http://researchspace.auckland.ac.nz

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author’s right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.
http://researchspace.auckland.ac.nz/feedback

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form.
THE ROLE OF TOPOISOMERASE II IN
AMSACRINE RESISTANCE

SANDRA J. RATTRAY (née DARKIN)

Department of Cellular and Molecular Biology
University of Auckland.

A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy.

May, 1989.
FRONTISPICE

There is no end, indeed, to making books or experiments, or, to travel, or to gathering wealth. Problem gives rise to problem. We may study for ever, and we are never as learned as we would. We have never made a statue worthy of our dreams. And when we have discovered a continent, or crossed a chain of mountains, it is only to find another ocean or another plain upon the farther side. In the infinite universe there is room for our swiftest diligence and to spare. It is not like the works of Carlyle, which can be read to an end. Even in a corner of it, in a private park, or in the neighbourhood of a single hamlet, the weather and the seasons keep so deftly changing that although we walk there for a lifetime there will be always something to startle and delight us.

Robert Louis Stevenson
(out of El dorado)
ABSTRACT

1. Antitumour agents from many different chemical classes including acridines, have been shown to induce topoisomerase II-associated DNA breaks both in cultured mammalian cells and in vitro in the presence of purified mammalian DNA topoisomerase II. However, mechanisms linking these protein-associated DNA breaks with drug cytotoxicity are poorly understood. Whether this type of DNA damage is responsible for drug cytotoxicity was investigated using a number of acridine derivatives belonging to the same chemical class but with varying potency in vivo. The modulation of topoisomerase II-mediated DNA breaks in whole L1210 cells, isolated nuclei and nuclear extract systems correlated with drug-induced cytotoxicity, at least for the drugs belonging to the amsacrine lineage. In contrast, there was no direct relationship between the inhibition of topoisomerase II strand-passing activity and the cytotoxic action of topoisomerase II-specific anticancer drugs.

It was concluded that drug-induced formation and stabilization of the topoisomerase II-DNA cleavable complex, rather than inhibition of its formation or of strand-passing functions, is responsible for DNA strand breakage and cell death caused by antitumour acridines such as amsacrine.

2. Two tissue culture systems (CHO-AA8 cells or P815 cell cycle mutants) were used as models to study the involvement of topoisomerase II in the resistance of non-cycling cells to amsacrine.

Plateau-phase CHO-AA8 cells with a G0/G1 DNA content are resistant to amsacrine and contain fewer DNA breaks than log-phase cells after drug treatment (Robbie et al., 1988). The observations of Robbie et al. (1988) were further investigated in this thesis. The phage P4 DNA
unknotting activity in CHO-AA8 cell nuclear extracts decreased 2-fold when the cells entered plateau-phase, but there was no difference in the sensitivity of unknotting to amsacrine between log- and plateau-phase nuclear extracts. Furthermore, drug stimulation of protein-DNA complex formation was similar in whole cells, isolated nuclei and nuclear extracts from either log- or plateau-phase cells. However, stimulation of complex formation in cells, nuclei or nuclear extracts was approximately 4-fold lower in plateau-phase than in log-phase. These results suggested that drug-enzyme interaction was altered in plateau-phase and there was a good correlation between the proliferative state of cells, amsacrine sensitivity, DNA breakage and topoisomerase II-DNA complex formation.

The second system used to study the effect of amsacrine was the cold-sensitive (proliferating at 39.5°C; reversibly arrested in Gl-phase at 33°C) cell-cycle mutant 21-Fb of the murine mastocytoma cell line, P815. The sensitivity of arrested 21-FB cells to amsacrine decreased less than two-fold in cell survival experiments when compared to proliferating cells. In contrast, DNA breakage and stimulation of protein-DNA complex formation in intact cells, lysed cells or isolated nuclei was reduced approximately 10-fold in amsacrine-treated arrested cells and DNA-topoisomerase II activity in arrested cells was only 5% of the activity in proliferating cells. Thus in contrast to the CHO-AA8 cell system, there was no correlation between cell survival and DNA damage or DNA topoisomerase II activity in drug treated 21-Fb cells.

The exact reasons for the differences in amsacrine sensitivity in growth arrested CHO and 21-Fb cells were not resolved. However, it was concluded that a complex relationship exists between amsacrine-induced DNA breakage, topoisomerase II-DNA complex formation, topoisomerase activity and drug cytotoxicity. Moreover, a very complex set of parameters can
influence drug-induced topoisomerase II-mediated lesions and cytotoxicity in different cells or even a single type of cell under different growth conditions.

3. Extracts of K21 murine mastocytoma cells were found to contain a factor that enhances formation of amsacrine-induced topoisomerase II-DNA complexes (PDC) when added to isolated K21 nuclei. The PDC enhancing activity was reduced in extracts from 2 or 6 h cycloheximide- or cordycepin-treated cells, implying that continuous protein synthesis is required to maintain the factor. Preliminary characterization showed that the factor was heat labile and proteinase-sensitive suggesting the factor was a labile protein which was distinct from the two known classes of topoisomerase.

The protein factor was present in at least four other cell lines and was substantially reduced in cells induced into a G1 state by temperature arrest or serum deprivation. Human Jurkat cells selected for resistance to amsacrine and displaying cross-resistance to other topoisomerase II-targeted drugs also exhibited significantly reduced PDC enhancing activity. These results suggested a contributing role for the PDC enhancing factor in mediation of drug resistance.

Fractionation of mouse mastocytoma cell cytoplasmic extracts by salt precipitation, DEAE-cellulose chromatography and SDS-PAGE resulted in a 3571-fold purification of the PDC enhancing activity. The PDC enhancing activity was shown to reside in a 70 kDa protein kinase with specificity for a casein kinase II substrate and sensitive to heparin and anti-casein kinase II antiserum.

This appears to be the first direct evidence of a protein factor that modulates amsacrine induced topoisomerase II action possibly by
phosphorylating topoisomerase II or proteins associated with topoisomerase II.
ACKNOWLEDGEMENTS

I gratefully acknowledge the Auckland Medical Research Foundation for financial support during the course of this study.

I would like to express sincere thanks to Professor Ray Ralph for his diligent supervision of this thesis and for the enthusiastic interest he has shown in all aspects of my research over the past 6 years. I would also like to express special thanks to Dr. Erasmus Schneider for his valuable comments on various aspects of this work and for collaborative studies.

Thanks also must be conveyed to other participants in collaborative studies, specifically Associate-Professor Bruce Baguley, Drs Graeme Finlay, Bill Wilson and Maxine Robbie (The Cancer Research Laboratory and Department of Pathology, Auckland School of Medicine).

To all those who have willingly shared their expertise I give my thanks, particularly to Philip Schofield and Terry Gruijters for their assistance with photography and to Stan Wojick for technical assistance willingly provided whenever required.

To other members of the Department of Cellular and Molecular Biology I probably should thank but appear to have neglected - I haven't, thanks for providing a friendly working environment.

I would like to thank my family, especially Jim and Thelma, for support and encouragement throughout the time I have devoted to this thesis.

To my parents, for their assistance and support in ways to numerous too mention I give my warmest thanks and love.

To my husband, Mark, who though coping with the trials of his own doctorate still found time to type the vast majority of this thesis and to provide general help and encouragement when needed, I give my love and heartfelt thanks, although it is barely sufficient. He above all understands and has shared the costs and rewards of experimental research.
# TABLE OF CONTENTS

| Frontispiece | i |
| Abstract     | ii |
| Acknowledgements | vi |
| Table of Contents | vii |
| List of Abbreviations | xiii |

## CHAPTER ONE: INTRODUCTION

1.1 Perspectives: Current status of cancer chemotherapy 1
1.2 Development of "broad spectrum" antitumour drugs: -
   the clinical antitumour drug AMSACRINE and
   related compounds. 3
1.3 The mechanism of action of amsacrine (and related 8
   derivatives.
   1.3.1 DNA binding 8
   1.3.2 DNA breakage 10
   1.3.3 Evidence for topoisomerase II-mediated cleavage of 12
   DNA
   1.3.4 Topoisomerase II reactions 13
1.3.5 Effects of amsacrine, other intercalating agents and 16
   epipodophyllotoxins on topoisomerase II reactions
   in vitro
1.4 Biological role of topoisomerase II 18
   1.4.1 Cellular regulation of topoisomerase II 26
1.5 The question of toxicity: Possible mechanisms of cell 27
   killing by mammalian topoisomerase II poisons.
1.6 Modes of action of anticancer drugs 29
1.7 Aims of this study 34

CHAPTER TWO: MATERIALS AND GENERAL METHODS
A Materials 36
B General Methods 40

CHAPTER THREE: CYTOTOXICITY AND DNA BREAKAGE PROPERTIES OF ANTITUMOUR ACRIDINES: ROLE OF DNA TOPOISOMERASE II
3.1 Introduction 57
3.2 Methods 61
3.3 Results 64
3.3.1 In vivo activity of related acridine derivatives against L1210 leukemia 64
3.3.2 In vitro growth inhibition by the acridine compounds in continuous exposure assays 66
3.3.3 The effect of related acridine derivatives on DNA strand breakage 66
3.3.4 Stimulation of protein-DNA complex formation by the acridine derivatives 70
3.3.5 Inhibition of topoisomerase II strand-passing activity: The P4 unknotting assay 76
3.4 Discussion 76
CHAPTER FOUR: THE ROLE OF TOPOISOMERASE II IN AMSACRINE RESISTANCE

4.1 Introduction 81

Part I: Mechanisms of resistance of non-cycling mammalian cells to amsacrine: The role of DNA topoisomerase II in Log- and Plateau-phase CHO cells

4.2 Introduction 82
4.3 Methods 83
4.4 Results 86
4.4.1 Quantitation of DNA topoisomerase II strand-passing activity in log- and plateau-phase AA8 cells 86
4.4.2 Inhibition of unknotting activity by amsacrine 88
4.4.3 Stimulation of covalent protein-DNA complex formation in log- and plateau-phase cells by amsacrine 88
4.4.4 In vitro stimulation of covalent DNA topoisomerase II complex formation with nuclear extracts from log- and plateau-phase cells 95
4.5 Part I Summary 95

Part II: The relationship between sensitivity to amsacrine and DNA topoisomerase II in a cold-sensitive cell cycle mutant of a murine mastocytoma cell line.

4.6 Introduction 98
4.7 Methods 98
4.8 Results 102
4.8.1 Amsacrine cytotoxicity in proliferating and arrested 102
CHAPTER FIVE: DEVELOPMENT OF A SYSTEM TO QUANTITATE PROTEIN-DNA COMPLEXES IN ISOLATED NUCLEI: EVIDENCE FOR A FACTOR THAT ENHANCES AMSACRINE-INDUCED TOPOISOMERASE II-DNA COMPLEX FORMATION

5.1 Introduction 123
5.2 Methods, Results and Discussion 124
5.2.1 High non-specific protein-DNA complex formation 125
5.2.2 Absence of drug-induced protein-DNA complexes in isolated K21 or L1210 nuclei 129
5.2.3 Preparation of cytoplasmic extracts from K21 cells 139
5.2.4 Preparation of isolated nuclei 139

4.8.2 Amsacrine-induced DNA breakage 102
4.8.3 Stimulation of protein-DNA complex formation 104
4.8.4 Quantitation of DNA topoisomerase II strand-passing activity 107
4.9 Part II Summary 112

4.10 Discussion 112
4.10.1 The relationship between sensitivity to amsacrine and DNA topoisomerase II in log- and plateau-phase CHO-AA8 cells 114
4.10.2 The relationship between sensitivity to amsacrine and DNA topoisomerase II in a cold sensitive cell-cycle mutant of a murine mastocytoma cell line 116
4.10.3 What is the role of topoisomerase II in cellular drug sensitivity? The CHO-AA8 cell system versus the 21-Fb cell cycle mutant system 118
5.2.5 Quantitation of PDC in nuclei with added cytoplasmic extract

5.3 Summary

CHAPTER SIX: A PROTEIN FACTOR THAT ENHANCES AMSACRINE MEDIATED FORMATION OF TOPOISOMERASE II-DNA COMPLEXES IN MURINE MASTOCYTOMA CELL NUCLEI: A PRELIMINARY CHARACTERIZATION

6.1 Introduction 144
6.2 Methods 146
6.3 Results 148

6.3.1 Facilitation and characterization of amsacrine-induced PDC formation in isolated nuclei by cytoplasmic extracts 148
6.3.2 The effect of inhibiting protein and RNA synthesis on cytoplasmic PDC enhancing activity 154
6.3.3 Physicochemical characterization of the cytoplasmic PDC enhancing activity 156
6.4 Discussion 160

CHAPTER SEVEN: GENERALITY OF THE PDC ENHANCING FACTOR AND ITS POSSIBLE INVOLVEMENT IN DRUG RESISTANCE

7.1 Introduction 164
7.2 Methods and Results 165

7.2.1 Generality of the PDC enhancing factor 165
7.2.2 The involvement of PDC enhancing activity in drug resistance

7.2.2.1 PDC enhancing activity in proliferating (39.5°C) and arrested (33°C) temperature sensitive 21-Fb 168
cells

7.2.2.2 PDC enhancing activity in K21 cells arrested with low serum 170

7.2.2.3 PDC enhancing activity in amsacrine sensitive and resistant human Jurkat cells 170

7.3 Discussion 174

CHAPTER EIGHT: ISOLATION AND PRELIMINARY IDENTIFICATION OF THE PDC ENHANCING FACTOR

8.1 Introduction 176
8.2 Methods 177
8.3 Results 185

8.3.1 Ammonium sulphate precipitation of cytoplasmic extracts 185

8.3.2 Purification of the PDC enhancing factor by column chromatography 187

8.3.3 Gel electrophoresis of column fractions 190

8.3.4 Elution and renaturation of the 70 kDa protein from SDS-polyacrylamide gels 193

8.3.5 Preliminary identification of the 70 kDa protein with PDC enhancing activity 196

8.3.5.1 Heparin-sensitivity of the PDC enhancing factor 196

8.3.5.2 Sensitivity of the PDC enhancing activity to anti rat-liver casein kinase II antiserum 199

8.3.5.3 Protein kinase activity of the 70 kDa protein 199

8.4 Discussion 201
CHAPTER NINE: CONCLUDING DISCUSSION

9.1 The question of cytotoxicity 204
9.2 The role of topoisomerase II in amsacrine resistance 206
9.3 A protein factor that enhances amscrine mediated formation of topoisomerase II-DNA complexes:
Speculations on its role in drug action, drug resistance and cancer cell growth. 211
9.4 General consideration 219

REFERENCES 220

ABBREVIATIONS

9-AAA  9-aminoacridine
ALL     acute lymphoblastic leukemia
α-MEM   alpha modified minimum essential medium
AMSA₂⁻ 4'-(9-acridinylamine)methanesulphonanilide
[γ²-³²P]-dATP deoxyadenosine 5'-[γ²-³²P] triphosphate
ATP     adenosine 5'-triphosphate
bisacrylamide N,N'-methylenebisacrylamide
BSA     bovine serum albumin
cDNA    complementary DNA
CHO     chinese hamster ovary cells
CI-921  N',5'-dimethyl-9-[(2-methoxy-4-methyl-sulphonylaniline)-phenylamino]-4-acridine carboxamide.
DAPI    4',6'-diamino-2-phenylindole
dATP    2'-deoxyadenosine 5'-triphosphate
ddH₂O   double distilled water
ddGTP   2'-deoxyguanosine 5'-triphosphate
DMSO    dimethyl sulphoxide
DSB     (DNA) double-strand break
DTT     1,4-dithiothreitol
dTPP    2'-deoxythymidine 5'-triphosphate
EDTA    ethylene diamine tetraacetic acid
EGTA    ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid
FADU    fluorescence assay for DNA unwinding
FCS     fetal calf serum
FPLC    fast pressure liquid chromatography
HMG     high mobility group (protein)
HPLC    high pressure liquid chromatography
hsp     heat shock protein
m-AMSA  4'-(9-acridinylamine)methanesulphon-m-anisidide
o-AMSA  4'-(9-acridinylamine)methanesulphon-o-anisidide
PBS     phosphate-buffered saline
PDC     protein-DNA-complex
POPOP   1,4-bis-[2-(5-phenyloxazolyl)] benzene
PPO     2,5-diphenyloxazole
QSAR    quantitative structure-activity relationship
SCE     sister chromatid exchange
SD      standard deviation
SDS     sodium dodecyl sulphate
SE      standard error
TBS     Tris buffered saline
TCA     trichloroacetic acid
TEMED   N,N,N',N'-tetramethylenediamine
Tris    tris(hydroxymethyl)aminomethane
CHAPTER ONE: INTRODUCTION

1.1 Perspectives: Current status of cancer chemotherapy.

During the past 15-20 years, significant advances have been made across a broad front in the assault against cancer. As a result, current concepts about cancer, the discovery and development of new therapeutic innovations and the clinical management of patients with cancer are radically different from those of only a few years ago.

Despite these developments the benefits of chemotherapy are still at present mainly palliative, although drugs can be used to achieve cures in at least a proportion of patients with 12 different types of cancer: two leukemias (acute lymphoblastic leukemia, ALL; acute myelogenous leukemia, AML), three lymphomas (Hodgkin's disease; diffuse histiocytic lymphoma; Burkitt's lymphoma), three childhood solid tumours (Wilm's tumour; rhabdomyosarcoma; Ewing's tumour) and two solid tumours (gestational choriocarcinoma; germ cell tumours). To the latter group may be added 5-15% of epithelial ovarian cancers and small cell carcinomas of the lung (Chabner et al., 1984; Whitehouse, 1984). Not withstanding this moderate success, the precise mechanisms by which most chemotherapeutic agents selectively eradicate malignant cells while retaining the functional integrity of normal cells remain unknown. Moreover, a depressingly large number of malignancies continue to be insensitive to existing chemotherapeutic approaches, while the most successful drugs are highly toxic and have low therapeutic indices.

Advances in understanding the cell biology of cancer and in various technologies have played a key role in the improvement of drug discovery
and development, but perhaps more important are the additional therapeutic targets identified as a result of the greater understanding of the molecular biology, immunology and biology of malignant cells. Although the opportunities presented by advances in molecular biology are exciting, it is difficult not to despair about the potential to understand and/or cure cancer. Each new discovery appears attractive as a key causal factor in the aetiology of cancer, yet invariably research shows that the relationship is much more complex than initially envisaged and the target is found to be merely associated with, rather than causing malignancy.

Over the past ten years, it has been established that the enzyme DNA topoisomerase II (EC 5.99.1.3) (Section 1.3.3) is the intracellular target of two families of clinically important and successful antitumour agents, namely the DNA intercalators including amsacrine and Adriamycin and the non-intercalating epipodophyllotoxins VM26 and VP16 (Liu, 1989)

DNA topoisomerase II as a target for neoplastic agents appears to partially satisfy the requirements for drug targets proposed by Crooke (1986), namely, that any target for anti-neoplastic agents should be

(a) qualitatively or quantitatively different from that found in normal cells and,

(b) that it is essential for the maintenance of the cancer phenotype (not necessary causal).

The elucidation of the mechanism of action of agents targeted at topoisomerase II has also been instrumental in advancing the understanding of the biological functions of topoisomerase II and it has provided new perspectives for the rational development of new antitumour drugs.

My research centered predominately on amsacrine, how this drug exerts its cytotoxic action through topoisomerase II and especially the role of topoisomerase II in cellular resistance to amsacrine. The following
section presents a synopsis of the development and current understanding of the mechanism of action of amsacrine, the cellular functions of eukaryotic topoisomerase II and its exploitation as a target for antitumour drugs. This leads logically into an area of present and future importance, the role of topoisomerase II in amsacrine resistance.

No attempt has been made to comprehensively review all of the available literature on amsacrine or other agents that target topoisomerase II. Rather, the readers attention is drawn to several excellent detailed reviews which consider intercalating drugs (Neidle and Waring, 1983), amsacrine (Marshall and Ralph, 1985), the epipodophyllotoxin VP16 (Issell et al., 1984), the molecular biology of topoisomerase II (Wang, 1985; Vosberg, 1985) and the general field of antitumour agents affecting topoisomerase II (Ralph and Schneider, 1987; Hancock et al., 1988; Liu, 1989).

1.2 Development of "broad spectrum" antitumour drugs: the clinical antitumour drug AMSACRINE and related compounds.

As mentioned above, significant advances have been made using antitumour drugs to treat malignant disease. However, several of the more prevalent tumours, including colorectal carcinomas, melanoma, oesophageal and nasal cancers do not respond to treatment with anticancer drugs (Tattersall, 1981). Clearly there is a need for the development of "broad spectrum" antitumour drugs with activity against these and other common solid tumours.

The anthracycline anticancer antibiotic Adriamycin (Figure 1.1), isolated from strains of Streptomyces peucetius, was the first agent displaying an exceptionally broad spectrum of antitumour activity (Goldin
et al., 1981; Arcamone, 1981) and having clinical applications in the
treatment of a number of solid tumours as well as acute leukemias. The
development and mechanisms of action of the anthracycline family of drugs
is reviewed in detail by Neidle and Waring (1983) and Liu (1989).

The anthracyclines, including adriamycin, induce severe, dose related,
cumulative and irreversible cardiac toxicity which limits their clinical
effectiveness. This has led to the search for new drugs with similar cell
killing properties but without the toxic side effects and eventually to
the development of the clinically active antitumour acridine derivative
amsacrine (mAMSA or 4'- (acridinylamino)methanesulfon-m-anisidide: Figure
1.1) by the Auckland Cancer Research Laboratories. Subsequently, their
major aim has been to synthesize modified 9-anilinoacridine derivatives
with improved antitumour activity, of which several hundred have now been
synthesized and tested (reviewed by Wilson, 1978; Denny et al., 1983;
Robbie, 1988).

It was the work of Cain and his co-workers in Auckland, beginning in
1972, which demonstrated the broad spectrum of biological activity of
9-anilinoacridines, and the outstanding experimental antitumour activity
of many of the analogues.

The rational development of compounds with extended biological
activities from quantitative structure-activity relationships then led to
the emergence of the AMSA compounds, as a particularly important group of
tumour active compounds. In 1974, the compound mAMSA was reported as a
highly active dose potent derivative against L1210 leukemia (Cain and
Atwell, 1974). mAMSA has since been the subject of extensive
investigation and it has been accepted for use in the clinic. It is
marketed by Warner-Lambert Parke-Davis as AMSACRINE and will be referred
to as such henceforth.
FIGURE 1.1: Structures of amsacrine and other antitumour drugs that inhibit topoisomerase II.

AMSACRINE  \( R_1 = OCH_3 \)  \( R_2 = H \)  \( R_3 = H \)  \( R_4 = H \)

\( g\)-AMSA  \( R_1 = H \)  \( R_2 = OCH_3 \)  \( R_3 = H \)  \( R_4 = H \)

CI-921  \( R_1 = OCH_3 \)  \( R_2 = H \)  \( R_3 = CH_3 \)  \( R_4 = CONHCH_3 \)

ADRIAMYCIN  \( R = OH \)

DAUNOMYCIN  \( R = CH_3 \)

VP-16  \( R = CH_3^- \)

VM-26  \( R = \text{S} \)
The spectrum of useful clinical antitumour activity of amsacrine, while not so broad as that of Adriamycin, closely resembles that of another anthracycline antibiotic, daunorubicin, with therapeutic activity against both myeloid and lymphoid acute leukemias (Legha et al., 1982) and lymphomas (Weick et al., 1983). However, treatment with amsacrine does not induce the severe irreversible cardiomyopathy associated with anthracycline antibiotics, although reversible congestive heart failure may develop after administration of the drug (Steinherz et al., 1982; Vorobiof et al., 1983).

Amsacrine does not have useful therapeutic activity against solid tumours (Legha, 1983). The reason for this limited activity is not yet fully understood. One aspect of "resistance" is investigated in this study, namely the refractory nature of non-cycling cells to amsacrine and the possibility of qualitative or quantitative alterations in the drug's target in non-cycling cells which constitute a major part of solid tumours.

One of the principal factors limiting the activity of amsacrine against solid tumours was assumed to be the inability of adequate drug to distribute to the tumour site. Screening 9-anilinoacridine derivatives for activity against remotely implanted tumours has identified a number of structural features that improve drug distribution, including high lipophilicity and the requirement of a methyl group at the carbon four position of the acridine ring (Cain and Atwell, 1976). Extended studies of the distribution properties of the AMSA series of drugs, have led to the development of a 4,5-disubstituted amsacrine analogue with superior activity to that of its parent compound against a variety of murine tumours. This agent, (N,5-dimethyl-9-[(2-methoxy-4-methylsulphonyl- amino)phenylamino-4-acridine carboxamide, CI-921; Figure 1.1), possesses
particularly good oral activity and has demonstrated similar potency to amsacrine against murine L1210, P388 and P388/ADR leukemia cell lines. Moreover, it is more active than amsacrine against several human tumour carcinoma cell lines in vitro including B16 melanoma, the CD8F1 mammary tumour and the colon 26 and 36 tumours (Leopold et al., 1987). The activity of CI-921 against Lewis lung carcinomas is particularly encouraging because this tumour is the least responsive mouse tumour in the National Cancer Institute main test panel (Goldin et al., 1981) and it is resistant to a number of other clinically useful agents including amsacrine. The results with CI-921 suggest that it may be superior to amsacrine in the treatment of human carcinoma, although the drug is still in the early stages of phase II clinical evaluation and it is too early to predict its success in treating the more common human solid tumours. More recent results look promising for another compound, acridine carboxamide and related substituted derivatives, which have also shown high activity against the solid Lewis lung carcinomas in mice (Atwell et al., 1987).

In addition to the relative ease with which acridines may be modified to overcome the barriers presented by resistant tumours, they also provide an ideal series of compounds to study their mechanism of action. One of the unusual features of the AMSA series of drugs is that only small changes in chemical structure often produce large changes in activity and potency. For example, the positional isomer of amsacrine, oAMSA (Figure 1.1) is completely inactive against both the L1210 leukemia and PY815 mouse mastocytoma cell lines in vivo and has growth inhibitory potency in the order of 50-fold lower than amsacrine in vitro (Wilson, 1978). The parent compound AMSA has a molar potency approximately 5-6 fold lower than amsacrine. The less active drugs therefore provide useful controls when investigating the interaction of active compounds with putative cellular
targets, as described in Chapter 3 of this thesis.

In summary, amsacrine has played a pivotal role in a successful drug development programme which has as its underlying strategy "that consideration of chemical properties of a clinically successful drug and the subsequent logical chemical modification of that drug may lead to compounds with improved broad spectrum antitumour activity and the ability to overcome barriers which prevent the parent compound from eliminating resistant tumour types". However, a greater understanding of both the basic mechanisms of action of the drug and tumour related mechanisms which limit the activity of amsacrine are of fundamental importance to the development of further amsacrine analogues with improved activity.

1.3 The Mechanism of action of amsacrine (and related derivatives).

1.3.1 DNA Binding.

The initial investigation of the action of any compound usually begins with attempts to identify its intracellular target. Numerous studies have shown that the 9-anilinoacridine derivatives, of which the AMSA series comprise a part, will bind to DNA in vitro (Waring, 1976; Baguley and Falkenhaug, 1978; Baguley et al., 1978; Baguley et al., 1981a; Hudecz et al., 1981; Wilson et al., 1981a).

Waring (1976) established an intercalative mode of binding for amsacrine. The acridine moiety is thought to intercalate into DNA between and parallel to the base pairs with the result that the double helix becomes extended and distorted. The methanesulphonanilide side chain does not interfere with binding to double-stranded DNA and is postulated to lie in the minor groove of the double helix. Amsacrine binds to native DNA
with a relatively low affinity when compared to inactive antitumour drugs such as proflavine and 9-aminoacridine which bind strongly to DNA. In fact, no clear correlation between DNA binding parameters and antitumour activity of compounds from the AMSA series of drugs has been demonstrated (Waring, 1976; Gormley et al., 1978; Wilson et al., 1981a). For example, the inactive positional isomer of amsacrine, oAMSA exhibits a 2-3 fold greater binding constant than amsacrine (Wilson et al., 1981a), although the association constants for both isomers are sufficiently high to ensure that essentially all the drug available for DNA binding in cells will in fact be bound to DNA (Wilson, 1978). The lower binding constant of amsacrine is attributed to the 3'-OCH₃ group sterically hindering the overlap of the acridine nucleus with the base pairs in DNA (Denny et al., 1983).

The intercalated drug causes the DNA helix to unwind by 26° per molecule and the unwinding angle is the same for AMSA, oAMSA and amsacrine. Amsacrine exhibits a more pronounced selectivity for duplex DNA or RNA over single-stranded nucleic acids than does its parent compound AMSA although there is no difference in base pair or base sequence binding sensitivities per se between the three congeners, AMSA, amsacrine and oAMSA (Robbie, 1983). Therefore, it appears that while the ability to intercalate into DNA appears to be a necessary condition, it is not alone sufficient to account for the antitumour activity of the AMSA series of drugs. Furthermore, recent studies indicate that there is a stronger correlation between the cytotoxicity of the amsacrine analogues and the kinetic stability of the drug-DNA complex than with the DNA binding affinity of these compounds (Denny and Wakelin, 1986).

In addition, evidence from structure-activity studies suggests that the effects of 1'-methanesulphonamide and 3'-methoxy substituents on the
antitumour activity of the AMSA drugs are mediated by mechanisms other than gross DNA binding (Robertson, 1980; Baguley et al., 1981b). So, if the broad quantitative features of amsacrine-DNA interactions cannot explain its antitumour effectiveness, what other factors might contribute to the molecular basis of the selective action of amsacrine?

1.3.2 DNA breakage

Early in vivo studies with mice bearing transplantable L1210 leukemia cells and in vitro studies led Burr-Furlong et al. (1978) to suggest that amsacrine caused cell death by inhibiting DNA replication as a result of its intercalative mode of binding to DNA. However, Wilson (1978) and Ralph (1980) using alkaline sedimentation methods showed that amsacrine produced single-strand breaks in the DNA of P815 cells. Later, these strand breaks were shown not to be an artefactual consequence of radiolabelling cells for sucrose density gradient studies or the scission of alkali-sensitive lesions, such as the loss of a base on exposure to the strong alkali used to denature DNA prior to gradient fractionation (Marshall and Ralph, 1982a). Instead strand breaks appeared to be a drug induced phenomenon. Subsequent studies with sucrose gradients and viscosity measurements showed that alkali was not required to detect DNA breakage by amsacrine providing that a sufficiently strong detergent was included in the cell lysis solution (Marshall and Ralph, 1982b; Marshall et al., 1983a; Darkin, 1985).

Kohn et al. (1979) also found breaks in the DNA of mouse leukemia L1210 cells after treating cells with adriamycin or ellipticine using the now well documented alkaline elution technique to assay for DNA breakage. The rationale behind this technique involves the ability of membrane
filters to retard the passage of large DNA strands in a size dependent manner under alkaline conditions. Thus, cells are lysed with alkaline detergent on filters to avoid shearing the DNA and the elution rate of denatured DNA is monitored.

No method used to measure DNA strand breaks is without pitfalls which may influence the interpretation of results and the reader is referred to Marshall (1983), Darkin (1985) and Robbie (1988) for detailed discussions of the relative advantages and disadvantages of various techniques used to quantify drug-induced DNA strand breakage.

Using filter elution or viscosity techniques along with sucrose density gradients (Marshall and Ralph, 1982c), it was eventually shown that agents such as amsacrine, adriamycin and ellipticine produced double-strand as well as single strand breaks in the DNA of treated cells (Ross and Bradley, 1981; Zwelling et al., 1981; Marshall and Ralph, 1982c). The resulting DNA fragments were very large with molecular weights in the order of $1-2 \times 10^8$, approximating in size to one double-strand break per replicon or chromosome loop (Zwelling et al., 1981; Marshall and Ralph, 1982c; Pommier et al., 1984a).

Further information on the nature of the DNA strand breaks induced by amsacrine was obtained by filter elution studies. Zwelling et al. (1981) showed that the amsacrine induced single- and double-strand breaks in DNA were tightly associated with protein and that the breaks could be unmasked by treating the DNA with proteinase K prior to elution. Moreover, after removal of amsacrine (Zwelling et al., 1981), or after treatment with 0.5 M NaCl (Liu et al., 1983), the breaks rapidly resealed. Zwelling et al. calculated that the ratio of DNA breaks to DNA-protein cross-links was nearly 1:1, suggesting that the two phenomena were related and that the protein was bound at, or near one end of the
broken DNA.

Enzymatic digestion of DNA fragments from amsacrine treated P815 cells with specific exonucleases showed that the 3'- ends of the DNA carried free nucleotide 3'-OH groups, whereas corresponding free 5'-phosphorylated nucleotide termini were not detected suggesting that the drug-induced cleavage of DNA phosphodiester bonds produced DNA fragments with blocked DNA 5'-termini (Marshall and Ralph, 1982a; Marshall et al., 1983c). Subsequently Ralph and Hancock (1985) used electron microscopy and SDS-PAGE to demonstrate the presence of a 175 kDa protein covalently bound to the broken DNA from amsacrine treated cells that was not detectable in untreated cells.

1.3.3 Evidence for topoisomerase II-mediated cleavage of DNA.

The phenomenon of drug induced DNA breakage, resealing and DNA protein cross-linking was demonstrated not only for amsacrine but for a variety of drugs including 5'-iminodaunomycin (Zwelling et al., 1982a), adriamycin (Zwelling et al., 1981; Ross and Smith, 1982) and ellipticine (Ross et al., 1978; Zwelling et al., 1982b) suggesting that it is a common feature of the action of DNA intercalating antitumour agents, as well as certain non-intercalating agents, for example epipodophyllotoxins (Issell, 1984). Similarities between the products produced by bacterial DNA topoisomerases which interconvert DNA topoisomers via covalently bound protein-associated intermediates (Gellert, 1981; Liu et al., 1983) and the protein-associated DNA fragments produced by intercalating drug action on mammalian cells (Liu et al., 1983) suggested that these drugs might interfere with the action of a mammalian topoisomerase.

