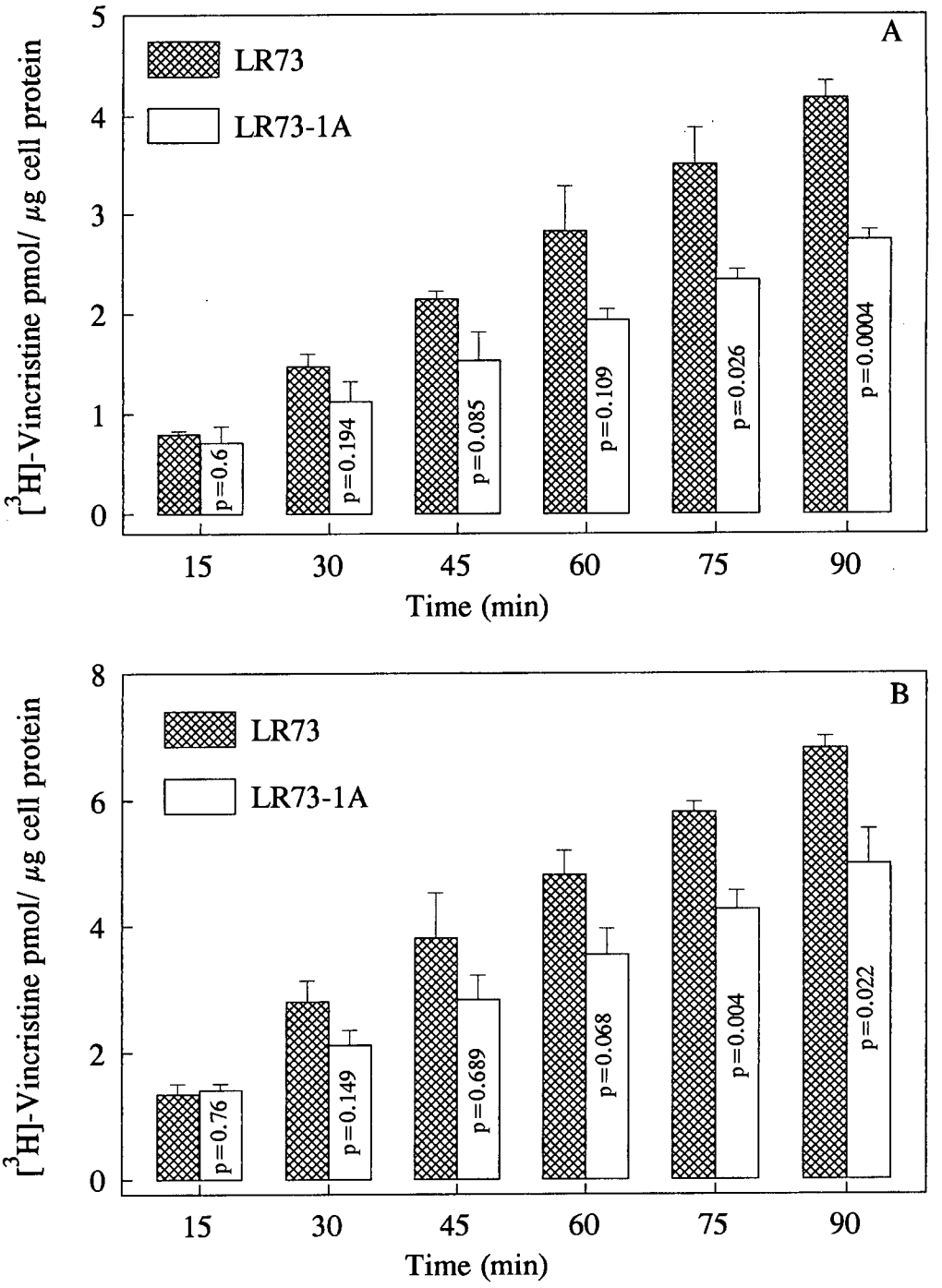

Figure 4. Comparison of vincristine accumulation by LR73 and LR73-1A cells cultured in the absence (A) or presence (B) of tunicamycin. Values are means ± S.E.M.(n=4). Student's two-tailed p values are presented within bars.

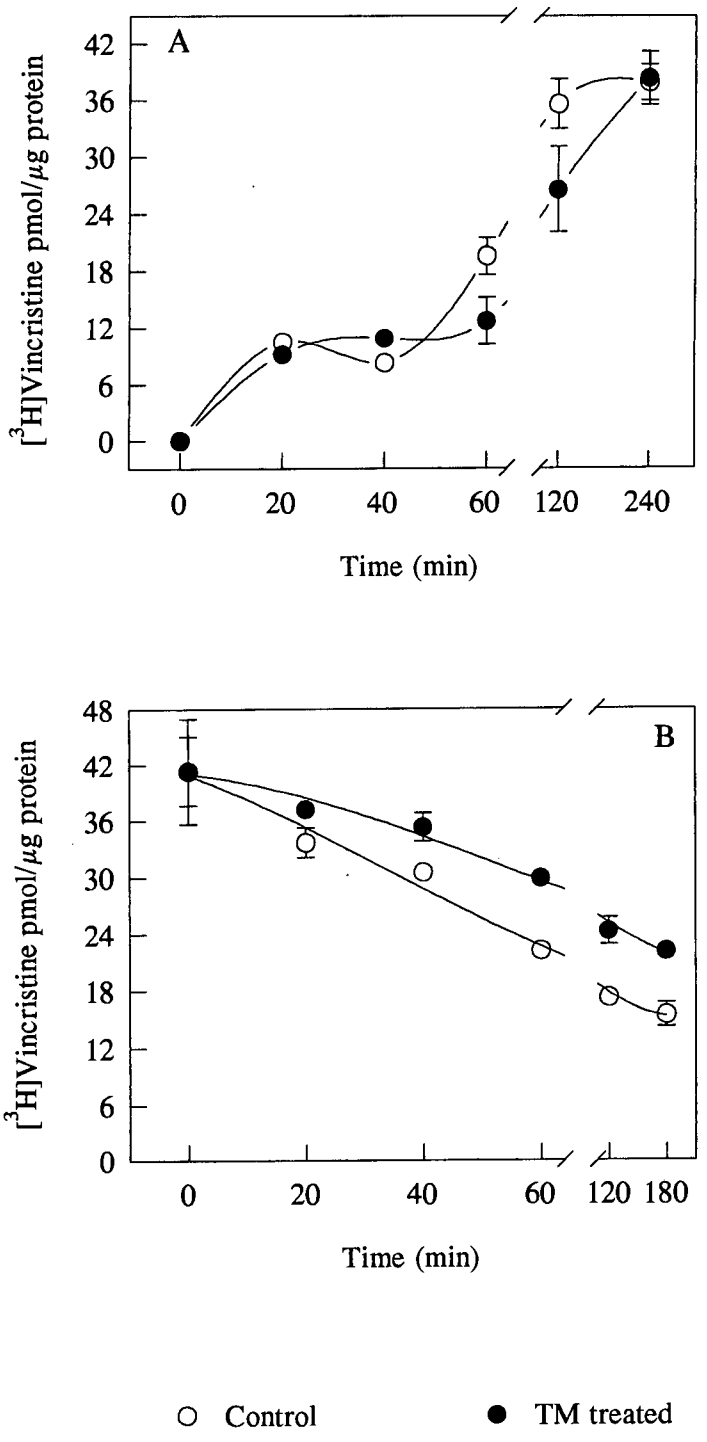

Figure 5. Time course of vincristine uptake (A) and efflux (B) in human UWOV2 ovarian carcinoma cells cultured in the absence or presence of tunicamycin following prior exposure to the antibiotic for 16h. Values are means ± S.E.M.(n=4).

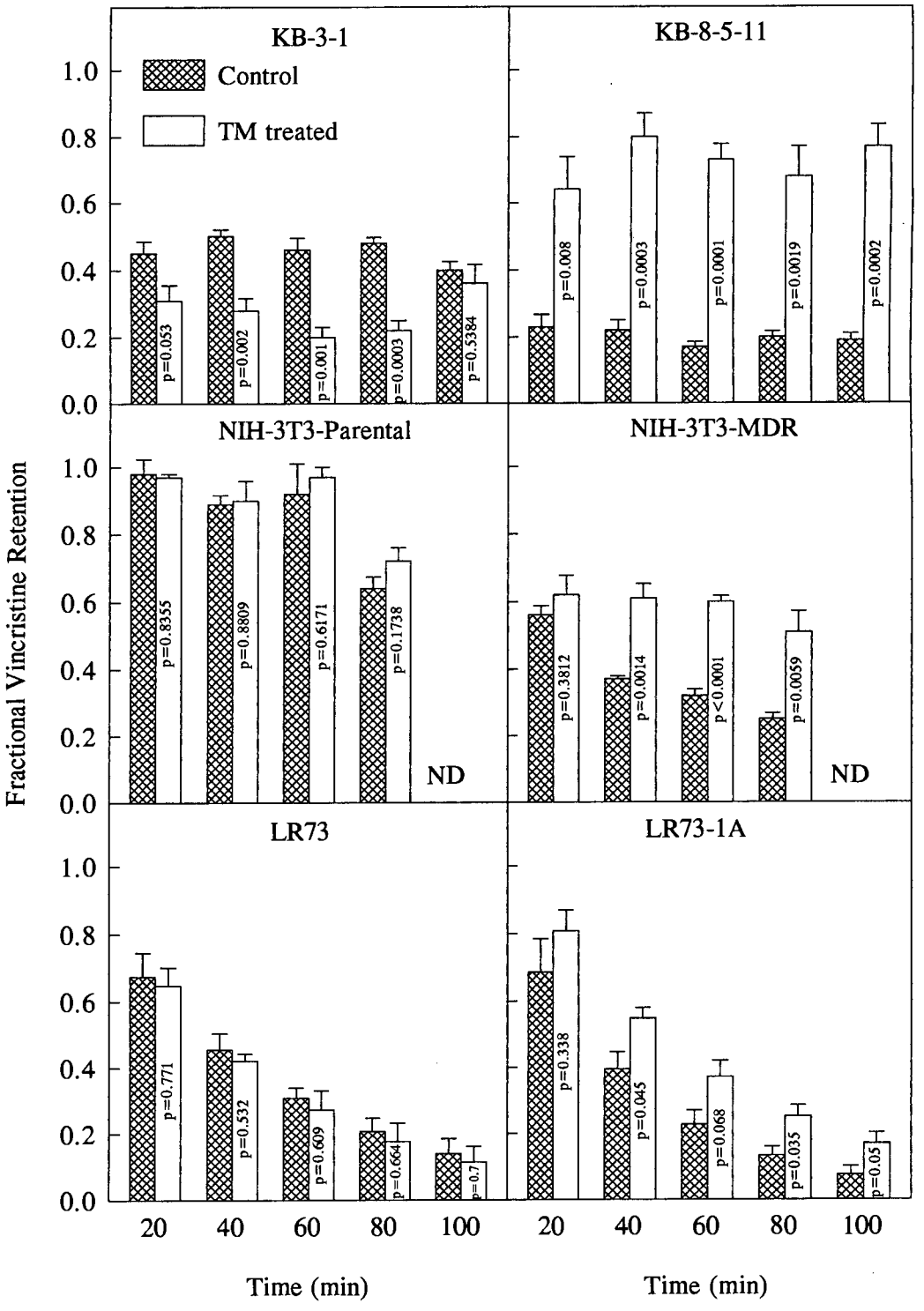

Figure 6. Comparison of the effects of tunicamycin on the retention of vincristine in various drug-sensitive and drug-resistant cell lines at different time intervals after a 1-hr preloading period with the drug and subsequent exposure to drug-free medium. Values are means ± S.E.M.(n=4). Student's two-tailed p values are presented within bars.

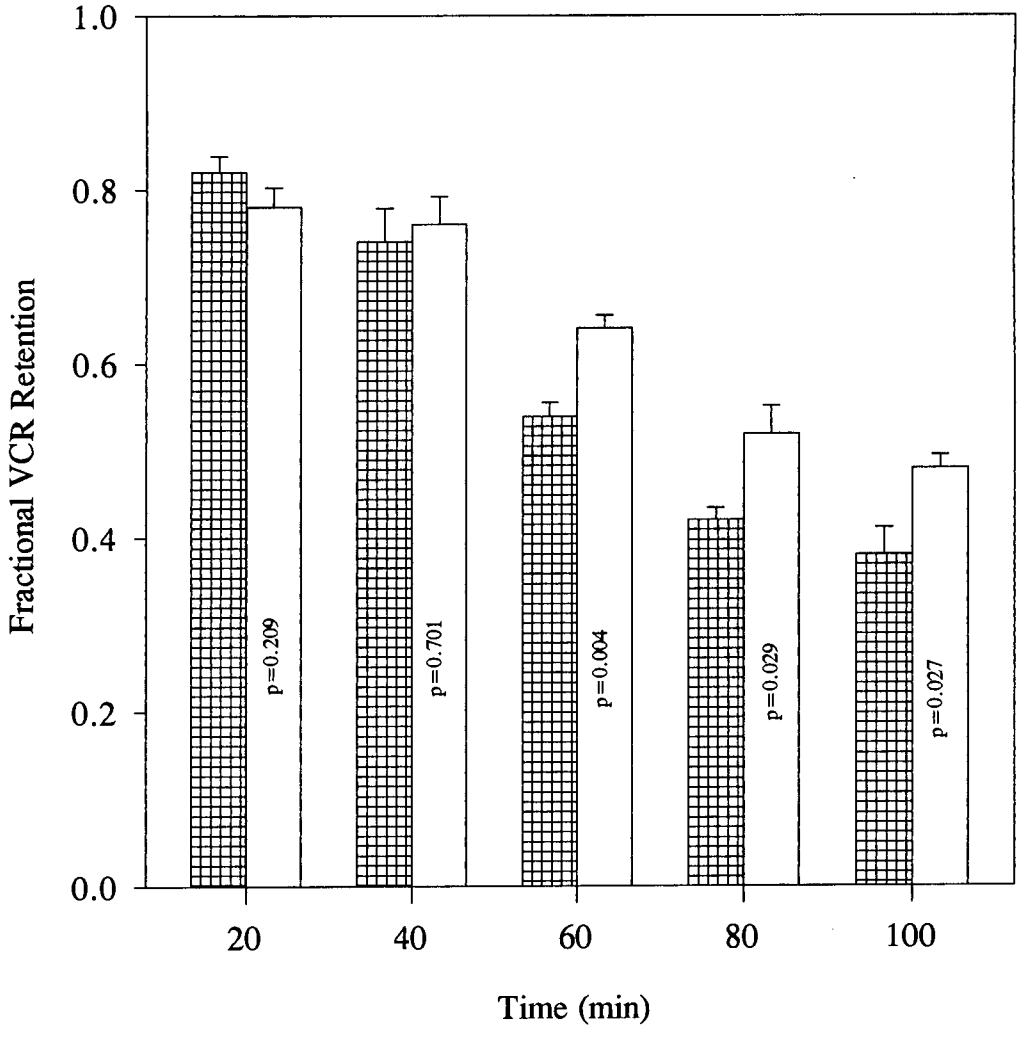

Figure 7. Effects of tunicamycin on the retention of vincristine in drug-resistant UWOV2 human ovarian carcinoma cells at different time intervals after a 1-hr pre-loading period with the drug and subsequent exposure to drug-free medium. Values are means ± S.E.M.(n=4). Student's two-tailed p values are presented within bars.

