INVESTIGATIONS OF MECHANISMS OF RESISTANCE TO ETOPOSIDE (VP-16) USING HUMAN TUMOUR CONTINUOUS CELL LINES

by

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ABSTRACT:

The aim of this project was to identify biochemical mechanisms associated with the significantly reduced responses to antitumour drugs observed in patients treated with initial chemotherapy or radiotherapy. In vitro exposure of a human tumour continuous cell line derived from a squamous cell carcinoma of the tongue (HN-1) to fractionated X-irradiation resulted in a subline (HN-1/DXR-11) which expressed resistance to VP-16 (Hill and Bellamy, 1984). In this study resistant sublines have been selected by continuous exposure to VP-16 (HN-1/VP-1 and HN-1/VP-2). Patterns of responses to antitumour drugs expressed by these independently-derived cell lines have been characterized and mechanisms associated with drug resistance have been studied.

Sublines expressing moderate orders of resistance (approximately 4-fold) to VP-16 can be derived readily by continuous exposure to VP-16 in vitro. These cells express cross resistance to structurally unrelated antitumour agents, including vincristine, vinblastine, adriamycin and daunomycin, consistent with the multidrug-resistant phenotype. The HN-1/DXR-11 subline, previously shown to be cross-resistant to vincristine but not adriamycin (Hill and Bellamy, 1984), proved marginally cross-resistant to vinblastine but showed an unaltered response to daunomycin. Therefore, patterns of drug sensitivities of these two independently-derived VP-16-resistant sublines differed.

Resistance to vincristine in HN-1/VP-2 cells was associated with reduced drug uptake and, consistent with the expression of multidrug resistance, there was overexpression of the P-glycoprotein. Neither of these characteristics was observed in HN-1/DXR-11 cells indicating that resistance mechanisms also differ depending upon the manner of resistance induction.

Modified VP-16 transport was not characteristic of these independently-derived VP-16-resistant sublines, neither did resistance appear to be due to alterations in levels of glutathione and associated enzymes. Resistance to VP-16 was associated with a reduction in VP-16-induced DNA single-strand breaks, the nature of which differed in the two independently-derived lines.

These studies provide evidence that patterns of responses to antitumour agents, and the mechanisms associated with these altered responses, may differ depending upon the agent employed to induce resistance. If confirmed, they may have major biological and clinical implications.

TABLE OF CONTENTS

		PAGE
TITLE PAGE		1
DEDICATION		2
ACKNOWLEDGEMENTS		3
ABSTRACT		4
TABLE OF CONTENTS		6
LIST OF FIGURES AND PLATES		7
LIST OF TABLES		13
LIST OF ABBREVIATIONS		16
CHAPTER 1:	GENERAL INTRODUCTION	19
CHAPTER 2:	MATERIALS AND METHODS	83
CHAPTER 3:	DERIVATION OF VP-16-TREATED HUMAN TUMOUR SUBLINES AND THEIR CHARACTERIZATION	124
CHAPTER 4:	*IN VITRO* DRUG RESPONSES OF HN-1 PARENTAL AND VP-16-TREATED CELL LINES	138
CHAPTER 5:	COMPARISON OF PATTERNS OF DRUG RESPONSES EXPRESSED BY TWO INDEPENDENTLY-DERIVED VP-16-RESISTANT HN-1 CELL LINES	159
CHAPTER 6:	INVESTIGATIONS OF MECHANISMS OF RESISTANCE TO VP-16 IN TWO INDEPENDENTLY-DERIVED VP-16-RESISTANT HN-1 CELL LINES	174
CHAPTER 7:	ANALYSES OF PLASMA MEMBRANE FRACTIONS FROM CHINESE HAMSTER OVARY AND HN-1 CELL LINES BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING	233
CHAPTER 8:	GENERAL DISCUSSION AND SUMMARY	251
REFERENCES		290
LIST OF PUBLICATIONS		313

LIST OF FIGURES AND PLATES

FIGURE No.	TITLE	PAGE No.
1-1:	Structural Formulae of VP-16, VM-26 and Podophyllotoxin.	38
1-2:	Structural Formulae of VP-16 and its Metabolites	42
1-3:	Proposed Formation of Complexes Between DNA Topoisomerase II and Double-Stranded DNA.	49
2-1:	Flow Microfluorimetric Determination of DNA Content of Cells.	100
2-2:	Standard Curve for the Fluorescence Detection of DNA using Hoechst 33258.	112
2-3:	Typical Alkaline Elution Profiles of HN-1/P Cells.	114
4-1:	Dose Response Curves following Exposure of HN-1/P, HN-1/VP-1 and HN-1/VP-2 Cells to VP-16.	142

FIGURE No.	TITLE	PAGE No.
4-2:	Dose Response Curves following Exposure of HN-1/P and HN-1/VP-2 Cells to Vincristine.	145
4-3:	Dose Response Curves following Exposure of HN-1/P and HN-1/VP-2 Cells to Adriamycin.	147
4-4:	Dose Response Curves following Exposure of HN-1/P AND HN-1/VP-2 Cells to Cisplatin.	149
5-1:	Survival of HN-1 Parental, VP-2 and DXR-11 Cells after 24h or 1h Exposures to VP-16.	163
6-1:	Uptake of [^{3}H]-VP-16 into Chinese Hamster Ovary AuxB$_1$ and CH^{R}C5 Cells Exposed as a Monolayer.	182
6-2:	Uptake of [^{3}H]-VP-16 into HN-1 Parental, VP-2 and DXR-11 Cells in Suspension.	183

FIGURE No.	TITLE	PAGE No.
6-3:	Uptake of [^{3}H]-VP-16 into HN-1 Parental, VP-2 and DXR-11 Cells Exposed as a Monolayer.	184
6-4:	Uptake of [^{3}H]-Vincristine into Chinese Hamster Ovary AuxB$_{1}$ and CH^{R}C5 Cells Exposed as a Monolayer.	188
6-5:	Uptake of [^{3}H]-Vincristine into HN-1 Parental, VP-2 and DXR-11 Cells in Suspension.	189
6-6:	Uptake of [^{3}H]-Vincristine into HN-1 Parental, VP-2 and DXR-11 Cells Exposed as a Monolayer.	190
6-7:	Uptake of [^{3}H]-Vinblastine into HN-1 Parental, VP-2 and DXR-11 Cells in Suspension.	192
6-8:	Uptake of [^{3}H]-Daunomycin into HN-1 Parental, VP-2 and DXR-11 Cells in Suspension.	194

FIGURE No.	TITLE	PAGE No.
6-9:	Uptake of cis-^{195m}Platinum into HN-1 Parental, VP-2 and DXR-11 Cells in Suspension.	196
6-10:	Schematic Represenation of the Role that Glutathione may Play in the Detoxification of Xenobiotics.	205
6-11:	DNA Single-Strand Breakage in HN-1 Parental, VP-2 and DXR-11 Cells after Exposure to VP-16.	215
6-12:	DNA Single-Strand Breakage in HN-1 Parental, VP-2 and DXR-11 Cells after Exposure to VP-16.	216
6-13:	Repair of DNA Single-Strand Breakage in HN-1 Parental, VP-2 and DXR-11 Cells after Exposure to VP-16.	220
6-14:	Repair of DNA Single-Strand Breakage in HN-1 Parental, VP-2 and DXR-11 Cells after Exposure to VP-16.	221

6-15: The Relationship between VP-16-Induced DNA Single-Strand Breakage and Cytotoxicity in HN-1/P, HN-1/VP-2 and HN-1/DXR-11 Cells. 225

6-16: The Relationship between VP-16-Induced DNA Single-Strand Breakage and Cytotoxicity in HN-1/P, HN-1/VP-2 and HN-1/DXR-11 Cells. 226

Plate No.	Title	Page No.
7-1:	SDS-PAGE of Plasma Membrane Fractions from Chinese Hamster Ovary $AuxB_1$ and CH^RC5 Cells.	238
7-2:	SDS-PAGE of Plasma Membrane Fractions from HN-1 Cell Lines.	240
7-3:	Detection of P-glycoprotein in Chinese Hamster Ovary and HN-1 Cell Lines by dot-immunoblot with ^{125}I-C219 Monoclonal Antibody.	241
7-4:	Detection of P-glycoprotein in Chinese Hamster Ovary and HN-1 Cell Lines by Western Blot with ^{125}I-C219 Monoclonal Antibody.	243

LIST OF TABLES

Table No.	Title	Page No.
1-1:	Summary of Patterns of Cross Resistance and Collateral Sensitivity in Experimental Tumour Cell Lines Expressing Resistance to VP-16.	63
3-1:	Characteristics of HN-1 Parental and Drug-Treated Cell Lines.	130
4-1:	Responses of HN-1 Parental and VP-2 Cells to a Range of Antitumour Agents.	144
4-2:	A Summary of the Differences in Cytotoxicity Assays employed for Estimations of Drug Resistance Parameters in Tumour Cell Lines.	151
4-3:	A comparison of the Degrees of Resistance to VP-16 shown by Drug-Resistant Tumour Cells.	155
4-4:	Summary of Responses Expressed by HN-1/VP-2 Cells to a Range of Antitumour Agents.	156

Table No.	Title	Page No.
5-1:	Survival Parameters of HN-1 Cells after Exposure to VP-16.	165
5-2:	Responses of HN-1 Parental and DXR-11 Cells to Podophyllotoxin, Vinblastine and Daunomycin.	166
5-3:	Summary of the Responses Expressed by HN-1/VP-2 and HN-1/DXR-11 Cells to the Cytotoxic Effects of a Range of Antitumour Agents.	169
6-1:	Uptake and Retention of VP-16, Vincristine and Daunomycin by HN-1 Cell Lines.	186
6-2:	Summary of Drug Uptake Characteristics Comparing HN-1/VP-2 and HN-1/DXR-11 Cells with Parental Cells.	199
6-3:	A Comparison between Levels of Resistance and Reduced Steady-State Accumulation of Drug Exhibited by a Range of Drug-Resistant Tumour Cell Lines.	202

Table No.	Title	Page No.
6-4:	Levels of Glutathione and Activities of Glutathione S-Transferase, Glutathione Reductase and Glutathione Peroxidase in HN-1 Cells.	208
6-5:	Comparison of Cell Survival and DNA Single-Strand Breakage and Repair Parameters of HN-1 Cell Lines Exposed to VP-16.	223
6-6:	A Comparison between the Reduction of VP-16-Induced Cytotoxicity and DNA Damage in a Range of Tumour Cell Lines.	229
7-1:	Plasma Membrane Protein Alterations in Multidrug-Resistant Cells.	245

LIST OF ABBREVIATIONS

Abbreviation	Full Title
Act D	Actinomycin D
ADR	Adriamycin
BSO	Buthionine sulphoximine
[^{14}C]	Carbon-14
CDNB	1-chloro-2,4-dinitrobenzene
CFA	Colony-forming assay
CHO	Chinese hamster ovary
Ci	Curie
Cisplatin)	cis-diamminedichloroplatinum II
Cis-Pt)	
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNR	Daunomycin
DPL	DNA-Protein crosslinks
DSB	Double-strand breaks
DXR	X-irradiation
E_4	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethylmethanesulphonate
FCS	Foetal Calf Serum
FMF	Flow microfluorimetry
GIA	Growth inhibition assay
GPO	Glutathione peroxidase
GR	Glutathione reductase

Abbreviation	Full Title
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GS-T	Glutathione S-transferase
Gy	Gray
[^{3}H]	Tritium
HBSS	Hank's balanced salt solution
9-OH-E	9-Hydroxy-Ellipticine
IC_{50}	Drug concentration resulting in 50% survival compared to untreated controls
IG_{50}	Drug concentration required to reduce cell numbers by 50% of untreated controls
IS	Internal standard
kb	Kilobase
kD	Kilodalton
m-AMSA	4'-(9-acridinylamino)methane-sulphon-m-aniside
mg	milligram
mRNA	messenger RNA
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PBSA	calcium- and magnesium-free phosphate-buffered saline

Abbreviation	Full Title
PMSF	Phenylmethylsulphonyl fluoride
P.T.I.	Post-treatment incubation
Rad-Eq.	Rad-equivalents
Resistance Index	Ratio of IC_{50} or IG_{50} values of drug-resistant cell lines to parental cells
RNA	Ribonucleic acid
r.t.	Room temperature
SCCHN	Squamous cell carcinoma of the head and neck
s.d.	Standard deviation
SDS	Sodium dodecyl sulphate
s.e.	Standard error
SSB	Single-strand breaks
$t^1/2$	Time taken for 50% of DNA lesions to disappear
Tris	Tris (hydroxymethyl) aminomethane
µg	Microgram
VCR	Vincristine
VLB	Vinblastine
VM-26	Teniposide or 4'-demethyl-epipodophyllotoxin-9-(4,6-O-thenylidene-β-D-glucopyranoside)
VP-16	Etoposide or 4'-demethyl-epipodophyllotoxin-9-(4,6-O-ethylidene-β-D-glucopyranoside).

CHAPTER 1 : GENERAL INTRODUCTION

CONTENTS:

1-1: CLINICAL BACKGROUND 22

a. Combined Modality Treatment of Cancer 22

(i) Historical background 22

(ii) Impact of prior radiotherapy on subsequent responses to antitumour drugs 25

b. Etoposide (VP-16) in the Treatment of Human Tumours 27

(i) VP-16 as a single agent 27

(ii) VP-16 in combination with other antitumour drugs 30

(iii) VP-16 in combination with radiotherapy 33

(iv) Effects of prior chemotherapy on subsequent responses to VP-16 35

1-2: ETOPOSIDE (VP-16): MODE OF CYTOTOXICITY 37

a. The Chemistry of VP-16 37

b. Effects of VP-16 on Chromosomal Aberrations, Cell Cycle Distribution and Macromolecular Synthesis 39

c. Metabolism and Macromolecular Binding Characteristics of VP-16 41

d. Effects of VP-16 on Microtubule Assembly, Nucleoside Uptake and DNA Damage 43

e. Structure-Activity Relationships 44

f. Characterization of VP-16-Induced DNA Damage 46

g. DNA Damage and Antitumour Effects 51

h. Resistance Studies 53

j. Summary 55

1-3: TUMOUR CELL RESISTANCE TO VP-16 55

a. The Origin of Drug-Resistant Phenotypes 55

b. Review of Experimental Model Systems used in VP-16 Resistance Studies 61

(i) Resistance developed following exposure to chemotherapeutic drugs 61

(ii) Resistance developed following exposure to mutagens or X-irradiation 66

(iii) Inherent resistance 67

(iv) Kinetic resistance relating to growth state 68

c. Patterns of Cross Resistance, Multidrug Resistance and Collateral Sensitivity with other Clinically Useful Antitumour Agents 68

d. Mechanisms of Resistance to VP-16 71

(i) Defective drug transport 71

(ii) Alterations in DNA damage after VP-16 treatment, not associated with reduced drug uptake but with intranuclear events 74

(iii) Alternative mechanisms including the role of intracellular glutathione and alterations in glutathione transferase, reductase and peroxidase 77

e. Circumvention of VP-16 Resistance 78

1-4: RATIONALE OF THESIS 81

1-1: CLINICAL BACKGROUND

a. Combined Modality Treatment of Cancer

(i) Historical background

Until the beginning of the twentieth century, surgery was the only treatment considered for cancer. However, following the discovery of X-rays by Roentgen in 1895, their biological effects became apparent, and since then, radiotherapy has evolved into one of the main scientific brances of medicine. Its role as the second line of attack, after surgery, has been firmly established in the treatment of primary cancers. Despite major advances in the surgical pre- and postoperative care of patients, surgical treatment of malignant disease remains a limited type of therapy, proving effective only against the primary tumour or its spread to regional lymphatics, but making no impact on the neoplasm outside the operative field. It was next appreciated that surgery and radiotherapy might be combined either as a preplanned strategy, or as a rescue technique when the surgical procedure proved incomplete or inadequate.

The first experimental use of the sulphur mustards as a form of chemotherapy resulted from observations during World War I that exposure to sulphur mustard gas caused bone marrow hypoplasia and severe damage to lymphoid organs (Adair and Bagg, 1931). Subsequently, many alkylating agents were developed during or shortly

after World War II, such as nitrogen mustard, busulphan, chlorambucil and melphalan. Then, in 1943, clinical trials with nitrogen mustard were initiated (Rhoads, 1946). In the 1940's folic acid antagonists were developed and Farber et al. (1948) showed aminopterin to be the first chemotherapeutic agent capable of regularly producing remissions in childhood lymphoblastic leukaemia. These initial successes led to the development of a large number of chemotherapeutic agents, so that today there are approximately 40 antitumour drugs in general clinical usage and many more under experimental investigation.

Over recent years the combined use of these various modalities of therapy has been evaluated in an attempt to produce superior response rates and survival figures from those obtained using any one modality alone.

Radiotherapy has proved very effective for controlling a variety of malignant tumours and consequently is widely used in the management of many cancer patients. It could, perhaps, be considered as a form of "localized chemotherapy", capable of highly accurate delivery to a specific area of the body, without direct dependence on blood supply or diffusion through tissues and without restriction based on anatomical factors, such as the location of blood vessels, nerves, or bones. The practice of treating patients with both radiation therapy and chemotherapy has increased strikingly over the past few years. Usually the objective is for each modality to prove complementary, with irradiation of tumour masses, often at the

primary site, coupled with systemic treatment aimed at widespread documented or imperceptible metastases. However, in addition, it is appreciated that the chemotherapeutic agents may modify the response of tumour and normal tissue to irradiation, and in particular, prior radiotherapy may exacerbate normal tissue response to subsequent chemotherapy. Phillips (1979) has proposed three main reasons for combining radiotherapy and chemotherapy:

(1) The combination may be administered so as to enhance local control of the primary tumour, which may not be optimal using only surgery and/or radiotherapy alone, or if a partial remission is induced, subsequent radiotherapy may eradicate the tumour at lower dose levels so decreasing the risk of late normal tissue injury.

(2) Chemotherapy is administered to sterilize distant metastases, either macro- or microscopic, which are outside the radiation field. Alternatively, pre-operative chemotherapy may be administered to decrease the size of the primary tumour which may enhance the possibility of local control with subsequent radiotherapy or surgery.

(3) Radiotherapy may be used to sterilize a pharmacological sanctuary where there is inadequate drug delivery, for example, the brain.

Therefore it is important to attempt to combine these modalities optimally, in an effort continually to enhance their interactions so leading to improved response rates and overall survival figures.

(ii) Impact of prior radiotherapy on subsequent responses to antitumour drugs

Primary treatment of the majority of human 'solid' tumours has tended to consist of surgery and/or radiotherapy, with chemotherapy relegated to palliation when local modalities have failed and the disease is advanced and disseminated (Carter and Soper, 1974). Since treatment of advanced disease is rarely curative, it is not surprising that the use of chemotherapy at this stage has failed to have any significant impact on cure rates. It is now becoming more widely accepted that the combined modality approach utilizing all available therapies early in the course of the disease, offers the best chance of increasing overall survival figures for several common 'solid' tumours, including those of the head and neck (Ervin et al., 1985). Furthermore, evidence is accruing which suggests that the efficacy of chemotherapy is significantly impaired by prior radiotherapy. Examples of the types of tumours where these reduced response rates have been reported include: squamous cell carcinoma of the head and neck (Price and Hill, 1981; Wolf and Chretein, 1981; Ervin et al., 1985), breast carcinoma (Holland et al., 1980), ovarian carcinoma (Young et al., 1982) and small-cell carcinoma of the lung (Cavalli et al., 1984).

Ervin and colleagues (1985) recently reviewed the combined modality treatment of advanced squamous cell carcinoma of the head and neck. Their findings indicated that prior radiotherapy decreased tumour responsiveness to chemotherapy and that, by the use of initial

induction chemotherapy, response rates for both single-agent and combination therapy have increased by 20-40%. It remains, however, to be established whether this approach will translate into any significant survival benefit. The precise mechanisms for the reduced response rates to chemotherapy recorded after radiotherapy are not understood, but a number have been proposed:

(i) Nutritional deficiency, altered immune status, and nonspecific immunosupression may affect both antitumour response to chemotherapy and host tolerance of the overall treatment plan (Thigpen et al., 1981).

(ii) Radiation-induced vascular fibrosis may reduce drug delivery to the tumour thereby creating a pharmacological sanctuary (Thigpen et al., 1981).

(iii) The presence of hypoxic cells may result in reduced tumour cell kill by antitumour drugs associated with resistance due to kinetic or epigenetic factors (Adams, 1981).

(iv) Radiotherapy may have induced the appearance of drug-resistant cells by mutations or specific radiation-induced genetic alterations (Bellamy, 1982).

It is difficult to provide a precise assessment of the effects of prior radiotherapy on subsequent responses to a range of single-agent therapies since predominantly drug combinations are now used to treat 'solid' tumours. However, this aspect was considered recently in an experimental setting, when a series of murine and

human tumour continuous cell lines were treated *in vitro* with fractionated X-irradiation, and the subsequent drug responses of these pretreated cell lines were evaluated (Bellamy and Hill, 1984; Hill and Bellamy, 1984; Hill et al., in press). The X-irradiation-pretreated lines consistently expressed resistance to vincristine and VP-16, indicating that, at least *in vitro* in experimental model systems, prior radiotherapy can induce the drug-resistant phenotype.

Reduced clinical response rates to chemotherapy after prior radiotherapy are not, however, invariable since, for example, patients with recurrent Hodgkin's lymphoma can be cured with combination chemotherapy (DeVita et al., 1980).

Therefore it would appear that investigations into the mechanisms associated with altered response rates to chemotherapy after prior radiotherapy are important and should be pursued in both experimental and clinical studies.

b. Etoposide (VP-16) in the Treatment of Human Tumours

(i) VP-16 as a single agent

VP-16 has single-agent activity in a variety of tumour types, including small-cell lung cancer, testicular cancer, lymphoma, leukaemia and Kaposi's sarcoma associated with the acquired immunodeficiency syndrome (AIDS) (O'Dwyer et al., 1985).

In small-cell carcinoma of the lung VP-16 has proved to be one of the most active single agents yet tested with overall response rates ranging between 40% and 60% (Pedersen and Hansen, 1983; Cavalli et al., 1984). However, less than 5% of patients are considered cured by this single-agent therapy (Cavalli et al., 1984). This has led to the further evaluation of VP-16 in these tumours in combination with other antitumour agents (discussed in Section 1-1:b(ii)).

The single-agent activity of VP-16 against testicular cancer was first reported in those patients who had relapsed after initial chemotherapy. VP-16 produced an overall response rate of 32% (Hainsworth et al., 1984) and of special interest was the observation that patients failing cisplatin-containing regimens sometimes responded to VP-16. The demonstration that VP-16 possessed antitumour activity as a single agent against refractory germinal neoplasms has led to its inclusion in a number of combination regimens.

VP-16 is active as a single agent, with an overall response rate of 38% reported both in Hodgkin's disease and in non-Hodgkin's lymphomas (O'Dwyer et al., 1985).

Reports of single agent activity for VP-16 in the treatment of leukaemia are few, with an indication of efficacy in patients with myelomonocytic or monocytic variants of this disease (O'Dwyer et al., 1985). Further definition of the role of VP-16 awaits results from

large-scale randomized trials.

VP-16 appears to be highly active as a single agent against Kaposi's sarcoma with an overall response rate of 89% (41% complete response) (Krigel, 1984). However, due to underlying immunodeficiency, the precise impact of chemotherapy on overall survival of these patients remains to be defined.

There are a number of tumours in which VP-16 has shown little single-agent activity, for example, tumours of the breast, bladder, ovary, cervix, colon, rectum, head and neck, and renal cancers, melanoma, or sarcoma (Louie et al., 1984). However, in a number of these examples there is insufficient clinical trial data available to substantiate a clear definition of inactivity (Louie et al., 1984). In the majority of trials evaluating VP-16 as a single agent the pretreatment status of the patients is not considered. Activity of a drug can be severely underestimated by the inclusion of patients who have relapse after prior chemotherapy. Indeed the few responses observed in trials involving very small numbers of patients with, for example, uterine cancer, Wilm's tumour, rhabdomyosarcoma,or prostate and thyroid cancer have served to provide justification for more extensive evaluation of the activity of VP-16 in these cancers (O'Dwyer et al., 1985).

In summary, VP-16 has shown a spectrum of antitumour activity as a single agent, and its activity may have been underestimated in heavily pretreated patients. Clinical trials are

now underway to establish the role of VP-16 in combination chemotherapy regimens in the range of tumours against which it has shown single-agent activity.

(ii) VP-16 in combination with other antitumour drugs

In experimental in vivo model systems VP-16 has been demonstrated to exert a synergistic antitumour effect in combination with cisplatin, cyclophosphamide or mitomycin C (Dombernowsky and Nissen, 1976; Schabel et al., 1979; Rose and Bradner, 1984). This effect was most pronounced when VP-16 was combined with cisplatin, as reported in at least three animal tumour models: P388 leukaemia (Burchenal et al., 1978; Mabel 1979; Schabel et al., 1979), B16 melanoma (Mabel and Little, 1979), and a carcinogen-induced bladder carcinoma (Soloway et al., 1980). These data have provided a basis for clinical trials evaluating the efficacy of VP-16 in combination with other clinically useful antitumour agents.

Initial evaluation has centred on those tumours where VP-16 has proven single-agent activity. For example, in small-cell carcinoma of the lung VP-16 has been tested in combination with cisplatin, cyclophosphamide, adriamycin and vincristine (Pedersen and Hansen, 1983; Alberto et al., 1986; reviewed by O'Dwyer et al., 1985). In previously untreated patients response rates of greater than 80% have been reported. These results compare favourably with those from regimens not including VP-16. However, these VP-16-containing regimens have not necessarily improved median survival

figures (Pedersen and Hansen, 1983; Alberto et al., 1986).

An alternative approach, adopted in the treatment of small cell carcinoma of the lung, and recently reviewed by Natale and Wittes (1985) has involved alternating effective chemotherapy regimens. A combination of cisplatin and VP-16 has been alternated with cyclophosphamide, adriamycin, and vincristine. The rational behind these trials was to test whether alternating non cross-resistant combination regimens could overcome clinical drug resistance, as proposed in the Goldie-Coldman hypothesis (1984). To establish whether this approach offers any survival advantage over standard non-alternating therapy or other regimens, results from prospective randomized trials are needed.

Although VP-16 has shown only slight single-agent activity in the treatment of non small-cell lung cancer (O'Dwyer et al., 1985), there is now some evidence of activity when it is used in combination with either cyclophosphamide, cisplatin or mitomycin C (Pederson and Hansen, 1983; Davis et al., 1986). However, larger clinical trials are necessary to establish definite response rates achieved with these combinations (Pedersen and Hansen, 1983, Davis et al., 1986; Pallares et al., 1986).

Few studies have evaluated VP-16 in combination chemotherapy regimens for the treatment of Hodgkin's disease, although activity has been claimed for a combination of VP-16,

prednisone and chlorambucil, alternating with the active regimen of chlorambucil, vinblastine, procarbazine and prednisone (McElwain and Selby, 1984). VP-16 has also been tested in acute non-lymphocytic leukaemia in combination with cytarabine, thioguanine, adriamycin and cyclophosphamide (Hurd et al., 1981; Bern et al., 1982). Combination regimens incorporating VP-16 have been evaluated extensively in non-Hodgkin's lymphoma. The most widely reported being prednisone, methotrexate, adriamycin, cyclophosphamide and VP-16 alternating with nitrogen mustard, vincristine, procarbazine and prednisone. The contribution of VP-16 in these effective treatment protocols is difficult to assess, although the results achieved are comparable with other successful combinations not including VP-16 (reviewed by O'Dwyer, 1985).

In some of these studies, therefore, a role for VP-16 has been sought in combination regimens for treating non-responsive tumours or those which have recurred after initial successful treatment. The combination of VP-16 and cisplatin has proved highly effective in these types of patients with testicular cancer (Williams et al., 1984; Hainsworth et al., 1985). The combination of VP-16, bleomycin and cisplatin has also proved highly active as initial therapy for testicular cancer (Horwich and Peckham, 1984) with 81% of patients remaining disease free after two years. Therefore it is apparent that a combination of VP-16 and cisplatin has value both in the initial treatment or as salvage therapy for testicular teratomas.

The activity of VP-16 in combination chemotherapy regimens used in the treatment of a number of other 'solid' tumours is less well documented. Blumenschein and colleagues (1984) have reported that VP-16 combined with adriamycin and cyclphosphamide, is an effective front-line treatment for metastatic breast cancer. More recently the combination of VP-16 and cisplatin has shown activity as second-line treatment of advanced ovarian cancer in patients who had failed initial treatment with adriamycin and cyclophosphamide (De Lena et al., 1986).

From this brief overview of the clinical activity of VP-16 in combination with other antitumour agents it is apparent that it already makes a major contribution to the treatment of small-cell lung cancer and testicular teratoma. Its role in significantly prolonging survival remains to be established, for which data from large scale randomized trials are necessary. Meanwhile its efficacy against other 'solid' tumours and its potential for 'salvage' therapy remains to be defined.

(iii) VP-16 in combination with radiotherapy

There is no experimental evidence to suggest that VP-16 exhibits radiosensitizing properties or that synergism, in terms of cytotoxicity, might result from the combined used of VP-16 and X-rays. Such a combination therefore has not been directly evaluated clinically. Although, clinical trials designed to evaluate the efficacy of VP-16 either as a single agent or in drug combinations

have incorporated radiotherapy as prior treatment or as palliation when patients relapse.

For example, patients with small cell lung carcinoma treated initially with radiotherapy and chemotherapy on relapse are entered into a clinical trial to evaluate the efficacy of VP-16 as second line treatment. The results of two such trials demonstrated that either VP-16 alone or in combination with cisplatin was less effective in patients who had received prior radiotherapy than in previously untreated patients (Cavalli et al., 1984; Batist et al., 1986). However, it is difficult to assess whether prior radiotherapy had a direct effect on the tumour cell response to chemotherapy or whether the reduced response rates were associated with tumour progression.

A recent report further highlights this dilemma, since VP-16 has shown little activity in the treatment of head and neck cancer (reviewed by O'Dwyer et al., 1985), although this agent demonstrated a low but definite level of activity in patients who had received prior radiotherapy (Grunberg et al., 1985). As with the majority of trials evaluating single-agent activity of a drug, heavily pretreated patients were used, and until adequate screening procedures are designed the activity of a new drug may be severely underestimated due to the pretreatment status of the patients.

(iv) Effects of prior chemotherapy on subsequent responses to VP-16

It is generally accepted that prior chemotherapy reduces the clinical response rates to subsequent chemotherapy. This also appears to apply to VP-16 used as a single agent since, for example, in the treatment of small-cell carcinoma of the lung, VP-16 gave a response rate of 48% (range 30%-65%) in previously untreated patients compared to 28% (range 4%-48%) in those who had received prior therapy (Cavalli et al., 1984).

However, in what may initially seem a contradiction, a major role is also now becoming apparent for VP-16 in combination with cisplatin, as second line treatment of small-cell lung carcinoma, testicular teratoma and possibly ovarian cancer. For example, Evans et al. (1985) reported that VP-16 plus cisplatin was effective against small-cell lung cancer in patients relapsing after initial therapy with cyclophosphamide, adriamycin and vincristine. However in attempting to interpret these data it is important to consider other studies in small-cell lung cancer which have shown a high overall response rate to this same combination in previously untreated patients (Pedersen and Hansen, 1983) and reduced single agent activity for VP-16 in patients treated with a combination of the same three drugs, cyclophosphamide, adriamycin and vincristine (Wolff et al., 1986). Furthermore, Batist and colleagues (1986) reported more recently that VP-16 plus cisplatin gave only a 12% overall response rate in their heavily pretreated patients with small-cell lung carcinoma, contrasting with the 55% overall response rate

described by Evans et al. (1985). One explanation put forward for this much lower response rate was that the aggressive initial therapy of a six-drug regimen plus radiotherapy, had not only reduced host tolerance to further treatment but also selected drug-resistant tumour populations (Batist et al., 1986). Therefore it may be concluded that the nature of prior treatment may be critical in determining subsequent responses to VP-16-containing regimens.

The combination of VP-16 and cisplatin is also an effective agent in the treatment of relapsed testicular cancer (Hainsworth et al., 1985). Overall response rates of 70% were achieved after relapse from the initial therapy with either cisplatin, vinblastine and bleomycin, or an adriamycin-containing modification of equal efficacy. Again it is difficult to assess whether this initial treatment has compromised the response rates to the VP-16-containing combinations, since this combination given as initial therapy has resulted in 81% of patients achieving a disease-free status (Horwich and Peckham, 1984).

It has recently been suggested that cisplatin plus VP-16 may also prove an effective treatment for patients with advanced ovarian carcinoma who have relapsed after initial treatment with adriamycin plus cyclophosphamide (De Lena et al., 1986). Again it is difficult to determine from available clinical data whether this combination would be more effective as an initial therapy and if prior treatment has significantly reduced the efficacy of this combination. Randomized trials are necessary to establish answers to

these questions. Furthermore, the individual contributions of cisplatin and VP-16 in these various studies are unclear.

Therefore, there is some clinical evidence, already available, indicating that responses to VP-16 may be modified in previously treated patients. Since experimental research into the development and mechanisms of resistance to VP-16 is an area which is receiving increasing attention, this may yield valuable information on optimal drug combinations and strategies for circumventing resistance, which may provide rationales for subsequent clinical evaluation.

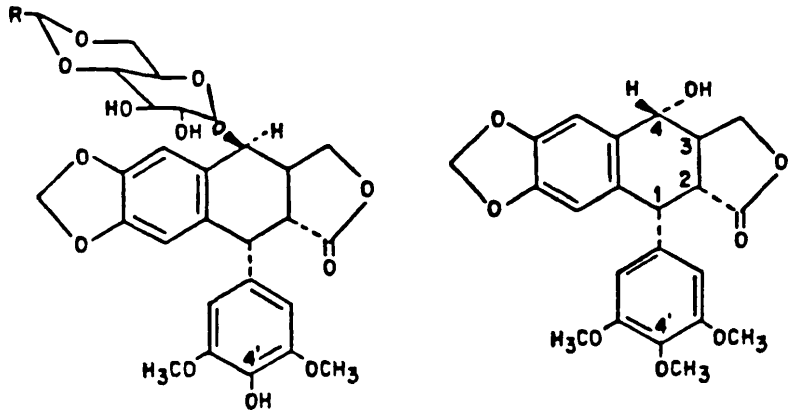
1-2: ETOPOSIDE (VP-16): MODE OF CYTOTOXICITY

a. The Chemistry of VP-16

Etoposide (VP-16) (4'-demethyl-epipodophyllotoxin-9-(4,6-O-ethylidene-β-D-glucopyranoside)) and its sister compound teniposide (VM-26) (4'-demethyl-epipodophyllotoxin-9-(4,6-O-thenylidene-β-D-glucopyranoside) were synthesized from the plant product podophyllotoxin in the early 1970's (Keller-Juslén et al., 1971, Stähelin, 1970) (Fig. 1-1). The three major structural differences between VP-16, VM-26 and podophyllotoxin are:

(i) The methoxy group at the 4' carbon of podophyllotoxin is replaced by a hydroxyl group in VP-16 and VM-26.

(ii) VP-16 and VM-26 both have an "epi" configuration at



Etoposide (VP16); R=H_3C-
Teniposide (VM26); R= 

Podophyllotoxin

Figure 1-1: Structural formulae of VP-16, VM-26 and podophyllotoxin

carbon 4 and consequently are collectively termed "epipodophyllotoxins".

(iii) VP-16 has an ethylidene glucoside moiety at carbon 4, whilst in VM-26 this is replaced by a thenylidene glucoside moiety

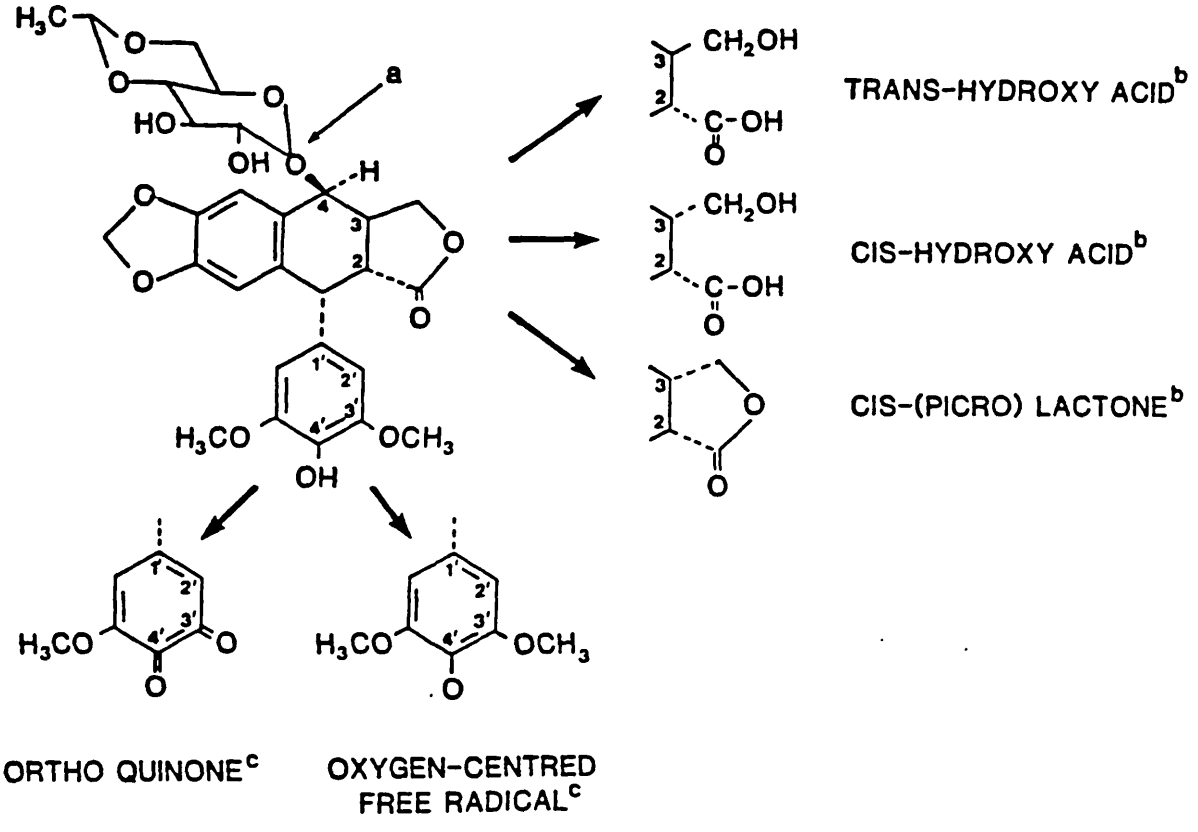
b. Effects of VP-16 on Chromosomal Aberrations, Cell Cycle Distribution and Macromolecular Synthesis

In 1973, Huang and colleagues observed multiple chromosomal aberrations after VP-16 treatment using four hematopoietic cell lines, two derived from the blood of healthy volunteers and two from patients with Burkitt's lymphoma. A correlation was demonstrated between the extent of chromosomal damage, and the concentration and time of drug exposure. The concentrations of VP-16 used in these experiments were of the same order as those used to assess cytotoxicity (Huang et al., 1973). More recently, Singh and Gupta (1983) working with Chinese hamster ovary cells measured mutation frequency and sister chromatid exchanges: VP-16 elicited a marked response in both of these parameters in a concentration-dependent manner. The mutation frequency at the hypoxanthine-guanine phosphoribosyl transferase locus correlated well with induction of sister chromatid exchanges. It was not possible, however, to explain the type of response elicited in terms of specific single base substitutions but rather to a series of deletions, frame-shifts, strand breaks, or chromosomal rearrangements.

Exposure of tumour cells to VP-16 both *in vitro* and *in vivo* has been shown to cause a rapid drop in mitotic index and accumulation of cells in the S and G_2 phase of the cell cycle, an effect typical of DNA damaging agents (Erba et al., 1983, Kalwinsky et al., 1983). Drewinko and Barlogie (1976), using synchronised cells of a human lymphoid (T1) cell line, showed that VP-16 exerted its greatest lethal effects in the S and G_2 phases. At higher concentrations of drug for exposure times exceeding 3 hours no accumulation was seen in either the S or the G_2 phase, but instead a 'frozen state' of cells in all phases of the cycle was noted. These workers suggested that this effect may be due to different target molecules being affected at higher drug concentrations. It appeared in this study that the lethal effects of VP-16 were not related to inhibition of nucleic acid or protein synthesis, as these were only inhibited at higher drug concentrations. Other studies using human leukaemic lymphoblasts have described an increase in cellular DNA, RNA and protein content, a decrease in cell proliferation, and an inhibition of incorporation of radioactive precursors into DNA after VP-16 treatment (Kalwinsky et al., 1983). These data are consistent with a block in the G_2 phase of the cell cycle, with DNA, RNA and protein content increasing in the S phase but the cells not going on to divide. It is possible to explain this G_2 block as the end-stage progression of cells that are lethally damaged earlier in the cell cycle, and, although able to complete DNA synthesis, they cannot proceed to chromosome condensation and mitosis.

c. Metabolism and Macromolecular Binding Characteristics of VP-16

Allen et al. (1976) demonstrated that 4'-demethylepidophyllic acid glucoside is the major urinary metabolite in man, indicating that the glucoside moeity is not cleaved. Van Maanen and colleagues (1982) studying metabolism of VP-16 *in vivo* by the rat and *in vitro* using rat liver homogenates and microsomes, observed that 45% of VP-16 was metabolised to the cis- or trans-hydroxy acid, but no aglycone was detected (Fig. 1-2). However, Dow and colleagues (1983), testing the cytotoxicity of these metabolites on human leukaemic lymphoblasts (CEM cells) observed that the picro-isomer was >100-fold less cytotoxic than VP-16 and the cis-hydroxy acid was >500-fold less cytotoxic than VP-16. These workers also observed that the epi-aglycone was extremely cytotoxic in their system. Sinha and Myers (1984) demonstrated the formation of an oxygen centred free radical when VP-16 was incubated with horseradish peroxidase and hydrogen peroxide (Fig. 1-2). These workers also observed O-demethylation of VP-16 in the presence of rat liver microsomes and NADPH. It was postulated that the irreversible binding of these derivatives to nucleic acid and protein would lead to inhibition of macromolecular synthesis and consequently cell death. In 1985, Van Maanen and colleagues demonstrated that the quinone derivative of VP-16 inactivated ØX174 DNA (Van Maanen et al., 1985a) (Fig. 1-2). These workers also showed that when VP-16 was incubated *in vitro* with rat liver microsomes 6% became irreversibly bound (Van Maanen et al., 1985b). However, Ross and colleagues (1984) had observed no significant binding of VP-16 to purified calf thymus DNA.



[a] - Cleavage point for epi-aglycone formation (Allen et al. 1976)
[b] - after Van Maanen et al. (1982)
[c] - after Sinha and Myers (1984)

Figure 1-2: Structural formulae of VP-16 and its metabolites

The observation that some free radical scavengers and dehydrogenase inhibitors repressed VP-16-induced DNA damage and cytotoxicity in L1210 cells, led to the proposal that VP-16 may undergo an oxidation-reduction reaction in order to exert cytotoxicity (Wozniak et al., 1984). However, this group later discounted the suggestion that VP-16 required activation (Glisson et al., 1984).

d. Effects of VP-16 on Microtubule Assembly, Nucleoside Uptake and DNA Damage

Studies in 1976 by Loike and Horwitz (1976a and b) compared the biochemical effects of VP-16 and its parent compound podophyllotoxin on HeLa cells. They showed that podophyllotoxin was a potent anti-mitotic agent and inhibited tubulin polymerization *in vitro*. VP-16, however, did not inhibit microtubular assembly *in vitro*, even at high drug concentrations, and, unlike the parent compound, did not arrest cells at mitosis. From studies with congeners of podophyllotoxin and VP-16 it was postulated that this lack of anti-mitotic activity was due to the glucoside moiety inhibiting binding of VP-16 to tubulin. In the same experiments VP-16 was shown to cause reversible DNA single-strand breakage in both a concentration-dependent and temperature-dependent manner. Podophyllotoxin, however, exerted no DNA damaging activity. Again, congener studies revealed that the 4'-hydroxyl group was necessary for this DNA damaging activity, with greater damage being attained if the substituents around carbon 4 were in the 'epi' configuration. This

VP-16-induced DNA damage was saturable. VP-16 did not, however, cleave purified adenovirus DNA and these results suggested that VP-16 either (i) activated an endonuclease, or (ii) was metabolised to a form which activated an endonuclease or directly cleaved DNA. Both podophyllotoxin and VP-16 were shown to inhibit nucleoside uptake in HeLa cells, however the VP-16 concentration required to elicit this response was some 50- to 200-fold higher than that required to produce DNA single-strand breakage. In addition, Yalowich and Goldman (1984) working with Ehrlich ascites cells showed that 50μM VP-16 caused inhibition of nucleoside uptake, however concentrations of lower than 1μM have been shown to cause DNA damage and inhibition of DNA synthesis (Kalwinsky et al., 1983). Furthermore, the observations that podophyllotoxin arrested cells in mitosis whilst VP-16 arrested cells in the S and G_2 phases of the cell cycle argue against inhibition of nucleoside transport being the main cytotoxic lesion (Loike and Horwitz, 1976b).

e. Structure-Activity Relationships

Studies of structure-activity relationships may yield important information on the mode of cytotoxicity of antitumour drugs. It can be seen from the studies of Loike and Horwitz (1976a and b) that a slight chemical alteration in the structure of podophyllotoxin has produced a drug with a different mode of cytotoxicity. The three chemical modifications responsible for this alteration are: (i) a glucoside moiety at the carbon 4 atom, (ii) the hydroxyl group at the 4'-carbon atom, and (iii) the substituents around the carbon 4

atom being of the 'epi' configuration. An excellent correlation was noted by Long and colleagues (1984a) when comparing cytotoxicity and DNA breakage activity of a number of congeners of VP-16 and podophyllotoxin. These workers also observed that the DNA single-strand breaks were rapidly formed, i.e. there was little increase in DNA single-strand breaks using drug exposure times of longer than 30 minutes, and that they were repaired rapidly, 90% being repaired within the first hour after drug removal. Therefore, if DNA damage is responsible for VP-16-induced cytotoxicity it must be exerted via a secondary response in this 'repaired' DNA or via residual DNA damage.

Evidence for DNA damage being the major cause of VP-16 induced cytotoxicity can be summarized as follows:

(i) VP-16 induces chromosomal aberrations,

(ii) VP-16 is a mutagen,

(iii) VP-16 induces sister-chromatid exchanges,

(iv) VP-16 arrests cells in the S and G_2 phases of the cell cycle, in common with other DNA damaging agents,

(v) VP-16 inhibits DNA synthesis,

(vi) VP-16 induces DNA single-strand breakage, and

(vii) structure-activity relationships correlate VP-16-induced cytotoxicity and DNA breakage activity.

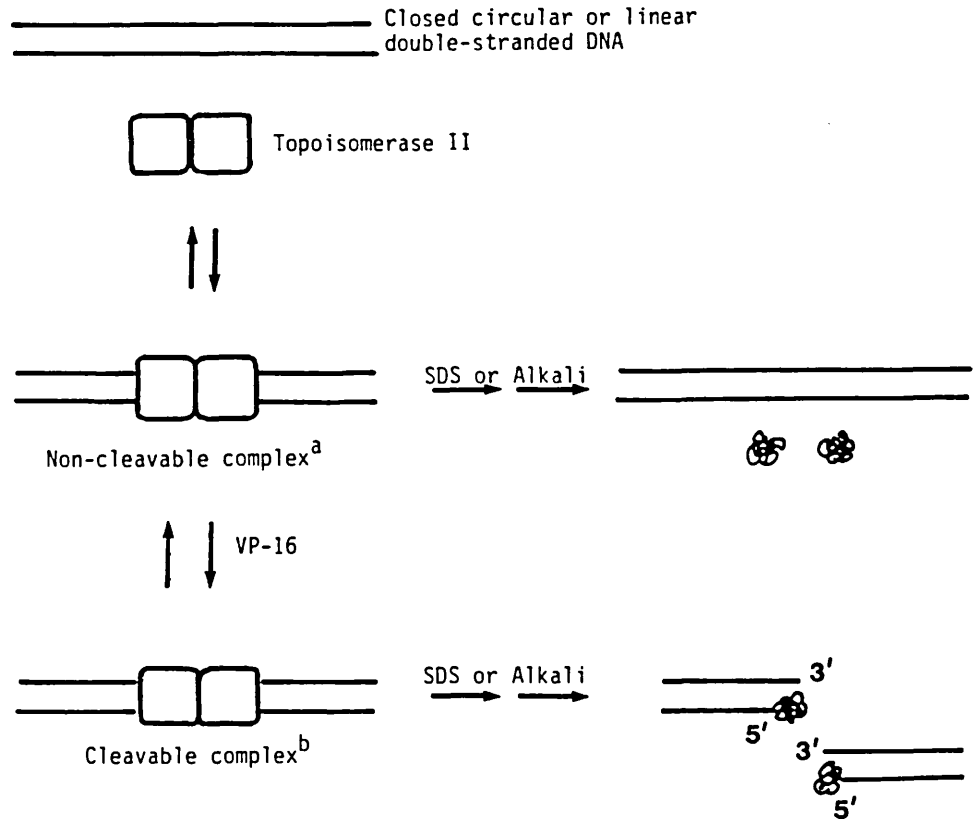
f. Characterization of VP-16-Induced DNA Damage

Loike and Horwitz (1976b) suggested, based on the nature of VP-16-induced DNA damage as measured by alkaline sucrose gradient sedimentation, that VP-16 either cleaved DNA via activation of an endonuclease or that VP-16 was metabolised to a form which directly cleaved DNA or activated an endonuclease. However, Kalwinsky and colleagues (1983), using the technique of alkaline elution to analyse VP-16-induced DNA damage in human leukaemic lymphoblasts, observed no loss of DNA in the lysis fraction which would be expected to occur if an endonuclease cleaved DNA. Further alkaline elution studies showed that VP-16 induced DNA single- and double-strand breaks in which the broken DNA strand was tightly associated with a protein (Wozniak and Ross, 1983). In 1984, Glisson and colleagues characterized VP-16-induced DNA damage in isolated nuclei from L1210 cells, therefore demonstrating that this activity required no cytosolic component. However, DNA damaging activity was dependent on pH and the presence of magnesium ions, and was also stimulated at low ATP concentrations. Additionally, a heat-labile activity in a nuclear non-histone protein extract was critical for VP-16-mediated DNA damage. This component of VP-16-induced DNA damaging activity could be extracted from nuclei with 0.35M sodium chloride. These characteristics are consistent with the involvement of a DNA topoisomerase in VP-16-induced DNA damage, and since VP-16 induces double-strand breaks it would be a type II topoisomerase. These studies, however, do not preclude the possibility that VP-16 could be metabolised to an active form within the nucleus.

The topological state of DNA, and the enzymes that control this state, play a crucial role in determining the function of DNA in cells. In eukaryotes, DNA supercoiling is intimately involved in the folding and organisation of the chromosome. DNA supercoiling is controlled by a class of enzymes called topoisomerases, which are defined by their *in vitro* reactions that interconvert various topological isomers of DNA (reviewed by Gellert, 1981). Because of these topological transformation reactions, DNA topoisomerases must interact intimately with DNA by transiently breaking one or both strands of the double helix. Enzymes that act by means of a transient double-strand break are termed type II DNA topoisomerases, while type I enzymes are those that make a transient single-strand break. Covalent enzyme-DNA intermediates are characteristic of all known DNA topoisomerases and a general rule is that type II topoisomerases bind to the 5' end of the DNA double helix whilst type I topoisomerases bind to the 3' end. Because of the strand-passing mechanism, type II DNA topoisomerases catalyze a number of novel topological isomerization reactions: they can knot and unknot double-stranded circular DNA; relax and supertwist closed circular DNA; and catenate and decatenate double-stranded DNA circles. Mammalian DNA topoisomerase II is a homodimer with a molecular weight of approximately 170kD (reviewed by Liu, 1984). Despite the fact that very little is known about the biological functions of eukaryotic DNA topoisomerases, it is expected that many genetic processes such as DNA replication, RNA transcription, recombination, chromosome condensation and decondensation, and DNA transposition may all require a DNA topoisomerase activity (adapted from reviews by Gellert

1981, and Lui 1984). It has recently been demonstrated that DNA topoisomerase II is required at the time of mitosis in yeast, possibly to allow the separation of intertwined chromosomal DNA molecules (Holm et al., 1985).