Early support for the conclusion that intercalating and certain
non-intercalating drugs affect topoisomerase action, but more specifically the action of a type II topoisomerase, came from our laboratory. Marshall et al. (1983a) showed that while strand breaks are present in the DNA extracted from isolated nuclei incubated with amsacrine, their formation is prevented by inhibitors (novobiocin and coumermycin) which block an early step in the topoisomerase II reaction (Gellert, 1981). A variety of other evidence has since been presented which unequivocally established that topoisomerase II is the target for drugs such as amsacrine (Reviewed in Ralph and Schneider, 1987; Hancock et al., 1988; Liu, 1989). Perhaps the most conclusive evidence was provided by Rowe et al. (1986a) who isolated and purified calf thymus DNA topoisomerase II and showed that a single amsacrine-dependent DNA cleavage activity copurified with the topoisomerase II at all chromatographic steps of enzyme purification. Moreover, Yang et al. (1985) were able to show that treatment of mammalian cells with amsacrine resulted in protein-associated DNA fragments that could be immunoprecipitated by a polyclonal antibody specific for type II topoisomerase.

1.3.4 Topoisomerase II reactions

Type II DNA topoisomerase belongs to a class of enzymes that alter the topological state of DNA by breaking and rejoining the phosphodiester backbone of DNA (Reviewed Gellert, 1981; Liu, 1983; Vosberg, 1985; Wang, 1985, 1987). Type II DNA enzymes change the topological state of DNA by making transient, enzyme-bridged, double-strand breaks in DNA through which it may pass the same (or another) molecule of DNA and consequently alter the linking number of DNA in multiples of two. This catalytic (strand-passing) activity of topoisomerase II enzymes is ATP-dependent.
The importance of topoisomerases in various cellular processes is discussed in Section 1.4.

A partial reaction of mammalian DNA topoisomerase II has been characterized whereby an enzyme dimer initiates its strand-passing reaction by forming a covalent association with DNA via a tyrosine linkage with the 5'-terminus of each DNA strand. Treatment with protein denaturants such as SDS, dissociates the enzyme's two subunits, and thus effectively cleaves the DNA. Both single and double strand breaks are produced (Liu, 1983). Based on the characteristics of the cleavage reaction, namely its speed, reversibility with 0.5 M salt (Liu, 1983) and lack of generation of free ends, a simple two state model has been proposed for the partial reaction (Figure 1.2) (Nelson, 1984). A topoisomerase II-DNA "cleavable complex", presumed to be the key covalent intermediate in the strand-passing reaction is proposed to be in rapid equilibrium with at least one other topoisomerase II-DNA complex, the "non-cleavable complex". In contrast to the non-cleavable complex, exposure of the cleavable complex to a strong protein denaturant results in DNA cleavage and the covalent linking of a topoisomerase II subunit to the 5'-phosphoryl end of each strand of the broken DNA. The 3-hydroxyl ends are recessed by four bases (Liu, 1983). Interaction of a second DNA segment with the cleavable complex presumably triggers the strand-passing reaction. The low level of cleavable complexes in a normal topoisomerase II reaction is consistent with its proposed transient nature.
FIGURE 1.2: A partial reaction of DNA topoisomerase II and a model for drug-induced cleavage by topoisomerase II.

Mammalian DNA topoisomerase II (A), a homodimer is proposed to form two different complexes with DNA that are in rapid equilibrium: the non-cleavable complex (B) and the cleavable complex (C). The cleavable complex is the transient covalent intermediate. The interaction of the second DNA with the cleavable complex (C) may trigger the translocation of the second DNA through the cleavable complex (not shown).

Mammalian DNA topoisomerase II poisons (e.g. amsacrine) stabilize the cleavable complex (C + drug) in the topoisomerase II reaction by forming a reversible drug-enzyme-DNA ternary complex as shown. Cellular processing of the drug-stabilized complex is required to trigger the lethal effect of topoisomerase II poisons (From Liu, 1989).
1.3.5 Effects of amsacrine, other intercalating agents and epipodophyllotoxins on topoisomerase II reactions in vitro.

Amsacrine, other intercalating agents and epipodophyllotoxins in low concentrations cause an increase in the frequency and number of sites at which topoisomerase II molecules integrate into defined DNA substrates. Nelson (1984) proposed that amsacrine for example, interfered with the breakage-reunion of DNA topoisomerase II by trapping the putative key reaction intermediate, the cleavable complex (Figure 1.2) described above. Amsacrine presumably stabilizes the cleavable complex by forming a non-productive drug-enzyme-DNA ternary complex. Amsacrine then can be considered as a stimulator of the topoisomerase II-DNA association, but an inhibitor of the strand-passing reaction. This "trapping" of the enzyme is not "inhibition" in a formal sense and has been termed "poisoning" (Nelson et al., 1984). The phenomenon explains the properties of DNA isolated from cells exposed to amsacrine because integrated topoisomerase II molecules are "trapped" in the cells DNA during their reaction and are denatured on DNA isolation, yielding DNA fragments bearing topoisomerase II polypeptides covalently bound to the 5'-termini (Marshall et al., 1983b; Ralph and Hancock, 1985).

As yet, there is no evidence that overt breaks exist in the DNA within cells exposed to these agents; the hidden or cryptic breaks are revealed and the DNA fragmented only during its isolation, as a consequence of denaturation of integrated topoisomerase II molecules. The relevance of these drug-induced protein-associated DNA breaks to the cytotoxic potency of a given compound must therefore be questioned and indeed is more fully examined in Chapter 3.

At the molecular level, two unusual features of the cleavage reaction
have been observed:

(1) intercalators from different chemical classes stimulated topoisomerase II-mediated DNA cleavage at different sites, whereas intercalators from the same chemical class stimulated cleavage at similar sites (Pommier et al., 1984b).

(2) high concentrations of intercalators inhibited topoisomerase II -mediated DNA cleavage (Pommier et al., 1984b).

The inhibition at high concentrations is presumably due to template blockage by intercalators. However, the sequence specificity reflected in different cleavage patterns of various drugs are more difficult to explain, although it suggests some selective influence of intercalating drugs on topoisomerase II cleavage specificity (Tewey et al., 1984a). This selectivity may reflect both specific drug-DNA interactions and enzyme-DNA interactions, consistent with the ternary complex hypothesis.

In recent studies, Spitzner and Muller (1988) derived a consensus sequence for cleavage by vertebrate DNA topoisomerase II, which was catalogued in the presence and absence of drugs that stabilize the cleavage intermediate. Moreover, 85% of all cleavages detected with amsacrine were identical to cleavages with the non-intercalating drug, VM26 and vice versa. The consensus is,

\[
-10 -8 -6 -4 -2 -1 1 3 5 7
5' A/G N T/C N N C N N G T/C V N G G/T T N T/C N T/C 3'
\]

where \(N\) is any base and cleavage occurs at the indicated mark between -1 and +1. The consensus accurately predicts topoisomerase II sites in vitro and is likely to apply in vivo as well, because in vivo sites have been reported to be a subset of the in vitro sites (Yang et al., 1985b).
Udvardy et al., 1986; Spitzner and Muller, 1988). The data indicated that DNA cleavage by topoisomerase II proceeds by 2 asymmetric and single-strand cleavage and resealing steps on opposite strands of the DNA (separated by 4 base pairs) and that the consensus is more accurate when both DNA strands are considered as it was shown that contributions from both strands affect the sites cleaved and the efficiency of the cleavage. This suggests that the action of topoisomerase II is not dictated by the sequence on a single strand of DNA but rather requires that each strand be recognized (either simultaneously or independently) by one topoisomerase II subunit. The vertebrate consensus sequence is not closely related to the Drosophila consensus sequence, but the two enzymes show some similarities in site recognition. However, topoisomerase II purified from human placenta cleaves DNA sites that are identical to the chicken enzyme, suggesting that vertebrate type II enzymes share a common catalytic sequence. Thus, Spitzner and Muller propose that the chicken topoisomerase II consensus sequence is a better predictor of cleavage sites by vertebrate compared to invertebrate enzymes.

As yet there are no detailed models explaining the molecular events involved in the trapping of topoisomerase II by topoisomerase II-targeted drugs or on the basis of apparent cleavage site selectivity. However, it is clear that development of such models is paramount to the understanding not only of drug action, but also possible mechanisms by which modifications of the enzyme (or other influencing factors) confer resistance.

1.4 Biological role of topoisomerase II.

Amsacrine and similarly targeted agents produce a spectrum of effects
on cells growing in culture and it is important to elucidate the singular lesion responsible for the cytotoxic action of these drugs. Correlations between the frequency of drug-induced trapping of topoisomerase II, resultant DNA damage and cytotoxicity (reviewed by Liu, 1989) of a number intercalating antitumour agents suggests that this "poisoning" of topoisomerase II on DNA may represent a form of lethal damage. I will return to the molecular basis of possible cell killing mechanisms in Section 1.5, but understanding this question is also dependent on knowledge of the role of the drug's target, the enzyme topoisomerase II, in the growth of mammalian cells.

The following is a review of the known biological roles of mammalian DNA topoisomerase II to April 1989.

Mammalian cells contain of the order of $10^6$ molecules of topoisomerase II per cell (Heck and Earnshaw, 1986). Mammalian DNA topoisomerase II, typified by human topoisomerase II (MW=170 kDa), is a homodimeric protein, encoded by a single copy gene on human chromosome 17q21-22 (Miller et al., 1981; Pflugfelder et al., 1988). It, like other eukaryotic topoisomerases and T4 topoisomerase, uses ATP to facilitate the strand passing of two DNA segments enabling it to relax superhelical twists in DNA molecules, to catenate and decatenate covalently closed double-stranded circular DNA molecules, and to knot and unknot DNA (Wang, 1985). Under ordinary circumstances, type I topoisomerase enzymes (which do not use ATP) perform only the relaxation of superhelical twist (Wang, 1983).

Sequence analysis of type II topoisomerases suggests that human or other eukaryotic type II DNA topoisomerases evolved from the bacterial equivalent, DNA gyrase, by fusion of the two gyrase subunits into a single polypeptide. Unexplicably however, purified eukaryotic type II enzymes do
not have the supercoiling activity, characteristic of the bacterial DNA gyrase. The partial reaction of mammalian topoisomerase II proposed by Nelson et al. (1984) is described in Section 1.3.4.

Until recently, the wealth of knowledge of the in vitro reactions of all classes of topoisomerases was in striking contrast to the limited understanding of the functions they serve in living cells. However, one of the significant achievements in the field of topoisomerase related research over the last year has been the progress made in understanding the biological role of these enzymes. The assignment of a number of in vivo functions to topoisomerase I and II has been greatly assisted by the exploitation of drugs specifically targeted at the enzymes and as a result of recent availability of prokaryotic and some eukaryotic mutants. However, even though defective mutants have recently been isolated in Saccharomyces and Schizosaccharomyces, the phenotypes they exhibit under restrictive conditions are complex. For example, mutants defective in topoisomerase I function are viable in both yeasts (Thrash et al., 1984; Uemura and Yanagida, 1984). In contrast, topoisomerase II is an essential gene (DiNardo et al., 1984; Uemura and Yanagida, 1984). In its absence, cells die in anaphase of mitosis (Holm et al., 1985; Uemura and Yanagida, 1986), implying that sister chromatid segregation involves the decatenation of topologically interlocked DNA domains (an activity that cannot be performed by type I topoisomerases).

Studies of the replication of SV40 DNA in vivo (Yang et al., 1987; Richter and Strausfeld, 1988) and of Cytomegalovirus DNA (Benson and Huang, 1988) suggest that topoisomerase II plays a role in the synthesis of the terminal DNA region as well as in the segregation of daughter chromosomes. For example, Richter and Strausfeld (1988) showed that the topoisomerase II poison, VM26, rapidly inhibits SV40 replication and
prevents the conversion to mature fully replicated SV40 DNA resulting in an accumulation of late replicative intermediates. Furthermore, Benson and Huang (1988) showed that Cytomegalovirus DNA replication was inhibited by amsacrine only if the drug was present well into the onset of replication, and their data were consistent with a role for topoisomerase II throughout the elongation and terminal phases of DNA replication. Moreover, these last steps involve the unwinding of the remaining terminal section of parental DNA, concomitant with separation of the progeny minichromosomes. Therefore, it is now thought that topoisomerase II is required as a SWIVELASE for the replication of a terminal segment of viral DNA (SV40 or Cytomegalovirus) genomes. It is likely that the termination of the replication cycle and the segregation of newly formed daughter DNA molecules occurs during one concerted topoisomerase II catalysed event leading to double-stranded DNA circles with single-strand gaps, the major class of early replication products. Such a role in replication is also consistent with the findings of Nelson (1986) that topoisomerase II was associated with daughter DNA molecules near replication forks.

The possible role for topoisomerase II in RNA transcription is uncertain. Topoisomerase II is uniformly distributed along chromosomes and is not enriched in actively transcribed regions (Earnshaw et al., 1985; Rowe et al., 1986b) compared to topoisomerase I (Stewart and Schutz, 1987; Zhang et al., 1988). However, Rowe et al. (1986b), mapping topoisomerase II cleavage sites using VM26, demonstrated strong cleavage sites on both 3' and 5' ends of the Drosophila heat shock gene hsp70. Upon induction of heat shock transcription, a significant increase in topoisomerase II cleavage sites at the 3' end of the hsp70 genes was observed suggesting a possible role for topoisomerase II in this region during active transcription. Strong topoisomerase II cleavage sites also
often occur adjacent to, or within gene enhancer regions (e.g. Yang et al., 1985a, b; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Jarman and Higgs, 1988). However, at best the evidence for the involvement of topoisomerase II in transcription is circumstantial and requires further investigation.

Recent studies have shown that mammalian type II topoisomerases, like other type II topoisomerases can promote illegitimate and homologous recombination in vitro (Bae, 1988; Christman et al., 1988; Nitiss and Wang, 1988). The suppression of homologous recombination in rDNA, by both topoisomerases I and II has been suggested by two independent lines of evidence (Christman et al., 1988; Nitiss and Wang, 1988). It was postulated that by introducing supercoils (under as yet unidentified conditions) topoisomerase I and II keep rDNA in a form not suitable for recombination. Consequently as ansacrine, but not oAMSA induces high levels of homologous recombination, this could be one mechanism through which the inhibition of topoisomerase II by ansacrine leads to cell death.

Ryoji and Worcel (1984) invoked the requirement for type II topoisomerases in the assembly of both active and static chromatin. In support of this idea, recent studies using topoisomerase II poisons have shown that topoisomerase II is needed during the post assembly stage involving the maturation of the assembled DNA (Newport, 1987). This maturation step involves the correct spacing of nucleosomes and stabilization of the chromatin in an ATP/Mg$^{2+}$ dependent reaction, consistent with the action of topoisomerase II (Almouzni and Mechali, 1988).

The biological functions assigned to topoisomerase II discussed so far have centered on the molecule's enzymatic activity. However, it is now becoming clear that topoisomerase II fulfills a dual catalytic/structural
role in cells, much like myosin and dynein. Earnshaw et al. (1985) have shown that the protein is one of the major components of the mitotic chromosome scaffold fraction, a biochemical fraction containing the most insoluble polypeptides of chromosomes (Adolph et al., 1977). In contrast, topoisomerase I is not found in the scaffold fraction, and it is quantitatively extracted by 1 M NaCl (McConaughy et al., 1981). Topoisomerase II appears concentrated in anchoring complexes at the base of the chromatin loop domains, and thus may fulfill a structural role by maintaining the loop domain architecture of the chromosomes (Laemmli et al., 1977). In addition, it may have an enzymatic role in manipulating the supercoiled state of chromatin loops which may be important in both replication and gene expression. Furthermore, topoisomerase II may have an important dual role throughout interphase as well since the enzyme is one of the major components of the nuclear matrix fraction in Drosophila melanogaster (Berrios et al., 1985).

Regions of attachment of DNA to the nuclear matrix or scaffold have recently become of great interest. They are known as Scaffold-associated-regions (SAR) and are characterized by short 500 bp A/T rich segments of DNA. Cockerill and Garrard (1986), Gasser and Laemmli (1986) and Jarman and Higgs (1988) have all shown that the consensus interaction sequence for topoisomerase II and SARs share certain, and in the case of Jarman and Higgs (1988) strong, sequence homologies. Often these sequences are closely associated with enhancer elements or within putative regulatory domains of genes. Thus, enhancers may be sites of interaction with the nuclear matrix mediated by topoisomerase II binding which further suggests a role for topoisomerase II in the control of gene expression.

Heck et al. (1988) examined the pattern of synthesis and degradation
of topoisomerase II around the cell cycle. They found that the expression
of topoisomerase II is tightly regulated, both as a function of cell cycle
position and as a function of proliferative state. Although the synthetic
rate remained high throughout S and G2 phase in log phase cells, the
stability of the enzyme varied significantly across the cell cycle. For
example, during the transition from mitosis to G1, the half-life of
topoisomerase II decreased approximately 7-fold to 2 hours. In other
words, chromosome condensation was accompanied by SYNTHESIS of
topoisomerase II, whereas chromosome decondensation was accompanied by a
programmed DEGRADATION of the enzyme. These observations suggest an
involvement of topoisomerase II in a new aspect of cellular control of the
dramatic structural alterations that occur at the onset of mitosis.

The roles of topoisomerase II in certain critical cellular events such
as DNA transcription, replication and mitotic segregation are becoming
better understood, although additional clarifications are still needed.
However, an involvement of topoisomerase II in excision repair is more
speculative and more controversial. I believe that this comes about
largely from the scant concrete knowledge of the workings of mammalian DNA
repair systems e.g. compared with the understanding of bacterial systems.
A number of workers have used topoisomerase II inhibitors, such as
novobiocin and nalidixic acid, to determine the involvement of
topoisomerase II in DNA repair (reviewed in Downes and Johnson, 1988).
However, these agents are not sufficiently specific for topoisomerase II
and they inhibit a wide range of other cellular functions, a number of
which are relevant to DNA repair. An alternative approach has been the
use of more specific topoisomerase II inhibitors, e.g. amsacrine and VP16,
in intact cells. By using this approach, Downes et al. (1987) and Snyder
(1987) found no correlation with UV excision repair and the inhibition of
topoisomerase II by these and other agents in intact cells. Moreover, it has been known for some time that amsacrine is without effect on UV excision repair in human cells (Wilkins, 1983). In contrast, work with cell-free systems or permeabilized cells (reviewed in Downes and Johnson, 1988) indicates that topoisomerase II inhibitors genuinely block excision repair at a pre-incision topoisomerase step. However, the contradiction may be explained by the peculiarities of cell-free systems or permeabilized cells which are discussed by Downes and Johnson. Studies with a eukaryotic cell mutant in which topoisomerase II is absent, deficient or inhibited are also suggestive of the involvement of topoisomerase II in repair (e.g. Singh et al., 1988). However, these studies are not by themselves conclusive with regard to the involvement of topoisomerase II per se in DNA repair, as there are two classes of DNA topoisomerasases and in many respects they are interchangeable (Uemura et al., 1987).

In summary, the general area of DNA repair in eukaryotes but more importantly mammalian cells, requires much more clarification and obviously is going to be a future area of importance. With respect to the role of topoisomerase II in DNA repair, there appears to be an equivalence in the data implying and negating its involvement in repair. Ideally, repair should be studied in eukaryotic temperature-sensitive topoisomerase mutants. In fact, a brief report by Boyce and Cox (1985) demonstrated that the removal of UV damage from DNA is normal in a temperature-sensitive yeast topoisomerase II mutant at the restrictive temperature, indicating that more research in this vein may be instrumental in elucidating the mechanics of DNA repair.
1.4.1 Cellular regulation of topoisomerase II.

The catalytic activity of purified topoisomerase II can be modified by phosphorylation (Ackerman et al., 1985; Sahouyn et al., 1986) and poly(ADP-ribosylation) (Darby et al., 1985). Indeed, kinase activity has also been shown to co-purify with topoisomerase II (Sander et al., 1984). However, demonstration of physiological modification of the enzyme has been much slower to appear and has only recently been reported in two studies. Kroll and Rowe (1988) using intact Hela cells have shown the existence of two differentially phosphorylated forms of the 170 kDa topoisomerase II, with pIs of 7.8 and >7.8. Only the more acidic form (pI 7.8) was phosphorylated and at either serine or threonine residues but not at tyrosine. $^{32}$P-Labeling of this protein was cell-cycle independent. However, a third high MW (>300 kDa) phosphorylated protein was detected in Western blots with anti-topoisomerase antiserum that was phosphorylated in a cell-cycle dependent S-M phase specific manner. It is not known whether this protein is structurally related to topoisomerase II. Ackerman et al. (1988) characterized the in vivo phosphorylation of topoisomerase II in Drosophila Kc cells. Several lines of evidence identified casein kinase II as the kinase primarily responsible for phosphorylating DNA topoisomerase II including, modification of a serine residue; inhibition of phosphorylation in cell homogenates by heparin (in the concentration range known to inhibit casein kinase II) and anti-Drosophila casein kinase II antiserum; the ability of cell homogenates to employ $^{[\gamma-^{32}P]}$GTP as a phosphate donor nearly as well as $[^{\gamma-^{32}P}]$ATP.

The role of these modifications in controlling the topoisomerase II's activity in vivo is not yet known, but it is becoming clear that post-transcriptional modifications have implications for the observed
heterogeneity of topoisomerase II and the efficacy of drugs targeted at the enzyme. Moreover, recent data suggests that there is a functional link between phosphorylation by protein kinase C, topoisomerase II activity and cellular differentiation (Constantinou et al., 1988; Gorsky et al., 1988). The influence of phosphorylation of topoisomerase II on drug action is discussed more fully in Chapter 9.

1.5 The question of cytotoxicity: Possible mechanisms of cell killing by mammalian topoisomerase II poisons.

Despite the development of a general model for the action of topoisomerase II poisons, there is still no clear understanding of the actual event(s) that leads to tumour cell killing by these drugs. However, correlations have been described between the cytotoxic action of topoisomerase inhibitors and the production of DNA double-strand breaks, chromosome breakage and sister chromatid exchanges (SCE) (Pommier et al., 1985a).

Substantially increased SCE and chromosome damage and rearrangement is observed in cells treated with drugs such as amsacrine or adriamycin suggesting that topoisomerase II is normally involved in chromosomal rearrangements (Vig, 1971; Deaven et al., 1978; Crossen, 1979).

Moreover, West et al. (1981) showed that equivalent concentrations of adriamycin and amsacrine produced similar toxicities in Chinese hamster V79 cells and that equivalent subtoxic doses of the drugs caused similar increases in SCE. Similarly, other workers (reviewed in Ralph and Schneider, 1987) have concluded that chromosome breakage and SCE induced by intercalating drugs (e.g., amsacrine) correlate with drug cytotoxicity. However, the precise nature of the cytotoxic event is unknown and may
result from general disruption of intracellular regulation following excessive chromosome damage or rearrangements.

Although cleavable complex formation was originally thought not to correlate with drug cytotoxicity (Ross et al., 1979; Zwelling et al., 1981, 1982a, b; Pommier et al., 1983), strong correlations have now been reported between the level of protein-linked breaks and cell death (Chapter 3; Rowe et al., 1986a; Covey et al., 1988). Therefore, it now seems likely that drug-induced formation of topoisomerase II-DNA cleavable complexes is responsible for cell killing, as well as other cellular responses such as SCE and chromosome aberrations. However, because of the reversibility of the DNA damage, the question of how the cell killing signal is triggered, is raised. Rowe et al. (1986a) proposed that drug stabilization of the cleavable complex is an important initial step in a chain of events involving additional factors which eventually causes cell death. In support of this proposal, a number of studies have now shown that other cellular processes must interact with the cleavable complex to trigger cell killing. For example, CHO cells in a thermo-tolerant state subsequent to heat shock treatment were protected from topoisomerase II poisons, although cellular levels of topoisomerase II and drug-induced cleavable complexes did not change (Li, 1987). Uncouplers of oxidative phosphorylation, specifically dinitrophenol, also protected L1210 cells against topoisomerase II poisons without observed changes in drug-induced cleavable complex formation (Kupfer et al., 1987). Furthermore, it was recently shown that inhibition of protein synthesis with cycloheximide diminished the cytotoxic action of anticancer drugs such as ansacrine or VPl6 without decreasing the level of topoisomerase II in cells (Chow et al., 1988; Schneider et al., 1988d). Although the mechanisms of protection are unclear, these
experiments suggest that the production or level of cleavable complexes alone is not sufficient to predict or confer cytotoxicity. Moreover, cellular processing of the cleavable complexes may be an important event in mediating the effects of cleavable complexes.

In summary, exactly how drugs that inhibit topoisomerase II kill cells is still unclear and the precise nature of the cytotoxic event(s) involved is unknown. Additional biochemical and genetic studies are needed to identify the primary event leading to cell death.

1.6 Modes of resistance to anticancer drugs.

Drug resistance in many common cancers has long been a stumbling block to successful chemotherapy and it presents one of the most challenging topics in cancer treatment research today. The long-term clinical effectiveness of amsacrine, other intercalating agents and the epipodophyllotoxins is severely limited by the inherent resistance of many tumours to these drugs, or by the emergence of resistant tumour cells after several months of treatment. Research with these drugs on cells in culture has revealed that drug resistance is a complex phenomenon which has multiple mechanisms.

Prolonged drug treatment of tumours often selects drug-resistant populations of cells. Similarly, the selection of resistant tumour cells in culture can be achieved by the step-wise increase of certain cytotoxic drugs. Mutant cells so selected often display altered drug transport or accumulation and cross-resistance to a number of structurally unrelated drugs, including topoisomerase II poisons (Riordan and Ling, 1985; Roninson, 1988). This multidrug resistance phenotype is apparently due to the overexpression in resistant cells of the MDR 1 gene which encodes a
170kD membrane P-glycoprotein (reviewed by Bradley et al., 1988).

Although the MDR phenotype due to this protein is frequently observed in cell lines, the resistance of many other cell lines in culture is NOT correlated with modifications of intracellular drug concentration or efflux rate (Glisson et al., 1986a, b; Pommier et al., 1986a; Danks et al., 1988; Mirski et al., 1987; Snow, 1988) or with the presence of overexpressed P-170 mRNA (Beck et al., 1987) which are characteristic of the MDR system. Moreover, drug resistant human ovarian cancer cells from untreated or refractory patients showed no difference in drug accumulation or metabolism indicating that other mechanisms of resistance may develop in vivo (Louie et al., 1985).

The identification of topoisomerase II as the target for a number of clinically important antitumour drugs (Chen et al., 1984; Nelson et al., 1984; Tewey et al., 1984a,b) has led to the re-investigation of drug-resistance mechanisms. In fact, in Chinese hamster ovary cells in culture, multidrug resistance has been linked to changes in topoisomerase II rather than drug transport (Glisson et al., 1986a; Pommier et al., 1986a). More recently evidence has been presented that both topoisomerase II and MDR related resistance can occur simultaneously within the same cell (Sinha et al., 1988; Danks et al., 1988), although the two in combination may still not be sufficient to explain extremely high degrees of drug resistance (Sinha et al., 1988).

Despite topoisomerase II being ubiquitous there are marked differences in the response of different tumours to the cytotoxic action of the topoisomerase II-specific anticancer drug amsacrine (Carroll et al., 1980; Casper et al., 1980; Schneider et al., 1980). Amsacrine has recorded clinical success against leukemia and lymphomas (Legha et al., 1982; Weick et al., 1983), but little activity against solid tumours
(Legha, 1983). The reasons for the resistance of solid tumours not only to amsacrine, but also other antitumor drugs, are not yet fully understood and not surprisingly are proving as complex as the nature of the solid tumours themselves.

By the time a tumour has grown to a detectable size the cancer cells and their local environments have often become heterogeneous. Subpopulations of cells may have developed a variety of growth and functional properties as well as diverse responses to therapeutic modalities (Sutherland, 1988). Inefficient vascular function within a tumour resulting in lack of oxygenation, nutrient deficiencies or growth factor depletion often brings about a state in which the interior of tumours becomes hypoxic and cells cease to cycle. There is now increasing evidence to suggest that in this non-cycling state the cells within a solid tumour develop resistance to the action of cell-cycle specific anticancer drugs, such as amsacrine. Major differences in sensitivity to drugs attributable to effects on quiescent cells as well as accessibility, uptake and local microenvironments have been demonstrated in tumour cells grown as spheroids. For example, Wilson et al. (1981b) in studies of the cytotoxic effects of amsacrine on cells in multicellular Chinese hamster spheroids demonstrated that cells at increasing depths from the spheroid surface were less susceptible to the cytotoxic effects of the drug and that a graded sequence of decreasingly actively cycling cells exists towards the interior of spheroids and presumably the interior of solid tumours. Similar conclusions were reached in other spheroid systems, for example with adriamycin and EMT6/Ro mouse mammary tumour spheroids (Sutherland et al., 1979; Sutherland, 1988 and references therein).

It has been suggested that the low numbers of actively cycling cells
in solid tumors may limit the tumour-killing activity of topoisomerase II-specific drugs, since non-cycling cells per se appear to be less sensitive to these drugs. For example, Wilson et al. (1981c) showed that the sensitivity of CHV79-171b cells to amsacrine decreased when the cells were grown to plateau phase and compared with early log-phase cells, apparently as a consequence of their non-cycling state. Drewinko et al. (1982) also found that non-proliferating human carcinoma cells were 10-fold less susceptible to the cytotoxic effects of amsacrine than proliferating cells. Recent evidence (Chow and Ross, 1987; Finlay et al., 1987a; Zwelling et al., 1987; Robbie et al., 1988) confirms and extends these earlier studies and it is now agreed that amsacrine and other intercalating drugs are most cytotoxic for cycling cells, although non-cycling cells may be killed at high drug concentrations. The resistance of non-cycling cells would appear to be a logical consequence of the selective action of these agents on dividing cells. The question is now, what are the molecular mechanisms underlying the resistance of non-cycling cells to drugs? Are qualitative or quantitative changes in intracellular targets in non-cycling cells causing or contributing to the observed drug resistance?

In a number of reports, the activity of topoisomerase II has been examined in relation to changes in the proliferative activity of cells and a tight coupling between topoisomerase II activity and the cell cycle has been implied. However, contradictory results suggest that generalizations may not hold for all tissues. For example, during terminal differentiation of erythroblasts into erythrocytes and of myoblasts into myotubes, a total loss of topoisomerase II antigen was observed (Heck and Earnshaw, 1986), although in brain tissue topoisomerase II activity has been shown to remain high in terminally differentiated cells (Tsutsui et
al., 1986). Furthermore, when peripheral blood lymphocytes (which lack detectable topoisomerase II) were stimulated to proliferate, de novo enzyme synthesis occurred and exactly paralleled the onset of DNA synthesis (Heck and Earnshaw, 1986; Taudou et al., 1984). Considerable increases in topoisomerase II activity were also observed in regenerating rat liver after partial hepatectomy (Duguet et al., 1983). In contrast, Tricoli et al. (1985) found that proliferating mouse embryo fibroblasts (C3H10T 1/2 cells) had four-fold increased topoisomerase I, but had similar topoisomerase II activity compared to confluent cells.

Recently, several studies investigating the relationship between the state of cell proliferation or phase of the cell cycle, DNA topoisomerase II and drug sensitivity found that both topoisomerase II activity and drug sensitivity vary as a function of cell proliferation (Sullivan et al., 1986, 1987; Markovits et al., 1987a; Zwelling et al., 1987; Chow and Ross, 1987; Estey et al., 1987a; Potmesil, 1988; Schneider et al., 1988b). In general, maximum drug sensitivity was observed in actively proliferating or S-phase cells, concomitant with a high topoisomerase II activity, although there was some variation between different cell lines. For instance, when Sullivan et al. (1986, 1987) studied drug-enzyme interactions in relation to the state of cell proliferation, they found little difference in drug-sensitivity between log- and plateau-phase HeLa or L1210 cells, but a marked decrease in the sensitivity of plateau-phase CHO cells towards amsacrine or the epipodophyllotoxin VP-16 compared with log-phase cells. The decreased drug sensitivity in plateau-phase cells was concomitant with decreased topoisomerase II activity and a reduced capacity to form cleavable complexes. Markovits et al. (1987a) reported higher frequencies of amsacrine- or VP-16 induced protein-DNA complex formation in nuclei from exponentially growing than from quiescent 3T3 or
L1210 cells, and maximum protein-DNA complex formation coincided with the peak of DNA synthesis and topoisomerase II strand-passing activity. On the other hand, a lack of correlation between maximal sensitivity to drug cytotoxicity and maximal DNA breakage was reported for HeLa and Balb/c 3T3 cells (Chow and Ross, 1987; Estey et al., 1987a). Together, these results suggested that there exists a complex relationship between the proliferative state of cells, their sensitivity to the cytotoxic activity of topoisomerase II-directed anticancer drugs, topoisomerase II and drug-induced DNA breakage.

1.7 Aims of this study.

At the outset of this study, topoisomerase II-associated DNA breaks induced by intercalating drugs were considered not to be cytotoxic (Section 1.3). However, this conclusion was based upon studies that compared intercalators from many different chemical classes. Therefore, I set out to assess whether this type of DNA damage was responsible for drug cytotoxicity by comparing the amounts of protein-linked breaks produced by a number of different acridine derivatives of varying potency in vivo, but belonging to the same chemical class. Secondly, I wished to clarify which of the activities of topoisomerase II after drug treatment, DNA binding or strand passing, was related to cell death.

Since evidence had accumulated that non-cycling cells were much more resistant to drugs affecting topoisomerase II, I also proposed to explore possible reasons for the resistance of G1(G0) phase cells to amsacrine at the molecular level, with particular reference to the role of topoisomerase II in this resistance. Eventually this became the major aim of the research described in this thesis. To achieve this goal, the
relationships between amsacrine sensitivity and DNA topoisomerase II were compared using two model non-cycling cell systems, namely (1) the CHO-AA8 cell system and (2) the cold sensitive cell cycle mutant (21-Fb) of the murine mastocytoma cell line P815.

In the course of developing an assay with which to quantitate drug-induced topoisomerase II-DNA complexes (PDC) in isolated nuclei, I discovered a cytoplasmic factor that enhances the drug-induced PDC lesion. Because it is conceivable that the resistance of some cancer cells to drugs such as amsacrine could be related to the absence or availability of protein factors that influence drug action, e.g. in non-cycling cells, the nature of the factor and its possible involvement in amsacrine action and resistance were investigated.