Table 1. Fold Increase in Vincristine Retention in Various Multidrug-Resistant Cell Lines Following Exposure to Tunicamycin

	KB-8-5-11		NIH-3T3-MDR		LR73-1A	
Time (min)	Fold FR[1]	95% CI[2]	Fold FR	95% CI	Fold FR	95% CI
20	2.78	1.74 to 5.86	1.11	0.93 to 1.32	1.18	0.92 to 1.52
40	3.63	2.55 to 6.06	1.64	1.42 to 1.93	1.78	0.83to 5.74
60	4.29	3.19 to 6.46	1.88	1.70 to 2.08	1.63	1.14 to 2.47
80	3.40	2.16 to 7.14	2.04	1.52 to 2.91	1.90	1.28 to 3.15
100	4.05	2.80 to 7.00	ND	ND	2.23	1.27 to 5.65

[1]Fold increase in vincristine retention induced by tunicamycin. [2]Confidence interval. [1,2]Calculated from fractional retention values presented in Figure 5 using Fieller's ratio-of- means test as described by Tallarida and Murray (1987). ND, not determined.

Table 2. Rate of Efflux of Vincristine from KB-8-5-11 and the Corresponding Level of Inhibition of Glycoprotein Synthesis After 16 Hours as a Function of Tunicamycin Concentration

Tunicamycin (μg/ml)	[^{3}H]Vincristine Efflux Rate (pmol.mg protein^{-1}·min^{-1})	% Inhibition of Efflux	Inhibition of [^{3}H]Glucosamine Incorporation (% of Control)
Control	1315±4.4	0	0
0.005	474±34.3[a]	64±4.6	0.59±0.005
0.05	571±51.3[a]	57±5.1	0.97±0.038
0.5	400±44.6[a]	70±7.8	43.2±1.507
5.0	395±29.7[a]	70±5.3	80.8±8.082

Values are means ± S.E.M. (n=4). [a]Differs significantly from control ($p < 0.0001$).

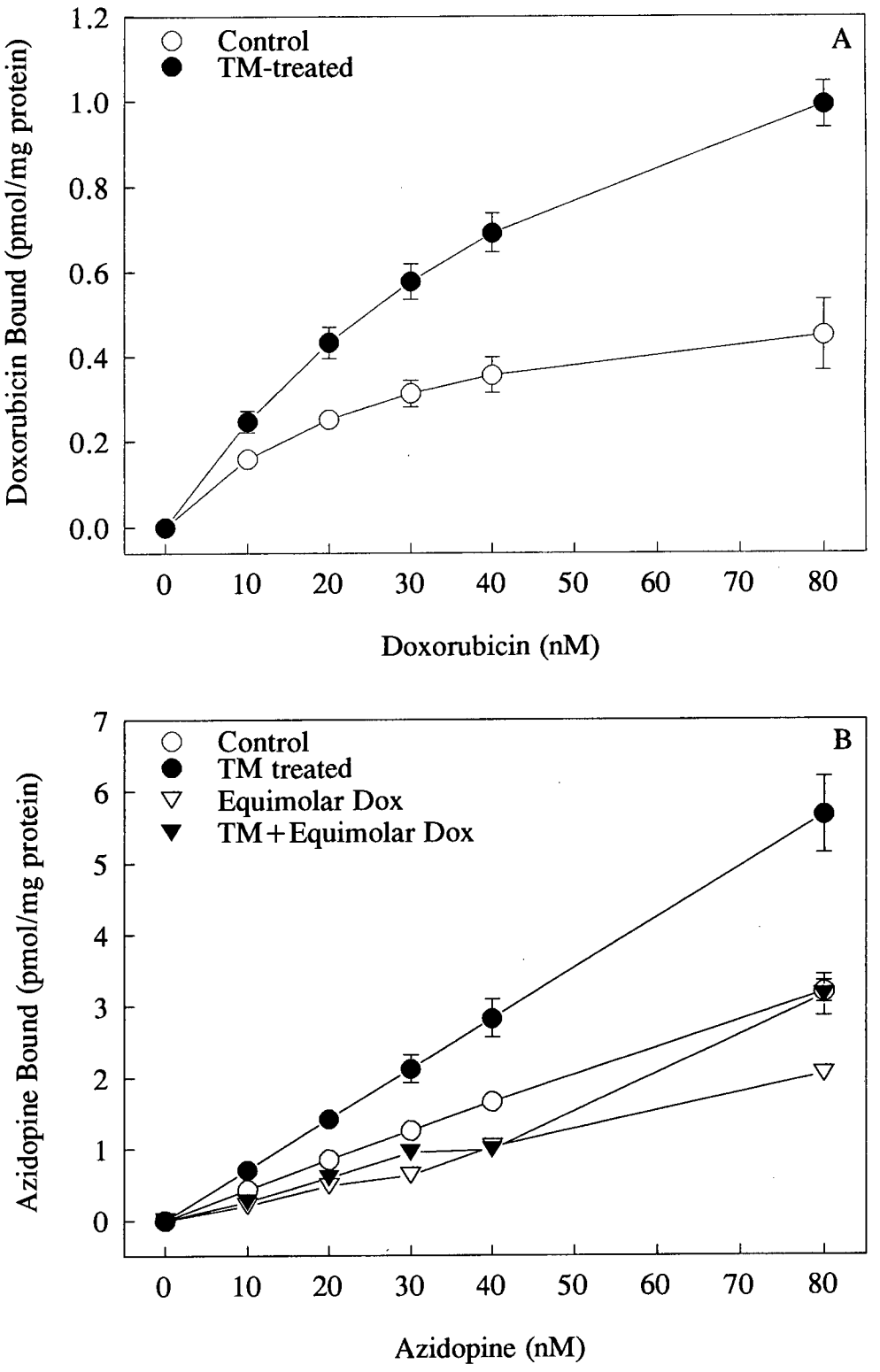

Figure 8. Specific binding of [^{14}C]doxorubicin (A) and [^{3}H]azidopine (B) to cultured UWOV2 cells at 4°C for 60 min in the absence (control) or presence (TM treated) of 5μg/ml tunicamycin following an initial 16-h pre-incubation at 37°C with or without TM. Specific binding of doxorubicin and azidopine was calculated by subtracting non-specific binding data obtained in the presence of 100 μM each of doxorubicin and azidopine, respectively, from total binding. Each point represents the mean$\pm$S.E.M. (n=4).

dihydropyridine calcium channel blocker known to bind to Pgp, and [^{14}C]doxorubicin to intact cells in culture at 4°C in the absence (control) or presence (TM-treated) of TM following an initial 16h pre-incubation at 37°C with or without the antibiotic. The binding of doxorubicin to these cells was saturable in the concentration range of 30-80 nM (Figure 8A), whereas that of azidopine remained linear between 10 and 80 nM (Figure 8B). In the presence of equimolar concentrations (range 30-80 nM) of doxorubicin, the binding of azidopine to UWOV2 cells was significantly reduced (Figure 8B). This reduction of azidopine binding to UWOV2 cells in the presence of doxorubicin was abolished by treatment of cells with 5μg/ml TM (Figure 8B). Tunicamycin increased the binding of both doxorubicin (Figure 8A) and azidopine (Figure 8B) to UWOV2 cells.

4. Discussion

The results show that TM enhances VCR retention in MDR cell lines by decreasing drug efflux. Drug-resistant cells appear to be more responsive to TM pretreatment, possibly due to the presence of Pgp. This observation is relevant in understanding the biochemical basis of MDR, since TM is thought to be selectively more toxic to transformed cells, and it could thus be useful in the design of anticancer agents for malignant cells while sparing non-transformed cells (Olden *et al.*, 1979b; Eren and Duksin, 1985). KB-8-5-11, LR73-1A and NIH-3T3-MDR cells share a common mechanism of drug resistance, *i.e.*, they all express amplified *mdr*1 genes which encode Pgp (Akiyama *et al.* 1985; Fojo *et al.*, 1985; Choi *et al.*, 1988).

It is possible that TM induces lesions in the oligosaccharide moiety of Pgp in MDR cells and that this contributes to the increased VCR retention that is linked with decreased drug efflux. Tunicamycin may exert its effect by the inhibition of Pgp synthesis and/or by independent alteration in efflux pump activity. In KB-8-5-11 cells, increasing concentrations of TM resulted in a concentration-related reduction in glycoprotein synthesis which did not correlate with inhibition of VCR efflux. Therefore, suppression of the efflux of this compound in these cells cannot be attributed to the inhibition of glycoprotein synthesis alone, since TM, even at low concentrations, can inhibit its efflux. This finding suggests that TM may act as a competitive inhibitor of VCR binding to Pgp for Pgp-mediated efflux, but this was not tested. Nevertheless, transient inhibition of both protein and glycoprotein synthesis during TM exposure may contribute to alterations in VCR efflux and retention observed in the MDR cell lines.

The UWOV2 cell line represents a cell line with *in-vivo* acquired MDR (Golombick *et al.*, 1990). Bio-Gel A-0.5M gel filtration chromatographic and sodium dodecyl sulphate polyacrylamide gel electrophoretic profiles of UWOV2 plasma membranes revealed the presence of a 170-kDa glycoprotein. The nature of this 170-kDa membrane glycoprotein has been analysed in our laboratory for immunoreactivity with Pgp-specific monoclonal antibodies (C219 and MRK16), and this is described in Chapter 5. In addition, the finding that both doxorubicin and azidopine bind to intact UWOV2 cells points to the presence of Pgp on their cell surfaces. It was also shown that the specific binding of azidopine to UWOV2 cells is inhibited (*ca.* 50%) by equimolar concentrations of doxorubicin. This is consistent with the notion that azidopine specifically binds to Pgp and that photolabelling with azidopine is inhibited by vinblastine, actinomycin D, doxorubicin and many MDR modulators and organic chemicals (Safa *et al.*, 1987; Yang *et al.*, 1989; Ichikawa *et al.*, 1991b). However, the information obtained from our binding studies must be supplemented with structural characterization of Pgp and comparison with cells expressing Pgp and their respective wild-type cells.

Thus, TM appears to modulate MDR by inhibition of enhanced drug efflux from MDR cells. TM inhibits both protein and glycoprotein synthesis and this may explain its action on efflux since the Pgp molecule has to be intact to be functional (Currier *et al.*, 1989). Tunicamycin may modify the molecular conformation of Pgp synthesized *de novo*, and its ability to engage in intermolecular interactions such as drug recognition, binding and efflux. A possible explanation as to why TM did not affect uptake of VCR in our experimental system is a rapid exchangeable pool with a steady flux of VCR in both directions. Since VCR reutilization was minimized in efflux experiments, actual retention of drug could be studied. Our results are in agreement with previous assertions of Beck and Cirtain (1982) that TM does not enhance drug uptake in resistant cells. However, our results do establish that TM increases drug retention in resistant cells.

In some MDR cell lines, the phosphorylation and glycosylation status of Pgp may or may not correlate with drug resistance (Center, 1983; Richert *et al.*, 1988; Mukhopadhyay and Kuo, 1989). We have not evaluated changes in MWs of MDR-associated glycoproteins upon TM treatment, but have assumed the known activity of the antibiotic on Pgp (Greenberger *et al.*, 1987; Greenberger *et al.*, 1988a; Ichikawa *et al.*, 1991a), at least in NIH-3T3-MDR, LR73-1A and KB-8-5-11 cells known to express Pgp. The function of the carbohydrate moieties of Pgp in MDR has not been evaluated adequately. Gervasoni *et al.* (1991) have proposed that anthracycline resistance in HL-60 human promyelocytic leukaemia cells may

be due to hypoglycosylation of cell-surface glycoproteins, albeit Pgp is not overexpressed in these cells. Several reports of differences between drug-sensitive and drug-resistant cells point to changes in glycosylation patterns of plasma membrane glycoproteins (Juliano and Stanley, 1975; Beck and Cirtain, 1982; Marsh and Center, 1987). Unglycosylated forms of Pgp have also been detected in certain MDR cell lines (Germann *et al.*, 1990). The fact that not all glycoproteins become unstable or non-functional in the unglycosylated form (Elbein, 1984; Elbein, 1987) suggests that Pgp may be synthesized at normal rates, but deficient in carbohydrate and yet retain its biological activity in MDR (Chou and Kessel, 1981). It is not clear, however, whether differential glycosylation of the *mdr*1 gene product (Greenberger *et al.*, 1989) is required for MDR.