In 1984, Ross and colleagues using highly purified calf thymus type II DNA topoisomerase demonstrated that VP-16 and VM-26 stimulated site-specific cleavage of purified DNA by topoisomerase II. The epipodophyllotoxins also inhibited the decatenation activity of this enzyme on knotted kinetoplast DNA. In this study relatively low concentrations of these agents were used to effect the reactions involving topoisomerase II. It was therefore proposed that VP-16 and VM-26 may exert their cytotoxic effects by 'poisoning' DNA type II topoisomerase (Ross et al., 1984). It was also suggested that the epipodophyllotoxins interfered with the breakage-reunion reaction of mammalian DNA topoisomerase II by stablizing a cleavable complex between DNA and the enzyme (Fig. 1-3), and that these agents also inhibited the strand passing or unknotting activity of topoisomerase II (Chen et al., 1984). Minocha and Long (1984) in a structure activity relationship study examined the cytotoxicity, DNA damaging activity and inhibition of type II topoisomerase decatenation activity by congeners of VP-16. The good correlation reported between these three parameters has strengthened the hypothesis that VP-16-induced cytotoxocity is mediated by a 'poisoning' of DNA topoisomerase II. In this latter study VP-16 was shown not to inhibit DNA topoisomerase I.



[a] - Non-cleavable complex of topoisomerase II non-covalently linked to DNA

[b] - Cleavable complex of topoisomerase II covalently linked to the 5' terminus of DNA via a tyrosine residue

Figure 1-3: Proposed formation of complexes between DNA topoisomerase II and double-stranded DNA (after Chen et al. 1984)

One study has identified DNA topoisomerase II as an intracellular target in Simian virus 40 (SV40) infected monkey cells treated with epipodophyllotoxins (Yang et al., 1985). When the infected cells were drug treated and the SV40 DNA extracted, the broken DNA was found to be associated with a protein. Three lines of evidence suggested that the protein linked to the SV40 DNA was topoisomerase II:

(i) The cleavage sites were remarkably similar to those produced when purified SV40 DNA was incubated with highly purified calf thymus topoisomerase II and the epipodophyllotoxins VP-16 or VM-26;

(ii) The protein linked to the DNA was specifically immuno-precipitated by an antibody prepared against topoisomerase II;

(iii) Novobiocin, an inhibitor of topoisomerase II, inhibited the epipodophyllotoxin-induced cleavage of SV40 DNA in this system.

These results are consistent with the hypothesis that VP-16 interferes with the breakage reunion reaction of DNA topoisomerase II by stabilizing an enzyme DNA complex in its cleavable state (Fig. 1-3). It is therefore possible that the epipodophyllotoxins induce chromosomal DNA breakage via DNA topoisomerase II. Further indirect evidence in favour of this idea was presented by Rowe and colleagues (1985) when analysing effects of ethidium bromide on VP-16-induced cytotoxicity, single-strand and double-strand DNA breakage in L1210 cells and VP-16-induced DNA cleavage via topoisomerase II <u>in vitro</u>.

Ethidium bromide, a weakly cytotoxic DNA intercalator inhibited VP-16-induced cytotoxicity and DNA single- and double-strand breakage in L1210 cells without altering drug uptake characteristics. This agent was also shown to block VP-16-induced DNA cleavage by topoisomerase II *in vitro*. Comparable concentrations of VP-16 were used in these series of experiments, therefore by inference the data add support to the proposal that topoisomerase II mediates VP-16-induced cytotoxicity.

g. DNA Damage and Antitumour Effects

To account for the kinetic and biochemical alterations observed following VP-16 treatment of tumour cells, Kalwinsky and colleagues (1983) proposed a mechanism whereby VP-16-induced DNA strand breakage mediated via topoisomerase II may inhibit replicon initiation. Replication would continue, however, although one round would take longer due to there being fewer replication sites. It may be that DNA formed in the presence of these blocked initiation sites would not contain the full complement of gene products necessary for chromosome condensation or movement through the G_2 phase of the cell cycle (Kalwinsky et al., 1983). However, VP-16-induced DNA strand breakage is rapidly reversible and therefore must be incompletely repaired or exert secondary effects in order to be the cytotoxic lesion. The arrest of cells in the G_2 phase of the cell cycle could be due to their attempts to repair damaged DNA prior to mitosis.

It is important, however, to relate the biochemical effects of VP-16 to its proven antitumour activity. In 1982, Arnold and Whitehouse using a murine lymphoma model (TLX 5), studied the *in vivo* interaction of VP-16 and the DNA repair inhibitor chloroquine. This particular tumour is responsive to both VP-16 and vincristine. A marked increase in life-span was observed in animals if chloroquine was administered with VP-16 compared to VP-16 treatment alone. Chloroquine had no effect when given 6 hours after VP-16 treatment, suggesting that DNA repair had been completed. Chloroquine had no effect when administered with vincristine. A less pronounced effect was observed when caffeine, a known DNA repair inhibitor, was administered with VP-16. These data infer that DNA repair inhibitors potentiate the antitumour effects of VP-16. This is, however, very indirect evidence relating VP-16-induced DNA damage to antitumour effect since chloroquine may be interacting in other ways with VP-16, for example, alterating its pharmacokinetics, either by delay in elimination of VP-16 or preventing its conversion to a non-active metabolite. Wozniak and Ross (1983) correlated VP-16-induced cytotoxicity in L1210 cells with DNA single-strand breakage, double-strand breakage and DNA-protein crosslinks. Further evidence linking DNA damage with antitumour effect has been obtained from studies investigating VM-26, which is 5- to 10-fold more cytotoxic on a molar basis than VP-16 clinically (Rozencweig et al., 1977). This same difference in potency is observed in epipodophyllotoxin-induced DNA strand breakage in both cells and isolated nuclei, as well as in topoisomerase II mediated DNA cleavage reactions *in vitro* (Ross et al., 1984).

It appears unlikely that the glucoside moiety of VP-16 is cleaved *in vivo* (Fig. 1-2), a reaction that would give the VP-16 metabolite anti-mitotic activity (Loike and Horwitz, 1976a), as the major metabolite in man was shown to be 4'-demethylepipodophyllic acid glucoside (Allen et al., 1976) and the aglycone has yet to be detected *in vivo* (Evans et al., 1982). However, this may not apply to VM-26, as approximately 8% of the administered drug dose was identified as aglycone in the urine of six patients (Rossi et al., 1984).

h. Resistance Studies

Investigations into mechanisms of resistance to VP-16 have yielded insights into the mode of cytotoxicity of this agent. Cross resistance is not observed between podophyllotoxin and VP-16, inferring that these agents have different modes of cytotoxicity (Gupta, 1981 and 1983). Long and colleagues (1984b), working with a series of human lung carcinoma cell lines exhibiting a range of sensitivities to VP-16, observed higher plateau levels for DNA single-strand breakage in the most sensitive lines compared to resistant lines after exposure to VP-16. A Chinese hamster lung cell line made resistant to 9-hydroxy-ellipticine and expressing cross resistance to VP-16 accumulated less DNA-protein crosslinks in cells and isolated nuclei compared to the sensitive parental line after exposure to VP-16 (Pommier et al., 1986a). These workers suggested that resistance may be due to a modification of DNA topoisomerase II activity in the resistant cells.

Glisson and colleagues (1986a) have further defined the relationship between DNA damage and cell death. Working with a mutant Chinese hamster ovary line expressing an approximate 20-fold level of resistance to VP-16 these workers have analysed DNA damage, by the technique of alkaline elution, after exposure to VP-16, and this correlated with VP-16-induced cytotoxicity in sensitive and resistant cell lines. Additionally, these workers have employed a number of topoisomerase II specific assays using crude nuclear extracts from both parental and resistant cell lines. Decatenation activity in the crude nuclear extracts was comparable and equally sensitive to inhibition by VP-16. However, the DNA cleavage activity in the nuclear extract from the resistant line was profoundly less sensitive to stimulation by VP-16. A 26-fold reduction in stimulation of type II topoisomerase-mediated DNA cleavage activity was observed in this topoisomerase II specific assay. A somatic cell hybrid formed between the parental and resistant cells exhibited recovery of VP-16 sensitivity in concert with reconstitution of DNA cleavage activity in this crude nuclear extract, indicating that stimulation of DNA cleavage rather than loss of topoisomerase II function was responsible for cytotoxicity. This observation that epipodophyllotoxin resistance is mediated by a loss of drug-stimulated DNA cleavage activity is perhaps the strongest evidence yet to link DNA strand scission directly with antitumour effects.

j. Summary

VP-16 has been shown to cause chromosomal aberrations, to be a mutagen, to induce reversible DNA single- and double-strand breakage, and to inhibit mammalian DNA topoisomerase II, all at concentrations which induce measurable levels of cytotoxicity in tumour cells. Thus, whilst the inhibition of topoisomerase II is the most attractive hypothesis to explain VP-16-induced cytotoxicity, its involvement in the antitumour effects of VP-16 is still only speculative.

1-3: TUMOUR CELL RESISTANCE TO VP-16

a. The Origin of Drug-Resistant Phenotypes

Despite the availability of at least 40 clinically-active antitumour agents, eventual treatment failure is the outcome when chemotherapy is used in the treatment of the majority of cancers. Even with tumours such as certain paediatric malignancies and testicular teratomas, which are considered very responsive to chemotherapy, a proportion may show poor initial responses or ultimately regrow on treatment. When patients die with metastatic cancer, their disease is characterized frequently by insensitivity to even the highest tolerated doses of available antitumour agents. It would appear therefore that resistance to drug treatment may be apparent at the very beginning of therapy, or it may be seen as a

late event, after initial beneficial or complete response has been attained.

There are two fundamental views regarding the process whereby a tumour becomes clinically resistant to therapy: The first proposes that the underlying mechanisms are essentially a combination of host and epigenetic factors. These may include processes such as a change in the overall growth kinetics of the tumour, resulting in a progressively declining growth fraction and longer intermitotic interval of the tumour cells (Steel and Lamerton, 1968). In addition, there may be alterations in the state of the immune response of the host (Harris et al., 1973) or growth by tumour cells in pharmacokinetic sanctuaries (McGuire et al., 1985). These factors may combine to produce a situation where drug-induced tumour cell kill is less than tumour regrowth between treatment cycles, thereby resulting in a neoplasm which is clinically drug-resistant. However, these alterations would not explain the occurrence of drug resistance after a drug-induced complete or partial remission. Neither would they explain why tumour cell sensitivity to antitumour agents may be observed to decrease as the tumour volume diminishes in size after each treatment cycle, whereas, if resistance was due solely to changes in growth kinetics, sensitivity might be expected to increase.

According to the second viewpoint, malignant cells, most of which are characterized by genetic instability (Nowell, 1982), exhibit drug resistance due to genetic alterations. These changes may be associated with altered, diminished, or increased gene

products which in some cases can be shown to be involved directly with the drug-resistant phenotype (Schimke et al., 1978). For example, using DNA transfection techniques, it has been possible to transfer DNA sequences coding for increased amounts of the enzyme dihydrofolate reductase from one cell line to another. The recipient cells were then shown to display stable and heritable resistance to methotrexate (Cline et al., 1980; Carr et al., 1983). Furthermore, in vivo experiments have shown that cells taken from tumours which are drug-resistant maintain their resistance when transferred to other host mice (Skipper et al., 1964; Skipper, 1978). This argues against involvement of host factors in the acquisition of drug resistance. Further studies by these workers using transplantable drug-resistant tumours have shown that, in many cases, the kinetics of growth of the resistant tumour line are not substantially different from those of the parental sensitive line from which they were obtained. This indicates that tumour cell resistance to drugs can be due to mechanisms unrelated to tumour growth properties.

Goldie and Coldman (1984) have proposed a hypothesis regarding antitumour drug resistance, using principles initially developed by Luria and Delbrück (1943) to account for the drug resistance patterns of bacteria. The model which they have proposed relates to the probability of one drug-resistant tumour cell being present (and therefore the probability of a recurrence) to both population size and mutation frequency. Resistant cells are not considered to be "induced" by the toxic agent, but rather to occur spontaneously as mutants at a predictable frequency in cell

populations. Drug exposure then provides a positive selection pressure, allowing resistant cells to outgrow the sensitive tumour cell population. The spontaneous and random nature of the appearance of resistant mutants also provides a theoretical basis for the variation in response to cancer chemotherapy seen in both clinical and experimental trials. According to their hypothesis Goldie and Coldman (1984) have predicted that, using the spontaneous mutation rate of 10^{-7} which is observed in many tumour cell populations, only small tumours (i.e. those having fewer than 10^5 cells) are likely to be cured with a single cytotoxic agent. Taken to its logical conclusion, the Goldie-Coldman hypothesis predicts optimal treatment results using drug combinations rather than single agents, and treating at a very early stage in the disease when the tumour burden is low.

In support of this hypothesis, studies using animal tumour models have shown that only small tumours were likely to be eradicated with a single cytotoxic agent, whereas for larger tumours, previously curative therapy would induce a remission in most animals, although the majority were not cured and subsequently died from their tumours (Skipper, 1983). The low cure rate could be attributed to the presence of large numbers of drug-resistant tumour cells at the start of therapy.

One proposed method of overcoming drug resistance is to alternate between two chemotherapy regimens of comparable efficacy, each of which individually produces a high response rate. This

approach has been employed successfully in the treatment of Hodgkin's disease, with the sequential use of nitrogen mustard, vincristine, procarbazine and prednisone, followed by adriamycin, bleomycin, vincristine, and dacarbazine (Santor et al., 1982). As individual treatment programmes, each of the combination regimens produced a complete remission rate of about 70%, whereas, when they were used alternatively, the complete remission rate was higher than 90%, and of major significance, resulted in improved disease-free survival. Presumably, each combination may eradicate malignant stem cells that are resistant to the other, thereby inhibiting regrowth of the tumour and increasing the frequency of cure.

However, a number of assumptions were made in defining the Goldie-Coldman hypothesis:

1. All cells within the tumour have stem cell capacity (i.e. they are capable of indefinite self renewal). This is unlikely to apply to spontaneous tumours in animals or man, where the stem cell population appears to be only a small subset of all of the cell types within the neoplastic tissue (Salmon et al., 1978). However, when mathematical adjustments taking this into account were made the qualitative conclusions of the hypothesis did not alter (Goldie and Coldman, 1984).
2. A resistant phenotype arising within the tumour population will be totally resistant to the maximum tolerated dose of drug. Again, with more complex

arguments, degrees of partial resistance did not alter the qualitative conclusions.

3. The kinetic behaviour of resistant phenotypes will be identical to that of the parental population. If these parameters are not identical then it is likely that one population will outgrow the other.

Recently, Mackillop (1986) suggested that the Goldie-Coldman hypothesis, although being able to account for tumour regrowth after an initial response to therapy, could not explain primary treatment failure. One fundamental difficulty in the Goldie-Coldman hypothesis is the assumption that in its natural state the tumour cell is "sensitive", and "resistance" is only subsequently acquired through stable heritable changes occurring during the evolution of the tumour. Therefore intrinsic variations which may occur in cellular chemosensitivity were not considered. However, the hypothesis could account for a neoplasm that is essentially 100% resistant at the time of treatment, which is likely to contain at least 10^9 cells, even if the initially transformed cell was "sensitive" (Coldman *et al.*, 1985). If the resistant mutant had a slight growth advantage compared to parental cells, this could yield a tumour that is composed of 10% to >90% resistant clonogenic cells when it reached a mature size.

In summary, antitumour drug resistance appears to have a genetic basis. The origin of drug-resistant phenotypes may be explained by random and spontaneous genetic mutations occurring

within an inherently unstable tumour cell population. Although unequivocal experimental evidence has yet to substantiate this hypothesis, it offers a basis by which tumour cell populations may "acquire" drug resistance.

b. Review of Experimental Model Systems used in VP-16 Resistance Studies

(i) Resistance developed following exposure to chemotherapeutic drugs

There are few examples in the literature where a drug-resistant tumour cell line has been developed following exposure to either VP-16 or VM-26 as the selective agent. The majority of studies investigating VP-16 resistance have used cell lines which express cross resistance to VP-16, although they have been derived by exposure to structurally unrelated drugs. Although VP-16 was synthesized in 1971 (Keller-Juslén et al., 1971) it is only within the last 10 years that it has undergone extensive clinical evaluation. Therefore tumour cell resistance to VP-16 is a relatively recent clinical phenomenon. The establishment of relevant experimental model systems to investigate VP-16 resistance is important, if effective methods are to be devised for its circumvention.

In 1982, Seeber and colleagues reported the development *in vivo* of murine Ehrlich ascites tumour cells resistant to VP-16

following biweekly intra-peritoneal injections of increasing doses of the drug. The level of resistance developed *in vivo* was at least 6-fold. Danks and Beck (1983) have derived a VP-16-resistant cell line from human leukaemic cells (CEM) by exposure to VM-26, whilst Long and colleagues (1985b) have selected both VP-16- and VM-26-resistant sublines from a human colon carcinoma derived cell line.

Tumour cell lines expressing cross resistance to VP-16 have also been derived (see Table 1-1) following: (i) exposure of human tumour cell lines *in vitro* to the Vinca alkaloids, vincristine and vinblastine (Beck, 1983; Conter and Beck, 1984; Slater et al., 1986) or to adriamycin (Fairchild et al., 1986; Harker et al., 1986), (ii) *in vitro* exposure of Chinese hamster lung cells to the DNA intercalator 9-hydroxy-ellipticine (Pommier et al., 1985), (iii) treatment of an L1210 murine leukaemia *in vitro* with mitomycin C (Dorr et al., 1985) or treatment of murine P388 cells *in vivo* with vinblastine (Wilkoff and Dulmadge, 1980).

Thus whilst existing model systems may well yield information as to how exposure to a structurally unrelated drug may cause the expression of resistance to VP-16, to complement these studies there is a need for a greater range of experimental systems in which drug-resistant tumour cells have been derived specifically by exposure to VP-16.

Table 1-1: Summary of patterns of cross resistance and collateral sensitivity in experimental tumour cell lines expressing resistance to VP-16

	Seeber et al. (1982)	Danks and Beck (1983)	Gupta (1983)	Dorr et al. (1985)	Pommier et al. (1986a)
Selecting Agent	VP-16	VM-26	VM-26	MMC	9-OH-E
Response to:					
Actinomycin D			PR	CR	CR
Adriamycin	CR		CR	CR	CR
Bleomycin			CS	NC	
Cisplatin	CS		CS	CR	
Colchicine			CR		
Daunomycin	CR		CR	CR	CR
Ellipticine			CR		CR
5-Fluorouracil			PR	NC	
9-Hydroxy-Ellipticine					R
m-AMSA			CR		
Methotrexate			NC	NC	CR
Mitomycin C				R	CR
Mitoxantrone			CR	CR	
Podophyllotoxin			NC		
Vinblastine			CR	CR	
Vincristine		NC		CR	CR
Vindesine	CR				
VM-26	CR	R	R		
VP-16	R	CR	CR	CR	CR

Note: CR, cross resistance; CS, collateral sensitivity; NC, no change; PR, partial cross resistance; R, resistance to the selecting agent.

Table 1-1: (continued)

	Wilkoff and Dulmadge (1980)	Conter and Beck (1984)	Slater et al. (1986)	Hill et al. (in press)	Beck (1983)
Selecting Agent	VCR	VCR	VCR	VCR	VLB
Response to:					
Actinomycin D	CR				
Adriamycin	CR	CR		CR	
Bleomycin					
Cisplatin				NC	
Colchicine					CR
Daunomycin	CR	CR	CR		CR
Ellipticine					
5-Fluorouracil					
9-Hydroxy-Ellipticine					
m-AMSA					
Methotrexate					PR
Mitomycin C					
Mitoxantrone					
Podophyllotoxin		NC			PR
Vinblastine		CR			R
Vincristine	R	R	R	R	CR
Vindesine		CR			CR
VM-26		CR			
VP-16	CR	CR	CR	CR	CR

Note: CR, cross resistance; CS, collateral sensitivity; NC, no change; PR, partial cross resistance; R, resistance to the selecting agent.

Table 1-1: (continued)

	Johnson et al. (1978)	Fairchild et al. (1986)	Harker et al. (1986)	Bellamy and Hill (1984)	Hill and Bellamy (1984)
Selecting Agent:	ADR	ADR	ADR	X-IRRAD.	X-IRRAD.
Response to:					
Actinomycin D	CR	CR	CR	CR	
Adriamycin	R	R	R	NC	NC
Bleomycin				CS	NC
Cisplatin	CS	NC		CS	CS
Colchicine			CR		
Daunomycin	CR		CR		
Ellipticine	CR				
5-Fluorouracil	NC			NC	CS
9-Hydroxy-Ellipticine					
m-AMSA	CR				
Methotrexate	NC			PR	NC
Mitomycin C	CS		CR		
Mitoxantrone			CR		
Podophyllotoxin				NC	NC
Vinblastine	CR	CR	CR	CR	
Vincristine	CR	CR	CR	CR	CR
Vindesine	CR				
VM-26	CR			CR	CR
VP-16	CR	CR	CR	CR	CR

Note: CR, cross resistance; CS, collateral sensitivity; NC, no change; PR, partial cross resistance; R, resistance to the selecting agent.

(ii) Resistance developed following exposure to mutagens or X-irradiation

It is apparent that many of the agents mentioned in the previous section are mutagenic (Singh and Gupta, 1983). It seems likely, therefore, that drug-resistant tumour cell lines have been derived in one of two ways: (i) as the product of a drug-induced mutation followed by selection of drug-resistant cells, or (ii) as the product of selecting resistant sub-populations already present and not being derived by a single drug-induced mutation (Goldie and Coldman, 1984). Complementary to this is the study of Gupta (1983) in which Chinese hamster ovary cells were exposed to the mutagen ethylmethanesulphonate and drug-resistant mutants selected for with VM-26. The resultant VM-26-resistant cell lines were fully cross-resistant to VP-16 and could be considered to result from a single mutation.

A novel approach to the derivation of drug-resistant cell lines has also been demonstrated recently whereby fractionated X-irradiation exposure specifically induced resistance to VP-16 in both a murine L5178Y lymphoma cell line (Bellamy and Hill, 1984) and in a human tumour cell line derived from a squamous cell carcinoma of the tongue (Hill and Bellamy, 1984). The rationale behind these studies was that the frequently reported reduced responses to chemotherapy noted in patients with a range of tumour types following fractionated radiotherapy might result from radiation-induced drug-resistance. Studies of this type are of interest since it is

possible that mechanisms of resistance to chemotherapeutic drugs may differ depending upon the agent employed to induce resistance.

(iii) Inherent resistance

A number of studies have used human tumour cell lines expressing a range of responses to chemotherapeutic drugs. This property, where a tumour cell line is less responsive to antitumour drugs than another cell line of the same tumour type, neither having experienced prior drug exposure, may be termed inherent resistance. Human lung carcinoma cell lines expressing a range of sensitivities to VP-16 were used by Long and colleagues (1984b) in a study attempting to correlate DNA damage induced by VP-16 with cytotoxicity. This study also demonstrated the heterogeneity within and between lung tumour types and was later extended to a colon carcinoma derived line and one subline selected for resistance to VP-16 (Long et al., 1985b).

Merry and colleagues (1984) have also demonstrated marked heterogeneity in response to VP-16 in six human glioma cell lines in vitro and they have suggested that differences in sensitivity observed in the clinic may have as their basis differences in sensitivity at the cellular level. This seems to highlight the need to identify the cellular mechanisms underlying this apparent heterogeneity of response.

Additionally, Yalowich and colleagues (1985) have studied the effects of VP-16 in both normal human bone marrow granulocyte-macrophage progenitors and human leukaemia cells *in vitro*. Taking into account that the major dose-limiting factor in the administration of VP-16 is myelosuppression, *in vitro* studies into the selective toxicity of VP-16, and enhancement thereof, in normal human bone marrow and human myelogenous leukaemia are important.

(iv) Kinetic resistance relating to growth state

Recent interest has been stimulated by observations on the differential cytotoxicity of VP-16 in proliferating and quiescent tumour cells, as this may be an important feature determining therapeutic outcome (Markovits et al., 1986; Sullivan et al., 1986). These studies, using both murine and human tumour cell lines, showed that quiescent cells were markedly more resistant to the cytotoxic effects of VP-16 compared to proliferating cells, and this differential sensitivity correlated with VP-16-induced DNA scission and regulation of cellular topoisomerase II content.

c. Patterns of Cross Resistance, Multidrug Resistance and Collateral Sensitivity with other Clinically Useful Antitumour Agents

As mentioned previously, the majority of experimental systems cited have employed a structurally unrelated antitumour agent to select multidrug-resistant sublines which expressed cross

resistance to VP-16. However, cell lines selected with VP-16 or VM-26 appear to follow the same general patterns of cross resistance and collateral sensitivity as those in which the selective agents were certain anthracyclines, Vinca alkaloids or ellipticine derivatives. Table 1-1 summarizes data derived from studies where resistance to VP-16 has been reported. The following general conclusions can be made:

(i) Cross resistance is observed between VP-16 and the anthracyclines adriamycin and daunomycin, and the antitumour antibiotic, actinomycin D. Exceptions are the X-irradiation-treated murine and human tumour cell lines expressing resistance to VP-16, which retained sensitivity to adriamycin (Bellamy and Hill, 1984; Hill and Bellamy, 1984).

(ii) Cross resistance can also be demonstrated between VP-16 and the Vinca alkaloids, vinblastine, vincristine and vindesine in cell lines derived by exposure to the epipodophyllotoxins VP-16 and VM-26, anthracyclines, X-irradiation or the Vinca alkaloids themselves. An exception is the study using human leukaemic lymphoblasts selected for resistance to VM-26, which did not express cross resistance to vincristine (Danks and Beck, 1983).

(iii) An interesting correlation between resistance to VP-16 and collateral sensitivity to cisplatin can be found in cell lines which have been selected by

exposure to VP-16 (Seeber *et al.*, 1982) or adriamycin (Johnson et al., 1978), mutagenized and selected by exposure to VM-26 (Gupta, 1983), or derived by exposure to fractionated X-irradiation (Bellamy and Hill, 1984; Hill and Bellamy, 1984). Exceptions to this general rule are cell lines derived by exposure to adriamycin (Fairchild et al., 1986) or mitomycin C (Dorr et al., 1985), which exhibited an unaltered response and resistance to cisplatin respectively.

(iv) Cell lines resistant to VP-16 are generally not cross-resistant to the parent compound podophyllotoxin. An exception is the highly resistant vinblastine-treated human leukaemic lymphoblastoid cell line of Beck (1983), which expressed partial cross resistance to podophyllotoxin.

(v) Full cross resistance is observed between VP-16 and VM-26.

Another facet of many of the cell lines expressing cross resistance to VP-16 is their high degrees of resistance to the selecting agent. For example, 420-fold vinblastine-resistant and 600-fold vincristine-resistant human leukaemic lymphoblasts (CEM cells) expressed 20-fold and 30-fold resistance to VP-16 respectively (Beck, 1983; Conter and Beck, 1984). Furthermore, human breast cancer cells (MCF-7) selected for resistance to adriamycin (190-fold) proved highly resistant to VP-16 (175-fold) (Fairchild et al., 1986).

It is questionable whether these levels of resistance, where biochemical differences between normal and resistant cells have been maximized, are relevant to clinical drug resistance. Because of the steep dose-response curve of antitumour agents it is considered that an increase in drug resistance of between 3- and 20-fold would lead to treatment failure in the clinic (Frei, 1982). In order to gain confidence in experimental studies being relevant to clinical drug resistance it is essential therefore also to use model systems in which only a moderate level of resistance is expressed. Examples of such levels of resistance are provided by the VP-16-resistant Ehrlich ascites cells (6-fold) of Seeber et al.(1982), 10-to 20-fold VP-16-resistant Chinese hamster ovary cells (Gupta, 1983, Glisson et al., 1986a), and the 2- to 20-fold level of VP-16 resistance reported for a human squamous cell carcinoma of the tongue (HN-1) cell line treated with fractionated X-irradiation (Hill and Bellamy, 1984). Interestingly, all of these cell lines expressing relatively low levels of resistance to VP-16 exhibit collateral sensitivity to cisplatin, which is a possible explanation for the reported synergism between these two agents both in experimental model systems in vivo, and in the clinic (discussed in Section 1-1:b(ii)).

d. Mechanisms of Resistance to VP-16

(i) Defective drug transport

As mentioned above (section 1-3:b) drug-resistant tumour cells can be selected with step-wise increases in drug concentration

and they may express cross resistance to a variety of structurally unrelated agents with different modes of action. This phenomenon of multidrug or pleiotropic resistance has been associated with reduced intracellular steady state levels of drug (Ling and Thompson, 1974; Skovsgaard, 1978a and b; Inaba and Johnson, 1978; Beck, 1983).

In 1982, Seeber et al. reported reduced steady state accumulation of VP-16 in Ehrlich ascites tumour cells resistant to VP-16. A non drug-specific energy-dependent pump was shown to cause rapid extrusion of VP-16 when the cells were released from metabolic block by the addition of glucose to the medium. Inaba and Johnson (1978), demonstrated enhanced efflux of adriamycin or daunomycin in a subline of P388 leukaemia developed by exposure to adriamycin in vivo. Cross resistance to VP-16 was demonstrated, however the mechanism involved in this altered response to VP-16 is unknown. Lee and Roberts (1984b) have analysed the flux of VM-26 across the plasma membrane of murine leukaemia cells (L1210) selected for VM-26 resistance. These resistant cell lines exhibited reduced rates of influx, enhanced rates of efflux, reduced steady-state contents of drug and reduced retention after incubation in drug-free medium, compared to sensitive cells. These observations suggested that resistance to the epipodophyllotoxin was associated with alterations in flux of drug across the cell membrane, however it appears that the precise mechanism of resistance to VM-26 in these cells is more complex, resulting from a summation of small changes (Roberts et al., 1986).

Newly expressed membrane proteins also appear to be phenotypic markers of pleiotropic drug resistance associated with reduced drug uptake. Juliano and Ling (1976) reported the overexpression of a plasma membrane glycoprotein with a molecular weight of approximately 170kD (the 'P-glycoprotein') in multidrug-resistant Chinese hamster ovary cells. The appearance of a similar molecular weight glycoprotein and the concomitant disappearance of a lower molecular weight (90kD) glycoprotein have since been observed in multidrug-resistant human leukaemic lymphoblasts selected for resistance to vinblastine (Beck et al., 1979). These cells expressed cross resistance to VP-16. In this study a direct relationship was demonstrated between the degree of vinblastine resistance and over-expression of the high molecular weight glycoprotein.

Other workers have demonstrated alterations in protein expression, including: overexpression of a 20kD membrane protein in multidrug-resistant MCF-7 cells (Fine et al., 1985); overexpression of a 19kD cytosolic protein in multidrug-resistant Chinese hamster fibroblasts (Myers and Biedler, 1981); and the loss of 72 and 75kD membrane proteins concomitant with the expression of P-glycoprotein in multidrug-resistant human epithelial carcinoma cells (Shen et al., 1986a). However, the precise role that these altered proteins play in the expression of multidrug resistance has yet to be defined.

(ii) Alterations in DNA damage after VP-16 treatment, not associated with reduced drug uptake but with intranuclear events

If, as is currently thought (Chen et al., 1984; Minocha and Long, 1984; Ross et al., 1984; Yang et al., 1985), DNA damage mediated via topoisomerase II is the main cytotoxic lesion in cells treated with VP-16, possibilities arise whereby VP-16-resistance could be associated with alterations in DNA damage or repair, or modified DNA topoisomerase II activity.

Long and colleagues (1985b) studying human lung and colon carcinoma cell lines exhibiting a range of responses to VP-16, demonstrated that the extent of VP-16-induced DNA single-strand break formation reflected sensitivity or resistance in all the cell lines. Additionally, the numbers of DNA breaks produced in isolated nuclei reflected those produced in intact cells suggesting that the expression of sensitivity or resistance was determined by factors localized in the nucleus and was not dependent upon differences in drug transport across the plasma membrane. Repair of VP-16-induced DNA strand breaks was rapid for all cell lines, so that differential repair did not appear to be a major factor in the varied responses to VP-16. It was proposed that variations in levels or activities of topoisomerase II may be responsible for cellular resistance to VP-16.

The mechanism associated with VP-16 resistance in a Chinese hamster ovary cell line, Vpm^{R}-5, selected for resistance to VM-26, may involve a multidrug-resistant phenotype not associated with

reduced drug uptake (Glisson et al., 1986a and b). These cells expressed cross resistance to structurally dissimilar drugs, but this was not associated with reduced drug uptake as in other, previously mentioned, multidrug resistance phenotypes (Inaba et al., 1981; Beck, 1983). The variety of agents to which these cells expressed cross resistance had in common the ability to stimulate type II topoisomerase-mediated DNA cleavage. The resistant cells were less responsive to the cytotoxic and DNA damaging effects of VP-16, adriamycin, m-AMSA and mitoxantrone. A crude nuclear extract from the resistant cells was profoundly less sensitive to topoisomerase II specific VP-16-induced DNA cleavage activity compared to extracts from sensitive cells. Actual topoisomerase II catalytic activity was, however, equivalent and so it would appear that the mutation in the resistant cell line resulted in a qualitative alteration in topoisomerase II which affected interaction of drug with the enzyme or enzyme-DNA complex. Recent purification of DNA topoisomerase II from parental and drug-resistant cell lines by Fast-Protein Liquid Chromatography (FPLC) has shown that Vpm^{R}-5 cells do indeed express a mutant form of the enzyme (W. Ross, personal communication).

A VP-16-resistant subline of Chinese hamster lung cells (DC3F/9-OHE) formed fewer protein-associated DNA strand breaks after exposure to VP-16 than did the sensitive parental cells. The same was true when isolated nuclei from these cells were examined, indicating that resistance was independent of drug uptake through the plasma membrane (Pommier et al., 1986a). Analyses of 0.35M sodium chloride extracts from nuclei of sensitive and resistant cells have

shown that the parental cells contained approximately twice the number of topoisomerase II molecules compared with the resistant cells. However, both topoisomerase I and II activities appeared similar in nuclear extracts from both parental and resistant cells. Further comparisons revealed that the DNA linking activity of the DC3F/9-OHE nuclear extract was not stimulated by drug, in contrast to that from sensitive cells. However, these drug-resistant cells did possess a VP-16-sensitive topoisomerase II. It was therefore suggested that the topoisomerase II in resistant cells was modulated in such a way that its trapping by topoisomerase II inhibitors was reduced in the total nuclear extract. The nature of this modulating factor is, as yet, unknown. However, in these resistant cells a drug-insensitive DNA linking activity, the presence of which is unusual, copurified with topoisomerase I and may be involved in modulation of topoisomerase II activity (Pommier et al., 1986b).

It has recently been suggested by Sullivan and colleagues (1986) that alterations in topoisomerase II activity may form the basis for the differential sensitivity of exponentially-growing and plateau-phase Chinese hamster ovary cells to VP-16-induced DNA damage and cytotoxicity. The activity of topoisomerase II was several fold higher in log-phase than in plateau-phase cells, and was more sensitive to stimulation by VP-16. Further studies are necessary to determine whether alterations in enzyme activity associated with proliferation state are due to qualitative or quantitative changes in topoisomerase II.

Markovitz and colleagues (1986) have proposed that the association of topoisomerase II with DNA during DNA synthesis may account for the increased sensitivity of proliferating murine L1210 leukaemic cells to VP-16 compared to quiescent cells. The yield of VP-16-induced DNA-protein crosslinks increased concomitantly with the onset of DNA synthesis after growth initiation. This increase in DNA-protein crosslinks correlated with VP-16-induced cytotoxicity.

(iii) Alternative mechanisms including the role of intracellular glutathione and alterations in glutathione transferase, reductase and peroxidase

The proposed metabolic activation of VP-16 to an oxygen centred free radical (Sinha and Myers, 1984) has led to the suggestion that resistance may be associated with elevated levels of intracellular free radical scavenging systems. Additionally, drug activity may be reduced by conjugation with glutathione within the cell.

In the adriamycin-resistant human breast cancer cell line (MCF-7/ADR^{R}) expressing a high level of cross resistance to VP-16 (175-fold) increased expression has been noted of glutathione S-transferase (45-fold), glutathione peroxidase (12-fold), and UDP-glucuronyl transferase (3-fold) compared to the parental drug sensitive line (Tulpule et al., 1986). This pleiotropic drug-resistant phenotype was also associated with reduced adriamycin

accumulation (2- to 3-fold) and amplification of specific DNA sequences (Fairchild et al., 1986).

The mitomycin C-resistant L1210 cell line, described by Dorr and colleagues (1985), and exhibiting cross resistance to VP-16, expressed a 2-fold increase in non-protein sulphydryl content compared to the parental sensitive cells. Exposure of these cells to non-cytotoxic concentrations of an inhibitor of γ-glutamyl synthetase, buthionine sulphoximine, substantially reduced levels of non-protein sulphydryl compounds and restored mitomycin C sensitivity in the resistant subline to that of the parental line. However, the significance, if any, of these observations to VP-16 resistance remains unclear.

e. Circumvention of VP-16 Resistance

By identifying mechanisms of resistance to VP-16 in model systems either *in vivo* or *in vitro* it may be possible to design new strategies to circumvent or prevent the emergence of drug-resistant tumour cells. Therapeutic effects may also be improved by the selective enhancement of drug cytotoxicity brought about by the identification of specific targets of drug action.

Tsuruo (1983) showed that the calcium influx blocker verapamil reversed acquired resistance to Vinca alkaloids and anthracycline antibiotics in a variety of tumour cell lines. In studies of this type resistance was associated with reduced

accumulation of drug brought about by an energy-dependent efflux pump (Inaba and Johnson, 1978). The precise mechanism by which verapamil reverses drug resistance is unknown but the implication was that it might interfere with the active efflux of drug raising the intracellular concentrations which therefore resulted in enhanced cytotoxicity (Tsuruo, 1983).

Slater and colleagues (1986) claimed that verapamil completely restored responsiveness of a pleiotropic drug-resistant human T cell acute lymphatic leukaemia cell line (GM3639) to VP-16, vincristine and daunomycin. However, their data indicated that the addition of verapamil shifted the VP-16 ID_{50} values in both the parental and resistant cell lines to a similar extent. Yalowich and Ross (1985) demonstrated that verapamil at a concentration of 10μM increased VP-16 accumulation in murine leukaemia (L1210) cells concomitant with enhanced VP-16-induced DNA single-strand breakage and cytotoxicity. However, Beck and colleagues (1986) reported that verapamil, whilst almost completely reversing Vinca alkaloid resistance, failed to reverse VP-16 resistance in human leukaemic lymphoblasts (CEM cells) selected for resistance to vinblastine.

In another series of studies using an adriamycin-selected pleiotropic drug-resistant human sarcoma cell line, Harker and colleagues (1986) demonstrated that verapamil only potentiated the cytotoxicity of drugs which produced DNA strand breakage mediated via topoisomerase II. The sensitizing effects of verapamil did not correlate well with its effects on intracellular drug accumulation

and therefore these workers suggested that verapamil may be acting to promote the formation or inhibit the repair of topoisomerase II-mediated DNA strand breakage.

One limitation of many of these in vitro studies is that verapamil concentrations employed were frequently far higher than those which are achievable clinically. Preliminary results have indicated that plasma levels in the range of 0.5 to 6.0μM can be attained, although these are limited by reversible cardiac toxicity (Ozols, 1985). Although, this point was considered in a recent study (Yalowich et al., 1985) where concentrations of verapamil approaching clinically relevant levels were shown selectively to enhance VP-16 cytotoxicity in human leukaemia cells compared to normal human bone marrow granulocyte-macrophage progenitors. However, the authors suggested that it was unlikely that the magnitude of the difference observed between the two cell types would lead to any therapeutic advantage.

In summary, identification of the proposed modulating factor of topoisomerase II activity in DC3F/9-OHE cells (Pommier et al., 1986b) and further studies on the mutant form of the enzyme present in CHO/Vpm^{R}-5 (W. Ross, personal communication) associated with resistance to VP-16 should serve to increase our knowledge of drug-enzyme interactions and help in the design of new methods of circumventing drug resistance and so enhancing therapeutic effect. The role that glutathione plays in resistance to VP-16 has yet to be defined. However, it seems likely that the expression of the

multidrug-resistant phenotype may be the summation of small changes associated with reduced drug uptake, alterations in expression or regulation of target molecules, and enhanced drug detoxification systems. It may well be that due to the heterogeneous nature of tumour cells a variety of resistance-circumventing agents may have to be employed to eradicate the recurrent drug-resistant tumour (Schnipper, 1986).

1-4: RATIONALE OF THESIS

The aims of the studies reported in this thesis were to identify mechanisms of VP-16 resistance, in human tumour continuous cell lines, which may underlie the reduced clinical response rates to this agent observed after prior chemotherapy or radiotherapy (see Section 1-1:b). Possible mechanisms of VP-16 resistance have been discussed (see Section 1-3:d) including reduced drug accumulation, elevated levels of intracellular drug detoxification systems, or reduced accumulation of VP-16-induced DNA damage, which may be associated with altered activity or mutant forms of DNA topoisomerase II.

From a review of the literature it was apparent that very few VP-16-resistant tumour cell lines had been derived by specific exposure to VP-16 (see Section 1-3:b). Therefore it was necessary first to develop an appropriate experimental model system for these investigations. In addition, there was the opportunity to use a novel human tumour continuous cell line which had been treated with

fractionated X-irradiation and shown to express resistance to VP-16 (Hill and Bellamy, 1984). This has enabled a comparison to be made of two independently-derived VP-16-resistant sublines following pre-exposure of the same parental cell line either to VP-16 itself or to fractionated X-irradiation and to address the question as to whether patterns of drug responses and their associated mechanisms differed depending upon the agent employed to induce resistance.

This thesis therefore describes a unique approach to investigations of <u>in vitro</u> drug resistance in human tumours and attempts to provide some insight into the biological and biochemical bases underlying the combined modality treatment of human cancers.

CHAPTER 2 : MATERIALS AND METHODS

CONTENTS:

2-1: MATERIALS 86

a. Antitumour Drugs 86

b. Radioactively-Labelled Drugs and Chemicals 87

(i) Radioactively-labelled drugs 87

(ii) Radioactively-labelled chemicals 87

c. Chemicals and Reagents 87

2-2: CELL CULTURE TECHNIQUES 89

a. Mammalian Tumour Cell Lines Used 89

(i) Subculture of human tumour cell lines 89

(ii) Subculture of murine tumour cells 90

b. Cloning of Human Tumour Cells in Soft Agar 90

c. Cryopreservation of Human Tumour Cell Lines 91

d. Cell Counting and Determinaton of Cell Volumes 92

e. Preparation of Chromosome Spreads 93

f. X-irradiation of Cells 94

2-3: ANALYTICAL TECHNIQUES AND EXPERIMENTAL PROTOCOLS 95

a. Cytotoxicity Assays 95

(i) Growth inhibition assays after drug treatment 95

(ii) Colony forming assays after drug treatment 95

b. Determination of Content of Cellular Macromolecules 96

(i) Protein 96

(ii) Deoxyribonucleic acid 97

c. Flow Microfluorimetric Analyses 98

d. Determination of Cellular Content of Reduced Glutathione 99

e. Determination of Cellular Enzyme Activities 102

(i) Glutathione S-transferase 102

(ii) Glutathione reductase 103

(iii) Glutathione peroxidase 104

f. Uptake of Radioactively-Labelled Drugs 106

(i) Drug uptake by monolayer cultures 106

(ii) Drug uptake by cells in suspension 107

g. Estimation of Drug-Induced DNA Single-Strand Breakage by the Technique of Alkaline Elution 108

(i) Alkaline elution of DNA 109

(ii) Data collection and processing 113

(iii) Quantitation of DNA single-strand breakage 113

h. Human Tumour Cell Fractionation and Analyses of Plasma Membrane Fractions by SDS-Polyacrylamide Gel Electrophoresis 115

(i) Cell fractionation and purification of plasma membrane fractions 115

(ii) Analyses of plasma membrane fractions by SDS-polyacrylamide gel electrophoresis 116

(iii) Staining of SDS-polyacrylamide gels with the Bio-Rad silver stain 118

(iv) Immunodetection of P-glycoprotein 119

2-4: COMPUTER AND STATISTICAL ANALYSES 122

a. Student's t-Test 122

b. Linear Regression 123

2-1: MATERIALS

a. Antitumour Drugs

VP-16 was obtained from Bristol-Myers Products (Basle, Switzerland) as a clinical preparation at a concentration of 20mg per ml and stored at 4°C. Each ml of carrier contained 30mg benzyl alcohol, 650mg polyethylene glycol 300, 2mg citric acid, 80mg Tween 80 and dehydrated alcohol. This was diluted in PBSA immediately prior to use and the final concentration of solvent in growth medium did not exceed 0.25%; adriamycin and daunomycin, obtained from Farmitalia Carlo Erba Ltd. (Hertfordshire, U.K.), and cisplatin obtained from Sigma Chemical Company Ltd. (Dorset, U.K.), were stored at 4°C, and dissolved in 0.8% saline immediately prior to use; vinblastine and vincristine, provided as gifts from Lederle Laboratories (Hampshire, U.K.) were dissolved in 0.8% saline to a concentration of 1mg per ml, and kept at 4°C and diluted prior to use; podophyllotoxin was purchased from Sigma Chemical Company Ltd. and stored at 4°C, dissolved in absolute ethanol to a concentration of 1mg per ml and diluted in 0.8% saline prior to use, the final concentration of the solvent in growth medium never exceeded 0.001%.

b. Radioactively-Labelled Drugs and Chemicals

(i) Radioactively-labelled drugs

[^{3}H]-VP-16 (400mCi/mmole) was purchased from Moravek Biochemicals Inc. (California, U.S.A.), stored at -20°C, and its purity was analysed by HPLC and shown to be 94%; [^{3}H]-vincristine (5.9Ci/mmole) and [^{3}H]-vinblastine (14Ci/mmole) were purchased from Amersham International plc (Amersham, U.K.), stored at -20°C, and were at least 97% pure as analysed by thin-layer chromatography; [^{3}H]-daunomycin (2.1Ci/mmol) purchased from New England Nuclear (Mass., U.S.A.) and stored at -20°C, was greater than 99% pure as measured by thin-layer chromatography; cis-^{195m}platinum was obtained from Dr. H.L. Sharma (Department of Medical Biophysics, University of Manchester, U.K.), and its purity, determined by thin-layer chromatography, was greater than 99%.

(ii) Radioactively-labelled chemicals

[Methyl-^{3}H]-thymidine (5.0Ci/mmole), [^{14}C]-thymidine (57mCi/mmole) and Iodine-125 (14.9mCi/μg) were purchased from Amersham International plc (Amersham, U.K.).

c. Chemicals and Reagents

Dulbecco's modified Eagle's (E_4) medium, Fischer's medium, trypsin (0.25% in Tris saline stored at -20°C), versene (0.02%

ethylenediaminetetraacetic acid (EDTA), disodium salt in PBSA), glucose (10% in distilled water), Hank's balanced salt solution (HBSS), calcium- and magnesium-free phosphate buffered saline (PBSA), and 0.8% saline, were all prepared by the Central Research Services of the Imperial Cancer Research Fund.

August rat blood was extracted by the technical staff of the Imperial Cancer Research Fund Animal Unit.

Foetal calf serum (FCS), horse serum (both virus and mycoplasma screened), and colcemid, were purchased from Gibco-Biocult (Paisley, U.K.).

The following chemicals were purchased: Difco-Bacto agar from Difco Laboratories (Michigan, U.S.A.); acetaldehyde from Griffin and George (Sales) Ltd. (London, U.K.); ethanol from James Burrough plc (London, U.K.); perchloric acid and 5-sulphosalicylic acid dihydrate from Aldrich Chemical Company Inc. (Milwaukee, U.S.A.); tetrapropylammonium hydroxide (20% in water) from Fluorochem (Glossop, U.K.), and were of the highest available grade.

The following chemicals were purchased from B.D.H. Chemicals Ltd. (Poole, U.K.): potassium chloride; potassium sodium tartrate; di-sodium hydrogen orthophosphate; sodium dihydrogen orthophosphate; sodium hydrogen carbonate and sodium hydroxide.

Mithramycin was purchased from Pfizer Ltd. (Sandwich, U.K.). Polycarbonate filters were purchased from Nuclepore (Pleasanton, U.S.A.). The Bio-Rad silver stain was purchased from Bio-Rad Laboratories (California, U.S.A.). All other chemicals were purchased from either Fisons plc (Loughborough, U.K.) or Sigma Chemical Company Ltd. (Poole, U.K.), and were of the highest analytical grade available.

2-2: CELL CULTURE TECHNIQUES

a. Mammalian Tumour Cell Lines Used

The HN-1 human tumour cell line used was that originally derived from a squamous cell carcinoma of the tongue by Easty et al., (1981). Also used were HN-1 cells which had been treated with fractionated X-irradiation for 11 fractions to a total dose of 50 Gy, and designated HN-1/DXR-11 cells, as previously described by Hill and Bellamy (1984).

Murine lymphoma (L5178Y) cells were used as an internal standard in alkaline elution studies after template labelling their DNA with [^{3}H]-thymidine (see section 2-3:g).

(i) Subculture of human tumour cell lines

HN-1 cell lines were grown as monolayers in E_4 medium, supplemented with 10% FCS. Subculturing of confluent cultures was

carried out once a week. After removing the medium, cells were washed with PBSA. 1-2.5ml of a solution of trypsin : versene (1:4) was added and the monolayers incubated at 37°C for approximately 2 min or until cells had detached from the surface of the vessel. Cells were then seeded out in fresh medium and serum at approximately 2×10^4 cells per ml into Falcon plastic flasks and incubated in a 10% CO_2-air atmosphere at 37°C.

(ii) Subculture of murine tumour cells

L5178Y cells were grown in suspension in Fischer's medium supplemented with 10% horse serum. Stock cultures were subcultured twice per week by dilution with fresh medium to cell numbers approximating 10^4 per ml. Cultures were incubated in a 5% CO_2-air atmosphere at 37°C.

b. Cloning of Human Tumour Cells in Soft Agar

For cytotoxicity assays HN-1 cells were drug treated as a monolayer, removed from the dish by incubation with trypsin : versene (1:4) and plated in soft agar as described by Courtenay et al. (1978). Incorporated into the agar (final concentration 0.3%) were heat inactivated August rat red blood cells diluted 1:8 with medium plus 10% serum, lethally irradiated (100 Gy) HN-1 feeder cells at 10^4 cells per ml and E_4 medium supplemented with 10% FCS. Tubes were incubated in an atmosphere of 5% oxygen, 5% carbon dioxide and 90% nitrogen at 37°C. Fresh medium was added after 6 and 12 days in

order to replenish depleted nutrients. After approximately three weeks, colonies of 50 cells and more were scored.

c. Cryopreservation of Human Tumour Cell Lines

Exponentially-growing cultures were harvested using trypsin/versene and resuspended in 10ml of medium plus serum. Cell suspensions were centrifuged (1000 rpm, 1 min) and resuspended in 6ml medium containing 10% DMSO to minimise any membrane damage due to osmotic change (Paul, 1975). 1ml aliquots were dispensed into sterile plastic freezing vials. Vials were suspended in liquid nitrogen and their temperature lowered to -40°C at a rate of 1° per minute, and then more rapidly to below -190°C, their storage temperature.

Upon retrieval from liquid nitrogen vials were thawed rapidly in a 37°C water bath and the contents resuspended in 10ml of medium plus serum. Cells were centrifuged immediately to remove DMSO and finally resuspended in medium plus serum.

d. Cell Counting and Determination of Cell Volumes

Cell counts were determined on a Model ZB1 Coulter Counter (Coulter Electronics Inc., Bedfordshire, U.K.) in filtered PBSA. The following settings were used:

Matching Switch	=	20K
Gain Trim	=	0
Lower Threshold	=	15
Upper Threshold	=	>100

The settings of $\frac{1}{\text{Amplification}}$ and $\frac{1}{\text{Aperture current}}$ were adjusted for each cell line to give an oscilloscope pattern filling approximately two thirds of the screen. Counts between 500 and 20,000 gave the greatest accuracy.

Cell volumes were determined using the Model ZB1 Coulter Counter. Using latex beads of known diameter (8.8 and 14.9µm) the machine was calibrated to establish a value for the machine constant.

$\frac{1}{\text{Amplification}}$ and $\frac{1}{\text{Aperture current}}$ were set for the cell line; Matching Switch = 20K; Gain Trim = 0; Lower Threshhold = 5; Upper Threshold = >100.