The bulk of the research presented in this thesis has been published: Parts of Chapter 3 are included in Schneider et al., 1988a; Chapter 4, Parts I and II comprise two independent publications, Schneider et al., 1988 b, c; the results in Chapters 5 and 6 are presented in Darkin and Ralph, 1989; and those in Chapters 7 and 8, are in preparation.
MATERIALS AND GENERAL METHODS

2. MATERIALS

2.1 CHEMICALS AND MATERIALS

2.1.1 Drugs and Inhibitors.

Amsacrine (mAMSA), isethionate salt NSC 249992; oAMSA, methane-sulfonate NSC 1563206; 9-aminoacridine hydrochloride SN 17764; acridine carboxamide NSC 601316; C1-921 isethionate NSC 343499, and C-6 bisacridine dihydrochloride NSC 210733 were generously provided by Associate Professor B.C. Baguley, Cancer Research Laboratory, University of Auckland School of Medicine. Quinacrine hydrochloride was obtained from Sigma Chemical Co. The drugs were stored as 1mM stock solutions in water at -20°C and working dilutions were made immediately prior to use.

Thymidine, amethopterin and hypoxanthine were purchased from Sigma Chemical Co.

Ethidium bromide (Sigma Chemical Co.) was stored in the dark at 4°C as a 10 mg/ml solution and diluted to 0.5 μg/ml as necessary.

The protease inhibitors aprotinin, leupeptin, α-2macroglobulin, phenylmethylsulfonylfluoride and diisopropylfluorophosphate and the protein and RNA synthesis inhibitors cycloheximide and cordecypin were obtained from Sigma Chemical Co.

2.1.2 Proteins and Enzymes.

Bovine serum albumin, Proteinase K and DNA Pol I Klenow fragment were obtained from Boehringer Mannheim Ltd. and the restriction enzyme EcoRI from Promega Biotec.
Enzymes on beaded agarose (bacterial protease type XXIV-A, 2 units per 2 ml packed gel; trypsin from bovine pancreas, 78 units per ml packed gel; papain, 825 units per g lyophilised solid) and ribonuclease A were from Sigma Chemical Co.

All enzymes were used according to the manufacturers' recommendations.

2.1.3 Radiochemicals.

[methyl-\textsuperscript{3}H]Thymidine (70-90 Ci/mmol) was from Amersham. [\textsuperscript{32}P]-Adenosine 5'-triphosphate (800 Ci/mmol) and [\textsuperscript{35}S]-Deoxyadenosine 5'-triphosphate (500 Ci/mmol) were from New England Nuclear.

All radiochemicals were stored at 4°C.

2.1.4 Tissue Culture Media.

Tissue culture media were from GIBCO (N.Z.) Ltd. or Sigma Chemical Co. Foetal calf serum, neonatal calf serum and horse serum were all purchased from GIBCO (N.Z.) Ltd.

2.1.5 General Chemicals.

General chemicals were from BDH Chemicals, Serva Biochemicals, Sigma Chemical Co., BRL or Merck.

2.1.6 Miscellaneous Materials.

Dextran Grade B 150-200, BDH.
Digitonin, BDH.
Hoechst 33258, Calbiochem Biochemicals.
p-iodonitrotetrazolium violet, Serva, Heidelberg, Germany.
Glass Fibre Filters (GF/B and GF/C), Whatman, U.K.
Dialysis membranes, Spectropor 6 (50,000 MW cut off), Spectrum
Medical Industries Inc. and Standard Visking (10,000 MW cut off), Serva, Heidelberg, Germany.
Cellulose nitrate and polyallomer centrifuge tubes, Beckman Instruments Ltd.
Photographic film and developer D19, Kodak (N.Z) Ltd.
Fixer (Amfix Rapid fixer), May and Baker Ltd., Lower Hutt, N.Z.
Nunc tissue culture flasks, petri dishes (60mm diameter)
and microtitre plates (24 and 96 well), GIBCO (N.Z.) Ltd.

2.2 BIOLOGICAL MATERIALS
2.2.1 Eukaryotic Cell Lines.
The clonal wild-type subline (K21) of the P815-X2 mouse mastocytoma cell line (Schindler et al., 1958) and the temperature sensitive cell cycle variant 21-Fb, derived from the parent K21 clone were a kind gift of Dr. R. Schindler, University of Berne, Switzerland.

L1210 mouse leukemia cells originating from Arthur D. Little Inc. Michigan were provided by Associate Professor B.C. Baguley, University of Auckland, School of Medicine.

The Jurkat cell line was derived in 1974 from the peripheral blood of a 14 year old boy with acute lymphoblastic leukemia (Schneider et al., 1977; Martin et al., 1982). The amsacrine sensitive and resistant Jurkat sublines used were selected and kindly provided by Dr. K. Snow, Auckland University, Department of Cellular and Molecular Biology.

CHO-AA8 cells were graciously provided by Drs. W.R. Wilson and M.A. Robbie, Oncology Department, Auckland University, School of Medicine.

Details of cell culture are outlined in section 2.5.

All cell lines used were stored as frozen stocks under liquid nitrogen
in growth medium supplemented with 10 % DMSO.

2.2.2 Bacteriophage and Host Bacterial Strains.

Bacteriophage P4 and host bacterial E.coli strains C-117 and C-2323 were a gift from Dr. R. Calendar, Department of Molecular Biology, University of California, Berkeley, California, U.S.A.

2.2.3 Plasmid Strains.

Plasmid pBR322 was kindly provided by Professor P. Berquist, Auckland University, Department of Cellular and Molecular Biology.

2.3 CULTURE MEDIA AND SUPPLEMENTS

2.3.1 RPMI 1640 Tissue Culture Medium.

RPMI 1640 medium was prepared as a twice strength concentrate by dissolving 19.18 g in 1 L of distilled H₂O. The pH of the medium was adjusted with CO₂ prior to filtration through a 0.45 μm Millipore filter. Sterile media was stored at 4°C.

A final 1 x strength RPMI medium was supplemented with 0.2 % Na₂HCO₃, 30 μg/ml penicillin, 50 μg/ml streptomycin and 10 % (v/v) of the appropriate serum as described in section 2.5.

2.3.2 Bacterial Culture Media and Solid Media.

Luria broth (L-broth): 1 % bactotryptone, 0.5 % yeast extract, 1 % NaCl (pH 7.0).

L-agar: L-broth with 2.2 % Davis Agar.
2.4 COMMONLY USED BUFFERS, SOLUTIONS AND REAGENTS

Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.6 mM KCl, 8 mM Na₂HPO₄,
1.4 mM KH₂PO₄ (pH 7.5).

Tris Buffered Saline (TBS): 25 mM tris-HCl (pH 7.5), 137 mM NaCl, 5 mM KCl.

TBE Buffer: 89 mM tris, 89 mM boric acid, 2 mM EDTA (pH 8.0).

TE Buffer: 10 mM tris-HCl (pH 8.0), 1 mM EDTA.

SSC: 150 mM NaCl, 15 mM tri-sodium citrate.

Bradford reagent: 100 mg Coomassie Brilliant blue G250 dissolved in 50 ml absolute ethanol, 55 ml phosphoric acid added and the solution diluted to 1 L.

Scintillation Fluid: 0.3 % PPO, 0.01 % dimethyl POPOP in toluene.

GENERAL METHODS

2.5 EUKARYOTE CELL CULTURE

The mastocytoma cell lines K21 and 21-Fb, the mouse leukemia L1210 line and human Jurkat lines were all cultured in RPMI 1640 medium, however they had different serum requirements. RPMI 1640 was supplemented with 10% horse serum for growth of the K21 and 21-Fb lines, 10% foetal calf serum and 50 µM 2-mercaptoethanol for the L1210 cell line and a mixture of 2.5% foetal calf serum / 7.5% neonatal calf serum for the Jurkat cell sublines.

Cell lines were grown in glass conical flasks (K21, 21-Fb, L1210) or Nunc tissue culture flasks (Jurkat sublines) in an atmosphere of 5% CO₂ plus 95% air at 37°C with the exception of the 21-Fb line which was cultured at the permissive temperature of 39.5°C.

Cultures were diluted every 24 h with fresh growth medium to 2x10⁵ cells/ml. The normal culture doubling times were 10-12 h for K21, 21-Fb and L1210 cell lines and 24 h for the Jurkat sublines.
For large scale culture, cells were grown in 1 L glass Schott bottles gassed with 5% CO₂ in air, sealed and incubated with magnetic stirring in a waterbath of the appropriate temperature (i.e. 37°C for K21, L1210 and Jurkat cells, 39.5°C or 33°C for 21-Fb cells).

CHO-AA8 cells were maintained as subconfluent monolayers and used to establish suspension cultures in glass spinner flasks. The growth medium was alpha-minimum essential medium containing 10% foetal calf serum, 100 μg/ml streptomycin sulphate and 100 iu/ml penicillin G. Flasks were gassed with 5% CO₂ in air, sealed and incubated with magnetic stirring in a 37°C waterbath (Robbie, 1988).

All glassware used for cell culture was sterilised by autoclaving. Solutions were sterilised by filtration through 0.45 μm millipore filters.

2.5.1 Induction of Growth Arrest of Temperature Sensitive cells.

21-Fb cells were cultured at the permissive temperature 39.5°C as described above. Growth arrest was induced by incubating cultures at the non-permissive temperature of 33°C. During growth arrest 25% of the culture medium was replaced per day by harvesting a portion of the cell culture by centrifugation and resuspending the cells in fresh growth medium to give a final density of 2x10⁵ cells/ml. Cultures were routinely arrested for 4 days at the non-permissive temperature. Cells were thawed and cultured for approximately 3 weeks then discarded to avoid any accumulation of revertants.

2.5.2 Growth Arrest of K21 Cells by Serum Deprivation.

Cell cultures were centrifuged (900 x g, 5min), washed once with RPMI 1640 and resuspended in 1 x RPMI 1640 medium supplemented with 0.1% (v/v) horse serum or neonatal calf serum at a density of 2x10⁵ cells/ml.
Details concerning periods of serum deprivation are given in the text.

2.5.3 Cell Density Determination.

Cell densities were determined by counting 1 ml samples diluted in 0.9 % (w/v) NaCl in a Coulter Counter or by counting aliquots in an improved Neubauer haemocytometer.

2.5.4 Cell Viability Determination.

Cell viability was determined by trypan blue exclusion. Aliquots (0.2 ml) of a filtered solution of trypan blue (0.16 % (w/v) in 0.9 % (w/v) NaCl) were mixed with aliquots (0.1 ml) of cells and the percentage of non-viable (blue-stained) cells was determined in a haemocytometer within 2 min.

2.6 DRUG TREATMENT

Cells were treated by adding drugs at the required concentration followed by incubation at the appropriate temperature. Drugs were added as sterile concentrated solutions in a volume not exceeding 1 % of the total cell culture volume. Precise details of individual treatments are given in the text.

2.7 ISOLATION OF BACTERIOPHAGE P4 CAPSIDS AS A SOURCE OF KNOTTED DNA

Bacteriophage P4 capsids are a source of highly knotted DNA. To facilitate the purification of knotted DNA, phage capsids must first be separated from the complete phage and phage tails, both of which contain unknotted DNA. The method of Liu et al. (1981) and a protocol provided by Dr. R. Calendar were used for large scale isolation of phage heads as follows.
Cultures of the bacterial strains C-117 and C-2323 were initiated by inoculating a series of tubes containing 1.5 ml L-broth with a scratch of the frozen bacterial stocks and incubating the tubes with aeration during the day at 37°C. When the bacteria had grown, C-117 starter cultures were prepared by inoculating 10 test-tubes containing 1.5 ml L-broth with 50 µl of the C-117 day culture and incubating overnight without shaking at 37°C.

Four 1:100 serial dilutions of phage P4 vir2de122 (Raimondi et al., 1985) were made in P4 buffer (80 mM MgCl₂, 10 mM tris-HCl (pH7.5), 1 % (w/v) ammonium acetate). 50 µl of the C-2323 day culture was added to 100 µl of each phage dilution and incubated at room temperature for 20 min. 5 ml of top agar (0.6 % agar in L-broth, 0.1 % glucose, 1.6 mM MgCl₂, 0.5 mM CaCl₂) at 41°C was added to each tube, mixed gently, then the mixture immediately poured onto 2 % L-agar plates. When set, the plates were turned upside down and incubated overnight at 37°C. The bacterial strain C-2323 was used as an indicator for P4 phage because it is efficient at the attachment of phage capsids to tails, which results in the formation of large plaques which can be easily picked.

After the overnight incubation, 10 plaques were picked and resuspended in 100 µl P4 buffer. To lyse and remove the C-2323 bacteria, 100 µl of chloroform:isoamyl alcohol (24:1, v/v) was mixed with the resuspended plaques and released phage was recovered after centrifugation for 5 s in a microfuge to remove debris. The top layers containing the phage were removed and added to the C-117 overnight starter cultures. 15µl of 100 mM CaCl₂ was added to aid phage adsorption and the cultures were incubated for 10 min at 37°C. The C-117 bacterial strain was used for large scale production of phage capsids as it is inefficient at tailing capsids, resulting in large numbers of incomplete phage molecules.
Ten 2 L flasks containing 400 ml L-broth, 0.1% glucose, 1.6 mM MgCl$_2$ and 0.5 mM CaCl$_2$ were then each inoculated with one of the C-117/P4 phage cultures and shaken at 37°C. Lysis occurred after approximately 4.5 h. At the onset of lysis, when the $A_{600}$ was between 0.7 and 1.5, 4 ml of 0.5 M EGTA (pH 8.8) was added to chelate the calcium and block phage readsorption. When lysis was complete (approx. 1 h after onset) 25 ml of 1 M MgCl$_2$ was added and the cellular debris was removed by centrifugation for 20 min at 11000 x g at 4°C. One third volume (1.3 L) of 2 M NaCl/40% PEG 6000 was added to the combined supernatants which were left overnight at 4°C to precipitate the phage.

The PEG sediment was collected by centrifugation for 30 min at 11000 x g at 4°C, drained very well after a second centrifugation and resuspended in P4 buffer by shaking GENTLY on ice. For every 5 ml of P4 buffer, 2.5 ml chloroform was added (i.e. 50% v/v) (a maximum of 40 ml total) and the centrifuge tubes were rolled until an emulsion formed. The phases were separated by centrifugation at 900 x g for 5 min before the aqueous (upper) layer was removed and its volume measured. CsCl was added to a final density of 1.2 g/ml (RI=1.3529), then the sample was divided and overlayed on 4 preformed gradients with steps of 1.35 (10 ml), 1.45 (10 ml) and 1.7 (5 ml) g CsCl/ml P4 buffer in 40 ml polyallomer tubes. The gradients were centrifuged at 22K in a TST 28.38 rotor overnight at 15°C. After centrifugation, three bands were evident in each of the four gradients, corresponding to densities of 1.28, 1.33 and 1.4 approximately. The bands were removed, like bands combined, their densities adjusted to 1.7 g/ml with CsCl then they were repurified separately by flotation up through a step gradient (as above). The resulting bands were removed and dialysed extensively against P4 buffer to remove the CsCl.
The band running at a density of 1.4 g/ml corresponded to untaiTed phage capsids.

2.7.1 Extraction of Knotted DNA from Isolated P4 Phage Capsids.

Phage P4 capsids were isolated as described in section 2.7. The band comprising phage capsids with a density of approximately 1.4 g/ml on the CsCl step gradient was used as a source of knotted DNA. The capsids were lysed by adding EDTA, Proteinase K and SDS to final concentrations of 20 mM, 100 μg/ml and 1 % respectively, followed by incubation at 37°C for 90 min before the solution was extracted with equal volumes of phenol, phenol-chloroform then chloroform (Maniatis, 1982). The aqueous sample containing DNA was concentrated to less than 1 ml with n-butanol and dialysed against TE buffer. Purity and concentration of the DNA were determined according to Maniatis (1982) and by the DAPI fluorescence test for DNA (section 2.12). The knotted DNA was diluted to 120 μg/ml and stored in aliquots at -90°C. Sufficient P4 knotted DNA for approximately 2000 assays (see section 2.14.1) was recovered from the starting material described.

An aliquot was electrophoresed in a 0.7 % agarose gel to confirm the knotted nature of the DNA.

2.8 ISOLATION OF PLASMID DNA

Plasmid pBR322 DNA was prepared by the alkaline-lysis method of Birnboim and Doly as described by Maniatis et al. (1982).

Contaminating chromosomal and linear plasmid DNA was removed from large scale plasmid DNA preparations by centrifugation in caesium chloride-ethidium bromide gradients. Plasmid DNA prepared by large scale lysis was resuspended in TE, ethidium bromide was added to a concentration
of 500 µg/ml and caesium chloride was added to a final concentration of 1 g/ml giving a density of 1.58 - 1.61 g/ml. After centrifugation for 12 h at 50K in a Sorvall TV865 vertical rotor, the band of covalently-closed circular DNA was removed under long wave length UV light. Ethidium bromide was removed by repeated extraction with water-saturated butan-2-ol, then the DNA was dialysed extensively against TE buffer at 4°C.

2.9 3'-END LABELLING OF pBR322 DNA

pBR322 DNA was 3'-end labelled for subsequent use in topoisomerase II assays (see section 2.14.2C).

EcoRI digested pBR322 DNA (14 µg) was 3'-end labelled using Polymerase I Klenow fragment and either [α32P]dATP or [α35S]dATP (500 Ci/mmol) according to Maniatis et al. (1982).

2.10 RADIOACTIVE LABELLING OF CELLULAR DNA

The specific requirements for labelling cellular DNA with [methyl-3H] thymidine differed for each cell line used in this study. Optimum conditions determined for each cell line (data not shown), are as follows:

The DNA of proliferating K21 and 21-Fb cells was labelled with 3 µCi/ml [methyl-3H]thymidine (70-90 Ci/mmol) in ATHG medium (growth medium supplemented with 0.01 mM amethopterin, 0.03 mM hypoxanthine, 0.01 mM non-radioactive thymidine, 0.1 mM glycine) for 16 h at 37°C for K21 cells and 24 h at 39.5°C for 21-Fb cells. The DNA of 21-Fb cells to be arrested was similarly labelled, but for the last 24 h of culture at the permissive temperature, 39.5°C, and for the first 24 h at the non-permissive temperature, 33°C.
Cellular DNA of CHO-AA8 cells in spinner cultures was radioactively labelled by incubating cells with 0.1 μCi/ml [methyl-³H]thymidine (20 Ci/mmol) plus 10⁻⁶ M non-radioactive thymidine for 24 h at 37°C. Radiolabel was added when cultures reached a density of approximately 6.5 x 10⁴ cells/ml for log-phase cultures (final density 2 x 10⁵ cells/ml) or a density of approx. 6.2 x 10⁵ cells/ml for plateau-phase cultures (final density 1.4 x 10⁶ cells/ml).

Jurkat and L1210 cell DNAs were labelled by incubating cells with 2 μCi/ml and 0.5 μCi/ml [methyl-³H]thymidine (70-90 Ci/mmol) respectively for 16 h at 37°C.

Using these protocols total acid-precipitable radioactivity per 1 x 10⁵ cells was routinely approximately 5 x 10⁴ cpm.

2.11 DETERMINATION OF RADIOACTIVITY INCORPORATED INTO CELLULAR DNA

1 mM Thymidine in 3 ml 1 x SSC was added to aliquots (1 ml) of cells that had been grown with [methyl-³H]thymidine. The cells were then centrifuged (900 x g, 5 min at 4°C), washed once with 1 x SSC and recovered by centrifugation. The pellets were resuspended in 0.5 ml SSC and 0.5 ml DNA carrier solution (0.6 mg/ml calf thymus DNA in 0.4 M NaOH) was added. After standing 30 min on ice, 1 ml of a solution of 12 % (w/v) sodium pyrophosphate in 1.5 M HCl was added and the samples were left for a further 15 min on ice. Radioactive precipitates were then collected on GF/C glass fibre filters under vacuum and washed 5 x with 0.5 % (w/v) sodium pyrophosphate in 0.5 M HCl. Filters were dried at 80°C for 10 min and the radioactivity on the filters measured in a liquid scintillation spectrometer (model LS3801, Beckman Instruments Inc., Irvine, California, U.S.A).
2.12 DAPI FLUORESCENCE ASSAY FOR DNA

The fluorescence of DAPI was used as a quantitative measure of DNA according to the method of Brunk et al. (1979).

The binding of DAPI to DNA is highly specific for A-T base pairs therefore it was essential that the DNA of the standard and sample had the same or similar base composition. Plasmid pBR322 DNA was commonly used as a standard when measuring phage or plasmid DNA samples.

2.13 CLONOGENICITY ASSAYS

Drug sensitivity of cultured 21-Fb cells was determined by colony formation in soft agarose. Cultures were treated with drugs as described in the text, washed twice with growth medium, resuspended and serially diluted with fresh medium to between 250 cells/ml and 400,000 cells/ml. Aliquots (0.25 ml) were mixed with 5 ml 0.2 % agarose in growth medium and poured onto a 10 ml layer of 0.3 % agarose in medium in 60 mm plastic petri dishes. The soft agarose was supplemented with 10 % horse serum, 0.1 mg/ml folic acid, 20 μm sodium pyruvate, 20 μm each L-alanine, L-aspartic acid, L-glutamic acid and L-proline and 0.2 mM cysteine. The plates were incubated at 39.5°C for 10-12 days. Colonies were stained overnight with p-iodonitrotetrazolium violet (1 mg/ml in dH₂O; 1 ml/plate) and the relative survival of drug treated cultures compared to untreated controls was determined.

2.14 TOPOISOMERASE II ASSAYS

The protocols for isolation of nuclei and preparation of nuclear extracts containing topoisomerase II activity are detailed in the text where they are relevant to the series of experiments under discussion. The following section describes the various assays for topoisomerase II used.
throughout this study.

2.14.1 The Phage P4 DNA Unknotting Assay.

The P4 unknotting assay (Liu et al., 1981) measures the ability of topoisomerase II to convert highly knotted P4 phage DNA to its simple circular form by a reaction in which the enzyme catalyses the topological passage of two crossing double-strand DNA chains.

Knotted phage P4 DNA was prepared as described in sections 2.7 and 2.7.1. Assay mixtures (20 µl) contained 50 mM tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM Na₂EDTA, 0.5 mM dithiothreitol, 30 µg/ml BSA, 1 mM ATP and 0.24 µg P4 DNA. After adding 4 µl of extract and adjusting the KCl concentration to 120 mM, the assays were incubated for 30 min at 37°C. To remove proteins, 1 µl 10 % SDS and 1 µl 10 mg/ml proteinase K were added and the mixture was incubated for a further 30 min at 37°C. Samples were electrophoresed in 0.7 % agarose gels in TBE buffer for 4 h at 100V before the gels were stained with ethidium bromide and photographed under UV illumination. Photographic negatives were then analysed by scanning on a microdensitometer. 1 Unit of topoisomerase II activity was defined as the amount of extract (in µg protein) that completely unknotted 0.24 µg phage P4 DNA. An example of a typical assay is shown in Figure 2.1.

2.14.2 Quantitation of Amsacrine-stimulated Covalent Protein-DNA Complex Formation.

Amsacrine-induced formation of protein-DNA complexes (PDC) was measured in intact cells, isolated nuclei and with cell extracts in vitro. Covalent protein-DNA complex formation in cells or nuclei after treatment with amsacrine was measured by determining the amount of radioactive
FIGURE 2.1: The P4 DNA unknottting assay. DNA topoisomerase II activities in cell extracts can be quantitated by the P4-DNA unknottting assay as described in Section 2.14.1. An example of a typical assay is presented. Reaction products were separated on 0.7% agarose gels, stained with ethidium bromide and photographed under UV light. Lane 1: P4 DNA; lanes 2-8: 2-fold decreasing amounts, as indicated, of K21 cell extracts (+ ATP).
LANE 1 2 3 4 5 6 7 8
P4 DNA 1 1 1 1 1 1 1 1
EXTRACT DILUTION 1 1/2 1/4 1/8 1/16 1/32 1/64

P4 circle

P4 knotted
cellular DNA co-precipitated with protein by the addition of 1 % SDS and 65 mM KCl. The method was based on the SDS/K⁺ precipitation assay originally described by Rowe et al. (1986a). The assay provides a non-specific method for measuring the formation of covalent protein-DNA adducts in cells, based on the ability of the ionic detergent, SDS, to neutralize cationic sites of weakly bound proteins, e.g. histones and DNA polymerases, thereby causing these proteins to dissociate from the DNA helix. Proteins that are covalently bound to DNA are not dissociated by SDS and precipitate along with their associated DNA after addition of KCl (Trask et al., 1984). The precipitated protein-DNA complexes can then be trapped on filters while free DNA washes through. The radioactivity remaining associated with the filters serves as a quantitative measure of drug-induced protein-DNA complexes. Cellular DNA was uniformly labelled with radioactive thymidine using the conditions for the various cell lines given in section 2.10.

A. In Intact Cells:

After labelling, cells were collected by centrifugation, washed with TBS, then resuspended in fresh growth medium to a final density of 1 x 10^5 cells/ml if a 24-well microtitre plate was to be used, or 5 x 10^7 cells/ml if a 96-well microtitre plate was to be used. Aliquots of 1 ml or 49 µl were distributed into either 24-well or 96-well plates respectively, then treated with various concentrations of drugs (1 µl) for 60 min at 37°C. It should be noted that refinements of the protocol were made as described in the text, in which the period of drug treatment was shortened to 10 min.

After drug treatment, the microtitre plates were centrifuged at 1200 x g for 10 min at room temperature, the supernatants were discarded and the
cells were lysed by adding prewarmed (65°C) lysis solution (1.25 % SDS, 5 mM EDTA, 0.4 mg/ml salmon sperm DNA; 1 ml or 200 μl of lysis solution were added to the 24-well and 96-well plates respectively). The lysates were transferred to Eppendorf tubes, and when using 96-well plates each well was rinsed with a further 200 μl of lysis solution which was then added to the corresponding Eppendorf tube. 1/4 Volume of prewarmed (37°C) 325 mM KCl was added (250 μl to the 1 ml lysates; 100 μl to the 400 μl lysates) and the tubes were immediately vortexed for 10 s and held at 0°C for 30 min. The precipitates were collected by centrifugation at 15,000 x g for 10 min at 4°C and then resuspended in 1 ml of ice-cold wash solution (10 mM tris-HCl (pH 7.5), 100 mM KCl, 2 mM EDTA) and collected on Whatman GF/C filters under gravity. Tubes were rinsed twice with 1 ml wash solution (0°C) and these washes were also passed through the corresponding filter under gravity. The filters were washed with 15 ml ice-cold wash solution (under vacuum), then dried with 90 % ethanol and finally with acetone, before drying and measuring associated levels of radioactivity in a liquid scintillation spectrometer.

B. In Isolated Nuclei:

The protocol developed for measuring drug-induced PDC in isolated nuclei is described in detail in the text (section 5.2.5 for final reaction conditions). Protein-DNA complexes were precipitated by SDS and K+ and samples were processed using the conditions given in section 2.14.2A for 96-well plates.

C. In Extract Systems:

The SDS/K+ precipitation assay described by Liu et al. (1983) was used to measure in vitro PDC formation in nuclear, soluble or cytoplasmic
extracts. $[^{32}\text{P}]$- or $[^{35}\text{S}]$3'-end labelled EcoRI digested pBR322 DNA was prepared as described in section 2.9 and used as a substrate. Reaction mixtures (50 µl) contained 20 mM tris-HCl (pH 7.5), 10 mM MgCl$_2$, 5 mM EGTA, 0.5 mM dithiothreitol, 30 ug/ml BSA, 2 mM ATP, 50 ng 3'-end labelled pBR322 and varying amounts of drug. The reactions were initiated by adding 10 µl of extract (KCl concentration was adjusted to 120 mM as appropriate) and then incubated for 30 min at 37°C. The reaction was stopped by adding 100 µl of a prewarmed (37°C) solution containing 0.2 M NaOH, 5 mM EDTA, 2 % SDS, 0.5 mg/ml herring sperm DNA for 10 min at 37°C, followed by addition of 50 µl of a solution containing 0.4 M tris-HCl (pH 7.9), 0.4 M HCl, 0.25 M KCl to precipitate covalent protein-DNA complexes for 10 min at 4°C. The precipitates were collected on GF/C filters under gravity and washed several times with ice-cold wash solution (10 mM tris-HCl (pH 7.5), 100 mM KCl, 2 mM EDTA) under vacuum. Radioactivity retained on the filters was measured in a liquid scintillation spectrometer.


A modified form of the Coombes-Pearson (1978) filter binding assay was also used to measure covalent DNA-protein complex formation after treatment of cells or nuclei with amsacrine.

The DNA in logarithmically growing or plateau phase cells was labelled with radioactive thymidine as described in section 2.10. Cells were collected by centrifugation, washed and resuspended in fresh growth medium at a density of 1 x $10^7$ cells/ml. Aliquots (2 ml) were transferred to a series of test-tubes, gassed with 5 % CO$_2$/95 % air and incubated at 37°C for 10 min prior to treatment with various concentrations of
ansacrine for 60 min at 37°C. After drug treatment, cells were harvested by centrifugation, washed once in chilled PBS and resuspended in PBS at 1 x 10^7 cells/ml. Cells were then lysed with 8 ml SDS lysis solution (1 M NaCl, 50 mM tris-HCl (pH 7.6), 1% SDS). Mercaptoethanol was added to 2% and the lysed cells were incubated for 60 min at 37°C. DNA was then purified by standard phenol extraction techniques (Maniatis et al., 1982), precipitated with 2 vols of 90% ethanol at -20°C and collected by centrifugation (3000 rpm for 30 min at 0°C). Recovered DNAs were washed with 70% ethanol containing 0.1 M NaAcetate and redissolved in 1 ml TE buffer. To digest associated RNA the nucleic acids were treated with 5 µg/ml of DNAse-free pancreatic ribonuclease at 25°C for 1 h. The DNA was then re-extracted with phenol, precipitated, washed (as above) and redissolved in 1 ml TE buffer. DNA yields were determined by absorbance at 260 nm according to Maniatis et al. (1982).

The total radioactivity associated with 10 µg DNA from each drug treatment was approximately 5-7 x 10⁴ cpm as determined by TCA precipitation.

As a measure of DNA-protein complex formation the radioactivity (i.e. DNA) associated with protein bound by filters was measured. Three aliquots of 10 µg DNA from each treatment were distributed into a series of test-tubes and the volumes adjusted to 1 ml with NaCl buffer (0.3 M NaCl, 10 mM tris-HCl (pH 7.6), 1 mM EDTA). To produce uniform shearing between the samples, they were vigorously vortexed for 10 s. NaCl buffer (2 ml) was passed through GF/B filters under gravity to establish a hydrostatic head. The 1 ml vortexed samples were then also filtered under gravity and the filters washed with a further 2 ml NaCl buffer to remove any DNA non-specifically bound to proteins by weak ionic interactions. The filters were dried and radioactivity determined as a percentage of the
total radioactivity in 10 μg DNA.

Studies using this assay provided very similar results to those obtained with the SDS/K⁺ assay (section 2.14.2 A) \((r=0.98, p=0.001)\), therefore it is probable that the same ansacrine induced lesion was observed.

2.15 DNA BREAKAGE STUDIES: FLUORESCENCE ASSAY FOR DNA UNWINDING (FADU)

DNA breakage induced by drugs in several of the cell lines used in this study was quantified by the fluorescence enhancement assay for DNA unwinding (FADU) described by Kanter and Schwartz (1982). In this technique, DNA breaks are detected through the enhancement of the rate of alkaline denaturation of DNA, using the bisbenzamide fluorophore H33258 as a probe for residual double-strand DNA after a fixed denaturation time. Cells were treated with drug for 60 min, centrifuged and resuspended to \(1 \times 10^6\) cells/ml in ice-cold PBS.

Three aliquots (0.5 ml; group A samples) were mixed with 1 ml of a solution of 0.1 M NaOH and 0.1 M HCl (1:1 v/v), and 0.5 ml FADU buffer (0.16 % sodium lauryl sarcosinate, 0.2 M \(K_2HPO_4\), 0.4 M Na₂EDTA, 1 μg/ml H33258, pH 7.4) to provide samples in which the DNA remained in duplex form. Another three aliquots (0.5 ml; group B samples) were mixed with 0.1 M NaOH (0.5 ml) and DNA was denatured for 30 min at room temperature before neutralizing with 0.1 M HCl (0.5 ml) and adding 0.5 ml FADU buffer. The extent of denaturation in these samples provided a measure of the size of the unwinding unit, i.e. the frequency of DNA strand breaks. Three samples (0.5 ml; group C samples) were treated similarly with 0.1 M NaOH (0.5 ml), then sonicated for 5 s to ensure complete unwinding during the 30 min denaturation period prior to neutralizing with 0.1 M HCl (0.5 ml) and adding 0.5 ml FADU buffer. After
adding FADU buffer ALL samples were sonicated for 15 s. Fluorescence intensities were determined using a Shimadzu RF-540 spectrofluorophotometer (excitation 351 nm, emission 451 nm). The fraction of residual double-stranded DNA, F, in group B samples after the fixed unwinding period was calculated using the relationship \( F = (B-C)/(A-C) \) where \( A \), \( B \) and \( C \) are the mean relative fluorescence intensities in groups \( A \), \( B \) and \( C \) respectively.

2.16 DETERMINATION OF PROTEIN CONCENTRATIONS

Protein concentrations were determined according to Bradford (1976).

2.17 SILICONIZATION OF GLASSWARE

All glassware used for protein renaturation experiments (section 8.2.5) was siliconized for 5 min with 2 % (v/v) dimethyldichlorosilane in carbon tetrachloride. The glassware was rinsed briefly with ddH\(_2\)O then baked for 20 min at 130°C. Care was taken not to siliconize too long, since this resulted in loss of the protein pellet.

2.18 COMMENT

A number of other methods have been used in this study, but as they have direct bearing on the development of protocols or specific series of experiments and are not in the category of "General Methods" they are detailed where relevant in the text.
CHAPTER THREE

CYTOTOXICITY AND DNA BREAKAGE PROPERTIES OF ANTITUMOR ACRIDINES: ROLE OF DNA TOPOISOMERASE II.