The MDR phenotype is complex (Cornwell, 1991; Fairchild and Cowan, 1991; Roninson, 1992b). Overexpression of distinct forms of Pgp may explain the observed diversity in the MDR phenotype (Hsu *et al.*, 1989). Different cell lines may display various patterns of drug resistance or cell-type specific factors may modulate the resistance. The *mdr*1-transfected cell lines provide a convenient tool for studying Pgp-mediated phenomena. The results presented here may be useful to study the effects of glycosylation on the properties of Pgp and its interaction with cytotoxic drugs. In the broader sense, it is probable that glycosylation regulates drug efflux in drug-resistant cells through an as yet undetermined mechanism. Recently, inhibition of N-linked glycosylation by TM has been shown to affect organic cation transport across the brush border membrane of opossum kidney cells (Ott *et al.*, 1992). This information, in association with the observation that Pgp binds certain organic chemicals in the adrenal gland and kidney, and presumably also functions in the excretion of these compounds (Ichikawa *et al.*, 1991b), and the knowledge that Pgps from KB-C2 cells, kidney and adrenal gland exhibit different lectin binding capacities (Ichikawa *et al.*, 1991a), may be applied effectively in strategies to overcome MDR. Moreover, TM interacts with lipophilic membrane components (Kuo and Lampen, 1976), which warrants consideration in such studies. However, the potential use of TM as an MDR modifier must await biochemical characterization of the precise relationship between the action of this antibiotic and Pgp together with an evaluation of its *in vivo* and *in vitro* cytotoxic spectrum (Vogel and McWilliam, 1987; Leaver, *et al.*, 1988).

5. Summary and Conclusions

Various drug-sensitive cell lines (NIH-3T3-parental mouse fibroblasts, LR73 Chinese hamster cells, and KB-3-1 human epidermoid carcinoma) and their

corresponding drug-resistant cell lines (NIH-3T3-MDR, LR73-1A and KB-8-5-11, respectively) as well as drug-resistant UWOV2 human ovarian carcinoma cells derived from an ovarian cystadenocarcinoma in a patient who had not responded to combined chemotherapy with actinomycin D, vincristine, cisplatin and doxorubicin, were studied for altered vincristine (VCR) transport in response to pretreatment with tunicamycin (TM), an inhibitor of protein N-glycosylation.

The uptake of VCR was not affected by prior exposure of cells to TM, except in LR73 and LR73-1A cells for which enhanced uptake was observed. A consistent association was found between treatment of drug-resistant cell lines with TM and reduced VCR efflux with resultant increased VCR retention. Thus, TM enhances VCR retention in MDR cell lines by inhibiting its efflux. Drug-resistant cells appear to be more responsive to TM pretreatment, possibly due to the presence of P-glycoprotein (Pgp). In KB-8-5-11 cells, increasing concentrations of TM resulted in a concentration-related reduction in glycoprotein synthesis which did not correlate with inhibition of VCR efflux. It is reported that TM affects *Vinca* alkaloid transport in drug-resistant cells and that transient inhibition of both protein and glycoprotein synthesis during TM exposure (see Chapter 2) may contribute to observed alterations in VCR efflux and retention.

The binding of [^{14}C]doxorubicin and [^{3}H]azidopine (a photoactive dihydropyridine calcium channel blocker known to interact with Pgp) to UWOV2 ovarian carcinoma cells was studied in order to partially characterize MDR in these cells. Binding of doxorubicin to UWOV2 cells was saturable in the concentration range 10-80 nM, whereas binding of azidopine remained linear between 10 and 80 nM. In the presence of equimolar concentrations of doxorubicin, the binding of azidopine to UWOV2 cells was significantly inhibited in the concentration range of 30-80 nM. Tunicamycin augmented the binding of both doxorubicin and azidopine to UWOV2 cells. In the presence of TM and equimolar concentrations of doxorubicin the binding of azidopine was unchanged. The interaction of both azidopine and doxorubicin to intact UWOV2 cells implies that Pgp is present on their cell surfaces. However, the increased binding of these drugs to intact UWOV2 cells in the presence of TM also suggests that TM may exert its effects by modulating cell surface binding activity. Collectively, these results show that TM is a resistance modifier in MDR, and that structural analyses should be beneficial in understanding the precise relationship between the effects of this antibiotic and Pgp-mediated drug binding and efflux. This will further aid in the design of strategies to overcome drug resistance in malignant tumours.

CHAPTER 5

PARTIAL CHARACTERIZATION OF HUMAN TUMOUR CELL SURFACE GLYCOPROTEINS, WITH SPECIAL REFERENCE TO UWOV2 OVARIAN CARCINOMA AND ACUTE LEUKAEMIA

1. INTRODUCTION

The aim of chemotherapy is the palliation and/or cure of of human cancers (Greenwald *et al.*, 1990; Edgington *et al.*, 1992). Tumours such as ovarian carcinoma, acute leukaemia, small-cell lung cancer and advanced breast cancer sometimes achieve high initial response rates and prolongation of survival after combination chemotherapy, only to be followed by relapse and death. The causes of failure of chemotherapy are multifactorial. The relapsed tumour phenotype, when it is associated with the development of drug resistance, is referred to as multiple drug resistance (MDR). The expression of MDR during chemotherapy, or after *in-vitro* exposure of tumour cells to chemotherapeutic drugs, is often not limited to the drugs to which the tumour cells have been exposed, but may include cross-resistance to other agents as well (Morrow and Cowan, 1988). Increased expression of a plasma membrane glycoprotein, Pgp, has been detected in certain human malignancies in response to chemotherapy (Chan *et al.*, 1991; Sanfilippo *et al.*, 1991; Murphy *et al.*, 1992). The use of cDNA encoding Pgp and mAbs raised against Pgp in the diagnosis and therapy of patients in the refractory stage, as well as in studies of clinical resistance mechanisms, has been described (Gottesman and Pastan, 1989; van Kalken *et al.*, 1991; Wallner *et al.*, 1991). P-glycoprotein expression has been reported in breast cancer (Fuqua *et al.*, 1987; Salmon *et al.*, 1989; Giai *et al.*, 1991), colon cancer (Dalton *et al.*, 1988), lung cancer (Scagliotti *et al.*, 1991; Holzmayer *et al.*, 1992; Shin *et al.*, 1992), ovarian carcinoma (Rubin *et al.*, 1990; Rutledge *et al.*, 1990; Murphy *et al.*, 1992), leukaemia (Campos *et al.*, 1992; Michieli *et al.*, 1992; Sonneveld *et al.*, 1992b), myeloma and lymphoma (Musto *et al.*, 1991a; Linsenmeyer *et al.*, 1992; Ucci *et al.*, 1992). However, there

is still uncertainty regarding the role of Pgp in tumour cells during the refractory stage of chemotherapy.

The use of chemosensitizers or resistance modifiers in tumours that are unresponsive to anticancer drugs is likely to be paramount to the prevention of MDR and the improvement of treatment outcome (Gottesman and Pastan, 1989; van Kalken, 1991). Furthermore, the increased expression of Pgp in diverse tumour cell types may be a prognostic indicator for tumour response to chemotherapy (Ling *et al.*, 1989; Rubin *et al.*, 1990; Chan *et al.*, 1991). Recent advances in cancer research indicate that clinically proven diagnostic tests and *in vitro* cytotoxicity assays are becoming increasingly applicable in the optimization of treatment protocols, the prediction of therapeutic responsiveness and the identification of potential candidates for alternative approaches to chemotherapy (Greenwald *et al.*, 1990).

This chapter describes the partial characterization of the MDR-associated 170 kDa glycoprotein in a continuous human ovarian tumour cell line and peripheral blood leukaemia blasts obtained from patients. The UWOV2 cells were isolated from an ovarian cystadenocarcinoma in a patient who had not responded to combined chemotherapy with actinomycin D, doxorubicin, vincristine and cisplatin (Golombick *et al.*, 1990). This cell line has not been studied previously with regard to its expression of Pgp, but was considered as a model to study *in-vivo* acquired (*i.e.*, clinical) drug resistance along with the *mdr*1-transfected and *in-vitro*-selected drug-resistant cell lines that were described in previous chapters. Evaluation of Pgp expression was performed in a randomly selected group of patients with acute leukaemia was by gel filtration, SDS-PAGE and by immunocytochemical detection methods using the P-glycoMab™ and P-glycoCHEK ™ kits.

2. Materials and Methods

2.1. Monoclonal Antibodies

MRK16, a mAb to Pgp, was a gift from Dr. Takashi Tsuruo (Japanese Foundation for Cancer Research, Tokyo, Japan). MRK16 (immunoglobulin subclass IgG2a) was generated by immunizing mice with intact adriamycin-resistant K562 cells (Hamada and Tsuruo, 1986a; Hamada and Tsuruo, 1986b). Indirect immunofluorescence studies with adriamycin-resistant K562 cells have shown that MRK16 reacts with a determinant on their surface that corresponds to Pgp (Hamada and Tsuruo, 1991). Unlabelled C219, CH^RC5 membrane standard

(prepared from multidrug-resistant CH^RC5 Chinese hamster ovary cells), P-glycoCHEK™ control slides (consisting of a drug-sensitive human acute lymphoblastic leukaemia cell line, CCRF-CEM, as the negative staining control and its highly drug-resistant derivative, CEM-VLB_{100}, as the positive Pgp staining control) and P-glycoMab™ (consisting of lyophilized C219 mAb and isotype-matched negative antibody, biotinylated anti-mouse antibody, avidin and biotinylated horseradish peroxidase visualization system) were purchased from Centocor Diagnostics, Tongeren, Belgium. The C219, a murine mAb isotype IgG_2A, was raised in mice immunized with multidrug-resistant Chinese hamster ovary and human leukaemia cell lines (Kartner *et al.*, 1985). C219 binds to the cytoplasmic domain of Pgp.

2.2. Radioisotopes and Biochemicals

Lymphoprep™ (Nyegaard and Co., Oslo, Norway), trypsin 1:250 (Difco Laboratories, Detroit, MI, USA), PBS Dulbecco 'A' [phosphate-buffered saline] (Oxoid, UK), tissue culture media and antibiotics (Gibco, UK), tunicamycin, *Dactylium dendroides* galactose oxidase, *Vibrio cholerae* neuraminidase (Boehringer Mannheim, Germany), D-[6-^{3}H]glucosamine hydrochloride (20-40 Ci/mmol), sodium boro[^{3}H]hydride [NaB^3H_4, 5-10 Ci/mmol] (Amersham, U.K.), polyethylene glycol compound MW 15,000-20,000 [PEG 20,000], trifluoromethanesulfonic acid [TFMS], 3,3'-diaminobenzidine tetrahydrochloride [DAB] and Protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO., USA), Bio-Rad™ protein assay dye reagent concentrate (Bio-Rad Laboratories Ltd., U.K.) and protein MW standards and kits (Amersham, Bio-Rad Laboratories, Boehringer Mannheim and Sigma Chemical Co.) were used in this study. All other reagents were of analytical grade and were obtained from either Merck Chemicals or Sigma Chemical Co.

2.3. Preparation of Human Peripheral Blood Leukaemia Cells

Peripheral blood specimens were obtained from patients with acute leukaemia and collected into sterile heparinized tubes. Leukaemia blasts were isolated by minor modification of the method described by Böyum (1968). Peripheral blood was mixed with RPMI-1640 medium 1:2 (v/v) and layered on top of lymphocyte separation medium (Lymphoprep™, $\rho = 1.077\text{-}1.078$) and centrifuged (650g, 30 min at room temperature). Mononuclear cells were removed from the interface, washed three times in RPMI-1640 medium containing 1 mM L-glutamine, 1% MEM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and

15% heat-inactivated fetal bovine serum (HIFBS), referred to as complete medium. Contaminating erythrocytes were eliminated from mononuclear cells by NH_4Cl lysis (Böyum, 1984). Briefly, pelleted cells were resuspended in 10 mM Hepes buffer, pH 7.4, containing 0.83% NH_4Cl and incubated for 10 min at 37°C. The cells were then centrifuged for 5 min at 650g and the supernatant fluid aspirated. This step was repeated three times. Mononuclear cells were suspended in complete medium and cultured at 37°C in 5%CO_2:air and relative humidity 85% for 24-72h to eliminate adherent monocytes. The leukaemia blasts in suspension were recovered and either used immediately or stored at -70°C in complete medium containing 10% dimethylsulphoxide (DMSO) as cryopreservative.

2.4. Assay of Tumour Cell Viability

The percentage viable cells in culture was assessed by the method of Shier (1988) using trypan blue. Uptake of trypan blue was evaluated after allowing cells to stand at room temperature for 5 min and viewing with a Nikon inverted microscope fitted with phase optics and a yellow filter. A minimum of 5 fields and 250 cells were scored. Only cells with a viability more than 95% were used.

2.5. Metabolic Labelling of Tumour Cell Membrane Glycoproteins

UWOV2 cells were grown in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum (HIFBS) and antibiotics. Leukaemia blast cells were cultured in complete RPMI-1640. For metabolic labelling, cells were incubated with complete RPMI-1640 containing 10 mM Hepes and 10 mM glucose for 72h at 37°C in the presence of 10 μCi/ml [^{3}H]glucosamine with (TM-treated) or without (control) 5μg/ml TM.

2.6. External Labelling of Cell-Surface Glycoproteins

The surface galactose and N-acetylgalactose residues of cells were labelled by a modification of the technique described by Gahmberg and Hakomori (1973). Solid NaB^3H_4 (250 mCi) was dissolved in 10 mM NaOH to a concentration of 1 mCi/50 μl, immediately frozen under liquid nitrogen and stored in aliquots (100 μl/tube) at -70°C until used. The contents of one tube at a time were diluted with 2 ml 10 mM NaOH and divided into 20 equal portions that were immediately frozen. Routinely, one of these tubes was used for labelling experiments. The isotope remained stable for at least 10 months when handled in this way. Cells were washed 3 times with Dulbecco's PBS, resuspended to a density of 10^8 cells/ml and incubated for 10 min

at 4°C with 1 mM sodium metaperiodate. The reaction was quenched by adding 400 μl 100 mM glycerol in PBS and washing 3 times with PBS. Galactose oxidase (5.25 U/ml) or galactose oxidase (5.25 U/ml) and neuraminidase (25 U/ml) were added to cells resuspended to 10^8 cells/ml and incubated for 30 min with gentle agitation at room temperature in the presence of 10 μCi/ml NaB^3H_4. The labelled cells were washed four times with PBS and solubilized in 2% SDS in buffer A. Sample viscosity due to the release of DNA from cells was abolished by brief sonication (20s) and subsequent centrifugation at 104,000g for 60 min at room temperature and the supernatant (solubilized plasma membrane fraction) was analysed by gel filtration chromatography on Bio-Gel A-0.5M.