Three counts were made of the first standard (<20,000) and these were averaged for T. T/2 was calculated and the lower

threshold (Th) adjusted to give counts of T/2 and then duplicate counts determined at Th/2 and 3Th/2. The true Th was calculated using the formula:

$$\text{Counts giving true Th} = \frac{\text{Mean counts for Th/2} + \text{mean counts for 3Th/2}}{2}$$

From equations $V = \frac{\pi d^3}{6}$ and $V = KAI\ Th$ (1) it is possible to obtain a value for K since:-

$$A = \frac{1}{\text{Amplification}}$$

$$I = \frac{1}{\text{Aperture Current}}$$

Th = True Threshold

d = Known diameter of Latex Beads

A mean value of K was calculated using different sized beads, and for the Coulter Counter used it was 7.70. To measure an unknown cell volume the above procedure was repeated and a value for Th obtained which, after substituting into equation (1), gave a value for the cell volume.

e. Preparation of Chromosome Spreads

The method described is essentially that of Worton and Duff (1979). Exponentially-growing cells were treated with colcemid (0.2μM) for approximately 3 h to block a significant number of cells in metaphase. Cells were harvested as for subculture (see section 2-2:a), retaining all PBSA washings. Cells were centrifuged (1000

rpm, 1 min) and washed twice with PBSA. After the final centrifugation cells were resuspended in 0.075M KCl (5ml) for 12 min. This hypotonic treatment leads to swelling and spreading of the chromosomes. Cells were centrifuged (800 rpm, 5 min), resuspended thoroughly in the residual volume, and fixative (methanol/acetic acid 3:1) was added dropwise to a final volume of 10ml. After 30 min cells were centrifuged (1200 rpm, 5 min) and resuspended in fixative. Cell suspensions were dropped onto clean microscope slides held at an angle of 45° to facilitate spreading of the chromosomes. After drying, metaphase spreads were examined under a Leitz Dialux 20 phase contrast microscope. A random sample of at least 20 metaphase spreads was counted and the mean chromosome number calculated.

f. X-irradiation of Cells

All X-irradiation was performed using a Pantac HF320 SR X-ray machine. Feeder cells for use in clonogenic assays (see section 2-2:b) were irradiated at a distance of 12" from the source with settings of 305kV and 10mA, which gave a radiation dose of approximately 6.8 Gy per min. For alkaline elution studies cells were irradiated at 4°C at a distance of 14" from the source with settings of 210kV and 8mA which gave a radiation dose of approximately 2.5 Gy per min.

2-3: ANALYTICAL TECHNIQUES AND EXPERIMENTAL PROTOCOLS

a. Cytotoxicity Assays

HN-1/P, HN-1/VP-1 and HN-1/VP-2 cells were set up at 2 x 10^4 cells per ml on Day 0. HN-1/DXR-11 cells were set up at 3 x 10^4 cells per ml on Day 0 as they exhibited a greater density-dependence than the other HN-1 lines.

(i) Growth inhibition assays after drug treatment

Exponentially-growing cultures were exposed to a range of drug concentrations starting on Day 2 and 72 h later cells were harvested (see section 2-2:a) and counted (see section 2-2:d). Cell numbers were calculated as a percentage of untreated controls. Growth inhibition was expressed as the concentration of drug required to reduce cell counts by 50% of untreated controls (IG_{50}).

(ii) Colony-forming assays after drug treatment

Exponentially-growing cultures were exposed to a range of drug concentrations on Days 2 or 3 for 24 or 1 h periods, before they were washed with PBSA and harvested (see section 2-2:a). For accurate colony-forming assays it was important to obtain a single-cell suspension. Therefore, cell suspensions were gently pipetted using a Pasteur pipette until microscopic examination revealed single cells present. The expected colony-forming

efficiency and extent of cell kill caused by drug treatment were estimated and the number of cells plated adjusted accordingly in order that sufficient numbers of colonies were obtained (50-100) for accurate determination of cell kill.

Cell survival was assessed by colony formation in soft agar (0.3%) (see section 2-2:b). Survival of drug-treated cells was expressed as a fraction of untreated controls. Cytotoxicity was expressed as concentration of drug resulting in only 50% cell survival compared to untreated controls (IC_{50}).

b. Determination of Content of Cellular Macromolecules

(i) Protein - according to the method of Lowry et al. (1951).

Reagents:

Reagent 'A' - 2% Na_2CO_3 in 0.1 N NaOH (made up freshly), 0.5% hydrated $CuSO_4$, 1% sodium potassium tartrate, in volume ratios of 100:1:1.

Reagent 'B' - Folin-Ciocalteau's reagent diluted 1:1 with distilled water, prepared at time of use.

Bovine plasma albumin (BPA), standard solution of 1mg/ml in 0.1 N NaOH.

Method:

1. Duplicate cell pellets of known cell number were washed with PBSA (x3) and solubilized by incubating with 1 N NaOH (1ml) for 1 h at room temperature.
2. Aliquots containing approximately 25-50µg of protein were removed in duplicate and made up to a final volume of 0.5ml with water and/or 0.1 N NaOH so that the final

solution was 0.1 N NaOH.

3. A standard curve of 0-100µg was prepared in duplicate and volumes made up to 0.5ml with 0.1 N NaOH.
4. 2.5ml of reagent 'A' was added to each sample and left for 10 min exactly.
5. 0.25ml of Reagent 'B was added to each sample which were mixed immediately on a vortex and left at room temperature for 30 min.
6. Optical density (O.D.) was measured spectrophotometrically at 750nm using glass cuvettes against a blank of distilled water.
7. Values for cellular protein content were estimated from the standard curve and expressed as µg protein per 10^6 cells.

(ii) Deoxyribonucleic acid (DNA) - according to the method of Burton (1956).

Reagents:

1N PCA

Reagent 'A' - 1.5g diphenylamine in 100ml glacial acetic acid plus 1.5ml concentrated sulphuric acid, prepared immediately prior to use.

Reagent 'B' - 16mg acetaldehyde per ml distilled water (i.e. 2ml acetaldehyde in 100ml distilled water). Can be stored at 4°C for 3-4 weeks.

Reagent 'C' - DNA reagent, containing reagents A and B in the ratio 20:0.1.

A standard solution of calf thymus DNA in distilled water. The concentration of DNA was determined by measuring the O.D. of the DNA at 260nm against a blank of distilled water. E_{260} x 48.01 x dilution = µg DNA/ml.

Method:

1. Duplicate cell pellets of known cell number were precipitated in 1N PCA.
2. Aliquots containing 10-50µg of DNA were removed in duplicate and volumes made up to 0.5ml with 1N PCA.
3. A standard curve of 0-100µg DNA was prepared in duplicate, volume being made up to 0.5ml with 1N PCA.
4. Both samples and standards were hydrolysed at 70°C for 20 min then placed on ice.
5. 1ml of DNA reagent was added to samples and standards which were incubated for 18 h out of direct light.
6. Optical density (O.D) was measured spectrophotometrically at 600nm using quartz cuvettes.
7. Values for DNA content of the samples were estimated from the standard curve and expressed as µg DNA per 10^6 cells.

c. Flow Microfluorimetric Analyses (FMF)

Cell cycle distribution was estimated by measurements of relative DNA content of individual cells using a fluorescence-activated cell sorter (FACS-1; Becton Dickinson, California, U.S.A.).

Reagents:

80% ethanol.

Staining solution - 50µg per ml mithramycin, 20mM $MgCl_2$ and 25% ethanol in distilled water.

Method:

1. Exponentially-growing cells were harvested (see section 2-2:a) and resuspended in PBSA. After centrifugation (1000 r.p.m., 1 min) cells were washed with PBSA (x 2).
2. After the final centrifugation the pellet was resuspended in the residual volume and 80% ethanol was added drop-wise to a total volume of 2ml, mixing well to avoid

aggregates. The cell suspension was incubated on ice for 30 min.

3. After centrifugation (1200 r.p.m., 5 min) cells were resuspended in 1.5ml of staining solution and incubated at room temperature for 30 min.
4. Cell suspensions were filtered through 30µm gauze to remove cell aggregates.

Percentages of cells in different phases of the cell cycle were calculated by analysis of DNA histograms according to the procedure recommended by Barfod (1977), as illustrated in Figure 2-1.

d. Determination of Cellular Content of Reduced Glutathione

Total glutathione content of HN-1 cells was determined using the enzymatic recycling assay based on glutathione reductase as described by Griffith (1980).

Reagents:

Buffer 'A' - 125mM sodium-phosphate buffer containing 6.3mM Na_2 EDTA, pH 7.5.
Buffer 'B' - 1M Triethanolamine-HCl buffer pH 8.0.
6mM DTNB in buffer A.
0.3mM NADPH in buffer A.
30% SSA and 3% SSA in distilled water.
Reduced glutathione (GSH), standard solution of 15µg per ml in 3% SSA.
Glutathione reductase 50 units per ml in distilled water, kept at 4°C.

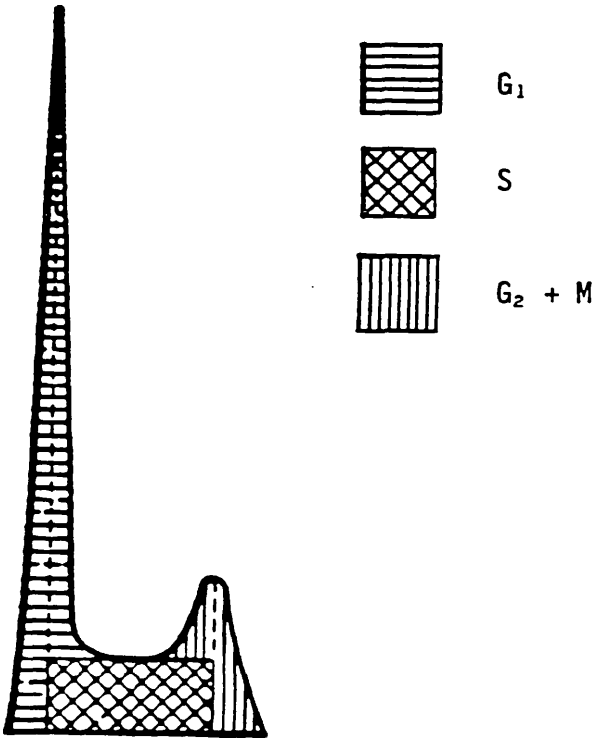


Figure 2-1: Flow Microfluorimetric Determination of DNA Content of Cells

Abscissa: channel number (relative fluorescence intensity)

Ordinate: cells per channel (relative units)

(after Barfod, 1977)

Method:

1. Exponentially-growing cultures were harvested with trypsin/versene (1:4) and resuspended in PBSA.
2. After centrifugation (1000 r.p.m., 1 min) cells were washed with PBSA (x 2) and the cell pellet was blotted dry.
3. 1.2ml of distilled water was added and cells were lysed by vortexing for 3-4 min. A 100µl aliquot was removed for protein determination (see section 2-3:b(i)) to which 20µl of 0.6N NaOH was added (final concentration 0.1 N NaOH).
4. To 900µl of cell lysate were added 100µl of 30% SSA and this was left on ice for 15 min, then centrifuged (12,000 r.p.m., 2 min)
5. The supernatant was removed and used for estimations of glutathione content.
6. The reaction mixture consisted of:
   200µl of cell lysate supernatant or GSH standard (0-3.0µg) made up with 3% SSA
   200µl buffer B
   1400µl 0.3mM NADPH
   100µl 6mM DTNB.
7. After equilibrating to 30°C, 20µl of glutathione reductase solution was added (final concentration 0.5 units per ml) and the rate of change in optical density monitored at 412nm.
8. Values for total reduced glutathione content in the cell lysate supernatant were estimated from the standard curve and expressed as nmole GSH per mg of total cellular protein.

e. Determination of Cellular Enzyme Activities

Exponentially-growing cultures were harvested with trypsin/versene and resuspended in PBSA. After centrifugation (1000 r.p.m., 1 min) cells were washed with PBSA (x2) and the cell pellet was blotted dry. The cell pellet was resuspended in distilled water to a concentration of approximately 10^7 cells per ml and cells were lysed by vortexing for 3-4 min. The lysate was left on ice for 15 min then centrifuged at 12,000 r.p.m. for 2 min. Aliquots of the supernatant were removed for determination of protein content (see section 2-3:b(i)) and estimation of enzyme activities.

(i) Glutathione S-transferase (GS-T) - according to the method of Habig and Jakoby (1981).

Principle:

A direct change in absorbance of the substrate is measured as glutathione-S-transferase catalyses the formation of a thioether between GSH and substrate.

Reagents:

Buffer 'A' - 0.1M sodium potassium phosphate buffer pH 6.5.

15mM 1-chloro-2,4-dinitrobenzene (CDNB). This substrate is first dissolved in absolute ethanol, then diluted with buffer A.

37.5mM reduced glutathione (GSH) in buffer A.

Method:

1. The reaction mixture contained:
   2400µl buffer A
   200µl CDNB
   200µl GSH
   200µl cell lysate or distilled water blank.
2. Increase in O.D. at 340nm and 25°C was monitored for at least 3 min where O.D. was less than 0.05 per minute. With high activity or non-linearity the enzyme preparation was diluted with distilled water.
3. From the equation

$$\frac{(\Delta \text{O.D. sample/min} - \Delta \text{O.D. blank/min}) \times 3 \times 10^3}{\varepsilon \times \text{volume of cell lysate}}$$

   glutathione S-transferase activity was calculated per ml cell lysate where:
   ε = Extinction coefficient of substrate at 340nm, $9.6 mM^{-1} cm^{-1}$
4. Activity was expressed as units per mg protein, where 1 unit = 1 nmole CDNB conjugated in 1 min at 25°C.

(ii) Glutathione reductase (GR) - according to the method of Horn (1965)

Principle:

Glutathione reductase (GR) catalyses the reaction:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

The reaction proceeds to completion and is irreversible. The measure of enzyme activity is the rate of decrease of O.D. due to the oxidation of NADPH.

Reagents:

Buffer 'A' - (see Section 2-3:e(i))

3mM NADPH in 1% $NaHCO_3$

15mM Oxidized glutathione (GSSG) in buffer A.

Method:

1. The reaction mixture contained:

   2400µl buffer A

   200µl NADPH

   200µl GSSG

   200µl cell lysate or distilled water blank.

2. Decrease in O.D. at 366nm and 25°C was monitored for at least 3 min. With high activity or non-linearity the enzyme preparation was diluted with distilled water.

3. From the equation:

$$\frac{(\Delta \text{ O.D. sample/min} - \Delta \text{ O.D. blank/min}) \times 3 \times 10^3}{\varepsilon \times \text{volume of cell lysate}}$$

   gluthatione reductase activity was calculated per ml cell lysate, where:

   ε = extinction coefficient of NADPH at 366nm, $3.3 mM^{-1} cm^{-1}$

Activity was expressed as units per mg protein, where 1 unit = 1 nmole NADPH oxidized in 1 min at 25°C.

(iii) Glutathione peroxidase (GPO) - determined with cumene hydroperoxide as substrate by a modification (Lawrence and Burk, 1976) of the coupled assay system originally described by Paglia and Valentine (1967).

Principle:

Glutathione peroxidase catalyses with high specificity the *in vitro* detoxification of hydrogen peroxide by the

oxidation of reduced glutathione as represented by the equation:

$$2GSH + H_2O_2 \xrightarrow{GPO} GSSG + 2H_2O \xrightarrow[NADPH \rightarrow NADP]{GR} 2GSH$$

Reagents:

Buffer 'A' - 50mM potassium phosphate Buffer, pH 7.0, containing 1mM EDTA.

Buffer 'B' - 100mM potassium phosphate buffer, pH 7.0, containing 2mM EDTA

100mM sodium azide in distilled water

10mM NADPH in buffer A

50mM GSH in buffer A

15mM cumene hydroperoxide in buffer A, prepared immediately prior to use

Type III yeast glutathione reductase (167 units/ml)

Method:

1. The reaction mixture contained:
   300µl cell lysate or distilled water blank
   300µl buffer B
   20µl NADPH
   50µl sodium azide
   20µl GSH
   200µl buffer A
   6µl glutathione reductase.
2. The reaction was started by the addition of 100µl cumene hydroperoxide and monitored by a decrease in O.D. at 340nm and room temperature for at least 3 min.
3. From the equation

$$\frac{(\Delta \text{ O.D. sample/min} - \Delta \text{ O.D. blank/min}) \times 10^3}{\varepsilon \times \text{volume of cell lysate}}$$

   glutathione peroxidase activity was calculated per ml cell lysate where:
   ε = extinction coefficient of NADPH at 340nm, $6.22 mM^{-1} cm^{-1}$

4. Activity was expressed as units per mg protein, where 1 unit = 1 nmole NADPH oxidized in 1 min at room temperature.

f. Uptake of Radioactively-Labelled Drugs

For HN-1 cell lines uptake was carried out using cells both as a monolayer and in suspension. However, no significant differences in drug uptake characteristics were observed under these different experimental conditions.

(i) Drug uptake by monolayer cultures

HN-1 cells were grown on 50mm glass petri dishes and exponentially-growing cultures were used for drug uptake studies (approximately 5 x 10^5 cells per dish). Cells were washed with HBSS plus 1% glucose and 1% $NaHCO_3$ (pH7.2) and incubated at 37°C with shaking in HBSS plus 1% glucose and 1% $NaHCO_3$ containing various concentrations of radio-labelled drug. Monolayers for zero time points were drug-treated on ice. At various time-points dishes were removed and washed with ice-cold PBSA (x3) and 1ml 0.1N NaOH added for 1 h at room temperature to solubilize the cells. Aliquots were then taken for determination of protein content (see section 2-3:b(i)) and liquid scintillation counting. Optiphase 'MP' (4ml containing 0.7% glacial acetic acid) was mixed with an aliquot of cell lysate and radioactivity determined by means of an LKB liquid scintillation counter (LKB Wallac, Finland).

Uptake was expressed as pmole radioactively-labelled drug per mg protein.

(ii) Drug uptake by cells in suspension

Exponentially-growing HN-1 cells were harvested using trypsin/versene and resuspended at a concentration of 5 x 10^5 cells per ml in E_4 medium plus 10% FCS and radio-labelled drug added to various final concentrations. Incubation, with shaking, was carried out at 37°C for 30 min, at which point aliquots were removed and cells washed with ice-cold PBSA (x3) with centrifugation (2000 r.p.m., 1 min). For efflux studies, after incubating with radio-labelled drug for 30 min an aliquot of cell suspension was removed and washed with ice-cold PBSA. After centrifugation (2,000 r.p.m., 1 min) cells were resuspended in pre-warmed (37°C) E_4 medium plus 10% FCS and incubated, with shaking, at 37°C for 30 min, after which an aliquot was removed and washed in ice-cold PBSA (x3) as described above.

For determination of VP-16, vincristine, vinblastine and daunomycin uptake, cell pellets were solubilized with 1ml of 0.1N NaOH for 1h at room temperature and aliquots taken for protein determination (see section 2-3:b(i)) and liquid scintillation counting (see section 2-3:f(i)). Uptake was expressed as pmole per mg protein.

For determination of cis-^{195m}platinum uptake; after the final wash in ice-cold PBSA cells were resuspended in 1ml PBSA, an aliquot taken and radioactivity determined on a Beckman 4000 Gamma Counter. Uptake was expressed as pmole per 10^6 cells.

g. Estimation of Drug-Induced DNA Single-Strand Breakage by the Technique of Alkaline Elution

The technique of alkaline elution utilizes filters to discriminate DNA single strands of different sizes. Cells are deposited directly onto the filters prior to lysis thereby avoiding mechanical fragmentation of DNA. After lysis, most of the cellular protein and RNA can be washed through the filter leaving double-stranded DNA which can be treated with enzymes, for example protease XI which digests DNA-protein cross links. An alkaline eluant of pH12.1 to 12.2 is then slowly pumped through the filter and DNA, now in single-strand form, will elute at a rate proportional to its size. DNA which contains single-strand breaks (SSB) will elute at a greater rate than DNA into which no single-strand breaks have been introduced (Kohn et al., 1981).

DNA is detected either by template labelling with radio-labelled thymidine or assayed fluorimetrically (Erickson et al., 1980).

Internal standard cells are usually incorporated into the assay. These cells are template labelled with [^{3}H]-thymidine and given a standard X-ray dose to introduce random DNA SSB, then mixed with sample cells labelled with [^{14}C]-thymidine (or unlabelled if to be detected fluorimetrically) and the mixture carried through the alkaline elution procedure. For example, in the alkaline elution studies described here the internal standard cells were suspension cultures of murine lymphoma (L5178Y) cells. The elution of internal standard cell DNA can be considered to define a time scale which will correct for any differences in flow rates between filters.

(i) Alkaline elution of DNA

Initial experiments revealed that it was possible to label HN-1 cells with [^{14}C]-thymidine to a maximum of only 0.015 dpm per cell. Attempts to increase the extent to which HN-1 cells were radioactively labelled by varying either the period of labelling or the 'chase' period, during which radio-labelled thymidine incorporated into low molecular weight DNA is 'chased' into higher molecular weight DNA by the addition of unlabelled thymidine, were without success. It was therefore necessary to detect sample cell DNA fluorimetrically. The procedures employed were as described by Kohn et al. (1981) and Bedford et al. (1984) with slight modifications.

After drug treatment (usually for 1 h) monolayer cultures were harvested with trypsin/versene and resuspended in ice-cold E_4 plus 10% FCS. For repair studies, after drug treatment medium containing drug was removed and the monolayer culture washed with 1ml PBSA. 5ml of pre-warmed (37°C) E_4 plus 10% FCS were added and cells were harvested after various post-treatment incubation times and resuspended in ice-cold E_4 plus 10% FCS. After centrifugation (1000 r.p.m., 1 min) cells were washed with ice-cold PBSA (x2), finally resuspended in 2.5ml ice-cold PBSA at a concentration of 2×10^5 cells per ml, and mixed with 10^4 internal standard (IS) cells which had previously received 300 Rad; the IS cells were suspensions of L5178Y murine leukaemia cells which had been prelabelled for 24h with [^{3}H]-thymidine (0.5µCi/ml). Cells were deposited onto polycarbonate filters (2.0µm pore size), washed with ice-cold PBSA (5ml) and lysed for 1h (flow stopped) with 5ml lysis solution pH10 (2% Sarkosyl, 2M NaCl, 0.04 Na_2 EDTA, containing 0.3mg/ml of Protease XI). The lysate was washed with 0.02M Na_2 EDTA (pH10.0) (2 x 3ml), the flow rate adjusted to 0.03-0.04ml per min and DNA eluted from the filters with 30ml of alkaline elution buffer (0.02M EDTA adjusted to between pH12.1 and 12.2 with tetrapropylammonium hydroxide), and 3ml fractions collected.

The filter holders had previously been covered with aluminium foil in order to exclude incident light which may induce DNA strand breaks or alkali labile sites (Kohn et al., 1981).

Flow rates were controlled by a Gilson Minipuls 2 (Anachem, Bedfordshire, U.K.) and fractions were collected on an LKB 2070 Ultrorac II (LKB Wallac, Finland).

After collection of fractions filters were finely minced in 6ml of alkaline eluant and incubated at 60°C with shaking for 1 h to remove non-eluted DNA from the filter. Filter holders and tubing were washed through with 3ml of alkaline eluant. Radioactivity was determined by mixing 1.5ml of fractions, filter supernatants and filter holder washes with 3ml of Optiphase 'MP' containing 0.7% glacial acetic acid. Samples were counted on an LKB liquid scintillation counter (LKB Wallac, Finland).

For the fluorimetric detection of DNA, 1.5ml of fractions, filter supernatants and filter holder washes were neutralized with 1.5ml of 0.1M sodium phosphate buffer (pH6.0). To this was added 1.5ml of a stock solution of Hoechst 33258 (1.5μM in filtered PBSA) and the mixture incubated out of direct light for 10 min. Relative fluorescence was measured on a Perkin-Elmer LS-5 Luminescence Spectrometer (Perkin-Elmer Ltd., Bucks., U.K.), excitation wavelength 360nm and emission wavelength 450nm, against a blank of alkaline eluant.

Figure 2-2 shows a typical standard curve using this fluorimetric technique to detect DNA. The relationship between DNA content and fluorescence is linear to below 40ng of DNA.

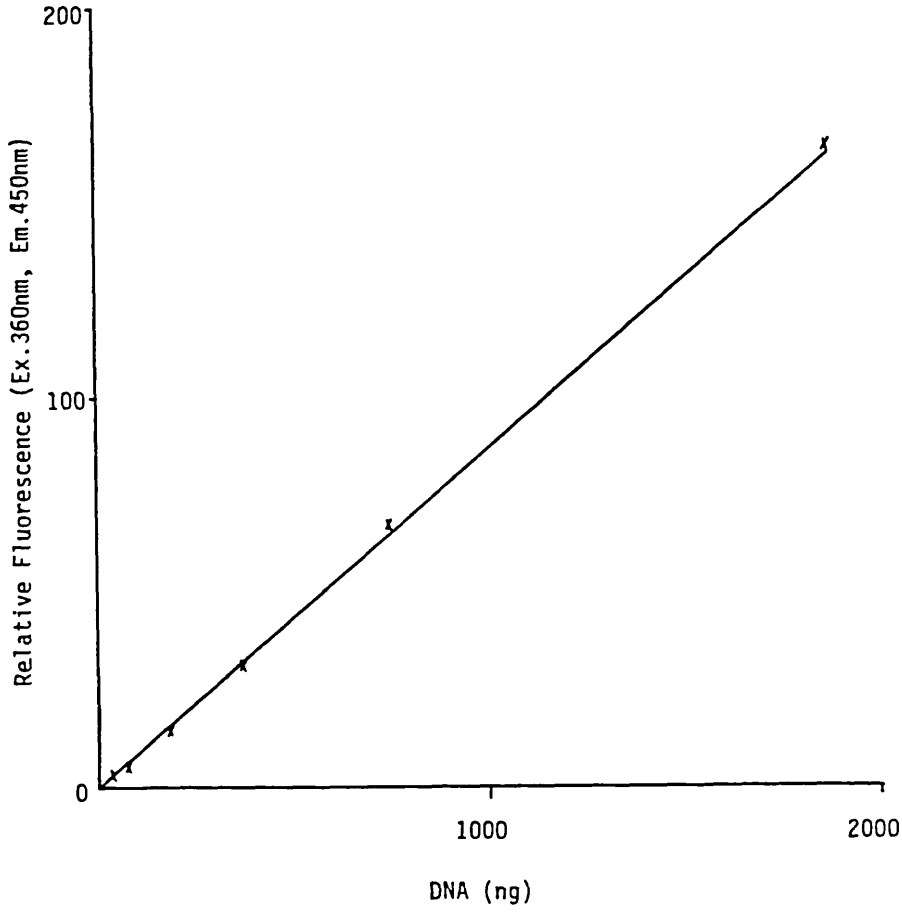


Figure 2-2: Standard curve for the fluorescence detection of DNA using Hoechst 33258. Data presented are from one experiment, each point represents the mean of three estimations.

(ii) Data collection and processing

Data from sample scintillation counting were stored on a BBC Microcomputer system, transferred to the I.C.R.F. computer system (DEC-2060) and corresponding relative fluorescence values for fractions, filter supernatants and filter holder washes were edited onto the file. Data were processed through the alkaline elution program "Duel" (K. Harris, I.C.R.F. Computer Department). Alkaline elution profiles were plotted manually.

(iii) Quantitation of DNA single-strand breakage

Figure 2-3 shows typical alkaline elution profiles of HN-1/P cells either untreated, treated with X-irradiation or with VP-16. It can be seen that HN-1/P cells treated with an X-irradiation dose of 300 Rad elute linearly with respect to elution of internal standard DNA (300R), whereas cells treated with VP-16 show an initial rapidly eluting species of DNA followed by a more stable slower eluting species.

It was therefore possible to quantitate VP-16-induced DNA single-strand breakage either by the slope of the initially eluting fraction or by the fraction of sample DNA retained at 35% retention of internal standard DNA, both compared to an irradiated control, as described by Kohn et al. (1981). DNA single-strand breakage was

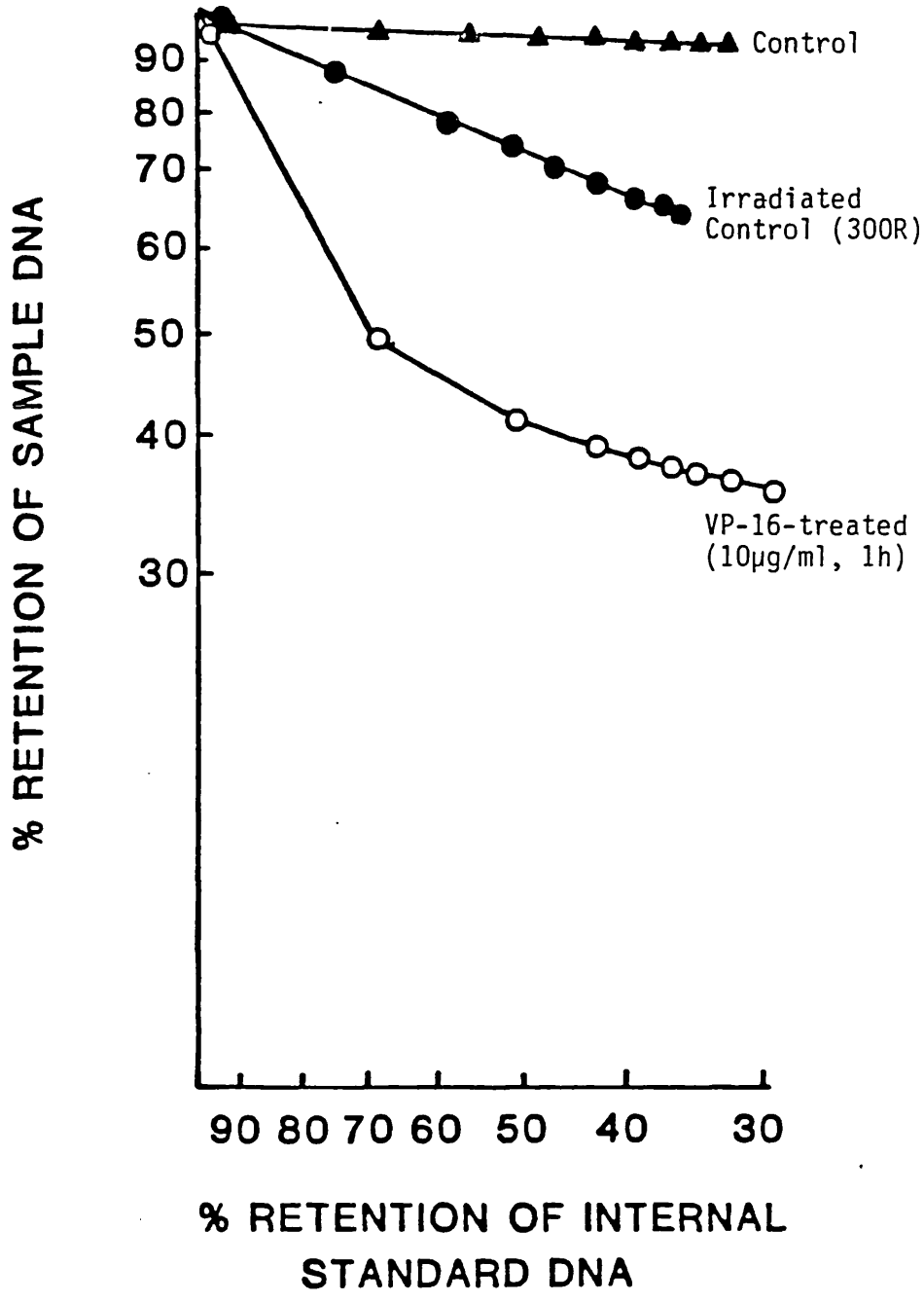


Figure 2-3: Typical alkaline elution profiles of HN-1/P cells

quantitated in terms of rad-equivalents using the equation:

$$P_{BD} = P_{BR} \left(\frac{\log(r_1/r_0)}{\log(R_0/r_0)} \right)$$

where R_0 and r_0 are the slopes or relative retentions for irradiated or unirradiated controls respectively, r_1 is the slope or relative retention for drug-treated cells, and P_{BR} is the dose of X-irradiation used to induce DNA single-strand breaks in the irradiated control, in this case 300 Rad.

h. Human Tumour Cell Fractionation and Analyses of Plasma Membrane Fractions by SDS-Polyacrylamide Gel Electrophoresis

(i) Cell fractionation and purification of plasma membrane fractions - according to the method of Riordan and Ling (1979).

All procedures were carried out in the presence of 2mM phenylmethylsulphonyl fluoride (PMSF) at 0-4°C unless stated otherwise.

1. Approximately $5x10^7$ HN-1 cells were harvested with trypsin/versene and cells resuspended in PBS, centrifuged (1000 r.p.m., 1 min), and washed with 20ml of PBS (x3). Cells were finally resuspended in 20ml of PBS.
2. Cells were disrupted using a Crumpton pump which lysed approximately 90% of cells.
3. Nuclei were pelleted at 300xg (I.E.C. Contra 7R, 3000 r.p.m., 10 min).
4. Mitochondria were pelleted at 8000xg (Sorvall RC5; HS4 rotor, 6000 r.p.m., 60 min).

5. Microsomal spin at 100,000xg (Beckman, SW41 rotor, 30,000 r.p.m., 60 min). The pellet from this centrifugation was resuspended in 2ml of PBS.
6. 1.5ml of the microsomal fraction was layered onto a discontinuous sucrose gradient consisting of 7.2ml of 60% (w/v), 14.4ml of 45%, 8.4ml of 31%, and 6.0ml of 16% sucrose in 5mM Tris-HCl (pH7.5). Centrifugation was performed in a SW28 rotor at 76,900xg (24,000 r.p.m.) for 18 h.
7. Material banded at the 16/31% sucrose interface was removed and diluted 1:5 with PBS and centrifuged in a SW14 rotor at 100,000xg (30,000 r.p.m.) for 60 min.
8. The pellet obtained was resuspended in 1.5ml of PBS and analysed by SDS-polyacrylamide gel electrophoresis. Protein content was determined by the method of Lowry et al. (1985) (see section 2-3:b(i)). This enriched microsomal fraction was stable stored under liquid nitrogen for 3 months.

(ii) Analyses of plasma membrane fractions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) - according to the method of Fairbanks et al. (1971)

Principle:

SDS binds to proteins giving them an overall negative charge due to sulphate groups. During SDS-PAGE proteins migrate towards the anode and separate according to size, low molecular weight proteins migrate further than high molecular weight proteins. The technique may be used to determine molecular weights of proteins.

Reagents for running gels:

Acrylamide bis concentrate containing 40% acrylamide (w/v) and 1.5% bisacrylamide in distilled water.

'10 times' buffer containing 40ml of 1M Tris, 10ml of 2M

sodium acetate, 10ml of 0.2M EDTA adjusted to pH7.4 with glacial acetic acid and made up to 100ml with distilled water.

20% SDS in distilled water (w/v).

Gel solution consisting of 6.3ml of acrylamide bis concentrate, 4.5ml of '10 times' buffer, 2.25ml of 20% SDS, 25.2ml of distilled water, 4.5ml of ammonium persulphate (2.5% w/v made up freshly in distilled water), and 2.25ml of NNN′N′-Tetramethylethylenediamine (TEMED) (diluted 1:100 in distilled water, made up freshly).

Sample buffer consisting of 20mM Tris-HCl, pH8.0, 2% SDS, 20% sucrose, 80mM dithiothreitol and 2mM EDTA.

Electrophoresis buffer containing 100ml of '10 times' buffer and 50ml of 20% SDS made up to 1L with distilled water.

Standards were prepared by mixing 10µl of each standard with 350µl of sample buffer and 10µl of 0.2% bromophenol blue, heating to 95-100°C for 2-3 min and then cooling to room temperature.

Samples were prepared by mixing an aliquot of the sample with an equal volume of sample buffer plus 5µl of 0.2% bromophenol blue.

Slab polyacrylamide gels (10cm x 13cm x 3mm) were prepared and run as follows:

1. All glass plates, spacers, gel formers and silicone rubber seals were washed by boiling in 1% SDS, rinsed in distilled water and dried.
2. Plates were assembled with spacers and clamped, then sealed with 1% agar.
3. Gel solution was prepared, adding the reagents in the order described to avoid premature polymerization. The gel was poured, minimizing air bubbles, after which the gel former was inserted and overlaid gently with

distilled water. Polymerization was complete in approximately 30 min.

4. Samples and standards (up to 60µl of each) were layered onto the gel and electrophresis was carried out at 100V and 100mA for approximately 2 h.

(iii) Staining of SDS-polyacrylamide gels with the Bio-Rad silver stain - according to the method of Merril et al. (1981)

Reagents:

Fixative '1' - 40% methanol, 10% glacial acetic acid (v/v) in distilled water.

Fixative '2' - 10% ethanol 5% glacial acetic acid (v/v) in distilled water.

Oxidizer - 10% of oxidizer concentrate (v/v) in distilled water, prepared immediately prior to use.

Silver reagent - 10% of silver reagent concentrate (v/v) in distilled water, prepared immediately prior to use.

Developer - 32g of developer concentrate were dissolved per litre with distilled water. This solution can be stored at 23-25°C for one month.

Stop solution of 5% glacial acetic acid (v/v) in distilled water.

Method:

All staining procedures were carried out in plastic luncheon boxes on an orbital shaker.

Reagent	Duration
Fixative 1	60 min
Fixative 2	30 min (x2)
Oxidizer	10 min
Distilled water	10 min (x3)
Silver reagent	30 min
Distilled water	2 min
Developer	30 sec
Developer	5 min (x2)
Stop solution	5 min

The gel was stored in stop solution but usually faded over a period of weeks and was therefore photographed immediately.

(iv) Immunodetection of P-glycoprotein

P-glycoprotein in plasma membrane fractions was detected using the monoclonal antibody C219 (Kartner et al., 1985) which was kindly provided by Dr. V. Ling (Ontario Cancer Institute, Toronto, Canada). All procedures using Iodine-125 were carried out by Mr. R. Whelan, Laboratory of Cellular Chemotherapy.

Iodination of monoclonal antibody:

1. The following were mixed together:
   100µg protein monoclonal antibody in 30µl PBS
   5µl DMSO
   10µl $Na^{125}I$ (100µCi/µl, carrier free)
   5µl Chloramine T (0.5mg/ml in PBS)

2. This mixture was incubated for 30 min at room temperature with occasional gentle agitation.

3. The reaction was stopped by the addition of sodium metabisulphite (1mg/ml in PBS) and the mixture was loaded onto a Sephadex G-25 column to separate the free $Na^{125}I$ from the labelled protein. Fractions were collected by adding 200µl volumes of PBS to the column.

4. 2µl samples from each fraction were counted on a gamma counter, the first peak pooled and the volume made up to 2.5ml with PBS.

5. For the second separation the pooled fractions were loaded onto a PD-10 column (Pharmacia), the run-through discarded (2.5ml) and 3.5ml PBS added to the column. The eluate was collected and mixed with 1ml 10% BSA in PBS, 0.1ml 1.5M Sodium azide, 5.4ml PBS, and stored at 4°C.

Immunodetection of immobilized antigen on nitrocellulose membrane filters:

1. Nitrocellulose membrane filters (0.45µm, Schleicher and Schuell) were blocked by presoaking in 3% (w/v) BSA in PBS containing 15mM sodium azide for 2 h at 37°C, and allowed to dry.

2. 5µg of protein from plasma membrane fractions were blotted onto the nitrocellulose and allowed to dry.

3. The nitrocellulose was placed in fresh blocking buffer (see 1. above) containing approximately $5x10^5$cpm/ml of ^{125}I-labelled monoclonal antibody and incubated for 16 h at 4°C.

4. The nitrocellulose was washed with PBS (20 min at room temperature, x 4) with shaking.

5. For exposure, the dried nitrocellulose was placed on Kodak X-AR5 film with intensifying screens and exposed at -70°C for 1 week.

Western blotting:

Following SDS-PAGE the protein profiles were replica blotted onto nitrocellulose and then probed with the iodinated monoclonal antibody.

1. The gel was rinsed with distilled water, clamped next to nitrocellulose paper and placed in the transfer apparatus containing transfer buffer (10mM Tris base, 8mM glycine, 20% (v/v) methanol in distilled water).

2. Electrophoretic transfer was carried out towards the anode at 10 V cm^{-1} for 4 h.

3. Probing of nitrocellulose was carried out as described for immunodetection of immobilized antigen on nitrocellulose membrane filters (omitting paragraph 2).

2-4: COMPUTER AND STATISTICAL ANALYSES

a. Student's t-Test

Replicate values of biochemical parameters were compared using the equations set out below. Biological statistical significance is considered to refer to where $p<0.05$, i.e. 95% confidence limits.

$$t = \frac{\text{mean sample 1 - mean sample 2}}{\sqrt{\left(\left(\frac{ss_1 + ss_2}{df_1 + df_2}\right) \times \left(\frac{1}{n_1} + \frac{1}{n_2}\right)\right)}}$$

where

n_1 = number in sample 1

n_2 = number in sample 2

$df_1 = n_1 - 1$

$df_2 = n_2 - 1$

$ss_1 = (\text{standard deviation}_1)^2 \times df_1$

$ss_2 = (\text{standard deviation}_2)^2 \times df_2$

The t value was calculated, and the degrees of freedom were calculated by:

D.F. = (no. values to obtain mean 1 + no. values to obtain mean 2) -2

The significance level, p, was then read off from the t-table using the appropriate values of t and D.F.

b. Linear Regression

Lines for uptake studies were fitted by a linear regression program contained in the package, MINITAB (Minitab, Inc., 1985).

Lines fitted by linear regression were also tested for statistical significance using the equations:

$$t = \frac{\text{Regression Coefficient}_1 - \text{Regression Coefficient}_2}{\sqrt{(\text{S.D. of coefficient}_1)^2 + (\text{S.D. of coefficient}_2)^2}}$$

$$\text{D.F.} = (\text{no. points for regression}_1 + \text{no. points for regression}_2) - 2.$$

The significance level, p, was then read from the t-table using the appropriate values of t and D.F.

CHAPTER 3 : DERIVATION OF VP-16-TREATED HUMAN TUMOUR SUBLINES AND THEIR CHARACTERIZATION

CONTENTS:

3-1: INTRODUCTION 126

3-2: DERIVATION OF VP-16-TREATED HUMAN TUMOUR SUBLINES 127

3-3: CHARACTERIZATION OF VP-16-TREATED HUMAN TUMOUR SUBLINES 128

a. General Description and Morphology 128

b. Growth Rates 129

c. Cellular Contents of Macromolecules 129

d. Karyotypic Analyses 131

e. Cell Volumes 132

f. Cloning Efficiencies 132

g. Cell Cycle Distributions 133

3-4: DISCUSSION AND SUMMARY 133

3-1: INTRODUCTION

VP-16 has a broad spectrum of clinical antitumour activity, proving one of the most active single agents in the treatment of small-cell carcinoma of the lung, and has activity as second line therapy of certain germ cell tumours in combination with other drugs (O'Dwyer et al., 1985). However, prior treatment appears to be a crucial factor determining therapeutic outcome. For example a number of combination chemotherapy regimens including VP-16 are capable of inducing rapid tumour regression and improved survival in patients with small-cell lung cancer, but death from recurrent chemotherapy-resistant disease remains the outcome in over 90% of cases (Natale and Wittes, 1985).

There are few reports in the literature where a drug-resistant human tumour cell line has been developed following specific exposure to either VP-16 or VM-26 (Danks and Beck, 1983; Long et al., 1985b). Therefore there remains a need to establish relevant experimental model systems to investigate VP-16 resistance.

This chapter describes the derivation of VP-16-treated HN-1 sublines, and their characterization in terms of certain cytological, biological and kinetic parameters.

3-2: DERIVATION OF VP-16-TREATED HUMAN TUMOUR SUBLINES

Initial experiments were carried out to determine the maximum concentration of VP-16 which permitted HN-1 cells to maintain their normal growth rate. Continuous exposure to VP-16 at 20 ng/ml induced no detectable growth inhibition in HN-1 cells. Drug-treated HN-1 cell lines were then selected by continuous exposure of exponentially-growing cultures to VP-16 concentrations ranging from 50 to 1000 ng/ml. Recovery was facilitated by addition of fresh medium at intervals and, providing the population appeared healthy, drug was re-added. Initially the maximum concentration of VP-16 permitting sufficient viable cells to repopulate was 100 ng/ml. However, after nine passages at 100 ng/ml of drug the cells appeared to have adapted to these conditions and it was possible to raise the drug concentration to 200 ng/ml. Attempts to further increase the drug concentration were, however, unsuccessful since the cultures deteriorated and viable populations could not be maintained.

HN-1 cells which had grown through eleven passages of VP-16 at 100 ng/ml were designated HN-1/VP-1, whilst those which had grown through nine passages of VP-16 at 100 ng/ml and two at 200 ng/ml were designated HN-1/VP-2. Adequate stocks of each subline were cryo-preserved (see section 2-2:c).

3-3: CHARACTERIZATION OF VP-16-TREATED HUMAN TUMOUR SUBLINES

HN-1 cells were characterized in terms of morphology, growth kinetics and certain biological parameters and compared to the VP-16-treated sublines, HN-1/VP-1 and HN-1/VP-2.

a. General Description and Morphology

HN-1/P cells grew as an epithelial-like monolayer which at high cell numbers (approximately 2.5 x 10^5 per 30 mm petri dish) covered the surface of the flask and became multilayered with cells floating off into the medium.

HN-1/VP-1 and HN-1/VP-2 cultures exhibited no signs of the debris, dead cells and giant cells which had been observed through the initial exposures to VP-16 and these sublines were morphologically identical to HN-1 parental cells.

The tumourigenicity of HN-1/P, HN-1/VP-1 and HN-1/VP-2 cell lines were tested by subcutaneous innoculation of 2 x 10^6 cells each into 5 nude mice. However, no tumours or subcutaneous nodules developed within a period of 3 months, as previously reported for HN-1/P cells by Easty et al. (1981) and Bellamy (1982).

b. Growth Rates

Growth curves were obtained by counting cells from replicate dishes on consecutive days as described in section 2-2:d. Cells were plated on day 0 and exponential growth continued between days 1 and 4, after which cell proliferation slowed between days 4 and 7 probably due to depletion of nutrients from the medium. The population doubling time for each cell line was estimated from these growth curves in terms of the number of hours taken for the cell population to double during exponential growth. The mean values from at least four separate experiments revealed no significant differences between the three cell lines (Table 3-1).

This finding is of importance since, when subsequently comparing drug responses and the biochemical mechanisms associated with altered drug responses, identical culture conditions and drug exposure times could be used for all these HN-1 cell lines. Any differences observed could not be attributed to alterations in growth rates.

c. Cellular Contents of Macromolecules

DNA and protein content of HN-1 cell lines were determined by the methods of Burton (1956) and Lowry et al. (1951) respectively, and results are shown in Table 3-1. No significant alterations in cellular contents of these macromolecules were found between parental and drug-treated cell lines. DNA contents ranged between 19.1 and

Table 3-1: Characteristics of HN-1 parental (P), and drug-treated (VP-1 and VP-2) cell lines. Data presented are the mean ± s.e. of at least three separate experiments.

Parameter	HN-1/P	HN-1/VP-1	HN-1/VP-2
Population doubling time (h) ± s.e.	28.3 ± 3.4	30.2 ± 4.2	28.7 ± 4.6
Protein content $\mu g/10^6$ cells ± s.e.	354 ± 37	415 ± 19	394 ± 36
DNA content $\mu g/10^6$ cells ± s.e.	20.6 ± 0.1	19.1 ± 0.9	21.7 ± 0.4
Mean chromosome number (range)	81 (56 - 87)	75 (62 - 85)	81 (62 - 86)
Colony forming efficiency % ± s.e.	13.6 ± 2.5	10.8 ± 2.3	12.4 ± 2.9
Cell volume μm^3 ± s.e.	3008 ± 129	2672 ± 223	2933 ± 142
Cell cycle distribution % ± s.e.			
G_1	56 ± 3	59 ± 4	57 ± 3
S	20 ± 1	17 ± 2	24 ± 5
G_2 + M	24 ± 2	24 ± 2	19 ± 3

21.7 μg/10^6 cells, whilst protein contents ranged between 354 and 415 μg/10^6 cells. These values are in close agreement with those reported by Hill and Bellamy (1984) for these macromolecular contents of HN-1 cells.

The apparent increase in cellular protein content in HN-1/VP-1 and HN-1/VP-2 cells compared to parental cells was not statistically significant and was not reflected by any increase in cell volume (see Section 3-3:e).

d. Karyotypic Analyses

Chromosome spreads of HN-1 cells were prepared as described by Worton and Duff (1979) (see Section 2-2:e). Data in Table 3-1 show that, in all the lines, cells were hypotetraploid with an extremely large variation in chromosome number from cell to cell. Mean chromosome numbers were 81, 75 and 81 for HN-1/P, HN-1/VP-1 and HN-1/VP-2 cells respectively. The mean chromosome number for HN-1/P cells in this study is in agreement with those published by Easty et al. (1981) and Hill and Bellamy (1984) for HN-1/P cells, with the slight variations between these studies being insignificant. The apparent shift in mean chromosome number noted for HN-1/VP-1 cells from 81 to 75, was not significantly different from HN-1/P cells due to the large range in chromosome number in all HN-1 cell lines (Table 3-1).

e. Cell Volumes

Cell volumes of HN-1 cell lines were determined as described in Section 2-2:d. As shown in Table 3-1, no significant differences in cell volume were observed between HN-1 parental and drug-treated cell lines, with values of 3008, 2672 and 2933 μm^3 for HN-1/P, HN-1/VP-1 and HN-1/VP-2 cells respectively.

f. Cloning Efficiencies

Colony-forming assays were carried out using the method described by Courtenay et al. (1978) (see Section 2-2:b). Data in Table 3-1 compare the colony-forming efficiencies of non drug-treated controls for HN-1 cell lines. These values did not differ significantly between parental and VP-16-treated sublines, with values ranging from 10.8 to 13.6 percent. Initial experiments demonstrated a linear relationship between cells plated per tube and number of colonies formed for each line. These observations are again important as they facilitate the direct comparison of drug responses exhibited by the three cell lines using identical colony-forming assay conditions.

g. Cell Cycle Distributions

Cell cycle distributions of HN-1 cell lines were estimated from measurements of relative DNA content of individual cells as described in Section 2-3:c. No significant differences were observed between exponentially-growing HN-1/P, HN-1/VP-1 and HN-1/VP-2 cells (see Table 3-1) with approximately 60% of cells in G_1, 20% in S and 20% in the G_2 + M phases of the cell cycle. These findings may have significance in this study since, when comparing responses of these cell lines to drugs which exert different lethal effects at certain phases of the cell cycle, any alterations in response may not be attributed to differences in cell cycle distribution between HN-1/P, HN-1/VP-1 and HN-1/VP-2 cells at the time of drug treatment.

3-4: DISCUSSION AND SUMMARY

In comparing HN-1 with other human tumour cell lines, particularly those derived from squamous cell carcinomas of the head and neck (SSCHN), it appears that growth characteristics may vary considerably. Easty et al. (1986) reported that seven out of ten SSCHN lines produced solid tumours after transplantation into nude mice whereas Rupniak et al. (1985) reported that only one out of four cell lines demonstrated unequivocal tumourigenicity in nude mice. Rheinwald and Beckett (1981) observed that three out of six squamous cell carcinoma cell lines formed progressively growing tumours. A degree of correlation was found in these three studies between tumourigenicity in nude mice and differentiation of the tumour which

may, in one case (Rupniak et al., 1985), have been associated with metastatic spread of the original tumour.

The growth rates of HN-1 cell lines are in general agreement with human tumour continuous cell lines derived from SCCHN (Easty et al., 1981; Rupniak et al., 1985); and cell lines derived from human colon carcinoma (Brattain et al., 1984), lung carcinoma (Lieber et al., 1976) and neuroblastoma (Schlesinger et al., 1976; Seeger et al., 1977) although considerable heterogeneity may be observed in this parameter with, as an extreme example, a SCCHN cell line with a population doubling time of 170 h (Easty et al., 1981).

A diploid mammalian cell contains approximately 10 pg of DNA, although literature values for DNA content in human tumour cell lines are few. A murine leukaemia cell line contained approximately 7 $\mu g/10^6$ cells (Bellamy and Hill, 1984) and the higher DNA content of HN-1 cells (approximately 20 $\mu g/10^6$ cells, Table 3-1) may be due to their high degree of aneuploidy. This characteristic is consistent with other literature reports of chromosomal analyses carried out on many cell lines derived from human 'solid' tumours, which have shown a high degree of aneuploidy with structural and numerical karyotypic abnormalities (Beidler, 1975; Trent, 1984). Interestingly, four of six SCCHN cell lines, established from tissues which had been exposed to preoperative radiotherapy and/or chemotherapy, contained a greater number of chromosomes per cell than cell lines derived from patients who were not pretreated (Easty et al., 1986), indicating that the

therapy itself may have led to further genetic instability within the tumour.