3.1 INTRODUCTION

As reviewed in Section 1.3.2, recent studies have indicated that certain antitumor drugs induce an unusual form of DNA damage in mammalian cells. Studies of the DNA from cells treated with these drugs, using the alkaline elution technique or alkaline sucrose gradient sedimentation, showed that significant levels of DNA strand breaks were protein linked (See reviews Ralph and Schneider, 1987; Liu, 1989) Moreover, removal of drugs from the culture media led to the disappearance of the protein-DNA crosslinks and restoration of the integrity of the DNA (Ross et al., 1979, Zwelling et al., 1981 and 1982a, Paolotti et al., 1979).

Amsacrine is one such drug that induces protein-linked DNA breaks in cultured mammalian cells, and it has been a useful tool for elucidating the nature of the protein-associated DNA lesion. Studies have shown that purified mammalian topoisomerase II can form a covalent association with DNA termed the "cleavable complex" (Liu, 1983). This complex dissociates upon treatment with protein denaturants resulting in single- and double-strand DNA breaks with the concomitant linking of a topoisomerase II subunit to the 5'phosphoryl end of each broken DNA strand (Liu, 1983). Amsacrine and a number of other antitumor drugs will stimulate the formation of this protein-DNA complex in vitro in a reversible reaction that is insensitive to DNA conformation (Nelson et al., 1984, Tewey et al., 1984a, b). Nelson et al. (1984) proposed that amsacrine
interferes with the breakage-reunion reaction of DNA topoisomerase II by trapping the putative key reaction intermediate, the cleavable complex (Section 1.3.4, Figure 1.2) and stabilising it by forming a non-productive drug-enzyme-DNA ternary complex.

The ability of certain antitumor drugs to stimulate topoisomerase II-mediated DNA cleavage in vitro suggested that DNA damage in cultured cells induced by antitumor drugs was mediated by topoisomerase II. Evidence presented in this chapter verifies this conclusion and extends the studies of Rowe et al. (1986a) in which topoisomerase II was identified as the protein-factor responsible for amsacrine-dependent DNA cleavage in cultured mouse L1210 cells. However, at the outset of this thesis, the mechanism linking protein associated DNA breaks with drug cytotoxicity was poorly understood and it still is.

In fact, the drug-induced protein-associated DNA breaks were for a time, considered not to be cytotoxic (Ross, et al., 1979, Zwelling et al., 1981, 1982a, b). However, this conclusion was based upon studies that compared intercalators from many different chemical classes and differences in cellular retention of drugs as well as additional effects on cells other than those involving topoisomerase II complicated the interpretation of results. Comparison of compounds belonging to the same chemical category may obviate some of the complicating factors. Therefore, to assess whether this type of DNA damage was responsible for drug cytotoxicity the amounts of protein-linked breaks produced by a number of different acridine derivatives (Figure 3.1) of varying potency in vivo but belonging to the same chemical class, were measured in whole cells, isolated nuclei and nuclear extract systems.

Because, it was unclear which activity of the enzyme (strand-passing or DNA-binding (Section 1.3.4)) was related to DNA strand breakage and
FIGURE 3.1: Structures of the free base forms of amsacrine and related acridine derivatives referred to in this chapter.

AMSACRINE

AMSACRINE

OAMS: OCH$_3$ in 2'

Acridinecarboxamide

Cl-921

C6-bisacridine

9-aminoacridine

Quinacrine
cell death a comparison was also made of the ability of different acridine
derivatives to inhibit formation of the topoisomerase II cleavable-complex
in the P4 unknotting assay (section 2.14.1), their ability to stimulate
the formation and stabilization of the protein-DNA complex in the SDS/K^+
assay (Section 2.14.2C) and their relation to drug IC_{50} values.

The action of seven acridines, with the structures shown in Figure
3.1, was compared. As previously discussed, amsacrine, which is in
clinical use for the treatment of acute leukemia (Arlin, 1983), is known
to be a specific inhibitor of topoisomerase II (Nelson et al., 1984).
oAMSA, an isomer of amsacrine, lacks antitumor activity. CI-921
(N,5-dimethyl-9-((2-methoxy-4-methyl-sulfonlamino)phenylamino)
-4-acridinecarboxamide), a derivative of amsacrine with improved solid
tumor activity (Baguley et al., 1984) is presently undergoing clinical
trial (Harvey et al., 1987). C-6 bisacridine, a bifunctional DNA
intercalator with a hexamethylene chain linking two 9-aminoacridine units
has experimental antileukemia activity (Chen et al., 1978).
Acridinecarboxamide (N-[2-(dimethylamino)-ethyl]acridine-4-carboxamide)
was recently synthesised in the Cancer Research Laboratory (Atwell et
al., 1987) and found to have curative activity against the Lewis lung
carcinoma in mice. The compounds 9-aminoacridine and quinacrine both lack
antitumor activity. A detailed discussion of the differences in chemistry
of the derivatives is not appropriate here. However, a collaborative
publication resulting from the research described in this section is
presented in Appendix A1, which gives a limited comparison of the
chemistry of the various compounds (Schneider et al., 1988a).
3.2 METHODS

3.2.1 Cell line

The mouse leukemia cell line L1210 was used throughout this section. The cells were maintained in culture as described in Section 2.5.

3.2.2 Growth Inhibition Assays (Performed by the Cancer Research Laboratory).

Growth inhibition assays were performed in 24-well microtitre trays. L1210 cells were seeded at $5 \times 10^4$ cells/well in growth medium. Drugs were added after 24 h, the cultures harvested after a further 3 days and the number of cells per well determined with an electronic cell counter. In experiments with amsacrine and CI-921, the RPMI 1640 medium was supplemented with 50 μM ascorbate to prevent aerial oxidation of the drug. Other compounds were resistant to oxidation (Appendix A1, Schneider et al., 1988a).

3.2.3 Clonogenicity Assays (Performed by the Cancer Research Laboratory).

L1210 cells were cultured at $5 \times 10^4$ cells/ml in alpha-MEM supplemented with Foetal Bovine serum (10 %) and 50 μM 2-mercaptoethanol. The cells were used after 24 h of culture, when the density was 1.5-2.0 $\times 10^5$ cells/ml. Cells were incubated with drug in growth medium (10 ml) at $37^\circ$C for 1 h. Cells were then centrifuged, washed once, counted and plated in 1 % methylcellulose in growth medium. Colony growth was determined after incubation at $37^\circ$C for 5 days (Finlay et al., 1987b). The $D_{50}$ was defined as the drug concentration required to reduce the number of surviving clonogenic cells in a culture by 50 % with respect to control cells.
3.2.4 DNA Breakage.

DNA breakage induced by the acridine drugs in L1210 cells was determined by the FADU method of Kanter and Schwartz (1985) as described in Section 2.1.

3.2.5 Preparation of Nuclear Extracts containing topoisomerase II activity from L1210 cells.

Aliquots of 2 x 10^7 logarithmic phase L1210 cells were collected by centrifugation, washed once in ice-cold TBS (section 2.4) and resuspended in 0.4 ml buffer A (20 mM Tris-HCl, pH7.2, 150 mM KCl, 5 mM MgCl_2, 1 mM EGTA, 10 mM Na_2S_2O_5, 2% (w/v) dextran 150-200) to a density of 4-5 x 10^7 cells/ml. Triton X-100 was added to a final concentration of 0.1% and the cells were placed for 10 min at 0°C with occasional gentle mixing, followed by centrifugation at 1200 x g for 10 min at 4°C. The supernatant was discarded and the nuclei pellet was resuspended in 0.4 ml buffer A, layered on 3 ml 30% (w/v) sucrose in buffer A and sedimented at 1200 x g for 10 min at 4°C. The nuclei were resuspended in buffer A (0.4 ml), centrifuged at 1200 x g for 10 min at 4°C and finally resuspended in 0.4 ml buffer B (buffer A without 5 mM MgCl_2). Nuclei were then treated with 20 mM 2-mercaptoethanol and 20 mM EDTA for 30 min at 4°C, followed by 3 x 15 sec bursts of sonication. One third volume of 24% PEG 6000, 2 M KCl, 10 mM Na_2S_2O_5 was added and the mixtures held for 30 min on ice. The resulting precipitates were removed by centrifugation at 30,000 x g for 30 min at 4°C to obtain clear supernatants with topoisomerase II activity. Nuclear extracts from log-phase cells were diluted to a protein concentration of approximately 100 µg/ml and stored in aliquots at -70°C.
3.2.6 Phage P4 DNA Unknotting Assay.

The ability of varying amounts of drugs to inhibit the topoisomerase II DNA strand-passing activity in L1210 nuclear extracts (3.2.5) was measured using the Phage P4 unknotting assay as described in section 2.14.1. Aliquots (1 µl) of concentrated drug stock solutions were added to give the desired final drug concentrations. One unit of enzyme activity was defined as the amount of extract (in µg protein) that completely unknotted 0.24 µg phage P4 DNA in the absence of drug.

3.2.7 Quantitation of Acridine-stimulated Covalent Protein-DNA complex (PDC) Formation in Whole Cells, Isolated Nuclei and Nuclear Extracts.

The Coombes-Pearson (1978) filter binding assay and the SDS/K⁺ precipitation assay of Rowe et al. (1986a) (as described in sections 2.14.2A and 2.14.3) were used to measure drug-induced PDC formation in intact cells. The method of Liu et al. (1983) (Section 2.14.2C) was used to quantitate in vitro drug stimulation of PDCs using nuclear extracts (section 3.2.5).

PDCs induced by the acridine derivatives were also measured in isolated nuclei by a protocol developed during the course of this study. This assay was based on the SDS/K⁺ precipitation method of Rowe et al. (1986a) and Liu et al. (1983) and the rationale behind the protocol is discussed in detail in Chapter 5. Briefly, radioactively labelled L1210 cells (Section 2.10) were collected and washed once in TBS by centrifugation, resuspended in nuclei buffer A (see Section 3.2.5) at 5 x 10⁷ cells/ml and lysed with 0.05 % triton X-100 for 10 min at 0°C with occasional mixing. Nuclei were harvested by centrifugation (1200 x g, 10
min at 4°C) and resuspended in nuclei buffer A to a density of 5 x 10^7 cells/ml. An aliquot was routinely examined under the microscope to check nuclei integrity and density. To initiate drug-stimulated PDC formation, 10^5 nuclei were added to the following reaction mixture in 96-well plates in a total volume of 50 µl/well: 50 mM tris-HCl, pH 7.5, 10 mM MgCl_2, 120 mM KCl, 5 mM EGTA, 2.25 mM EDTA, 0.5 mM dithiothreitol, 30 µg/ml bovine serum albumin, 2 mM ATP, 0-50 µm drug and additional cytoplasmic extract. The cytoplasmic extract was prepared by digitonin extraction of corresponding L1210 cells as described in Chapter 5. No PDC forming activity was detected in this cytoplasmic extract. The 96-well plates were incubated for 10 min at 37°C then the nuclei were collected by centrifugation, lysed and the protein-DNA complexes were precipitated and quantitated as described in Section 2.14.2B.

3.3 RESULTS

3.3.1 In vivo activity of related acridine derivatives against L1210 leukemia.

The in vivo activity of the related acridine derivatives, amsacrine, acridinecarboxamide, oAMSA, CI-921, C-6 bisacridine, 9-aminoacridine and quinacrine against L1210 leukemia was tested at the Cancer Research Laboratory, Auckland School of Medicine. The results, in part summarizing previously reported data (Denny et al., 1987; Baguley et al., 1984), are presented in Table 3.1. Amsacrine and CI-921 showed the highest activity against L1210 leukemia, with acridinecarboxamide showing marginal activity. The other compounds were not significantly active.
**TABLE 3.1:** In vivo antitumour activity of acridine derivatives.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>L1210 CELLS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>OD&lt;sup&gt;a&lt;/sup&gt; (mg/kg)</strong></td>
<td><strong>ILS&lt;sup&gt;b&lt;/sup&gt; (%)</strong></td>
<td></td>
</tr>
<tr>
<td>CI-921</td>
<td>20</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>Amsacrine</td>
<td>13.4</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Acridinecarboxamide</td>
<td>65</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Quinacrine</td>
<td>100</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>oAMSA</td>
<td>65</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>C-6 bisacridine</td>
<td>8.9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Optimal dose, administered 3 times at 4 day intervals.

<sup>b</sup> Increase in lifespan with respect to control animals.
3.3.2 In vitro growth inhibition by the acridine compounds in continuous and short term exposure survival assays.

Growth inhibition was tested using a panel of cultured mouse cell lines (see Appendix A1, Schneider et al., 1988a), however only the IC\textsubscript{50} values (i.e. the drug concentration required to reduce the number of cells in a culture by 50% with respect to control cultures) for L1210 leukemia cells are presented here (Table 3.2). The compounds listed in order of decreasing cytotoxic potency against L1210 leukemia were: CI-921 > amsacrine > acridinecarboxamide > C-6 bisacridine > oAMSA > 9-aminoacridine > quinacrine.

The cytotoxicity of amsacrine, CI-921 and acridinecarboxamide was also compared following a short term incubation. Logarithmic L1210 cells were exposed to drug for 1 h, then washed free of drug and assayed for ability to form colonies in vitro. D\textsubscript{50} values are shown in Table 3.2 and the results indicate that CI-921 and amsacrine were more cytotoxic towards L1210 cells than acridinecarboxamide, in keeping with the continuous exposure assays.

3.3.3 The effect of related acridine derivatives on DNA strand breakage.

The FADU assay (Section 2.15) was used to determine the relative capacity of the acridine derivatives to cause DNA breakage. In this technique DNA breaks are detected through the enhancement of the rate of alkaline denaturation of DNA, using the bisbenzamide fluorophore Hoechst 33258 as a probe for residual double-stranded DNA after a fixed denaturation time in alkali. The FADU assay has been shown to give the same information as alkaline elution techniques (Tanigher et al., 1987; Pommier et al., 1985b).

As can be seen in Figure 3.2, only the two most cytotoxic compounds,
**TABLE 3.2:** In vitro growth inhibitory activity and cytotoxicity of acridine derivatives.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>L1210 CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$IC_{50}$ (nM)</td>
</tr>
<tr>
<td>CI-921</td>
<td>4.2</td>
</tr>
<tr>
<td>Amsacrine</td>
<td>6.8</td>
</tr>
<tr>
<td>Acridinecarboxamide</td>
<td>98</td>
</tr>
<tr>
<td>C-6 bisacridine</td>
<td>110</td>
</tr>
<tr>
<td>oAMSA</td>
<td>660</td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>1900</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>2400</td>
</tr>
</tbody>
</table>

ND = Not determined.
FIGURE 3.2: Fluorescence assay for DNA unwinding (FADU) after treatment of L1210 cells with drugs. Logarithmic phase L1210 cells were diluted with fresh growth medium to $2 \times 10^5$ cells/ml before exposure to the various drugs from 0-1 $\mu$M for 1 h at 37°C. Cells were then processed for the FADU assay (Section 2.15). $F$ is the fraction of DNA still in the duplex form after an alkaline unwinding time of 30 min. Values given are means from three independent experiments. Amsacrine (○); acridinecarboxamide (▼); oAMSA (▲); CI-921 (■); C6-bisacridine (▲); 9-aminoacridine (◇); quinacrine (○).
### TABLE 3.3: Quantitation of acridine drug-induced covalent DNA-protein complexes (Coombes-Pearson Filter Binding Assay)

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>% DNA RETAINED ON FILTER b, c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no drug)</td>
<td>0.4</td>
</tr>
<tr>
<td>oAMSA</td>
<td>0.6</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>0.6</td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>1</td>
</tr>
<tr>
<td>C-6 bisacridine</td>
<td>1</td>
</tr>
<tr>
<td>Acridinecarboxamide</td>
<td>8</td>
</tr>
<tr>
<td>CI-921</td>
<td>17</td>
</tr>
<tr>
<td>Amsacrine</td>
<td>18</td>
</tr>
</tbody>
</table>

a Concentration of drugs used, 4 μM.

b % DNA Retained on filter = Radioactivity on filter (cpm) x 100

Total Radioactivity

c Data are means of three or more individual experiments, in which each compound was tested in triplicate.
amsacrine and CI-921, induced a dose-dependent increase in DNA breakage during a 60min treatment of L1210 cells. At low drug concentrations, acridinecarboxamidine produced a similar amount of DNA breaks to amsacrine and/or CI-921. However, acridinecarboxamidine induced only limited additional DNA breakage at higher drug concentrations. None of the other compounds used produced significant DNA breakage although 6AMS A appeared to have a slight effect. Therefore, the level of strand DNA breaks induced by the acridine series strongly correlated with their cytotoxicity.

3.3.4 Stimulation of protein-DNA complex formation by the acridine derivatives.

To determine whether protein-linked DNA breaks correlated with drug cytotoxicity, the effect of the related acridine compounds on the induction of topoisomerase II-linked DNA breaks was measured in cultured L1210 cells, isolated L1210 nuclei and in a nuclear extract system.

Initially, the formation of protein-linked DNA breaks in drug-treated L1210 cells was measured using the Coombes-Pearson filter binding assay. The results presented in Table 3.3 show that the compounds which induced the highest level of DNA breakage (Figure 3.2), amsacrine and CI-921, also induced the highest level of covalent protein-DNA complexes in L1210 cells, although acridinecarboxamidine also induced a reasonable level of PDC formation at the concentration (4 \( \mu \text{M} \)) tested. As this assay was extremely laborious, requiring DNA isolated from drug-treated cells, only one concentration of each compound could be managed at any one time. While this work was in progress Rowe et al. (1986a) published a quicker, more convenient and sensitive assay for monitoring cellular levels of protein-linked DNA complexes based on the SDS/K\(^+\) assay of Liu et al.
(1983) (see section 2.14.2A). Therefore, the modified SDS/K+ assay was adopted and the induction of PDCs in whole L1210 cells in response to varying amounts of the acridine derivatives after a 60 min treatment were quantitated. The results are shown in Figure 3.3. As in the DNA breakage assay, only amsacrine and CI-921 (and to a smaller extent, oAMSA) induced dose-dependent protein-DNA complex formation while very little complex forming activity was observed with the other drugs used. However, the unusual behaviour of acridinecarboxamide should be noted. At very low drug concentrations (1 μM) acridinecarboxamide consistently produced a higher level of PDC formation than amsacrine, but there was a gradual decline in complex formation at higher drug concentrations, with no stimulation at, or above 20 μM drug. This compares to the significant level of PDC induced by 4 μM acridinecarboxamide in the Coombes-Pearson filter binding assay (Table 3.3) and the initial DNA breakage observed at low drug concentrations with the FADU assay (Figure 3.2).

To overcome the possibilities of the cell membrane or cellular drug metabolism influencing the interaction of the drugs with potential intracellular targets such as topoisomerase II, PDC induction by the acridines was also measured in nuclei isolated from L1210 cells using a protocol described in this thesis (see Section 2.14.2B, Chapter 5 for details). In general, the overall pattern was the same as that observed in whole cells, although at high drug concentrations the levels of drug-induced PDC did not reach those achieved in intact cells (Figure 3.4), possibly because the period of incubation of nuclei with drugs was 10 min rather than 60 min to reduce non-specific nuclease activity on DNA.

Results of a similar experiment using nuclear extracts from L1210 cells and [32P]3'-end labelled pBR322 DNA are shown in Figure 3.5. The pattern of PDC formation paralleled those observed in whole cells and
FIGURE 3.3: Stimulation of protein-DNA complex formation in whole L1210 cells. Drug-stimulated formation of protein-DNA complexes was measured in L1210 cells with 3H-thymidine labelled cellular DNA as a substrate. Protein-DNA complexes formed were precipitated by SDS/K+ (Section 3.2.6).

Values given are means from 3 independent experiments. Amsacrine (●); acridinecarboxamide (▲); oAMSA (▲); CI-921 (■); C6-bisacridine (△); 9-aminoacridine (◇); quinacrine (○).
FIGURE 3.4: Stimulation of protein-DNA complex formation in nuclei isolated from L1210 cells. Drug-stimulated formation of protein-DNA complexes was measured in nuclei isolated from L1210 cells. Protein-DNA complexes were precipitated by SDS/K+ (Section 3.2.6). Values given are means from 3 independent experiments. Amsacrine ( ), acridine-carboxamide ( ▼ ); oAMSA ( ▲ ); CI-921 ( □ ); C6-bisacridine ( △ ); 9-aminoacridine ( ◊ ); quinacrine ( ○ ).
FIGURE 3.5: Stimulation of protein-DNA complex formation in nuclear extracts of L1210 cells. This was determined using 50 ng 3' end-labelled pBR322 DNA as a substrate (Sections 3.2.4 and 3.2.5). Values given are means of at least 3 independent experiments. Amsacrine (●); acridine-carboxamide (▼); oAMSA (▲); CI-921 (■); C6-bisacridine (△); 9-aminoacridine (◇); quinacrine (○).
FIGURE 3.6: Drug effects on the P4 unknotting activity in nuclear extracts of L1210 cells. The minimal amount of nuclear extract (Section 3.2.4) from L1210 cells required for complete unknotting of 0.24 μg P4 DNA (1 unit) was used in an unknotting assay (section 3.2.5) with varying amounts of drug (0-100 μM) added as indicated. Reaction products were separated on 0.7 % agarose gel, stained with 0.5 μg/ml ethidium bromide and photographed under UV illumination.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4 DNA</td>
<td>2 5 10 20 50 100</td>
</tr>
<tr>
<td>mAMSA</td>
<td>2 5 10 20 50 100</td>
</tr>
<tr>
<td>CI-921</td>
<td>2 5 10 20 50 100</td>
</tr>
<tr>
<td>oAMSA</td>
<td>2 5 10 20 50 100</td>
</tr>
<tr>
<td>Acridinecarboxamide</td>
<td>2 5 10 20 50 100 μM</td>
</tr>
<tr>
<td>C6-bisacridine</td>
<td>2 5 10 20 50 100</td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>2 5 10 20 50 100</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>2 5 10 20 50 100</td>
</tr>
</tbody>
</table>
isolated nuclei. However, the stimulation of protein-DNA complex
formation by acridinecarboxamide occurred at higher drug concentrations
and over a wider range than with intact cells or nuclei. A delayed but
significant increase in complex formation observed with C-6 bisacridine in
nuclei extracts was not found using whole cells or nuclei.

3.3.5 Inhibition of topoisomerase II strand-passing activity: the P4
unknotting assay.

To test the effect of the acridine compounds on the strand-passing
activity of topoisomerase II, various concentrations of drugs were
incubated with L1210 nuclear extract containing approximately 1 unit of
topoisomerase II activity (20–40 ng protein) in P4 unknotting assays and
analysed by gel electrophoresis (Figure 3.6). The following minimal
concentrations of drug inhibited topoisomerase II P4 unknotting activity:
amsacrine, 20 µM; CI-921, 20 µM; acridinecarboxamide, 50 µM; oAMSA, 100
µM; C-6 bisacridine, 20 µM; 9-aminoacridine, 20 µM and quinacrine, 50 µM.

3.4 DISCUSSION

A body of evidence (reviewed in Chapter 1) has accumulated that
implicates DNA topoisomerase II as a cellular target for a number of
intercalating agents including amsacrine and the non-intercalating
epipodophyllotoxins, VP16 and VM26. At the outset of this study,
significant progress had been made with understanding the biochemical
effects of topoisomerase II inhibitors, but a causal relationship between
drug-induced protein-linked strand breaks and cytotoxicity had not been
unequivocally established.

A number of experimental approaches were used to establish a
quantitative relationship between drug-induced topoisomerase II inhibition and cytotoxicity. For example, Pommier et al. (1986a, b) and Glisson et al. (1986a) showed that Chinese hamster lung or ovary cells resistant to topoisomerase II inhibitors produced fewer intercalator-induced protein-linked DNA strand breaks than did the sensitive parent lines and that resistance was not due to drug uptake. These observations were consistent with a causal relationship between topoisomerase II-mediated damage to DNA and the cytotoxicity of the drugs tested.

An alternative approach was to examine this relationship by comparing DNA lesion frequency with survival in a single drug-sensitive cell line using compounds from different classes of intercalating topoisomerase II inhibitors (e.g. Ross et al., 1979; Zwelling et al., 1981; Zwelling et al., 1982 a, b). In these types of studies, drugs from different chemical classes routinely produced distinctly different curves when either single-stranded breaks or double-stranded breaks were plotted against cell kill. The results suggested that the level of protein-linked breaks did not correlate with cytotoxicity. However, it is known that intercalating antitumour drugs from different chemical classes induce topoisomerase II-linked breaks at different sites on DNA (Tewey et al., 1984a). This site specificity may be one of the factors responsible for the lack of correlation between protein-linked DNA breaks and cytotoxicity among structurally dissimilar drugs. Differences in cytotoxic potential of some drugs may also have been explained by simultaneous operation of additional cytotoxic mechanisms such as membrane effects, free radical production and inhibition of topoisomerase I or polymerases.

It was thought possible that studies of structure-activity relationships within a single chemical class of topoisomerase II inhibitors may have provided additional insights by eliminating some of
the variability factors listed above. Therefore, to determine whether protein-linked breaks correlated with drug cytotoxicity the effect of a number of synthetic acridines on induction of topoisomerase II-associated DNA breaks were measured using whole cells, isolated nuclei and nuclear extract systems. The results presented in this chapter extend those of Rowe et al. (1986a) and provide additional evidence that mammalian DNA topoisomerase II is responsible for amsacrine-induced breaks in cultured mammalian cells. Furthermore, these results demonstrate, at least for drugs belonging to the amsacrine lineage (i.e. substituted 9-amino-acridines), that topoisomerase II-linked DNA breaks (Figures 3.3-3.5) correlate well with drug cytotoxicity as measured by IC$_{50}$ assays (Table 3.2).

The effect of acridinecarboxamide on DNA breakage (Figure 3.2) and on the formation of protein-DNA complexes (Figures 3.3-3.5), together with the cross-resistance of a cell line with altered topoisomerase II activity to acridinecarboxamide (see Appendix A1, Schneider et al., 1988a) suggested that topoisomerase II is the intracellular target of acridinecarboxamide (see Appendix A1 for a more detailed discussion of acridinecarboxamide and its effects). However, there were obvious differences between acridinecarboxamide and amsacrine in the dose-dependence of formation of DNA-protein complexes (Figures 3.3-3.5). The D$_{50}$ concentration (0.85 µM) of acridinecarboxamide induced approximately an 11-fold increase in complex formation in intact cells (Figure 3.3), while cytotoxic concentrations of amsacrine (0.046 µM) or CI-921 had no significant effect on complex formation. In contrast, all three drugs caused similar DNA breakage (30-40 % reduction in fluorescence) at these concentrations. The reason for the inhibition of drug-stimulated formation of protein-DNA complexes by high concentrations
of acridinecarboxamide (Figures 3.3-3.5), which is similar to that reported for 2-methyl-9-hydroxyellipticine (Pommier et al., 1985b), has not been elucidated. Nevertheless, a quantitative or qualitative difference in the interaction of acridinecarboxamide and amsacrine with topoisomerase II, DNA, or other factors is indicated.

It is clear that the capacity to introduce DNA breaks is not a universal feature of acridine derivatives, since C-6 bisacridine has been reported to have biological activity (Chen et al., 1978). All the more curious, the delayed but substantial increase in topoisomerase II-DNA complex formation induced by C-6 bisacridine in nuclear extracts with exogenous DNA as substrate was not observed in either whole cells or isolated nuclei! This may be a reflection of greater availability of DNA binding sites in a uniform DNA substrate such as pBR322, or a barrier to drug uptake presented by the nuclear membrane. Markovits et al. (1978) have suggested a different mechanism of action for the anticancer agent ditercalinium, which is also a bifunctional intercalator, and this mechanism may apply to C-6 bisacridine. Whatever the mechanism, the results obtained with C-6 bisacridine highlight the possibility of generating misleading results when extract systems alone are used to predict structure-activity relationships.

In addition, an assessment was also made of which activity of topoisomerase II (strand passing or DNA binding) correlated with cytotoxicity when affected by drugs. It was found that although the drug concentrations needed to inhibit strand-passing activity varied (Figure 3.6) there was no correlation with IC$_{50}$ values in Table 3.2. For instance, 9-aminoacridine has low cytotoxic activity but shows the same inhibition of topoisomerase II as amsacrine in the phage P4 unknotting assay. A qualitatively similar result was also obtained using a
catenation assay for the measurement of topoisomerase II activity (Hutchins, 1986). The results, in agreement with Nelson et al. (1984), show that there was no direct relationship between the inhibition of topoisomerase II strand-passing activity and the cytotoxic action of topoisomerase II-specific anticancer drugs.

In conclusion, drug induced formation and stabilization of the topoisomerase II-DNA cleavable complex, rather than inhibition of its formation or of strand-passing functions, appears to be related to DNA strand breakage and cell death caused by acridines such as amsacrine.
CHAPTER FOUR

THE ROLE OF TOPOISOMERASE II IN AMSACRINE RESISTANCE

4.1 INTRODUCTION

As discussed in Chapters 1 and 3, the anticancer drug amsacrine has been shown to act on the enzyme DNA topoisomerase II and cells treated with this drug exhibit an increased number of topoisomerase II-associated DNA breaks. The drug-induced protein-DNA complex can be isolated and, as described in the previous chapter, it serves as a measure for drug activity.

Despite topoisomerase II being ubiquitous there are marked differences in the response of different tumours to the cytotoxic action of amsacrine. Solid tumours in particular are refractory to its action. It is possible that the high number of non-cycling cells in solid tumours which appear to be less sensitive to topoisomerase II-specific drugs, and in general contain reduced topoisomerase II activity, may limit the tumour-killing activity of these drugs. Topoisomerase II activity appears to be a function of the cell cycle and in general it increases when cellular proliferation is stimulated.

The major aim of this thesis was to examine the role of DNA topoisomerase II in amsacrine resistance with particular reference to resistance in non-cycling cells. In this chapter studies are described that investigate the interaction between the sensitivity to amsacrine and DNA topoisomerase II in two model systems for non-cycling cells, namely (i) the CHO-AA8 cell system and (ii) the cold sensitive cell-cycle mutant (21-Fb) of the murine mastocytoma cell line P815.
The experiments conducted and results achieved with these model systems are presented independently in Parts I and II of this Chapter, although Parts I and II are discussed together at the conclusion of the chapter.

PART I

MECHANISMS OF RESISTANCE OF NON-CYCLING MAMMALIAN CELLS TO AMSACRINE: THE INVOLVEMENT OF DNA TOPOISOMERASE II IN LOG- AND PLATEAU-PHASE CHO CELLS.

4.2 INTRODUCTION

Robbie et al. (1988) demonstrated that CHO-AA8 cells gradually lose their sensitivity towards amsacrine upon entry into plateau-phase. Their studies showed that this was not due to reduced drug uptake or differences in intracellular drug concentrations. Furthermore, no evidence for drug metabolism or conversion into more active compounds was found in either log- or plateau-phase cells. However, there was a close relationship between amsacrine-induced cytotoxicity and DNA breaks, suggesting that the differences in drug-induced cell killing of log- and plateau-phase cells may have been due to differences in drug-induced DNA breakage.

Using the assay systems available in this laboratory, the investigations of Robbie et al. (1988) were extended to study the role of DNA topoisomerase II in the differential sensitivity of log- and plateau-phase AA8 cells to amsacrine. Therefore the aims of research described this section were to compare the topoisomerase II activities in nuclear extracts from log- and plateau-phase cells as well as the formation of protein-DNA complexes to identify possible reasons for
differences in cell killing by amsacrine.

The studies described were performed in collaboration with Dr E. Schneider (Department of Cellular and Molecular Biology, Auckland University) and Drs M.A. Robbie and W.R. Wilson (Oncology Department, Auckland School of Medicine) to whom I am especially grateful for providing the CHO-AA8 cells and for conducting the initial studies (described in Robbie et al., 1988) which provided the foundation for this work in 1986.

4.3 METHODS

4.3.1 Cell Culture

AA8 cells, a subline of the CHO cell line, were grown in spinner culture by Drs M.A. Robbie and W.R. Wilson, as described in Section 2.5. The cell kinetics and drug sensitivity of the log- and plateau-phase suspension cultures of CHO-AA8 cells have been described in detail by Robbie et al. (1988). Briefly, the growth rate declined when the cell density exceeded approximately $8 \times 10^5$ cells/ml, with a concomitant decrease in thymidine incorporation and accumulation of cells in G1 phase. Maximum cell density was reached at $2 \times 10^6$ cells/ml. Dose-response survival curves for cell killing by amsacrine exhibited a continuous increase in drug resistance up to 4- to 8- fold during growth from log-phase ($2 \times 10^5$ cells/ml) into plateau-phase ($1.4 \times 10^6$ cells/ml). Unless stated otherwise, spinner cultures with the above cell densities were used as a source of log- and plateau-phase cells for mechanistic studies described in this thesis. Although some further increase in amsacrine resistance was observed at higher cell densities (see Figure 4.4 of this thesis and Figures 3 and 4 of Robbie et al., 1988), the latter
cell density was chosen as a maximum because at higher cell densities survival of non-drug treated cells was impaired (Robbie et al., 1988).

4.3.2 Extraction of DNA topoisomerase II activity from log- and plateau-phase CHO-AA8 cells.

Topoisomerase II activity was extracted from nuclei isolated from log- and plateau-phase CHO-AA8 cells as described in Chapter 3 (Section 3.2.2.4) except that the CHO-AA8 cells were lysed with Triton X-100 added to a final concentration of 0.2 % and incubated for 20 min at 0°C with occasional gentle mixing.

Nuclear extracts from log- and plateau-phase cells were diluted to equal protein concentrations of approx. 100 µg/ml and stored at -70°C.

4.3.3 The phage P4 DNA-unknotting assay.

The phage P4 DNA-unknotting assay was performed as described in section 2.14.1.

Four microliters of nuclear extract from log- or plateau-phase cells (see section 4.3.2) provided 120 mM KCl when added to the assay mixture (see section 2.14.1).

One unit of enzyme activity was defined as the amount of extract that completely unknotted 0.24 µg phage P4 DNA.

4.3.4 Quantitation of amsacrine-stimulated covalent protein-DNA complex formation in log- and plateau-phase CHO-AA8 cells, isolated nuclei and nuclear extracts.