2.7. Preparation of Tumour Cell Membranes

Tumour cell membranes were prepared by minor modifications of procedures described previously (Riordan and Ling, 1979; Ujhazy *et al.*, 1990). All operations were carried out at 0-4°C. Tumour cells (control and TM-treated) were suspended to a density of 10^8 cells/ml in cold hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$ 1 mM DTT, 2 mM PMSF, 1% aprotinin) and incubated for 15 min in an ice-bath. The swollen cells were then disrupted with 20-50 strokes in a tight-fitting Dounce homogenizer. Phase-contrast microscopy revealed ~15% intact cells and disruption was continued with 10 more strokes followed by 4 cycles (45-s bursts with a 30-s interval) of sonication. This treatment disrupted >90% of the cells. Nuclei were removed by centrifugation of the cell lysate at 400g for 10 min. The supernatant was then centrifuged at 4000g for 10 min to eliminate mitochondria, and the supernatant so obtained subjected to high-speed centrifugation (104,000g, 60 min). The pellet was retained and resuspended by sonication in preservation buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM KCl, 150 mM NaCl, 25 mM $MgCl_2$, 250 mM sucrose, 1 mM EDTA, 10 mM NEM, 1 mM PMSF, 5 mM DTT, 5 mM 2-mercaptoethanol, 5 mM benzamidine-HCl, and 5 μg/ml each of pepstatin A, aprotinin and leupeptin) and stored in 1 ml aliquots at -70°C. This preparation yielded ~50 mg membrane wet weight that was suspended as 10 mg membrane /ml preservation buffer.

2.8. Detergent Solubilization of Membrane-Associated Glycoproteins

Plasma membranes (~50 mg protein) derived from tumour cells incubated for 72h in the absence (control) or presence (TM-treated) of 5 μg/ml TM was solubilized by boiling at 60°C for 5 min in 2% SDS in buffer A (50 mM Tris-HCl, pH 7.5, containing 7 mM 2-mercaptoethanol, 5 mM dithiothreitol (DTT), 1 mM

phenylmethylsulphonyl fluoride (PMSF), 10 mM N-ethylmaleimide (NEM), 20 mM ethylene diaminetetraacetic acid (EDTA), 5 mM benzamidine-HCl, 10 μg/ml leupeptin and 5 μg/ml pepstatin). The solution (~5 ml in Beckman Ultra-Clear™ tubes) was centrifuged for 60 min at 104,000g in a Beckman model SW 55 Ti swing-out rotor and model L8-80 ultracentrifuge at room temperature and the supernatant used in subsequent steps. An initial solubilization trial for UWOV2 cell-associated glycoproteins involved the extraction of [^{3}H]glucosamine-labelled material from cell lysates (prepared as described in section 3.7) with increasing concentrations of SDS in buffer A and centrifugation at 104,000g for 60 min at room temperature. The 104,000g supernatant and pellet fractions were assayed for radioactivity by liquid scintillation spectrometry.

2.9. Gel Filtration Chromatography

Dry Sephadex G-150, G-200 and Bio-Gel A-0.5M beads were suspended in buffer B (50 mM Tris-HCl, pH 7.5, containing 0.1% SDS, 10 mM mercaptoethanol, 2 mM EDTA, 2 mM PMSF and 10 mU/ml aprotinin) and allowed to swell at 90°C for 5h. The slurry was cooled to room temperature, washed twice in buffer B and poured into a column (1.5 X 85 cm) using a packing reservoir. The packed column was washed with 5 column volumes of buffer B described above and calibrated with buffer C (buffer B containing a micro-spatula tip each of blue dextran 2000 and phenol red to mark the void and the total volume, respectively). Solubilized tumour membranes (1 ml, ~100 μg protein) were applied on the top of the column. Elution was achieved with buffer B at room temperature. Fractions were standardized and aliquots counted by liquid scintillation spectrometry. Molecular weight calibration proteins were assayed by recording the absorbance at 280 nm. Peak fractions were pooled, dialyzed extensively against distilled water at 4°C and lyophilized before SDS-PAGE analysis. Relative MWs were determined from a plot of log MW vs K_{av} where K_{av} is given by the following relationship: $K_{av} = (V_e-V_o)/(V_t-V_o)$; V_e=elution volume, V_o=void volume (marked by elution position of blue dextran 2000) and V_t=total volume (marked by elution position of phenol red).

2.10. Deglycosylation of UWOV2 Cell-Surface Glycoproteins

Chemical deglycosylation of UWOV2 cell-surface glycoproteins was performed by the method described by Kalyan and Bahl (1981). Lyophilized samples containing between 10 and 30 μg/ml plasma membrane glycoprotein were treated with 50 μl trifluoromethane sulphonic acid (TFMS) in the presence of 25 μl anisole as

scavenger. After digestion at room temperature, the reaction was stopped at various time intervals by adding 250 μl pyridine diluted 1:4 (v/v) in distilled water and by cooling samples on ice for 15 min. Samples were then dialyzed for 48h against several changes of distilled water and concentrated by either reverse dialysis against PEG 20,000 or lyophilization, before neutralization and SDS-PAGE.

2.11. Immunoprecipitation of UWOV2 Cell-Surface Glycoproteins

UWOV2 plasma membranes were immunoprecipitated according to the method described by Foxwell *et al.* (1989). Whole cells (10^8) or purified plasma membranes (100 μg protein) were solubilized in 200 μl 50 mM Tris-HCl, pH 7.4, containing 0.2% SDS, 1.25% Nonidet P-40 (NP-40), 150 mM NaCl and 2 mM PMSF, and centrifuged at 12,000g for 10 min at 4°C. Solubilized samples (supernatants) were incubated for 16h at 4°C with either 10 μg C219 mAb or 25 μg MRK-16 mAb, specific for Pgp. Control samples were treated with normal mouse serum. Protein A-Sepharose CL-4B (20%, v/v in the above buffer) was added to the precipitates and incubated at 4°C for a further 120 min. The protein A-Sepharose-mAb complexes were washed 5 times with 50 mM Tris-HCl, pH 7.4, containing 0.1% NP-40, 0.03% SDS, 150 mM NaCl, 1 mg/ml BSA, 2 mM PMSF and once with Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl), before processing for SDS-PAGE.

2.12. Immunocytochemical Detection of P-Glycoprotein

Immunohistochemical detection of MDR-associated Pgp with P-glycoCHEK™ was performed as recommended by the manufacturer. UWOV2 cells and acute leukaemia blast cells were centrifuged at 150g for 5 min onto slides and either stored at -20°C as acetone-fixed slides or used immediately as cytospin slide preparations. The following criteria were considered necessary for a valid test, which if not met, made the results of unknown specimens suspect: positive staining with P-glycoCHEK™ (brown or dark brown) in more than 40% of positive cells on the control slide, staining should be localized typically on the cell membrane, and in immunoperoxidase reactions in the cytoplasm as well, giving a heterogeneous and often polarized pattern, minimal or no staining (purple) of negative cells with glycoCHEK C219™, and minimal or no staining with negative control antibody in either cell preparations. Cytospin slides were incubated with 100 μl of 5% normal horse serum in PBS/1% BSA for 10 min at 4°C. Normal serum was removed and replenished with 100 μl 10 mg/ml P-glycoCHEK C219™ in normal horse serum

and incubated for 60 min on ice. The slides were then rinsed 3 times (duration of 5 min each) in cold PBS and excess liquid on the slides was blotted off before application of 100 μl biotinylated anti-mouse IgG solution (5 μl biotinylated IgG plus 2.5 ml 5% normal horse serum) for 30 min. The slides were again rinsed in PBS as described above and exposed to 100 μl of avidin-biotinylated horseradish peroxidase conjugate for 60 min. Following another rinse in PBS, slides were covered for 10 min with DAB (3,3'-diaminobenzidine tetrahydrochloride, prepared by dissolving 10 mg DAB 0.1 M Tris-HCl, pH7.2 and adding 3 μl 30% H_2O_2 prior to filtering) as chromogen substrate. After removal of the chromogen solution, the slides were rinsed once in PBS, counterstained with Mayer's haematoxylin for 45-60s and mounted in aqueous mounting media. Peroxidase activity was visualized according to the manufacturer's instructions and expressed either as positive or negative according to the above criteria.

2.13. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a Bio-Rad model MiniProtean™ mini-gel apparatus or a Hoeffer model SE-600 vertical slab gel apparatus using the discontinuous buffer system described by Laemmli (1970). Slab gels (1-1.5 mm thick) consisted of a linear 5-15% acrylamide gradient supporting gel and a 3.5% stacking gel. Samples, equalized for protein and radioactivity, were prepared for electrophoresis by boiling for 5 min in an equal volume of sample treatment buffer consisting of 0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol and 0.002% bromophenol blue. Electrophoresis (usually 30 mV, constant current, 12-16h) was continued until the tracking dye reached the bottom of the gel. Gels were stained with Coomassie brilliant blue R-250, dried on a Bio-Rad model 583 gel dryer, and photographed. Molecular weights of samples were estimated by comparing the relative mobilities with those of standard proteins.

2.14. Densitometry of SDS Gels

Densitometric scans of dried gels were obtained on a Shimadzu model CS-9000 dual wavelength flying-spot densitometer. Intensities of the UWOV2 plasma membrane glycoprotein bands in the 170-kDa region of control and TFMS-deglycosylated samples were compared.

2.15. Protein Estimation

The total protein content in solubilized cells and cell lysates was estimated by utilizing the Bio-Rad™ protein dye-binding assay kit based on the method described by Bradford (1976) with bovine serum albumin as standard.

2.16. Patient Studies

Patients with acute myeloblastic leukaemia were selected for this study. Only cell samples with more than 95% viability were studied. The clinical and haematological data of the patients are presented in Table 2.

3. RESULTS

Leukaemia blast cells isolated from peripheral blood of patients with AML, and the human ovarian carcinoma cell line (UWOV2), were characterized for their expression of the 170-kDa MDR-associated Pgp by gel filtration chromatography, SDS-PAGE and immunohistochemical localization using Pgp-specific mAbs. Typical elution profiles and MW calibration curves obtained for Sephadex G-150 (Figure 1), Bio-Gel A-0.5M (Figure 2A) and Sephadex G-200 (Figure 2B) are shown. The chromatographic properties of the protein MW markers eluted from the different gel types are summarized in Table 1.

Bio-Gel A-0.5M filtration profiles of NaB^3H_4-labelled plasma membrane glycoproteins isolated from leukaemia blast cells prepared from peripheral blood of patients L24 and L100 are presented in Figures 3A and 3B, respectively. Tritiated plasma membrane glycoproteins prepared from both patients eluted as distinct peaks in the regions between 450-158 kDa and 158-44 kDa. The peaks that eluted beyond V_t (marked by phenol red) represent free or degraded radioactive material of low MW. Fractions constituting the peaks that eluted in the high MW region (*i.e.*, 450-158 kDa) of the column profile were pooled, lyophilized and prepared for SDS-PAGE. Coomassie blue staining of the gels revealed the presence of a 170-kDa band in samples prepared from both patients L24 and L100 (Table 2). This finding was confirmed in a separate experiment involving SDS-PAGE of L100 plasma membranes solubilized directly with SDS (Figure 8). In similar experiments, SDS-PAGE analysis of pooled, lyophilized high MW peak fractions (eluted from Sephadex G150, Sephadex G-200 or Bio-Gel A-0.5M columns) or purified plasma membranes, resolved a 170-kDa band in samples prepared from patients L19, L23, L24, L28, L31, L45, L84, L94, L95, and L97, but not from