The ability of HN-1 cells to clone in soft agar (Table 3-1) is considerably higher than those of other SCCHN cell lines in semisolid medium with colony-forming efficiencies ranging from 2 to less than 0.0001% (Rheinwald and Beckett, 1985; Rupniak et al., 1985). Although the problem of low cloning efficiency of some SCCHN cell lines in soft agar has been overcome by the use of plastic surfaces (Rupniak et al., 1985), this characteristic of HN-1 cells allows extensive analyses of their responses to antitumour agents by colony formation in soft agar.

Methods employed for the derivation of drug resistant tumour cell lines by specific exposure to either VP-16 or VM-26 can be summarized as follows:

(i) biweekly injections of increasing doses of VP-16 into mice carrying the Ehrlich ascites tumour (Seeber et al., 1982),

(ii) exposure of murine L1210 cells to step-wise increases in VM-26 concentration <u>in vitro</u>, followed by isolating clonal populations in the presence of higher VM-26 concentrations (Lee and Roberts, 1984a),

(iii) mutagenizing CHO cells followed by selection of VM-26-resistant clonal populations (Gupta, 1983), and

(iv) exposure of human tumour continuous cell lines to sublethal VP-16 or VM-26 concentrations (Danks and Beck, 1983; Long et al., 1985b).

HN-1/VP-1 and HN-1/VP-2 cell lines were selected by exposure to sublethal VP-16 concentrations in an attempt to simulate in vitro the conditions which might exist in an in vivo setting. From a purely biochemical point of view it is desirable to maximize any potential differences between sensitive and resistant cell lines, and the use of clonal populations which have grown through high drug concentrations may facilitate this. In the study by Gupta (1983) the probability of isolating drug-resistant clonal populations was increased by pretreatment with the mutagen ethylmethanesulphonate. Although this experimental system may appear to bear little relevance to clinical drug resistance, the important mechanistic findings of this study may yet prove relevant in the clinic (Glisson et al., 1986a and b). This may also be true of clonal populations of L1210 cells which are highly resistant to VM-26 (Lee and Roberts, 1984a). VP-16-resistant HN-1 cells were selected with a view to obtaining clinically relevant degrees of drug resistance (3- to 20-fold; Frei, 1982) using uncloned cell populations in common with the studies of Danks and Beck (1983) and Long et al. (1985b). This is the first detailed description and characterisation of such VP-16-resistant human tumour cell lines.

Continuous exposure of HN-1 cells to VP-16 for up to eleven passages has selected populations which exhibited no significant differences in the following cellular parameters:

Morphology

Growth rate

Macromolecular content

Chromosome number

Cell volume

Cloning efficiency

Cell cycle distribution

These observations permitted a direct comparison of the responses of HN-1/P, HN-1/VP-1 and HN-1/VP-2 cells to a range of antitumour agents using identical culture conditions and drug exposure times. Any differences in the sensitivities of HN-1/VP-1 and HN-1/VP-2 cells to these agents may be attributed to biochemical alterations caused or selected by continuous exposure to VP-16.

CHAPTER 4 : *IN VITRO* DRUG RESPONSES OF HN-1 PARENTAL AND VP-16-TREATED CELL LINES

CONTENTS:

4-1: INTRODUCTION 140

4-2: DRUG RESPONSES OF HN-1 PARENTAL AND VP-16-TREATED CELL LINES 140

a. VP-16 141

b. Podophyllotoxin 143

c. Vincristine and Vinblastine 143

d. Adriamycin and Daunomycin 146

e. Cisplatin 148

4-3: DISCUSSION AND SUMMARY 148

4-1: INTRODUCTION

In the clinical treatment of human cancers it is apparent that prior radiotherapy or chemotherapy are major factors in determining the therapeutic outcome of subsequent drug treatment. For example, VP-16 as a single agent in the treatment of small-cell bronchogenic carcinoma gave a response rate of 48% (range 30-65%) in previously untreated patients compared to only 28% (range 4-48%) for patients who had received prior chemotherapy or radiotherapy (Cavalli et al., 1984). It was decided, therefore, to investigate whether prior exposure of human tumour cells to VP-16 *in vitro* would also lead to alterations in subsequent responses to chemotherapeutic agents. This chapter compares the responses of these VP-16-treated and parental HN-1 cell lines to the selecting agent, VP-16 and provides a detailed evaluation of the responses of one of these VP-16-treated cell lines (HN-1/VP-2) and the parental line to a range of other antitumour agents.

4-2: DRUG RESPONSES OF HN-1 PARENTAL AND VP-16-TREATED CELL LINES

Cytotoxicity was assessed either by growth inhibition assay after a 72 h exposure to drug (see Section 2-3:a(i)), or by colony-forming assay after a 24 h exposure to drug (see Section 2-3:a(ii)). In all cases cytotoxicity assays were carried out in parallel on parent and VP-16-treated cell lines under identical experimental conditions. IC_{50} or IG_{50} values were estimated from survival curves or growth inhibition curves respectively.

The parameters used for quantitative comparisons of drug responses were:

(i) For growth inhibition assays the IG_{50} was defined as the drug concentration required to reduce cell numbers by 50% of untreated control values,

(ii) For colony-forming assays the IC_{50} was defined as the drug concentration resulting in 50% cell survival compared to untreated controls, and

(iii) The Resistance Index was calculated for each drug tested by dividing either the IC_{50} or the IG_{50} values for the drug-treated cell line by the corresponding value for the parental line.

Survival curves presented are the mean ± s.e. of at least two experiments, each experiment was the mean of at least four estimations.

a. VP-16

Responses of HN-1/P, HN-1/VP-1 and HN-1/VP-2 cells to VP-16, assessed by colony-forming assay after a 24 h exposure to drug, are shown in Figure 4-1. It can be seen that both of the VP-16-pretreated cell lines expressed significant resistance to this agent compared to parental cells. IC_{50} values were 0.10, 0.40 and 0.39 μg/ml for HN-1/P, HN-1/VP-1 and HN-1/VP-2 cells respectively,

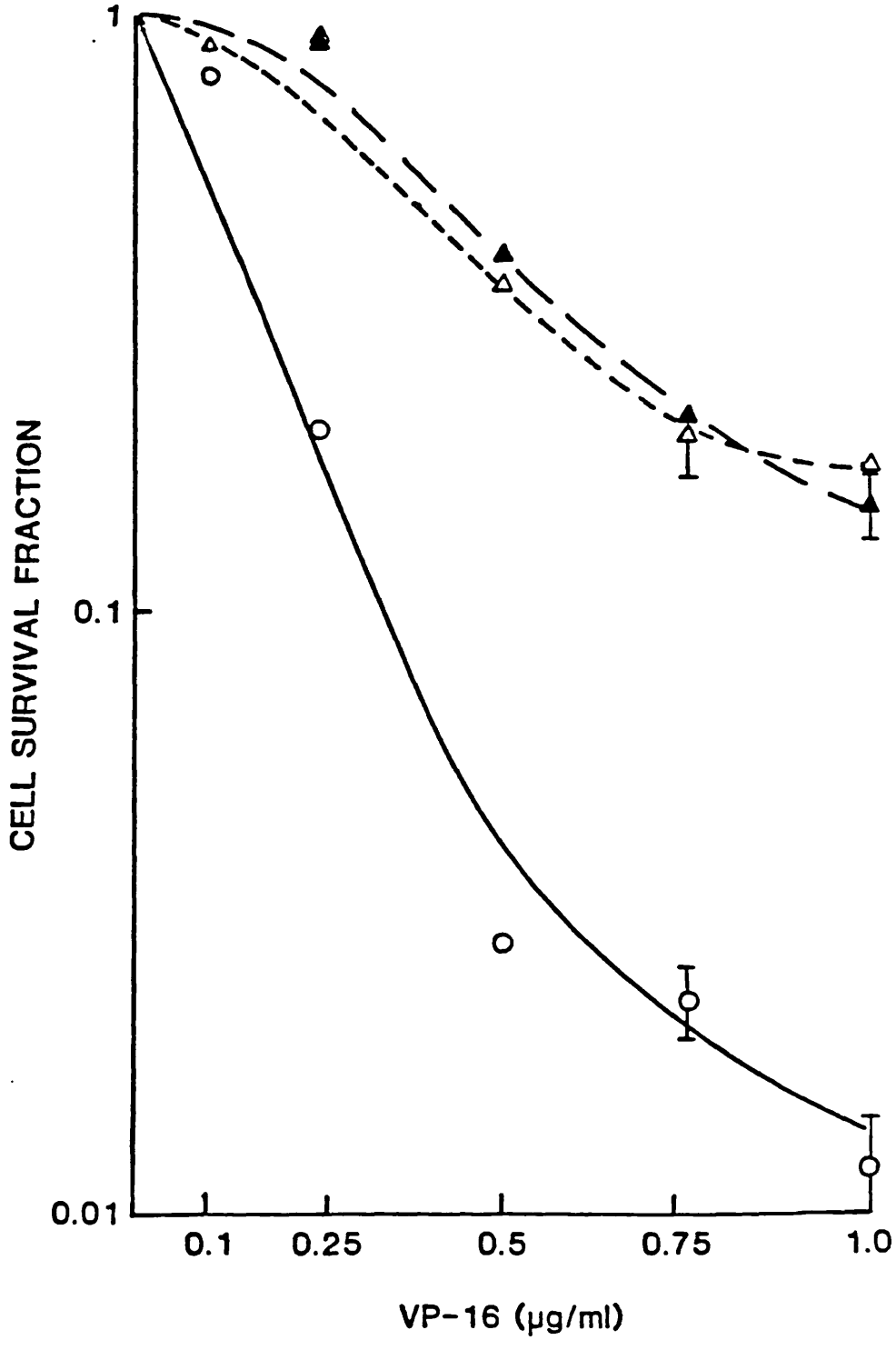


Figure 4-1: Dose response curves following 24 h exposures of exponentially-growing HN-1/P (○), HN-1/VP-1 (▲) and HN-1/VP-2 (△) cells to VP-16. Survival was assessed by colony formation in soft agar. Each point represents the mean of two experiments ± s.e.
Error bars are drawn where s.e. >10%

which gave Resistance Indices to VP-16 of 4.0 and 3.9 for HN-1/VP-1 and HN-1/VP-2 cells respectively. HN-1/VP-2 cells were also shown to express a Resistance Index of 2.1 to VP-16 when this parameter was assessed by the short-term growth inhibition assay (see Table 4-1). Due to the similarity in response to VP-16 expressed by HN-1/VP-1 and HN-1/VP-2 cells, it was decided to investigate extensively the patterns of response expressed to a range of antitumour agents, using only the HN-1/VP-2 cell line.

b. Podophyllotoxin

HN-1/VP-2 cells expressed an unaltered response to podophyllotoxin compared to parental cells, as estimated by growth inhibition assay. IG_{50} values were 10.4 and 9.7 ng/ml for HN-1/VP-2 and HN-1/P cells respectively, which gave an insignificant Resistance Index of only 1.1 (see Table 4-1).

c. Vincristine and Vinblastine

Figure 4-2 shows the responses of HN-1/P and HN-1/VP-2 cells to vincristine. Survival was assessed by colony formation after a 24 h exposure to drug. It can be seen that HN-1/VP-2 cells expressed resistance to this agent compared to parental cells. At the two highest concentrations of vincristine used, survival of HN-1/VP-2 cells was significantly greater ($p<0.01$) than parental cells. IC_{50} values were 1.8 and 4.6 ng/ml for HN-1/P and HN-1/VP-2 cells respectively, giving a Resistance Index of 2.6 for HN-1/VP-2

Table 4-1: Responses of HN-1 parental and VP-2 cells to a range of antitumour agents, assessed by colony-forming assay (CFA) or growth inhibition assay (GIA).

Antitumour Agent	HN-1 cell line	IC_{50} by CFA (ng/ml)	IG_{50} by GIA (ng/ml)	Resistance Index by: CFA	GIA
VP-16	P	100	290	3.9	2.1
	VP-2	390	600		
Podophyllo-toxin	P	nt[1]	9.7	nt	1.1
	VP-2	nt	10.4		
Vincristine	P	1.8	3.2	2.6	2.2
	VP-2	4.6	6.9		
Vinblastine	P	nt	1.8	nt	1.4
	VP-2	nt	2.6		
Adriamycin	P	8.8	42	2.7	2.6
	VP-2	23.4	110		
Daunomycin	P	nt	11.9	nt	1.8
	VP-2	nt	21.1		
Cisplatin	P	230	nt	1.3	nt
	VP-2	300	nt		

[1] - Not tested

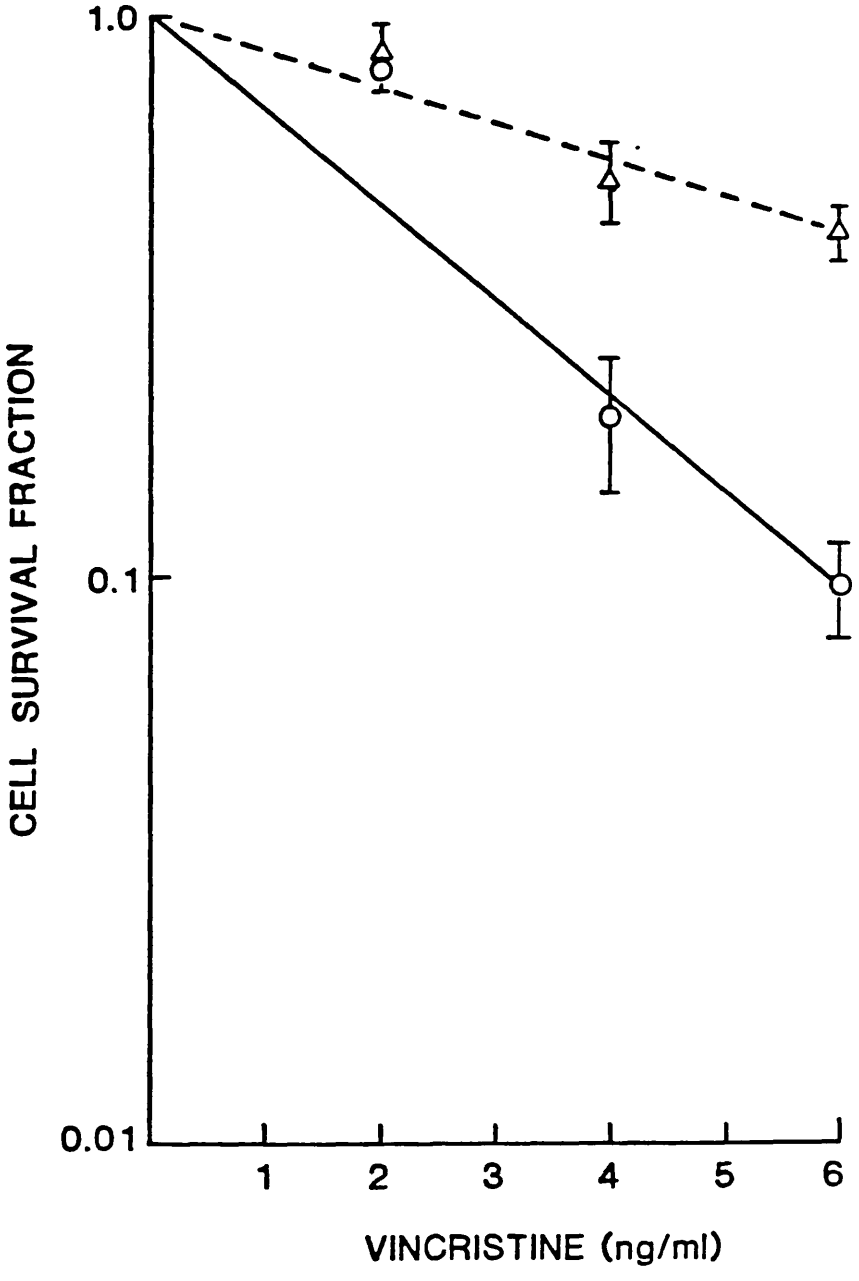


Figure 4-2: Dose response curves following 24 h exposures of exponentially-growing HN-1/P (○) and HN-1/VP-2 (△) cells to vincristine. Survival was assessed by colony formation in soft agar. Each point represents the mean of two experiments ± s.e.

cells to this agent (see Table 4-1). IG_{50} values for vincristine were 3.2 and 6.9 ng/ml for HN-1/P amd HN-1/VP-2 cells respectively, indicating that HN-1/VP-2 cells expressed a 2.2-fold level of resistance to vincristine compared to parental cells, when this parameter was assessed by growth inhibition assay (Table 4-1).

HN-1/VP-2 cells also expressed cross resistance to vinblastine as estimated by growth inhibition assay: IG_{50} values were 1.8 and 2.6 ng/ml for HN-1/P and HN-1/VP-2 cells respectively, giving a Resistance Index of 1.4 (see Table 4-1). However, in contrast to vincristine this was only considered as marginal cross resistance.

d. Adriamycin and Daunomycin

The survival of HN-1/P and HN-1/VP-2 cells after a 24 h exposure to a range of adriamycin concentrations is shown in Figure 4-3. HN-1/VP-2 cells expressed resistance to this agent compared to parental cells. Cell survival at adriamycin concentrations of 30 and 50 ng/ml was significantly different ($p<0.01$) between HN-1/VP-2 and parental cells, with IC_{50} values being 8.8 and 23.4 ng/ml for HN-1/P and HN-1/VP-2 cells respectively (see Table 4-1). These IC_{50} values indicate that HN-1/VP-2 cells showed a 2.7-fold level of resistance to adriamycin compared to HN-1/P cells. The responses of HN-1/P and HN-1/VP-2 to adriamycin were also estimated by growth inhibition assay. IG_{50} values for HN-1/P and HN-1/VP-2 were 42 and 110 ng/ml respectively (see Table 4-1). HN-1/VP-2 cells therefore expressed a

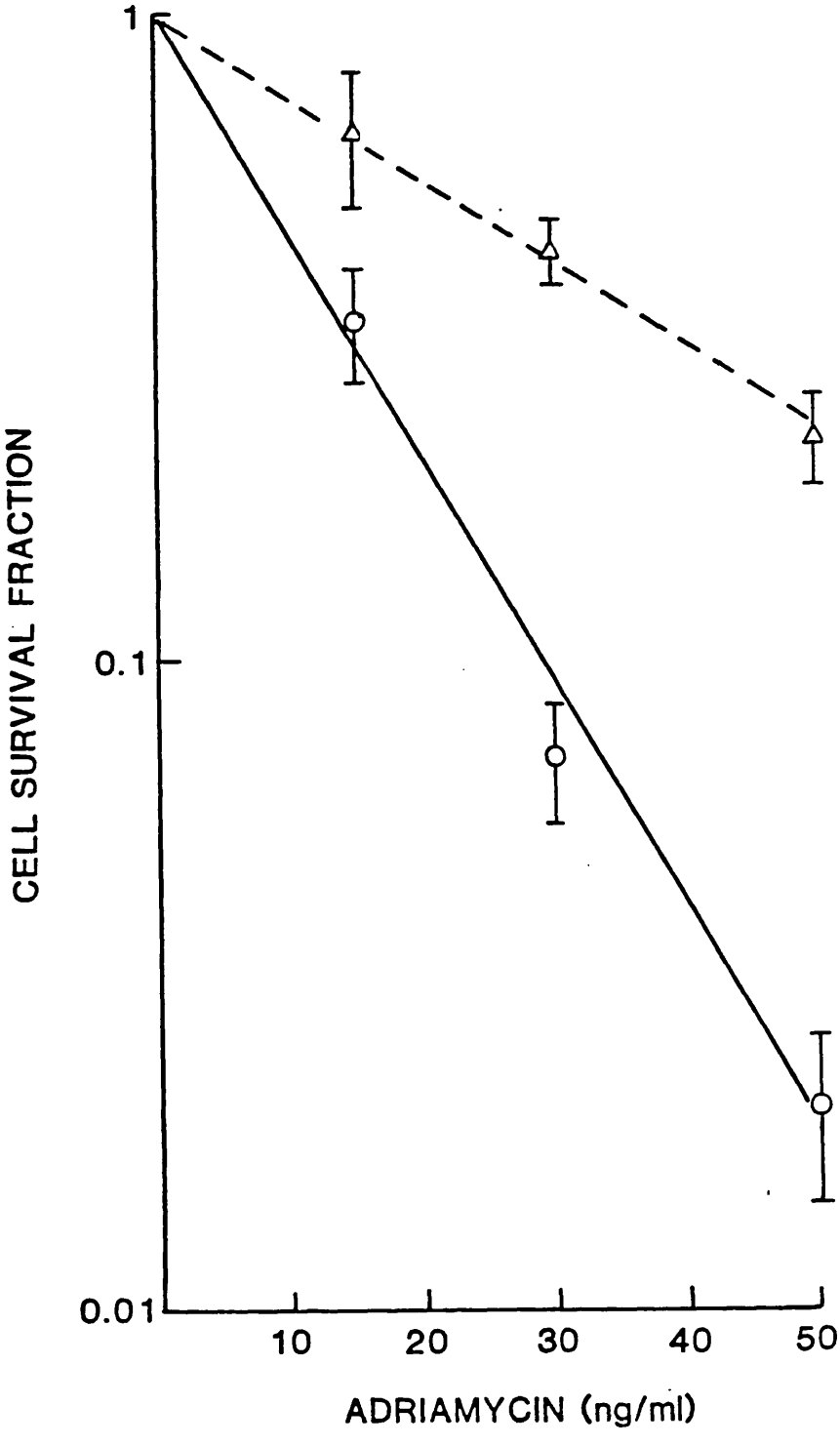


Figure 4-3: Dose response curves following 24 h exposures of exponentially-growing HN-1/P (○) and HN-1/VP-2 (△) cells to adriamycin. Survival was assessed by colony formation in soft agar. Each point represents the mean of two experiments ± s.e.

2.6-fold level of resistance to adriamycin compared to parental cells when this parameter was assessed by growth inhibition assay.

Cross resistance was also exhibited by HN-1/VP-2 cells to daunomycin, estimated by growth inhibition assay. IG_{50} values of 11.9 and 21.1 ng/ml for HN-1/P and HN-1/VP-2 cells respectively gave a Resistance Index of 1.8 for HN-1/VP-2 cells to this agent (see Table 4-1).

e. Cisplatin

Figure 4-4 shows the survival of HN-1/P and HN-1/VP-2 cells after a 24 h exposure to a range of cisplatin concentrations. It can be seen that HN-1/VP-2 cells appeared to express marginal cross resistance to this agent, as at the three highest concentrations of cisplatin used, cell survival differed significantly ($p<0.01$) between HN-1/VP-2 and parental cells. The IC_{50} values were 0.23 and 0.30 µg/ml for HN-1/P and HN-1/VP-2 cells respectively, which gave a Resistance Index of only 1.3 to this agent (see Table 4-1) indicating by the criteria used in this study, that HN-1/VP-2 cells expressed only marginal resistance to this agent.

4-3: DISCUSSION AND SUMMARY

The cytotoxicity of the various antitumour agents tested was assessed either by colony-forming assays after a 24 h exposure to drug or growth inhibition assay after a 72 h exposure to drug. From

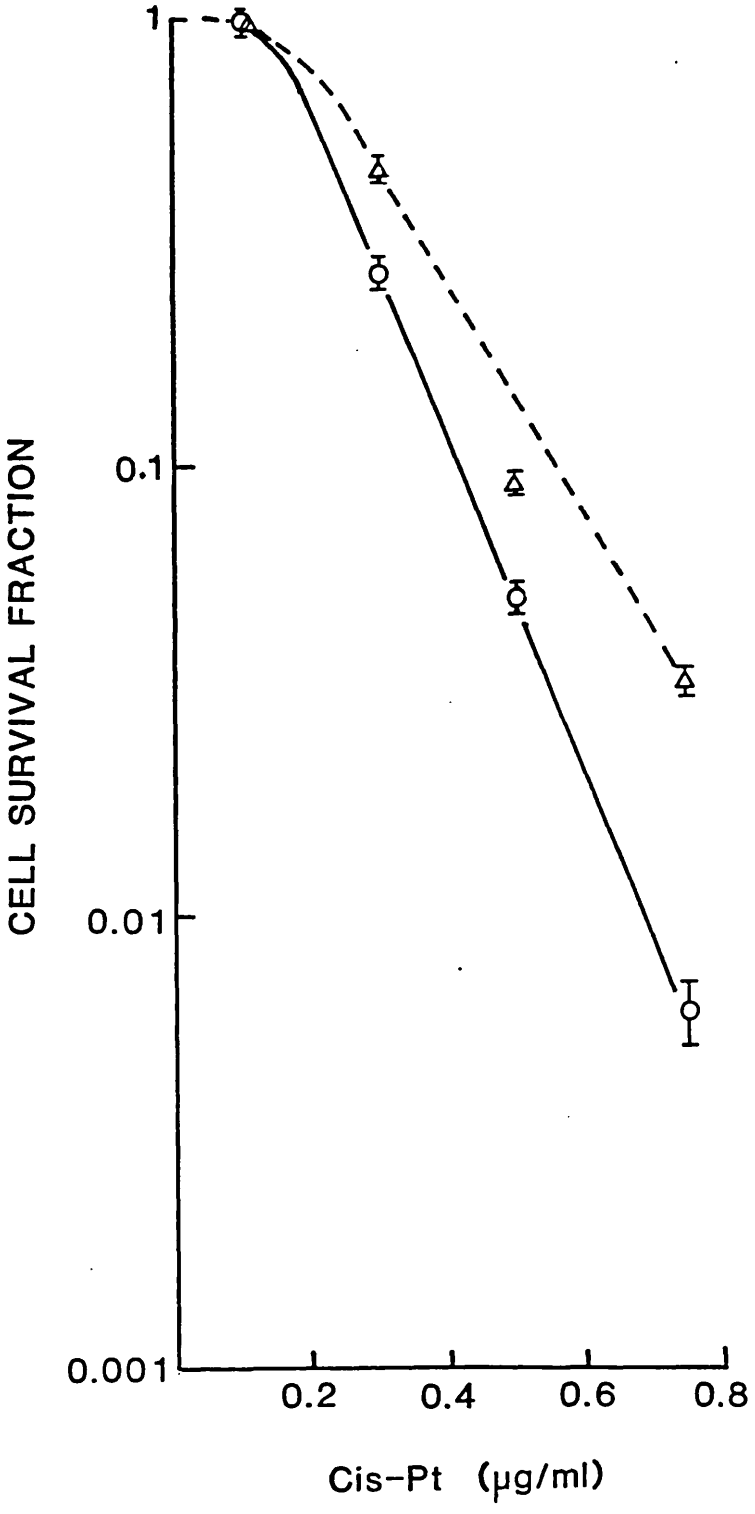


Figure 4-4: Dose response curves following 24 h exposures of exponentially-growing HN-1/P (○) and HN-1/VP-2 (△) cells to cisplatin. Survival was assessed by colony formation in soft agar. Each point represents the mean of two experiments ± s.e.

the literature there appears no standard method for comparing drug responses of sensitive and resistant cell lines and some of the various methods employed are summarized in Table 4-2, which serves also to illustrate the range of exposure times utilized in both clonogenic and growth inhibition assays. In the design of cytotoxicity assays it was necessary to take into account three basic requirements:

(i) that drug concentrations and exposure times were balanced between attaining a level of cytotoxicity in the tumour cells *in vitro*, and clinical considerations of peak plasma concentrations and decay constants of the drug,

(ii) that the assay was accurate enough to detect drug resistance in cells which had intentionally been derived to express moderate levels of drug resistance, and

(iii) in order to investigate patterns of cross resistance and collateral sensitivity to a variety of antitumour agents, the assay should be rapid and reproducible.

Direct comparisons between different drug exposure times and methods of estimating cytotoxicity are difficult to make since, for each tumour cell line, data presented are usually confined to one particular assay, antitumour drug or drug exposure time. However, for HL60 cells exposed to adriamycin, the IC_{50} value for a 1 h exposure was some 37-fold higher than the corresponding value for a

Table 4-2: A summary of the differences in cytotoxicity assays employed for estimations of drug resistance parameters in tumour cell lines (excluding isotope incorporation studies).

Tumour cell line	Drug exposure time [1]	Method used to assay cytotoxicity	Reference
Chinese hamster lung cells	30 min	Clonogenic assay	Pommier et al., 1986a
Human sarcoma (MES-SA) cells	1 h	Clonogenic assay	Harker and Sikic, 1985
Chinese hamster ovary cells		Clonogenic assay	Glisson et al., 1986a and b
Human leukaemia (HL60) cells		Clonogenic assay	Odaimi et al., 1986
Murine leukaemia (L5178Y) cells	24 h	Clonogenic assay	Bellamy and Hill, 1984
Human SCCHN (HN-1) cells		Clonogenic assay	Hill and Bellamy, 1984
Human epidermal carcinoma (KB) cells	Continuous	Clonogenic assay	Akiyama et al., 1985
Human SCCHN cells		Clonogenic assay	Rosowsky et al., 1985
Human leukaemia (HL60) cells		Clonogenic assay	Odaimi et al., 1986
Human lung carcinoma cell lines	1 h	Growth inhibition assay	Long et al., 1986
Murine leukaemia (L1210) cells	24 h	Growth inhibition assay	Lee and Roberts, 1984a
Human leukaemia (CCRF-CEM) cells	48 h	Growth inhibition assay	Danks et al., 1985
Chinese hamster lung cells	72 h	Growth inhibition assay	Biedler and Riehm, 1970; Salles et al., 1982

[1]- cytotoxicity assays involved exposure of tumour cell lines to a range of chemotherapeutic drugs, including: anthracyclines, antimetabolites, cisplatin, epipodophyllotoxins and Vinca alkaloids.

continuous exposure, as measured by colony-forming assay (Odaimi et al., 1986). Hill et al. (1985) recently compared the effects of different antitumour agents on a series of human tumour continuous cell lines by clonogenic assay and suggested that the relationship between drug concentration and exposure time differed depending upon the individual lines tested and the drug used. For Chinese hamster lung cells exposed to 2-methyl-9-hydroxyellipticine the IC_{50} value for a 30 min drug exposure was approximately 10 μM as measured by clonogenic assay, compared to an IC_{50} value of 1.2 μM for a 72 h drug exposure as measured by growth inhibition assay, only an 8-fold difference (Pommier et al., 1986a; Salles et al., 1982). This discrepancy, as well as illustrating differences between drug exposure times, also serves to highlight the between-assay variations of colony-forming assays and growth inhibition assays.

Therefore it was decided to estimate cell survival by colony-forming assay after a 24 h exposure to drug as this approximated to a population doubling time for HN-1 cells (see Table 3-1), and estimations of cytotoxicity could be made using clinically achievable drug concentrations (Alberts and Chen, 1980). Furthermore, it was observed that a more rapid growth inhibition assay, employing a 72 h drug exposure, also induced measurable growth inhibition at these concentrations. For the cell lines employed in this study, and the antitumour drugs tested, the results obtained either by colony-forming assay or growth inhibition assay generally correlate. This indicates that the growth inhibition assay remains useful for rapid assessment of patterns of response to antitumour agents, since

results were obtained in 5 days whereas colony-forming assay data were only obtained after 25-30 days.

Table 4-1 indicates however that IC_{50} concentrations were consistently lower (approximately 3-fold) than IG_{50} concentrations. The most likely explanation for this was that, although the drug exposure time was only 24 h, the colony-forming assays, by scoring colonies of 50 cells or more, measured the survival of cells which underwent at least 7 rounds of replication. However, the growth inhibition assays, although using a 72 h drug exposure time, involved counting all cells which were still attached to the surface of the dish and took no account of the fact that a proportion may be incapable of undergoing further replication. Certain discrepancies do appear to arise, however, upon detailed analysis of the Resistance Indices for a given drug when estimated by these different methodologies. Criteria for defining full cross resistance, marginal cross resistance or an unaltered response were therefore adjusted to take into account the order of resistance expressed by HN-1/VP-2 cells to the selecting agent VP-16 (3.9-fold by CFA) which was low compared to other in vitro experimental systems. For example, Beck (1983) considered that the highly vinblastine-resistant (>400-fold) human leukaemic lymphoblast cells were not (or only minimally) cross-resistant to podophyllotoxin as they only expressed a Resistance Index of 4.5 to this agent. However this figure is itself higher than the degree of resistance exhibited by HN-1/VP-2 cells to VP-16 indicating that these parameters should be adjusted according to the degree of resistance shown to the selecting agent.

A comparison of the degrees of resistance to VP-16 expressed by a range of drug-resistant tumour cell lines which have been derived by exposure to VP-16 or VM-26 either *in vivo* or *in vitro* (see Table 4-3), indicates that they are of the same order as that exhibited by HN-1/VP-2 cells (from approximately 4-fold to 20-fold). This may signify a difficulty in selecting highly VP-16-resistant mammalian tumour cell lines as reported in Chapter 3. Alternatively, as in this study, other workers may have deliberately intended to select VP-16-resistant cells in order to study resistance mechanisms at moderate but clinically relevant degrees of resistance.

The HN-1/VP-2 cell line not only expressed resistance to VP-16 itself, but also exhibited altered responses to structurally unrelated antitumour agents with different modes of cytotoxicity (see Table 4-4). A comparison of Resistance Indices, estimated by colony-forming assays, for the three major classes of antitumour agent to which HN-1/VP-2 cells were resistant, demonstrates that the highest level of resistance was exhibited towards the selecting agent, VP-16. This phenomenon, reported previously in multidrug-resistant cell lines, has led to questions being posed as to the relevance of the multidrug-resistant phenotype in the clinic where degrees of drug resistance may be slight. In the studies by Glisson et al. (1986a and b), Chinese hamster ovary cells selected for VM-26 resistance and fully cross resistant to VP-16 (20-fold) exhibited lower levels of resistance to adriamycin (5-fold), mitoxantrone (5-fold) and m-AMSA (3-fold). Human leukaemic lymphoblasts (CEM cells) selected for varying degrees of resistance to vincristine

Table 4-3: A comparison of the degrees of resistance to VP-16 shown by drug-resistant tumour cells derived by *in vivo* or *in vitro* exposure to either VP-16 or VM-26.

VP-16-resistant tumour cell line	Cytotoxicity assay used after VP-16 exposure (time)	IC_{50} or IG_{50} of parental cells (µg/ml)	Degree of resistance to VP-16	Reference
ET_{VP-16}	Increase in life span following biweekly injections of NMRI mice bearing Ehrlich ascites tumours	2mg/kg were effective in wild type tumours	>6	Seeber et al. (1982)
CEM/VM	GIA (48h)	0.12	10	Danks and Beck (1983)
HCT116 VP	GIA (1h)	ns[1]	12	Long et al. (1985b)
$CHO/Vpm^{R}-5$	CFA (1h)	1.47	20	Glisson et al. (1986a)
HN-1/VP-2	CFA (24h)	0.01	3.9	This study

[1]- not stated

Table 4-4: Summary of responses expressed by HN-1/VP-2 cells to a range of antitumour agents.

Antitumour Agent Tested	Postulated Mode of Cytotoxicity (Reference)	Response of HN-1/VP-2	Resistance Index by CFA	Resistance Index by GIA
VP-16	Topoisomerase II inhibitor (Yang et al., 1985)	Resistant	3.9	2.1
Vincristine	Microtubule inhibitor (Bender and Chabner, 1982)	Resistant	2.6	2.2
Vinblastine	Microtubule inhibitor (Bender and Chabner, 1982)	Marginally resistant	nt[1]	1.4
Adriamycin	Free radical formation and chelation of metal ions with resultant damage to DNA and cell constituents (Myers, 1982)	Resistant	2.7	2.6
Daunomycin	Free radical formation and chelation of metal ions with resultant damage to DNA and cell constituents (Myers, 1982)	Resistant	nt	1.8
Cisplatin	DNA cross-linker (Erickson et al., 1981)	Marginally Resistant	1.3	nt

[1]- not tested

began to exhibit patterns of multidrug resistance only at orders of resistance to the selecting agent of greater than 50-fold (Conter and Beck, 1984). Antitumour agents to which these cells began to develop resistance included the epipodophyllotoxins and the anthracyclines. A similar phenomenon was observed when vinblastine-resistant cells were selected from the same parental population (Beck, 1983), indicating that the expression of multidrug resistance may depend upon the order of resistance shown to the selecting agent. Similarly, human sarcoma (MES-SA) cells selected for adriamycin resistance were markedly less resistant to other classes of antitumour agents including VP-16 (Harker and Sikic, 1985). The studies reported here are the first indication that multidrug resistance may be expressed in cell lines which are less than 5-fold resistant to the selecting agent.

The majority of <u>in vitro</u> studies investigating antitumour drug resistance have maximized differences between sensitive and resistant cells so that biochemical differences may be studied under these conditions. It remains questionable whether the high degrees of resistance attained bear relevance to clinical drug resistance. The Chinese hamster ovary cells which were originally used by Ling's group to define the multidrug-resistant phenotype were highly resistant (180-fold) to the selecting agent colchicine (Bech-Hansen et al., 1976). The Vinca alkaloid-resistant CEM cells used to describe this phenotype in human tumour cell lines were greater than 400-fold resistant to their respective selecting agents (Beck, 1983; Conter and Beck, 1984). More recently though studies have been

instigated using cell lines exhibiting low levels of drug resistance. Frei (1982) proposed that a 3- to 20-fold degree of drug resistance in the clinic would translate into treatment failure, and his group have recently derived human head and neck squamous cell carcinoma lines which exhibited low levels (<20-fold) of resistance to methotrexate or cisplatin (Rosowsky et al., 1985; Teicher et al., 1986). HN-1/VP-2 cells may therefore prove useful for investigations of multidrug resistance expressed by cells exhibiting low but probably more clinically relevant degrees of drug resistance.

In summary, HN-1 cells which were selected by continuous exposure to VP-16, HN-1/VP-2, have been shown to exhibit patterns of response to a range of antitumour drugs consistent with the multidrug-resistant phenotype, that is, cross resistance is exhibited to a variety of structurally unrelated antitumour agents (Bech-Hansen et al., 1976). In the following Chapter, these patterns of response are compared with those expressed by a VP-16-resistant HN-1 cell line derived by fractionated X-irradiation treatment of HN-1 cells (Hill and Bellamy, 1984) in order to test the hypothesis that patterns of responses to antitumour agents may differ depending upon the agent employed to induce resistance.

CHAPTER 5 : COMPARISON OF PATTERNS OF DRUG RESPONSES EXPRESSED BY TWO INDEPENDENTLY-DERIVED VP-16-RESISTANT HN-1 CELL LINES

CONTENTS:

5-1: INTRODUCTION 161

5-2: PATTERNS OF DRUG RESPONSES EXPRESSED BY TWO INDEPENDENTLY-DERIVED VP-16-RESISTANT HN-1 CELL LINES 162

a. VP-16 162

b. Podophyllotoxin 164

c. Vincristine and Vinblastine 164

d. Adriamycin and Daunomycin 167

e. Cisplatin 167

5-3: DISCUSSION AND SUMMARY 168

5-1: INTRODUCTION

Prior radiotherapy or chemotherapy significantly reduces clinical response rates to subsequent chemotherapy in a number of tumour types, including:

(i) Squamous cell carcinoma of the head and neck (Price and Hill, 1981; Wolf and Chretien, 1981),

(ii) Breast carcinoma (Holland et al., 1980),

(iii) Ovarian carcinoma (Young et al., 1982), and

(iv) Small-cell bronchogenic carcinoma (Cavalli et al., 1984).

A possible explanation for these altered response rates is the outgrowth of chemotherapy-resistant tumour cells. However it remains to be established whether there are consistent patterns of drug resistance exhibited irrespective of the initial treatment administered. To address this question in this chapter the patterns of drug responses expressed by the HN-1/VP-2 cell line derived by continuous exposure to VP-16 (see Chapter 4) are compared with those of the previously reported HN-1/DXR-11 cell line derived by *in vitro* exposure of HN-1/P cells to fractionated X-irradiation, which also proved resistant to VP-16 (Hill and Bellamy, 1984).

5-2: PATTERNS OF DRUG RESPONSES EXPRESSED BY TWO INDEPENDENTLY-DERIVED VP-16-RESISTANT HN-1 CELL LINES

The fundamental growth characteristics exhibited by HN-1/P cells and the two independently-derived resistant sublines were essentially similar (see Chapter 3 and Hill and Bellamy, 1984). The responses of HN-1/VP-2 cells to the cytotoxic effects of a range of antitumour agents have been reported in Chapter 4. HN-1 cells which were pretreated with fractionated X-irradiation have been characterized in terms of their responses to VP-16, vincristine, adriamycin and cisplatin by colony-forming assay (Hill and Bellamy, 1984). The response of these cells to VP-16, podophyllotoxin, vinblastine and daunomycin have, in this study, been examined by either colony-forming assays (see Section 2-3:a(ii)) or growth inhibition assays (see Section 2-3:a(i)).

a. VP-16

Figure 5-1 compares the survival of HN-1/P, HN-1/VP-2 and HN-1/DXR-11 cells after a 24 h (a) or 1 h (b) exposure to VP-16. Survival was assessed by colony formation in soft agar. In all cases increased cytotoxicity resulted from prolonging the exposure duration, with IC_{50} values differing by factors of 16, 22 and 20 in the HN-1/P, HN-1/VP-2 and HN-1/DXR-11 lines respectively. However data from both series of survival curves indicate that both HN-1/VP-2 and HN-1/DXR-11 cells consistently showed significant resistance to VP-16 compared to HN-1/P cells. Cell survival parameters are listed

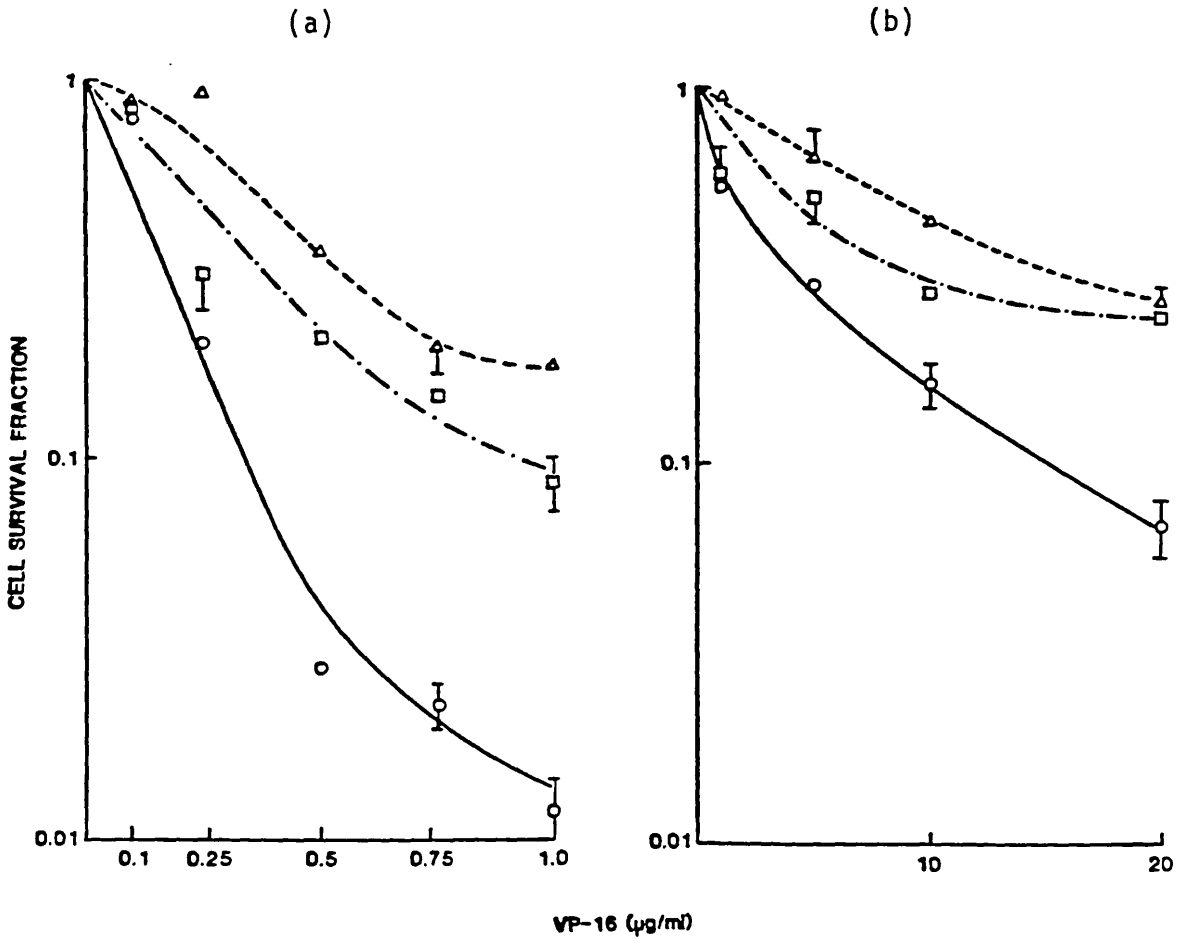


Figure 5-1: Survival of HN-1 parental (○), VP-2 (△) and DXR-11 (□) cells after 24 h (a) or 1 h (b) exposures to VP-16. Survival was assessed by colony formation in soft agar. Each point represents the mean of at least two experiments ± s.e. Bars are drawn where s.e. >10%.

in Table 5-1. It can be seen that HN-1/VP-2 and HN-1/DXR-11 cells expressed the same order of resistance to VP-16 irrespective of whether cytotoxicity was assessed after a 24 h or 1 h exposure to the drug.

b. Podophyllotoxin

HN-1/VP-2 cells exhibited an unaltered response to podophyllotoxin compared to parental cells (see Section 4-2:b). HN-1/DXR-11 cells also showed an unaltered response to podophyllotoxin compared to parental cells, assessed by growth inhibition assay. IG_{50} values were not significantly different, being 9.7 and 9.1 ng/ml for HN-1/P and HN-1/DXR-11 cells respectively, which gave a Resistance Index of 0.9 (see Table 5-2).

c. Vincristine and Vinblastine

HN-1/VP-2 cells have been shown to express cross resistance to vincristine and marginal cross resistance to vinblastine (see Section 4-2:c). Hill and Bellamy (1984) demonstrated that HN-1/DXR-11 cells were cross-resistant to vincristine compared to parental cells, which was of the same order as that shown by HN-1/VP-2 cells to this agent. From Table 5-2 it can be seen that HN-1/DXR-11 cells also exhibited marginal cross resistance to vinblastine. Responses to this agent were assessed by growth inhibition assay and IG_{50} values for HN-1/P and HN-1/DXR-11 cells were 1.8 and 2.3 ng/ml respectively, giving a Resistance Index of 1.3.

Table 5-1: Survival parameters of HN-1 cells after exposure to VP-16

HN-1 cell line	24h exposure				1h exposure			
	IC_{50} (µg/ml)	Resistance Index at IC_{50}	Survival fraction at 1.0µg/ml ± s.e.	Resistance Index at 1.0µg/ml	IC_{50} (µg/ml)	Resistance Index at IC_{50}	Survival fraction at 20µg/ml ± s.e.	Resistance Index at 20µg/ml
P	0.10	1.0	0.012 ±0.003	1.0	1.6	1.0	0.069 ±0.001	1.0
VP-2	0.39	3.9	0.17±0.01*	14.2	8.5	5.3	0.27±0.03*	3.9
DXR-11	0.22	2.2	0.071 ±0.01**	5.9	4.3	2.7	0.25±0.03*	3.6

* Statistically significant difference from HN-1/P ($p<0.01$)
** Statistically significant difference from HN-1/P ($p<0.02$)

Table 5-2: Responses of HN-1 parental and DXR-11 cells to podophyllotoxin, vinblastine and daunomycin, assessed by growth inhibition assay.

Antitumour Agent Tested	HN-1 cell line	IG_{50} (ng/ml)	Resistance Index
Podophyllotoxin	P	9.7	0.9
	DXR-11	9.1	
Vinblastine	P	1.8	1.3
	DXR-11	2.3	
Daunomycin	P	11.9	1.0
	DXR-11	12.0	

d. Adriamycin and Daunomycin

HN-1/VP-2 cells were cross resistant to both adriamycin and daunomycin compared to parental cells (see Section 4-2:d). However, HN-1/DXR-11 cells have been shown previously to express an unaltered response to adriamycin (Hill and Bellamy, 1984) and data in Table 5-2 indicate that HN-1/DXR-11 cells also exhibited an unaltered response to daunomycin compared to parental cells. IG_{50} values were 12.0 and 11.9 ng/ml for HN-1/DXR-11 and HN-1/P respectively which were not significantly different.

e. Cisplatin

HN-1/VP-2 cells expressed marginal cross resistance to cisplatin compared to parental cells (see Section 4-2:e). However, Hill and Bellamy (1984) demonstrated that HN-1/DXR-11 cells were collaterally sensitive to this agent compared to parental cells, although this was only marginal since survival curve parameters between HN-1/P and HN-1/DXR-11 cells exposed to cisplatin were not significantly different.

5-3: DISCUSSION AND SUMMARY

Drug resistant human tumour cell lines have been derived from HN-1 cells either by continuous exposure to VP-16 (HN-1/VP-2) (see Chapters 3 and 4) or by fractionated X-irradiation treatment (11 fractions to a total dose of 50 Gy, designated HN-1/DXR-11) (Hill and Bellamy, 1984). The responses of these two independently-derived cell lines to a range of antitumour drugs have been compared with those of HN-1 parental cells and are summarized in Table 5-3. These data clearly indicate that, in this unique experimental model system, whereby two VP-16-resistant cell lines have been derived independently from the same parental cells, patterns of responses to antitumour drugs differ depending upon the agent employed to induce resistance.

Gupta (1983) derived Chinese hamster ovary cell lines resistant to VP-16 and VM-26, primarily by mutagenizing the cells with ethylmethanesulphonate and then selecting resistant mutants with VM-26. In agreement with the patterns of responses to antitumour agents exhibited by HN-1/VP-2 cells, these cells proved cross resistant to daunomycin but not to podophyllotoxin and were only marginally cross-resistant to vinblastine. However, these VP-16-resistant CHO mutants expressed collateral sensitivity to cisplatin. This response is in agreement with that of the HN-1/DXR-11 cells. HN-1/VP-2 cells were selected without prior exposure to a potent mutagenizing agent such as ethylmethanesulphonate although VP-16 itself has been shown to be mutagenic (Singh and Gupta, 1983).

Table 5-3: Summary of the responses expressed by HN-1/VP-2 and HN-1/DXR-11 cells to the cytotoxic effects of a range of antitumour agents compared with HN-1 parental cells.

Antitumour Agent Tested	HN-1/VP-2 Cells	HN-1/DXR-11 Cells
VP-16	Resistance	Resistance
Podophyllotoxin	No Change	No Change
Vincristine	Resistance	Resistance
Vinblastine	Marginal Resistance	Marginal Resistance
Adriamycin	Resistance	No Change
Daunomycin	Resistance	No Change
Cisplatin	Marginal Resistance	Marginal Collateral Sensitivity

Therefore the HN-1/VP-2 cell line may have been derived either by a mutation induced by VP-16 which was subsequently selected for, and/or by the selection of resistant sub-populations already present in the parental cell population (Goldie and Coldman, 1984). The derivation of the HN-1/DXR-11 cell line involved exposure of HN-1/P cells to a known mutagen, X-irradiation, although further selection of VP-16-resistant mutants did not take place (Hill and Bellamy, 1984), unlike the study of Gupta (1983). Therefore it is possible that these differences in patterns of responses to antitumour drugs expressed between VP-2 and DXR-11 cells may be due to the method by which resistance was derived. This hypothesis is strengthened when considering other in vitro studies where drug-resistant cell lines have been derived by selection either with drug or by exposure to X-irradiation: in the study by Hill et al. (1984) murine leukaemia (L5178Y) cell lines resistant to vincristine were derived either by exposure to the drug itself or to 10 equal fractions of X-irradiation. The cell line derived by vincristine pretreatment was resistant to vincristine, vinblastine, adriamycin and bleomycin, but exhibited unaltered responses to VP-16 and cisplatin. However, the cell line derived by X-irradiation pretreatment expressed resistance to vincristine, vinblastine, and VP-16, an unaltered response to adriamycin and collateral sensitivity to cisplatin and bleomycin. These studies have also now been extended to three human tumour cell lines, derived from a breast carcinoma, a bladder carcinoma and a testicular teratoma, in order to test this hypothesis further (Hill et al., in press). Preliminary data indicate that, in this cross section of human tumour cell lines used, resistant cell lines derived

by exposure to either VP-16 or vincristine exhibit patterns of responses to antitumour agents consistent with the multidrug-resistant phenotype (Bech-Hansen et al., 1976), whereas those derived by fractionated X-irradiation treatment, although consistently expressing resistance to VP-16 and vincristine and unaltered responses to adriamycin, differ markedly in their responses to cisplatin (Hill et al., in press). Therefore it appears that specific drug-radiation interactions may have occurred in these in vitro derived lines although the mechanisms associated with these altered responses after fractionated X-irradiation treatment are, as yet, unresolved.