The Coombes-Pearson filter binding assay and the SDS/K+ precipitation assay of Rowe et al. (1986a) (as described in Sections 2.14.3 and 2.14.2 A, B) were used to measure amsacrine-induced PDC
formation in intact cells and isolated nuclei. The method of Liu et al. (1983) (section 2.14.2C) was used to quantitate in vitro drug stimulation of PDCs using nuclear extracts (section 4.3.2) from log- and plateau-phase CHO-AA8 cells.

For experiments requiring isolated nuclei, nuclei were prepared as follows. Washed log- or plateau-phase cells were resuspended in nuclei buffer 1 (20 mM Tris.HCl, pH7.2, 150 mM KCl, 5 mM MgCl₂, 100 mM dithiothreitol, 10 mM Na₂S₂O₅, 2 % (w/v) dextran grade B) at 2 x 10⁷ cells/ml and lysed with 0.1 % Triton X-100 for 10 min at 0°C.

Nuclei were collected by centrifugation at 1200 x g for 10 min at 4°C, resuspended in the same volume of buffer 1 and overlaid onto 3 ml 30 % (w/v) sucrose in buffer 1. Following centrifugation (1200 x g for 10 min) the nuclei were resuspended in 1 ml buffer 1, recentrifuged and resuspended in 1 ml buffer 2 (buffer 1 with 5 mM EGTA and no dextran). An aliquot of nuclei from each population was examined with a microscope and the nuclei were counted using a haemocytometer. (It should be noted that although the conditions of cell lysis described here successfully lysed and stripped the plasma membrane from log- and plateau-phase cells, significant remnants of cytoplasmic material remained closely associated with the nuclei, even after repeated washings).

For use in the Coombes-Pearson binding assay, nuclei were diluted to 1 x 10⁷ nuclei/ml with buffer 2 and treated with various concentrations of amsacrine for 60 min at 37°C.

For use in the SDS/K⁺ precipitation assay nuclei were diluted to 1 x 10⁵ nuclei/ml with buffer 2 and treated with various concentrations of amsacrine as described in section 2.14.2A. In this instance, the SDS/K⁺ precipitation assays with isolated nuclei were performed in 24-well plates and processed as described in section 2.14.2A.
PDC formation in nuclear extracts from log- and plateau-phase cells was performed using 50 ng $^{32}$P 3'-end labelled pBR322 DNA (as described in section 2.14.2C). The reactions were initiated by adding 5 μl of extract containing approx. 0.2 μg protein (which provided an additional 60 mM KCl to make a final concentration of 120 mM KCl).

4.4 RESULTS

4.4.1 Quantitation of DNA topoisomerase II strand-passing activity in log- and plateau-phase AA8 cells.

To determine whether the increased resistance of plateau-phase cells to the action of amsacrine was due to a decrease in topoisomerase II strand-passing activity, the P4-unknotting activities of nuclear extracts from log- and plateau-phase cells were compared. Serial 2-fold dilutions of both extracts were prepared and a P4-unknotting assay was performed with each dilution to determine the amount of extract that contained one unit of topoisomerase II activity (defined above).

Results from a typical experiment presented in Figure 4.1, show a slight reduction of ATP-dependent (lanes 2 and 9) topoisomerase II activity in plateau phase nuclear extracts (compare lanes 5 and 12). The apparent decrease in topoisomerase II strand-passing activity was also reflected in the specific activities of log- and plateau-phase nuclear extracts of 15.5 +/- 1.4 and 10.9 +/- 1.9 units/μg protein (+/- S.E., six independent experiments), respectively. Together with the consistently observed reduction in protein content of plateau-phase nuclei of approximately 30 % (data not shown), this amounted to an overall decrease in nuclear topoisomerase II activity of 50 % in plateau-phase cells.
FIGURE 4.1: Quantitation of DNA topoisomerase II strand-passing activities. Nuclear extracts from log- and plateau-phase cells (Section 4.3.2) were serially 2-fold diluted and with each dilution a P4 DNA unknotting assay was performed as described (Section 2.14.1). Reaction products were separated on a 0.7% agarose gel, stained with ethidium bromide and photographed under UV light. Lane 1, P4 DNA alone; lane 2, undiluted nuclear extract from log-phase cells, without ATP; lanes 3 - 8, serially 2-fold diluted extracts as indicated from log-phase cells; lane 9, undiluted nuclear extract from plateau-phase cells, without ATP; lanes 10 - 15, serially 2-fold diluted extracts as indicated from plateau-phase cells.
4.4.2 Inhibition of unknotting activity by amsacrine.

To test for reduced sensitivity of topoisomerase II strand-passing activity to inhibition by amsacrine, P4-unknotting assays were performed with different amsacrine concentrations and 1 unit of log- or plateau-phase nuclear extract. As shown in Figure 4.2, inhibition of unknotting was observed with concentrations of 10 μM amsacrine or more, and there was no apparent difference in sensitivity between nuclear extracts from log- or plateau-phase cells (compare lanes 5 and 12, Figure 4.2).

4.4.3 Stimulation of covalent protein-DNA complex formation in log- and plateau-phase cells by amsacrine.

The amsacrine-induced formation of PDCs in whole cells and isolated nuclei was measured by the Coombs-Pearson filter binding assay (see Section 2.14.3) and the SDS/K+ precipitation assay (see Section 2.14.2A and B). For reasons already stated in Section 2.14.3, it is believed that both assays measured the same amsacrine-induced lesion. However, although the overall trends demonstrated by the two assays may be compared the data are calculated and presented differently so that a direct comparison between actual amounts of PDCs measured in the two assays cannot be made.

Initially formation of amsacrine-induced PDCs in AA8 cells and isolated nuclei was measured using the Coombs-Pearson filter binding assay. By comparing the concentrations of drug required to produce 10% DNA retention on glass fibre filters, it was calculated that log-phase cells were approximately 6-fold more sensitive to amsacrine-induced formation of covalent protein-DNA complexes than plateau-phase cells (Figure 4.3). This difference in sensitivity was similar to that for amsacrine-induced DNA breakage in log- and plateau-phase AA8 cells.
FIGURE 4.2: Inhibition of the unknotting reaction by amsacrine. The minimal amount of each extract required for complete unknotting of 0.24 μg P4 DNA (1 unit) was used in an unknotting assay with varying amounts of amsacrine (0-100 μM) added. Reaction products were analysed as in Figure 4.1. Lane 1, P4 DNA alone; lanes 2 - 8, 1 unit of nuclear extract from log-phase cells with 0, 2, 5, 10, 20, 50, 100 μM amsacrine respectively; lanes 9 - 15, 1 unit of nuclear extract from plateau-phase cells with 0, 2, 5, 10, 20, 50, 100 μM amsacrine respectively.
FIGURE 4.3: Stimulation of protein-DNA complex formation in log- and plateau-phase AA8 cells and nuclei. Amsacrine-induced formation of PDCs was measured in log- and plateau-phase AA8 cells and isolated nuclei by the Coombes-Pearson binding assay as described in Section 4.3.4. Intact log-phase cells, ( ); nuclei from log-phase cells, ( ); intact plateau-phase cells, ( ); nuclei isolated from plateau-phase cells, ( ).
determined by the FADU assay after treating cells with similar drug concentrations (Robbie et al., 1988). Together with the rapid reversibility of the lesions (Robbie et al., 1988) this provides further evidence that amsacrine induced lesions detected by the FADU assay probably reflect topoisomerase II-mediated DNA strand breaks detected by alkaline elution techniques.

Although there was no apparent difference in the levels of drug-stimulated PDCs between log-phase cells and nuclei, there was at first inspection a marked difference between plateau-phase cells and nuclei. For example, at 4 μM amsacrine only 2% of the DNA from plateau-phase nuclei was retained on filters compared with approx. 15% of the DNA from intact plateau-phase cells. This difference was attributed to the degree to which cells had progressed into plateau-phase at the time they were harvested for use in individual experiments. For example, the plateau-phase cells used for these initial studies on cells and isolated nuclei were taken from independent populations of AA8 cells which had grown to different stages within plateau-phase. Isolated nuclei were prepared from cells at a density of 1.53 x 10^6 cells/ml, whereas AA8 cells for intact cell studies were harvested when they had reached a density of 1.3 x 10^6 cells/ml. Such a difference in the induction of PDCs (Figure 4.3) within such a narrow range of plateau-phase was not anticipated and warranted further investigation. Due to the laborious and expensive nature of the filter binding assay, the SDS/K^+ assay of Rowe et al. (1986a) was adopted and the induction of PDCs in cells from early to late plateau-phase was examined.

As shown in Figure 4.4, the transition of cells from early (1.2 x 10^6 cells/ml) to late (1.53 x 10^6 cells/ml) plateau-phase was associated with a considerable reduction in amsacrine-induced protein-DNA
FIGURE 4.4: Stimulation of protein-DNA complex formation in AA8 cells on progression through plateau-phase. AA8 cells were harvested at various points within plateau-phase (early to late plateau-phase) and then amsacrine-induced formation of PDCs were measured by SDS/K⁺ precipitation as described (Section 2.14.2A). Plateau-phase cell harvested when culture densities were: $1.2 \times 10^6$ cells/ml ($\blacksquare$); $1.3 \times 10^6$ cells/ml ($\triangle$); $1.4 \times 10^6$ cells/ml ($\cdot$); $1.53 \times 10^6$ cells/ml ($\bigcirc$).
formation. This reflects the decrease in sensitivity of AAB cells to the cytotoxic effects of amsacrine as they progress through into plateau-phase (see Figure 5 of Robbie et al., 1988). Furthermore, it supports the conclusion of Robbie et al. (1988) that the change in drug sensitivity is not a direct consequence of lack of cell-cycle progression per se, but rather due to biochemical changes related to entry into a noncycling state.

The results of this series of experiments stressed the importance of using AAB cell cultures of identical densities for all future experiments. Therefore, all experiments thereafter were performed with log-phase AAB cell cultures with densities of exactly $2.1 \times 10^5$ cells/ml and plateau-phase cultures with cell densities of exactly $1.4 \times 10^6$ cells/ml.

When the SDS/K+ precipitation assay was used to measure amsacrine-induced PDCs in intact cells and isolated nuclei (Figure 4.5) a marked dose-dependent increase in covalent-protein-DNA complex formation was observed. Up to approximately 14-fold stimulation with 20 μM amsacrine occurred in log-phase cells with little difference between whole cells and isolated nuclei. On the other hand, complex formation in plateau-phase cells and nuclei gradually increased 4-fold with 20 μM amsacrine, and only a slight increase was observed at higher drug concentrations. Again, there were only minor differences between whole cells and isolated nuclei. When the initial slopes of the graphs were compared, it was calculated that plateau-phase cells required 12-fold greater amsacrine concentrations to produce the same stimulation of complex formation as that in log-phase cells. This value was comparable to the 4- to 8-fold increase in amsacrine concentration needed to produce the same amount of cell-killing in plateau-phase cultures as in log-phase cultures (Robbie
FIGURE 4.5: Stimulation of protein-DNA complex formation in whole cells and isolated nuclei. Amsacrine-stimulated formation of protein-DNA complexes was measured in whole cells and isolated nuclei from log- and plateau-phase cultures with $^3$H-thymidine labelled cellular DNA as substrate. Protein-DNA complexes formed were precipitated by SDS/K+ as described (Section 4.3.4). Log-phase whole cells (●); plateau-phase whole cells (○); isolated nuclei from log-phase cells (▲); isolated nuclei from plateau-phase cells (▲). Values given are means from 2 - 4 independent experiments with standard errors.
et al., 1988).

4.4.4 In vitro stimulation of covalent DNA-topoisomerase II complex formation with nuclear extracts from log- and plateau-phase cells. Results of an experiment similar to those described in section 4.4.3 with nuclear extracts from log- and plateau-phase cells and using [α-32p]3'-end labelled DNA as a substrate for complex formation are presented in Figure 4.6. Amsacrine induced stimulation of protein-DNA complex formation with nuclear extracts from log-phase cells was 3-fold greater than that obtained with nuclear extracts from plateau-phase cells with 20 μM amsacrine. Although the initial stimulation with low amsacrine concentrations was similar in both extracts, saturation of stimulation was attained at considerably lower drug concentrations in plateau-phase extracts, and only a slight additional stimulation was observed at higher drug concentrations. This somewhat unusual result is as yet unexplained.

4.5 SUMMARY

(i) A 2-fold reduction of topoisomerase II catalytic activity was found in plateau-phase CHO-AA8 cells. However, this difference in strand-passing activity was unlikely to account solely for the 4- to 8-fold decrease in amsacrine cytotoxicity (Robbie et al., 1988).

(ii) There was no detectable decrease in sensitivity of the unknotting reaction to amsacrine in nuclear extracts from plateau-phase cells. In accord with Chapter 3 and other reports (Nelson et al., 1984) the inhibition of the unknotting reaction of topoisomerase II by amsacrine was not directly related to the cytotoxic action of the drug. Therefore, it
FIGURE 4.6: Stimulation of topoisomerase II-DNA complex formation in nuclear extracts. Stimulation by amsacrine of the topoisomerase II-DNA complex formation in nuclear extracts from log- and plateau-phase cells was determined using 50 ng 3'end-labelled pBR322 DNA as substrate. Assay conditions were as described in Section 2.14.2C. Nuclear extracts from log-phase cells, ( ); nuclear extracts from plateau-phase cells ( ).
is unlikely that alterations in topoisomerase II strand-passing catalytic activity contributed to the observed drug-resistance of plateau-phase AA8 cells.

(iii) Plateau-phase cells and nuclei were much less susceptible to amsacrine stimulated formation of PDC than log-phase cells or nuclei from log-phase.

(iv) Results of experiments using nuclei extracts and an exogenous DNA, 3'-end labelled pBR322, as a uniform substrate for PDC formation reflected those obtained with whole cells and isolated nuclei. This indicates that the decreased sensitivity of plateau-phase cells to amsacrine-induced PDC formation is not related to changes in chromatin conformation.

(v) There was a correlation between the emergence of drug resistance in non-cycling cells and drug-induced topoisomerase II-DNA complex formation, but not topoisomerase II catalytic activity.

(vi) The data taken together suggested that drug-enzyme and/or enzyme-DNA interactions were altered in plateau-phase CHO-AA8 cells.

A more detailed discussion of these results in combination with the results presented in Part II of this chapter is provided at the conclusion of Part II.
PART II: THE RELATIONSHIP BETWEEN SENSITIVITY TO AMSACRINE AND DNA TOPOISOMERASE II IN A COLD-SENSITIVE CELL CYCLE MUTANT OF A MURINE MASTOCYTOMA CELL LINE.

4.6 INTRODUCTION

The complex relationship between the cytotoxic effects of amsacrine, topoisomerase II and drug-induced DNA breakage was also investigated using 21-Fb cells, a cold-sensitive (proliferating at 39.5°C, reversibly arrested in G1 at 35°C) cell cycle mutant of the P815-X2 murine mastocytoma cell line. After cell cycle arrest 21-Fb cells have been shown to exhibit a dominant phenotype in cell fusion experiments, to resemble normal mast cells and to differentiate (Zimmerman et al., 1981, 1983; Laeng et al., 1985). The aim of this study was to compare the sensitivity of proliferating and arrested cells to amsacrine by measuring cell survival, DNA breakage, protein-DNA complex formation and topoisomerase II strand-passing activities.

The studies presented in this section were performed in collaboration with Dr E. Schneider (Department of Cellular and Molecular Biology, Auckland University).

4.7 METHODS

4.7.1 Cell culture and drug toxicity assays.

Details of cell culture and induction of growth arrest of temperature sensitive 21-Fb cells are given in Chapter 2, section 2.6 and 2.6.1 respectively.

The protocol for the drug toxicity assays is given in section 2.13.
4.7.2 Extraction of DNA topoisomerase II activity.

Topoisomerase II activity was extracted from nuclei isolated from proliferating and growth arrested 21-Fb cells essentially as described in Chapter 3, section 3.2.2.4, with a few minor modifications. The following protease inhibitors were added at the indicated final concentrations to all buffers immediately prior to use: aprotinin (1 % v/v), leupeptin, 0.1 mg/ml; α2-macroglobulin, 0.01 mg/ml; phenylmethyl-sulfonyl fluoride, 1 mM; diisopropylfluorophosphate, 0.1 mg/ml. This effectively inhibited a strong proteolytic activity observed mainly in arrested 21-Fb cells, but did not adversely effect topoisomerase II activities per se. Conditions for cell lysis were as in Section 3.2.2.4, but the supernatant resulting from the centrifugation step immediately following Triton X-100 treatment of cells was retained for extraction of soluble topoisomerase II activity. Nuclei isolation buffer A (see section 3.2.2.4) also contained 2 mM CaCl₂ and 0.1 mM dithiothreitol, whereas buffer B was buffer A with 5 mM MgCl₂ and without CaCl₂. After the final centrifugation in buffer B, nuclei were resuspended in 0.4 ml buffer C (20 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM MgCl₂, 0.1 mM dithiothreitol, 10 mM Na₂S₂O₅). Topoisomerase II activity was recovered separately from the nuclei and cytoplasmic supernatant as described in section 3.2.2.4.

Extracts from proliferating and arrested cells were diluted to equal protein concentrations of approx. 0.1 mg/ml (nuclear) and 3 mg/ml (soluble) and stored at -70°C.

4.7.3 The phage P4 unknotted assay.

The phage P4 DNA unknotted assay was performed as described in Section 2.14.1 with 4 μl of nuclear or soluble extracts (providing 120 mM
One unit of activity was defined as the amount of extract (in µg protein) that completely unknotted 0.24 µg phage P4 DNA.

4.7.4 Quantitation of amsacrine-stimulated covalent PDC formation in proliferating and arrested 21-Fb cells, lysed cells, isolated nuclei and nuclear extracts.

The SDS/K⁺ precipitation assays described by Rowe et al. (1986a) and Liu et al. (1983) were used to measure drug-induced formation of protein-DNA complexes in intact 21-Fb cells and nuclear or soluble extracts from proliferating and arrested 21-Fb cells.

A protocol developed during the course of the study, based on the methods of Rowe et al. (1986a) and Liu et al. (1983) was used to quantitate amsacrine-induced PDCs in lysed cells and isolated nuclei. A full description of the protocol is given in Chapter 5, with only an outline of the assay provided below.

For studies with cells, the DNA of proliferating and arrested 21-Fb cells was labelled with [³H]-thymidine as described in section 2.10. The cells were collected by centrifugation and washed once with TBS. One half of the cells were kept on ice until required for cell lysate or isolated nuclei preparation (see below). The rest were resuspended in fresh growth medium to a final density of 2 x 10⁶ cells/ml, distributed in 49 µl aliquots (10⁵ cells) into 96-well microtiter plates and treated with 0-20 µM amsacrine for 10 min at 37°C. Drug-induced protein-DNA complexes were then measured as described in section 2.14.2 A.

PDCs were also measured in cell lysates or isolated nuclei to overcome the plasma membrane barrier. The cells that had been retained on ice were collected by centrifugation, resuspended in nuclei buffer (20 mM Tris-HCl,
pH 7.2, 150 mM KCl, 5 mM MgCl$_2$, 10 mM Na$_2$S$_2$O$_3$ and 2% (w/v) dextran 150-200) at 5 x 10$^7$ cells/ml and lysed with 0.05% Triton X-100 for 10 min at 0°C. For experiments requiring cell lysates, triton X-100 treated cells were added directly to the assay mixture (see below). For isolated nuclei experiments, the lysed cells were centrifuged (1200 x g, 10 min 4°C) and harvested nuclei were resuspended in nuclei buffer at 2 x 10$^7$ cells/ml. To initiate drug-stimulated protein-DNA complex formation, cell lysates containing 10$^5$ nuclei (2 μl) or 10$^5$ isolated nuclei (5 μl) were added to the reaction mixture in 96-well plates in a total volume of 50 μl/well together with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 120 mM KCl, 5 mM EGTA, 2.25 mM EDTA, 0.5 mM dithiothreitol, 30 μg/ml bovine serum albumin, 2 mM ATP, 0-20 μM amsacrine and additional cytoplasmic extract. The cytoplasmic extract was prepared by digitonin extraction of corresponding proliferating or arrested 21-Fb cells (as described in Chapter 5.2.3), and added in predetermined optimal amounts of 20 μg and 40 μg extract protein prepared from proliferating or arrested cells, respectively. No protein-DNA complex forming activity was detected in this digitonin extract. Incubation was for 10 min at 37°C and PDCs were measured as described in section 2.14.2A for 96-well plates.

PDC formation was also measured in nuclear and soluble extracts from proliferating or arrested 21-Fb cells using [α$^{35}$S]3'-end labelled EcoR1 digested pBR322 DNA as substrate according to section 2.14.2C. The reactions were initiated using 10 μl of nuclear or soluble extract containing approximately 0.3 μg or 10 μg protein respectively (which provided 120 mM KCl).

4.7.5 DNA breakage assay.

DNA breakage induced by amsacrine in proliferating or arrested 21-Fb
cells was determined by the fluorescence enhancement assay for DNA unwinding described in section 2.15.

4.8 RESULTS

4.8.1 Amsacrine cytotoxicity in proliferating and arrested 21-Fb cells.

To determine whether arrested 21-Fb cells were less sensitive to amsacrine cytotoxicity than their proliferating counterparts, proliferating (at 39.5°C) and arrested (4 days at 33°C) cells were exposed to various drug concentrations for 60 min at their respective culture temperatures and cell survival was assessed by colony formation in soft agarose. As shown in Figure 4.7, arresting 21-Fb cells at 33°C decreased their sensitivity to amsacrine only slightly, increasing the C_{10} (the drug concentration needed to produce 90% cell killing) by 50% from 1.2 to 1.8 µM amsacrine. To confirm that the difference in drug sensitivity between proliferating and arrested 21-Fb cells was not due to the different incubation temperatures, wild-type K21 cells were used as a control. Both the proliferation characteristics and the amsacrine sensitivity of K21 cells remained unchanged when cells were cultured at 33°C, 37°C or 39.5°C or when K21 cells cultured at 39.5°C were shifted to 33°C for four days or vice versa (data not shown). Therefore it was concluded that the difference in drug sensitivity of 21-Fb cells was unlikely to be due to the temperature shift used to arrest cell proliferation.

4.8.2 Amsacrine-induced DNA breakage.

Since it has been shown that amsacrine-induced cell death is generally accompanied by DNA breakage (Ralph and Schneider, 1987) drug-induced DNA
FIGURE 4.7: Effect of amsacrine on survival of proliferating and arrested 21-Fb cells. Aliquots of cells continuously grown at 39.5°C or arrested for four days at 33°C were incubated with 0-10 μM amsacrine for 60 min at 39.5°C or 33°C respectively. Cells were washed, then plated in soft agarose and incubated for 10-12 days at 39.5°C. Surviving fractions were determined relative to non-drug treated controls. All values are means of two separate experiments with at least four different cell densities per amsacrine dose. The mean cloning efficiencies of non-drug treated cells were: ( ) 21-Fb cells grown at 39.5°C, 95%; ( ) 21-Fb cells arrested for four days at 33°C, 40%.
breakage was measured in proliferating and arrested 21-Fb cells, using the fluorescence enhancement assay for DNA unwinding (FADU) described by Kanter and Schwartz (1982). This assay has been shown to yield the same information as alkaline elution (see Chapter 4, Part 1; Robbie et al., 1988; Taningher et al., 1987). Figure 4.8 shows that arrested 21-Fb cells were substantially less susceptible to amsacrine-induced DNA breakage than non-arrested cells. Since F appeared to be a logarithmic function of amsacrine concentration, the sensitivity of 21-Fb cells to amsacrine-induced DNA breakage could be described by a single parameter, $C_{1/2}$, defined as the drug concentration required to reduce the fraction of residual duplex DNA to half the control value under the conditions of the assay. $C_{1/2}$ values for proliferating and arrested 21-Fb cells were 0.12 $\mu$M and 0.78 $\mu$M respectively, indicating a more than 6-fold increase in the resistance of arrested cells. This value was in contrast to the 1.5-fold increase in drug resistance found in cell survival experiments.

4.8.3 Stimulation of protein-DNA complex formation.

The amsacrine-induced formation of protein-DNA complexes with cellular DNA in intact and lysed cells and isolated nuclei was measured by the SDS/K+ precipitation assay (Figure 4.9). A marked amsacrine dose-dependent stimulation of protein-DNA complex formation up to approx. 9-fold stimulation with 10 $\mu$M amsacrine was observed in proliferating cells, with little difference between intact or lysed cells and isolated nuclei. After an initial steep increase paralleling that in non-arrested cells, stimulation of complex formation in arrested cells reached saturation at approximately half the amount of stimulation found in proliferating cells with 10 $\mu$M amsacrine. This was followed by a slight drop in complex formation at higher drug concentrations in both arrested
FIGURE 4.8: Effect of amsacrine on DNA breakage in proliferating and arrested 21-Fb cells. Aliquots of cells continuously grown at 39.5°C or that were arrested for four days at 33°C, were treated with 0-1 μM amsacrine for 60 min at 39.5°C or 33°C respectively. Drug-induced DNA breakage was measured by the fluorescence enhancement assay for DNA unwinding (FADU), and F was calculated as described in Section 2.15. 21-Fb cells grown at 39.5°C, (○); 21-Fb cells that were arrested for four days at 33°C, (▲). Values given are means with standard errors from three independent experiments.
FIGURE 4.9: Stimulation of protein-DNA complex formation in intact and lysed cells and isolated nuclei. Amsacrine-stimulated formation of protein-DNA complexes was measured in intact and lysed 21-Fb cells and in nuclei isolated from 21-Fb cells that were continuously grown at 39.5°C or that were arrested for four days at 33°C. Cellular DNA was prelabelled with [3H]thymidine and protein-DNA complexes formed were precipitated by SDS/K+ (4.7.4). Intact cells grown at 39.5°C, (■); lysed cells grown at 39.5°C, (□); nuclei isolated from cells grown at 39.5°C, (▲); intact cells arrested for four days at 33°C, ( ▼ ); lysed cells arrested for four days at 33°C, ( ▼ ); nuclei isolated from cells arrested for four days at 33°C, ( ▼ ). Values given are means with standard errors from three independent experiments.
and proliferating cells. In contrast to the results with intact cells, lysed arrested cells and isolated nuclei exhibited less than 2-fold stimulation of protein-DNA complex formation at 10 μM amsacrine. Little additional stimulation in isolated nuclei was observed at higher drug concentrations although a slight increase in PDC formation was observed with lysed cells at 20 μM amsacrine. Thus the differences in amsacrine stimulation of protein-DNA complex formation between proliferating and arrested cells were comparable to the differences in DNA breakage, indicating a 6-fold increase in resistance to drug-mediated DNA damage in arrested cells.

Results of a similar experiment with nuclear extracts from proliferating and arrested cells using [α-35S]3'-end labelled DNA as substrate are presented in Figure 4.10. With 10 μM amsacrine, stimulation of protein-DNA complex formation with nuclear extracts from proliferating cells was almost 10-fold greater than that obtained with nuclear extracts from arrested cells, with little further stimulation at higher drug concentrations. When the same experiment was performed with soluble extracts (see below), the result was essentially the same as with nuclear extracts. However some stimulation was apparent in soluble extracts from arrested cells at or above 5 μM amsacrine. Thus the differences in protein-DNA complex formation between soluble and nuclear extracts from arrested cells seemed to be similar to those between intact and lysed arrested cells and nuclei.

4.8.4 Quantitation of DNA topoisomerase II strand-passing activity.

To determine whether there was any relationship between the state of proliferation, sensitivity to amsacrine and DNA topoisomerase II activity in 21-Fb cells, the strand passing activities of nuclear extracts from
FIGURE 4.10: Stimulation of topoisomerase II complex formation in nuclear and soluble extracts from proliferating or arrested 21-Fb cells. Determination of amarscine of the topoisomerase II-DNA complex was determined using 50 ng 3'-end labelled pBR322 DNA as substrate (Section 4.7.4). Nuclear extract from cells arrested for four days at 39.5°C, ( ); nuclear extract from cells grown at 39.5°C, ( ). Soluble extract from cells arrested for four days at 33°C, ( ); soluble extract from cells grown at 33°C, ( ). Values given are means with standard errors from four experiments.

AMSACRINE CONCENTRATION (µM)

FOLD INCREASE IN PRECIPITATED 3'[35S]-DNA

0 5 10 15 20
proliferating and arrested cells were compared. After preparing serial
2-fold dilutions of nuclear extracts from proliferating and arrested
cells, a P4 unknotting assay was performed with each dilution to determine
the amount of extract that contained 1 unit of topoisomerase II activity.
The results of a typical experiment presented in Figure 4.1 show at least
a 16-fold reduction in topoisomerase II activity in extracts from arrested
cells (compare lanes 5 and 8). Essentially the same result was obtained
using a second assay for topoisomerase II catalytic activity, the PM2
catenation assay (performed by A.M. Hutchins, Department of Cellular and
Molecular Biology, data presented in Schneider et al., 1988c; Appendix
A3). The same differences in activity were reflected in the specific
activities of the nuclear extracts used to measure catenation activity of
8.1 and 0.5 units/µg protein and to measure unknotting activity of 40 and
approx. 2.5 units/µg protein for extracts from proliferating and arrested
cells respectively. Thus, the nuclear topoisomerase II activity in
arrested 21-Fb cells was only approx. 5% of the activity in proliferating
cells. Furthermore, the fact that the two different methods gave the same
results makes it unlikely that the observed reduction in activity was due
to different extract composition (eg. salt concentration), which might
alter the assay specificity (eg. catenation in low salt vs. decatenation
or unknotting in high salt).

When cells were lysed with Triton X-100 and the nuclei were separated
by centrifugation the remaining soluble fraction contained ATP-dependent
P4 unknotting and amsacrine-stimulated protein-DNA complex-forming
activity with 3'-end labelled DNA as substrate. Therefore, it was
concluded that this activity was a type II topoisomerase (Rowe et al.,
1986a; Liu et al., 1981). To determine whether there was any difference
in the relative amounts of soluble and nuclear topoisomerase II activities
FIGURE 4.11: Quantitation of nuclear DNA topoisomerase II strand-passing activities by P4 DNA unknotting assays in nuclear and soluble extracts from proliferating or arrested 21-Fb cells. Nuclear and soluble extracts were serially 2-fold diluted and with each dilution a P4 DNA unknotting assay was performed (Section 2.14.1). Reaction products were analysed as in Figure 4.1. (A) Nuclear extracts; Lanes 1-7: 2-fold decreasing amounts as indicated of nuclear extract from cells grown at 39.5°C; lanes 8-14: 2-fold decreasing amounts as indicated of nuclear extract from cells arrested for four days at 33°C. (B) Soluble extracts; Lanes 1-7: 2-fold decreasing amounts as indicated of soluble extract from cells grown at 39.5°C; lanes 8-14: 2-fold decreasing amounts as indicated of soluble extract from cells arrested for four days at 33°C.
### A. Nuclear Extract Dilution

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
</table>

**39.5°C**
- 1
- 2
- 4
- 8
- 16
- 32
- 64

**33°C**
- 1
- 2
- 4
- 8
- 16
- 32
- 64

### B. Soluble Extract Dilution

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
</table>

**39.5°C**
- 1
- 2
- 4
- 8
- 16
- 32
- 64

**33°C**
- 1
- 2
- 4
- 8
- 16
- 32
- 64

Lane
before and after cell-cycle arrest the P4 unknotting activity in the soluble fraction of proliferating and arrested cells was also assayed. Soluble extracts from proliferating or arrested cells were serially 2-fold diluted and a P4 unknotting assay was performed with each dilution. As shown in Figure 4.11B) unknotting activity was clearly detected in soluble extracts from both proliferating and arrested cells. An approx. 4- to 8-fold (lanes 5 and 10) decrease in soluble topoisomerase II activity in cell-cycle arrested 21-Fb cells was obtained. This decrease was less than the reduction in enzyme activity in nuclear extracts which raises the question whether the two activities were due to different enzymes. Recently Drake et al. (1987) reported two distinct forms of topoisomerase II in mouse leukemia cells and suggested that they had different sensitivities to amsacrine and were present in different relative amounts in sensitive and drug-resistant cells. Also Heller et al. (1986) found multiple forms of type II topoisomerase in Drosophila. At present it is not known whether the soluble and nuclear activities in P815 cells are different. However, in initial attempts to isolate both enzymes by DEAE column chromatography the two activities eluted at different salt concentrations, suggesting that they might be different (B. Gallaher, unpublished results).

To determine whether the reduction in enzyme activity in arrested 21-Fb cells was caused by an inhibitor mixing experiments were performed with extracts prepared from proliferating and arrested cells. When increasing amounts of extracts from arrested cells were added to the corresponding extracts from proliferating cells and P4 unknotting and protein-DNA complex formation assays were performed there was no evidence for the presence of an inhibitor in either nuclear or soluble extracts from arrested 21-Fb cells (data not shown). Therefore it is likely that
the reduction in topoisomerase II activity was due to a decrease in enzyme content, although inactivation due to enzyme modification in vivo cannot be excluded.

4.9 PART II SUMMARY.

(i) The sensitivity of arrested 21-Fb cells to amsacrine decreased less than 2-fold in cell survival experiments when compared to proliferating cells.

(ii) In contrast, DNA breakage and stimulation of protein-DNA complex formation in intact cells, lysed cells or isolated nuclei was reduced approx. 10-fold in arrested cells.

(iii) Using 3'-end labelled DNA as a uniform substrate specific for the topoisomerase II-DNA complex it was found that the differences in amsacrine-stimulated PDC formation between extracts from proliferating and arrested cells were similar to those with intact cells, lysed cells and isolated nuclei.

(iv) A 16-fold reduction of DNA topoisomerase II catalytic activity occurred in arrested 21-Fb cells.