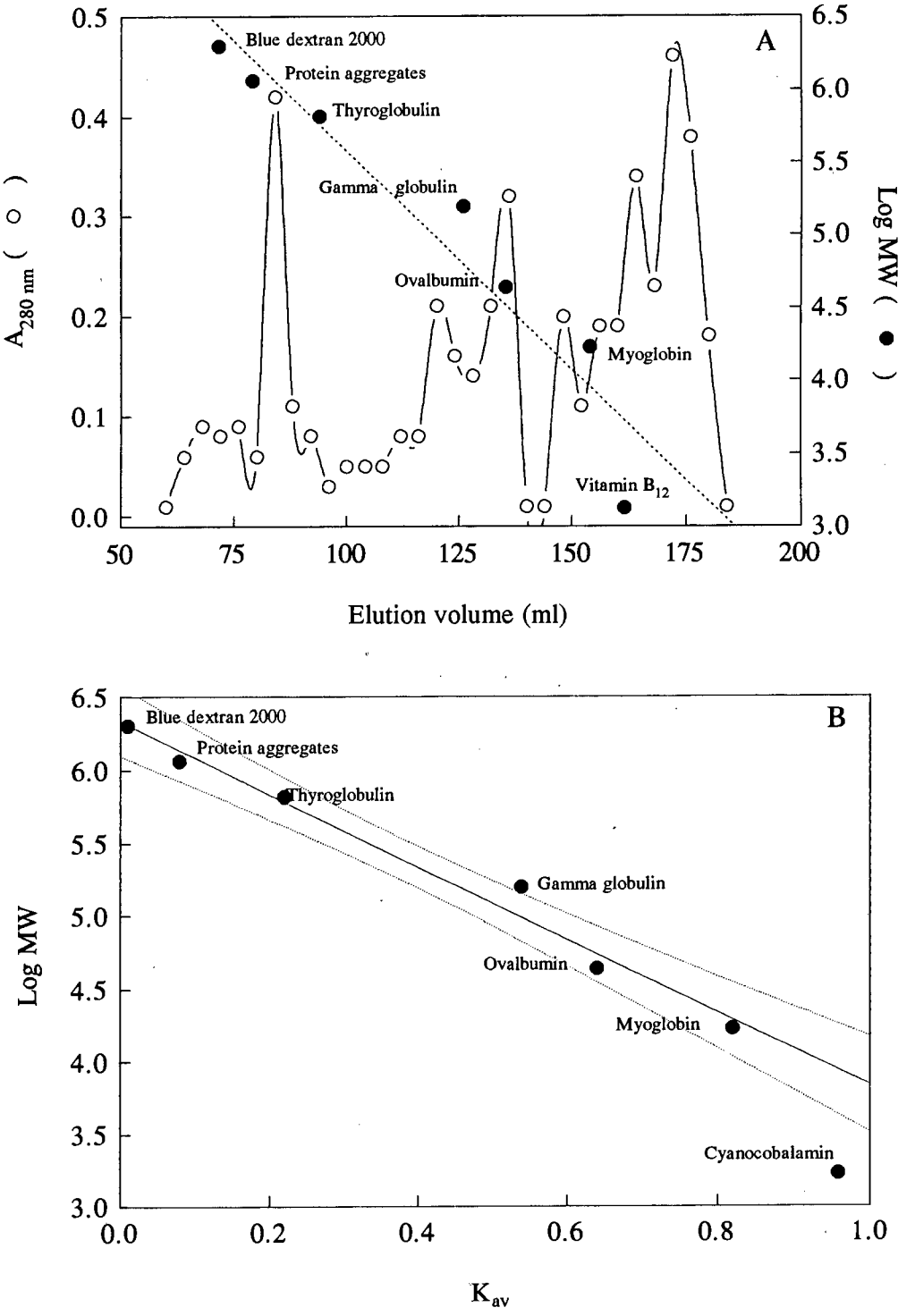

Figure 1. Molecular weight calibration of Sephadex G-150. Calibration proteins were solubilized and eluted at room temperature with 50 mM Tris-HCl, pH 7.5, containing 0.1% SDS, 10 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM PMSF and 10 mU/ml aprotinin. Positions of protein peaks were obtained by measuring elution volumes and recording absorbances at 280 nm (A). A plot of log MW against the partition coefficients (K_{av}) of the standard proteins (B) shows linearity of elution. Vitamin B_{12} (cyanocobalamin) eluted anomalously (B, K_{av} lies outside the 95% confidence band indicated by the dotted line). Pharmacia column: 1.5 X 85 cm; Pharmacia P1 peristaltic pump constant flow rate: 1 x 10 pulses; Pharmacia Frac-100 fraction collector setting: 60 min/fraction. Run time: ~96h.

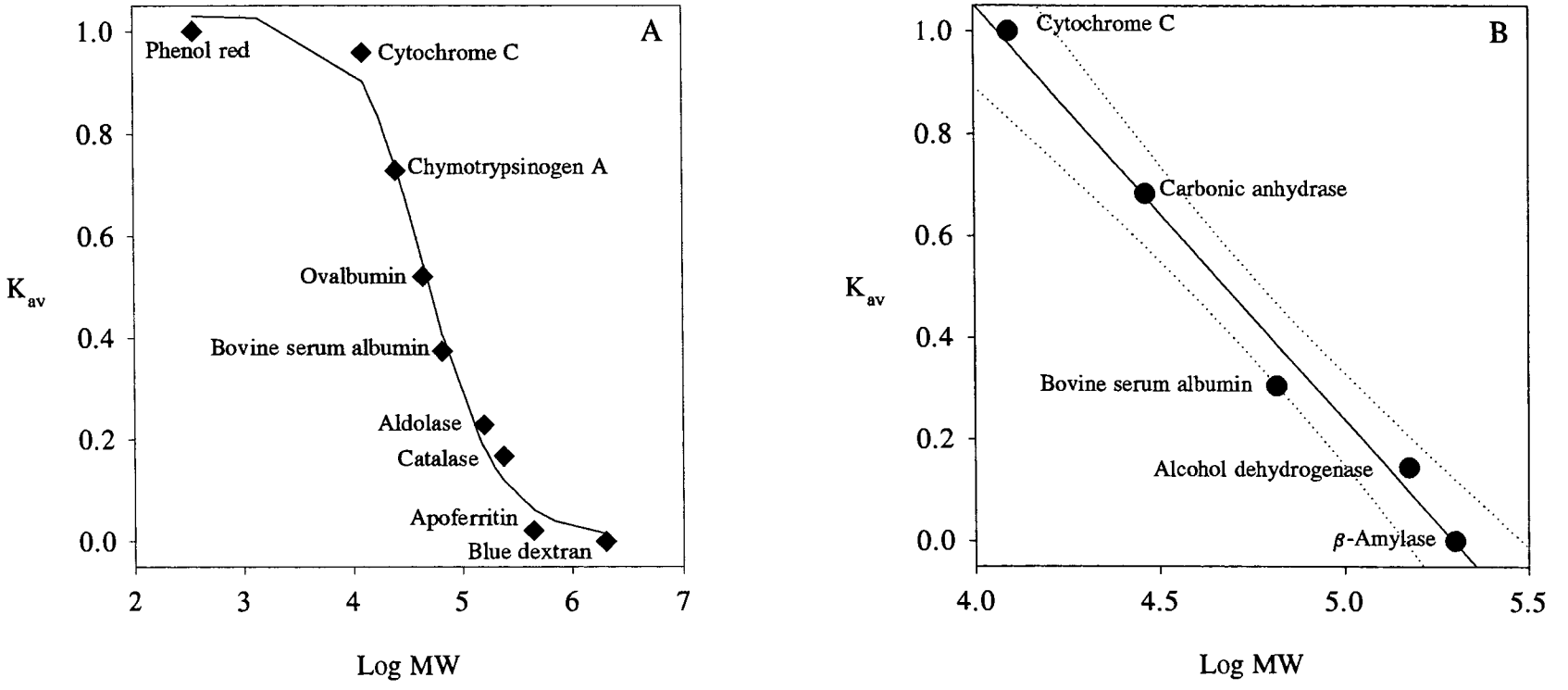

Figure 2. Calibration curves typically obtained for proteins separated on (A) Bio-Gel A-0.5M and (B) Sephadex G-200. See Figure 1 for details. The partition coefficients (K_{av}s) for standard proteins eluted from Bio-Gel A-0.5M fall within the 95% confidence limits (not shown). Dotted lines indicate the 95% confidence band of K_{av} estimates for protein standards eluted from Sephadex G-200.

Table 1. Relationship Between Molecular Weight and Elution Behaviour of Various Calibration Proteins on Sephadex G-150 Sephadex G-200 and Bio-Gel A-0.5M Gel Filtration Columns

		K_{av} Values/Gel type		
Molecular weight markers	MW (kDa)	Sephadex G-150	Sephadex G-200	BioGel A-0.5M
Blue dextran	2000	$V_o(0)$		$V_o(0)$
Thyroglobulin	669	0.220		
Apoferritin	450			0.021
Catalase	240			0.167
β-Amylase	200		$V_o(0)$	
Aldolase	158			0.229
γ-Globulin	158	0.540		
Alcohol dehydrogenase	150		0.144	
Bovine serum albumin	66		0.304	0.374
Ovalbumin	44	0.640		0.521
Carbonic anhydrase	29		0.682	
Chymotrypsinogen A	25			0.729
Myoglobin	17	0.820		
Cytochrome C	12.4		$V_t(1.0)$	0.958
Cyanocobalamin	1.35			
Phenol red	0.354	$V_t(1.0)$		$V_t(1.0)$

K_{av}, partition coefficient; kDa, kilodaltons; V_o, void volume marked by phenol red; V_t, total column volume marked by blue dextran 2000.

Table 2. Analysis of P-Glycoprotein Expression in Human Peripheral Blood leukaemia Cells by Gel Filtration, SDS-PAGE and the P-glycoMab™ Diagnostic Kit

Patient	Gender	Age	Diagnosis (FAB)	Course/Management	Sephadex G-150*/G-200	BioGel A0.5M	SDS-PAGE	P-glycoMab™ reaction
L19	M	33	AML	No chemotherapy	0.052-0.224	ND	+	-
L21	M	22	AML, M1	VP-16, single dose	ND	ND	-	-
L23	M	39	ALL	Ara-C, VP-16, MTX	0.017-0.224	ND	+	-
L24	M	19	AML resistant to treatment	Resistant to VP-16, Ara-C, MTX	ND	0.009-0.138	+	+
L26	F	69	AML	Paliative therapy	0.086-0.284	ND	-	-
L28	M	42	AML, M1	VP-16, DNR, Ara-C	ND	ND	+	-
L31	F	82	ANLL	No chemotherapy	ND	ND	+	-
L42	M	31	AML	Terminal care	V_o-0.120*	ND	-	-

(Continued)

Table 2. (continued)

Patient	Gender	Age	Diagnosis (FAB)	Course/Management	Sephadex G-150*/G-200	BioGel A0.5M	SDS-PAGE	Monoclonal antibody C219 Reaction
L45	M	44	AML, M3	Ara-C, VP-16, DNR	ND	ND	+	-
L55	M	67	AML	Terminal care	ND	0.009-0.157	-	-
L84	F	46	AML, M1	CTR4 induction therapy	ND	ND	+	-
L94	M	21	AML, M2	CTR4 induction therapy	ND	ND	+	
L95	M	44	AML, M1	Palliative therapy	ND	ND	+	-
L97	M	50	AML	VP-16, ADR, MTX	ND	ND	+	-
L100	M	49	Relapsed AML	Ara-C, VP-16, DNR	ND	0.009-0.138	+	+

Abbreviations: FAB, French-American-Bristish Co-operative Group classification of the acute leukaemias (Bennett *et al.*, 1976; Bennett *et al.*, 1981); VP-16, etoposide (4'-dimethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); Ara-C, cytosine arabinoside; MTX, methotrexate; DNR, daunorubicin; ADR, adriamycin; CTR4, cytosine arabinoside-etoposide-daunorubicin (Cape Town regimen 4).

samples prepared from patients L21 and L26 (Table 2 and Figure 8) and patients L42 and L55 (Table 2).

The use of SDS in the solubilization of plasma membrane glycoproteins was evaluated in UWOV2 ovarian carcinoma cells. The solubilization trial was conducted in an attempt to optimize the extraction of cell-surface glycoproteins from these cells. Figure 4 illustrates the recovery of [^{3}H]glucosamine-labelled glycoproteins from the 104,000g supernatant and pellet fractions as a function of detergent concentration. The increased recovery of radiolabel from the supernatant fraction was concentration-related, whereas that recovered from the pellet remained constant at all detergent concentrations. Optimal solubilization, relative to control (0% SDS), was achieved at SDS concentrations in excess of 0.1 %. The 104,000g supernatant fraction was taken to represent the plasma membrane fraction and an SDS concentration of 2% was considered sufficient to extract the majority of cell-surface glycoproteins.

Separation of cell-surface glycoproteins derived from UWOV2 cells metabolically labelled with [^{3}H]glucosamine on a column of Bio-Gel A-0.5M, yielded high MW peak fractions in the 240-158 kDa region of the column profile (Figure 5), suggesting, but not confirming, the presence of a 170-kDa glycoprotein. This finding was corroborated by Sephadex G-200 fractionation of [^{3}H]glucosamine-labelled plasma membrane glycoproteins isolated from UWOV2 cells following 72h incubation in the absence (control) and presence (TM-treated) of 5 μg/ml TM (Figure 6). High MW UWOV2 plasma membrane glycoproteins eluted as a broad peak in the 200- and 150 kDa range of the Sephadex G-200 chromatographic profile (Figure 6). This peak was reduced to approximately 53% (obtained by integration of area under peak) of control in the presence of TM, indicating inhibition of glycoprotein synthesis. This presence of the purported 170-kDa glycoprotein in UWOV2 cells (Figure 7A and Figure 8) and its disappearance as a result of prolonged TM exposure (Figure 8) were confirmed by SDS-PAGE analysis of purified plasma membrane samples. Chemical deglycosylation of the 170-kDa protein with TFMS for 30 min reduced its band intensity by about 5%, but did not alter the MW of the 170-kDa protein (Figure 7B). At later times, the band intensity was reduced by more than 80%, indicating cleavage of peptide bonds. Since the deglycosylation was not optimized, no conclusion regarding the contribution of oligosaccharaide moieties to the overall MW of the 170 kDa glycoprotein could be made. When UWOV2 plasma membranes were immunoprecipitated with Pgp-specific mAbs MRK16 and C219, the 170-kDa band was again present, although contaminating bands were also noted (Figure 8). This

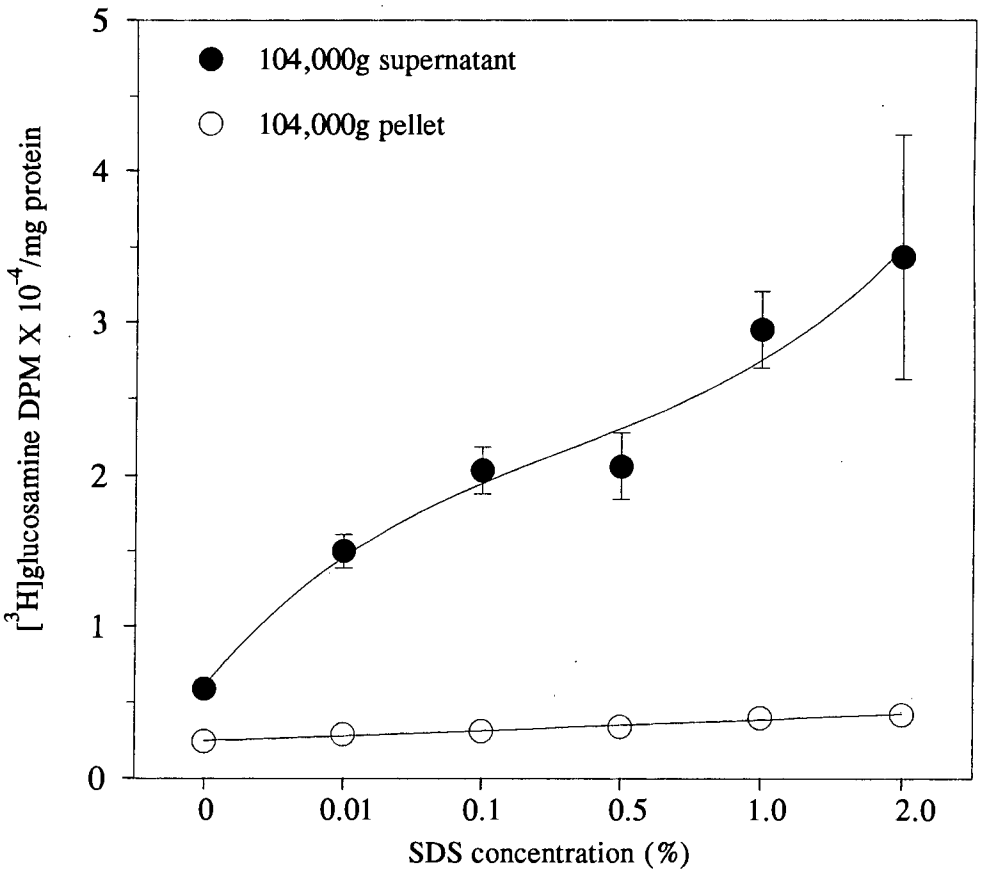

Figure 4. Solubilization profiles for [^{3}H]glucosamine-labelled glycoproteins extracted from UWOV2 ovarian carcinoma cells. Cells were metabolically labelled with [^{3}H]glucosamine and lysed in hypotonic buffer. Cell lysates, from which nuclei and mitochondria were removed by differential centrifugation, were solubilized with increasing concentrations of SDS in buffer A and re-centrifuged for 1h at 104,000g at room temperature as described in "Materials and Methods". The supernatant and pellet fractions so obtained were equalized for protein and assayed for radioactivity by liquid scintillation spectrometry.