HN-1/VP-2 and HN-1/DXR-11 cell lines expressed cross resistance to vincristine, but only marginal cross resistance to vinblastine. Conter and Beck (1984) also demonstrated a lack of full cross resistance to vinblastine in human leukaemic lymphoblasts (CEM cells) selected for resistance to vincristine, although these workers explained that this may have been due to the parental cell line being inherently less sensitive to vinblastine than vincristine. However, other workers have also reported higher degrees of cross resistance to vincristine than vinblastine in tumour cell lines selected for resistance to adriamycin or colchicine (Fojo et al., 1985; Harker and Sikic, 1985; Yamaguchi et al., 1986). In these reports there were no indications that the parental cells were inherently less sensitive to vinblastine than vincristine, and from Table 4-1 it would appear that HN-1 parental cells are more sensitive to vinblastine than vincristine. The reason for this anomaly is unresolved, but, in the

case of Conter and Beck's human leukaemic lymphoblasts, may relate to a difference in efficacy of these drugs against the original tumour (Conter and Beck, 1984).

The term multidrug resistance has been used to describe the phenomenon whereby drug-resistant tumour cell lines, having been selected for by a particular drug, may exhibit cross resistance to a variety of structurally unrelated antitumour agents to which they have had no prior exposure (Bech-Hansen et al., 1976). This phenomenon was originally described in the 1970's in mammalian tumour cells selected for resistance in vitro to: colchicine (Bech-Hansen et al., 1976); vincristine (Wilkoff and Dulmadge, 1978); daunomycin (Kessel et al., 1968); and actinomycin D (Biedler and Riehm, 1970). More recently multidrug resistance has been shown in human tumour continuous cell lines in vitro after selection with: adriamycin (Fairchild et al., 1986; Harker et al., 1986); colchicine (Akiyama et al., 1985); vinblastine (Beck, 1983) and vincristine (Conter and Beck, 1984). HN-1/VP-2 cells, having been selected for by exposure to VP-16, may be considered to express the multidrug-resistant phenotype as they are cross resistant to the variety of structurally unrelated antitumour agent which are included in this phenomenon (Beck, 1983). A unique form of the multidrug-resistant phenotype may be exhibited by HN-1/DXR-11 cells as they are cross resistant to VP-16, vincristine and vinblastine although the agent employed to induce resistance was X-irradiation.

Further investigations of these independently-derived drug-resistant human tumour sublines have centred on the biochemical mechanisms involved in their altered drug responses. These studies have been facilitated by the demonstration that the HN-1/VP-2 and HN-1/DXR-11 cells exhibited approximately the same orders of resistance to either VP-16 or vincristine.

CHAPTER 6 : INVESTIGATIONS OF MECHANISMS OF RESISTANCE TO VP-16 IN TWO INDEPENDENTLY-DERIVED VP-16-RESISTANT HN-1 CELL LINES

CONTENTS:

6-1: GENERAL INTRODUCTION 177

6-2: PATTERNS OF DRUG UPTAKE AND EFFLUX 178

a. Introduction 178

b. Patterns of Drug Uptake and Efflux 180

(i) VP-16 180

(ii) Vincristine 187

(iii) Vinblastine 191

(iv) Daunomycin 193

(v) Cisplatin 195

c. Discussion and Summary 195

6-3: CELLULAR LEVELS OF GLUTATHIONE AND ASSOCIATED ENZYMES INVOLVED IN DETOXIFICATION MECHANISMS 204

a. Introduction 204

b. Cellular Levels of Glutathione, Glutathione S-transferase, Glutathione Reductase and Glutathione Peroxidase 206

(i) Glutathione 206

(ii) Glutathione S-transferase 207

(iii) Glutathione reductase 209

(iv) Glutathione peroxidase 209

c. Discussion and Summary 209

6-4: ANALYSES OF DNA DAMAGE AND REPAIR IN PARENTAL HN-1 CELLS AND RESISTANT SUBLINES AFTER EXPOSURE TO VP-16 212

a. Introduction 212

b. Formation of DNA Single-Strand Breaks in Parental HN-1 Cells and Resistant Sublines After Exposure to VP-16 213

c. Repair of DNA Single-Strand Breaks in Parental HN-1 Cells and Resistant Sublines After Exposure to VP-16. 219

d. Discussion and Summary 222

6-5: OVERALL SUMMARY 230

6-1: GENERAL INTRODUCTION

Tumour cell resistance to VP-16 and VM-26, discussed in Section 1-3:d, has been associated with the following cellular alterations:

(i) Reduced steady state accumulation of drug in murine tumour cells selected for resistance to VP-16 *in vivo* (Seeber et al., 1982) or to VM-26 *in vitro* (Lee and Roberts, 1984b),

(ii) Elevated activities of glutathione S-transferase, glutathione peroxidase and UDP-glucuronyl transferase in a human tumour cell line selected for a high level of resistance to adriamycin (192-fold) which proved highly cross-resistant to VP-16 (175-fold) (Tulpule et al., 1986),

(iii) Elevated non-protein sulphydryl content in murine leukaemia cells selected for resistance to mitomycin C and exhibiting cross resistance to VP-16 (Dorr et al., 1985),

(iv) Reduced accumulation of VP-16-induced DNA damage, independent of any differences in drug transport across the plasma membrane, in human lung carcinoma cells which were naturally resistant to VP-16 (Long et al., 1986), Chinese hamster ovary cells selected for resistance to VM-26 which were cross-resistant to VP-16 (Glisson et al., 1986a), and Chinese hamster lung cells selected for 9-hydroxyellipticine resistance which expressed cross resistance to VP-16 (Pommier et al., 1986a). In the

latter two examples modified DNA topoisomerase II activity was considered responsible for the reduction of VP-16-induced DNA damage in resistant cells (Glisson et al., 1986a; Pommier et al., 1986b).

In this study, therefore, it was decided to examine the following parameters in an attempt to elucidate the mechanism(s) associated with VP-16 resistance in HN-1/VP-2 and HN-1/DXR-11 cells:

(i) VP-16 uptake and efflux characteristics,

(ii) Cellular contents of glutathione and activities of glutathione S-transferase, glutathione reductase and glutathione peroxidase, and

(iii) Formation and repair of VP-16-induced DNA single-strand breakage.

In addition, vincristine, vinblastine, daunomycin and cisplatin uptake and efflux characteristics of HN-1 cell lines were also investigated in an attempt to correlate uptake and efflux parameters with the altered responses exhibited by HN-1/VP-2 and HN-1/DXR-11 cells to these agents.

6-2: PATTERNS OF DRUG UPTAKE AND EFFLUX

a. Introduction

Antitumour drug resistance has been associated with reduced steady state accumulation of either the agent with which the resistant cells were selected, or a drug to which they exhibited cross resistance, for example:

(i) Ehrlich ascites cells specifically selected for VP-16 resistance *in vivo* and L1210 cells specifically selected for VM-26 resistance *in vitro* showed impaired uptake of the respective selecting agent (Seeber et al., 1982; Lee and Roberts, 1984b),

(ii) Vinblastine- and vincristine-resistant human leukaemic (CEM) cells and vincristine-resistant L5178Y cells all exhibited a reduced steady-state accumulation of the selecting drug (Beck, 1983; Danks and Beck, 1983; Hill et al., 1984),

(iii) Chinese hamster ovary mutants selected for VM-26 resistance by Gupta expressed impaired uptake of daunomycin associated with cross resistance to this agent (Gupta, 1983), although with higher extracellular drug concentrations no differences in daunomycin uptake were detected (Glisson et al., 1986b). However no differences in VP-16 uptake were observed between these parental and resistant cell lines,

(iv) Reduced accumulation of adriamycin was associated with cross resistance to this agent in Chinese hamster lung cells selected for resistance to 9-hydroxyellipticine, although cross resistance to VP-16 was not associated with any alterations of VP-16 uptake by these cells (Pommier et al., 1986a).

It would appear then that resistance to VP-16 may or may not be associated with modified drug uptake, and that cross resistance to daunomycin or adriamycin may be associated with reduced

uptake of these agents even though the resistant cells expressed mechanisms of resistance to the selecting agent which were independent of differences in drug transport across the plasma membrane. Therefore it was decided to examine the patterns of drug uptake and efflux expressed by the differently-derived VP-16-resistant HN-1 cell lines in order to determine whether altered drug uptake or efflux was associated with their altered responses to a range of antitumour agents.

Drug uptake studies were carried out in parallel on HN-1 cell lines as a monolayer or in suspension and efflux studies were performed using a cell suspension (see sections 2-3:f(i) and (ii)). No significant differences in drug uptake characteristics were observed using either of these two methodologies. Values of uptake at zero time were deducted, since these were considered to represent instantaneous non-specific surface binding (Beck, 1984). Straight lines were fitted by linear regression and compared statistically, as described in Section 2-4.

b. Patterns of Drug Uptake and Efflux

(i) VP-16

In order to test whether the methodology employed was sensitive enough to detect differences in VP-16 uptake, initial experiments were carried out using the $AuxB_1$ and CH^RC5 Chinese hamster ovary cell lines. The CH^RC5 cell line originally defined the

multidrug-resistant phenotype associated with impaired drug uptake (Bech-Hansen et al., 1976; Juliano and Ling, 1976), and expressed an 8-fold level of cross resistance to VP-16 when comparing IC_{50} values derived from colony-forming assays of $CH^{R}C5$ and $AuxB_1$ (parental) cells after a 24 h exposure to drug (R. Whelan, personal communication). The uptake of [^{3}H]-VP-16 into these Chinese hamster ovary cells growing as a monolayer exposed to 1.84 µg/ml (3.1µM) for 30 or 60 min is shown in Figure 6-1. It can be seen that $CH^{R}C5$ cells accumulated significantly less [^{3}H]-VP-16 than parental $AuxB_1$ cells at both time points. These data are therefore consistent with the previously demonstrated transport defect for daunomycin and colchicine reported in these cells (Riordan and Ling, 1985). They also indicate that for an 8-fold level of cross resistance an approximate 2-fold reduction in drug uptake can be detected in these cells. This methodology therefore should be sensitive enough to detect any significant alterations in drug uptake in the HN-1 resistant sublines expressing an approximate 4-fold order of resistance (see Table 5-1).

[^{3}H]-VP-16 uptake was therefore examined in HN-1 cell lines. Figure 6-2 shows that the HN-1 lines exposed as cell suspensions to a range of extracellular [^{3}H]-VP-16 concentrations exhibited no differences in extent of drug uptake. Furthermore, HN-1 cells exposed as a monolayer to 1.84 µg/ml VP-16 for 15 or 30 min also showed no significant differences in VP-16 uptake between cell lines (Figure 6-3) with values ranging from 19.7 to 20.4 pmole/mg protein for a 15 min exposure, and from 18.3 to 20.1 pmole/mg protein

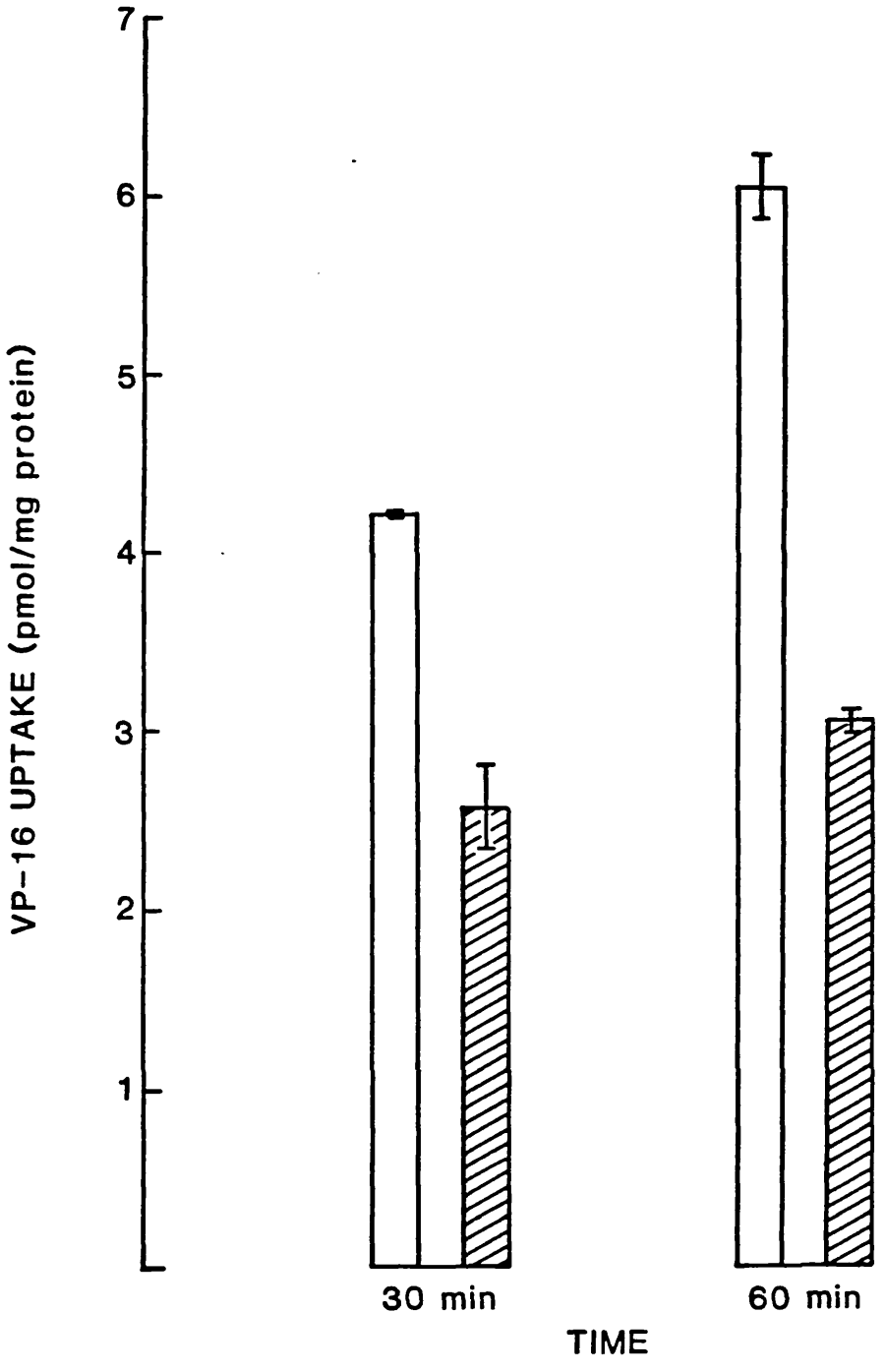


Figure 6-1: Uptake of [^{3}H]-VP-16 into Chinese hamster ovary $AuxB_1$ (□) and CH^RC5 (▨) cells exposed as a monolayer to VP-16 (1.84μg/ml) for 30 or 60 min. Each experiment was the mean of two estimations. Histograms shown represent the mean of two experiments ± s.e.

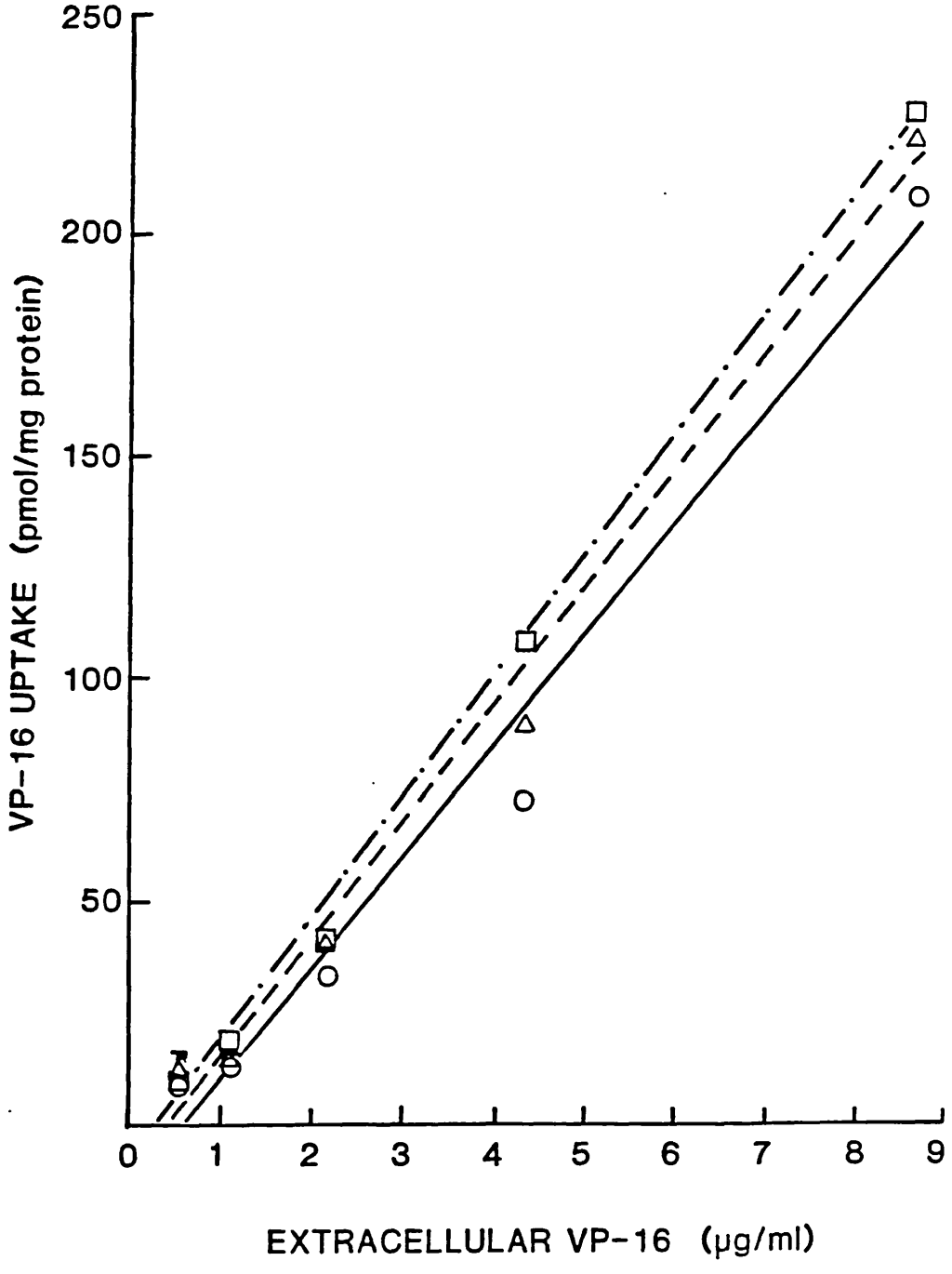


Figure 6-2: Uptake of [^{3}H]-VP-16 into HN-1 parental (○——○), VP-2 (△- - -△) and DXR-11 (□—·—□) cells in suspension exposed to a range of VP-16 concentrations for 30 min. Each experiment was the mean of four estimations. Each point represents the mean of two experiments ± s.e. Bars are drawn where s.e.>5%.

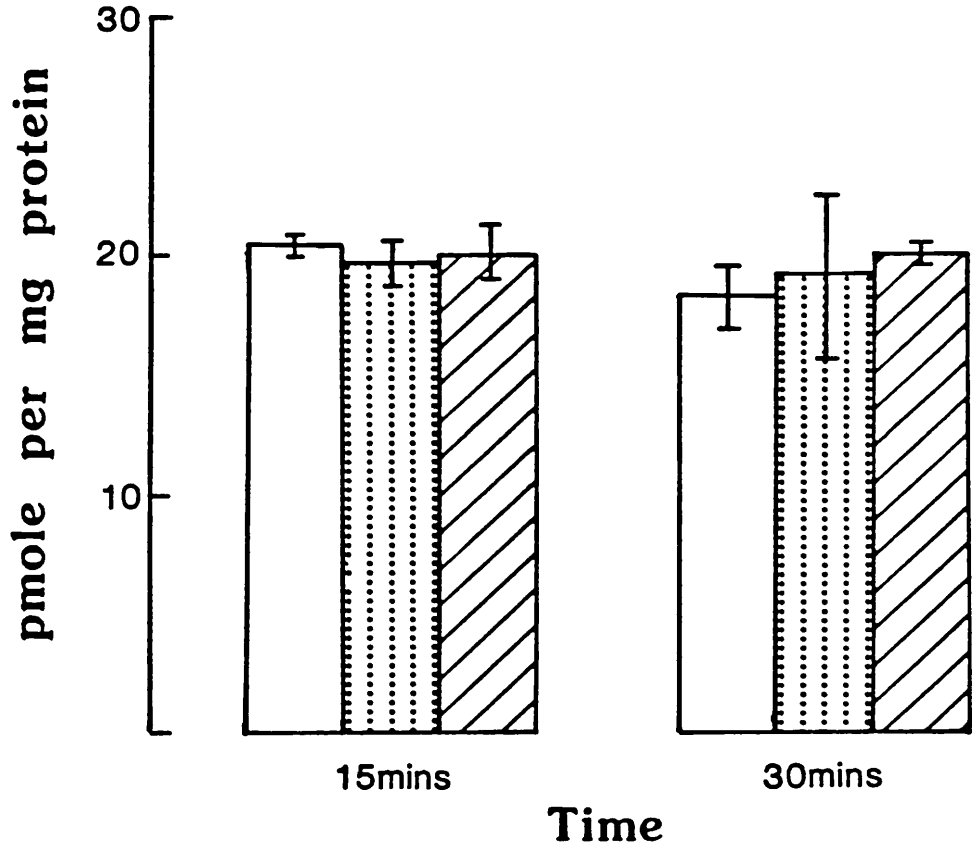


Figure 6-3: Uptake of [^{3}H]-VP-16 into HN-1 parental (□), VP-2 (⊞) and DXR-11 (▨) cells exposed as a monolayer to VP-16 (1.84μg/ml) for 15 or 30 min. Each experiment was the mean of two estimations. Histograms shown represent the mean of four experiments ± s.e.

for a 30 min exposure to drug. From Figures 6-2 and 6-3 it can be seen that the extent of drug uptake into HN-1 cells was comparable using either a suspension of cells or cells attached as a monolayer.

These values of [^{3}H]-VP-16 uptake are of the same order as those reported by Danks and Beck (1983) using CCRF-CEM cells, since approximately 650 pmole/10^6 cells were accumulated when the extracellular VP-16 concentration was much greater (100 µg/ml). It would appear in the HN-1 cells that steady state levels of VP-16 have been attained within a 15 min exposure to drug, as no significant increases in uptake were observed by extending the incubation time to 30 min. These data are in agreement with those of Allen (1978) and Yalowich and Ross (1985) who reported steady state accumulation of VP-16 after 10 to 20 min incubations of L1210 cells with drug, although direct comparisons of levels of VP-16 uptake are difficult between these studies as their units of uptake were expressed in terms of nmole/g dry weight.

HN-1/P cells which had been exposed to VP-16 (2.2 µg/ml) for 30 min as a cell suspension retained approximately 10% of radioactivity after being resuspended in drug-free medium and reincubated for 30 min at 37°C (see Table 6-1). This is consistent with data from Yalowich and Ross (1985) who observed that murine leukaemia (L1210) cells retained less than 5% of radioactivity when resuspended in drug-free medium for 30 min after a 25 min incubation with medium containing VP-16 (8.8 µg/ml). This extent of [^{3}H]-VP-16 efflux was comparable in all three HN-1 lines tested, indicating that

Table 6-1: Uptake and retention of VP-16, vincristine and daunomycin by HN-1 cell lines.

ANTITUMOUR DRUG TESTED	EXPERIMENTAL CONDITIONS OF DRUG UPTAKE OR EFFLUX at 37°C	HN-1 CELL LINE		
		P	VP-2	DXR-11
VP-16	VP-16 UPTAKE (2.2µg/ml 30 min) (pmole/mg protein)	32.6±1.6	40.2±2.0	41.5±1.6
	VP-16 RETENTION after 30 min incubation in drug-free medium (pmole/mg protein)	4.8±1.0	5.3±0.8	4.2±1.0
	% VP-16 RETAINED	14.7	13.2	10.1
VCR	VCR UPTAKE (1µg/ml, 30 min) (pmole/mg protein)	55.8±2.4	35.9±1.8*	51.4±0.9
	VCR RETENTION after 30 min incubation in drug-free medium (pmole/mg protein)	22.3±2.2	14.5±1.2**	27.5±2.1
	% VCR RETAINED	40.0	40.4	53.5
DNR	DNR UPTAKE (1µg/ml, 30 min) (pmole/mg protein)	681±39	524±13**	658±31
	DNR RETENTION after 30 min incubation in drug-free medium (pmole/mg protein)	452±19	369±15**	481±13
	% DNR RETAINED	66.4	70.4	73.1

* significantly different from HN-1/P and HN-1/DXR-11 ($p<0.01$)
** significantly different from HN-1/P and HN-1/DXR-11 ($p<0.05$)

modifications in this parameter were not associated with drug resistance.

(ii) Vincristine

CH^RC5 cells, which expressed a 13-fold level of resistance to vincristine when their IC_{50} value, derived from colony-forming assays, was compared to that of $AuxB_1$ cells after a 24 h exposure to drug (R. Whelan, personal communication), were again used as a positive control to test the sensitivity of this methodology. Figure 6-4 indicates that CH^RC5 cells had a strikingly reduced uptake (approximately 7-fold) of [^{3}H]-vincristine compared to parental cells when exposed as a monolayer to vincristine (0.92 μg/ml, 1μM) for either 30 or 60 min.

[^{3}H]-vincristine uptake characteristics of HN-1 cells are shown in Figures 6-5 and 6-6. It can be seen that HN-1/VP-2 cells exhibited reduced accumulation of vincristine compared to HN-1/P and HN-1/DXR-11 cells. In Figure 6-5 where HN-1 cells in suspension were exposed to a range of vincristine concentrations for 30 min, the difference between regression coefficients of the straight lines for HN-1/P and HN-1/VP-2 cells was highly significant ($p<0.01$). However, the difference in these parameters between HN-1/P and HN-1/DXR-11 cells was not significant ($p>0.1$).

Vincristine uptake into HN-1 cells exposed to 0.92 μg/ml vincristine as a monolayer for either 30 or 60 min is shown in Figure

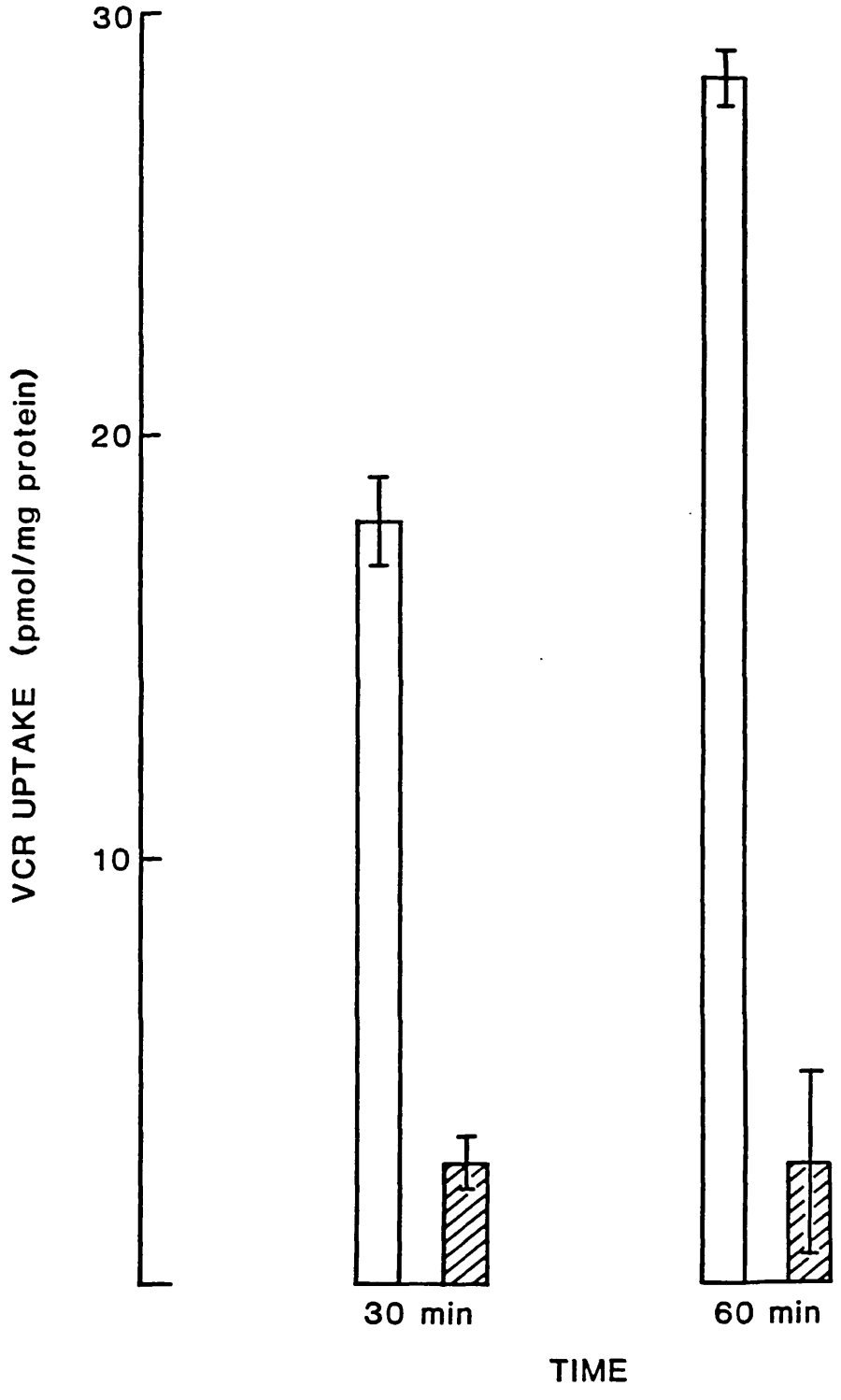


Figure 6-4: Uptake of [^{3}H]-vincristine into Chinese hamster ovary AuxB$_1$ (□) and CH^{R}C5 (▨) cells exposed as a monolayer to vincristine (0.92µg/ml) for 30 or 60 min. Each experiment was the mean of two estimations. Histograms shown represent the mean of two experiments ± s.e.

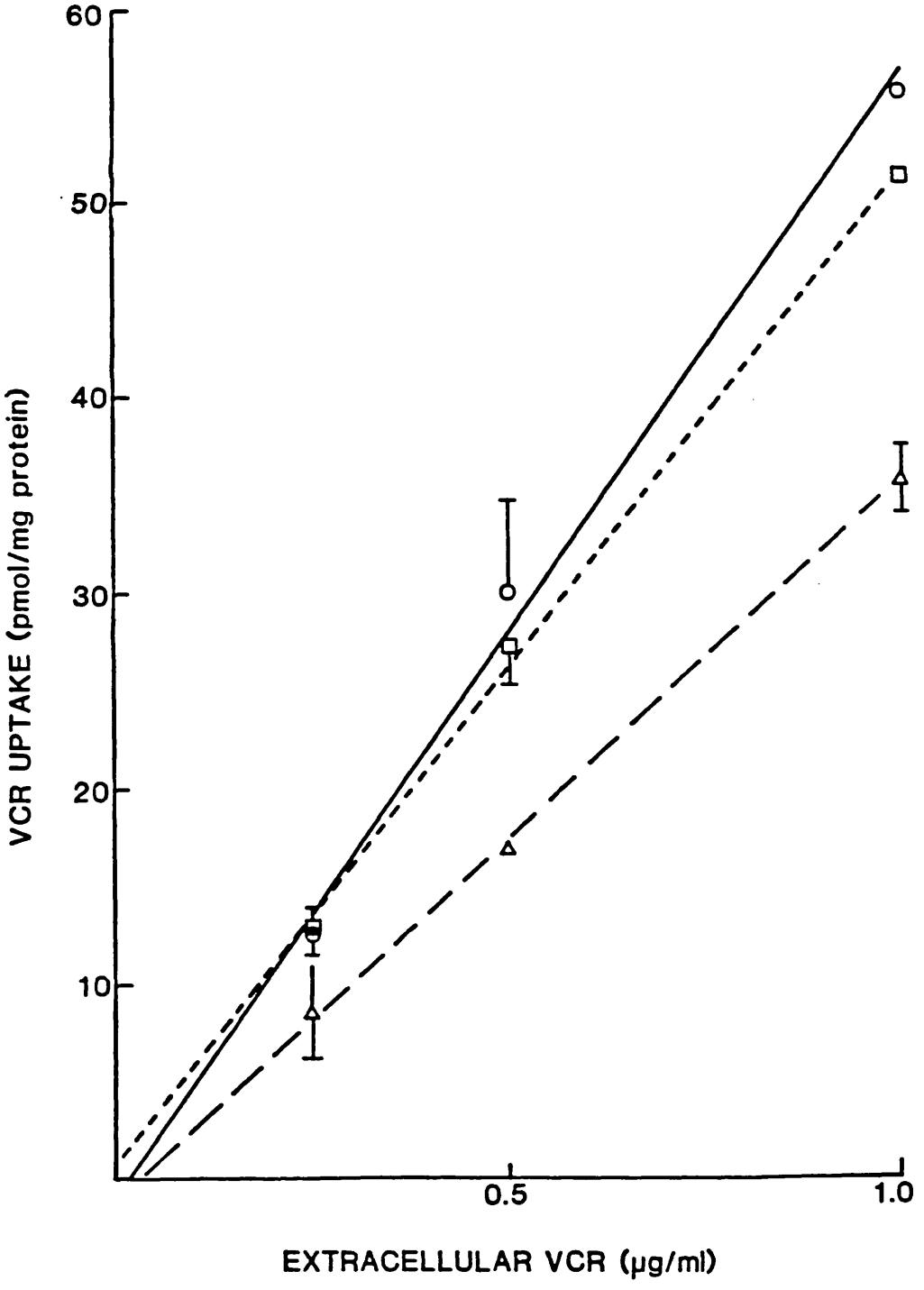


Figure 6-5: Uptake of [^{3}H]-VCR into HN-1 parental (○——○), VP-2 (△– – –△) and DXR-11 (□—·—□) cells in suspension exposed to a range of VCR concentrations for 30 min. Each experiment was the mean of four estimations. Each point represents the mean of two experiments ± s.e. Bars are drawn where s.e.>5%.

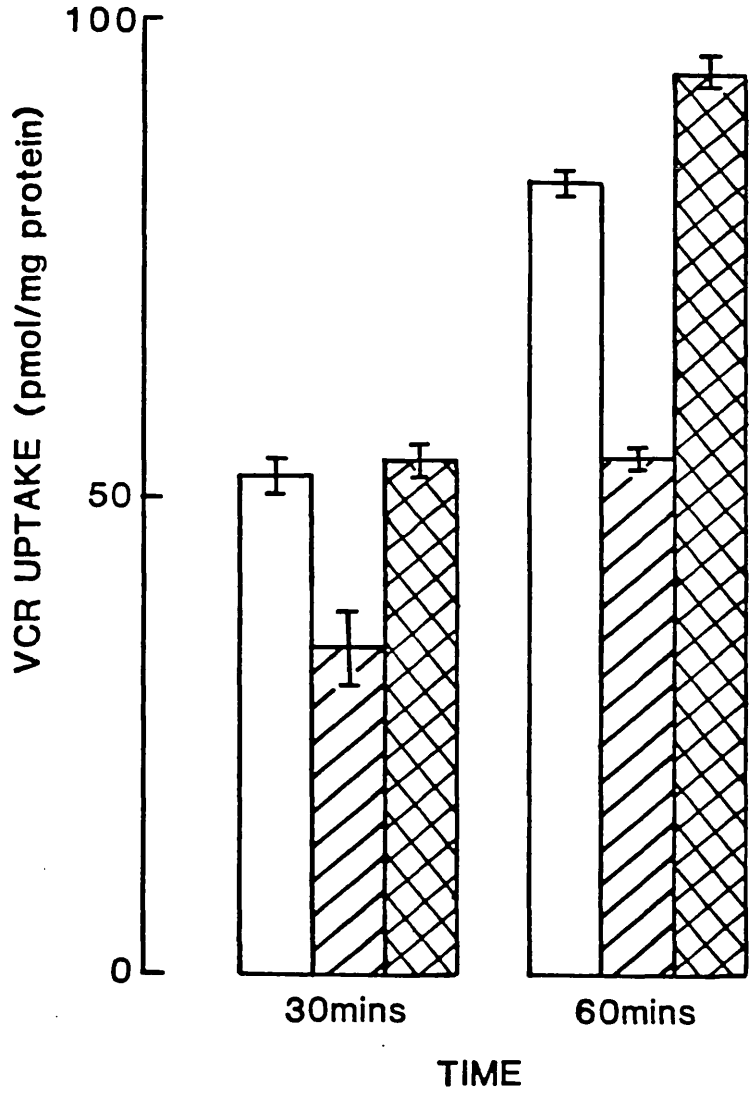


Figure 6-6: Uptake of [^{3}H]-VCR into HN-1 parental (□), VP-2 (▨) and DXR-11 (⊠) cells exposed as a monolayer to VCR (0.92µg/ml) for 30 or 60 min. Each experiment was the mean of two estimations. Histograms shown represent the mean of four experiments ± s.e.

6-6. HN-1/VP-2 cells exhibited significantly ($p<0.01$) impaired uptake of vincristine compared to HN-1/P and HN-1/DXR-11 cells after both 30 and 60 min exposures to drug. In contrast there was no impaired uptake by HN-1/DXR-11 cells and they accumulated comparable levels of [^{3}H]-vincristine to the parental cells after either 30 or 60 min drug exposures.

These data are in general agreement with values of [^{3}H]-vincristine uptake published for CCRF-CEM cells, which accumulated approximately 15 pmole/10^6 cells after a 60 min exposure to 0.19µg/ml vincristine. Also, CEM cells selected for vincristine resistance (600-fold) accumulated approximately 6 pmole/10^6 cells of vincristine under the same experimental conditions (Danks and Beck, 1983).

HN-1 cells which had been exposed to [^{3}H]-vincristine (1 µg/ml, 30 min) and were resuspended in drug-free medium for 30 min at 37°C retained approximately 40-50% of the total radioactivity (see Table 6-1), with HN-1/DXR-11 cells retaining a slightly higher percentage than parental and HN-1/VP-2 cells. HN-1/VP-2 cells which had taken up less drug during the initial incubation retained significantly less [^{3}H]-vincristine than parental and HN-1/DXR-11 cells after this period of drug efflux.

(iii) Vinblastine

The [^{3}H]-vinblastine uptake characteristics of HN-1 cell lines are shown in Figure 6-7. No significant differences in uptake

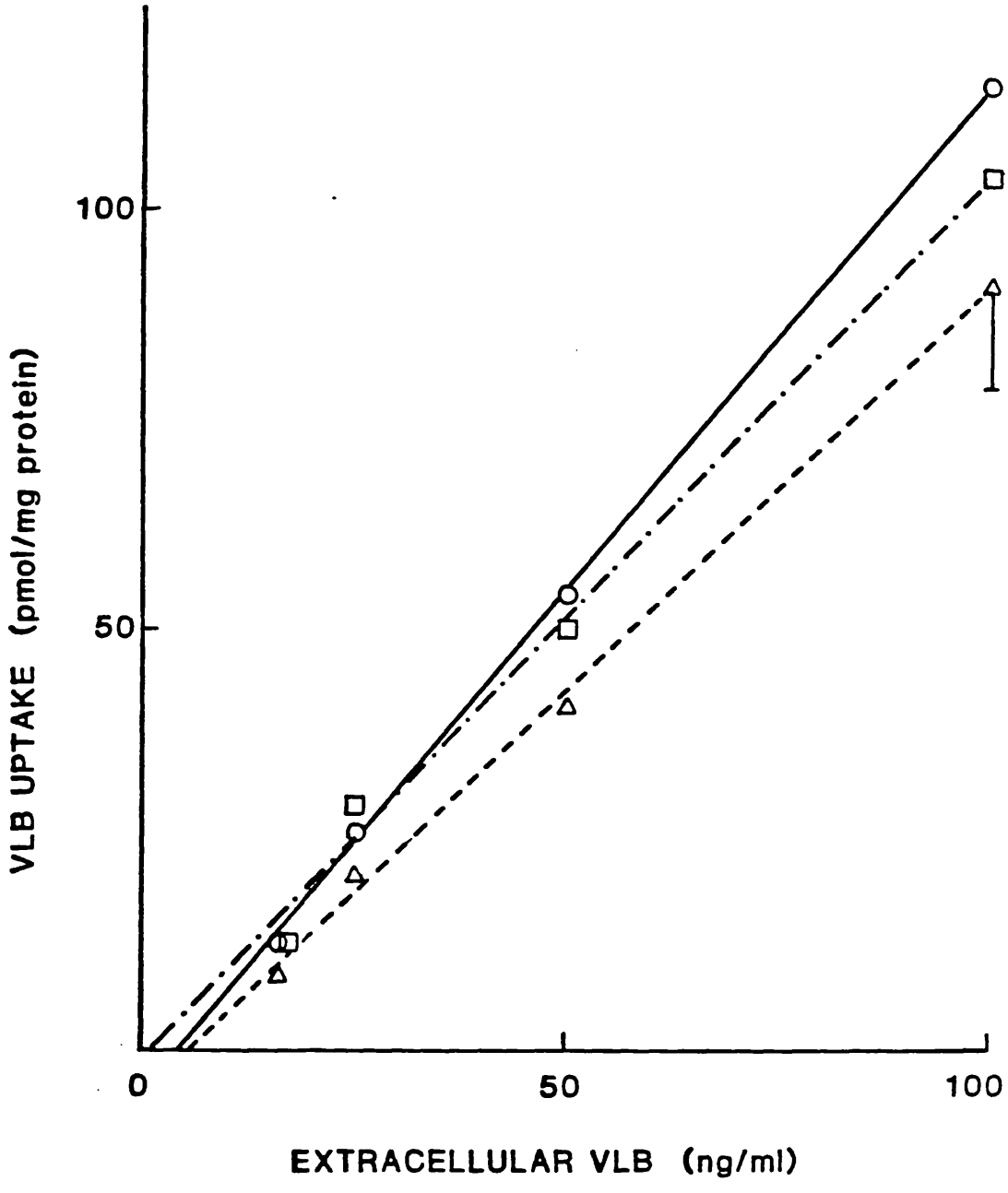


Figure 6-7: Uptake of [^{3}H]-VLB into HN-1 parental (○——○), VP-2 (△----△) and DXR-11 (□—·—□) cells in suspension exposed to a range of VLB concentrations for 30 min. Each experiment was the mean of four estimations. Each point represents the mean of two experiments ± s.e. Bars are drawn where s.e. >5%.

of this drug were observed between parental and DXR-11 cells. HN-1/VP-2 cells, which exhibited marginal cross resistance to this agent, showed an overall reduction in vinblastine uptake compared to parental cells although the difference between regression coefficients for the two straight lines was not statistically significant ($p=0.24$).

(iv) Daunomycin

HN-1/VP-2 cells exhibited impaired uptake of [^{3}H]-daunomycin compared to HN-1/P and HN-1/DXR-11 cells, as shown in Figure 6-8. The regression coefficients for straight lines fitted for the daunomycin uptake experiments with HN-1/P and HN-1/VP-2 cells were significantly different ($p<0.01$), but those for HN-1/P and HN-1/DXR-11 cells were comparable and not statistically different ($p>0.50$).

For efflux studies HN-1 cells were loaded with [^{3}H]-daunomycin at an extracellular concentration of 1 µg/ml for 30 min. Cells were then resuspended in drug-free medium at 37°C for a further 30 min. HN-1 cells retained between 65 and 75% of the total radioactivity after this drug efflux period, with HN-1/VP-2 cells retaining significantly less total [^{3}H]-daunomycin than both parental and HN-1/DXR-11 cells (Table 6-1). In the study of Inaba and Johnson (1978), murine leukaemia (P388) cells which had been incubated in [^{3}H]-daunomycin for 1h and resuspended in drug-free medium at 37°C for 30 min retained approximately 55% of radioactivity, which is

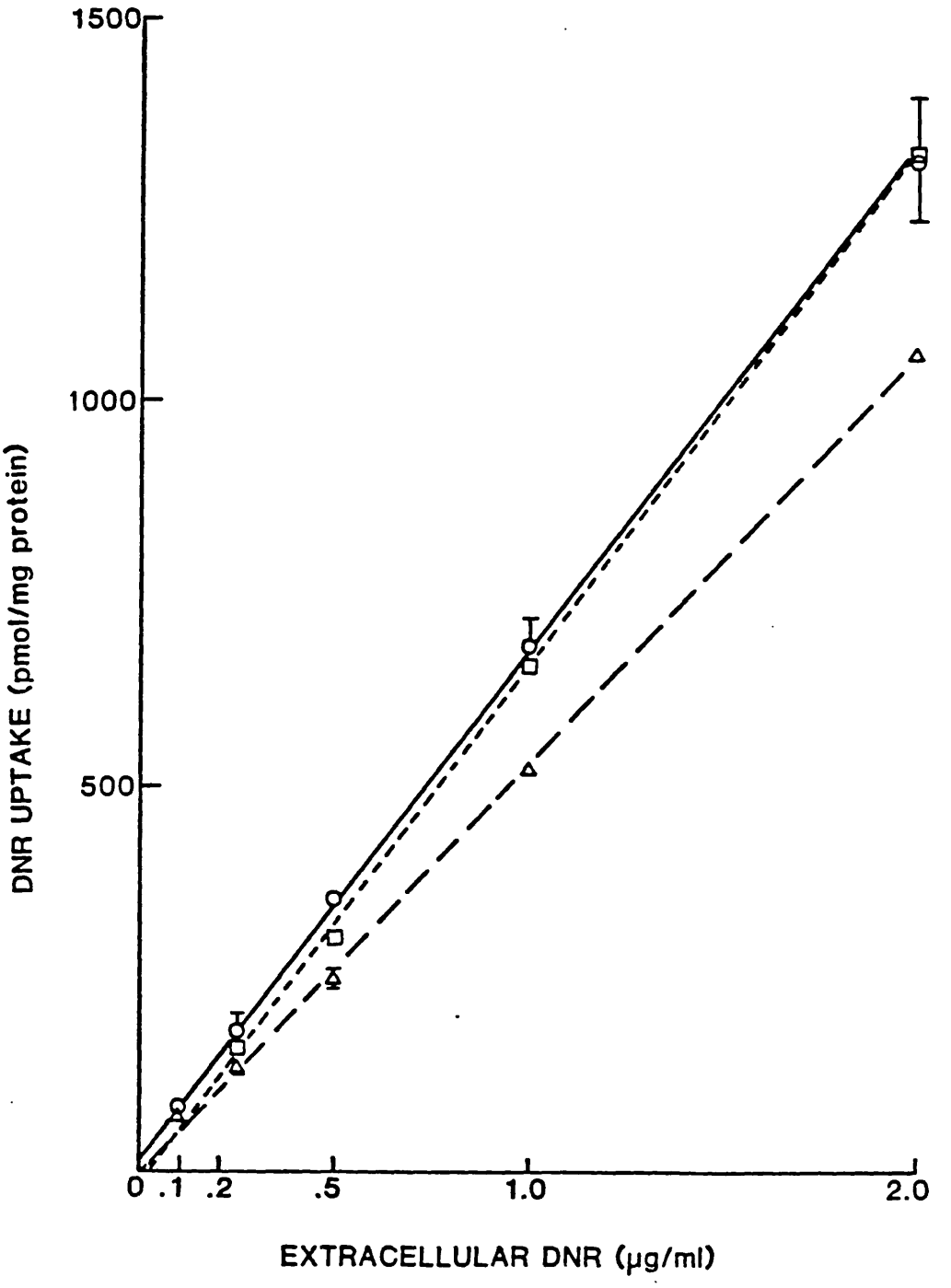


Figure 6-8: Uptake of [^{3}H]-DNR into HN-1 parental (○——○), VP-2 (△– – –△) and DXR-11 (□- - -□) cells in suspension exposed to a range of DNR concentrations for 30 min. Each experiment was the mean of four estimations. Each point represents the mean of two experiments ± s.e. Bars are drawn where s.e.>5%.

slightly lower than that retained by HN-1/P cells. However, in contrast to HN-1/VP-2 resistant cells, daunomycin-resistant P388 cells retained only about 20% of radioactivity after the same period of efflux. These results may reflect the considerably higher level of resistance exhibited to daunomycin (56-fold) by these P388 cells compared to the 1.8-fold cross resistance shown by the HN-1/VP-2 cells.

(v) Cisplatin

Uptake of cis-^{195m}platinum into HN-1 cells in suspension exposed to a range of cisplatin concentrations for 30 min is shown in Figure 6-9. Neither HN-1/VP-2 cells ($p>0.50$) nor HN-1/DXR-11 cells ($p>0.20$) exhibited any significant differences in uptake of this agent compared to parental cells.

c. Discussion and Summary

Drug uptake studies were carried out using cells attached as a monolayer or in suspension. Preliminary experiments did not indicate that there was any significant differences in the extent of drug uptake by HN-1 cells using either of these methodologies and this becomes apparent when comparing Figures 6-2 and 6-5 with Figures 6-3 and 6-6 respectively. Other studies have used monolayer cultures for drug uptake experiments (Gupta, 1983; Glisson et al., 1986b) and since the process of detachment may damage the cells it would appear unnecessary to remove HN-1 cells from monolayer culture in order to

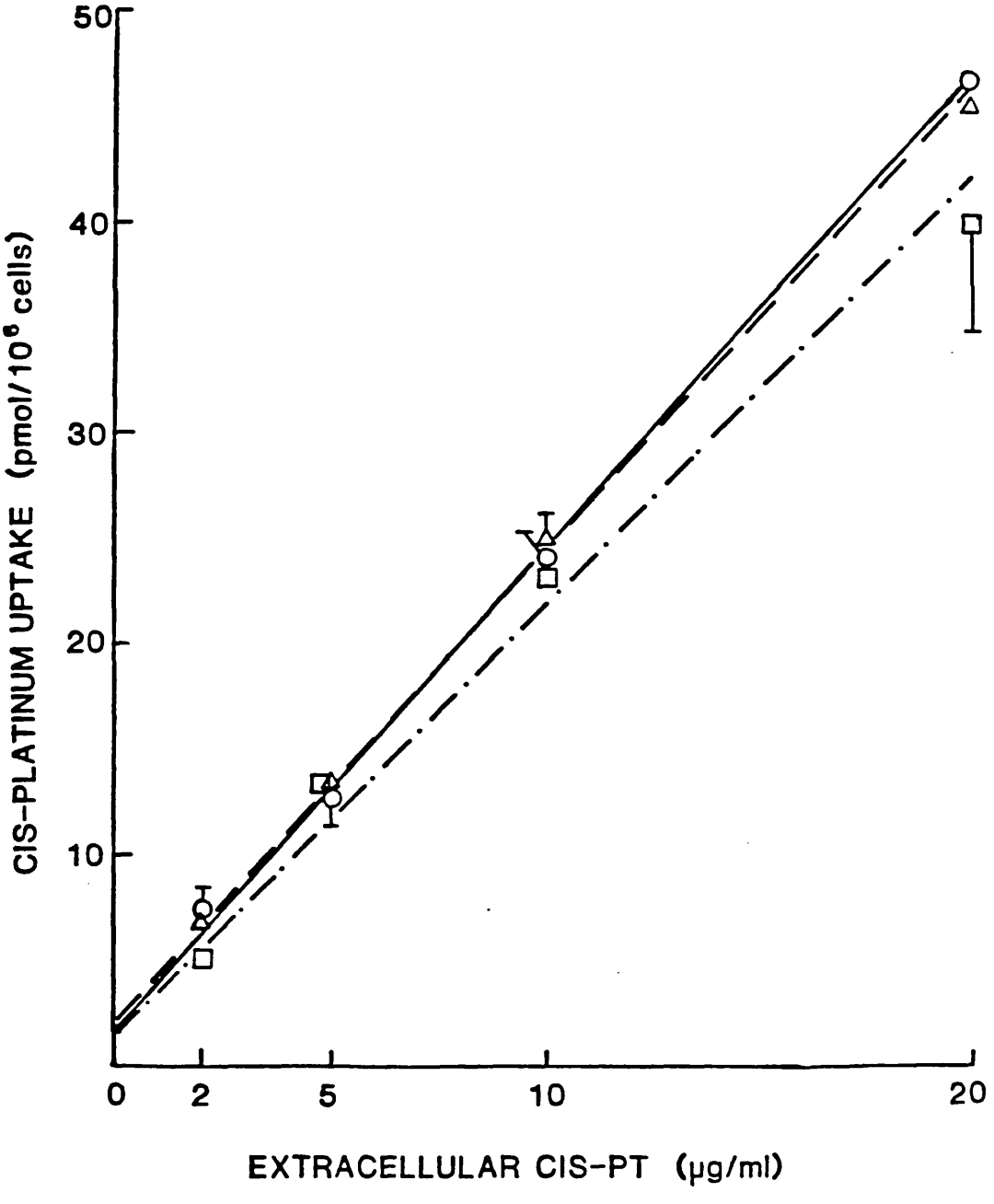


Figure 6-9: Uptake of cis-^{195m}Pt into HN-1 parental (○——○), VP-2 (△– – –△) and DXR-11 (□–·–□) cells in suspension exposed to a range of cis-Pt concentrations for 30 min. Each experiment was the mean of four estimations. Each point represents the mean of two experiments ± s.e. Bars are drawn where s.e.>5%.

carry out these experiments. However, the technical problems associated with handling three cell lines, using up to five extracellular drug concentrations including zero time-points, and estimations of drug efflux, were better solved by using cells in suspension rather than attached to a monolayer. For example, when handling a large number of samples, it was easier to take a 1ml aliquot from a suspension of cells and wash with ice-cold PBSA than it was to remove the radioactive medium from petri-dishes and wash each three times with ice-cold PBSA. Furthermore, suspensions of cells were obtained from stock culture flasks, whereas for drug uptake studies using monolayer cultures each petri-dish of cells had to be set up individually, thereby increasing the chances of contamination or of an unequal number of cells per dish. Nevertheless it was no more economical to use cells in suspension as the same quantities of radioactive drugs and approximately the same numbers of cells were used in both methodologies.