4.10 DISCUSSION

The question of resistance of mammalian cells to amsacrine and other topoisomerase II-specific anticancer drugs has attracted considerable attention over recent years. Almost simultaneously several authors
suggested that the stabilisation of the topoisomerase II-DNA cleavable complex rather than the inhibition of the strand passing reaction might be responsible for the cytotoxic action of these drugs (Nelson et al., 1984; Tewey et al., 1984a,b) and that the pharmacological effects of the drugs are not due to the inactivation of topoisomerase II but are the consequence of converting the enzyme into a DNA damaging agent (Wang, 1987). Using a drug resistant CHO cell line, Glisson et al. (1986a) showed that the topoisomerase II strand passing activity was identical and equally sensitive to inhibition by etoposide (VP16) in the sensitive parent and resistant mutant cell lines. In contrast, only nuclear extracts from the resistant cell line were refractory to drug-stimulated formation of the cleavable complex. Similar results were also reported by others (Bakic et al., 1986; Pommier et al., 1986a and Estey et al., 1987a). Further support for the cleavable complex hypothesis has come from results presented in Chapter 3, Rowe et al. (1986a) and Covey et al. (1988) providing evidence of a causal relationship between the in vitro cytotoxicity of a series of amsacrine derivatives and their potential to stimulate protein-DNA complex formation. Finally, while this study was in progress, Sullivan et al. (1986) and Markovits et al. (1987a) reported a decrease in topoisomerase II strand passing activity and a decreased formation of drug-stimulated protein-DNA complexes in cell-cycle arrested cells. In fact, a positive correlation between cytotoxicity, DNA damage and PDC formation is generally found (Pommier et al., 1985a; Sullivan et al., 1986, 1987; Markovits et al., 1987a; Robbie et al., 1988; Sinha et al., 1988), although some exceptions have been reported (Zwelling et al., 1982, 1987; Estey et al., 1987b; Ishida et al., 1988).

The results in this chapter represent further investigations into the
role of DNA topoisomerase II and DNA damage in amsacrine-mediated cell killing and a possible role for topoisomerase II in drug resistance using two model systems for non-cycling cells.

4.10.1 The relationship between sensitivity to amsacrine and DNA topoisomerase II in log- and plateau-phase CHO-AA8 cells.

The first system studied (Part 1) was the CHO-AA8 system of Robbie et al. (1988) in which a gradual loss of sensitivity towards amsacrine and a concomitant decrease in drug-induced DNA breakage had been demonstrated upon accumulation of cells in G1 phase (plateau-phase). Moreover, the decrease in sensitivity of plateau-phase AA8 cells to amsacrine was not attributed to altered drug accumulation and metabolism, changes in either the rate or extent of repair of drug-induced lesions, or lack of cell cycle progression per se. Rather, the primary determinant of amsacrine sensitivity appeared to reflect some biochemical change which began well before cells entered a non-cycling state and continued to develop after cells became growth arrested (Robbie et al., 1988; Figure 4.4). The close relationship between amsacrine induced cytotoxicity and reversible DNA breaks suggested that the differences in drug-induced cell killing of log- and plateau-phase cells may have been related to drug-induced topoisomerase II-mediated DNA breakage. Therefore, a possible role of topoisomerase II in this drug resistance was examined.

Results from P4 unknotting assays indicated an approx. 2-fold reduction in DNA topoisomerase II catalytic activity in plateau-phase cells. The drug resistance of the arrested cells was unlikely to be explained simply by a drop in topoisomerase II activity, as the 4-8 fold increase in resistance (Robbie et al., 1988) was substantially higher than the 50% reduction in topoisomerase II activity. This decrease in
activity was smaller than that reported by Sullivan et al. (1986) in a
different CHO sub-line or in the arrested temperature sensitive P815 cells
described in Part II of this Chapter. Possible reasons for this difference
are discussed below, however it is unlikely that the relatively high
residual activity in plateau-phase cell nuclei was due to contamination of
the nuclei with cytoplasmic topoisomerase II because the last supernatant
from the nuclei preparation contained no detectable DNA unknotting
activity.

To determine whether the decreased DNA breakage in plateau-phase cells
seen by Robbie et al. (1988) was in fact due to a modified action of
topoisomerase II, the formation of protein-DNA complexes in whole cells
was examined using two different assay systems, the Coombes-Pearson filter
binding assay and SDS/K+ precipitation assay. The results clearly
showed that plateau-phase cells were much less susceptible to amsacrine-
stimulated formation of protein-DNA complexes than log-phase cells.
Although Robbie et al. (1988) found no difference in drug uptake by log-
or plateau-phase cells, the possibility could not be completely excluded
that the activity of amsacrine against plateau-phase cells might be
limited by drug availability at its site of action, on topoisomerase II
molecules in the nucleus. Therefore, amsacrine-stimulated formation of
protein-DNA complexes was also measured in isolated nuclei to circumvent
possible effects at the plasma membrane. The results confirmed the
difference in drug-stimulated protein-DNA complex formation detected with
intact cells. Furthermore, no significant differences were observed
between whole cells and isolated nuclei from either log- or plateau-phase
respectively. This clearly indicated that (i) these cells did not have to
be intact for the formation of the protein-DNA complex and (ii) reduced
drug sensitivity of plateau-phase cells was unlikely to be due to altered
intracellular drug concentrations.

Zwelling et al. (1987) suggested that chromatin condensation might influence intercalator-induced protein-associated DNA cleavage, and Riou et al. (1986a, b) have shown that amsacrine stimulates DNA breakage preferentially in transcriptionally active, DNase I-hypersensitive sites. Therefore, it is conceivable that in arrested cells, there are less potentially cleavable DNA sites for topoisomerase II to act. To eliminate the possibility that altered DNA in non-proliferating cells influenced the results of the protein-DNA complex formation studies, 3'-end labelled DNA was used as a uniform substrate for the complex-formation reaction with nuclear extracts. The 3'-end labelled DNA substrate is specific for topoisomerase II (Rowe et al., 1986a) and therefore its use also eliminates the contribution of non-specific PDC's, for example due to topoisomerase I. As for intact cells and isolated nuclei, protein-DNA complex formation with the exogenous DNA substrate was more pronounced using log- than plateau-phase nuclear extracts. These results suggest that differences in the amounts of the protein-DNA complex formed by log-versus plateau-phase cells were due to alterations in topoisomerase II.

4.10.2 The relationship between sensitivity to amsacrine and DNA topoisomerase II in a cold-sensitive cell-cycle mutant of a murine mastocytoma cell line.

The second cell system used to investigate the complex relationship between the sensitivity to amsacrine, topoisomerase II and drug-induced DNA breakage was the cold-sensitive cell cycle mutant of the P815 murine mastocytoma cell line (PART II). Upon incubation at the non-permissive temperature, these cells arrest in G1 and develop a differentiated, normal mast cell-like phenotype (Zimmermann et al., 1981, 1983; Laeng et al.,
1985). In cell survival experiments (Figure 4.7) only a marginal decrease in amsacrine sensitivity was observed in arrested 21-Fb cells, which was similar to the results of Sullivan et al. (1987) with log- and plateau-phase L1210 cells, but contrasts with the CHO-AA8 system of Robbie et al. (1988) used in the studies described in Part I of this chapter. Possible reasons for these differences are discussed below.

To determine whether the marginal response of 21-Fb cells to amsacrine was reflected at the molecular level, DNA breakage and cleavable complex formation were examined. In contrast to the only slight decrease in amsacrine sensitivity in arrested cells, a marked reduction in DNA breakage was seen in arrested cells after amsacrine treatment which was paralleled by a similar reduction in amsacrine-stimulated protein-DNA complex formation. To circumvent possible plasma membrane related effects such as altered drug uptake or efflux influencing the interaction between amsacrine and its intracellular target, topoisomerase II, protein-DNA complex formation was also measured in lysed cells and isolated nuclei. These results confirmed the decrease in drug-stimulation of protein-DNA complex formation detected with intact cells after cell-cycle arrest. Therefore, it was concluded that (i) reduced intracellular drug concentrations were not the cause of the decreased response of arrested 21-Fb cells to amsacrine and (ii) that intact cells were not necessary for the formation of the protein-DNA complex. However, the fact that I was unable to detect drug-induced protein-DNA complex formation in nuclei from P815 cells without adding an excess of cytoplasmic extract, prepared by digitonin extraction of cells (see Section 5.2.5) and which itself did not contain any detectable topoisomerase activity (Section 6.3.1) suggested that some additional factor(s) might be involved in protein-DNA complex formation and possibly cell killing. This possibility was investigated
further and the results are presented later in this thesis.

The results of experiments using 3'-end labelled DNA as a uniform substrate for the specific detection of topoisomerase II-DNA complexes in nuclear and soluble extracts from proliferating and arrested cells were similar to those obtained with intact cells, lysed cells and isolated nuclei. Therefore, the possibility that altered DNA in arrested cells influenced the results of the protein-DNA complex formation studies could be eliminated. Taken together these results suggested that differences in the amounts of protein-DNA complex formed were due to a reduction or alteration in topoisomerase II. Therefore, topoisomerase II strand-passing activity in nuclear extracts was measured using the P4 unknotting assay (Figure 4.11) and the PM2 catenation assay (data not shown here, but included in Appendix A3, Schneider et al., 1988c). Both methods showed that there was an approx. 16-fold reduction in topoisomerase II activity when the cells became arrested, similar to the reduction in protein-DNA complex formation. Consequently, it is likely that the reduction of amsacrine-induced DNA breakage was due to lower enzyme activity present in arrested cells. An alteration of topoisomerase II activity by enzyme modification, such as phosphorylation (Ackerman, 1985; Sahyoun et al., 1986) or ADP-ribosylation (Darby et al., 1985; Mattern et al., 1987) cannot be excluded. Alternatively, other as yet unidentified factor(s) that modify the topoisomerase II-amsacrine interactions in arrested 21-Fb cells could be involved.

4.10.3 What is the role of Topoisomerase II in cellular drug sensitivity?:

The CHO-AA8 cell system versus the 21-Fb cell cycle mutant system.

The contrasts between the two cell systems studied in terms of sensitivity to amsacrine, DNA damage or DNA topoisomerase II activity
further highlight the complexity of the mechanisms of drug-induced cell killing and drug resistance.

The influence of cell proliferative state on the magnitude of topoisomerase II-mediated DNA cleavage was established in both Parts I and II above and by other workers (reviewed in Chapter 1). However, a causal relationship between drug-induced protein-associated DNA cleavage and cytotoxicity is still not conclusively proven. Data supporting (Chapters 3 and 4, Part I) and opposing (Chapter 4, Part II) such a relationship has been presented here and in the published literature. I can only conclude again that a simple relationship does not necessarily exist between the frequency of protein-associated DNA cleavage produced by a given drug dose and the cytotoxic potency of that drug dose.

The exact reasons for the differences in amsacrine sensitivity of the growth arrested CHO and 21-Fb cells used in this study are not known. However, it is possible that differences in cellular phenotype e.g. the degree of transformation or malignancy, are important in determining sensitivity to anticancer drugs. A few comparative studies to date (Sullivan et al., 1986; Markovits et al., 1987b; Sullivan et al., 1987; Zwelling et al., 1987) raise the possibility that some cells of malignant origin are intrinsically more susceptible to anticancer drugs. Moreover, the degree of malignancy may affect the quality of the arrested state and hence the decrease in enzyme activity when cells enter stationary phase. The studies reported also tend to suggest that less-malignant cells become more resistant to anticancer drugs when entering quiescence or plateau-phase (Sullivan et al., 1986; Markovits et al., 1987; Sullivan et al., 1987; Zwelling et al., 1987). In agreement with this possibility, little change was found in drug sensitivity after cell-cycle arrest in the 21-Fb cells, a mutant of the
highly malignant P815 cell line. However, the method used to arrest cells may differentially affect topoisomerase II activity (or expression) or extractability as only a 2-fold decrease in topoisomerase II strand-passing activity was detected in cells arrested by serum deprivation or DBcAMP (Hutchins, A.M., unpublished results) compared to the 16-fold decrease in topoisomerase II activity detected in the temperature arrested mutants in the present study. Therefore, it is possible that the different methods of growth arrest used in individual studies may also contribute to the enigmatic relationship between topoisomerase II and drug sensitivity illustrated by the data presented here and elsewhere.

Taken together the data I have presented suggest that a very complex set of parameters can influence amsacrine-induced DNA cleavage and cytotoxicity in different cells or even a single type of cell under different growth conditions.

With the 21-Fb cells used in this study there was a lack of correlation between the small increase in resistance to amsacrine in cell survival and the large decrease in sensitivity to DNA breakage and protein-DNA complex formation and in topoisomerase II activity when cells entered quiescence. Similarly, Chow and Ross (1987) and Estey et al. (1987a) found no correlation between amsacrine-induced DNA cleavage and amsacrine-induced cell killing using synchronised Balb/c 3T3 or HeLa cells. They observed the lowest DNA cleavage frequency in late G1 and S phase cells, but maximal cytotoxicity of amsacrine in early S phase cells. Furthermore, there was only a small increase in survival of G1 phase cells as compared to asynchronously proliferating cells. It is possible that 21-Fb cells used here arrest in G1 at or near the G1/S phase border with a concomitant low sensitivity to amsacrine-induced DNA breakage but
significant sensitivity to amsacrine cytotoxicity, which might explain the poor correlation between cytotoxicity and DNA cleavage induced by amsacrine. It has recently been suggested (Pommier et al., 1986a, 1988) that the formation of cleavable complexes is an early step in a cascade of events eventually leading to cell death and that the DNA breaks must be modified or elicit secondary lethal events to kill cells. If so, it is conceivable that the breaks in arrested 21-Fb cells, though fewer in number, are of a different quality being more susceptible to modification by additional factors or events. For instance it is possible that in arrested cells topoisomerase II predominantly binds to actively expressed genes whose cleavage is lethal to the cell, while in proliferating cells cleavable complex formation also occurs at many non-essential sites. From this reasoning it follows that the location of the cryptic breaks formed in the cleavable complex may be more important for cell survival than their actual formation. Alternatively, it is possible that topoisomerase-mediated DNA breaks in arrested cells persist longer due to a delayed resealing, thus being more susceptible to secondary events which turn them into lethal lesions. However, although this possibility cannot be completely excluded given the fact that the precise nature of the cold-sensitive mutation is not known, it is thought to be unlikely because no difference in resealing of DNA breaks after drug removal was observed between log- and plateau-phase CHO-AA8 cells (Robbie et al., 1988) or in different phases of the cell-cycle in HeLa cells (Estey et al., 1987a).

The experiments described in this chapter and published literature using in vitro assays measuring only topoisomerase II activity (eg. P4 unknotting, PM2 cation and drug-induced topoisomerase II-DNA binding) need to be interpreted with caution. Differences in enzyme activity in proliferating and arrested cells need not necessarily reflect changes in
enzyme content but may also arise from altered extractability of the enzyme or from post-translational mechanisms which modify enzyme-DNA binding or activity. For example, it is interesting to note that in plateau-phase CHO-AA8 cells (Part I) exhibiting only a 2-fold drop in topoisomerase II strand-passing activity, minimal drug-stimulated protein-DNA complex formation occurred and this remained limited even at high drug concentrations. Therefore, it is tempting to speculate that alterations in drug-enzyme or enzyme-DNA interactions are involved, which limit the amount of protein-DNA complex formed. As previously mentioned, possibilities include alteration of topoisomerase II activity by phosphorylation (Ackerman, 1985; Sahyoun et al., 1986), ADP-ribosylation (Darby et al., 1985; Mattern et al., 1987) or as yet unidentified additional factor(s) that modify amsacrine binding to topoisomerase II. Furthermore such phenomena cannot be excluded when considering the 21-Fb system described here.

Unfortunately, neither antisera to topoisomerase II nor the cloned gene were available to me at the time these studies were conducted and I was unable to investigate these questions further. Nevertheless, it is clear that further biochemical and genetic studies are needed to determine the extent to which a decrease in topoisomerase II activity relates to topoisomerase II content and provide the connection between the complex topoisomerase II-DNA damage drug-cytotoxicity relationship.

The results in Parts I and II of this Chapter comprise two independent collaborative publications in Biochemica et Biophysica Acta which are included in Appendices A2 and A3.
DEVELOPMENT OF A SYSTEM TO QUANTITATE PROTEIN-DNA COMPLEXES IN ISOLATED NUCLEI: EVIDENCE FOR A FACTOR THAT ENHANCES AMSACRINE-INDUCED TOPOISOMERASE II -DNA COMPLEX FORMATION.

5.1 INTRODUCTION

Chapter 4 described the successful use of isolated CHO cell nuclei to examine the relationship between the cytotoxicity of anticancer drugs and topoisomerase II with particular reference to drug resistance (Chapter 4: Part I) and salt extracted murine mastocytoma cell nuclei to study the relationship between the cytotoxic effects of topoisomerase II directed anticancer drugs, topoisomerase II and drug-induced DNA breakage (Chapter 4: Part II). However, in contrast to results with CHO cells, I was unable to detect any amsacrine-induced topoisomerase II-DNA complex formation with intact nuclei isolated from murine K21 cells or L1210 leukemia cells. As a number of other workers had successfully employed isolated nuclei systems to study drug action, examining specifically drug-induced protein-associated DNA breaks by alkaline elution, it was of considerable interest to delineate the reasons for differences between the unsuccessful K21 or L1210 nuclei systems, the successful CHO nuclei system and those of other researchers. Experiments detailed in this chapter show that the inability to detect amsacrine-induced PDCs in isolated nuclei was not due to experimental or technical artifacts, but was attributed to the absence or reduction in isolated nuclei of a component which facilitates formation of amsacrine-induced topoisomerase II-DNA complexes.

The development of a system in which to assay drug-induced PDC's in
isolated nuclei is presented in this chapter with methodology and results described and discussed together. It should be noted that the basic SDS/K⁺ precipitation technique of Rowe et al. (1986a) (Section 2.14.1A) employed to detect PDC's provides the foundation of the assay for PDC's in isolated nuclei developed and described in this chapter.

5.2 METHODS, RESULTS AND DISCUSSION.

As previously mentioned, isolated CHO cell nuclei were successfully used as a system in which to measure drug-induced topoisomerase II-DNA complexes, free from complications due to drug metabolism and transport phenomena. The unsuccessful use of the identical protocol with a different cell system, namely K21 cells (or L1210 cells) was not anticipated.

The SDS/K⁺ precipitation technique of Rowe et al. (1986a) (section 2.14.2A) was initially adapted to measure drug-induced protein-DNA complexes in isolated nuclei as follows. Briefly, nuclei from radioactively labelled cells were isolated (as described in Section 4.3.4), treated with various concentrations of amsacrine for 60 min at 37°C and collected by centrifugation at 1200 x g for 10 min at 4°C. Supernatants were discarded and the nuclei lysed with a solution containing SDS, EDTA and salmon sperm DNA (prewarmed to 65°C). Proteins were precipitated by the addition of KCl and after an incubation on ice the precipitates were collected by centrifugation at 0°C. The resulting pellet contained covalent protein-DNA complexes, while free DNA remained in the supernatant. The supernatants were discarded and the complexes collected and washed on GF/C filters. The amount of radioactivity associated with the filters was then determined to provide a quantitative
measure of drug-induced PDC's.

This method provided excellent reproducible results when intact cells were used. In contrast, with isolated nuclei two major difficulties were encountered:

1. High background levels (i.e. in non-drug treated nuclei) of non-specific PDC were routinely precipitated and no difference between non-drug treated and drug treated samples could be detected. It was thought that this non-drug-specific complex formation obscured the drug-specific PDC's formed. This situation prevailed for nuclei from all cell lines used, including CHO nuclei.

2. After resolution of the problems leading to high backgrounds, it became apparent that there was in fact little discernible drug-induced PDC formation by isolated nuclei and no difference in the amounts of complexes induced by varying concentrations of drugs were detected. This problem was peculiar to K21 or L1210 cell nuclei as drug-stimulated formation of topoisomerase II-DNA complexes was readily detected with isolated CHO nuclei.

Because the experiments conducted to overcome the problems associated with the precipitation of high levels of non-specific PDC's were fundamental to the development of final reaction conditions they are briefly described.

5.2.1 High non-specific protein-DNA complex formation.

Routinely in non-drug treated samples, up to 25% of total acid precipitable radioactivity per $10^5$ nuclei was non-specifically precipitated by SDS/K$^+$ compared with less than 1% in intact cells (e.g. Table 5.1). No repeatable differences between drug treated and control samples could be detected in isolated nuclei.
TABLE 5.1: Protein-DNA complex formation in non drug- and drug-treated K21 cells and isolated nuclei.

<table>
<thead>
<tr>
<th>AMSACRINE CONCENTRATION (μM)</th>
<th>% RADIOACTIVITY PRECIPITATED (^a)</th>
<th>INTACT CELLS</th>
<th>ISOLATED NUCLEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.8</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.5</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.6</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.6</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) % of total acid precipitable counts/assay (10^5 cells or nuclei)
It was possible that such high background complex formation was attributable to inadequate dispersal of nuclei and nuclear scaffolds during the lysis step. In attempts to overcome this many approaches were tried, including variation of SDS or EDTA concentrations in the lysis buffer and the inclusion of 2-mercaptoethanol which disrupts nuclear scaffolds. None were successful.

As shown in Table 5.2(A), the increase in background complex formation became evident immediately upon resuspension and lysis of cells in the first nuclei buffer. The composition of the nuclei buffer was then examined critically. Dextran was included in the buffers to stabilise the nuclei, however the dextran used at this point had a polymer length of 500. It was thought that over-stabilisation of the nuclei or nuclear scaffolds by the Dextran 500 may have contributed to the non-specific aggregation of protein and DNA, resulting in high backgrounds. When Dextran 150 replaced Dextran 500 in the buffers, background radioactivity was reduced substantially from 25% to 2-4% of total precipitable counts (Table 5.2, B). The omission of Ca\(^{2+}\) from nuclei buffers resulted in a further drop in background PDC levels (Table 5.2, C), bringing them into the (1% range expected from intact cell data. Ca\(^{2+}\) (and heavy metals such as Cu\(^{2+}\)) is known to irreversibly stabilise nuclear scaffolds (Lebkowski and Laemmli, 1982a, b; Lewis and Laemmli, 1982) and unless Ca\(^{2+}\) was omitted from nuclei buffers topoisomerase II activity per se (determined by the P4 unknotting assay) could not even be detected in nuclei after extraction with 0.6 M salt. Therefore, high backgrounds resulting from the non-specific binding of proteins to DNA were partially attributed to the irreversible stabilisation of scaffold protein binding to DNA by Ca\(^{2+}\) in the nuclei preparation buffers.

In addition to the above conditions the use of flat-bottomed 24- or
TABLE 5.2: Non-specific protein-DNA complex formation in non-drug treated nuclei at various stages and conditions of preparation measured by the SDS/K⁺ assay.

<table>
<thead>
<tr>
<th>STAGE OF NUCLEI</th>
<th>% RADIOACTIVITY PRECIPITATED WITH⁺⁺ DIFFERENT BUFFER INCLUSIONS b</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PREPARATION</td>
<td>Growth A. Dextran 500 B. Dextran 150 C. Dextran 150 Medium + Ca²⁺ + Ca²⁺ -Ca²⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Intact Cells</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II. Lysed Cells</td>
<td>27</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>III. Nuclei, first wash.</td>
<td>24</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>IV. Nuclei, second wash.</td>
<td>24</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>V. Nuclei, final resuspension.</td>
<td>24</td>
<td>2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

a % of total acid precipitable counts/10⁵ cells or nuclei in the absence of drug.

b see Section 4.3.4. for standard buffer conditions.
96-well microtitre plates was crucial for reproducible background levels of complex formation in both intact cells and isolated nuclei. Presumably, flat-bottomed plates provided a larger surface area over which cells and nuclei were dispersed during centrifugation, which prevents clumping of protein-DNA aggregates during the subsequent lysis step.

5.2.2 Absence of drug-induced protein-DNA complexes in isolated K21 or L1210 nuclei.

Using the assay conditions established in section 5.2.1, the effect of amsacrine concentration on PDC formation in isolated CHO cell nuclei was successfully determined (section 4.4.3). However, an unanticipated result was obtained when identical assay conditions were used to quantitate drug-induced PDC's in isolated K21 or L1210 nuclei. As shown in Table 5.3, the induction of PDC's by amsacrine was not detected in these nuclei. For example, whereas a 12-fold stimulation in PDC's (i.e. 9.6 % of total radioactivity) induced by 10 µM amsacrine was observed in intact cells, only approximately 1.0 - 1.9 fold stimulation was achieved in isolated nuclei at the same drug concentration. This result was highly repeatable with nuclei from both K21 and L1210 cell lines.

As drug-induced topoisomerase II-DNA complexes were readily detected in isolated CHO cell nuclei, their absence in K21 and L1210 nuclei was attributed merely to the use of conditions that were not optimal for drug action in nuclei isolated from these cell lines. The following is a description of extensive attempts to optimise conditions for PDC formation in K21 or L1210 nuclei.

Previous studies (Darkin, 1985) had shown that the DNA in isolated nuclei is particularly sensitive to the action of nucleases during prolonged periods of drug treatment. Such nuclease activity could
**TABLE 5.3: Amsacrine-induced protein-DNA complexes in intact K21 cells and isolated nuclei.**

<table>
<thead>
<tr>
<th>AMSACRINE CONCENTRATION (μM)</th>
<th>FOLD STIMULATION OF PDC FORMATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INTACT CELLS</td>
</tr>
<tr>
<td>0</td>
<td>1&lt;sup&gt;a&lt;/sup&gt; (0.8)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>7 (5.6)</td>
</tr>
<tr>
<td>10</td>
<td>12 (9.6)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are expressed as fold-stimulation compared with non-drug treated samples which = 1

<sup>b</sup> Data are % of total acid precipitable counts/10<sup>5</sup> cells or nuclei.
conceivably have negated the SDS/K+ assay used to detect PDC formation because it is extremely sensitive to DNA fragment size. To determine whether nuclease action negated drug-induced PDC formation in K21 nuclei, PDCs were quantitated in nuclei incubated with 10 μM amsacrine from 0-120 min at 37°C. Figure 5.1 shows that maximum complex formation was achieved with only a 10 min incubation with amsacrine, after which PDC levels steadily decreased, reaching background levels by 60 min. In contrast to the 10.8 fold increase in PDC's achieved after 10 min in intact cells, only a maximum 2.5 fold stimulation in PDC's was seen in isolated nuclei at equivalent drug concentrations. Therefore a 10 min drug incubation period was used for all subsequent experiments.

A number of parameters, shown by others (Pommier et al., 1982) to influence topoisomerase II activity in nuclei, were altered in efforts to develop optimal conditions for complex formation, e.g. pH, nuclei density, salt concentration, Mg2+ ion concentration and ATP. Typical results are shown in Table 5.4, A-E. Results are expressed as "fold-stimulation of PDC" in K21 nuclei treated with 10 μM amsacrine for 10 min at 37°C (Similar series of experiments were performed for nuclei isolated from both K21 and L1210 cells (data not shown)). It can be seen that these alterations in the reaction conditions did not markedly improve quantitation of drug effects in isolated K21 nuclei.

Thus far, it was known that intact cells possessed the ability to form protein-DNA complexes in response to drug and that the failure of nuclei to do so was not due to ion balance, pH or co-factor requirements. Therefore, at what precise stage during their preparation the nuclei lost the ability to form drug-induced PDC's was more closely examined.

The conditions giving maximal PDC formation for each parameter described in Table 5.4, namely pH 7.2, 120 mM KCl, 5 mM MgCl2, 0.5 mM
FIGURE 5.1: The effect of the length of treatment of isolated K21 nuclei with amsacrine on protein-DNA complex formation. Protein-DNA complexes were quantified by SDS/K+ precipitation in intact K21 cells and isolated nuclei after incubation with 10 μM amsacrine from 0 - 120 min. at 37°C. Intact K21 cells, (●): Isolated K21 nuclei (◇).
TABLE 5.4: The effect of varying reaction conditions on drug-inducible PDC formation.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>FOLD INCREASE IN PDC&lt;sup&gt;a&lt;/sup&gt; AT A GIVEN REACTION CONDITION.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. pH</td>
<td>pH 6.4 7.2 7.5 8.0</td>
</tr>
<tr>
<td></td>
<td>1 2.6 2.5 1</td>
</tr>
<tr>
<td>B. Nuclei Density. (X 10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>20 10 5 2.5 1.2 0.62</td>
</tr>
<tr>
<td></td>
<td>ND&lt;sup&gt;b&lt;/sup&gt; 2.3 1 1 ND ND</td>
</tr>
<tr>
<td>C. [KCl] (mM)</td>
<td>0 50 100 120 150 200</td>
</tr>
<tr>
<td></td>
<td>1.2 1.4 2.5 2.5 2.5 1.6</td>
</tr>
<tr>
<td>D. [Mg&lt;sup&gt;2+&lt;/sup&gt;] (mM)</td>
<td>0 2 5 10 20 50</td>
</tr>
<tr>
<td></td>
<td>1.1 1.3 2.3 1 1 1</td>
</tr>
<tr>
<td>E. [ATP] (mM)</td>
<td>0 0.5 1 2 5 10</td>
</tr>
<tr>
<td></td>
<td>1.3 1.3 2.5 3.1 1.3 1</td>
</tr>
<tr>
<td>F. [EGTA] (mM)</td>
<td>0 0.5 1 5 10 20</td>
</tr>
<tr>
<td></td>
<td>1.3 2.1 2.1 2 1.2 1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fold increase in PDC at the given reaction condition with 10μM amscarine for 10 min at 37°C.

<sup>b</sup> ND, not detectable.
EGTA, 2 mM ATP, $1 \times 10^5$ nuclei/sample and a drug treatment of 10 min at 37°C, were adopted and amsacrine-induced PDC formation in nuclei at various stages of preparation were determined. As shown in Table 5.5, the ability of nuclei to respond to amsacrine and form PDC's equivalent to levels seen in intact cells was lost as soon as the cell membrane was disrupted by detergents. Consequently, conditions of cell lysis were examined critically.

K21 cells were lysed with various concentrations of Triton X-100 for 0 (intact cells) - 30 min on ice. The extent of cell lysis was determined by Trypan blue exclusion (section 2.5.4) and the stimulation of PDC's by 10 μM amsacrine was followed. As shown in figure 5.2, both the concentration of Triton X-100 and the period of lysis were critical to the ability to stimulate PDC's in nuclei. There was little difference in PDC stimulation in nuclei isolated with either 0.01 % or 0.05 % Triton X-100 for up to 10 min, however a steady decline in complex formation occurred with prolonged periods of lysis. Very little complex was formed in nuclei prepared by lysis with 0.1 % or 0.3 % detergent. Trypan blue exclusion data indicated that the less disruptive the detergent was on the cell, the greater the retention of complex forming ability. For example, after treatment with Triton at 0.01 % for up to 10 min K21 cell cultures remained only "partially lysed" (25 %) and the stimulation of PDC's was at a maximum (3-fold), although a similar increase in complex formation was observed after treatment of cells for 10 min with 0.05 % Triton X-100 at which point cell lysis had reached 100 %. However, there was a qualitative difference in the nuclei prepared with 0.05 % compared to 0.1 % or 0.3 % detergent. Namely, large amounts of cytoplasmic material were associated only with nuclei prepared by 0.01 % or 0.05 % Triton and it remained even after repeated washings.
**TABLE 5.5:** PDC formation in isolated nuclei at various stages of preparation.

<table>
<thead>
<tr>
<th>STAGE OF NUCLEI PREPARATION</th>
<th>FOLD INCREASE IN PDC FORMATION WITH INCREASING AMSACRINE CONCENTRATION (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>I. Intact cells</td>
<td>9.2</td>
</tr>
<tr>
<td>II. Lysed cells</td>
<td>2.5</td>
</tr>
<tr>
<td>(0.1% TX-100)</td>
<td></td>
</tr>
<tr>
<td>III. Nuclei, first wash.</td>
<td>2.1</td>
</tr>
<tr>
<td>IV. Nuclei, second wash.</td>
<td>1.9</td>
</tr>
<tr>
<td>V. Final resuspension.</td>
<td>1.3</td>
</tr>
</tbody>
</table>
FIGURE 5.2: The effect of cell lysis conditions on protein-DNA complex formation. K21 cells were lysed with various concentrations of triton X-100 for 0 (intact cells) to 30 min. on ice, then protein-DNA complexes stimulated by 10 μM amsacrine were determined by SDS/K+ precipitation. The solid line (——) represents PDC formation and the dashed line (-----) represents the % of cells lysed or permeabilised after detergent treatment. Concentrations of triton X100 used: 0.01% (●); 0.05% (X); 0.1% (▼); 0.3% (○).
Together these results suggested that lysis conditions, generally, were critical to the quantitation of drug effects in K21 (or L1210) nuclei. Therefore, a comparison was made between a number of different detergents, permeabilising agents and methods of mechanical disruption for their ability to lyse cells and yet retain in nuclei the ability to produce drug-induced PDC's. These studies, with the exception of mechanical disruption, were performed as time courses (0-10 min) and the extent of cell lysis was followed by trypan blue exclusion. The experiments were performed on both K21 and L1210 cells.

Table 5.6 outlines the detergents and permeabilising agents tested and the concentrations and minimum period of incubation required to permeabilise or lyse >90% of cells, in comparison to the fold increase in PDC formation achieved with the resulting nuclei upon incubation with 10 μM amsacrine for 10 min at 37°C. To summarise the data, none of the permeabilising agents or detergents tested significantly improved the ability to detect drug-induced protein-DNA complexes in nuclei from either cell line. At best, a 3-fold increase in PDC formation was achieved on lysis of K21 cells with 0.05% Triton X-100. Generally, there was no correlation between the degree of lipophilicity of detergents (data not shown) and their ability to lyse or permeabilise cells. For example, Triton X-100 is more lipophilic, i.e. stronger, than Brij-58 but in terms of cell lysis Triton X-100 was much weaker. In toto, it was clear that the ability to produce protein-DNA complexes in response to amsacrine was lost immediately upon perturbation of the cell membrane with any reagent or method tried. This suggested that a cytoplasmic or membrane component might be necessary for K21 and L1210 nuclei to produce the levels of PDC formation achieved in intact cells in response to amsacrine.
TABLE 5.6: The effect of lysis conditions on PDC formation.