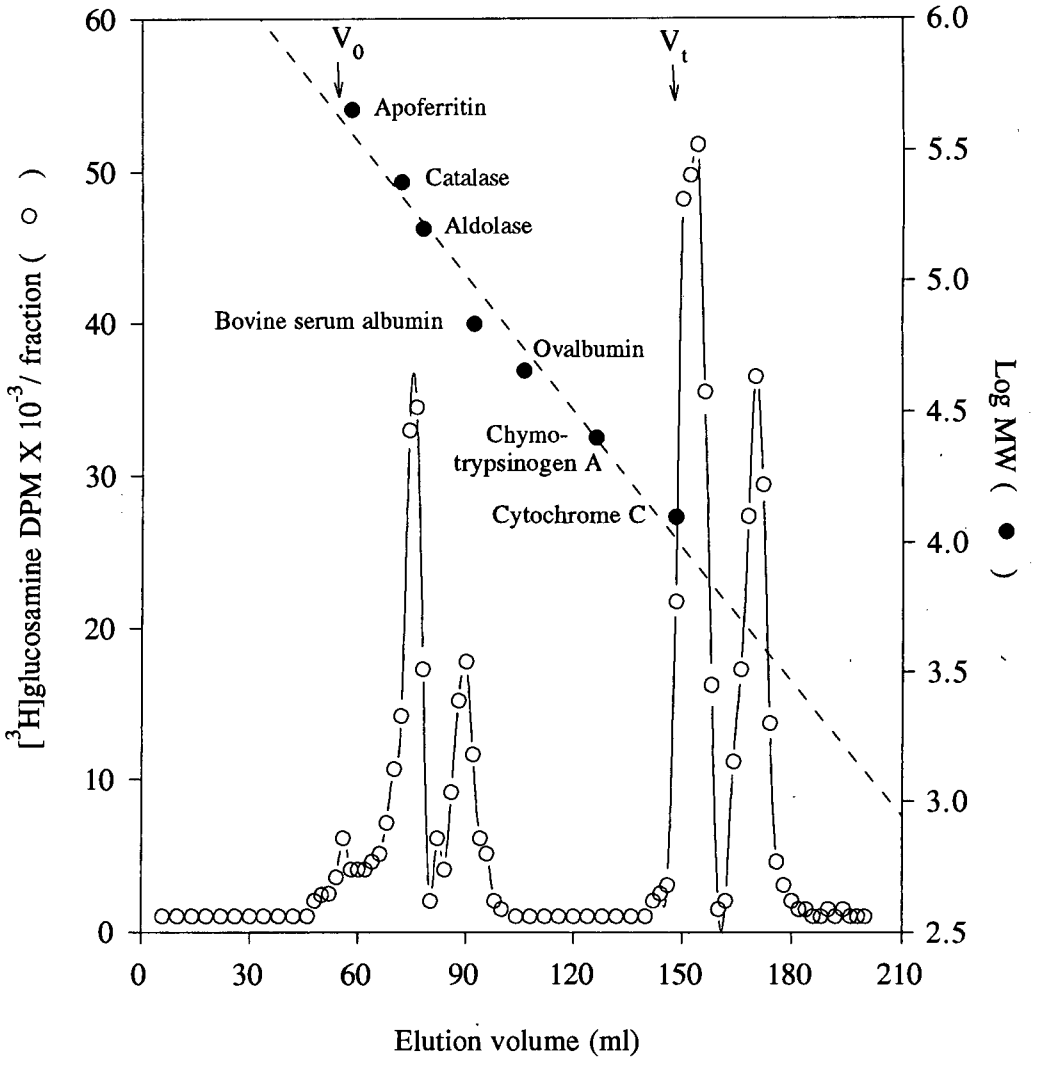

Figure 5. BioGel A-0.5M chromatography of UWOV2 ovarian carcinoma cell-surface glycoproteins. Cells, metabolically labelled with [^{3}H]glucosamine, were lysed in hypotonic buffer and the nuclei and mitochondria removed by differential centrifugation as described in "Materials and Methods". The resultant cell lysate was solubilized in 2% SDS/buffer A and centrifuged at 104,000g for 1h at room temperature. The 104,000g supernatant, containing radiolabelled plasma membrane glycoproteins, was mixed with calibration proteins and a spatula tip (~0.5 mg) each of blue dextran and phenol red. The mixture was layered on top of the column and allowed to enter the gel prior to elution with buffer B. The elution of calibration proteins was monitored by recording the absorbance at 280 nm and the elution volumes plotted against the respective log MWs (see Table 1 for MWs and K_{av} values). Arrows mark the elution positions of blue dextran (V_0) and phenol red (V_t).

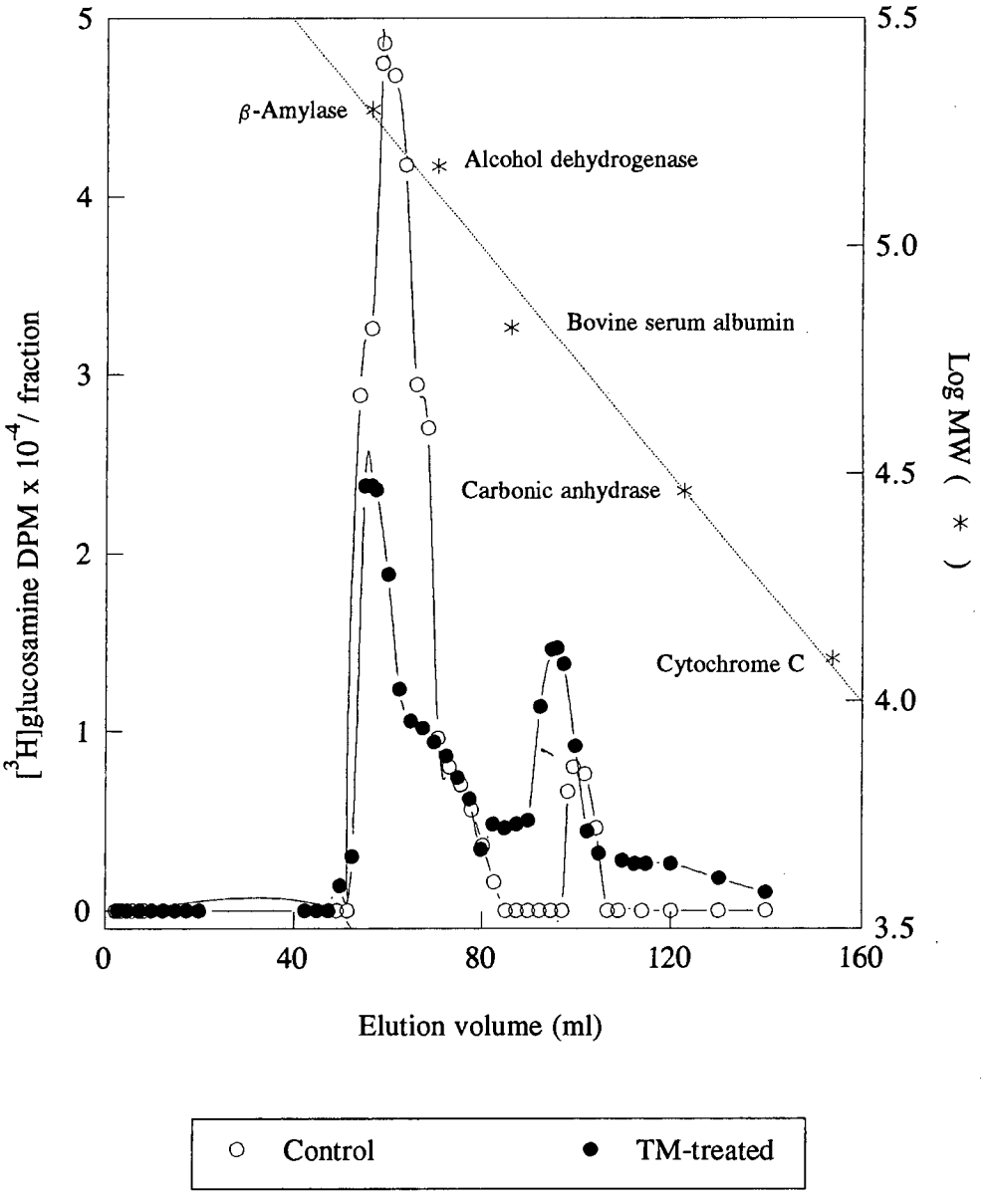

Figure 6. Sephadex G-200 chromatography of UWOV2 ovarian carcinoma cell-surface glycoproteins. Cells, metabolically labelled with [^{3}H]glucosamine in the absence (control) or presence (TM-treated) of 5 μg/ml tunicamycin (TM), were lysed in hypotonic buffer and the nuclei and mitochondria removed by differential centrifugation as described in "Materials and Methods". For details see the legend to Figure 5.

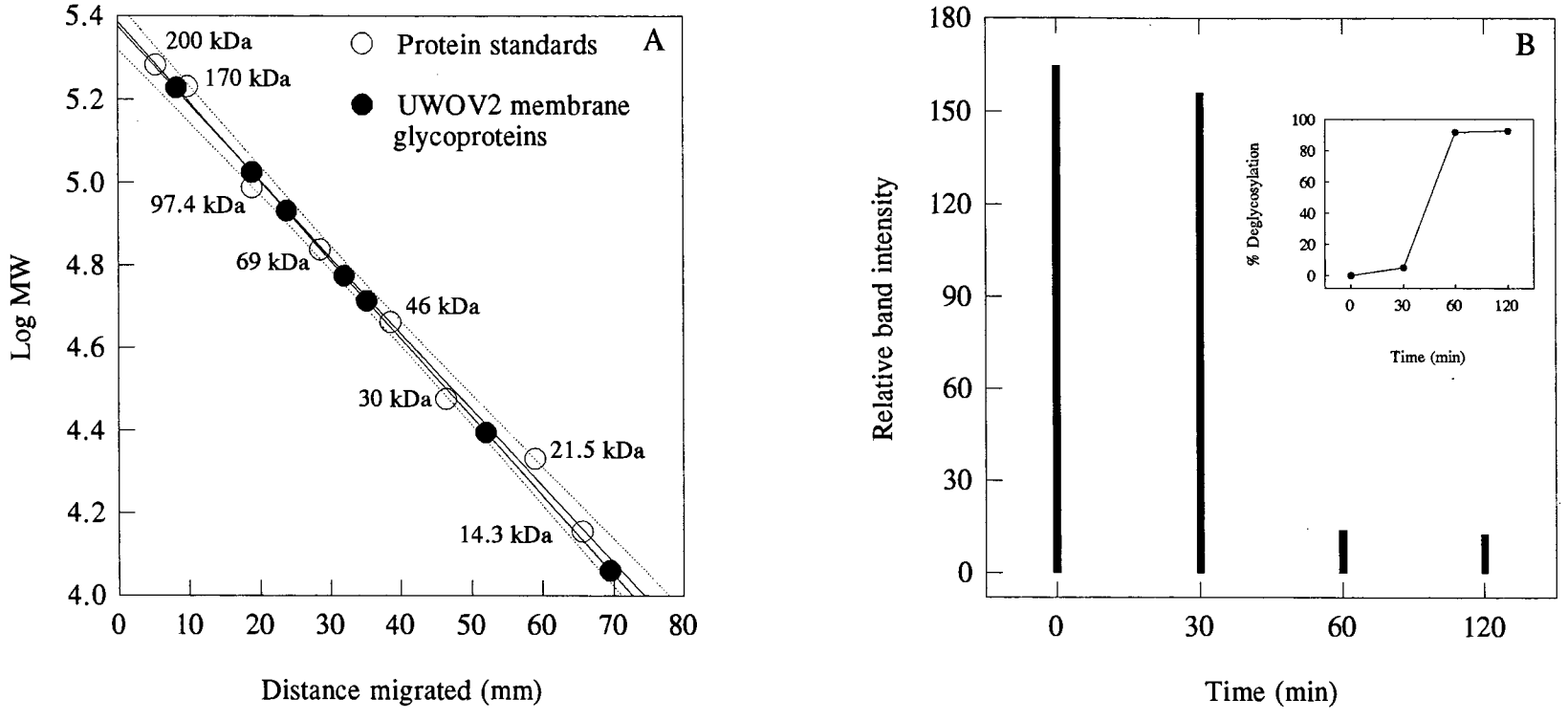

Figure 7. Electrophoretic mobilities (A) of UWOV2 plasma membrane glycoproteins. Dotted line represents the 95% confidence interval of MW estimation. Relative band intensity (obtained by densitometric tracing of dried gels) and % deglycosylation (inset) of the 170-kDa UWOV2 plasma membrane glycoprotein at various time periods after chemical deglycosylation with TFMS are shown (B). Protein standards[§]: myosin (200 kDa), α_2-macroglobulin[¶] (non-reduced 340 kDa, reduced 170 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa). [§]Rainbow™ protein molecular weight markers (Amersham), [¶]Combithek® calibration proteins (Boehringer Mannheim).