Studies described in this section using HN-1 cells only included measurements of drug uptake following either a 15 or 30 min incubation. Therefore any differences in uptake which may have occurred outside of these time-points would not have been detected, and in particular no attempts were made to measure initial rates of uptake. However it appeared from the literature that steady-state levels of the antitumour agents to be tested would have been achieved by 30 min (Allen, 1978; Tsuruo et al., 1982; Beck, 1983; Hill et al., 1984; Yalowich and Ross, 1985), and that any gross differences in drug uptake between parental and resistant cell lines would be

revealed at this time-point. Furthermore, in those studies where a reduction in initial rate of drug uptake was demonstrated in resistant cells compared to parental cells, there was a concomittant reduction in steady-state accumulation of drug (Tsuruo et al., 1982; Hill et al., 1984; Lee and Roberts, 1984b). In addition to attempting to correlate the varied responses exhibited by the HN-1 cell lines to a range of antitumour agents with the steady-state levels of drug uptake, we also planned to attempt a similar comparison with the amount of drug retained after a representative period of efflux. Again a search of the literature revealed that drug efflux generally was reported to reach a plateau after cells had been resuspended in drug-free medium at 37°C for 30 min (Allen, 1978; Beck, 1983; Hill et al., 1984; Lee and Roberts, 1984b; Yalowich and Ross, 1985). Therefore drug retention by HN-1 cells was estimated after a 30 min efflux period. Data obtained under these experimental conditions could then be related to the cytotoxicity data available.

Comparisons of drug uptake characteristics between HN-1/P cells and the two resistant sublines, HN-1/VP-2 and HN-1/DXR-11 (summarized in Table 6-2), revealed the following patterns:

(i) HN-1/VP-2 cells exhibited impaired uptake of vincristine, vinblastine and daunomycin, agents to which these cells showed various degrees of cross resistance (see Chapter 4), but showed no apparent transport defect under these experimental conditions, for the selecting agent, VP-16. These cells, which were also marginally cross resistant to cisplatin had unaltered uptake of this drug. For each drug tested, any differences detected were consistently

Table 6-2: Summary of drug uptake characteristics comparing HN-1/VP-2 and HN-1/DXR-11 cells with parental cells.

Antitumour Agent Tested	HN-1/VP-2 Cells	HN-1/DXR-11 Cells
VP-16	Unaltered	Unaltered
Vincristine	Impaired	Unaltered
Vinblastine	Marginally Impaired	Unaltered
Daunomycin	Impaired	Unaltered
Cisplatin	Unaltered	Unaltered

shown both in terms of altered steady-state levels and altered retention.

(ii) HN-1/DXR-11 cells which expressed resistance to VP-16, vincristine, marginal resistance to vinblastine, an unaltered response to daunomycin and some collateral sensitivity to cisplatin (see Chapter 5) failed to exhibit alterations in uptake or efflux of any of these agents compared to parental cells. In addition they did not express any altered uptake or efflux characteristics of [^{3}H]-VP-16.

The expression of cross resistance to vincristine by HN-1/VP-2 cells, which has been shown to be associated with a reduced steady-state accumulation of drug, accompanied by reduced drug retention after a period of efflux, is in agreement with reports describing other drug resistant human tumour cell lines derived by exposure to drug which express the MDR phenotype (Beck, 1983; Conter and Beck, 1984). Although mechanisms of resistance to vincristine have mainly been reported to involve reduced cellular uptake of drug (Beck, 1984) other mechanisms have been proposed independent of cellular uptake modifications which may involve alterations in target proteins (Cabral et al., 1980; Gupta et al., 1982). HN-1/DXR-11 cells expressed no transport alterations for vincristine or vinblastine under these experimental conditions and the mechanisms of resistance in these cells to these agents are, as yet, unknown. Therefore, in this respect, at least one of the mechanisms of

resistance to vincristine expressed by HN-1/VP-2 and HN-1/DXR-11 cells appear to differ depending upon whether resistance has been derived by continuous exposure to VP-16 or by fractionated X-irradiation treatment.

Furthermore, HN-1/VP-2 cells which were cross-resistant to daunomycin exhibited reduced uptake and retention of [^{3}H]-daunomycin, a characteristic of Chinese hamster ovary cells selected for VP-16 and VM-26 resistance (Gupta, 1983), although HN-1/DXR-11 cells, which were not cross-resistant to daunomycin, had, not unexpectedly, no transport defect for this agent.

A further question which may arise is: are the differences in drug uptake characteristics observed in HN-1/VP-2 cells large enough to account for their levels of cross resistance to vincristine, vinblastine and daunomycin? Table 6-3 summarizes a series of studies where steady-state accumulation of drug has been estimated in cells exhibiting different levels of resistance to certain antitumour agents. It should be noted that different cytotoxicity assays were used to estimate Resistance Indices in these cell lines, including colony-forming or growth inhibition assays. It can be seen that even for the cells expressing the highest level of resistance (1227-fold) steady-state accumulation of drug was reduced only 4.5-fold compared to parental cells. However these workers have recently suggested that resistance to VM-26 in these cells may be multifaceted, so that altered transport alone may not account for the degree of resistance expressed (Roberts et al., 1986). Conversely

Table 6-3: A comparison between levels of resistance and reduced steady-state accumulation of drug exhibited by a range of drug-resistant tumour cell lines

Drug-resistant tumour cell	Antitumour agent tested	Resistance Index compared to parental cells	Reduction in steady-state accumulation of drug compared to parental cells	Reference
L1210/L1a5μM	VM-26	1227	4.5	Lee and Roberts, 1984b
CEM/VCR_{100}	VCR	600	2.5	Danks and Beck, 1983
CEM/VLB_{100}	VLB	420	2.1	Beck, 1983
L1210/L17.5	VM-26	136	2.2	Lee and Roberts, 1984b
$CHO/CH^{R}C5$	VCR	13	6.6	This study
$CHO/CH^{R}C5$	VP-16	8	2.0	This study
DC-3F/9-OH-E	Act D	7	1.6	Salles et al., 1982
ET_{VP-16}	VP-16	6	1.8	Seeber et al., 1982
$L5178Y/VCR_{R}$	VCR	3.7	3.7	Hill et al., 1984
HN-1/VP-2	VCR	2.6	1.5	This study
$CHO/Vpm^{R}-5$	DNR	2.4	1.3	Gupta, 1983
HN-1/VP-2	DNR	1.8	1.3	This study
HN-1/VP-2	VLB	1.4	1.2	This study

the largest uptake defect, a factor of 6.6 in CHO/$CH^{R}C5$ cells with vincristine, was noted in cells which were only 13-fold resistant to the drug. This may also indicate that in cells expressing higher degrees of resistance to vincristine, but with a correspondingly smaller uptake defect, multiple mechanisms of resistance operate. In only one study (L5178Y/VCR_{R}) was there a direct correlation between Resistance Index and reduced steady-state accumulation of drug, although in this series of studies listed in Table 6-3 there is a general correlation between the degree of drug-resistance and extent of reduction in drug accumulation. Therefore it appears that HN-1/VP-2 cells show the same trend, and it may be postulated that the extent of reduction in steady-state accumulation of vincristine, vinblastine and daunomycin detected in HN-1/VP-2 cells compared to parental cells may account for their degree of cross resistance to these agents.

However, in neither of the VP-16 resistant HN-1 cell lines was VP-16 uptake or efflux altered which indicates that the mechanisms of resistance to VP-16 expressed by HN-1/VP-2 and HN-1/DXR-11 cells involves processes which are independent of VP-16 uptake through the plasma membrane. Although no attempts were made to characterize initial rates of drug uptake, it is thought that VP-16 uptake occurs by passive diffusion (Allen, 1978) and therefore this parameter may not be expected to differ between HN-1 sublines.

6-3: CELLULAR LEVELS OF GLUTATHIONE AND ASSOCIATED ENZYMES INVOLVED IN DETOXIFICATION MECHANISMS

a. Introduction

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is present in all mammalian cells and plays a role in a number of cellular functions, including protection against the effects of free radicals and reactive oxygen intermediates, and the toxic effects of drugs (Meister, 1983). These functions may be relevant to the expression of drug resistance, and Figure 6-10 outlines the pathways by which drug detoxification may occur. Drug-induced cytotoxicity may be decreased by facilitating drug metabolism to an inactive species or by the detoxification of any drug-induced free radical species. The former reaction is catalyzed by glutathione S-transferases, whereas detoxification of free radicals may occur via the oxidation-reduction cycle of glutathione involving the enzymes glutathione reductase and glutathione peroxidase. The proposed metabolic activation of VP-16 to an oxygen centred free radical (Sinha and Myers, 1984) has led to the suggestion that resistance to VP-16 may be associated with elevated activities of intracellular free radical scavenging systems. Furthermore, antitumour drug resistance has been associated with enhanced levels of non-protein sulphydryl compounds and amplification of glutathione S-transferase and glutathione peroxidase activities, as discussed in Section 1-3:d(iii). For example, a mitomycin C-resistant murine leukaemia (L1210) cell line, which expressed cross resistance to VP-16, had a 2-fold elevation in non-protein sulphydryl

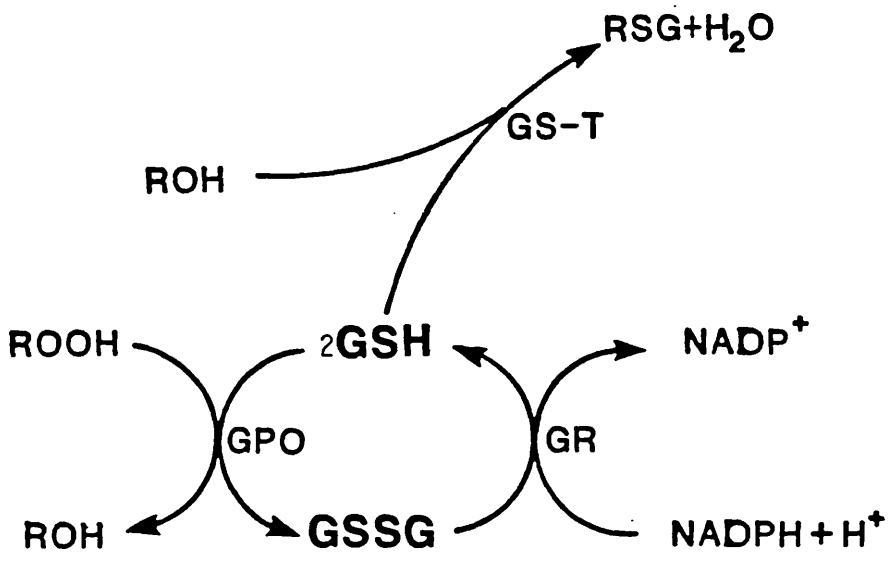


Figure 6-10: Schematic representation of the role that glutathione may play in the detoxification of xenobiotics. GSH and GSSG, reduced and oxidized glutathione; GPO, reaction catalysed by glutathione peroxidase; GR, reaction catalysed by glutathione reductase; GS-T, reaction catalysed by glutathione S-transferase; NADPH and NADP+, reduced and oxidized nicotinamide adenine dinocleotide phosphate; ROH, xenobiotic; ROOH, toxic free radical intermediate.

content compared to parental cells (Dorr et al., 1985). Also, a human breast carcinoma (MCF-7) cell line selected for a high level of resistance to adriamycin (190-fold) and exhibiting cross resistance to VP-16 (175-fold) had elevated activities of glutathione S-transferase (45-fold) and glutathione peroxidase (12-fold) (Tulpule et al., 1986).

Levels of reduced glutathione, and the activities of glutathione S-transferase, glutathione reductase and glutathione peroxidase were estimated in HN-1 cell lines. All experiments were carried out in parallel on HN-1 cell lines using exponentially-growing cultures, 3 days after initial plating.

b. Cellular Levels of Glutathione, Glutathione S-transferase, Glutathione Reductase and Glutathione Peroxidase

(i) Glutathione

Glutathione levels were estimated and the data are summarized in Table 6-4. HN-1/VP-2 cells exhibited a slight decrease in glutathione content compared to parental cells which, however, was not statistically significant. HN-1/DXR-11 cells contained unaltered levels of glutathione compared to parental cells.

(ii) Glutathione S-transferase

The activities of glutathione S-transferase in these HN-1 cells are listed in Table 6-4. HN-1/VP-2 cells expressed unaltered activities of glutathione S-transferase compared to parental cells. However, in HN-1/DXR-11 cells glutathione S-transferase activity was elevated compared to parental cells. Although this elevation in activity appears marginal, being only 17% greater than in parental cells, it was a statistically significant difference ($p<0.05$). However, in another series of experiments VP-16 at concentrations of up to 1 mg/ml was shown not to compete with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate for, or inhibit, purified equine glutathione S-transferase, observations which question the significance of altered levels of glutathione S-transferase in mechanisms of resistance to VP-16. If glutathione S-transferase detoxified VP-16 by catalyzing its conjugation with GSH, VP-16 may have been expected to compete with other substrates of glutathione S-transferase, for example CDNB.

Western blot analyses of these HN-1 cell lines have revealed that they all express only the acidic glutathione S-transferase sub-units, which have been shown to be widely distributed in human tissues (Mannervik, 1985), and not the basic or the neutral sub-units (C.R. Wolf, personal communication). However, analyses of this type were not sensitive enough to identify whether the elevated level of glutathione S-transferase in the HN-1/DXR-11

Table 6-4: Levels of glutathione and activities of glutathione S-transferase, glutathione reductase and glutathione peroxidase in exponentially-growing HN-1 cells. Each experiment consisted of at least two estimations. Data shown are the mean of at least four experiments ± s.e.

Parameter	HN-1 Cell Line		
	P	VP-2	DXR-11
Glutathione (nmole/mg protein)	53.9±3.0	46.9±2.1	52.8±2.6
Glutathione S-transferase (Units[1]/mg protein)	467±16	441±43	544* ±20
Glutathione reductase (Units[2]/mg protein)	61.5±8.2	72.4±5.3	73.1±11.8
Glutathione peroxidase (Units[3]/mg protein)	1.83±0.24	2.60±0.31	2.62±0.22

[1]-1 unit = 1 nmole CDNB conjugated in 1 min at 25°C

[2]-1 unit = 1 nmole NADPH oxidized in 1 min at 25°C

[3]-1 unit = 1 nmole NADPH oxidized in 1 min at r.t.

* - Statistically significant difference from HN-1/P ($p<0.05$)

cell line was associated with amplification of specific glutathione S-transferase sub-units.

(iii) Glutathione Reductase

The activities of glutathione reductase, shown in Table 6-4, in HN-1/VP-2 and HN-1/DXR-11 cells were not significantly different from HN-1/P cells with values ranging between 61.5 and 73.1 units/mg protein.

(iv) Glutathione Peroxidase

Activities of glutathione peroxidase range from 1.83 to 2.62 units/mg protein for HN-1 cell lines. Both HN-1/VP-2 cells and HN-1/DXR-11 cells contained elevated levels of glutathione peroxidase compared to HN-1/P cells (see Table 6-4), although these were not statistically significant differences.

c. Discussion and Summary

The activities of glutathione S-transferase and glutathione reductase cited for HN-1 cells are somewhat higher than those cited by Hamilton and colleagues (1985) who quote values of 118 and 39 units/mg of protein for glutathione S-transferase and glutathione reductase activities respectively, in a tumour cell line (A2780) derived from a human ovarian carcinoma. Levels of reduced

glutathione in the A2780 cell line were 1.89 nmoles/10^6 cells which again is considerably lower than in HN-1 cells, although levels of 12.3 nmole/10^6 cells have recently been cited for human ovarian tumour cells grown as a xenograft (Hamilton et al., 1986). Furthermore, non-protein sulphydryl content of two human prostate tumour cell lines (PC3MA2 and DU145) was of the same order as the GSH content of HN-1 sublines (Metcalfe et al., 1986).

HN-1 cells which had been treated either with fractionated X-irradiation, HN-1/DXR-11, or continuous exposure to VP-16 expressed no significant alterations in the following parameters:

Glutathione content

Glutathione reductase activity

Glutathione peroxidase activity

However, HN-1/DXR-11 cells expressed a slight (17%), but significant, elevation in glutathione S-transferase activity compared to HN-1/P and HN-1/VP-2 cells. The biochemical significance of these observations in terms of VP-16 resistance are unknown, but it would appear unlikely that this marginal increase in enzymatic activity could account for the significant expression of resistance to VP-16 by HN-1/DXR-11 cells, and that other biochemical alterations may account for this. The possibility of multiple biochemical changes within these cells cannot, at this point, be discounted. However, the significance of glutathione S-transferase in VP-16 resistance could be further tested by investigating the effects of depleting

glutathione levels on VP-16 cytotoxicity in HN-1 cell lines. This may then provide less substrate for glutathione S-transferase to conjugate with VP-16. The inhibition of γ-glutamylcysteine synthetase by buthionine sulphoximine (BSO) has been shown to deplete cellular glutathione and potentiate adriamycin, melphalan and cisplatin cytotoxicity in human ovarian carcinoma cell lines (Hamilton et al., 1985), although its effect on VP-16 cytotoxicity has yet to be established. A role in VP-16 resistance for the slight elevation of glutathione S-transferase activity exhibited by HN-1/DXR-11 cells could then be postulated if this BSO induced potentiation of VP-16 cytotoxicity was demonstrated.

In summary, no alterations of levels of glutathione or related enzymes were detected in HN-1/VP-2 cells, and the association of a slight elevation in glutathione S-transferase activity and resistance to VP-16 expressed by HN-1/DXR-11 cells is tenuous. Therefore, expression of VP-16 resistance by these differently-derived HN-1 sublines appears likely to be associated with biochemical modifications not dependent upon postulated VP-16 detoxification mechanisms.

6-4: ANALYSES OF DNA DAMAGE AND REPAIR IN PARENTAL HN-1 CELLS AND RESISTANT SUBLINES AFTER EXPOSURE TO VP-16

a. Introduction

VP-16 has been shown, using the technique of alkaline elution, to possess potent DNA damaging activity in the form of DNA single-strand breaks (SSB), double-strand breaks (DSB) and DNA-protein crosslinks (DPL) which correlated with cytotoxicity in L1210 cells (Wozniak and Ross, 1983). In addition Long et al. (1986) studying human lung carcinoma cell lines, observed that the extent of formation of VP-16-induced DNA SSB reflected their responses to the cytotoxic effects of VP-16. However, the rate of repair of DNA SSB was rapid, with a $t1/_2$, the time taken for 50% of DNA lesions to disappear, of 20-30 min, and the rates did not differ significantly between the sensitive and resistant cell lines.

It was decided, therefore, (i) to estimate VP-16-induced DNA SSB in the HN-1 cell lines, and (ii) to follow the rates of repair of these lesions, in an effort to identify biochemical mechanisms responsible for VP-16 resistance in HN-1/VP-2 and HN-1/DXR-11 cells.

The technique of alkaline elution was employed to estimate drug-induced DNA SSB. All experiments were performed in parallel on exponentially-growing HN-1 cultures. The biphasic nature of alkaline elution profiles from cells treated with VP-16 permitted quantitation

of DNA SSB either by measuring the relative retention of sample cell DNA at 35% retention of IS DNA, or by measuring the slope of the initially eluting fraction (see Section 2-3:g(iii)). It has been postulated by Long and colleagues (1985a) using human lung adenocarcinoma cells that the initial rapidly eluting component is related to DNA DSB, and these workers have also demonstrated a stronger correlation between VP-16-induced cytotoxicity and DNA DSB compared to SSB in a series of human lung carcinoma cell lines which exhibited a range or responses to VP-16 (Long et al., 1986). Therefore it was decided to quantitate VP-16-induced DNA damage in HN-1 cells in these two ways, and to attempt to correlate the extent of DNA damage with cytotoxicity. In both cases the formation of DNA SSB were expressed in rad-equivalents.

b. Formation of DNA Single-Strand Breaks in Parental HN-1 Cells and Resistant Sublines After Exposure to VP-16

Hill and Bellamy (1984) previously demonstrated no major differences in alkaline sucrose gradient sedimentation profiles between HN-1 and HN-1/DXR-11 cells exposed to VP-16 (1 μg/ml) for 24 h. It was suggested that shorter drug exposure times may reveal differences in initial DNA damage between HN-1 cell lines. Therefore it was decided to use alkaline elution, a highly sensitive technique whose results have been shown to correlate with those of alkaline sucrose gradient sedimentation (Pera et al., 1981), and which allows the rapid processing of a large number of samples. Alkaline elution studies have shown that, from the time of exposure, VP-16-induced DNA

damage increases rapidly and reaches a plateau at approximately 30 min (Long et al., 1985a). Therefore, HN-1 cells were exposed to VP-16 for 1 h after which DNA SSB were estimated.

Dose-response curves of VP-16-induced DNA SSB in HN-1 cells exposed to a range of VP-16 concentrations for 1h are shown in Figures 6-11 and 6-12. Concentrations of VP-16 at 1, 5, 10 and 20 µg/ml were chosen for alkaline elution studies as they were relevant to VP-16-induced cytotoxicity evaluated in HN-1 cell lines (see section 5-2:a, Figure 5-1(b), Table 5-1) and also produced a measurable quantity of DNA SSB. It can be seen that both HN-1/VP-2 and HN-1/DXR-11 cells sustained less VP-16-induced DNA SSB at VP-16 concentrations of 5, 10 and 20 µg/ml, compared with HN-1/P cells irrespective of whether this parameter was estimated in terms of the fraction of sample cell DNA retained at 35% retention of IS DNA (Figure 6-11) or by the slope of the initially eluting fraction (Figure 6-12). The reduction of DNA SSB sustained by HN-1/VP-2 cells was significantly different from that of HN-1/P cells at VP-16 concentrations of 1µg/ml ($p<0.01$), 5µg/ml ($p<0.05$) and 10µg/ml ($p<0.01$) (see Figure 6-11). The difference between VP-16-induced DNA SSB between HN-1/DXR-11 cells and HN-1/P cells was significant at VP-16 concentrations of 10 and 20 µg/ml ($p<0.01$) (Figure 6-11). However, both Figures 6-11 and 6-12 indicate a marked difference in dose response profiles of DNA SSB between HN-1/P and HN-1/DXR-11 cells, namely, that as the VP-16 concentration was increased beyond 5µg/ml DNA SSB in HN-1/DXR-11 cells reached a plateau, whereas in HN-1/P cells DNA SSB began to plateau only at a higher VP-16

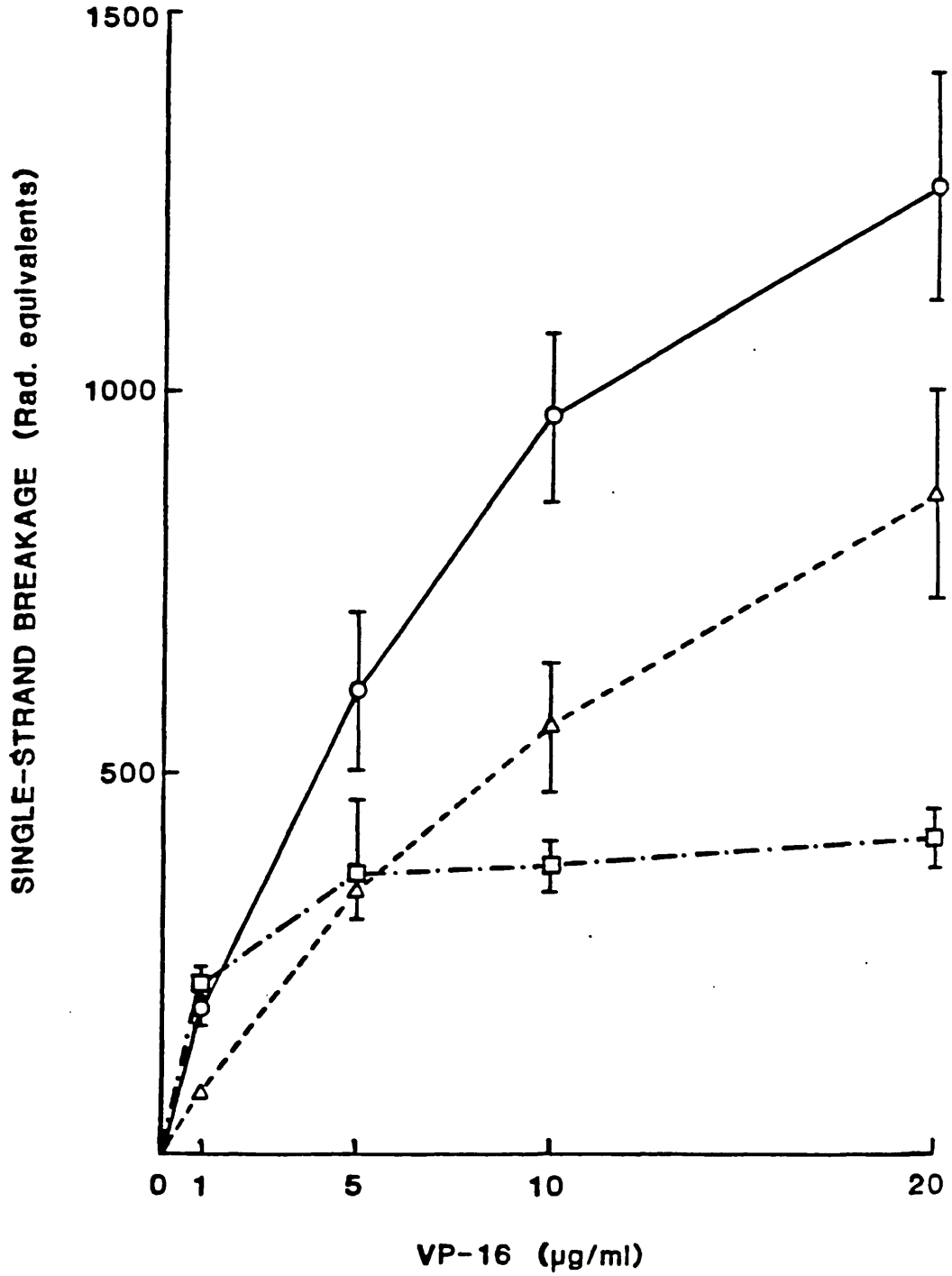


Figure 6-11: DNA single-strand breakage in HN-1 parental (○——○), VP-2 (△- - -△) and DXR-11 (□—··—□) cells after exposure to VP-16 (1h), as measured by the fraction of sample cell DNA retained at 35% retention of IS DNA (see section 2-3:g(iii)). Each experiment was the mean of two estimations. Each point represents the mean of at least three experiments ± s.e. Bars are drawn where s.e.>5%.

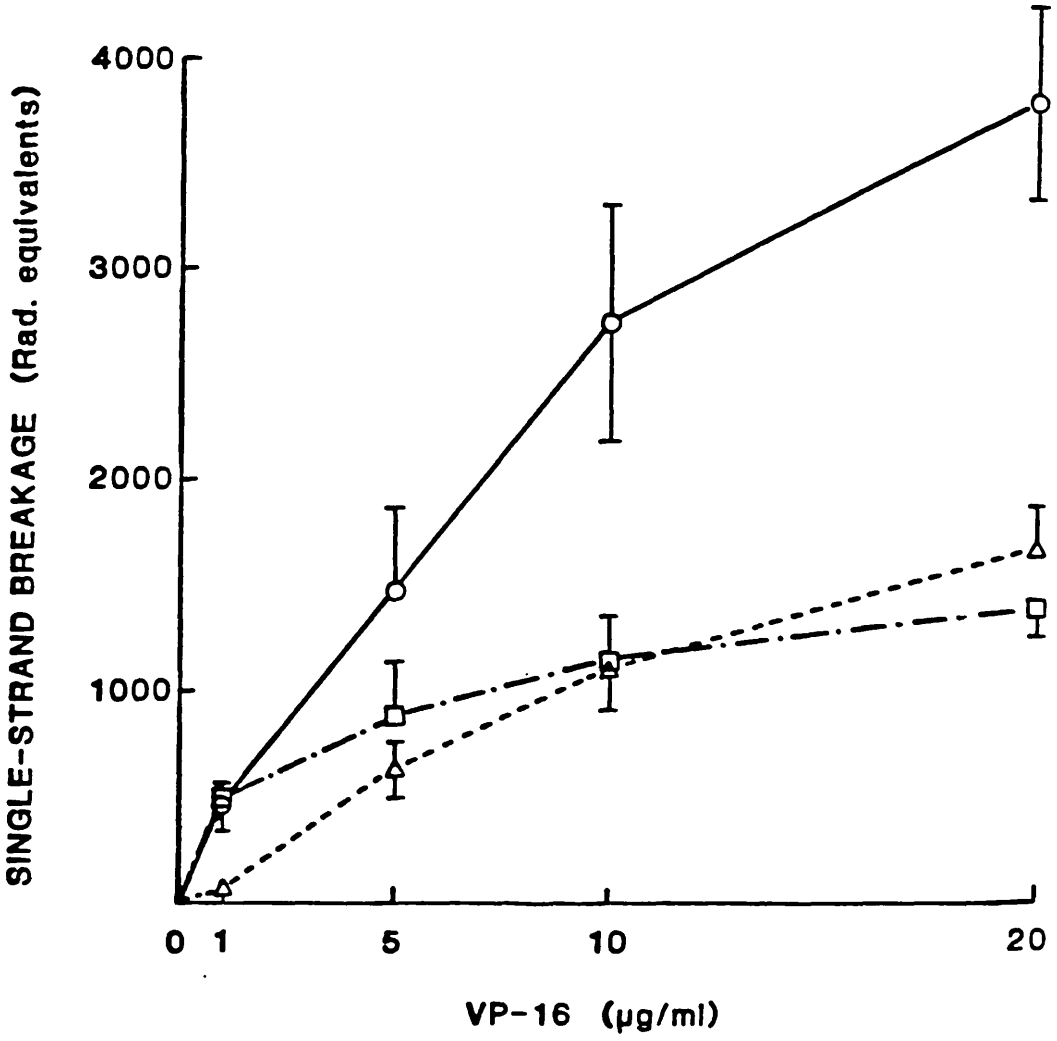


Figure 6-12: DNA single-strand breakage in HN-1 parental (○——○), VP-2 (△- - -△) and DXR-11 (□—··—□) cells after exposure to VP-16 (1h), as measured by the slope of the initially eluting fraction (see section 2-3:g(iii)). Each experiment was the mean of two estimations. Each point represents the mean of at least three experiments ± s.e.

concentration (between 10 and 20μg/ml). VP-16-induced DNA SSB in HN-1/VP-2 cells, as shown in Figure 6-11, showed no evidence of the marked plateau exhibited by HN-1/DXR-11 cells, whereas in Figure 6-12, DNA SSB, as measured by the slope of the initially eluting fraction, reached a plateau in both HN-1/VP-2 and HN-1/DXR-11 cells. However, at a concentration af 1μg/ml of VP-16, DNA SSB in HN-1/VP-2 cells were markedly reduced compared to both HN-1/P and HN-1/DXR-11 cells (Figures 6-11 and 6-12). These apparent differences in extent of VP-16-induced DNA SSB between HN-1/P, HN-1/VP-2 and HN-1/DXR-11 cells are discussed later (Section 6-4:d), and attempts are made to correlate cytotoxicity and DNA SSB estimated by these two different methods.

Due to the large variations inherent in measuring the slope of the initially eluting fraction of sample cell DNA, only the differences between VP-16-induced DNA SSB in HN-1/VP-2 and HN-1/DXR-11 cells at 10 and 20 μg/ml of VP-16, shown in Figure 6-12, were significantly different statistically from HN-1/P cells. However, Figure 6-12 does present an overall trend of a reduction in VP-16-induced DNA SSB in HN-1/VP-2 and HN-1/DXR-11 cells compared to HN-1 parental cells. If the nature of the initially eluting fraction is DNA DSB, as has been postulated (Long et al., 1985a), a more accurate quantitation of this lesion may be obtained by neutral elution as described by Kohn et al., (1981).

The extent of DNA SSB induced in HN-1 cells is in agreement with those of Long and colleagues (1985a) when studying the cytotoxic

and DNA damaging effects of VP-16 on human adenocarcinoma (A549) cells. These workers, using the technique of alkaline elution, showed that DNA SSB reached a plateau as the VP-16 concentration was increased. Also rad-equivalent values at IC_{50} concentrations of VP-16 were approximately equal between HN-1 and A549 cells: values were approximately 260 and 158 rad-equivalents for HN-1 and A549 cells respectively, as measured by the retention of sample cell DNA at 35% retention of IS, and approximately 620 and 780 rad-equivalents respectively, as measured by the slope of the initially eluting fraction. Long et al. (1985a) postulated that the initially eluting fraction of DNA in these A549 cells represented double-strand breakage. It was not possible to estimate VP-16-induced DNA double-strand breakage in HN-1 cells as, due to an inability to incorporate significant amounts of radioactively-labelled thymidine into these cells (see section 2-3:g(i)), technical difficulties arose when attempting neutral elution with detection of DNA fluorimetrically. For example, the Hoechst 33258 fluorochrome exhibits differential fluorescence with single-stranded DNA compared to double-stranded DNA (Cesarone et al., 1979) and the difficulty in removing double-stranded DNA from minced filters, coupled with denaturing the DNA into single-stranded form gave variable results when this methodology was attempted.

These observations of reduced DNA SSB in HN-1/VP-2 and HN-1/DXR-11 cells compared to HN-1/P cells after exposure to VP-16 are in agreement with other studies which used cell lines expressing inherent or acquired resistance to VP-16. Long and colleagues (1986)

observed higher plateau levels for DNA SSB in VP-16-sensitive-, and lower plateau levels for DNA SSB in VP-16-resistant-, human lung carcinoma cell lines upon exposure to VP-16. Impaired formation of VP-16-induced DNA SSB has also been reported in a human colon carcinoma cell line selected for resistance to VP-16 (Long et al., 1985b), Chinese hamster ovary mutants selected for resistance to VM-26 which expressed full cross resistance to VP-16 (Glisson et al., 1986a), and Chinese hamster lung cells selected for 9-hydroxyellipticine resistance which also expressed cross resistance to VP-16 (Pommier et al., 1986a).

c. Repair of DNA Single-Strand Breaks in Parental HN-1 Cells and Resistant Sublines After Exposure to VP-16

DNA repair studies were carried out by following the disappearance of DNA SSB after exposure of HN-1 cells to equimolar concentrations of VP-16 (10 μg/ml, 1 h). This concentration of VP-16 for a 1 h exposure induced a measurable level of cytotoxicity (see section 5-2:a, Figure 5-1(b)) and DNA SSB (see section 6-4:b, Figures 6-11 and 6-12) in HN-1 cell lines.

Time-courses of repair of VP-16-induced DNA SSB in HN-1 cell lines are shown in Figures 6-13 and 6-14. Figure 6-13 shows DNA SSB quantitated in terms of rad-equivalents and in Figure 6-14 DNA SSB was normalized to a percentage of the zero post-treatment incubation time. It can be seen that, for all HN-1 cell lines, VP-16-induced DNA lesions rapidly disappeared. Indeed, after a 6 h post-

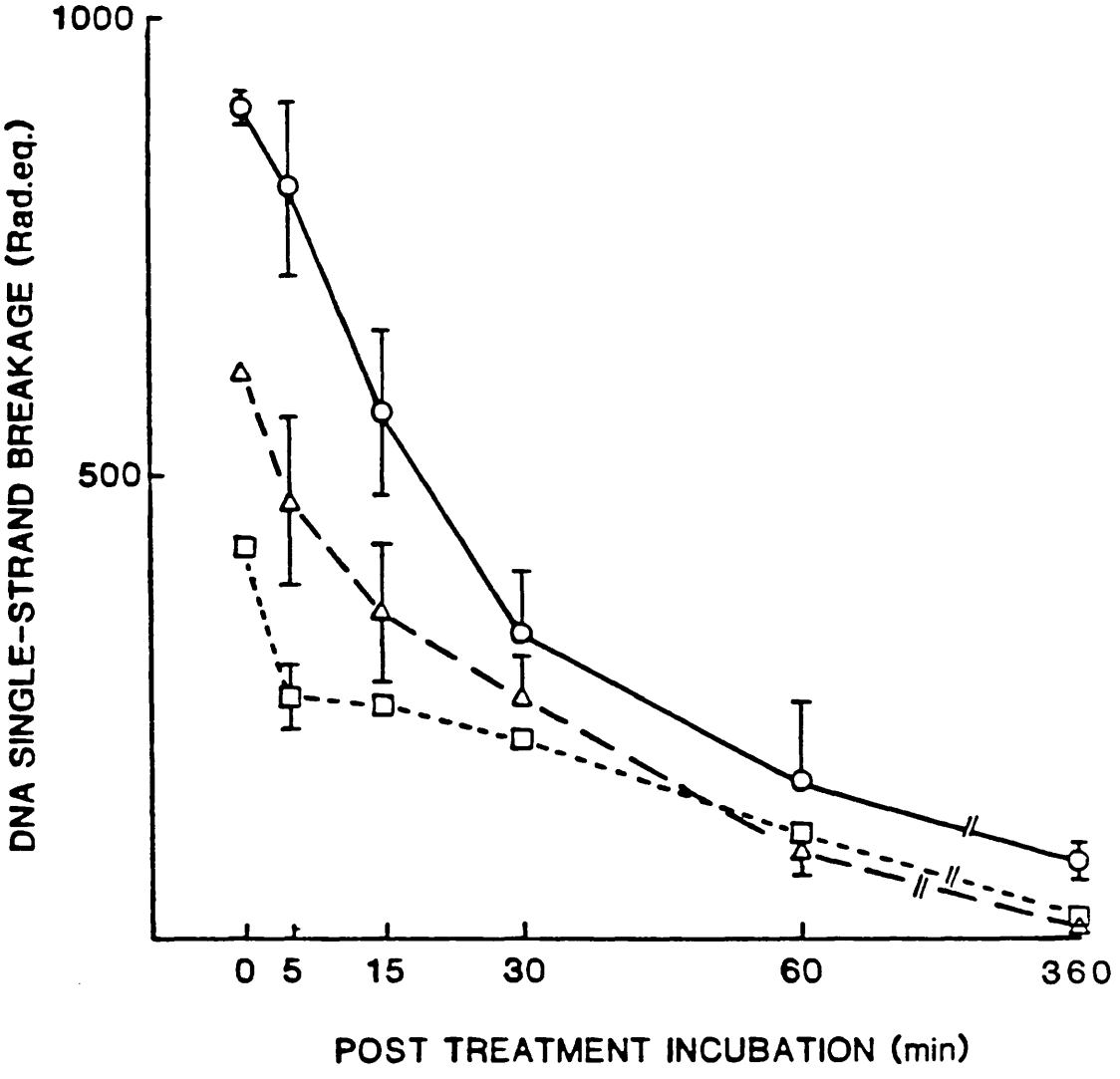


Figure 6-13: Repair of DNA single-strand breakage in HN-1 parental (○——○), VP-2 (△— — △) and DXR-11 (□----□) cells after exposure to VP-16 (10μg/ml, 1h), as measured by the fraction of sample cell DNA retained at 35% retention of IS DNA (see Section 2-3:g(iii)). Each experiment was the mean of two estimations. Each point represents the mean of two experiments ± s.e. Bars are drawn where s.e. >5%.

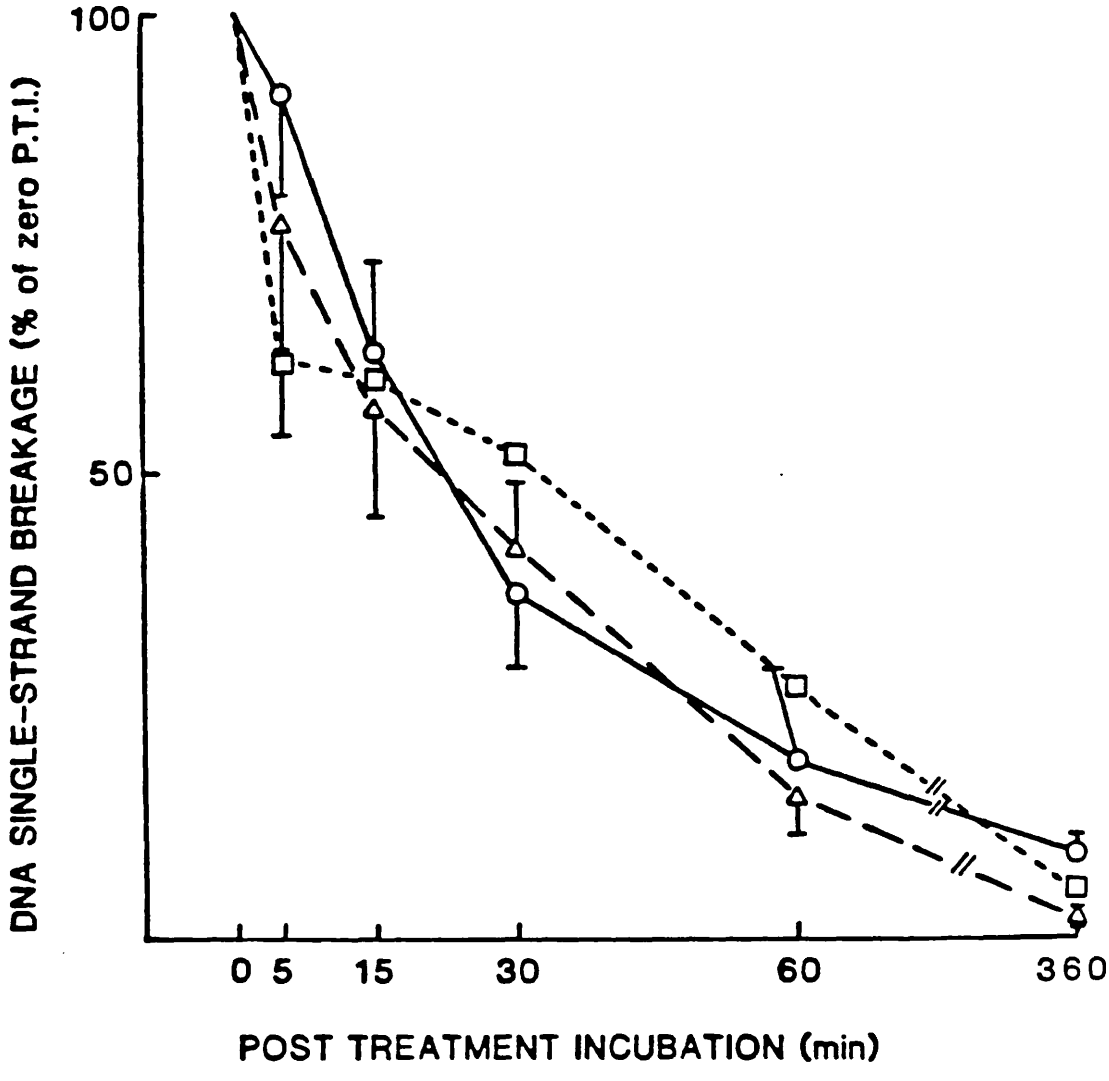


Figure 6-14: Repair of DNA single-strand breakage in HN-1 parental (○——○), VP-2 (△— —△) and DXR-11 (□---□) cells after exposure to VP-16 (10μg/ml, 1h), as measured by the fraction of sample cell DNA retained at 35% retention of IS DNA (see Section 2-3:g(iii)). Rad-equivalent values were corrected to a percentage of the zero post-treatment incubation time. Each experiment was the mean of two estimations. Each point represents the mean of two experiments ± s.e. Bars are drawn where s.e. >5%.

treatment incubation these lesions were difficult to detect. However, this disappearance of DNA lesions within 6 h of post-treatment incubation was not likely to result from cell death as there was no observable loss of cells from the surface of the flasks and total fluorescence values of DNA from the alkaline elution data were not significantly lower than for untreated controls.

The half lives of repair ($t1/_2$) of VP-16-induced DNA SSB were estimated for HN-1 cell lines. These values ranged between 20.1 and 25.5 min and were not statistically different for these lines (Table 6-5). These data are in agreement with those of Long and colleagues (1985b) who observed $t1/_2$ values for the disappearance of VP-16-induced DNA SSB of between 20 and 30 min in human lung and colon carcinoma cell lines, which expressed a range of responses to VP-16.

Since altered rates of repair had not been detected in these three HN-1 cell lines at equimolar concentrations of VP-16, it was considered unlikely that differences would be detected using equitoxic drug concentrations. Therefore these studies were not extended to investigate rates of repair of VP-16-induced DNA SSB in HN-1 cell lines using equitoxic concentrations of VP-16.

d. Discussion and Summary

HN-1 cells which have been made resistant to VP-16 by either fractionated X-irradiation treatment or continuous exposure to

Table 6-5: Comparison of cell survival and DNA single-strand breakage and repair parameters of HN-1 cell lines exposed to VP-16 (10μg/ml, 1h).

HN-1 Cell Line	Cell Survival at 10μg/ml VP-16 (1h) (% ± s.e.)	DNA SSB[1] at 10μg/ml VP-16 (1h) (Rad-Eq ± s.e.)	DNA SSB[2] at 10μg/ml VP-16 (1h) (Rad-Eq ± s.e.)	$t1/_2$ of repair of DNA SSB at 10μg/ml VP-16 (1h) (min ± s.e.)
P	16.2±2.2	969±112	2738±557	22.2±3.5
VP-2	43.8*±3.9	560*±84	1105**±194	20.1±2.2
DXR-11	28.2**±2.5	380*±33	1137**±218	25.5±3.5

[1]- As measured by the fraction of sample cell DNA retained at 35% retention of IS DNA (see section 2-3:g(iii)).

[2]- As measured by the slope of the initally eluting fraction (see section 2-3:g(iii))

* - Statistically significant difference from HN-1/P ($p<0.01$).

** - Statistically significant difference from HN-1/P ($p<0.05$).

VP-16 sustained less DNA SSB than HN-1 parental cells after a 1h exposure to VP-16. These observations are in agreement with other in vitro studies investigating mechanisms of acquired or inherent VP-16 resistance (Long et al., 1986; Glisson et al., 1986a; Pommier et al., 1986a). Furthermore, data presented here also indicate that the differences in responses to VP-16 expressed by HN-1/VP-2 and HN-1/DXR-11 cells were not due to enhanced rates of DNA repair, in agreement with data from Long and colleagues (1985b).

A comparison of cell survival and DNA SSB and repair parameters of HN-1 cell lines exposed to equimolar VP-16 concentrations for 1h is shown in Table 6-5. It can be seen that at 10μg/ml of VP-16 for a 1h exposure, cell survival and DNA SSB in HN-1/VP-2 and HN-1/DXR-11 cells differed significantly from parental cells, whereas the $t1/_2$ values of repair of DNA lesions were not significantly different between HN-1 cell lines. The implications of these observations are that resistance to VP-16 in these differently-derived sublines is associated with reduced formation of DNA SSB after exposure to VP-16. In addition, it would appear that the nature of these lesions may differ between HN-1/VP-2 and HN-1/DXR-11 cells, since a better correlation was observed between DNA SSB estimated by the slope of the initially eluting fraction and cytotoxicity than DNA SSB estimated by the fraction of sample cell DNA retained at 35% retention of IS DNA (Figures 6-15 and 6-16, Table 6-5). In Figure 6-15 it can be seen that VP-16-induced DNA SSB in HN-1/DXR-11 cells reached a plateau at a concentration of 5μg/ml, whereas cytotoxicity increased up to 10μg/ml indicating that an additional cytotoxic

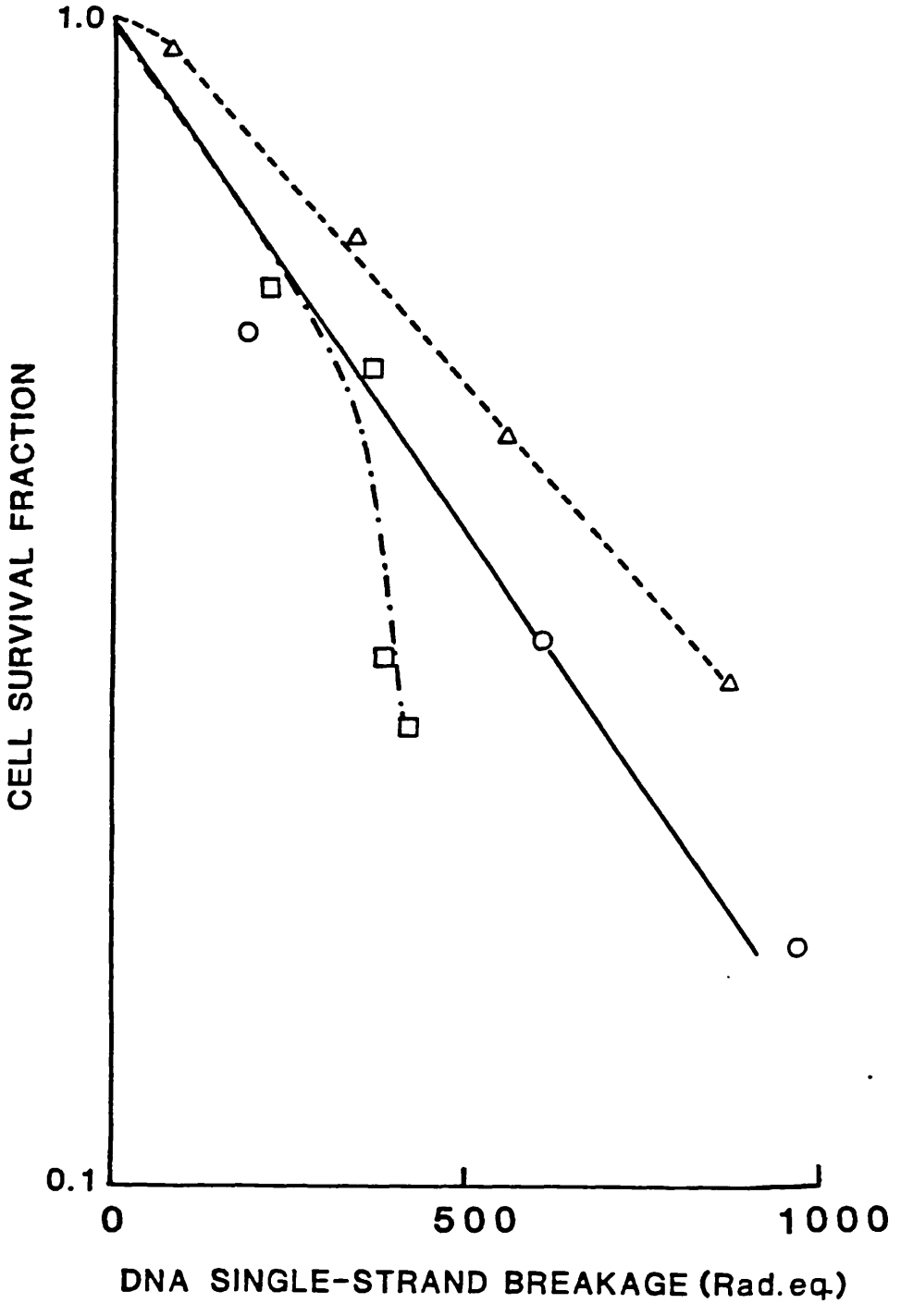


Figure 6-15: The relationship between VP-16-induced DNA single-strand breakage and cytotoxicity in HN-1/P (○——○), HN-1/VP-2 (△- - -△) and HN-1/DXR-11 (□—·—□) cells. Cytotoxicity was estimated by colony formation in soft agar (see Section 2-3:a(ii)), DNA single-strand breakage was estimated as the fraction of sample cell DNA retained at 35% retention of IS DNA (see Section 2-3:g). Error bars are as for Figures 5-1(b) and 6-11.