<table>
<thead>
<tr>
<th>METHOD OF CELL LYSIS</th>
<th>L1210 CELLS [%]/min&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PDC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>K21 CELLS [%]/min</th>
<th>PDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (intact cells)</td>
<td>10.2</td>
<td>2.0</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>Brij-58</td>
<td>0.05/10</td>
<td>2.2</td>
<td>0.05/10</td>
<td>1.8</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.05/10</td>
<td>1.7</td>
<td>0.05/10</td>
<td>2.9</td>
</tr>
<tr>
<td>Triton X-114</td>
<td>0.05/10</td>
<td>2.1</td>
<td>0.05/10</td>
<td>1.6</td>
</tr>
<tr>
<td>NP-40</td>
<td>0.3 /10</td>
<td>1.4</td>
<td>0.3 /10</td>
<td>2.1</td>
</tr>
<tr>
<td>Tween-40</td>
<td>0.3 /10</td>
<td>1.0</td>
<td>0.3 /10</td>
<td>1.5</td>
</tr>
<tr>
<td>Tween-80</td>
<td>mg/ml/min</td>
<td>PDC</td>
<td>mg/ml/min</td>
<td>PDC</td>
</tr>
<tr>
<td>Digitonin</td>
<td>0.25/10</td>
<td>1.5</td>
<td>0.1 /10</td>
<td>7.5</td>
</tr>
<tr>
<td>Saponin</td>
<td>0.08/10</td>
<td>2.5</td>
<td>0.08/10</td>
<td>2.5</td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>0.25/ 1</td>
<td>1.4</td>
<td>0.25/ 1</td>
<td>1.8</td>
</tr>
<tr>
<td>Freeze/Thaw</td>
<td>1.9</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Results expressed as final concentration (% or mg/ml) of lysing agent per minutes required to permeabilise or lyse >90% of the cells.

<sup>b</sup> Fold increase in protein-DNA complex formation with 10μM amsacrine for 10 min at 37°C.
5.2.3 Preparation of cytoplasmic extracts from K21 cells.

Blank-Liss and Schindler (1985) published a procedure for the preparation of concentrated cytoplasmic extracts from the P815-X2 murine mastocytoma cell line. The method involved the release of cytoplasmic proteins from cultured cells by treatment with digitonin. Digitonin extraction of cells has been shown to result in the release of cytosolic proteins without releasing lysosomal or mitochondrial enzymes (Mackall et al., 1979).

A cultured suspension of K21 cells containing approximately $5 \times 10^8$ cells was centrifuged (900 x g for 10 min at $4^\circ$C) and the cells were washed twice with ice-cold TBS (Section 2.4). After the final wash, the cells were resuspended in 10 ml of TBS and cell number determined. The cells were recentrifuged and suspended in digitonin solution (17 mM MOPS (pH 7.0); 250 mM sucrose; 2.5 mM EDTA; 400 units/ml aprotinin; 0.1 mg/ml leupeptin; 0.01 mg/ml α2-macroglobulin; 1 mM PMSF; 0.1 mg/ml diisopropylfluorophosphate (DFP) and 2 mg digitonin/ml) to obtain a cell density of $1.2 \times 10^8$ cells/ml. Freshly prepared protease inhibitors and digitonin were added to each preparation. After treatment with digitonin for 10 min at room temperature, the suspension was centrifuged (30,000 x g for 10 min at $0^\circ$C). The protein content of the cytoplasmic supernatant was determined (Section 2.16) before it was diluted to 2.5 mg protein/ml and stored in 100μl aliquots at $-90^\circ$C.

Cytoplasmic extracts were then added to nuclei using the reaction conditions described below to quantitate drug-induced PDC formation.

5.2.4 Preparation of Isolated Nuclei.

The DNA of proliferating K21 cells was labelled with $[^3]$H thymidine as described in Section 2.10. The cells were harvested by centrifugation,
washed once with TBS (Section 2.4), then resuspended in nuclei buffer (20 mM Tris-HCl (pH7.2); 150 mM KCl; 5 mM MgCl$_2$; 10mM Na$_2$S$_2$O$_3$; 2 % (w/v) dextran 150-200) at 5 x 10$^7$ cells per ml. Cells were then lysed with 0.05 % triton X-100 for 10 min at 0°C, the nuclei harvested by centrifugation (1200 x g for 10 min at 4°C) and resuspended in nuclei buffer at 2 x 10$^7$ cells/ml.

5.2.5 Quantitation of PDC in nuclei with added cytoplasmic extracts.

To determine whether a cytoplasmic component was necessary for PDC formation in nuclei, 10$^5$ nuclei were added to the following reaction mixture in flat-bottomed 96 well plates in a total volume of 50μl/well: 50 mM Tris-HCl (pH7.5); 5 mM MgCl$_2$; 120 mM KCl; 1 mM EGTA; 0.5 mM DTT; 30 μg/ml BSA; 2 mM ATP; 10 μM amsacrine and additional cytoplasmic extract (0 - 100 μg protein). The nuclei were incubated for 10 min at 37°C, then collected by centrifugation at 1200 x g for 10 min at room temperature. Protein-DNA complexes were precipitated with SDS and K+ and quantitated as described in Section 2.14.1A for 96-well plates. The total acid precipitated radioactivity per assay (i.e. per 10$^5$ nuclei) was routinely approximately 5 x 10$^4$ cpm.

Figure 5.3 shows that the addition of cytoplasmic extracts to isolated K21 nuclei restored the level of PDC formation to 80 % of that induced by 10μM amsacrine action on whole cells, which was approximately four-fold greater than that observed in nuclei without added extract. A peak in complex formation at 30μg protein was observed, with a sharp decline in PDC production at higher extract concentrations. This profile of activity in nuclei conferred by cytoplasmic extracts was highly reproducible.

Taking this peak of activity as an optimum, the reaction conditions (i.e. final concentrations of ions, ATP etc in the reaction buffer) were
FIGURE 5.3: The effect of cytoplasmic extracts on amsacrine-induced protein-DNA complex formation in isolated nuclei. Protein-DNA complexes induced by 10 μM amsacrine in isolated K21 nuclei in the presence of 0 - 100 μg of cytoplasmic extracts (Section 5.2.3) were measured by SDS/K+ precipitation (non-optimised conditions, see Section 5.2.5). K21 nuclei with 0 - 100 μg cytoplasmic protein (●); Control, PDC formation induced by 10 μM amsacrine in intact K21 cells (○).
reassessed (data not shown). The following optimal conditions for PDC formation by $10^5$ nuclei were determined as described in Section 5.2.4 in a total volume of 50 µl/well containing final concentrations of:

- 50 mM Tris-HCl (pH 7.5)
- 10 mM MgCl₂
- 120 mM KCl
- 5 mM EGTA
- 2.25 mM EDTA
- 0.5 mM DTT
- 30 µg/ml BSA
- 2 mM ATP

Together with 0 – 20 µM amsacrine, and with added cytoplasmic extract (0 – 100 µg protein). Because such a sharp peak in cytoplasmic activity was routinely observed (defined "PDC enhancing activity"), it was necessary to determine optimal amounts of extract (µg protein) for each fresh extract prepared. Drug treatments were for 10 min at 37°C and samples were processed for PDC's as described above. Under these optimised conditions, cytoplasmic extracts fully reconstituted PDC formation in nuclei to levels achieved with equivalent numbers of intact cells (see Chapter 6).

5.3 SUMMARY

In contrast to results with CHO nuclei (Chapter 4, Part I), only a low level of amsacrine induced topoisomerase II-DNA complexes was detected in K21 or L1210 cell nuclei.

Using a systematic approach the reasons for this difference were resolved. It was found that a component of cytoplasmic extracts made with digitonin restored drug-induced PDC formation in these nuclei to levels
induced in whole cells.

Further characterization of the cytoplasmic factor(s) is described in Chapter 6.
CHAPTER SIX

A PROTEIN FACTOR THAT ENHANCES AMSACRINE MEDIATED FORMATION OF TOPISOMERASE II-DNA COMPLEXES IN MURINE MASTOCYTOMA CELL NUCLEI: A PRELIMINARY CHARACTERIZATION.

6.1 INTRODUCTION

A wealth of evidence (see Chapter 1) has shown that DNA topoisomerase II is the nuclear target of several anticancer drugs, including amsacrine. The drugs interfere with DNA breakage-rejoining by stabilizing a cleavable complex between DNA and topoisomerase II (Wang, 1985; Ralph and Schneider, 1987). The amount of drug-induced complex serves as a measure for drug activity (Rowe et al., 1986a) and the "frozen" topoisomerase II-DNA complex is thought to be a key factor in drug mediated cytotoxicity (e.g. Chapter 3). Recently, a correlation between sister chromatid exchange and cytotoxicity was reported and attributed to the double-strand DNA breaks induced by anticancer drugs (Pommier et al., 1985a). However, others have found no correlation between DNA breakage and cytotoxicity (Chow and Ross, 1987; Estey et al., 1987b; Chow et al., 1988; Schneider et al., 1988d; Chapter 4, Part II). For example, in studies on amsacrine or etoposide-mediated cytotoxicity and DNA breakage in relation to cell cycle stage, Estey et al. (1987b) and Chow and Ross (1987) found maximal cytotoxicity in S phase cells, whereas maximal DNA breakage occurred in G2/M phase cells concomitant with maximal topoisomerase II activity. These and other studies have led to the conjecture that stabilisation of the cleavable complex is an essential initial step in a cascade of events
eventually leading to cell death and Rowe et al. (1986a) have proposed that cleavable complexes are disrupted by additional factors which reveal cryptic DNA breaks and generate open double-strand breaks that are lethal and/or recombinogenic. In support of the need for other factors, recent studies have shown that inhibiting protein synthesis with cycloheximide in Balb/c3T3, CCRF-CEM and L1210 cells (Chow et al., 1988) and in K21 mouse mastocytoma cells (Schneider et al., 1988d) diminishes the cytotoxic action of etoposide or amsacrine. Moreover, the cytoprotection conferred by cycloheximide was not a result of decreased topoisomerase II. Prolonged treatment of proliferating splenocytes by etoposide and other topoisomerase II inhibitors induces DNA fragmentation by a mechanism which does not directly involve topoisomerase II (Jaxel et al., 1988).

Previously (Chapter 4, Part I), isolated CHO-cell nuclei were used successfully to examine the relationship between the cytotoxicity of anticancer drugs and topoisomerase II, with particular reference to drug resistance. In contrast to results with CHO cells, I was unable to detect amsacrine induced topoisomerase II-DNA complexes in the nuclei from K21 or L1210 cells. However, in the preceding chapter, I have described the development of an assay employing specific conditions which facilitated the formation and detection of drug-induced PDCs in isolated K21 cell nuclei. It was demonstrated that the inability to detect PDCs in isolated nuclei was due to the absence or reduction in K21 cell nuclei of a component which enhances the formation of amsacrine-induced topoisomerase II-DNA complexes. This component or factor appeared to reside in the cytoplasm of cells which when added to isolated nuclei restored full complex formation.

As already mentioned other workers have proposed a role for additional factors in the stabilization and processing of the cleavable complex and
suggested their involvement may eventually cause cell death. Therefore, it was of obvious importance to pursue the conclusions of Chapter 5. In this chapter, I demonstrate that the "PDC enhancing activity" in K21 cells resides in a protein distinct from topoisomerases I and II. A preliminary characterization of the factor is presented.

6.2 METHODS

6.2.1 Preparation of cytoplasmic extracts.

Cytoplasmic extracts were prepared by digitonin extraction of log phase K21 cells as described in Section 5.2.3. The resulting extracts were diluted to 2.5 mg protein/ml and stored in 100 µl aliquots at -90°C.

6.2.2 Quantitation of amsacrine-stimulated covalent protein-DNA complex formation.

An SDS/K⁺ precipitation assay, based on that of Rowe et al. (1986a), was developed as described in Chapter 5 (optimal conditions given in Section 5.2.5) and used to measure the drug-induced factor-enhanced formation of protein-DNA complexes in isolated nuclei. It was necessary to determine optimal amounts of each cytoplasmic extract (µg protein) therefore a concentration range (0 - 100 µg protein) was tested for the ability to enhance amsacrine-stimulated PDC formation. The amount of extract (µg protein) that produced maximal protein-DNA complex formation with 10 µM amsacrine was defined as one unit.

In each experiment, the stimulation of PDC formation in whole cells by 10 µM amsacrine was determined as an internal control. This was also performed in a 96 well plate, however cells were distributed in 49 µl
aliquots (10^5 cells) in fresh growth medium (see Section 2.14.2A). Conditions of drug incubation and the precipitation of PDCs were as described in Section 2.14.2A. A 10 to 12-fold stimulation of PDC formation in whole cells was routinely achieved with 10 μM amsacrine.

6.2.3 Dialysis of cytoplasmic extracts through selective membranes.

In one series of experiments K21 cytoplasmic extracts were dialysed against 2000 volumes of 1.1-fold concentrated reaction buffer for 6 h at 4°C using selective membranes with 10 and 50 kDa molecular weight cut-offs. Dialysed extracts were then tested for PDC formation enhancing activity.

6.2.4 Heat inactivation of cytoplasmic extracts.

The thermal stability of the PDC enhancing component of K21 cytoplasmic extracts was tested by heating extracts at 65°C or 95°C for 0 - 120 minutes. Heat treated extracts were subsequently tested for ability to enhance drug stimulated PDC formation in isolated nuclei.

6.2.5 Treatment of cytoplasmic extracts with immobilized enzymes.

Gels with immobilized bacterial protease, trypsin or papain (Section 2.1.2) were washed several times with water then extract buffer (without digitonin and protease inhibitors) according to the manufacturers instructions. For enzymatic digestion, undiluted cytoplasmic extracts (freshly prepared without protease inhibitors) were incubated with the enzyme gels at 30°C for 2 h with constant gentle agitation. As controls, extract buffer was also incubated with the gels. The extracts or extract buffer were then separated from the gels by centrifugation. The protein concentration of each extract was determined and the extracts
were diluted to 2.5 mg/ml protein with non-enzymatically treated extract buffer containing the protease inhibitor cocktail described in Section 5.2.3. Protease inhibitors were also added to the extract buffer that had been incubated with the enzyme gels. Both the extracts and the control buffers (enzyme treated) were stored at -90°C prior to assay.

6.3 RESULTS.

6.3.1 Facilitation and characterization of amsacrine-induced PDC formation in isolated nuclei by cytoplasmic extracts.

When protein-DNA complexes (PDCs) in K21 cells and nuclei were measured by the SDS/K+ precipitation assay there was routinely an approximately 10-fold stimulation of PDC formation in whole cells with 10 μM amsacrine, whereas there was less than or equal to 3-fold stimulation of PDCs in isolated nuclei at equivalent or higher drug concentrations (Chapter 5, Table 5.3). As shown in Chapter 5, attempts to increase PDC formation in nuclei by altering the assay conditions (e.g. pH, ion composition of buffers, period of drug incubation, nuclei density and cell lysis conditions) were unsuccessful, although it was observed that full complex forming activity was lost immediately after disrupting the plasma membrane with detergents or by freeze-thawing. This suggested that a cytoplasmic or membrane component might be necessary for nuclei to achieve the levels of PDC formation obtained with intact K21 cells. In Chapter 5 (Figure 5.3), the addition of cytoplasmic extract to isolated K21 nuclei was shown to restore the level of PDC formation induced by 10 μM amsacrine to that of whole cells. The effect was repeated with several independently prepared extracts and a peak of PDC enhancing activity was consistently obtained with 20 - 30 μg of cytoplasmic protein (e.g. Figures
FIGURE 6.1: The effect of cytoplasmic extracts on amsacrine induced protein-DNA complex formation in isolated nuclei. Protein-DNA complexes induced by 10 μM amsacrine in isolated nuclei were measured by SDS/K+ precipitation. Cytoplasmic extracts and reaction conditions are described in Section 5.2.5. K21 nuclei with 0-100 μg cytoplasmic protein, (■); control: PDC formation induced by 10 μM amsacrine in intact K21 cells, (□). Values are means with standard errors from four independent experiments. Error bars are shown when greater than the size of the symbol.
The amount of each extract (μg protein) that induced maximal PDC formation was defined as one unit.

To exclude the possibility that the PDC enhancing activity was an alternative form of topoisomerase II such as described by Schneider et al. (1988c) or Drake et al. (1987), cytoplasmic extracts were tested for topoisomerase II-DNA binding activity, using \(^{35}\)S\(_{3}'\)-end labelled pBR322 DNA as substrate (Section 2.14.2) and topoisomerase II strand-passing activity using the P4 unknotting assay (Section 2.14.1). Results of typical assays for topoisomerase II activity in cytoplasmic extracts are shown in Figures 6.2 and 6.3. No topoisomerase II activity was detected in any cytoplasmic extract prepared with digitonin, although these assays readily detected topoisomerase II activity in more dilute extracts prepared with buffers containing higher salt concentrations and triton X-100 (Chapter 4, Part I and II; Schneider et al., 1988c).

Topoisomerase I was not responsible for the PDC enhancing activity in cell extracts because topoisomerase I activity is not increased by amsacrine (Hsiang et al., 1985). Furthermore, topoisomerase I produces protein complexes with nicked DNA in the absence of drugs and nicks in DNA produced during nuclei isolation (Pommier et al., 1985b) would have provided the appropriate substrates. However, there was no stimulation of PDC formation by cytoplasmic extracts added to isolated nuclei in the absence of amsacrine and the radioactive DNA precipitated by SDS/K+ with non-drug treated nuclei in the presence or absence of cytoplasmic extract was routinely approximately 1% of the total radioactive DNA in the nuclei, which was the same as the background value obtained with untreated whole cells. More conclusively, adding antibody against topoisomerase I to cytoplasmic extracts did not inhibit PDC enhancing factor in these extracts (Figure 6.4).
FIGURE 6.2: Topoisomerase II-DNA complex formation in cytoplasmic extract from K21 cells. Topoisomerase II binding activity in K21 cell cytoplasmic extracts was determined using 50 ng 3'-end labelled pBr322 as substrate. Reactions were initiated with 10 µM amsacrine and 2-fold serial dilutions of cytoplasmic extract as indicated. Decreasing amounts of 2-fold serially diluted (1/1 - 1/32) cytoplasmic extract (■); Control (●) decreasing amounts of 2-fold serially diluted (1/1 - 1/32) K21 nuclear extract with known topoisomerase II activity.
FIGURE 6.3: Quantitation of topoisomerase II activity by P4-DNA unknotting assays in cytoplasmic extracts from K21 cells. Cytoplasmic extracts (Section 5.2.3) were serially 2-fold diluted and with each dilution, a P4 DNA unknotting assay was performed (Section 2.14.1). Reaction products were analysed on 0.7 % agarose gels, stained with ethidium bromide and photographed under UV light. Lane 1, P4 DNA; Lane 2, Control, 1/4 dilution of K21 nuclear extract with known topoisomerase II activity; Lanes 3 - 9, 2-fold decreasing amounts (1/1 - 1/64) of cytoplasmic extract from K21 cells.
FIGURE 6.4: The effect of anti-topoisomerase I antibody on PDC enhancing activity. Amsacrine-induced protein-DNA complex formation in K21 nuclei incubated for 10 min. at 37°C with cytoplasmic extracts in the absence or presence of various dilutions (as indicated) of anti-topoisomerase I antibody was determined by SDS/K+ precipitation (Section 5.2.5).
The PDC enhancing factor showed a striking decrease in activity with increasing concentrations of the cytoplasmic extract (Figures 5.3, 6.1). Because cytoplasmic extracts prepared with or without protease inhibitors showed identical activity profiles this effect did not appear to be due to proteolysis of topoisomerase II unless the protease was not affected by the potent inhibitor cocktail employed. Moreover, a corresponding decrease in background PDC formation in non-drug-treated controls did not occur at high extract concentrations (data not shown). However, competition between proteins at high concentration might explain the decrease in PDC enhancing activity. For example, the factor could modulate topoisomerase II by phosphorylation which is negated by phosphatases in high concentrations of cytoplasmic extract.

It was conceivable that a component(s) of the buffer used to prepare cytoplasmic extracts could produce the observed PDC enhancing effect when added to the nuclei. However, the inclusion of protease inhibitors during nuclei isolation and in the assay reaction mixture did not affect the amsacrine-induced increase in PDC formation with or without cytoplasmic extract (Figure 6.5). Similarly, the extract buffer with or without added protease inhibitors did not stimulate drug-inducible PDC formation in isolated nuclei (data not shown). Therefore, reconstitution of full PDC formation in K21 nuclei by cytoplasmic extracts was not due to the protease inhibitors or any other component of the extract buffer in the added extracts.

6.3.2 The effect of inhibiting protein and RNA synthesis on cytoplasmic PDC enhancing activity.

To determine whether inhibiting protein or RNA synthesis affected the PDC enhancing activity, cytoplasmic extracts were prepared from K21 cells
FIGURE 6.5: The effect of protease inhibitors on the ability to induce protein-DNA complexes in isolated K21 nuclei. Amsacrine induced protein-DNA complex formation in K21 nuclei incubated with or without the protease inhibitor cocktail described in Section 5.2.3 was assessed by SDS/K+ precipitation (Section 5.2.5). Controls are as indicated: PDC formation in intact K21 cells (treated with 10 μM amsacrine) or K21 nuclei with 1 unit of PDC enhancing activity (30 ug cytoplasmic extract).
before and after treating the cells for 2 or 6 h with 10 μg/ml cycloheximide (protein synthesis inhibitor) or cordycepin (RNA synthesis inhibitor) (These concentrations were found to inhibit $^{35}$S methionine incorporation by at least 95%, and $^3$H uridine by at least 80% respectively; data not shown). The extracts were then tested for ability to enhance amsacrine-induced PDC formation in isolated K21 nuclei. As shown in Figure 6.6, inhibition of protein synthesis substantially reduced PDC enhancing activity, with reductions in PDC formation to approximately 40% or 20% that of the untreated extract after 2 and 6 h cycloheximide treatment respectively. Cordycepin treatment for 2 h did not appreciably affect levels of PDC enhancing activity in cytoplasmic extracts compared to control extracts. However, inhibition of RNA synthesis for 6 h reduced PDC enhancing activity to approximately 40% of the untreated extract (Figure 6.7). Since cycloheximide and cordycepin have previously been shown not to affect the assay of topoisomerase II per se (Schneider et al., 1988d), these results suggested that the PDC stimulating activity was associated with a labile protein, with a half life of 2 h or less.

6.3.3 Physicochemical characterization of the cytoplasmic PDC enhancing activity.

The properties of the PDC enhancing activity in cytoplasmic extracts from proliferating K21 cells were investigated in a preliminary characterization.

The cytoplasmic PDC enhancing activity was rapidly lost at 65°C and 95°C. At 65°C, only 40% of the activity remained after 30 minutes and all activity was lost within 2 h whereas incubation at 95°C caused a more rapid loss of activity, with less than 50% remaining after one minute (Figure 6.8). The activity was retained by size selective dialysis
FIGURE 6.6: The effect of inhibiting protein synthesis on PDC enhancing activity. Cytoplasmic extracts were prepared from K21 cells grown for 2 h or 6 h with or without 10 μg/ml cycloheximide. The enhancement of amsacrine induced topoisomerase II-DNA complexes in K21 nuclei by extracts (0-60 μg protein) from untreated and cycloheximide treated cells was determined by SDS/K+ precipitation (Section 5.2.5). Nuclei with untreated cell extract, (○); nuclei with extract from cells grown for 2 h with cycloheximide, (■); nuclei with extract from cells grown for 6 h with cycloheximide, (▲).
FIGURE 6.7: The effect of inhibiting RNA synthesis on PDC enhancing activity. Cytoplasmic extracts were prepared from K21 cells grown for 2 h or 6 h with or without 10 μM cordycepin. The enhancement of amsacrine induced topoisomerase II-DNA complexes in K21 nuclei by extracts (0-60 μg protein) from untreated and cordycepin treated cells was determined by SDS/K⁺ precipitation (Section 5.2.5). Nuclei with untreated cell extract, (●); nuclei with extract from cells grown for 2 h with cordycepin, (■); nuclei with extract from cells grown for 6 h with cordycepin, (▼).
FIGURE 6.8: The effect of heat on PDC enhancing activity. K21 cell extracts were heated at 65°C or 95°C for 0-120 min and the remaining amsacrine-induced PDC enhancing activity was determined with isolated K21 nuclei by SDS/K2 precipitation. Whole cells, ( ); nuclei without cytoplasmic extract, (x) or with cytoplasmic extract heated at 65°C, (■) or 95°C, (▼).
membranes with molecular weight cut offs of 10 and 50 kDa indicating that it was associated with material above 50,000 molecular weight (Figure 6.9).

Treatment of extracts with immobilized proteases destroyed the PDC enhancing activity. Figure 6.10 shows that proteolytic digestion of cytoplasmic extracts with trypsin or papain reduced its activity to the basal two-fold increase seen with nuclei alone. The bacterial protease XXIV-A was less effective, reducing the activity by 54%. Control experiments showed that the loss of PDC enhancing activity from enzymatically treated extracts was not due to digestion of topoisomerase II per se in the assays by contaminating proteases leached from the immobilized enzymes (Figure 6.10). These properties all suggest that the PDC enhancing activity is a protein.

6.4 DISCUSSION

Previously, I and collaborators have used cell free systems and isolated nuclei to examine the relationship between the cytotoxic effects of topoisomerase II-directed anticancer drugs and topoisomerase II (Chapter 4, Parts I and II; Schneider et al., 1988b, c). However, initial attempts to quantitate amsacrine-induced topoisomerase II-DNA complexes in isolated K21 cell nuclei were unsuccessful and only low levels of PDC could be detected (Chapter 5). As shown in Chapter 5 and above, a factor present in cell extracts made with digitonin restored drug-induced PDC formation in K21 nuclei to the level induced in whole cells. Since digitonin extraction of cells releases cytosolic proteins without releasing lysosomal or mitochondrial enzymes (Mackall et al., 1979), the PDC enhancing activity is presumably located in the cytoplasm,
FIGURE 6.9: Dialysis of K21 cytoplasmic extracts through size selective membranes. K21 cytoplasmic extracts were dialysed through size selective (10 kDa and 50 kDa cut off) membranes as described (Section 6.23) and the remaining amsacrine-induced PDC enhancing activity was determined with isolated K21 nuclei by SDS/K\textsuperscript{+} precipitation (Section 5.2.5). Control: K21 nuclei with non-dialysed cytoplasmic extract.
Nuclei + cytoplasmic extracts digested with immobilised proteases OR control extracts diluted with extract buffer previously incubated with immobilised proteases.

FIGURE 6.10: The effect of protease digestion on PDC enhancing activity. Undiluted K21 cytoplasmic extracts or extract buffer were incubated with immobilised proteases (10 units of trypsin or papain or 0.25 units of protease XXIV-A per 300 μl of extract or extract buffer) at 30°C for 2 h. After removing the proteases by centrifugation protease inhibitors were added and the cytoplasmic extract or control extracts diluted with protease-digested buffer were added to isolated K21 nuclei to determine the effect on PDC complex formation.
although leakage from nuclei cannot be excluded. Treating cells with cycloheximide prior to preparing extracts substantially reduced the PDC enhancing activity. Moreover, qualitatively similar results were achieved with 10 µM cordycepin indicating that continuous RNA and protein synthesis was required to maintain the enhancing factor. In view of its properties the possibility that the factor was type I or II topoisomerase was eliminated. A preliminary physicochemical characterization of the factor showed that it was sensitive to heat, non-dialysable and destroyed by protease. Together, these results indicate that the PDC enhancing activity resides in a labile cytoplasmic protein in excess of 50,000 MW that is distinct from the two known classes of eukaryotic topoisomerases.

Topoisomerase II has been shown to be modified by protein kinase C (Sahouyn et al., 1986), casein kinase II (Ackerman et al., 1985, 1988) or ADP-ribosylation (Darby et al., 1985). However, this is the first direct evidence for a protein that stimulates drug-induced topoisomerase II action. Further elucidation of the nature of the factor and its association with topoisomerase II should facilitate greater understanding of its in vivo function and the control of topoisomerase II. Moreover, because of its apparent association with the action of a topoisomerase II-targeted drug, alterations to the factor may contribute to topoisomerase II-related drug resistance.

Speculations on the in vivo role(s) of the PDC enhancing factor and models attempting to explain its involvement in drug action and resistance are considered in the concluding chapter of this thesis.

The results presented in this chapter are published in Darkin and Ralph, (1989) (Appendix A4). In Chapter 7, the generality of the factor and a possible role in drug resistance is investigated.
CHAPTER SEVEN.

GENERALITY OF THE PDC ENHANCING FACTOR AND ITS POSSIBLE IN VolVEMENT IN DRUG RESISTANCE.

7.1 INTRODUCTION.

The mechanism(s) by which amsacrine induced topoisomerase II-DNA complexes translate into a lethal event is not well understood although stabilization of the cleavable complex appears to be an important step in a chain of events involving additional factors which eventually causes cell death (Rowe et al., 1986a). Accordingly, inhibition of protein synthesis with cycloheximide protects cells against the cytotoxic action of anticancer drugs such as amsacrine or etoposide without decreasing the level of topoisomerase II per se in cells (Chow et al., 1988; Schneider et al., 1988d). Furthermore, in Chapters 5 and 6, I demonstrated and partially characterized a factor that enhanced the formation of amsacrine-induced topoisomerase II-DNA complexes in isolated nuclei of K21 murine mastocytoma cells. This factor appeared to be a labile protein, distinct from topoisomerases I and II, that was rapidly lost from cells grown with cycloheximide or cordycepin. Because the resistance of some cancer cells, e.g. non-cycling tumour cells, to drugs such as amsacrine could be related to the absence or availability of the protein factor(s) that enhances cleavable complex formation or stabilizes preformed complexes, I further investigated the nature of the protein from K21 cells that increases amsacrine-induced protein-DNA complex formation. Evidence is presented below that this, or a similar factor(s), is reasonably widespread, being present in four murine cell lines and one
human cell line tested. Detectable factor activity appeared to be associated with the state of the cell cycle, with temperature arrested or serum starved cells having reduced factor activity. Moreover, greatly reduced factor activity in extracts from amsacrine resistant human Jurkat cells suggests a role for the factor in drug resistance.

7.2 METHODS AND RESULTS.

Details of cell culture are given in Section 2.5. The preparation of cytoplasmic extracts and assessment of PDC enhancing activity was as described in Sections 5.2.3 and 5.2.5 respectively.

7.2.1 Generality of the PDC enhancing factor

The generality of the PDC enhancing factor described in Chapters 5 and 6 was assessed using five cell lines readily available to me. These were, the mouse mastocytoma cell lines K21, P815 and the temperature sensitive 21-Fb cells, mouse leukemia L1210 cells, and human leukemia Jurkat cells.

Cytoplasmic extracts were prepared from each cell line and their ability to stimulate amsacrine-induced protein-DNA complexes in "factor-depleted" K21 nuclei was assessed. None of the extracts contained topoisomerase II activity per se as assessed by P4 unknotting and the ability to stimulate topoisomerase II-DNA complexes with 3'-end labelled DNA as a substrate (data not shown). Figure 7.1 (A, B and C) shows that P815, 21-Fb and L1210 cells possess a PDC enhancing activity qualitatively and quantitatively similar to that in K21 cells (Figure 6.1). In each of these three cell lines, PDC levels in nuclei were reconstituted to levels routinely observed in whole cells at the same drug concentration (10µM amsacrine) and peak activities were reached within the 20-30 µg protein
FIGURE 7.1: The generality of the PDC enhancing activity. Stimulation of amascrine-induced PDC formation in isolated K21 nuclei by cytoplasmic extracts from four cell lines was measured by SDS/K+ precipitation (Section 5.2.5). Cytoplasmic extracts were prepared as described in Section 5.2.3. K21 nuclei with cytoplasmic extracts from: A. (▼), P815 cells; B. (■), 21-Fb cells; C. (■), L1210 cells; D. (▲), Jurkat cells. Control, PDC formation induced by 10 μM amascrine in intact K21 cells, (◇). Values are means with standard errors from 3 or more independent experiments. Error bars are shown when greater than the size of the symbol.
range normally observed for K21 extracts. With extracts from human Jurkat cells (Figure 7.1 D) a profile of PDC enhancing activity was obtained that resembled that of the other cell lines tested, although drug-induced protein-DNA complexes were not stimulated to levels equivalent to those achieved in whole cells and K21 nuclei with added murine cytoplasmic extracts. Possibly this result may be attributed to limited cross-reactivity between the factor in human Jurkat cell extracts and interacting proteins in murine K21 nuclei.

Nuclei from all the murine cell lines examined exhibited reduced ability to form PDCs, which could be stimulated with cytoplasmic extracts prepared from the same or other mouse cells. In contrast, Jurkat nuclei prepared using identical conditions demonstrated complex forming activity almost equivalent to that of intact cells in the absence of added Jurkat cytoplasm (data not shown). This result was reminiscent of the situation with CHO cell nuclei (Chapter 4, Part I) which produced amsacrine-induced PDCs without added cytoplasmic extract. Again, as discussed in Chapter 6, this may be a reflection of the differential retention of the factor in nuclei prepared from cells which differ in their response to nuclei isolation procedures. Nevertheless, cytoplasmic extract prepared from Jurkat cells possessed an activity that enhanced drug-induced PDC formation in factor-depleted K21 nuclei (Figure 7.1D).

7.2.2 The involvement of PDC enhancing activity in drug resistance.

Collectively, the results presented in Chapters 5 and 6 and Section 7.3.1 imply an association between the PDC enhancing factor and amsacrine-induced topoisomerase II activity. Such a second factor might be required for drug-enzyme or enzyme-DNA interactions.
It is conceivable that an absence, or reduced availability of required additional factor(s) could contribute to drug resistance, for example in non-cycling cells or cell lines selected for resistance to topoisomerase II-targeted drugs.

To investigate a possible involvement of the PDC enhancing factor in drug resistance, the ability of cytoplasmic extracts prepared from cells in drug sensitive and resistant states to stimulate protein-DNA complexes in isolated nuclei was compared. To avoid the compounding influence of differing topoisomerase II levels in nuclei from drug sensitive and resistant cells, PDC enhancing activities in extracts were assessed using factor-depleted K21 nuclei (Section 5.2.4), which maintained a standard level of topoisomerase II activity.

7.2.2.1 PDC enhancing activity in proliferating (39.5°C) and arrested (33°C) temperature sensitive 21-Fb cells.

Incubation of temperature sensitive 21-Fb cells at the non-permissive temperature (33°C), arrests these cells in G1. In this arrested state the 21-Fb cells exhibit only a marginal increase in resistance to amsacrine but a significant decrease in ability to form topoisomerase II-DNA complexes not attributed to drug metabolism or transport effects, and also a 16-fold reduction in topoisomerase II catalytic activity (Chapter 4, Part II).