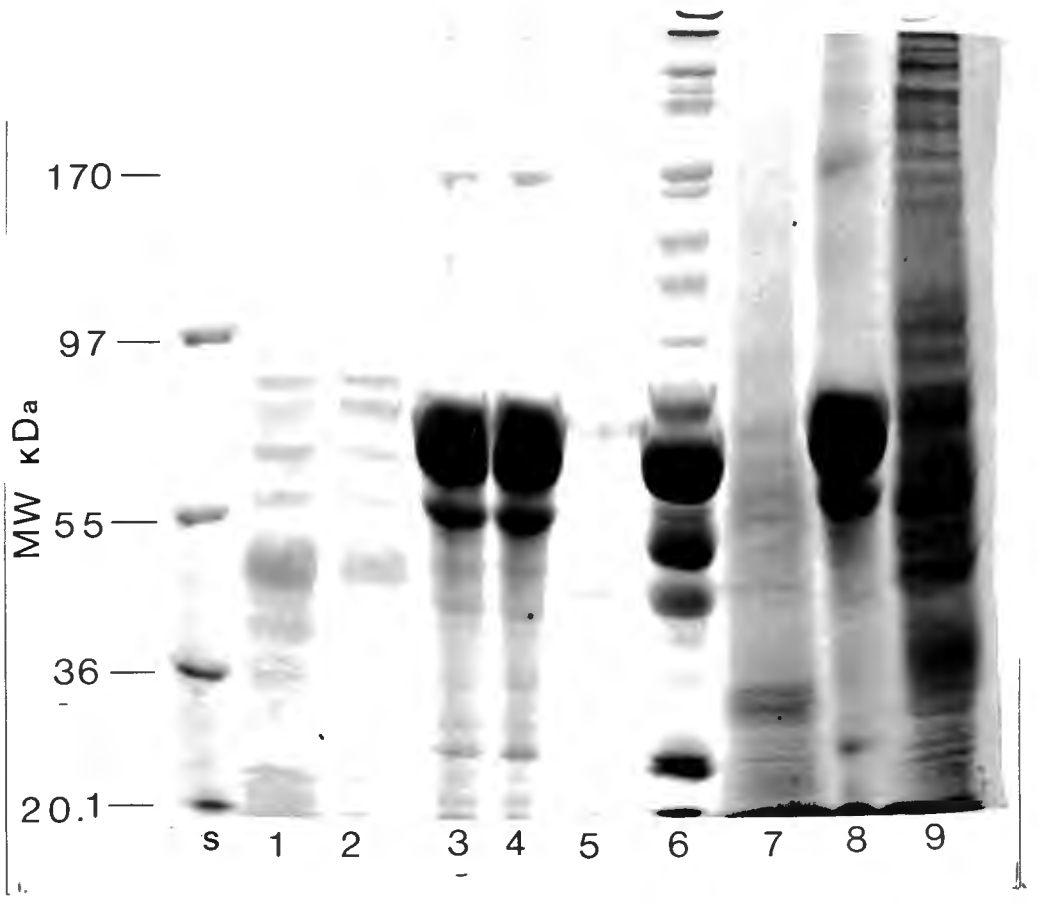

Figure 8. SDS polyacrylamide gel electrophoretic profiles of samples prepared from patient material and the human UWOV2 ovarian carcinoma cell line. Samples were processed for SDS-PAGE as described in "Materials and Methods". Lane s, molecular weight standards: α_2-macroglobulin (170 kDa, reduced), phosphorylase b (97.4 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), trypsin inhibitor (20.1 kDa); lanes 1 and 2, plasma membranes from patients L21 and L26, respectively; lanes 3 and 4, UWOV2 plasma membranes immunoprecipitated with mAbs MRK16 and C219, respectively; lanes 5 and 6, plasma membranes derived from UWOV2 cells after a 72-h incubation in the presence (TM-treated) and absence (control) of tunicamycin, respectively; lanes 7 and 8, negative and positive control for mAb C219, respectively; lane 9, 104,000g supernatant fraction recovered after solubilization of cell lysates prepared from patient L100.

is probably due to non-specific binding and/or insufficient removal of proteins that might have entered the Sepharose CL-4 beads during the immunoprecipitation step.

The expression of Pgp was examined in UWOV2 cells and peripheral blood leukaemia blast cells prepared from a randomly selected group of patients with AML. The expression of mdr1 gene product, Pgp, in the aforementioned cells was compared by means of immunocytochemistry, using the mAb C219 and the avidin-biotin-immunoperoxidase method. The drug-sensitive human acute lymphoblastic leukaemia cell line, CCRF-CEM (Pgp-negative; purple) and its drug-resistant derivative, CEM-VLB_{100} (strongly Pgp-positive; dark brown) were used as controls (Plate 1). Immunoperoxidase staining showed expression of C-219 reactive Pgp in the UWOV2 cell line (Plate 2). Two out of the 15 samples prepared from patients with AML that were analysed with the P-glycoCHEK™ diagnostic kit stained positively for Pgp (Table 2, Plate 2). Although SDS-PAGE analysis of some samples from AML patients, who had either received chemotherapy or not, showed the presence of a 170-kDa membrane protein, this could not be confirmed by the C219 mAb reaction using the standard exclusion criteria (Table 2).

4. Discussion

Effective management of ovarian carcinomas is often limited by their clinical non-responsiveness to chemotherapy. Cell lines derived from these tumors manifest aspects of the MDR phenotype to various extents (Finstad *et al.*, 1990; Sekiya *et al.*, 1992; Wu *et al.*, 1992). However, the development of resistance to drugs that are most active against ovarian carcinoma may occur through mechanisms other than the expression of Pgp (Rubin *et al.*, 1990; Misawa *et al.*, 1992). Despite this and the large variability in Pgp levels observed in patients with ovarian cancer (van der Zee *et al.*, 1991), the insensitivity of this malignancy to chemotherapy can be explained, in part, by the expression of the *mdr*1 gene product (Rutledge *et al.*, 1990; Holzmayer *et al.*, 1992; Sekiya *et al.*, 1992). Thus, ovarian carcinoma cell lines derived from clinically drug-resistant patients will facilitate *in-vitro* anticancer drug screening which may allow the identification of potentially valuable treatment regimens (Wu *et al.*, 1992). The results from this study show that UWOV2 ovarian carcinoma cells express the 170-kDa membrane Pgp that is synthesized at reduced rates following TM exposure. Thus, TM may be used *in vitro* as a resistance modifier in MDR. The appearance of a 170-kDa membrane glycoprotein in the plasma membranes of the UWOV2 carcinoma cell line is further in agreement with reports of Pgp expression in human ovarian tumours and cell lines (Bourhis *et al.*, 1989; Bradley *et al.*, 1989; Volm *et al.*, 1989).

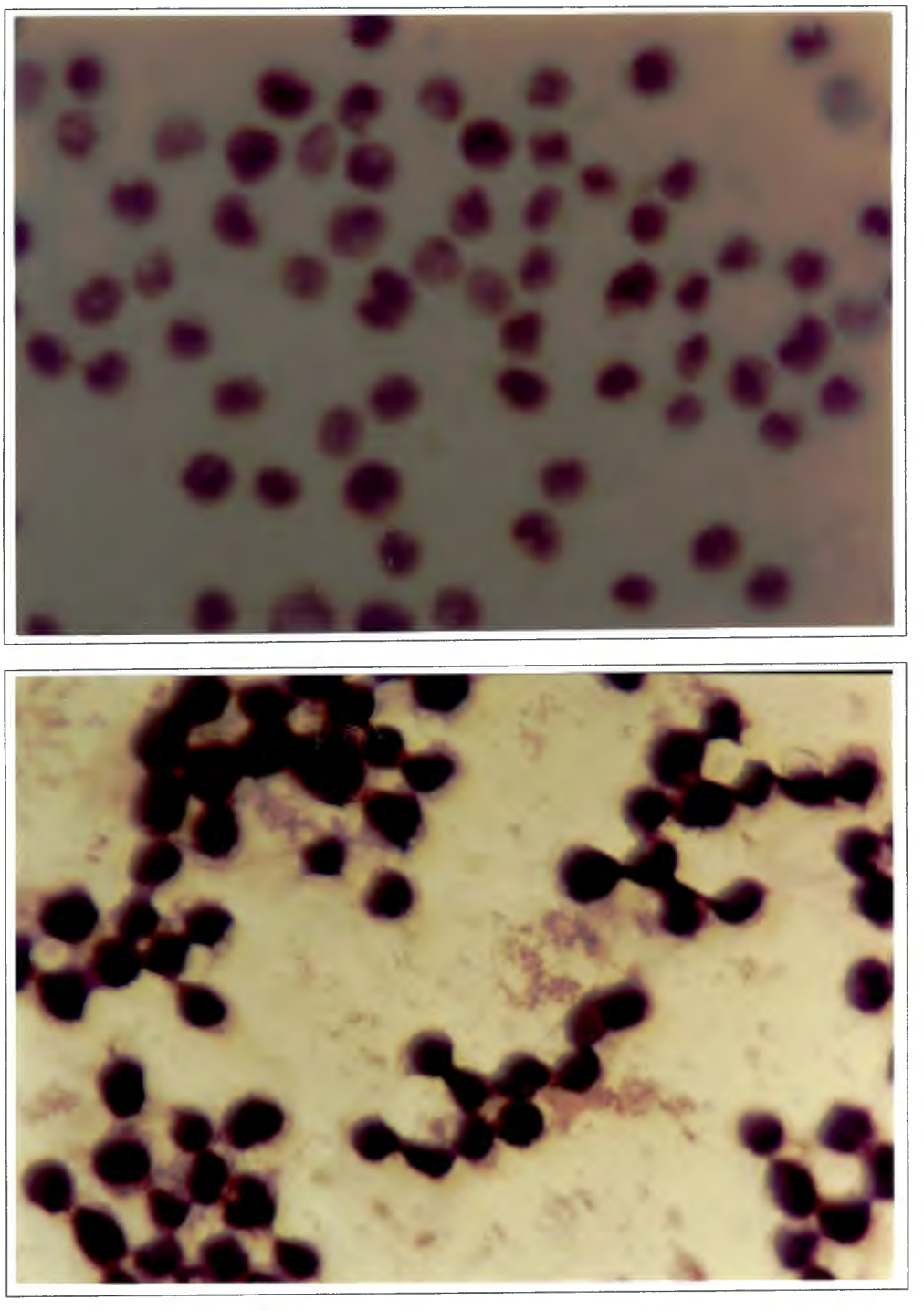

Plate 1. Phase-contrast micrographs of the drug-sensitive human acute lymphoblastic leukaemia cell line, CCRF-CEM (P-glycoprotein-negative control slide, top) and its drug-resistant derivative, CEM-VLB_{100} (P-glycoprotein-positive control slide, bottom) stained with the P-glycoMab™ mAb C219 avidin-biotin-immunoperoxidase technique.

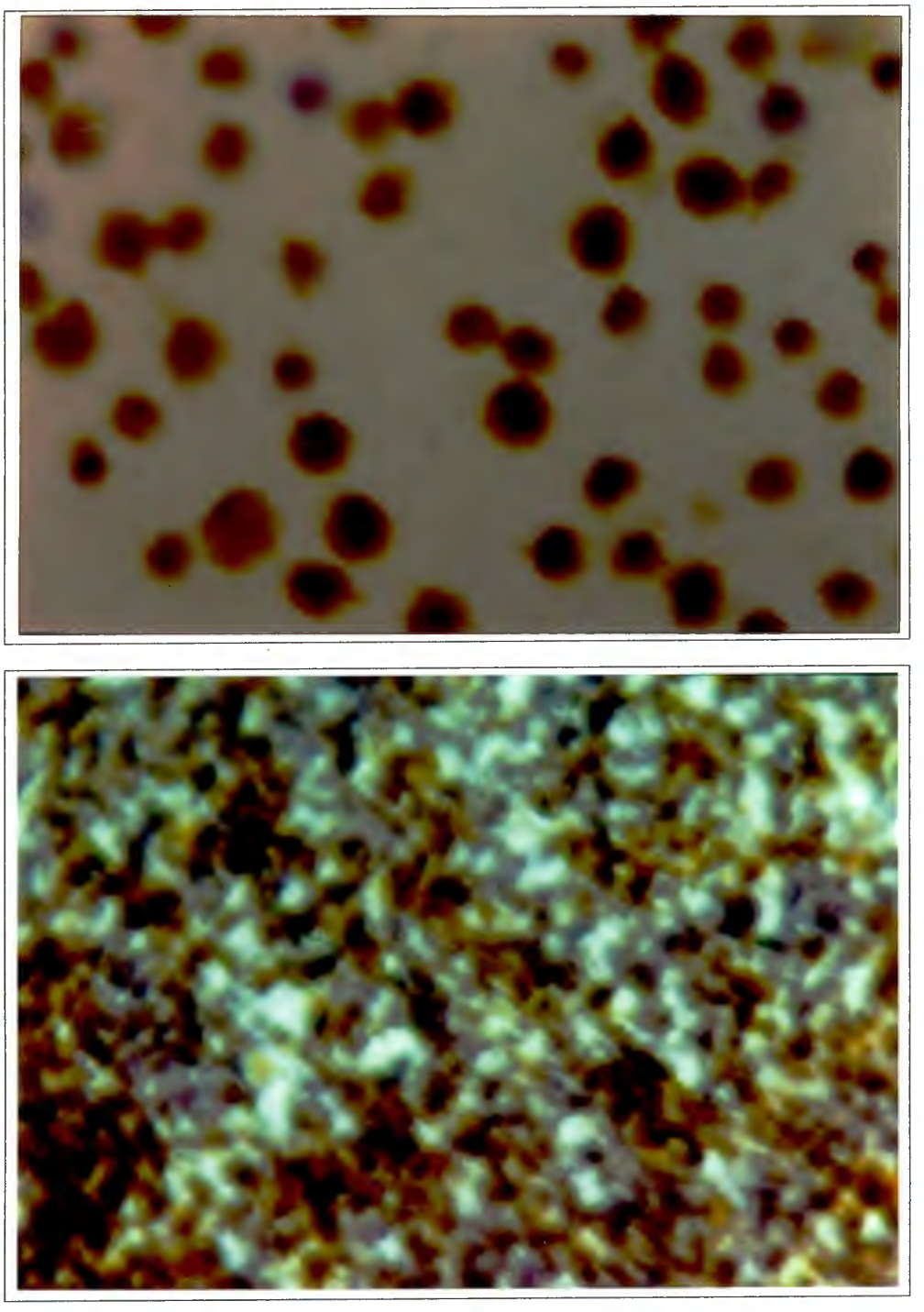

Plate 2. Phase-contrast micrographs of cytocentrifuge preparations of the human ovarian carcinoma cell line, UWOV2 (top) and peripheral blood leukaemia blast cells isolated from patient L24 (bottom) exhibiting positive staining with the P-glycoMab™ mAb C219 avidin-biotin-immunoperoxidase complex (compare with staining patterns of +ve and -ve controls in Plate 1).