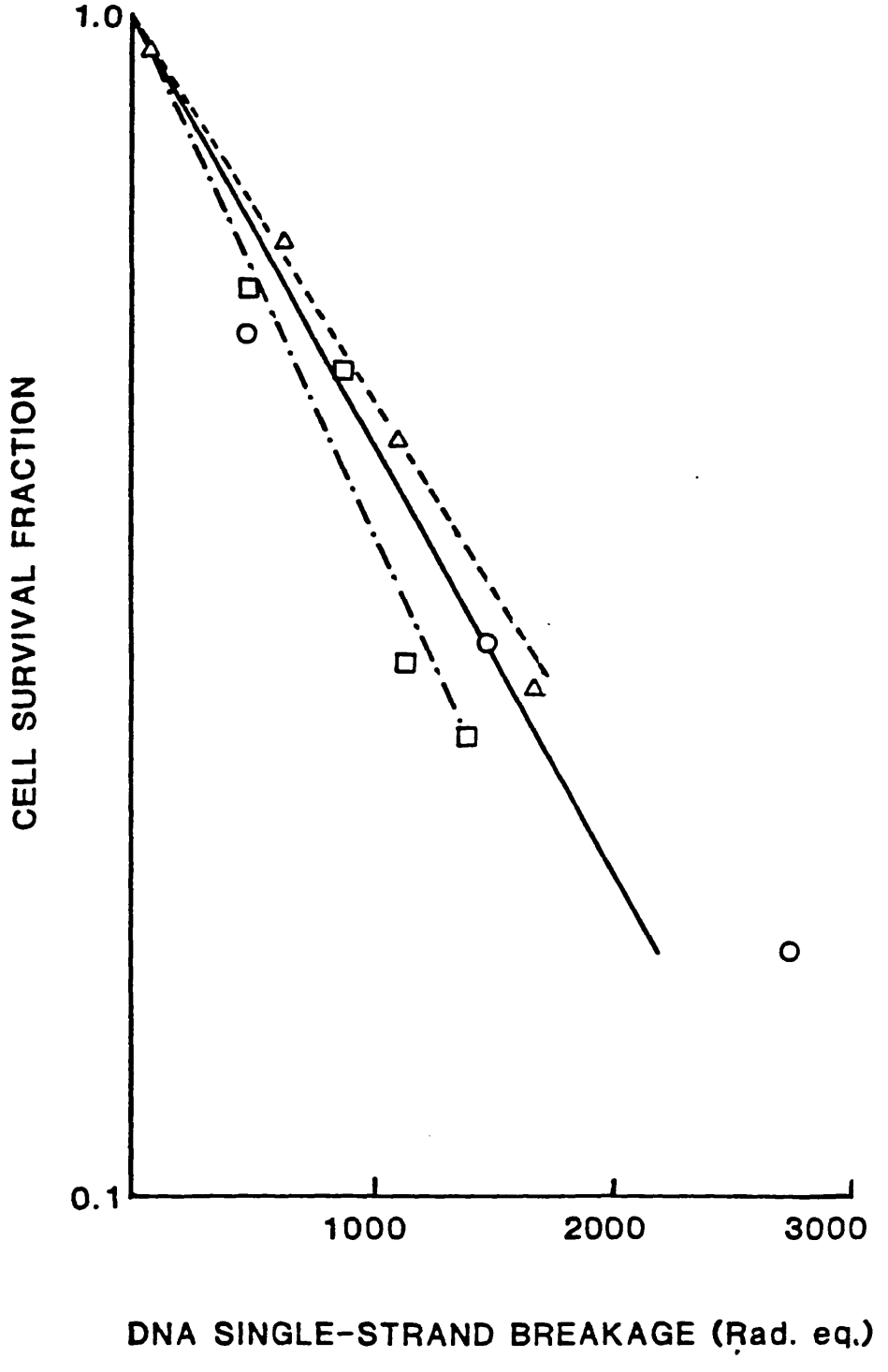


Figure 6-16: The relationship between VP-16-induced DNA single-strand breakage and cytotoxicity in HN-1/P (○——○), HN-1/VP-2 (△- - -△) and HN-1/DXR-11 (□—·—□) cells. Cytotoxicity was estimated by colony formation in soft agar (see Section 2-3:a(ii)), DNA single-strand breakage was estimated by the slope of the initially eluting DNA fraction (see Section 2-3:g). Error bars are as for Figures 5-1(b) and 6-12.

lesion may be involved in these cells at the higher drug concentrations. HN-1/VP-2 cells, on the other hand, showed an initial shoulder which may represent sublethal DNA SSB, followed by a slope which did not differ significantly from that of parental cells. VP-16-induced DNA SSB and cytotoxicity correlated in HN-1/P cells when DNA SSB was estimated by either of the two methods selected. Figure 6-16 also shows that DNA SSB, as estimated by the slope of the initially eluting fraction, correlated with cytotoxicity in HN-1/VP-2 and HN-1/DXR-11 cells to a similar extent as parental cells. This initially eluting fraction may represent DNA DSB (Long et al., 1985a) which is generally thought to be more cytotoxic than DNA SSB, and this may explain the differences between Figures 6-15 and 6-16. In support of this Long et al., (1986) demonstrated a closer correlation between VP-16-induced cytotoxicity and DSB than SSB in a series of human lung carcinoma cell lines. However, it has already been mentioned that estimation of DNA SSB by the slope of the initially eluting fraction is rather less accurate than by the fraction of sample cell DNA retained at 35% retention of IS DNA, and estimations of DNA DSB by neutral elution are necessary to clarify whether VP-16-induced DNA SSB with respect to cytotoxicity in HN-1/DXR-11 cells reaches a plateau (Figure 6-15) whereas DNA DSB does not (Figure 6-16).

Interestingly at a VP-16 concentration of 1μg/ml DNA SSB in HN-1/VP-2 cells were reduced compared to both HN-1/P and HN-1/DXR-11 cells (see Figures 6-11 and 6-12). This difference is also apparent in the cytotoxicity induced by 1μg/ml of VP-16 (see Figure 5-1(b))

and may be a reflection of the manner in which these resistant cell lines were derived. In selecting cells by exposure to VP-16, HN-1/VP-2, sensitive cells may be killed, leaving a population which experienced less DNA SSB than the parental population even at the lowest VP-16 concentration employed. However, it is possible that the HN-1/DXR-11 cell line, not having been selected by exposure to VP-16, may contain VP-16-sensitive and resistant sub-populations which gave an overall response of DNA SSB equivalent to parental cells at the lowest VP-16 concentration used, which then reached a plateau as the VP-16 concentration was increased. Alternatively the HN-1/DXR-11 cells may contain an altered target molecule, such as DNA topoisomerase II, which is rapidly saturated by VP-16 to give this plateau effect of DNA SSB.

The significance of these observations can be tested by comparing the reduction of VP-16-induced cytotoxicity and DNA SSB in HN-1/VP-2 and HN-1/DXR-11 cells with those reported for other tumour cell lines exhibiting acquired or natural resistance to VP-16. Table 6-6 compares Resistance Indices and extent of reduction in VP-16-induced DNA damage in a series of VP-16-resistant cell lines. An observation which appears unusual is that CHO/Vpm^{R}-5 cells appear to exhibit a reduction of VP-16-induced SSB which is greater than their level of resistance. This may be a result of these authors having expressed differences between parental and resistant cell lines as the concentration of drug required to induce equivalent DNA SSB, which contrasts with other studies where differences were estimated in terms of DNA lesions induced in parental and resistant cells at

Table 6-6: A comparison between the reduction of VP-16-induced cytotoxicity and DNA damage in a range of tumour cell lines exhibiting acquired or natural resistance to VP-16.

VP-16-resistant cell line	Resistance Index	Extent of reduction in DNA damage	Reference
CHO/Vpm^R-5	20[a]	30[b]	Glisson et al., 1986a
DC-3F/ 9-OH-E	12[c]	4.9[d]	Pommier et al., 1986a
SW900	5.9[a]	4.0[e]	Long et al., 1986
HN-1/VP-2	3.9[c]	2.3[f]	This study
HN-1/DXR-11	3.6[c]	2.7[f]	This study

[a] - Ratio of IC_{50} values of VP-16-resistant and parental cell lines after a 1h exposure to VP-16.

[b] - Ratio of concentration of VP-16 required to induce equivalent DNA SSB in VP-16-resistant and parental cells.

[c] - Ratio of survival of VP-16-resistant cells and parental cells after exposure to equimolar VP-16 concentrations.

[d] - Ratio of DPL induced in parental and VP-16-resistant cells after exposure to equimolar VP-16 concentrations.

[e] - Ratio of DNA DSB induced in VP-16-sensitive and VP-16-resistant cells after exposure to equimolar VP-16 concentrations.

[f] - Ratio of DNA SSB induced in parental and VP-16-resistant cells after exposure to equimolar VP-16-concentrations, as estimated by the slope of the initially eluting alkaline elution fraction.

equimolar drug concentrations. This collation indicates that the reduction in VP-16-induced DNA damage observed in HN-1/VP-2 and HN-1/DXR-11 cells could indeed account for the level of resistance exhibited by these cells to VP-16. Alterations in the activities of DNA topoisomerase II have been detected in two of the VP-16-resistant mammalian tumour cell lines compared in Table 6-6 (Glisson et al., 1986a; Pommier et al., 1986b), indeed a mutant form of DNA topoisomerase II has recently been purified from the CHO/Vpm^{R}-5 cell line (W. Ross, personal communication). Characterization of DNA topoisomerase II activity in HN-1 cell lines may reveal, not only modifications between parental and resistant cells, but also any divergence in activity or mutant forms of the enzyme between differently-derived resistant sublines which may be associated with the manner in which resistance was induced.

6-5: OVERALL SUMMARY

Human tumour cell lines resistant to VP-16 have been derived either by exposure to VP-16, HN-1/VP-2, or by fractionated X-irradiation treatment, HN-1/DXR-11. The responses expressed by these cell lines to a range of antitumour agents have been evaluated and shown to differ depending upon the agent employed to induce resistance (see Chapter 5). Expression of multidrug resistance by HN-1/VP-2 cells was associated with reduced uptake of vincristine, vinblastine and daunomycin, but not of the selecting agent, VP-16 (see Section 6-2). In contrast to these observations HN-1/DXR-11 cells expressed unaltered drug uptake characteristics which indicate

that mechanisms of resistance to the Vinca alkaloids, vincristine and vinblastine, may differ depending upon the manner in which resistance was induced. Further studies may indicate whether HN-1/VP-2 cells express the 170kD molecular weight P-glycoprotein associated with the multidrug-resistant phenotype, in which cells express impaired uptake of structurally unrelated antitumour agents to which they are cross resistant (Juliano and Ling, 1976; Beck et al., 1979). These studies may also reveal whether HN-1/DXR-11 cells have overexpression of the P-glycoprotein, which would be an unusual observation, since drug-resistant cell lines which express P-glycoprotein generally express a drug transport defect and have been derived by exposure of parental cells to chemotherapeutic drugs.

Alterations in intracellular detoxification mechanisms involved in glutathione metabolism appeared unlikely to account for the level of VP-16-resistance expressed by HN-1/DXR-11 cells (see Section 6-3). HN-1/VP-2 cells expressed unaltered activities of glutathione and related enzymes compared to parental cells.

The most likely explanation for the expression of VP-16 resistance by HN-1/VP-2 and HN-1/DXR-11 cells appears to be reduced formation of DNA SSB compared to parental cells after exposure to VP-16 (see Section 6-4). The extent of the reduction of VP-16-induced DNA SSB in both VP-16-resistant HN-1 cell lines is consistent with other literature reports (Table 6-6) and therefore could account for the level of VP-16 resistance exhibited by these cells. However, the nature of these drug-induced lesions appeared to differ between

the two VP-16-resistant cell lines indicating that mechanisms of resistance to VP-16 may differ depending upon the agent employed to induce resistance. The evidence presented indicates that VP-16-induced DNA SSB reached a plateau in HN-1/DXR-11 cells, as the VP-16 concentration was increased, which did not correlate with cytotoxicity (see Figure 6-15), unlike the HN-1/VP-2 cells in which this lesion did correlate directly with cytotoxicity. Although by inference it was proposed that DNA DSB, which may be a more cytotoxic lesion than DNA SSB, did correlate with cytotoxicity in all HN-1 cell lines (see Figure 6-16). Therefore, although it appeared that the extent of VP-16-induced DNA SSB differed between HN-1/VP-2 and HN-1/DXR-11 cells with respect to cytotoxicity, DNA DSB may be the major cytotoxic lesion. Characterization of DNA topoisomerase II activity from HN-1 parental and resistant sublines may yield more evidence in support of these hypotheses.

CHAPTER 7 : ANALYSES OF PLASMA MEMBRANE FRACTIONS FROM CHINESE HAMSTER OVARY AND HN-1 CELL LINES BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

CONTENTS:

7-1: INTRODUCTION 235

7-2: ANALYSES OF PLASMA MEMBRANE FRACTIONS FROM CHINESE HAMSTER OVARY AND HN-1 CELL LINES BY SDS-PAGE AND WESTERN BLOTTING 237

7-3: DISCUSSION AND SUMMARY 244

7-1: INTRODUCTION

Juliano and Ling (1976) originally described the overproduction of a 170kD membrane glycoprotein (the P-glycoprotein) in colchicine-resistant (180-fold) Chinese hamster ovary cells ($CH^{R}C5$) which exhibited reduced permeability to the drug. One distinguishing feature of these membrane-altered mutants was their pleiotropic cross resistance to a wide range of apparently unrelated compounds such as the Vinca alkaloids, colcemid, the anthracyclines and puromycin (Bech-Hansen et al., 1976). Similar alterations have subsequently been described in actinomycin D-resistant and vincristine-resistant Chinese hamster lung cells (Peterson and Biedler, 1978; Biedler and Peterson, 1981), in vinblastine-resistant human leukaemic lymhoblasts (Beck et al., 1979), and in daunomycin-resistant Chinese hamster ovary cells (Kartner et al., 1983). Common characteristics of all these cells included expression of multidrug resistance and reduced drug accumulation. Initially the P-glycoprotein was detected by surface labelling or by metabolic labelling with carbohydrate precursors followed by SDS-PAGE. Alternatively, in the isolated plasma membranes of highly resistant Chinese hamster ovary cells the P-glycoprotein could be detected, following SDS-PAGE, by protein staining alone (Riordan and Ling, 1979). The specificity and sensitivity of detection of the P-glycoprotein was improved by the use of monoclonal antibodies specific for P-glycoprotein (Kartner et al., 1985). Using the monoclonal antibody, C219, and Western blotting techniques, Kartner et al. (1985) compared plasma membranes isolated from different multidrug-resistant cell lines. They

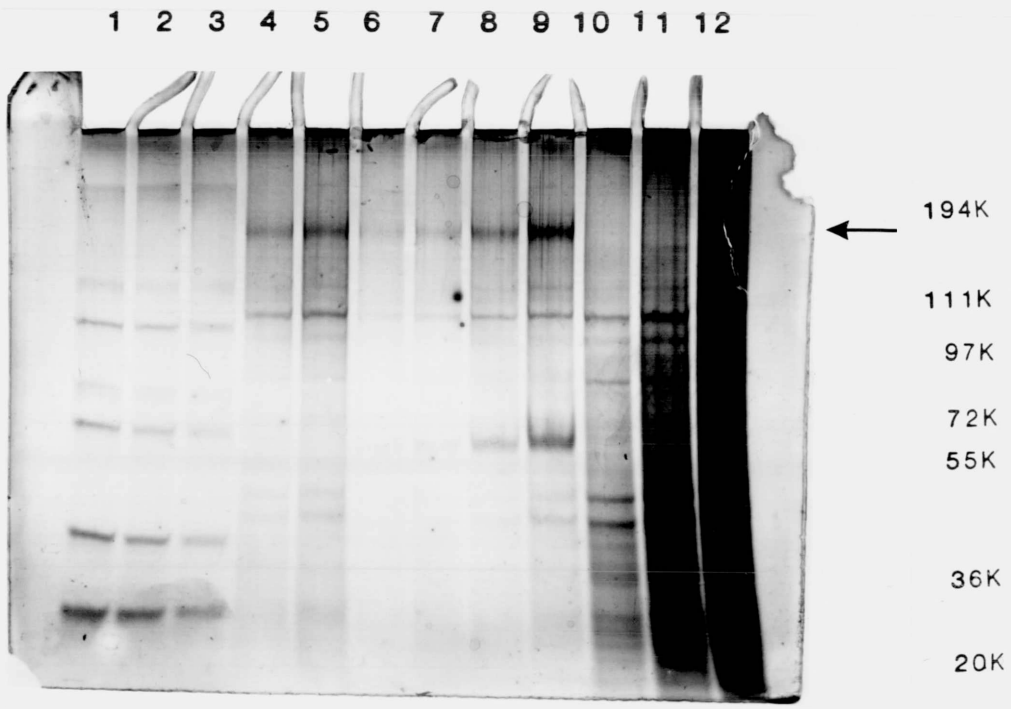
demonstrated that multidrug resistance-associated glycoproteins, which had been identified by different groups in a variety of multidrug-resistant cell lines, were conserved in molecular size and in antigenic structure. Thus, it would appear that a homologous P-glycoprotein is expressed in multidrug-resistant cells of different origins (Kartner et al., 1985).

In this study it was decided initially to confirm the presence of the P-glycoprotein in the highly colchicine-resistant (180-fold) Chinese hamster ovary cells ($CH^{R}C5$) by SDS-PAGE of plasma membrane fractions as described by Riordan and Ling (1979), followed by silver staining of proteins. Then this methodology could be applied to analyze plasma membrane fractions of HN-1 cell lines. A greater degree of sensitivity and specificity in detecting the P-glycoprotein was facilitated subsequently by the use of the monoclonal antibody, C219, which was kindly provided by Dr. V. Ling (Ontario Cancer Institute, Toronto, Canada). Following iodination of this monoclonal antibody with ^{125}I (see Section 2-3:h(iv)) the expression of P-glycoprotein in parental and drug-resistant Chinese hamster ovary cells, and in HN-1 cell lines, was studied either by detection of immobilized antigen on nitrocellulose membrane filters or by Western blotting (see Section 2-3:h(iv)). Cell fractionation, purification of plasma membrane fractions, and SDS-PAGE, were carried out as described in Sections 2-3:h(i) and (ii).

7-2: ANALYSES OF PLASMA MEMBRANE FRACTIONS FROM CHINESE HAMSTER OVARY AND HN-1 CELL LINES BY SDS-PAGE AND WESTERN BLOTTING

Plasma membrane fractions were prepared from $CH^{R}C5$ cells and stored at -20°C for 1 month in order to establish whether the P-glycoprotein was stable under these conditions. In addition, plasma membrane fractions were freshly prepared from $AuxB_1$ (parental) and $CH^{R}C5$ cells. These samples were analyzed for P-glycoprotein overexpression by SDS-PAGE. From Plate 7-1 (lanes 4-9) it can be seen that, in both of the $CH^{R}C5$ preparations, P-glycoprotein was consistently overexpressed compared to $AuxB_1$ cells (lanes 10-12), indicating that the P-glycoprotein did not undergo degradation when stored at -20°C for 1 month. When the gel was calibrated using proteins of known molecular weight (lanes 1-3), the position of this overexpressed protein corresponded to 170kD. The degree of overexpression of the P-glycoprotein in $CH^{R}C5$ cells was illustrated when equivalent amounts of total protein from $CH^{R}C5$ and $AuxB_1$ preparations were loaded onto the gel (compare lane 8 with lane 11 in Plate 7-1). Using the silver stain, P-glycoprotein overexpression could be detected when as little as 0.13µg of total protein (lane 6) were loaded onto the gel. In the study by Riordan and Ling (1979) approximately 100µg of total protein from plasma membrane fractions were loaded onto each gel in order to detect the P-glycoprotein, however in their study proteins were stained with Coomassie blue, which is known to be less sensitive than the silver stain.

Plate 7-1: SDS-PAGE of plasma membrane fractions from Chinese hamster ovary $AuxB_1$ and CH^RC5 cells. Cell fractionation, purification of plasma membrane fractions and SDS-PAGE were carried out as described in Section 2-3:h. Proteins were stained using the Bio-Rad silver stain (see Section 2-3:h(iii)). Lanes 1-3, protein standards of known molecular weight (kD); lanes 4 and 5, CH^RC5 preparation stored at -20°C for 1 month (0.3 and 0.45µg of total protein respectively); lanes 6-9, CH^RC5 (0.13, 0.25, 0.5 and 1.0µg respectively); lanes 10-12, $AuxB_1$ (0.25, 0.5 and 0.75µg respectively). The position of the 170kD P-glycoprotein is marked with an arrow.


1 2 3 4 5 6 7 8 9 10 11 12
194K
111K
97K
72K
55K
36K
20K

Following this detection of the P-glycoprotein in these highly drug-resistant $CH^{R}C5$ cells, the HN-1/VP-2 and HN-1/DXR-11 cells were next used to examine whether any overexpression could be detected in these cells expressing relatively low levels of drug resistance (see Chapters 4 and 5). Plate 7-2 indicates that any differences in the expression of membrane proteins in HN-1/VP-2 (lane 6) or HN-1/DXR-11 (lane 3) cells were undetectable from HN-1/P (lanes 4 and 5) cells at this level of resolution. This finding was not unexpected since Riordan and Ling (1979) showed that, by the Coomassie blue staining procedure, the P-glycoprotein was undetectable in a Chinese hamster ovary cell line which, although being a revertant, expressed a 5-fold level of resistance to colchicine compared to $AuxB_1$ cells.

Although the quality of the gel shown in Figure 7-2 was rather poor, it was apparent that overexpression of the P-glycoprotein could not be detected in HN-1 cell lines using the silver stain. In an attempt to increase the sensitivity of detection of the P-glycoprotein plasma membrane fractions were dotted onto nitrocellulose membrane filters, dried, and probed with the iodinated monoclonal antibody, C219. Plate 7-3 shows a dot immunoblot using 5μg of total protein from Chinese hamster ovary or HN-1 cells lines. Dots 1 and 2 show the positive expression of P-glycoprotein in two separate $CH^{R}C5$ preparations, whilst an $AuxB_1$ preparation (dot 4) gave background radioactivity. The difference in intensity between these two $CH^{R}C5$ samples may be attributed to either variability during membrane fractionation or to variations in the dotting procedure

Plate 7-2: SDS-PAGE of plasma membrane fractions from HN-1 cell lines. Cell fractionation, purification of plasma membrane fractions and SDS-PAGE were carried out as described in Section 2-3:h. Proteins were stained using the Bio-Rad silver stain (see Section 2-3:h(iii)). Lanes 1 and 2, protein standards (as in Plate 7-1); lane 3, HN-1/DXR-11 (1.3µg of total protein); lanes 4 and 5, HN-1/P (1.3 and 0.7µg respectively); lane 6, HN-1/VP-2 (1.3µg). The position where the P-glycoprotein would occur (170kD) is marked with an arrow.

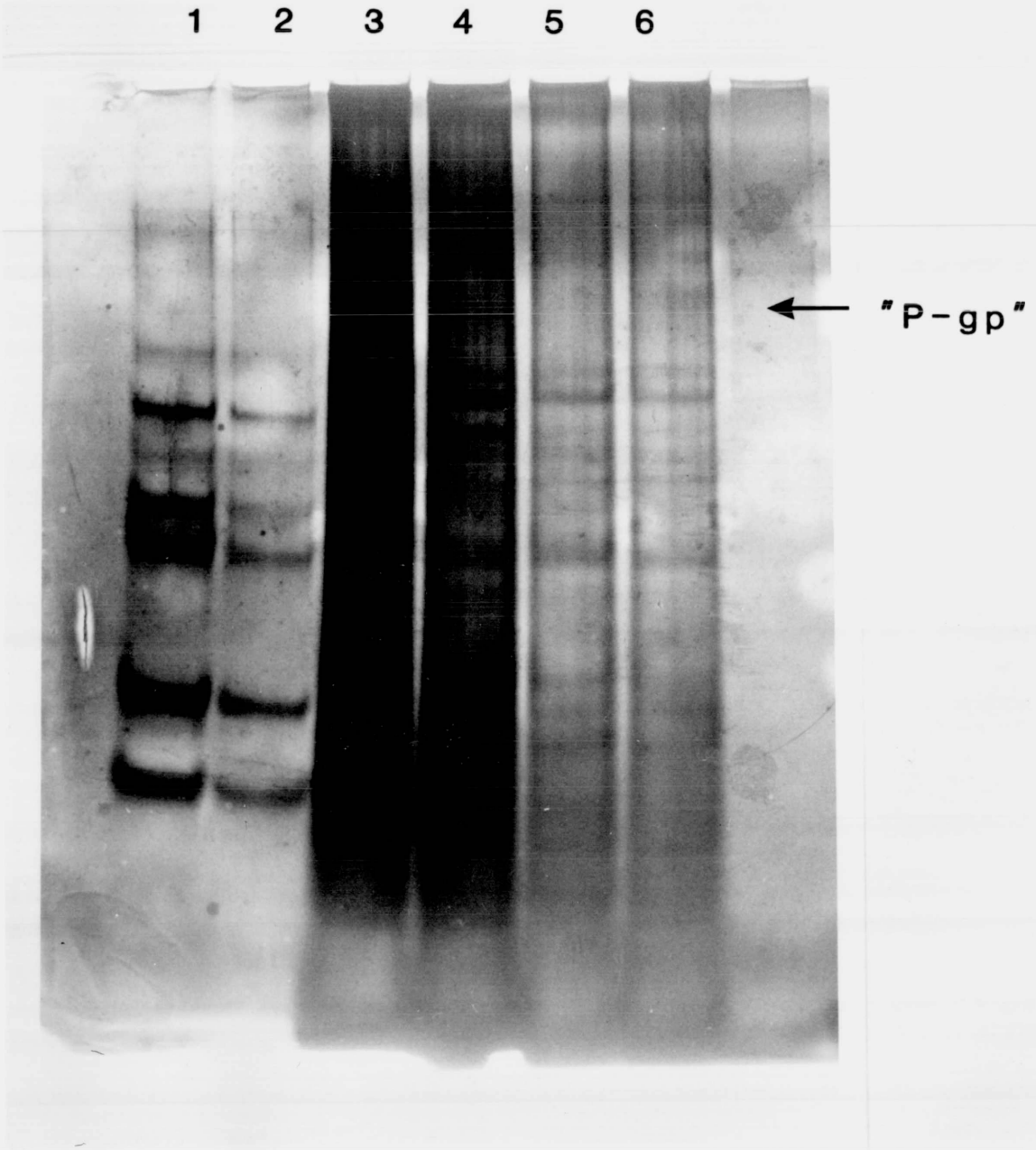
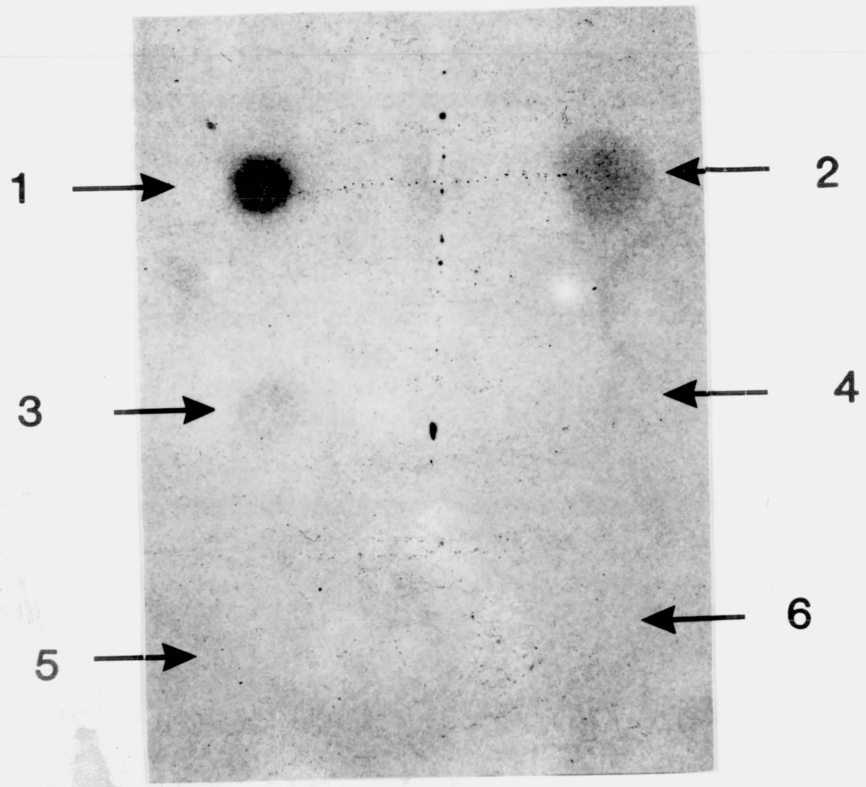

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5
6
"P-gp"

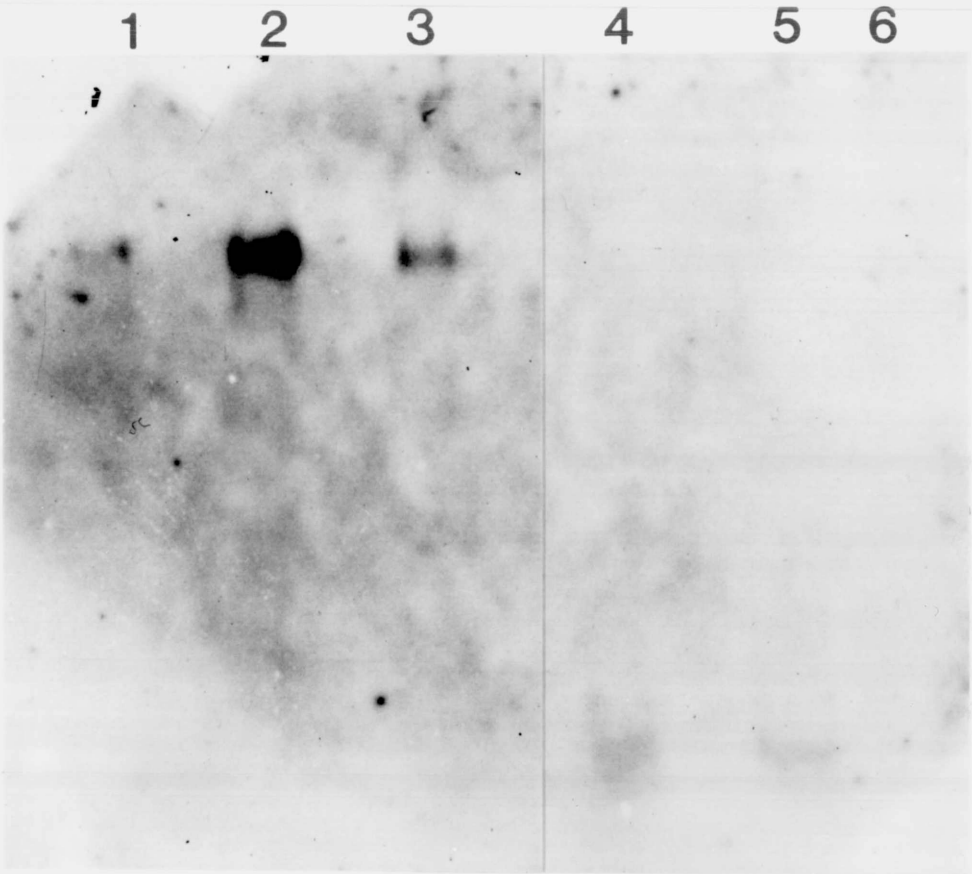
Plate 7-3: Detection of P-glycoprotein in Chinese hamster ovary and HN-1 cell lines by dot immunoblot with ^{125}I-C219 monoclonal antibody. All procedures were carried out as described in Section 2-3:h. 5µg of total protein were used for each dot. (1) and (2) separate preparations from $CH^{R}C5$ cells; (3) HN-1/VP-2; (4) $AuxB_1$; (5) HN-1/P; (6) HN-1/DXR-11.


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since the $CH^{R}C5$ sample in dot 2 appears to be rather more diffuse than the $CH^{R}C5$ sample in dot 1. Plate 7-3 also indicates that HN-/VP-2 cells exhibited overexpression of the P-glycoprotein compared to HN-1/P and HN-1/DXR-11 cells when equivalent amounts of protein were dotted onto the nitrocellulose filter (compare dot 3 with dots 5 and 6 in Plate 7-3), although it should be noted that these are preliminary results. The intensity of the blot was considerably less than either of the two $CH^{R}C5$ samples (dots 1 and 2) which indicates that the degree of expression of the P-glycoprotein was much lower in HN-1/VP-2 cells.

Further evidence that the protein overexpressed in HN-1/VP-2 cells, detected by dot immunoblot, is the 170kD molecular weight P-glycoprotein is shown in Plate 7-4. This Western blot indicates that the proteins overexpressed in membrane fractions of both $CH^{R}C5$ and HN-1/VP-2 cells conform in antigenic structure and in molecular weight to those described by Kartner et al. (1985) in a series of multidrug-resistant mammalian tumour cell lines. Although 7-times the amount of total protein from HN-1/VP-2 cells (7µg, lane 3) was electrophoresed compared to $CH^{R}C5$ cells (1µg, lane 2), equivalent amounts of protein were loaded for HN-1 cell lines. Therefore, these results, although preliminary, indicate that P-glycoprotein is overexpressed in HN-1/VP-2 cells, however the degree of overexpression is considerably less than in $CH^{R}C5$ cells.

Plate 7-4: Detection of P-glycoprotein in Chinese hamster ovary and HN-1 cell lines by Western blot with ^{125}I-C219 monoclonal antibody. All procedures were carried out as described in Section 2-3:h. Lanes 1 and 2, $CH^{R}C5$ (0.25 and 1µg of total protein respectively); lane 3, HN-1/VP-2 (7µg); lane 4, HN-1/P (7.2µg); lane 5, HN-1/DXR-11 (8µg); lane 6, $AuxB_1$ (1µg).


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7-3: DISCUSSION AND SUMMARY

The expression of multidrug resistance and impaired drug uptake by HN-1/VP-2 cells is associated with overproduction of the 170kD P-glycoprotein compared to parental and HN-1/DXR-11 cells. However, additional alterations in membrane proteins of these cells cannot be ruled out although to detect them it may require procedures more sensitive than general silver staining. The major alterations in plasma membrane proteins, which have been shown to occur in multidrug-resistant cells, are summarized in Table 7-1. An increase in the 170kD glycoprotein in multidrug-resistant Chinese hamster ovary cells, human leukaemic lymphoblasts, and human myeloma (8226/Dox) cells, has been detected by the C219 monoclonal antibody (Kartner et al., 1985; Dalton et al., 1986) which was used to detect overexpression of this glycoprotein in HN-1/VP-2 cells in this study. This indicates that the molecular weight and antigenic structure of the P-glycoprotein are conserved between species and between different tissue types. Fine and co-workers (1985) have identified increased levels of a 20kD phosphoprotein in the plasma membranes of colchicine- and adriamycin-resistant MCF-7 cells which may be characteristic of the multidrug-resistant phenotype in breast carcinoma cells. Shen et al. (1986a) observed, along with an increase in the 170kD P-glycoprotein, a decrease in two membrane proteins of molecular weight 72 and 75kD in highly drug-resistant human KB carcinoma cells. In addition Beck et al. (1979) reported the disappearance of a 90kD membrane glycoprotein concomitant with the appearance of the P-glycoprotein in multidrug-resistant human

Table 7-1: Plasma membrane protein alterations in multidrug-resistant cells

Cell line	Selecting agent	Resistance Index	M. Wt. of altered protein (kD)	Reference
CHO/$CH^{R}C5$	Colchicine	180	170↑[a]	Juliano and Ling, (1976)
CHO/$DNR^{R}51$	Daunomycin	41	170↑	Kartner et al. (1983)
CEM/VLB_{100}	Vinblastine	420	170↑ 90↓[b]	Beck et al. (1979)
8226/Dox	Adriamycin	17	170↑	Dalton et al. (1986)
MCF-7/COL	Colchicine	30-100	20↑	Fine et al. (1985)
MCF-7/ADR	Adriamycin	10-100	20↑	Fine et al. (1985)
KB-C4	Colchicine	260	170↑ 75↓ 72↓	Shen et al. (1986a)
HN-1/VP-2	VP-16	4	170↑	This study

[a] - increased expression

[b] - decreased expression

leukaemic lymphoblasts. It would appear, then, that multiple alterations in the plasma membrane may be associated with multidrug resistance, although the most consistently reported modification is overexpression of the P-glycoprotein.

There is evidence to suggest that the P-glycoprotein has an intimate role in multidrug resistance: the degree of overexpression has been shown to correlate with the degree of resistance to the selecting agent (Kartner et al., 1985). Furthermore, transfection studies, where genomic DNA from highly drug-resistant cells has been transferred to drug-sensitive recipient cells, have shown that the recipient cells were multidrug-resistant and expressed increased levels of the P-glycoprotein (Debenham et al., 1982).

Studies of the molecular biology of the P-glycoprotein have indicated that it was conserved and encoded by a gene family, members of which were amplified in multidrug-resistant cells (Riordan et al., 1985). These studies by Riordan and colleagues identified a mRNA of approximately 4.7kb which was specific to multidrug-resistant cells. More recently Van der Blick and co-workers (1986) showed that five genes were amplified in the $CH^{R}C5$ cell line, one of which encoded for the P-glycoprotein, and the others may only have shared in the amplification due to the initial amplicon being so large. Gros and colleagues (1986), working independently with two multidrug-resistant Chinese hamster ovary cell lines, have demonstrated that over-expression of a single gene is sufficient to confer drug resistance, and that this gene encodes an approximate 5kb mRNA. Therefore it is

possible that the gene identified by Gros et al. (1986) may be identical to one of the members of the putative gene family reported by Riordan et al. (1985) and Van der Blick et al. (1986).

Shen et al. (1986b) recently reported that, at low levels of colchicine resistance, elevated expression of a 4.5kb mRNA occurred without amplification of the corresponding genomic sequences (the multidrug resistance locus, mdr1). However, during selection for increased levels of resistance, expression of this mRNA increased simultaneously with amplification of mdr1 DNA. These workers suggested that increased expression of mdr1 mRNA may be a common mechanism for multidrug resistance, and that activation of the mdr1 gene by mutations or epigenetic changes may precede its amplification during the development of drug resistance.

The mechanism whereby P-glycoprotein is overexpressed in HN-1/VP-2 cells is unresolved and may involve increased expression of the appropriate mRNA without amplification of the corresponding DNA sequences, which would correlate with the low degree of drug resistance exhibited by these cells. One problem with studies attempting to correlate the relative degree of amplification of mRNA or DNA with moderate levels of drug resistance is the limitation imposed by the sensitivity of the available probes. DNA sequences may be amplified, for example, two or three-fold in cells expressing low levels of drug resistance, but such levels could not be reliably detected.

In this chapter it was demonstrated that $CH^{R}C5$ cells overexpressed P-glycoprotein to a considerably greater degree than HN-1/VP-2 cells. This correlates with the Resistance Index of 180 shown by $CH^{R}C5$ cells to the selecting agent, colchicine (Juliano and Ling, 1976), compared to the 4-fold degree of resistance exhibited by HN-1/VP-2 cells to VP-16. Furthermore, this relationship between the degree of resistance and extent of overexpression of the P-glycoprotein agrees with previously reported studies, although overexpression of the P-glycoprotein had only been detected at a 7-fold level of resistance to the selecting agent (Kartner et al., 1985).

The possible functions that the P-glycoprotein may perform have been speculated to involve: modulating a property of the cell surface, such as membrane fluidity, thereby altering drug transport; acting as an efflux pump in order to transport cytotoxic drugs out of the cell; or having an active role in being phosphorylated and dephosphorylated (reviewed by Gerlach et al., 1986). Despite the fact that a precise function has yet to be assigned to this glycoprotein, Kartner et al. (1985) have proposed that it may eventually serve as a biochemical marker for clinically unresponsive tumours. Indeed, P-glycoprotein has been identified in tumour samples from certain patients with ovarian cancer or sarcoma (Bell et al., 1985a and b).

Overexpression of the P-glycoprotein was not detected in HN-1/DXR-11 cells. This observation was not entirely unexpected since these cells had not been selected by exposure to a

chemotherapeutic agent, as in all other instances where overexpression of the P-glycoprotein has been reported. Furthermore, HN-1/DXR-11 cells do not express the "classic" pattern of multidrug resistance (Bech-Hansen et al., 1976) i.e. they are resistant to vincristine but not to anthracyclines. Finally, they exhibited no patterns of altered drug uptake under the experimental conditions of this study (see Section 6-2). The possibility does arise, however, that these cells may overexpress P-glycoprotein to such a limited extent that it was undetectable with the probe used in this study. If HN-1/DXR-11 cells were conclusively demonstrated to have no amplification of the genes associated with multidrug resistance, this would endorse the possibility that clinical biopsies considered negative for P-glycoprotein expression may still exhibit drug resistance to a range of agents. If this is the case, therefore, effective screening for drug resistance in human tumours is likely to involve the use of multiple probes.

In summary, the studies reported in this chapter have shown that a multidrug-resistant human tumour cell line, HN-1/VP-2, which has been selected by exposure to VP-16 to exhibit a 4-fold level of resistance to this agent, overexpresses the P-glycoprotein, considered a marker for multidrug resistance (Kartner et al., 1985). This appears to provide the first demonstration that P-glycoprotein is overexpressed in human tumour cells which have been selected by exposure to VP-16, although the mechanisms involved are, as yet, unresolved. No overexpression of the P-glycoprotein was detected in HN-1/DXR-11 cells, which further indicates that, in this experimental

model system, at least one of the biochemical alterations associated with antitumour drug resistance is differentially expressed depending upon the agent employed to induce resistance.

CHAPTER 8 : GENERAL DISCUSSION AND SUMMARY

CONTENTS:

8-1: DEFINITION OF IN VITRO EXPERIMENTAL MODEL SYSTEMS 253

8-2: ANTITUMOUR DRUG RESISTANCE IN HUMAN TUMOUR CONTINUOUS CELL LINES 259

8-3: MECHANISMS INVOLVED IN THE ALTERED RESPONSES TO ANTITUMOUR DRUGS EXPRESSED BY TWO VP-16-RESISTANT HUMAN TUMOUR CONTINUOUS CELL LINES 273

8-4: FUTURE STUDIES 285

8-1: DEFINITION OF IN VITRO EXPERIMENTAL MODEL SYSTEMS

The *in vitro* model system employed for investigating mechanisms of antitumour drug resistance in these studies consisted of tumour cells derived from a human tumour biopsy, which had been adapted to continuous growth *in vitro* (Easty et al., 1981). In the last 5 years success rates in establishing continuous lines from human tumour material have increased with the result that more have become available for investigations of drug resistance. A number of advantages and disadvantages in the use of these systems are apparent, for example: (i) the lines are of a human origin, so that data obtained may be more analogous to clinical drug resistance than those from animal model systems, (ii) studies using a particular tumour type may be related back to the tissue of origin although, due to heterogeneity within the tumour, extrapolation of results derived from studies using only one or a few cells lines of a given tumour type should be avoided, (iii) the major disadvantage may be that the tumour cells established as lines may not be truly representative of malignant cell types found in tumours in patients, and (iv) tumour cells may adapt or change in long term culture and therefore it is considered preferable to use them over only a limited number of *in vitro* passages.

In an attempt to justify using *in vitro* tumour cell cultures to provide data applicable to clinical drug resistance it is worthwhile examining those *in vitro* findings where biochemical differences have been demonstrated between parental and

resistant cells which have been correlated subsequently with clinical experience, for example:

(i) the use of small-cell lung carcinoma cells derived directly from biopsies or from continuous cell lines have proved equally useful as screening systems for clinical correlations of drug sensitivity or resistance (Carney et al., 1983),

(ii) the P-glycoprotein, considered a marker for the multidrug-resistant phenotype (Kartner et al., 1985), has been detected in multidrug-resistant human leukaemia cell lines in vitro and in some tumour specimens obtained directly from patients with either ovarian carcinomas or sarcomas who had failed to respond to chemotherapy (Beck, 1983; Bell et al., 1985a and b), and

(iii) resistance to methotrexate, associated with gene amplification, has been identified in drug-resistant human tumour sublines (Morandi and Attardi, 1981), in continuous cell lines derived from squamous cell carcinoma of the head and neck (Frei et al., 1984) and in tumour cells from patients with small-cell lung carcinoma, acute leukaemia or ovarian adenocarcinoma who had been treated with methotrexate (Curt et al., 1983; Carman et al., 1984; Horns et al., 1984; Trent et al., 1984).

Therefore it appears that certain *in vitro* studies have some relevance to the *in vivo* situation, although only a few such correlative studies have been reported (as reviewed by Hill, 1986). When continuing these types of investigations it is essential to evaluate a range of tumour cell lines derived not only from the same tumour type but from a variety of types before being justified in drawing any overall conclusions.

Host factors may also be important in evaluating tumour response to chemotherapeutic agents. Host factors which include: decreased delivery of drug to the tumour due to vascular fibrosis or altered pharmacokinetics of the drug; decreased host tolerance due to prior treatment; or in the case of X-irradiation treatment, an increased proportion of hypoxic cells, have been implicated in the reduced responses to subsequent chemotherapy *in vivo* (Thigpen et al., 1981). In experimental model systems *in vitro* host factors are effectively removed. This presents certain advantages when attempting to identify specific biochemical mechanisms associated with drug resistance, although criticisms have been made as to the clinical relevance of these systems, since the derivation of human tumour continuous cell lines involves the selection of subpopulations of tumour cells which may not be representative of the *in situ* tumour. However, an important characteristic exhibited by tumour stem cells, that is the ability for indefinite replication under the appropriate conditions, is exploited in these *in vitro* studies and it may be assumed that these particular cells would contribute to the

growth and responses to antitumour agents exhibited by the tumour *in vivo*.

Further confidence in the relevance of drug resistance studies to the clinic may be achieved by using human tumour xenografts growing in immune suppressed mice (as reviewed by Houghton and Houghton, 1983). Another possibility is to work directly with biopsy specimens from treated and untreated patients, although this is frequently severely hampered by a shortage of material of adequate quality (Salmon et al., 1980). Therefore it appears logical to direct initial investigations towards *in vitro* model systems and, when useful or significant data are derived, these findings can then be evaluated further using human tumour xenografts or biopsy material directly.

The procedures used to assess cytotoxicity in this study may also be questioned for clinical relevance. Perhaps the most basic discrepancy is drug exposure time which may not correlate with *in vivo* conditions, where plasma concentrations of drugs administered as intravenous stat doses gradually decrease over a period of hours. However, in this *in vitro* study, where drug responses of parental and drug-resistant cell lines were compared, it appears justified to standardize drug exposure times. Therefore, for reasons discussed in Chapter 4, one, 24 and 72 h drug exposure times were used to compare responses of HN-1 cell lines. By employing a clonogenic assay, which has been shown to attain a degree of predictive chemosensitivity testing (Salmon et al., 1980), to test for drug sensitivity or

resistance, only reproductively viable cells were scored as survivors. These cells have been claimed to be analagous to the postulated 'stem cells' in vivo, i.e. those cells which individually have the capacity for regrowth if not killed. One of the major problems of predictive chemosensitivity testing on biopsies, namely that of disaggregating the tumour into single cells, is considerably reduced by the use of in vitro cell cultures.

Alternative cytotoxicity assays include those short-term procedures which estimate incorporation of radioactive precursors into DNA or RNA, or cell viability by vital dye exclusion (Volm et al., 1979). It would appear that, despite the gulf between the short-term inhibition of some biochemical processes and the eventual outcome in terms of cell death, clinical correlations using isotope incorporation assays may have limited predictive capabilities, although these in vitro correlates tend to be inferior to those achieved with clonogenic assays (Mattern and Volm, 1982).

These in vitro studies may also be discussed in terms of the levels of resistance exhibited by drug-resistant human tumour cell lines. Clinical quantitation of drug resistance is complicated by host factors such as drug tolerance and variable schedules of drug administration. The accepted definition of a clinical response is a reduction of at least 50% in the product of two perpendicular diameters of all measurable lesions. Measurement of response in terms of the change in volume of a solid tumour may be an indicator

of drug activity, but takes little account of the residual tumour cell population, which may contain a significant proportion of cells resistant to further therapy. The degree of resistance expressed by a non-responsive tumour can only be quantitated in terms of the maximum tolerated dose which can be administered to the host. However, this problem does not present itself in vitro where levels of drug resistance in human tumour continuous cell lines may frequently rise above 100-fold compared to parental cells (Beck, 1983; Conter and Beck, 1984; Akiyama et al., 1985; Harker and Sikic, 1985; Dalton et al., 1986) which obviously could not be quantitated in vivo. This is not to say that, by the spontaneous mutation model proposed by Goldie and Coldman (1979), highly drug-resistant mutants could not arise which would be co-selected upon subsequent drug exposure, along with mutants expressing low levels of drug resistance. However, the majority of in vitro drug resistance studies appear to maximize differences between parental and resistant cells in order to analyze biochemical alterations, and therefore do not take into account that different mechanisms of resistance may operate at the low levels of drug resistance which may occur in the patient. Studies now being instigated are necessary to establish whether different mechanisms of resistance do predominate at different levels of resistance. For example, recent reports have suggested that different mechanisms of methotrexate resistance may operate in human tumour cell lines selected for differing degrees of resistance to methotrexate (Alam et al., 1983; Frei et al., 1984). However, studies using highly resistant cloned cell populations are still important as they may yield specific information on precise

biochemical mechanisms of resistance, which can subsequently be studied in uncloned, moderately-resistant tumour cells. In this present study the resistant lines were not cloned, in order to simulate *in vitro* the conditions which might exist in an *in vivo* setting, where multiple resistant subpopulations of cells may be present in a tumour. Evidence has been presented which suggests that most malignant cell populations are characterized by genetic instability which may lead to the spontaneous generation of variant phenotypes or genotypes (Nowell, 1982). It may seem advantageous to use clonal populations when studying mechanisms of drug resistance, although in view of the vast number of cell doublings required to expand a clonal population to provide significant numbers for biochemical analyses, it is likely that a certain degree of heterogeneity will have again been introduced.

Therefore, *in vitro* studies which are designed to investigate mechanisms of drug resistance at moderate levels of resistance using uncloned cell populations may yield valuable information on clinically non-responsive tumours.

8-2: ANTITUMOUR DRUG RESISTANCE IN HUMAN TUMOUR CONTINUOUS CELL LINES

The studies reported in this thesis were designed to identify a biochemical basis for the clinically-observed reduced response rates to chemotherapy in patients previously treated with either chemotherapy or radiotherapy. In order to address this problem, VP-16-resistant human tumour cell lines were developed by

continuous drug exposure, where the degree of resistance was relatively low and analogous to the VP-16-resistant cell line previously derived by fractionated X-irradiation treatment of the same parental cells (Hill and Bellamy, 1984). Furthermore, the patterns of responses exhibited by these independently-derived VP-16-resistant cell lines, and some of the associated mechanisms, were compared in order to determine whether they differed depending upon the agent employed to induce resistance.