Analysis of Pgp expression in patient samples using the P-glycoCHEK™ immunocytochemical diagnostic kit, stained positively only 2 out of 15 samples. The 2 samples that stained positively were obtained from patients with acute leukaemia in relapse and who were unresponsive to combination chemotherapy with cytosine arabinoside, daunorubicin and methotrexate. These data would suggest a significant relationship between failure of chemotherapy in these patients and expression of the *mdr*1 gene product. No difference in staining patterns could be discerned between samples prepared from treated and untreated patients. In some cases, SDS-PAGE analysis of plasma membrane proteins prepared from AML patients who had received no prior chemotherapy as well as those who had received either single-dose or combination chemotherapy revealed the presence of a 170-kDa protein. However, no clinical correlation could be made of the presence of this 170-kDa protein with clinical status because Pgp was not identifiable in these samples by immunocytochemical staining using the C219 mAb reaction. Samples that were diffusely positive were regarded as negative. Since the C219 antibody cannot distinguish between the *MDR*1 and the *MDR*2 gene products (Georges *et al.*, 1990a), it cannot be concluded that Pgp is not present in the samples that yielded a 170-kDa band by SDS-PAGE analysis.

The low incidence of AML patients expressing Pgp was expected since the samples were selected at random and the progression of the condition during chemotherapy was not followed. Perhaps a different approach to the analysis of Pgp expression in AML patients would be necessary, including staging of the disease following chemotherapy. Presumably the variations detected in staining of Pgp for the various AML cells are the result of patient differences. Nevertheless, other studies have demonstrated that resistance to chemotherapy remains an important cause of treatment failure in acute leukaemias (Kuwazuru *et al.*, 1990; Marie *et al.*, 1991; List and Spier, 1992). P-glycoprotein is detected with high frequency in AML and in prospective studies its overexpression is an independent determinant of response to treatment and overall survival with conventional dose-induction regimens (Chitnis *et al.*, 1991; Musto *et al.*, 1991b; Pirker *et al.*, 1991). Therapeutic studies aimed at testing various MDR-reversal agents, such as verapamil, quinine and several others, to restore drug sensitivity will imminently improve treatment outcome in high-risk acute leukemias (Sonneveld and Nooter, 1990; Marie *et al.*, 1992b; Solary *et al.*, 1992).

Recently, Pieters *et al.* (1991) reported that *in-vitro* drug sensitivity assays (*e.g.*, MTT assays) which measure gross cellular resistance may have prognostic value in relation to initial clinical drug resistance and long-term outcome of

chemotherapy. Many of the differences in cell-biological and clinical characteristics in human cancers (*e.g.*, age, gender, immunological and chromosomal phenotype) are of prognostic value and may reflect differences in clinical response to chemotherapy. Therefore, the identification of common mechanisms of resistance (clinical and experimental) in subsets of patients is an important consideration in the design of effective clinical trials and treatment modalities. Long-term follow-up studies might also add to our understanding of the prognostic value of *in vitro* drug-sensitivity assays and analysis of Pgp expression in clinical drug resistance. Non-Pgp-mediated drug resistance mechanisms have been described (Estey *et al.*, 1987; Danks *et al.*, 1988; Baas *et al.*, 1990). Consequently, it will be important to explore further which mechanisms are responsible in cases of clinical drug resistance where Pgp is undetectable. Finally, models of broad-spectrum drug resistance (Pgp and non-Pgp-mediated MDR) and clinical trials (Millward *et al.*, 1992; Trump *et al.*, 1992; Yahanda *et al.*, 1992) are being implemented to address the problems associated with MDR.

5. Summary and Conclusions

Human ovarian carcinoma cells (UWOV2, established from a patient refractory to combination chemotherapy with actinomycin D, doxorubicin, cisplatin and vincristine), and blast cells isolated from peripheral blood of 15 patients with acute myeloid leukaemia (AML), were characterized for their expression of the multidrug resistance (MDR)-associated 170 kDa P-glycoprotein (Pgp) by gel filtration chromatography, sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunocytochemistry using Pgp-specific monoclonal antibodies (mAbs). Plasma membrane glycoproteins from patients L24 and L100 eluted from a Bio-Gel A-0.5M column as distinct peaks in the regions between 450-158 kDa and 158-44 kDa. Analysis of pooled lyophilized 450-158 kDa fractions from patients L24 and L100, respectively, by SDS-PAGE revealed a 170-kDa band, the presence of which was also confirmed in purified plasma membranes extracted directly with SDS.

In similar experiments, high molecular weight (≥150 kDa) peaks (eluted from Sephadex G150, Sephadex G-200 or Bio-Gel A-0.5M columns) and SDS-solubilized plasma membranes showed a 170-kDa band in samples derived from patients L19, L23, L28, L31, L45, L84, L94, L95, and L97, but not in those obtained from patients L21, L26, L42 and L55. The expression of Pgp in peripheral blood leukaemia blast cells prepared from a randomly selected group of patients with AML was analysed by the P-glycoCHEK C219™

immunohistochemical method. Only 2 of 15 samples from patients with AML stained positively for Pgp. This was expected since one patient (L24) was resistant to combination chemotherapy with etoposide, cytosine arabinoside and methotrexate, and the other patient (L100) presented with AML in relapse following chemotherapy with cytosine arabinoside, etoposide and daunorubicin. SDS-PAGE analysis of samples from AML patients, who had received either chemotherapy or no chemotherapy, showed the presence of a 170-kDa membrane protein, but this could not be confirmed by immunoperoxidase staining with the mAb C219. No difference in staining patterns could be discerned between cytospin preparations from the treated and the untreated patients in this group. Therefore, no clinical correlation could be made with the presence of the 170-kDa protein using this technique.

The expression of *mdr*1 gene product, Pgp, was also shown in UWOV2 cells by means of immunocytochemistry, using the mAb C219 and the avidin-biotin-peroxidase method. Separation of cell-surface glycoproteins derived from UWOV2 cells metabolically labelled with [^{3}H]glucosamine on a column of Bio-Gel A-0.5M yielded high MW (240-158 kDa) peak fractions, suggesting but not confirming the presence of a 170-kDa glycoprotein. This was again demonstrated by Sephadex G-200 fractionation of [^{3}H]glucosamine-labelled UWOV2 plasma membrane glycoproteins isolated after 72h incubation in the absence (control) and presence (TM-treated) of 5 μg/ml tunicamycin (TM). High MW UWOV2 cell-surface glycoproteins eluted as a broad peak in the 200-150 kDa range of the Sephadex G-200 chromatographic profile. This peak was reduced to approximately 53% of control in the presence of TM, indicating inhibition of glycoprotein synthesis. The presence of a 170-kDa glycoprotein in UWOV2 cells and its disappearance as a result of prolonged TM exposure were confirmed by SDS-PAGE of purified plasma membranes. When UWOV2 plasma membranes were immunoprecipitated with Pgp-specific mAbs MRK16 and C219, the 170-kDa band was again present, although contaminating bands were also noted. The results from this study show that UWOV2 ovarian carcinoma cells express Pgp and that TM exposure reduces its rate of synthesis. Thus, TM may have implications as a resistance modifier in *in vitro* studies related to the mechanisms and prevention of MDR.

CHAPTER 6

CONCLUSIONS AND PERSPECTIVES

Resistance to chemotherapeutic agents represents a major clinical obstacle in the control of most tumours. P-glycoprotein (Pgp) confers multidrug resistance by functioning as an energy-dependent drug efflux pump. To fully realize the therapeutic potential associated with manipulation of the multidrug transporter, it will be necessary to understand the mechanisms of action of the transporter and its regulation. Recent advances have provided insights into the complexity of this multifunctional transport system. Chemosensitizers like verapamil modulate multidrug resistance by interfering with the efflux action of Pgp and thus can decrease drug resistance or can re-establish drug sensitivity by restoring normal drug accumulation and distribution within the multidrug-resistant tumour cell. Clinical trials using verapamil have been prompted for leukemia and ovarian cancer. In addition, other approaches are the subject of current preclinical investigations.

If it can be demonstrated that tumours are dependent upon an increased expression of Pgp for resistance, the critical issues will be the regulation of such an expression and the mechanism by which the steady-state levels and biochemical characteristics of Pgp are elevated or altered in the absence and presence of drug selection pressure. These ideas suggest that the regulation of Pgp gene expression at both the transcriptional and translational levels is a promising area for future research. It is possible that post-translational processing such as phosphorylation and glycosylation may regulate the insertion and activity of Pgp in the plasma membrane.

The aim of the work described in this thesis was to establish whether tunicamycin could be used *in vitro* as a chemosensitizer to reverse the MDR phenotype. A correlation was sought between the MDR phenotype (drug resistance and toxicity, drug transport and retention) and the perturbation of tumour cell-surface glycoproteins *in vitro* by TM.

The data indicate that the rates of glycoprotein synthesis in certain MDR cell lines are increased compared with the corresponding parental cell lines. Tunicamycin inhibited the synthesis of proteins and glycoproteins in the various cell lines studied. No constant pattern of altered glycosyltransferases between drug-resistant and drug-sensitive cells was observed. The lack of inhibition of any of the glycosyltransferases by TM observed in KB-8-5-11-MDR cells, for example, indicates that inhibition of N-linked oligosaccharide synthesis may not necessarily interfere with the surface expression of certain glycoproteins. The results obtained suggest that the degree of inhibition by TM of glycosyltransferases and of total protein and glycoprotein synthesis in drug-sensitive and drug-resistant cells, respectively, may depend on their specific complement of oligosaccharide structures. From these considerations it may be that changes induced by TM in the biosynthesis of glycoproteins in drug-resistant cells are relevant in research into the nature of multiple drug resistance.

Tunicamycin enhances the cytotoxicity of various anticancer agents in drug-resistant cell lines to a greater extent than in their corresponding parental cells. The extent of the potentiation of cytotoxicity of anticancer drugs by TM differed in the various cell lines that have been studied. These variations in response can perhaps be attributed to variations in cross-resistance to the anticancer drugs which, in turn, may be determined by differences in genetic regulation and biochemical mechanisms involved in the protection of cells against cytotoxic agents. The consistent pattern of increased drug cytotoxicity that was observed in all MDR cell lines suggests a common mechanism(s) of reversal of drug resistance. Our findings do not directly demonstrate that the enhancing effects of TM on drug cytotoxicity are mediated by inhibition of Pgp, but they do establish that TM is an *in vitro* biological response modifier (chemosensitizer) in MDR.

The uptake of vincristine (VCR) was not affected in the various cell lines following exposure to TM. Cellular VCR loading was significantly greater in certain parental (drug-sensitive) cells compared with their corresponding MDR cells. The decreased VCR accumulation in MDR cells can perhaps be ascribed to an enhanced VCR efflux, since these cells overexpress Pgp. Treatment of MDR cell lines with TM consistently limited VCR efflux with resultant increase in VCR retention. Tunicamycin did not alter drug efflux and consequently VCR retention in some parental cells. In other parental cells, however, TM reduced the level of VCR retention. Tunicamycin probably enhances VCR retention in MDR cell lines by inhibiting drug efflux. Drug-resistant cells were more responsive to TM pretreatment, possibly due to the presence of raised Pgp levels. This observation is

relevant in understanding the biochemical basis of MDR. Tunicamycin may exert its effect by the inhibition of Pgp synthesis and/or by alteration in efflux pump activity. Transient inhibition of both protein and glycoprotein synthesis during TM exposure may contribute to alterations in VCR efflux and retention observed in the MDR cell lines. Tunicamycin may modify the molecular conformation of *de novo* synthesized Pgp, and its ability to engage in intermolecular interactions such as drug recognition, binding and efflux. The results presented here may have important implications for the potential use of TM as an MDR modifier, particularly to study the effects of glycosylation on the properties of Pgp and its interaction with cytotoxic drugs. The *in vivo* and *in vitro* cytotoxic spectrum of this antibiotic, above all, remains to be determined.

P-glycoprotein expression was demonstrated in UWOV2 ovarian carcinoma cells and in acute leukaemia blasts from patients using the P-glycoCHEK™ immunohistochemical diagnostic kit. It is concluded that the identification of common mechanisms of resistance (clinical and experimental) in subsets of patients is an important consideration in the design of clinical trials and treatment modalities. Long-term follow-up studies might also aid in the acceptance of the prognostic value of *in vitro* drug-sensitivity assays and analysis of Pgp expression in clinical drug resistance. It would be interesting to explore further which mechanisms are responsible in cases of clinical drug resistance where Pgp is undetectable.

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