At the outset of this study there were few reports of drug-resistant tumour cell lines which had been derived by specific exposure to either VP-16 or VM-26 (Seeber et al., 1982; Gupta, 1983) and none of these related to human tumour systems. Therefore it was necessary first to establish a relevant experimental model system for investigating the development and expression of VP-16 resistance, with the ultimate aim of defining effective means of overcoming or circumventing clinical resistance to this agent. The first detailed description of the selection and characterization of drug-resistant human tumour cell lines derived by exposure to VP-16 is provided in Chapter 3. Following continuous exposure to VP-16 for 11 passages *in vitro*, the growth characteristics of the two VP-16-treated established sublines showed no significant differences from HN-1 parental cells. This allowed a direct comparison of their responses to a range of antitumour agents using identical cell culture conditions and drug exposure times. Analyses of the responses of these two sublines to VP-16 showed that they exhibited an approximate 4-fold level of resistance compared to HN-1 parental cells (Chapter

4). This degree of resistance to the selecting agent is low compared to most other drug-resistant tumour cell lines derived by *in vitro* exposure to, for example: adriamycin (50-fold) (Harker and Sikic, 1985); colchicine (180-fold) (Bech-Hansen et al., 1976); vinblastine (420-fold) (Beck, 1983); vincristine (5- to 600-fold) (Conter and Beck, 1984) or VM-26 (50-fold, Danks and Beck, 1983; and >100-fold, Lee and Roberts, 1984a). It is, however, comparable to the approximate 6- to 12-fold degree of resistance expressed by other tumour cell lines which have been derived by exposure to VP-16 (Seeber et al., 1982; Long et al., 1985b). Drug-resistant tumour cell lines which exhibit high levels of resistance to the selecting agent (Bech-Hansen et al., 1976; Beck, 1983; Gupta, 1983; Conter and Beck, 1984; Harker and Sikic, 1985; Fairchild et al., 1986; Pommier et al., 1986a) generally express higher levels of cross resistance to VP-16 (ranging from 8- to 175-fold) than those cell lines derived by specific exposure to VP-16 (Seeber et al., 1982; Long et al., 1985b; this study). This may be the result of: (i) differences between the model systems employed, (ii) differences in the manner in which drug-resistant cell lines were derived, or (iii) differences in the mode of cytotoxicity of the selecting agent. A combination of all these possibilities is the most likely explanation for this disparity in levels of drug resistance since: (i) Both animal and human tumour model systems have been used to select drug-resistant lines following exposure to VP-16 or a range of other antitumour agents, although the same parental cell line has not been used to test whether it is possible to develop high levels of resistance with other agents, but only relatively low levels with VP-16. (ii) In certain studies tumour

cells have been mutagenized and subsequently selected for resistance with, for example, colchicine (Bech-Hansen et al., 1976; Akiyama et al., 1985) or VM-26 (Gupta, 1983). This approach has yet to be employed in selecting VP-16-resistant cells. However, the procedure whereby drug-resistant human tumour cells have been selected by exposure of uncloned parental populations to increasing concentrations of adriamycin (Harker and Sikic, 1985; Fairchild et al., 1986), vinblastine (Beck et al., 1979), vincristine (Conter and Beck, 1984), VM-26 (Danks and Beck, 1983) or VP-16 (Long et al., 1985b; this study) has led to the conclusion that the degree of resistance established was lowest when the selecting agent was VP-16. This suggests therefore that differences in levels of drug resistance were unlikely to be due solely to the selection method adopted. (iii) Selection of drug-resistant cells may be easier with one antitumour agent than another. There are many reports in the literature of drug-resistant tumour cell lines obtained by selection, for example, with the anthracyclines or Vinca alkaloids. However, relatively few lines expressing resistance to cisplatin or the nitrosoureas have been described. In this study VP-16-resistant cells expressing a modest order of resistance were readily obtained, although it was not possible to select further for highly resistant human tumour cells as viable populations could not be maintained in higher drug concentrations. VP-16 appears to be an agent to which drug resistance readily develops, although the reported degree of resistance to this selecting agent is generally less than 15-fold. However, as in this study, Long and colleagues (1985b) may have intentionally set out to select VP-16-resistant tumour cells which

exhibited moderate levels of resistance. It therefore remains to be firmly extablished whether it is possible to develop highly VP-16-resistant tumour cell lines, comparable with the 180-fold resistance to colchicine reported in the Chinese hamster ovary $CH^{R}C5$ cells (Bech-Hansen et al., 1976) or the 420-fold level of vinblastine resistance reported in human leukaemic lymphoblasts (Beck, 1983).

These human tumour cells selected for resistance to VP-16, HN-1/VP-2, were shown to express cross resistance to a variety of structurally unrelated antitumour agents (Chapter 4) consistent with the multidrug-resistant phenotype (Bech-Hansen et al., 1976). The highest level of resistance was exhibited to the selecting agent in agreement with other in vitro studies (Bech-Hansen et al., 1976; Salles et al., 1982; Gupta, 1983; Conter and Beck, 1984; Akiyiama et al., 1985;). However this present study appears to provide the first demonstration that the multidrug-resistant phenotype is expressed at levels of resistance to the selecting agent of less than 5-fold. Frei (1982) has suggested that, because of the steep dose response curve of antitumour agents, a 3- to 20-fold order of drug resistance in the clinic would lead to treatment failure. Therefore, if the multidrug-resistant phenotype emerged only at high levels of resistance to the selecting agent it may not be frequently observed clinically. This was one of the conclusions made by Conter and Beck (1984) in human leukaemic lymphoblasts (CEM cells), selected for vincristine resistance, since expression of the multidrug-resistant phenotype emerged only at high levels of resistance (>50-fold) to the selecting agent. However data presented here for the HN-1/VP-2 cells

appear to indicate that multidrug resistance is a clinically relevant phenomenon. Furthermore, the 170kD membrane protein (P-glycoprotein), considered a 'marker' of the multidrug-resistant phenotype, has been detected in tumour samples from certain patients with ovarian cancer or sarcoma (Bell et al., 1985a and b), and evidence presented in Chapter 7 suggests that this 'marker' protein is also overexpressed in HN-1/VP-2 cells.

The patterns of responses exhibited by two independently-derived VP-16-resistant HN-1 cell lines to a range of antitumour agents were compared in Chapter 5. These cell lines had been derived either by continuous exposure to VP-16, HN-1/VP-2 (see Chapter 3), or by fractionated X-irradiation treatment, HN-1/DXR-11 (Hill and Bellamy, 1984). A comparison of the responses shown by these two cell lines to VP-16 revealed that they expressed a similar order of resistance of approximately 4-fold. HN-1/VP-2 cells showed cross resistance to adriamycin, daunomycin and vincristine, and marginal cross resistance to vinblastine and cisplatin. HN-1/DXR-11 cells also proved cross-resistant to vincristine and marginally cross-resistant to vinblastine, with the same order of resistance shown by HN-1/VP-2 cells to these agents. The finding of a lack of full cross resistance between vincristine and vinblastine in both VP-16-resistant cell lines is in agreement with studies by Conter and Beck (1984) using a vincristine-resistant human leukaemic lymphoblast cell line, although the authors explained that this lack of full cross resistance was due to the parental cells being inherently less sensitive to vinblastine than vincristine. Studies where adriamycin

or colchicine were employed to select resistant cells have also shown higher degrees of resistance to vincristine than vinblastine (Fojo et al., 1985; Harker and Sikic, 1985; Yamaguchi et al., 1986), although in these reports there were no indications that the parental cells were less sensitive to vinblastine than vincristine, which appears to apply to HN-1 cells (see Table 4-1). However, these HN-1/DXR-11 cells showed very different responses, from the HN-1/VP-2 cells, to the other antitumour drugs tested, since they had unaltered responses to adriamycin and daunomycin, and showed marginal collateral sensitivity to cisplatin.

The present results therefore provide evidence that, in this experimental model system, patterns of responses expressed by two independently-derived VP-16-resistant human tumour cell lines to a range of antitumour agents differ depending upon the manner in which drug resistance was induced. These observations also confirm that patterns of multidrug resistance may be expressed after selection of drug-resistant human tumour cells with VP-16, and that X-irradiation-induced interactions may result in the expression of resistance to VP-16 and vincristine.

Attempts were next made to identify any biochemical alterations which may be associated with the differential patterns of drug responses exhibited by HN-1/VP-2 and HN-1/DXR-11 cells (Chapters 6 and 7). Mechanisms which have been associated with tumour cell resistance to VP-16 have included: reduced accumulation of drug associated with an energy-dependent efflux pump in the <u>in vivo</u> study

by Seeber et al. (1982); drug detoxifiation associated with enhanced activities of glutathione-related enzymes (Tulpule et al., 1986); and a reduction of VP-16-induced DNA damage which may be associated with altered activity or mutant forms of DNA topoisomerase II (Long et al., 1986; Pommier et al., 1986a and b; W. Ross, personal communication). Mechanisms of resistance to anthracyclines and Vinca alkaloids, however, are mainly thought to involve alterations in cellular uptake and/or efflux of drug (Hill and Whelan, 1982; Tsuruo et al., 1982; Beck, 1984; Curt et al., 1984; Harker and Sikic, 1985; Dalton et al., 1986), frequently associated with overexpression of the P-glycoprotein (Kartner et al., 1985). Since the anthracyclines appear to have multiple potential sites of action, other mechanisms of resistance have also been proposed: these include a decrease in the activity of enzymes which may activate the drug, an increase in cellular glutathione which may scavenge and detoxify free radical species, a decreased affinity of the cell membrane for drug (reviewed by Curt et al., 1984) and, since adriamycin has been shown to inhibit mammalian DNA topoisomerase II (Tewey et al., 1984), alterations in topoisomerase II. Similarly for the Vinca alkaloids, which are thought to exert cytotoxicity by an inhibition of tubulin polymerization brought about by binding to the β-subunit of tubulin (Bender and Chabner, 1982), alternative mechanisms of resistance include alterations in tubulin subunit composition (Cabral et al., 1980; Gupta et al., 1982).

Therefore, in this study the following parameters were investigated: (i) drug uptake and efflux characteristics, (ii) levels of reduced glutathione and activities of glutathione-related enzymes,

(iii) VP-16-induced DNA single-strand breakage and repair, and (iv) expression of the 170kD P-glycoprotein.

The results showed that selection of VP-16-resistant HN-1 cells by continuous exposure to VP-16 has also selected for the expression of impaired uptake and retention of structurally unrelated antitumour agents, to which HN-1/VP-2 cells were cross-resistant (see Section 6-2). Despite the finding that these cells had unaltered uptake and retention of the selecting agent, VP-16, compared to parental cells, reduced accumulation of [^{3}H]-daunomycin, [^{3}H]-vinblastine and [^{3}H]-vincristine was demonstrated. In contrast, HN-1/DXR-11 cells exhibited no alterations in drug uptake or efflux characteristics compared to HN-1 parental cells. It can therefore be concluded that, for vincristine and vinblastine, mechanisms of resistance may differ depending upon the manner in which resistance was induced. Gupta (1983) showed that Chinese hamster ovary cells selected for VM-26 resistance, which expressed cross resistance to daunomycin, exhibited reduced accumulation of [^{3}H]-daunomycin compared to parental cells. However, Glisson et al., (1986a and b) later showed in these cells, which were cross-resistant to VP-16 and had unaltered uptake of this agent, that daunomycin uptake at higher extracellular concentrations was not modified. In a similar study, Chinese hamster lung cells selected for resistance to 9-hydroxy-ellipticine expressed impaired uptake of adriamycin, an agent to which they were cross resistant, but no impairment with the selecting agent (Pommier et al., 1986a). These workers also demonstrated that the biochemical alterations associated with the expression of cross

resistance to VP-16 by these cells occurred independently of differences in VP-16 uptake through the plasma membrane. The study of Seeber et al. (1982), using VP-16-resistant Ehrlich ascites tumour cells derived *in vivo*, has provided the only published evidence of reduced accumulation of [^{3}H]-VP-16, although the multidrug-resistant Chinese hamster ovary cells, CH^{R}C5, have also now been shown to have impaired VP-16 uptake (Chapter 6). However, these cells have been shown to be highly resistant (180-fold) to the selecting agent, colchicine (Bech-Hansen et al., 1976) and 8-fold cross-resistant to VP-16 (Chapter 6). These studies indicate that different mechanisms of resistance to VP-16 may be expressed depending not only upon the antitumour agent used to select resistant cells, but also, apparently on the degree of resistance expressed to the selecting agent. *In vitro* studies have therefore predominantly provided evidence indicating that resistance to VP-16 is mediated by factors which are independent of differences in drug uptake through the plasma membrane (Danks and Beck, 1983; Long et al., 1985b; Glisson et al., 1986a; Pommier et al., 1986a) and this has been confirmed in this present study. The finding that selection of a VP-16-resistant cell population has also selected cells which exhibited transport alterations for structurally unrelated antitumour agents, but not the selecting agent, raises the possibility that multiple mechanisms of resistance operate in these resistant cells. Furthermore, HN-1/VP-2 cells overexpressed the P-glycoprotein compared to HN-1 parental and HN-1/DXR-11 cells, which is consistent with the reported association between amplification of this protein and reduced drug accumulation in multidrug-resistant cells (Kartner et al., 1985). This difference

between expression levels of P-glycoprotein between HN-1/VP-2 and HN-1/DXR-11 cells therefore strengthens the hypothesis that different mechanisms of resistance may operate in resistant cell lines derived by independent procedures.

Since [^{3}H]-VP-16 uptake was unaltered in either of the differently-derived VP-16-resistant HN-1 sublines, the possibility that resistance to this agent may involve drug detoxification was next investigated. Levels of reduced glutathione and the activities of glutathione S-transferase, glutathione reductase and glutathione peroxidase were not found to differ significantly between parental and HN-1/VP-2 cells (Chapter 6). The role that glutathione plays in VP-16 resistance has yet to be defined. It has been postulated that this intracellular detoxification system could be involved in the inactivation of VP-16 or of any free radical intermediates formed by VP-16 which have been shown to occur <u>in vitro</u> (Sinha and Myers, 1984). Under these conditions elevated activity could confer VP-16 resistance. HN-1/DXR-11 cells, although containing unaltered levels of reduced glutathione and activities of glutathione reductase and glutathione peroxidase, showed a slight elevation in glutathione S-transferase activity compared to parental cells. A human breast carcinoma (MCF-7) cell line selected for a high level of resistance to adriamycin and exhibiting cross resistance to VP-16 showed multiple biochemical alterations including elevated glutathione peroxidase and glutathione S-transferase activities (Tulpule et al., 1986). The significance of these changes in terms of VP-16 resistance remains unclear. It is possible, therefore, that a role

may yet be found for the slight, but significant, elevation of glutathione S-transferase activity in HN-1/DXR-11 cells, although it appears unlikely that the magnitude of this elevation would totally account for the significant level of VP-16 resistance expressed. Recent studies have shown that inhibition of γ-glutamylcysteine synthetase by buthionine sulphoximine leads to depletion of cellular glutathione and potentiation of the cytotoxic effects of adriamycin, melphalan and cisplatin in human ovarian carcinoma cell lines (Hamilton et al., 1985). This agent is currently under consideration for clinical trials (Ozols and Cowan, 1986). A role for the slight elevation of glutathione S-transferase activity exhibited by HN-1/DXR-11 cells in the mechanisms of resistance to VP-16 may yet be postulated, if buthionine sulphoximine was demonstrated to potentiate VP-16 cytotoxicity.

The major cytotoxic lesion induced by VP-16 is generally considered to be protein-associated DNA strand breakage mediated via a 'poisoning' of DNA topoisomerase II (see Section 1-2). The expression of resistance to VP-16 by mammalian tumour cell lines has mainly been attributed to alterations of VP-16-induced DNA lesions, independent of any differences in cellular drug uptake (Glisson et al., 1986a; Long et al., 1986; Pommier et al., 1986a). Furthermore, rates of repair of VP-16-induced DNA single-strand breakage, although rapid, did not differ significantly between a series of human lung carcinoma cell lines exhibiting a range of responses to VP-16 (Long et al., 1986). Similarly, in HN-1 cell lines the extent of VP-16-induced DNA single-strand breakage correlated with the expression of

VP-16 resistance, with a reduction of these lesions in HN-1/VP-2 and HN-1/DXR-11 cells compared to parental cells after exposure to equimolar concentrations of VP-16 for 1 h. Rates of repair of VP-16-induced DNA single-strand breaks were not significantly different between the three cell lines. The relationships between VP-16-induced cytotoxicity, DNA single-strand breakage and DNA double-strand breakage were analyzed by Long et al. (1986) in a series of human lung carcinoma cells. A greater degree of correlation existed between cytotoxicity and DNA double-strand breakage than cytotoxicity and DNA single-strand breakage, indicating that VP-16-induced DNA double-strand breakage was likely to be the major cytotoxic lesion. By inference, in Chapter 6, it was suggested that this may also be the case with HN-1 cells, although confirmation of this awaits quantitation of VP-16-induced DNA double-strand breakage in HN-1 cells by the technique of neutral elution. Such studies may reveal differences not only between parental and resistant cells, but also differences in dose response profiles between the two independently-derived VP-16-resistant cell lines.

In discussing the spectrum of drug responses exhibited by these differently-derived VP-16-resistant cell lines, and the mechanisms associated with their altered drug responses, it is important to attempt to relate these to the clinical treatment of human cancers. It has been shown that prior radiotherapy or chemotherapy may significantly reduce clinical response rates to subsequent chemotherapy in a number of tumour types, including squamous cell carcinoma of the head and neck (Price and Hill, 1981;

Wolf and Chretien, 1981). A possible explanation for these altered response rates, which is supported by results presented in this thesis and by Hill and Bellamy (1984), is the outgrowth of chemotherapy-resistant tumour cells. These results indicate that prior VP-16 treatment may select multidrug-resistant cells which exhibit altered uptake of structurally unrelated antitumour agents and overexpress the P-glycoprotein, consistent with other multidrug-resistant tumour cell lines derived in vitro (Kartner et al., 1985). Furthermore it is possible that mechanisms of drug resistance expressed by differently-derived resistant cells may differ and consequently treatments designed to circumvent or overcome resistance may need to take into account prior therapy.

Although VP-16 is not considered an active agent for the treatment of squamous cell carcinoma of the head and neck (O'Dwyer et al., 1985) it is important to remember that most Phase II clinical studies involve heavily pretreated patients and so single-agent activity may be underestimated. However, vincristine is incorporated into combination chemotherapy regimens for the treatment of head and neck cancer (Wolf and Chretien, 1981). The main intention of this study though was to define an experimental model system in order to study the interactions between X-irradiation and VP-16. The level of VP-16 resistance expressed after fractionated X-irradiation treatment defined the extent of resistance to be established by drug exposure. This approximate 4-fold order of resistance was considered to fall within the level that might be expressed in the clinic (Frei, 1982).

8-3: MECHANISMS INVOLVED IN THE ALTERED RESPONSES TO ANTITUMOUR DRUGS EXPRESSED BY TWO VP-16-RESISTANT HUMAN TUMOUR CONTINUOUS CELL LINES

Selection of a multidrug-resistant cell line, HN-1/VP-2, by continuous exposure to VP-16 may have followed one of two possible pathways:

(i) cells may have been mutagenized by VP-16, a known mutagen (Singh and Gupta, 1983), and VP-16-resistant mutants subsequently selected, or

(ii) VP-16-resistant cells may have already existed in the parental population, according to the spontaneous mutation theory of Goldie and Coldman (1984), and these were preferentially selected by continuous exposure to VP-16.

In both cases the selection pressure exerted by VP-16 would have confered a survival advantage upon resistant cells.

Multidrug-resistant HN-1/VP-2 cells sustained less VP-16-induced DNA single-strand breakage than parental cells, which did not appear to be associated with reduced drug uptake, enhanced drug efflux, or elevated activities of glutathione-related enzymes. Glisson et al. (1986a and b) have proposed that multidrug resistance may be acquired by a qualitative change in DNA topoisomerase II in VM-26-resistant Chinese hamster ovary cells, since the agents to which significant cross resistance was exhibited were restricted to those which have been suggested to exert their cytotoxic action by

inhibiting DNA topoisomerase II. Indeed, these workers have recently identified a mutant form of DNA topoisomerase II in these multidrug-resistant Chinese hamster ovary cells (W. Ross, personal communication). It is possible that resistance to VP-16, adriamycin and daunomycin shown by HN-1/VP-2 cells is also associated with alterations of DNA topoisomerase II activity, since these agents have all been proposed to exert their lethal effects by an inhibition of this enzyme (Chen et al., 1984; Tewey et al., 1984). However, multiple mechanisms of resistance must be expressed by HN-1/VP-2 cells since cross resistance to vincristine and daunomycin, and marginal cross resistance to vinblastine was associated with reduced drug uptake and overexpression of the P-glycoprotein. The concept of multiple mechanisms operating is consistent with data from Pommier et al. (1986a) who reported that mechanisms of resistance to VP-16, m-AMSA and 2-methyl-9-hydroxyellipticine, expressed by a subline of Chinese hamster lung cells, were dependent upon factors contained in the nuclei of resistant cells, whereas adriamycin and actinomycin D resistance was associated with reduced cellular uptake of drug (Salles et al., 1982; Pommier et al., 1986a).

The various proposed and documented mechanisms of resistance to anthracyclines and Vinca alkaloids have been discussed earlier (see Section 8-2). These are mainly considered to involve alterations in cellular drug accumulation associated with enhanced expression of the P-glycoprotein (reviewed by Curt et al., 1984; Kartner et al., 1985). To support the proposal that resistance to vincristine, adriamycin and daunomycin, and marginal resistance to

vinblastine in HN-1/VP-2 cells was brought about by reduced intracellular drug accumulation, data were presented (Table 6-3) which indicated that the relationship between the degree of resistance and extent of reduction in steady state accumulation of these drugs in HN-1/VP-2 cells was similar to that reported with other multidrug-resistant cell lines. Various alterations in the expression of plasma membrane proteins have been documented in multidrug-resistant cell lines (see Table 7-1). However, the most consistent observation has been overexpression of the P-glycoprotein associated with reduced cellular drug accumulation (reviewed by Gerlach et al., 1986). HN-1/VP-2 cells were also shown to overexpress the P-glycoprotein (Chapter 7). These data further support the concept that reduced cellular drug accumulation, associated with overexpression of the P-glycoprotein, confers the multidrug-resistant phenotype on HN-1/VP-2 cells.

Previously described multidrug-resistant tumour cell lines which express P-glycoprotein have been derived either by exposure to colchicine, the Vinca alkaloids or the anthracyclines (reviewed by Gerlach et al., 1986). Therefore this is the first demonstration of overexpression of P-glycoprotein in human tumour cells selected for resistance to VP-16.

Data presented here favour the proposal that multiple mechanisms of resistance are involved in this VP-16-resistant cell line. It remains to be established whether all the cells express multiple mechanisms of resistance, including alterations in DNA topoisomerase II activity and overexpresssion of the P-glycoprotein.

Support for this contention comes from studies by Roberts et al. (1986) who proposed that VM-26-resistance, expressed by clonal populations of murine leukaemia (L1210) cells, was multifaceted and probably resulted from a summation of small changes exhibited by drug-resistant cells. Alternatively, different sub-sets of drug-resistant populations may be present within the HN-1/VP-2 cell line, including not only cells expressing multiple mechanisms, but also cells which are resistant to VP-16 only by virtue of either an alteration in DNA topoisomerase II activity or overexpression of P-glycoprotein. It is difficult to distinguish between these alternatives unless detailed analysis were possible on individual cells.

A precise function for the P-glycoprotein has yet to be defined although it has been proposed that it may: modulate the permeability of the plasma membrane, act as an efflux pump, or play an active role in being phosphorylated and dephosphorylated (reviewed by Gerlach et al., 1986). However, it appears to be intimately involved in the expression of multidrug resistance, for example: in a variety of multidrug-resistant tumour cell lines the degree of P-glycoprotein overexpression correlated with the level of drug resistance (Kartner et al., 1985). Furthermore, transfection experiments have shown that, by transferring genomic DNA from highly resistant cells to drug-sensitive recipient cells, the multidrug-resistant phenotype emerged along with overexpression of the P-glycoprotein (Debenham et al., 1982). Analyses of multidrug-resistant cells derived from different mammalian species have also

indicated that homologous P-glycoproteins are expressed (Kartner et al., 1985).

Molecular analyses of the P-glycoprotein originally showed that there may be a number of different genes that code for P-glycoprotein, however it was not known which of the different members of the putative P-glycoprotein gene family were transcribed, or whether they encoded functionally distinct isotypes of P-glycoprotein (Riordan et al., 1985). More recently, Gros and colleagues (1986) have demonstrated that overexpression of a single gene is sufficient to confer drug resistance. These workers also suggested that, since the cDNA was isolated from a drug-sensitive cell, mutations in the sequence of the gene would not be required to produce a multidrug-resistant phenotype. Whatever the precise mechanism by which P-glycoprotein is amplified it would appear that by selection of drug-resistant cells with VP-16, in which the mechanisms of resistance to VP-16 may involve alterations in DNA topoisomerase II activity (Long et al., 1985b), the presence of a modulating factor which may influence drug-topoisomerase II interactions (Pommier et al., 1986b), or a mutant form of DNA topoisomerase II (W. Ross, personal communication), a process has been initiated which also results in overexpression of the P-glycoprotein.

The first possible explanation for this proposed co-selection of drug resistance mechanisms may be due to the different transport mechanisms which are thought to predominate for

VP-16 and the Vinca alkaloids and anthracyclines. Allen (1978) using murine leukaemia (L1210) cells showed that VP-16 uptake occurred by passive diffusion whereas efflux, which was temperature-dependent, probably occurred via an energy-dependent pathway. Carrier-mediated and passive diffusion have been proposed for the process of Vinca alkaloid uptake into cells (Carlsen et al., 1976; Sirotnak et al., 1986) and both energy-dependent efflux pumps and passive efflux of unbound drug have been postulated for the efflux process (Skovsgaard, 1978a; Beck, 1984). Furthermore, whilst VP-16 is loosely bound within the cell and rapidly washed out (Yalowich and Ross, 1985) tumour cells may retain up to 50% of the Vinca alkaloids or anthracyclines (Inaba and Johnson, 1978; Beck, 1983). These fundamental differences, if they applied to HN-1 cells, may indicate that reduced uptake or binding of VP-16, or enhanced efflux, may not be a general property exhibited by multidrug-resistant cells. This hypothesis is, however, not consistent with the results in Chapter 6, where multidrug-resistant Chinese hamster ovary cells were shown to exhibit reduced uptake of VP-16. However, these cells were highly resistant to the selecting agent, colchicine, (180-fold) (Bech-Hansen et al., 1976) and, as mentioned earlier, it is possible that different mechanisms operate depending on the level of resistance expressed. This matter may be resolved if it were possible to select highly drug-resistant cells from the HN-1/VP-2 population by further exposure to VP-16 and then re-examine their drug uptake and efflux characteristics to determine whether VP-16 accumulation was impaired at higher levels of resistance. It is therefore proposed that at low levels of drug resistance alterations in DNA topoisomerase II

activity confer VP-16 resistance, although by a process of co-selection these cells exhibit multidrug-resistance and over-expression of P-glycoprotein. Whereas at higher levels of VP-16 resistance there is impaired transport of VP-16 associated with a greater degree of amplification of the P-glycoprotein. The fact that the highly resistant $CH^{R}C5$ cells overexpressed the P-glycoprotein to a greater degree than HN-1/VP-2 cells (see Chapter 7) is consistent with this hypothesis.

A second possible explanation for co-selection of drug-resistance mechanisms may be derived from results of studies of heat-shock protein synthesis in eukaryotic cells. This series of proteins is transiently expressed by prokaryotic or eukaryotic cells in response to various conditions of stress, including heat (Bienz, 1985). By analogy, overexpression of the P-glycoprotein by HN-1/VP-2 cells could be a cellular response to the stress exerted upon them by exposure to VP-16. Furthermore, Rowe and colleagues (1986) have recently suggested that the inhibition of DNA topoisomerase II by VM-26 may lead to heat-shock transcription on Drosophila heat shock chromatin. However, the analogy ends there since heat-shock proteins are only transiently expressed while the cell recovers, whereas P-glycoprotein expression remains stable, along with the multidrug-resistant phenotype. Furthermore, heat-shock proteins fall into two categories, of molecular weight 15-30kD and 68-100kD (Nover and Scharf, 1984) whereas the P-glycoprotein is considerable larger (170kD). This second explanation, although unsubstantiated, could explain how exposure of HN-1 cells to VP-16 selected a resistant population which accumulated less VP-16-induced DNA single-strand

breakage than parental cells, possibly due to alterations in DNA topoisomerase II, but also selected for or induced overexpression of the P-glycoprotein in a protective response, which is associated with multidrug resistance.

A third explanation for co-expression of VP-16 resistance and multidrug resistance in HN-1/VP-2 cells to be considered is that amplification of DNA topoisomerase II in these cells may be associated with co-amplification of P-glycoprotein genes. However one of the unusual properties of DNA topoisomerase II is that, unlike other targets of antitumour drugs, elevated activity of this enzyme is generally considered to lead to greater cytotoxicity, or sensitivity, resulting from a greater number of drug-enzyme-DNA interactions (Ross, 1985). This proposal is therefore unlikely.

Mechanisms associated with the altered responses to antitumour agents exhibited by HN-1/DXR-11 cells appear to be rather more complex in nature than those which may exist in HN-1/VP-2 cells. It has been proposed that by treating the parental cell population with 11 fractions of X-irradiation, each of 4.5Gy, a total of between 248 and 495 possible mutants may have been induced, assuming an average mutation frequency of $5x10^{-7}$ mutants per viable cell per 0.01Gy (Bellamy, 1982). In order to survive, these mutants would have to be subjected to positive selection pressure, either by selection of resistant cells with drug or X-irradiation, or under normal conditions of growth exhibit a survival advantage over parental non-irradiated cells. However, subsequent to the

fractionated X-irradiation procedure selection of drug-resistant mutants with VP-16 or vincristine did not take place. Furthermore the resultant cell line, HN-1/DXR-11, exhibited significant sensitivity to X-irradiation compared to parental cells (Hill and Bellamy, 1984) which, upon treatment with further X-irradiation, would have resulted in negative selection pressure. A more likely proposition may be that specific radiation-induced changes may have occurred in these cells which, upon repopulation, overgrew the parental cells. Support for this hypothesis comes from studies analyzing the subsequent drug responses of a series of animal and human tumour cell lines which had been treated in vitro with fractionated X-irradiation (Hill et al., in press). In all of the lines tested, fractionated X-irradiation treatment induced resistance to VP-16 and vincristine and an unaltered response to adriamycin. These data argue against a random process of mutation induction in HN-1 cells and instead support the hypothesis that specific X-irradiation-induced alterations may have occurred. An alternative possibility is that fractionated X-irradiation treatment has mutagenized HN-1 cells, leaving a heterogeneous mixture of mutants in the HN-1/DXR-11 population. Highly drug-resistant mutants may be present in this population but their antitumour drug responses detected using cytotoxicity assays may be masked by the predominantly sensitive population. However, this mutant population would have to co-exist with the sensitive cells with neither experiencing a survival advantage, since resistance in these HN-/DXR-11 cells has remained stable in the absence of selection pressure (Hill and Bellamy, 1984).

X-irradiation-induced DNA damage is a random process and a dose of 4.5Gy would cause a large number of single- and double-strand breaks such that it is unlikely that the cell would survive without having repaired considerable damage to its DNA (Elkind, 1984). However, studies using Chinese hamster ovary cells, which showed similarities to the method described to develop the HN-1/DXR-11 cell line, have indicated that neither single-strand nor double-strand DNA breaks were propagated in the progeny of the survivors of fractionated X-irradiation treatment, although the survivors were marginally less sensitive to X-irradiation (Elkind, 1984). Therefore it is possible that, although X-irradiation-induced DNA damage is a random event, mis-repair may have occurred at specific sites giving rise to mutations. There is no body of evidence to suggest that one particular region of the genome may be more sensitive to mutation than another, although studies by Sagi et al. (1978) have indicated that X-irradiation-induced chromosomal breakage, 24 h after treatment, was a non-random event with certain 'hot-spots' for chromosome breakage observed. This observation raises the possibility, therefore, that successive insults with X-irradiation may have induced mutations in genes encoding DNA topoisomerase II and β-tubulin leading to a reduction in VP-16-induced DNA damage and vincristine-induced cytotoxicity.

The drug transport characteristics of HN-1 cells were not altered following fractionated X-irradiation exposure, and no overexpression of the P-glycoprotein was detected in HN-1/DXR-11 cells. VP-16-induced DNA damage, however, is markedly reduced

compared to parental cells and this may be associated with the expression of VP-16 resistance. In the study by Gupta (1983), Chinese hamster ovary cells were mutagenized with ethylmethanesulphonate prior to selection of resistant cells with VM-26. Cross resistance was exhibited by these cells to the anthracyclines, with partial resistance to vinblastine and collateral sensitivity to cisplatin, although their response to vincristine was not reported. More recently, the reduction in VP-16-induced DNA strand breakage exhibited by these cells compared to parental cells has been attributed to a mutant form of DNA topoisomerase II (W. Ross, personal communication). Therefore, by analogy, HN-1/DXR-11 cells, which are resistant to VP-16 and VM-26, partially resistant to vinblastine and collaterally sensitive to cisplatin, may also be found to express a mutant form of DNA topoisomerase II. However, the divergence of the Chinese hamster ovary mutants and HN-1/DXR-11 cells with respect to their responses to the anthracyclines may indicate a different point of mutation on the enzyme. Currently, very little is known about the sites of drug-topoisomerase II interactions in human tumour cells, although it may be surmised that VP-16 and adriamycin bind to different sites since VP-16 does not intercalate with DNA (Ross et al., 1984) whereas the anthracyclines are known to bind tightly to DNA (Myers, 1982).

Tumour cell resistance to the Vinca alkaloids appears mainly to involve alterations in drug uptake and retention (Beck, 1984). The HN-1/DXR-11 cells are therefore unusual in exhibiting no alterations in these parameters. Vinca alkaloid-resistant mutants of

Chinese hamster ovary cells which were mutagenized with ethylmethanesulphonate and subsequently selected for colchicine resistance had an altered β-tubulin (Cabral et al., 1980), whilst Chinese hamster ovary cells which were mutagenized with ethylmethanesulphonate and selected for podophyllotoxin resistance were cross-resistant to vinblastine and had an altered microtubule-associated protein (Gupta et al., 1982). However, if tubulin is to be implicated in the mechanism of resistance to vincristine expressed by these HN-1/DXR-11 cells, their lack of cross-resistance to podophyllotoxin would have to be explained, although podophyllotoxin and the Vinca alkaloids are currently considered to bind to different sites on β-tubulin (Bender and Chabner, 1982).

Finally, P-glycoprotein has been shown to be overexpressed in a number of multidrug-resistant animal and human tumour cell lines and is considered a marker for this phenotype (Kartner et al., 1985). HN-1/DXR-11 cells show an apparently unique pattern of multidrug resistance in that they are resistant to the epipodophyllotoxins and the Vinca alkaloids but not to the anthracyclines. Furthermore, overexpression of P-glycoprotein was not detected in these cells, and Gros and colleagues (1986) have proposed that mutations in the primary sequence of the putative gene coding for P-glycoprotein are not required to produce a multidrug-resistant phenotype. Therefore by treating cells with a mutagen such as X-irradiation, mechanisms of resistance other then those associated with overexpression of the P-glycoprotein, may be expected to predominate. Attempts to screen for overexpression of this protein as a marker for clinical multidrug

resistance, as suggested by Kartner et al. (1985), may need to take into account these *in vitro* findings, which indicate that cross resistance can be exhibited to structurally unrelated antitumour agents, without any accompanying overproduction of the P-glycoprotein.

8-4: FUTURE STUDIES

Further analyses of the degree of expression of the P-glycoprotein in HN-1/VP-2 cells should reveal whether overproduction of the protein is associated with elevated mRNA expression or gene amplification. It has been proposed that in human multidrug-resistant cells overexpression of mdr1 (the multidrug resistance locus) mRNA may precede amplification of the specific DNA sequences as the level of resistance is increased (Shen et al., 1986b). HN-1/VP-2 cells, since they express relatively low levels of multidrug resistance, may exhibit overexpression of this mRNA without gene amplification. At this time, however, it is not known whether these are real differences in degrees of expression of the mRNA and gene amplification, or whether the present probes lack sufficient sensitivity to detect low levels of gene amplification. HN-1/DXR-11 cells should also be included in these analyses of mRNA overexpression and gene amplification since it is possible that they may overexpress P-glycoprotein but only at a level undetectable with the probes used in this present study.

It has already been proposed that different mechanisms of resistance may operate at different levels of drug resistance.

Therefore, attempts should be made to increase the level of resistance to VP-16 expressed by HN-1/VP-2 cells. Subsequent characterization of their VP-16 uptake and efflux parameters, in concert with analyses of the degree of expression of the P-glycoprotein, may strengthen this hypothesis.

Estimations of VP-16-induced DNA double-strand breakage may reveal differences not only between parental and resistant cell lines, but may also show differences in dose-response profiles between HN-1/VP-2 and HN-1/DXR-11 cells, which may be dependent upon the way in which VP-16 resistance was induced. Molecular biological analyses of the DNA topoisomerase II of HN-1 cell lines, including estimation of enzymic activity, sensitivity to DNA cleavage by VP-16, and purification of the enzymes from the different cell lines by fast-protein liquid chromatography to identify mutant forms in drug-resistant cells, would be a likely drug target to examine in order to identify the biochemical lesion(s) responsible for the expression of VP-16 resistance.

Studies aimed at selecting clonal populations from HN-1/VP-2 and HN-1/DXR-11 cell lines may resolve some of the above issues including whether multiple mechanisms of resistance are expressed within a single human tumour cell, findings that may have major implications for the combined modality treatment of cancer. In addition, by increasing the sensitivity of the probes available to detect biochemical markers of drug resistance, it may be feasible to carry out analyses on individual cells. The implications of results

from studies using individual cells would be much greater than those derived from cell populations of clonal origin.

Investigations of methods to circumvent antitumour drug resistance *in vitro* have led to clinical trials using the calcium influx blocker, verapamil, in an attempt to overcome adriamycin resistance in human ovarian cancer (Ozols, 1985). More recently Hamilton and colleagues (1986) demonstrated that buthionine sulphoximine, which depletes cellular glutathione, increased the survival of nude mice carrying human ovarian cancer xenografts when treated with melphalan or carboplatin. Verapamil has also been shown to reverse Vinca alkaloid and anthracycline resistance in animal tumours by raising intracellular drug levels (Tsuruo, 1983), although Beck and colleagues (1986) recently showed that verapamil reversed Vinca alkaloid resistance, but not multidrug resistance, in human leukaemic lymphoblasts (CEM cells). This agent has also been shown to potentiate VP-16 cytotoxicity in a number of *in vitro* model systems, although complete reversal of drug resistance has yet to be demonstrated (Yalowich et al., 1985; Harker et al., 1986; Slater et al., 1986). Harker et al. (1986) noted that the antitumour agents which were responsive to the sensitisation of human sarcoma (MES-SA) cells were restricted to those which were proposed to exert their cytotoxic effect via DNA topoisomerase II. This prompted the authors to suggest that verapamil may be acting by either promoting the formation or inhibiting the repair of topoisomerase II-mediated DNA strand breaks. Studies of this type may not only indicate which agents may be used to circumvent resistance to specific drugs, but

may also offer an insight into the resistance mechanisms operating. The effects of verapamil in reversing VP-16 and vincristine resistance in HN-1/VP-2 and HN-1/DXR-11 cells should be examined for this reason. A recent report demonstrated that depletion of polyamines, which in prokaryotic systems have a known role in mediating topoisomerase II strand breakage, potentiated the cytotoxic effects of VP-16 in human myeloma (8226) cells and murine leukaemia (L1210) cells (Dorr et al., 1986), observations which may offer alternative strategies for circumventing VP-16 resistance.

Finally, more confidence in the findings of *in vitro* model systems may be attained if the data could be reproduced *in vivo*. HN-1 cell lines have been found not to xenograft in nude mice (Easty et al., 1981; Bellamy, 1982; Chapter 3) and therefore it would first be necessary to develop drug-resistant cell lines analogous to HN-1/VP-2 and HN-1/DXR-11, which can be successfully xenografted. Alternatively, it may be valuable to establish whether irradiation of tumour-bearing animals also leads to the expression of drug resistance. Furthermore, it may be more appropriate when deriving this *in vivo* model system to use as the tumour of origin, one in which VP-16 has been shown to have clinical activity, for example: small-cell lung carcinoma, testicular teratoma, lymphoma or leukaemia (O'Dwyer et al., 1985). An attractive candidate for these studies therefore appears to be provided by the series of SuSa cell lines, derived from a testicular teratoma, recently established in this laboratory (Hill et al., in press).

In conclusion, as combinations of radiotherapy and chemotherapy are increasingly employed in the treatment of human cancers, the type of studies which have been reported in this thesis may increase our understanding of how these modalities may be combined more successfully in order to increase overall response rates and improve survival figures.

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LIST OF PUBLICATIONS

Abstracts

1. Investigations of mechanisms of resistance to etoposide in a human tumour continuous cell line. Br. J. Cancer 52: 425-426, (1985). R.B. Lock and B.T. Hill

2. In vitro drug resistance; interactions between X-irradiation and etoposide. Br. J. Cancer 54: 184, (1986). R.B. Lock and B.T. Hill

Full Publications

1. Drug resistance in vitro: interactions between X-irradiation, etoposide and vincristine. Biochem. Soc. Trans. 14: 618-619, (1986). Richard B. Lock and Bridget T. Hill*

2. Tumor cell resistance to etoposide (VP-16) - A review. In: Resistance to Antineoplastic Drugs (Ed: Kessel, D). CRC Press Boca Raton, Florida. (In Press). Bridget T. Hill and Richard B. Lock

3. Interactions between antitumor drugs and radiation in mammalian tumor cell lines: differential drug responses and mechanisms of resistance following fractionated X-irradiation or continuous drug exposure in vitro. NCI Monographs (in press). Bridget T. Hill, Richard D.H. Whelan, Louise K. Hosking, Sharon, A. Shellard, Philip Bedford and Richard B. Lock

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4. Effects of fractionated X-irradiation on subsequent response to acute X-irradiation in two human tumour cell lines in vitro. Int. J. Rad. Biol. (in press). Angela S. Bellamy, R.D.H. Whelan, R.B. Lock and Bridget T. Hill

5. Differential patterns of cross resistance and collateral sensitivity to certain antitumour drugs in a series of independently-derived VP-16 resistant human tumour cell lines. Richard B. Lock and Bridget T. Hill. Manuscript submitted to J. Natl. Cancer Inst.

6. Investigations of mechanisms of resistance to VP-16 in two independently-derived VP-16-resistant human tumour continuous cell lines in vitro. Richard B. Lock and Bridget T. Hill. Manuscripted to be submitted to Cancer Res.

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Drug resistance *in vitro*: interactions between X-irradiation, etoposide and vincristine

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Reduced response rates to chemotherapy have been observed in a number of human 'solid' tumours after prior treatment, not only with chemotherapy, but also with radiotherapy (Holland *et al.*, 1980; Price & Hill, 1981; Thigpen *et al.*, 1981; Young *et al.*, 1982). In an effort to discover the underlying basis for these clinical observations a human tumour continuous cell line (HN-1), derived from an epithelioid carcinoma of the tongue, was treated with fractionated X-irradiation *in vitro* and the resultant sub-line (HN-1/DXR) was shown to express resistance to etoposide (VP-16-213) and vincristine (Hill & Bellamy, 1984).

VP-16-213, a semi-synthetic derivative of podophyllotoxin, has a broad spectrum of clinical anti-tumour activity, proving one of the most active single agents in the treatment of small-cell carcinoma of the lung and has activity as second line therapy of germ-cell tumours in combination with other drugs (O'Dwyer *et al.*, 1985). Its mechanism of cytotoxicity appears to involve DNA strand breakage (Loike & Horwitz, 1976; Long *et al.*, 1984; Wozniak & Ross, 1983) and subsequent chromosomal aberrations (Huang *et al.*, 1973) brought about by a poisoning of DNA topoisomerase II (Ross *et al.*, 1984).

Vincristine has also shown considerable clinical anti-tumour activity in combination with other drugs in a variety of human tumours, including those of the head and neck (Price & Hill, 1982). It has considerable anti-mitotic activity *in vitro*, and its cytotoxicity is thought to be manifested in an inhibition of tubulin polymerization (Watanabe & West, 1982). Resistance to vincristine *in vitro* has been associated predominantly with reduced cellular accumulation of drug (reviewed by Beck, 1984).

The biochemical mechanisms associated with these altered drug responses after X-irradiation are being studied in order to determine whether the preponderant mechanism differs depending upon the agent and procedure employed to induce drug resistance. We have therefore compared the X-irradiated sub-line (HN-1/DXR) with a line in which drug resistance has been established by continuous exposure of the parental HN-1 cells to VP-16-213 (HN-1/VP-2). This line also expresses cross-resistance to vincristine.

The following parameters have been studied: drug uptake, glutathione levels, and extent of DNA damage after drug treatment.

Drug responses were established by assessing cell survival by colony formation in soft agar (Courtenay & Mills, 1978). IC_{50} values for the HN-1, HN-1/DXR and HN-1/VP-2 cells were 3.2, 5.0 and 9.7 μM respectively after a 1 h exposure to VP-16-213. The order of resistance expressed by the HN-1/DXR and HN-1/VP-2 cells to vincristine was 2.8 and 3.4 respectively compared with the HN-1 line after exposure to 8 nM-vincristine for 24 h.

Drug uptake studies using [^{3}H]VP-16-213 at 3.1 μM for a 30 min exposure have shown no significant differences between the three lines, with values of 18.3 $\pm$ 1.9, 20.1 $\pm$ 0.5 and 19.1 $\pm$ 4.6 pmol/mg protein for the HN-1, HN-1/DXR and HN-1/VP-2 cells respectively. However, using [^{3}H]vincristine at 1 μM for 30 min there was reduced uptake in the drug-treated sub-line (198 $\pm$ 14 pmol/mg of protein) compared with the parental cells (300 $\pm$ 2 pmol/mg of protein) and the X-irradiation-treated (387 $\pm$ 14 pmol/mg of protein) sub-line.

Levels of non-protein sulphydryl compounds have been estimated and shown not to differ significantly between the sub-lines, total cellular contents being 55 $\pm$ 5, 57 $\pm$ 2 and 47 $\pm$ 2 nmol/mg of protein for the HN-1, HN-1/DXR and HN-1/VP-2 cells respectively. The drug-treated sub-line also showed no alteration in glutathione *S*-transferase activity compared with the parental line with activities of 249 $\pm$ 24 and 234 $\pm$ 17 nmol/mg per min respectively.

Alkaline elution studies have revealed a reduction in total DNA breakage and estimated single-strand breakage in the drug treated sub-line (HN-1/VP-2) compared with the parental line after exposure to equimolar concentration of VP-16-213. Rad-equivalent values for total DNA breakage are 346 $\pm$ 35 and 461 $\pm$ 11, and for single-strand breakage are 625 $\pm$ 131 and 1252 $\pm$ 309 for HN-1/VP-2 and HN-1 cells respectively (8.5 μM-VP-16-213, 1 h).

These data indicate that different mechanisms of resistance may be implicated in these differently derived resistant cell lines. Further studies using isolated nuclei should reveal whether the alteration in DNA damage in the drug-treated sub-line is independent of drug uptake through the plasma membrane, a finding that would suggest that resistance to VP-16-213 in these cells may be associated with reduced activity of DNA topoisomerase II (Pommier *et al.*, 1985).

Abbreviation used: IC_{50}, drug concentration required to reduce cell survival to 50% of the control.

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Synergistic effect of two anti-cancer drugs on the growth of HTC cells in culture

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The three major polyamines, putrescine, spermidine and spermine, are small aliphatic polycations found in all living cells. It has been shown repeatedly that high concentrations of these molecules are a characteristic of rapidly growing cells and that transformed cells contain greatly elevated polyamine concentrations (Wallace & Keir, 1982).

In view of the central role of polyamines in cell growth, recent interest has centred on inhibitor studies where it has been suggested that polyamine anti-metabolites may be exploited to improve cancer chemotherapy. Indeed, it has been shown that depleting tumour cells of polyamines has resulted in an enhanced anti-tumour effect from some anti-cancer drugs (Cavanaugh *et al.*, 1984).

In this study two drugs were examined, melphalan, a bifunctional alkylating agent, and methylglyoxal bis-(guanylhydrazone) (MGBG), a polyamine biosynthesis inhibitor. Both of these drugs have been used clinically in the treatment of some types of cancer. The aim of the study was to determine the effect of both drugs, used singly and in combination, on the proliferative and DNA synthetic activity of HTC cells grown in culture.

HTC cells were seeded at a density of 1.6×10^4 cells/cm^2 and grown at 37°C in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) horse serum in an atmosphere of 5% CO_2/95% O_2. MGBG at a concentration of 10 μM in 0.9% (w/v) NaCl and melphalan at a concentration of 15 μM in 0.1 M-HCl were added to the appropriate cell cultures at the time of plating. Cell growth was determined by the change in protein content with time in culture by the method of Lowry *et al.* (1951).

MGBG had a cytostatic effect on HTC cells at the concentration used (10 μM) as indicated by no change in the protein content over the 48 h of the experiment. The protein content of the cells treated with melphalan increased with time in culture but this increase was only 60% of the increase observed in the untreated cells indicating inhibition of cell growth. MGBG and melphalan used in combination appeared to be cytotoxic to the HTC cells, the protein content at 48 h being less than that found at 4 h and only 50% of that measured after 24 h in culture.

DNA synthesis was determined by pulse-labelling the cells with [^{3}H]thymidine (2.5 μCi/ml) for 30 min and measuring both total and acid-insoluble radioactivity. Both MGBG and melphalan individually suppressed DNA synthesis (Fig. 1). Melphalan was more inhibitory than MGBG but the combination of the two drugs produced the greatest effect (Fig. 1). In the untreated control cells, 78% of the total radioactivity in the cells was present as acid-insoluble material at 48 h. This compared with 61% and 34% acid-insoluble material in the MGBG- and melphalan-treated cells respectively. A markedly enhanced effect was observed in the cells treated with both drugs, where only 8% of the total radioactivity was acid-precipitable. In other words, the drug treatments progressively inhibited DNA synthesis.

Inhibition of DNA synthesis by melphalan is almost certainly related to its ability to alkylate DNA causing strand breakage and cross-linking and thus preventing normal replicative synthesis. It has been shown in mouse leukaemia L1210 cells that melphalan induced DNA damage at the concentration used in this study (Ross *et al.*, 1978). In the case of MGBG, studies have been unable to show a similar DNA-damaging effect after treatment (Wallace *et al.*, 1984) and preliminary experiments during this study also showed no change in the DNA integrity with MGBG treatment. Inhibition of DNA synthesis by MGBG has been suggested to be related to its polyamine anti-metabolite properties. Treatment of cells with this drug resulted in a depletion of spermidine and spermine and an accumulation of putrescine (results not shown). It is not known, however, how this inhibition of polyamine biosynthesis relates to the inhibition of DNA replication. There is some evidence in lymphocytes that MGBG acts through inhibition of thymidine kinase activity (Otani *et al.*, 1977), possibly also related to polyamine depletion.

It is known that polyamines are important in maintaining the condensed structure of DNA (Gosule *et al.*, 1978). It may be then, that cells treated with MGBG and melphalan, in combination, show a synergistic inhibition of DNA synthesis as a result of the former depleting the cellular polyamines hence loosening the structure, exposing more sites for alkylation and thereby increasing the effect of melphalan.

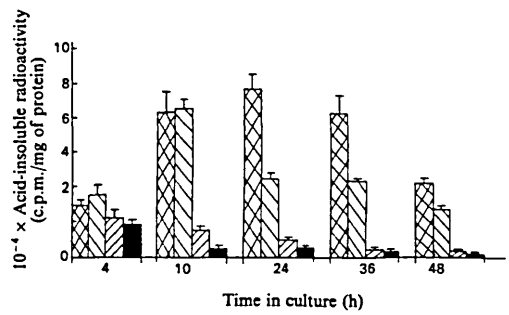


Fig. 1. *Effect of the anti-cancer drugs MGBG and melphalan on DNA synthesis in HTC cells in culture*

HTC cells were grown as described in the text, in the absence of drugs (▨), or in the presence of 10 μM-MGBG (▧), 15 μM-melphalan (▧) or 10 μM-MGBG + 15 μM-melphalan (■). Cells were pulse-labelled with [^{3}H]thymidine for 30 min, and the acid-insoluble radioactivity determined as described previously (Wallace & Keir, 1981). Values are expressed as mean ± S.D. (n = 3).

Abbreviation used: MGBG, methylglyoxal bis(guanylhydrazone).