To determine whether induction of growth arrest affected PDC enhancing activity, cytoplasmic extracts were prepared from proliferating (39.5°C) and arrested (33°C) 21-Fb cells. The extracts were then tested for ability to enhance amsacrine-induced PDC formation in isolated K21 nuclei. As shown in Figure 7.2, a 1.3-fold increase in the amount of extract from arrested cells was required to produce a peak stimulation of
FIGURE 7.2: PDC enhancing activity in proliferating and arrested temperature sensitive 21-Fb cells. Cytoplasmic extracts were prepared from proliferating and temperature arrested 21-Fb cells. The enhancement of amsacrine induced PDCs in K21 nuclei by extracts (0-100 µg protein) from proliferating (39.5°C) and arrested (33°C) 21-Fb cells was determined as described in Section 5.2.5. Nuclei with extract from 21-Fb cells grown at 39.5°C (●), or 33°C (■). Controls: PDC formation induced by 10 µM amsacrine in intact cells (○), or in K21 nuclei with extract from K21 cells (◆).
PDC formation in nuclei although the extract exhibited substantially reduced PDC enhancing activity compared to proliferating cell extract. For example, with extracts from arrested cells, there was approximately a 47% reduction in the maximum levels of stimulated complex, relative to extracts from proliferating cells (Figure 7.2).

7.2.2.2 PDC enhancing activity in K21 cells arrested with low serum.

It has been shown that growth of mastocytoma cells in low serum induces growth arrest in G1, presumably because of a requirement for serum factors for cells to cycle (Knightbridge, 1981). Other work from our laboratory (Hutchins, 1986) has also shown that under conditions of serum deprived growth arrest, K21 cells exhibit a 16-fold increase in resistance to amsacrine, but only a two-fold decrease in topoisomerase II catalytic activity. Topoisomerase II cleavage (i.e. complex forming) activity was not measured.

Figure 7.3, shows that cytoplasmic extracts prepared from K21 cells grown in 0.1% calf serum for 4 or 16 h contained substantially reduced PDC-enhancing activity, with (considering only the stimulatory contribution of the added extract) reductions in PDC formation to approximately 20% or 14% of the activity with control extracts prepared from cells grown in 10% serum.

Taken together, the data in Figures 7.2 and 7.3 suggests that the PDC enhancing factor is rapidly and markedly reduced in non-cycling cells.

7.2.2.3 PDC enhancing activity in amsacrine sensitive and resistant human Jurkat cells.

Mechanisms other than those related to the proliferative state of cells contribute to the phenomenon of drug resistance. For instance, cell
FIGURE 7.3: PDC enhancing activity in K21 cells growth arrested by serum deprivation. Cytoplasmic extracts were prepared from cells grown in 10% calf serum or growth arrested with 0.1% serum for 4 or 16 h. The enhancement of amsacrine-induced PDCs in K21 nuclei by extracts (0-60 μg protein) from cells grown in normal serum (10%) or serum deprived (0.1%) conditions was determined by SDS/K+ precipitation as described in Section 5.2.5. Nuclei with extract from cells grown in 10% serum (○); 0.1% serum for 4 h (■); 0.1% serum for 16 h (▼). Control: PDC formation induced by 10 μM amsacrine in intact K21 cells, (○).
lines can be selected in vitro which exhibit resistance to anticancer
drugs such as amsacrine or adriamycin and often the resistance is
associated with the overexpression of a membrane glycoprotein (GP170),
related to drug transport, or with altered topoisomerase II activity (See
Section 1.6).

A clonal subline of the Jurkat human T lymphoblastoid cell line
selected for resistance to amsacrine by Dr K. Snow (Department of Cellular
and Molecular Biology, University of Auckland) was used to further
investigate the possible involvement of the PDC enhancing factor in drug
resistance. The cell line used, designated Little AMSA (Resistance
factor, R=46) exhibits cross resistance to other topoisomerase II-targeted
drugs but does not display classical multidrug resistance characterized by
the overexpression of the 170 kDa membrane glycoprotein (Snow, 1988). The
mechanism of amsacrine resistance in this cell line is not yet clear, but
appears to involve qualitative alteration of topoisomerase II (K. Snow,
pers. comm.).

Cytoplasmic extracts prepared from amsacrine-sensitive and resistant
Jurkat cells were tested for their ability to stimulate protein-DNA
complexes in factor depleted K21 nuclei. As shown in Figure 7.4, a
four-fold increase in the amount of cytoplasmic extract from amsacrine-
resistant Jurkat cells was required to produce levels of PDCs in K21
nuclei, equivalent to those induced by extracts from drug sensitive cells
(peak activity with 80μg protein from resistant: c.f. 20 μg protein from
sensitive-cell lines).

These results suggest that quantitative or qualitative alterations in
the PDC enhancing activity may also contribute to mechanisms of drug
resistance in cells other than those associated with their proliferative
state.
FIGURE 7.4: PDC enhancing activity in cytoplasmic extracts from amsacrine sensitive and resistant Jurkat cells. Cytoplasmic extracts were prepared from amsacrine-sensitive or -resistant human Jurkat cells. The enhancement of amsacrine-induced PDCs in K21 nuclei by extracts (0-100 µg protein) from drug sensitive and resistant cells was determined by SDS/K+ precipitation as described in Section 5.2.5. K21 nuclei, with extract from amsacrine-sensitive Jurkat cells (●) or amsacrine-resistant Jurkat cells (▼); Control: PDC formation induced by 10 µM amsacrine in intact K21 cells, (○), or in K21 nuclei with extract from K21 cells, (♦).
7.3 DISCUSSION.

The generality of the PDC enhancing factor described and partially characterized in Chapter 6 was investigated in a preliminary study using five cell lines described in Section 7.2.1. Quantitatively and qualitatively similar PDC enhancing activity was found in all cell lines tested. Furthermore, the factor from human cells could stimulate drug-induced topoisomerase II-DNA complexes in mouse nuclei, suggesting that it may be functionally conserved, at least in mammalian cell systems. Although the occurrence of this protein factor in many other cell lines needs to be tested, the data presented here indicates that it is reasonably widespread.

The possible involvement of the PDC enhancing factor in resistance to amsacrine was also investigated. Factor activity was reduced in extracts from growth arrested cells and in cells selected for resistance to amsacrine (Figures 7.2 - 7.4), although in the latter case, when sufficient factor was added, full complex forming ability was restored to nuclei (Figure 7.4). In contrast, this restoration of PDC formation was not seen with high concentrations of extracts from non-cycling cells (Figures 7.2 and 7.3) suggesting that levels of PDC enhancing activity may be cell-cycle regulated and thus may also have significance in relation to cancer cell growth.

Generally, the PDC enhancing activity increased to a maximum then declined dramatically when increasing amounts of cytoplasmic extracts were added to K21 cell nuclei and possible explanations for this effect were discussed in Chapter 6. However, when the activity of the purified 70 kDa PDC enhancing factor from K21 cells was tested, the steep decline in PDC
formation with higher concentrations was not observed, therefore some other component(s) in the crude cytoplasmic extract appeared to negate the action of the factor at high extract concentrations. Apparently less of these components were present in drug-resistant Jurkat cells since high concentrations of Jurkat cytoplasmic extracts were neccasry to stimulate PDC formation (Figure 7.4). At present, I have no adequate explanation for these phenomena and the difference in activities of extracts from drug sensitive and resistant cells.

A number of workers have postulated the influence of secondary factors on topoisomerase II-targetted drug action and cytotoxicity. The PDC enhancing factor is the first of such additional factors identified that enhances drug-induced topoisomerase II action. It is quite conceivable that resistance could be mediated by alterations to the PDC enhancing factor which might impair topoisomerase II-DNA interaction or drug-enzyme interaction.

At this point, considerations of the involvement of the PDC enhancing factor in the mediation of drug resistance are at best speculative (as indeed they are for drug action) and this question will be more fully discussed in the concluding chapter of the thesis. Without doubt, more definitive knowledge of the nature of the factor would greatly facilitate the understanding of its role both in drug action and resistance. The following chapter describes some progress made with the purification and preliminary identification of the PDC enhancing factor.
CHAPTER EIGHT.

ISOLATION AND PRELIMINARY IDENTIFICATION OF THE PDC ENHANCING FACTOR.

8.1 INTRODUCTION

In the previous three chapters, I considered evidence for, and partially characterised a factor that enhanced the formation of amsacrine-induced topoisomerase II-DNA complexes in isolated nuclei of K21 murine mastocytoma cells and which seems to be present in at least four other cell lines. This factor appeared to be a labile protein, distinct from topoisomerases I and II, that was rapidly lost from cells grown with protein and RNA synthesis inhibitors. Furthermore, the PDC enhancing factor was substantially reduced in cells induced into a G1 state by temperature arrest or serum deprivation. Human Jurkat cells selected for resistance to amsacrine and displaying cross-resistance to other topoisomerase II-targeted drugs also exhibited significantly reduced PDC enhancing activity. Together, these results suggested that quantitative or qualitative alterations in PDC enhancing factor may be a contributing factor in mediation of drug resistance. Possible cell-cycle associated regulation of the factor was also indicated.

In this chapter, some progress made with isolation of the factor is described. The PDC enhancing activity is shown to reside in a 70 kDa protein kinase. A preliminary identification of the protein is made and its nature and general significance in relation to drug action and drug resistance is discussed.
8.2 METHODS

8.2.1 Ammonium sulphate precipitation of cytoplasmic extracts

As an initial step in the purification of the PDC enhancing activity from mouse mastocytoma cytoplasmic extracts (Section 5.2.3), four broad ammonium sulphate fractions (0 - 20 %, 20 - 60 %, 60 - 80 %, >80 % supernatant) were recovered at 0°C according to Scopes (1987).

The following equation was used to calculate g of solid \((\text{NH}_4)_2\text{SO}_4\) added to give the desired final % saturation

\[
G = \frac{0.533 (S2 - S1) \times V}{100 - 0.3 \times S2}
\]

where

- \(S1\) = initial % saturation
- \(S2\) = final % saturation
- \(V\) = volume in mls

The fractions were centrifuged at 10,000 x g for 30 min at 4°C and the precipitates were redissolved in 1-2 pellet volumes of "isolation buffer" (20 \(\mu\)M Tris-HCl (ph 7.5), 1 % (v/v) aprotinin, 10 mM \(\text{Na}_2\text{S}_2\text{O}_5\), 0.1 mg/ml DFP, 1 mM PMSF. To remove the \((\text{NH}_4)_2\text{SO}_4\), the precipitates and the final supernatant were dialysed against 2000 vols of the above solution for 6 h at 4°C. After dialysis, the protein concentration of each sample was determined and an aliquot of each fraction was assayed for PDC enhancing activity. The remainder of the fractions were stored at -90°C.
8.2.2 Purification of the PDC enhancing factor by column chromatography.

FPLC-superose 12 and DEAE-cellulose column chromatography were used to purify the PDC enhancing activity.

8.2.2.1 FPLC column chromatography

Chromatography was performed using a Pharmacia FPLC system composed of two P-500 pumps and a LCC-500 controller at room temperature. All buffers and protein samples were filtered through 0.22 µm glass fibre filters prior to use.

FPLC was performed on a Pharmacia Superose 12 (HR 10 x 30 cm) column. This analytical grade column size-fractionates proteins of 300,000 MW. The column was equilibrated with isolation buffer (Section 8.2.1) for 4 h prior to use. An aliquot (100 µl, i.e. 100 µg) of the dialysed and filtered 20-60 % (NH₄)₂SO₄ fraction from the cytoplasmic extract was loaded onto the column. FPLC was performed at a flow rate of 0.5 ml/min, and 30 x 1 ml fractions were collected. The void volume of the column (Vo) (6 ml) was discarded. Protein absorption at A₂₈₀ was monitored continuously by an on-line UV analyzer. As successive column runs produced nearly identical A₂₈₀ profiles, like fractions from more than one run were combined and selected fractions pooled. After concentrating (Section 8.2.3), the fractions were assayed for PDC enhancing activity (Section 5.2.5).

8.2.2.2 DEAE-anion exchange chromatography

Trial DEAE-cellulose (Celex-D, Biorad) columns (1 x 2.5 cm) were prepared in plastic disposable syringes. Columns were equilibrated for several hours with isolation buffer (Section 8.2.1) before samples (200 µl) of the dialysed 20 - 60 % (NH₄)₂SO₄ cytoplasmic extract
fraction, containing approximately 2 - 3 mg protein, were loaded. The columns were eluted with either a linear 0.01 - 0.2 M KCl gradient (50 ml) in isolation buffer followed by a 0.5 - 1 M KCl wash (10 ml) or by step-wise elution over the same salt concentration range. The flow rate was 30 ml/h and 1 ml fractions were collected. The first fraction was collected after the emergence of the void volume (2 ml).

Salt concentrations and protein content of fractions were followed by refractive index and absorbance at A\textsubscript{280} nm, respectively. After chromatography, selected fractions were pooled, concentrated, dialysed against isolation buffer (Section 8.2.1) including 20 % glycerol and then assayed for PDC enhancing activity (Section 5.2.5).

8.2.3 Concentration of column fractions.

Dilution factors and low protein content necessitated that pooled column fractions were concentrated prior to assays for activity.

The pooled fractions were transferred to dialysis bags and dehydrated with Aquacide (Calbiochem) until the desired concentration factor was achieved. Routinely, fractions were 10-fold concentrated after dehydration with aquacide for 2 h at 4°C. To hasten the concentration process, at 30 min intervals rehydrated Aquacide gel was removed and the dialysis bags were covered with fresh, dry Aquacide powder.

This method of concentration proved rapid, economical and it resulted in minor losses of sample compared to all other methods of concentrating tried.
8.2.4 Polyacrylamide gel electrophoresis (SDS-PAGE) and gel staining techniques

8.2.4.1 Solutions

Deionized water was used to prepare all solutions used in PAGE.

- **Acrylamide solution**: 30 % (w/v) acrylamide (Serva)
- 0.8 % (w/v) bisacrylamide (Serva)
- **Separating Gel Buffer**: 1 M Tris-HCl (pH 8.8)
- **Stacking Gel Buffer**: 1 M Tris-HCl (pH 6.8)
- **SDS solution**: 20 % (w/v) SDS in H2O
- **Ammonium persulphate**: 10 % (w/v) prepared immediately prior to use
- **TEMED**: 10 % (v/v) diluted immediately prior to use
- **PAGE sample buffer**: 5% SDS
  - 0.1 M Tris-HCl (pH 6.8)
  - 15 % glycerol
  - 0.002 % Bromophenol blue
  - 5 % (v/v) 2-mercaptoethanol (final concentration) added immediately prior to use.
- **Running Buffer (10 x)**: 0.25 M Tris
  - 1.9 M glycine
  - 1 % (w/v) SDS in water

8.2.4.2 Molecular Weight Markers

A combination of Sigma high and low molecular weight standards were used. Molecular weights were 14 kDa, 20.1 kDa, 24kDa, 29kDa, 36 kDa, 45
kDa, 66 kDa, 97 kDa, 116 kDa and 205 kDa.

8.2.4.3 Gel pouring and running.

All gels were formed in a Biorad Protean II gel apparatus and were 18 cm x 15 cm x 1 mm thick. Separating and stacking gel lengths were approximately 18 cm and 5 cm respectively. Routinely, 7 - 15 % separating gels (7 or 15 % acrylamide, 370 mM Tris-HCl (pH 8.8), 0.1 % SDS, 0.025 % TEMED, 0.03 % ammonium persulphate) were prepared. A two chamber gradient maker was used to produce linear gradient gels. All gel solutions were degassed prior to the addition of SDS.

Poured gels were immediately overlaid with water-saturated butan-2-ol, and after gel polymerization the butan-2-ol was removed and the gel surface rinsed extensively with water. Degassed stacking gel solution (4 % acrylamide, 123 mM Tris-HCl (pH 6.8), 0.1 % SDS, 0.05 % TEMED and 0.05 % in ammonium persulphate) was poured on to the separating gel and a comb inserted to form sample wells. On removal of the comb, the wells were rinsed with distilled water, then filled with running buffer.

Protein samples were prepared by adding sample buffer to give a total volume of 50 µl. The samples were sonicated briefly (5 s) then heated to 100°C for 4 min before being placed in sample wells. Gels were then placed in the Protean II apparatus and electrophoresed for 2.5 h at 50 V/gel.

8.2.4.3 Gel Staining

A. Coomassie blue staining

Gels were placed in gel fix (15 % Methanol / 7 % Acetic Acid (v/v) in \( H_2O \)) for 5 to 10 mins, then stained with Coomassie blue (0.5 % (w/v) Coomassie Blue (Serva Blue R-250) / 7 % (v/v) Glacial Acetic Acid / 50 %
(v/v) Ethanol) for 15 to 20 mins only. To visualise bands, the gels were then destained in repeated changes of 7.5 % (v/v) Glacial Acetic Acid / 15 % (v/v) Methanol, until background traces of stain not associated with protein were removed.

B. Silver staining

If a gel was to be silver stained, either immediately after electrophoresis or staining with Coomassie blue, it was placed in 500 ml of 50 % MeOH / 10 % Acetic acid for 12 - 16 h. The gel was then washed extensively (24 h) in many changes of distilled water. After washing, the gel was covered with freshly prepared ammoniacal silver (see below) and agitated for 20 min in the dark. The silver stain solution was then discarded and the gel was again washed thoroughly with four, 10 min washes in 500 ml water. The gel was then covered with freshly made developer (see below) and agitated until sufficiently developed. To terminate the development process, the gel was placed in 50 % (v/v) methanol for at least 30 min, then washed thoroughly in water.

Ammoniacal silver solution: Mix 1 ml aqueous ammonia (25 %) with 0.2 g of AgNO₃ dissolved in 1 ml of water. Five ml of 4 % NaOH is then added and the mixture diluted to 200 ml in deionized water.

Developer: 270 µl of a 37 % formaldehyde solution and 2.5 ml of a 1 % citric acid solution are added to 500 ml of deionized water.
8.2.5 Elution and renaturation of proteins from SDS-polyacrylamide gels.

Enzymatically active proteins were recovered from SDS-PAGE gels essentially as described by Hager and Burgess (1980).

8.2.5.1 Solutions

**Elution Buffer**: 0.1% SDS; 0.05 M Tris-Cl (pH 7.9 at 25°C):

- 0.1 mM EDTA
- 5 mM DTT
- 0.1 mg/ml BSA
- 0.15 M NaCl

**Dilution Buffer**: 0.05 M tris-Cl (pH 7.9); 20% glycerol:

- 0.1 mg/ml BSA
- 0.15 M NaCl
- 1 mM DTT
- 0.1 mM EDTA

SDS-PAGE gels were prepared and electrophoresed as described in Section 8.4.

8.2.5.2 Procedure

Following electrophoresis, gels were removed to a clean tray avoiding contact with fingers, rinsed with cold H2O, then stained for 5 - 15 min with ice-cold 0.25 M KCl and 1 mM DTT. If white bands (i.e. proteins precipitated by the combination of SDS and K+) were not evident after 15 minutes, additional KCl was added and the gel box held on ice. Once white bands appeared, the gels were rinsed again and destained for 30 - 60 min with cold H2O containing 1 mM DTT. The protein band of interest was excised (3 - 5 mm gel piece) and transferred to a 2 ml siliconized Dounce homogenizer. The gel pieces were soaked in two changes of 1 ml cold H2O and 1 mM DTT for 15 min.

To elute the protein, the destain was decanted and discarded, 1 ml of
elution buffer (see above) was added and the gel crushed with 3 to 4 strokes of a snugly fitting Teflon pestle. The protein was then eluted overnight at room temperature (although elution for 1 h at 25°C with occasional agitation is sufficient for some proteins). After elution, the mixture was transferred to an Eppendorf centrifuge tube and the crumbled gel was pelleted by centrifugation for 1 min in a microfuge. The supernatant was then placed in a siliconized 15 ml Corex tube.

To remove SDS, 4 volumes of cold acetone (-20°C) was added to the gel eluate and the protein precipitated for 30 min in a dry ice-ethanol bath. The precipitate was collected by centrifugation at 10000 x g for 10 min at 4°C, the acetone supernatant poured off and the tube inverted to drain. The pellet was rinsed once, gently, with 1 ml of ice-cold 80% acetone: 20% elution buffer to remove the last traces of residual SDS, then air dried for 10 - 15 minutes.

When dry, the protein precipitate was dissolved in 20 ul of 6 M guanidine-HCl in dilution buffer for a minimum of 20 minutes at room temperature. The solution was then diluted 50-fold with dilution buffer and the protein permitted to renature for 12 h at room temperature.

Prior to testing in enzyme activity assays, the renatured protein solution was concentrated (Section 8.2.3) and dialysed against 2000 vols of isolation buffer (described in Section 8.2.1)

This procedure also worked with protein excised from a gel stained for 5 minutes with Coomassie blue and then destained for 15 minutes in 7.5% acetic acid. In fact, staining with Coomassie blue was extremely useful for the detection and excision of low amounts of protein.

8.2.6 Protein kinase activity assays.

The acidic peptide Ser-Glu-Glu-Glu-Glu was purchased from Dr. D.
Harding (Massey University, Palmerston North, N.Z). This synthetic peptide is a specific substrate for the protein kinase referred to as Casein kinase II (Agostinis et al., 1986). Phosphorylation of the peptide was assayed at 37°C in 50 μl containing 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 30 μM [γ³²P]ATP (6951 cpm/pmol), 0.5 mg of substrate/ml and an aliquot (2 - 5 μl) of crude cytoplasmic extract (Section 5.2.3), column fraction or renatured protein. Reactions were terminated with glacial acetic acid added to a final concentration of 30 % then the volume was adjusted to 500 μl with 30 % acetic acid. To recover the radioactive peptide, approximately 0.4 g of Dowex 1-8 anion exchanger (equilibrated in 30 % acetic acid but vacuum filtered) was added and gently mixed with the reaction mixture for 5 - 10 minutes. The tubes were then centrifuged for 20 s in a microfuge. By following this procedure, more than 95 % of the free ATP and Pi remained tightly bound to the Dowex gel while the protonated phosphopeptide remained in the supernatant. The supernatants were transferred directly to glass scintillation vials and the excess acetic acid was removed by evaporation before 5 ml of aqueous scintillant (ACS II) was added and radioactivity associated with peptide measured.

8.3 RESULTS

8.3.1 Ammonium sulphate precipitation of cytoplasmic extracts.

A typical example of a salt fractionation of cytoplasmic extracts is shown in Figure 8.1. The 20 - 60 % ammonium sulphate fraction contained the bulk of the PDC enhancing activity, with one unit of activity (see Section 6.3.1 for definition) achieved with only 5 μg of added protein. When dilution factors were taken into consideration, this equated to a
FIGURE 8.1: PDC enhancing activity in ammonium sulphate fractionated K21 cytoplasmic extracts. K21 cytoplasmic extracts were fractionated with ammonium sulphate as described (Section 8.2.1) and amsacrine-induced PDC enhancing activity in each fraction was determined with isolated K21 nuclei by SDS/K\(^+\) precipitation (Section 5.2.5). Ammonium sulphate fractions: 0-20 %, (♦); 20-60 %, (■); 60-80 %, (○); 80 % supernatant, (▽); control extract, (○).
14-fold purification, as shown in Table 8.1. Approximately 0.5 units of activity was achieved with 30 µg of protein from the 60 - 80 % fraction, and no activity was detected in either the 0 - 20 % or >80 % supernatant fractions (Figure 8.1).

Typically, 40 -45 % of the total protein was precipitated in the 20 - 60 % fraction and as most of the PDC enhancing activity was reproducibly recovered in this fraction, the 20 -60 % cut was routinely taken for subsequent chromatographic steps.

8.3.2 Purification of the PDC enhancing factor by column chromatography.

Initially I wished to establish conditions with which to purify the factor using only small scale chromatography.

FPLC-gel filtration chromatography was performed (as described in Section 8.2.2.1) on salt fractionated cytoplasmic extracts (20 - 60 % fraction). The elution profile is shown in Figure 8.2. PDC enhancing activity eluted as one peak, with a molecular weight of >400 kDa. It can be seen from the A280 nm profile that proteins were poorly separated and non-specific aggregates of high molecular weight had formed in which the protein of interest appeared to be trapped. This aggregation of proteins was later overcome by including 1 % mercaptoethanol in isolation buffers.

A 119-fold purification was achieved with one initial chromatography step (Table 8.1) and 90 % of the protein loaded was removed. However, there was a considerable loss of material during an obligatory filtration of the cytoplasmic extract through a 0.22 µm filter prior to column chromatography, and the amount of material that could be loaded on the FPLC column was restricted. Consequently, there was insufficient material recovered for further purification. As an alternative more efficient approach, it was decided to obtain a preliminary fractionation of the
### TABLE 8.1: Purification of the PDC enhancing factor.

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>TOTAL PROTEIN (mg)</th>
<th>TOTAL ACTIVITY (units)</th>
<th>SPECIFIC ACTIVITY (units/mg)</th>
<th>PURIFICATION (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>6</td>
<td>83.3</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4) 20-60%</td>
<td>2.7</td>
<td>540</td>
<td>200</td>
<td>14.2</td>
</tr>
<tr>
<td>FPLC</td>
<td>0.02</td>
<td>33.3</td>
<td>1665</td>
<td>119</td>
</tr>
<tr>
<td>DEAE</td>
<td>0.17</td>
<td>340</td>
<td>2000</td>
<td>143</td>
</tr>
<tr>
<td>Renatured 70kDa protein</td>
<td>0.0002</td>
<td>10</td>
<td>50000</td>
<td>3571</td>
</tr>
</tbody>
</table>
FIGURE 8.2: Purification of PDC enhancing activity by FPLC. Dialysed and filtered 20-60 % (NH₄)₂SO₄ fraction (100 µg protein) from cytoplasmic extracts was applied to a Pharmacia Superose 12 (HR 10x30 cm) column. Proteins were eluted with isolation buffer (Section 8.2.1) at 0.5 ml/min and 30 x 1 ml fractions collected. Absorbance at 280 nm was monitored continously. Fractions were pooled as indicated (——) on the basis of protein content, concentrated then dialysed prior to assessment of PDC enhancing activity (●). Arrows indicate elution positions of MW markers.
factor using DEAE-ion exchange chromatography, then further fractionate the semi-purified product by FPLC.

Therefore, 20 - 60 % ammonium sulphate extract fraction was applied to a DEAE anion exchange column (see 8.2.2.2). The elution profile from one such column is shown in Figure 8.3. The PDC enhancing activity eluted with 120 - 160mM KCl in a single peak with a 143-fold purification (Table 8.1). With the column loadings described, this provided sufficient active material remaining after activity tests to purify further by other chromatography steps if desired.

SDS-PAGE electrophoresis of active fractions from DEAE columns indicated that separation of proteins by DEAE chromatography was more effective with linear gradients than stepwise-elutions (data not shown).

It was concluded that if the DEAE cellulose columns were scaled up using the conditions established, reasonable yields of PDC enhancing activity could be expected.

8.3.3 Gel electrophoresis of column fractions.

Figure 8.4A shows a representative Coomassie blue stained SDS-PAGE gel of protein samples taken from various stages of the purification process. Although a 14-fold purification of PDC enhancing activity was achieved on fractionation of cytoplasmic extract with 20 - 60 % ammonium sulphate, there was little change in total proteins (c.f. lanes 2 and 3). Lane 4 merely highlights the loss of proteins on filtration through 0.22 μm filters. With Coomassie blue staining, no protein bands were detected in the DEAE column active fraction (lane 5). However, Figure 8.4B shows the same gel (lanes 5 and 6 only) after silver staining when a predominant band at approximately 68 - 70 kDa was highlighted, together with some minor bands of lower molecular weight. When active fractions from other
FIGURE 8.3: Purification of PDC enhancing activity by DEAE-cellulose chromatography. Dialysed 20-60 % (NH₄)₂SO₄ cytoplasmic extract fraction (2-3 mg protein) from K21 cells was applied to a DEAE-cellulose (1x2.5 cm) column. Proteins were eluted with a linear 10-200 mM KCl gradient in isolation buffer (Section 8.2.1) (50 ml), followed by a 0.5-1 M KCl wash (10 ml). The flow rate was 30 ml/h and 1 ml fractions were collected. Selected fractions were pooled as indicated (---), concentrated, dialysed then tested for PDC enhancing activity, (●).
FIGURE 8.4: SDS-PAGE electrophoresis of cytoplasmic extracts at various stages of fractionation. Cytoplasmic extracts were fractionated by several procedures (as described in sections 8.2.1 - 8.2.2.2) and proteins in aliquots from successive stages were separated by SDS-PAGE (7-15% acrylamide gradient).

A. Coomassie blue-stained gel:
   Lane 1 = MW standards.
   Lane 2 = Crude cytoplasmic extract from K21 cells.
   Lane 3 = 20-60% (NH₄)₂SO₄ cytoplasmic extract fraction.
   Lane 4 = 20-60% (NH₄)₂SO₄ fraction after filtration through 0.22 μm filters.
   Lane 5 = DEAE-cellulose column fraction 6, containing maximum PDC enhancing activity.
   Lane 6 = MW standards.

B. Gel A, after silver staining. Lanes 5 and 6 only.
DEAE column runs where fractionated by gel electrophoresis and silver stained, a similar band at approximately 70 kD appeared to predominate.

8.3.4 Elution and renaturation of the 70 kD protein from SDS-polyacrylamide gels.

After samples of the DEAE column fraction containing PDC enhancing activity were electrophoresed on a 7 - 15% polyacrylamide gel, with BSA alongside as a 66 kDa MW marker, the protein region at 70 kD was excised and renatured as described in Section 8.2.5. The renatured 70 kDa protein was then tested for PDC enhancing activity. As shown in Figure 8.5, the 70 kDa protein reconstituted PDC forming activity in nuclei to levels seen with intact cells, crude cytoplasmic extract and the salt fractionated extract. Moreover, the sharp decline in PDC enhancing activity normally observed with crude extracts at higher concentrations was not seen. This may indicate that the renatured protein was relatively free from contaminating proteins that might negate its action. To estimate the concentration and purity of the renatured protein, aliquots were electrophoresed alongside known amounts of BSA on a 7 - 15% SDS gel and then stained with silver. The resulting gel is shown in Figure 8.6 and it was quite clear that the PDC enhancing activity resided in a single protein of 70 kDa MW. By comparison with the known amounts of BSA electrophoresed on the same gel, the concentration of the renatured 70 kDa protein and the amount required for one unit of PDC enhancing activity was estimated. It was calculated that successive (NH₄)₂SO₄ precipitation, DEAE column chromatography and excision of 0.2 μg of 70 kDa protein from gels resulted in a 3571-fold purification (Table 8.1). Without doubt the yields could be improved by scale-up procedures.
FIGURE 8.5: The PDC enhancing activity of the renatured 70 kDa protein. The 70 kDa protein region was excised from an SDS polyacrylamide gel and the protein eluted and renatured as described (Section 8.3.4). The enhancement of amsacrine (10 μM)-induced topoisomerase II-DNA complexes in K21 nuclei by the 70 kDa protein (0-0.2 μg) was determined by SDS/K+ precipitation. K21 nuclei with: 70 kDa protein, (●); controls: 1 unit (30 μg protein) of crude extract (○); 1 unit (5 μg protein) of 20-60% (NH₄)₂SO₄ fraction, (■); intact cells, (×).
FIGURE 8.6: SDS-PAGE of the renatured 70 kDa protein. An aliquot of the 70 kDa protein (Section 8.3.4) possessing PDC enhancing activity was electrophoresed alongside known amounts of BSA on a 7-15 % SDS-PAGE gel and the gel then stained with silver.

Lanes 1-5 = 0.1, 0.2, 0.5, 1.0 and 2.0 μg BSA.
Lane 6 = Renatured 70 kDa protein (10 μl).
Lane 7 = MW standards
8.3.5 Preliminary identification of the 70 kDa protein with PDC enhancing activity.

Topoisomerase II activity can be modulated by phosphorylation in *Drosophila* cell free systems (Ackerman *et al.*, 1985) and *in vivo* with *HeLa* or *Drosophila* cell lysates (Knoll and Rowe, 1988; Ackerman *et al.*, 1988). However, although topoisomerase II was phosphorylated in *Drosophila* cell homogenates under conditions which specifically stimulated a variety of protein kinases, Ackerman *et al.* (1988) found that modification of the enzyme was always sensitive to inhibitors specific for casein kinase II (CKII), namely heparin and anti-casein kinase II antiserum. Furthermore, the modification of topoisomerase II by casein kinase II is known to stimulate topoisomerase II activity, at least *in vitro* (Ackerman *et al.*, 1985). Therefore, I endeavoured to determine whether the 70 kDa PDC enhancing factor isolated from K21 cell cytoplasmic extracts (previously shown to stimulate drug induced topoisomerase II activity) bore any relationship to the casein kinase family of enzymes.

8.3.5.1 Heparin-sensitivity of the PDC enhancing factor.

The sensitivity to heparin of the PDC enhancing activity in crude cytoplasmic extracts was tested over a range of heparin concentrations known to specifically inhibit CKII (0 - 0.3 μg heparin / ml). As shown in Figure 8.7, heparin significantly inhibited the action of the PDC enhancing activity with 0.12 μg heparin / ml reducing PDC formation approximately 8-fold. It should be noted that heparin reduced complex forming activity in nuclei in the presence of cytoplasmic extract to levels lower than those achieved with nuclei alone. This suggests that
FIGURE 8.7: The effect of heparin on PDC enhancing activity. The enhancement of amsacrine-induced PDC formation by crude K21 cytoplasmic extracts in the presence of 0-0.3 µg heparin/ml was determined with isolated K21 nuclei by SDS/K+ precipitation. K21 nuclei with cytoplasmic extracts and 0-0.3 µg/ml heparin, (●); K21 nuclei only, (□).
FIGURE 8.8: The sensitivity of PDC enhancing activity at successive stages of purification to heparin and anti-casein kinase II antiserum. PDC enhancing activity (1 unit) from crude cytoplasmic extracts (A), the DEAE-cellulose column active fraction (B) and the renatured 70 kDa protein (C) were incubated with or without 50 μg of anti-CKII antibody (Ab) or 0.2 μg/ml heparin (H) under standard reaction conditions (Section 5.2.5). PDCs induced by 10 μM emascrine in isolated K21 nuclei were measured by SDS/K+ precipitation. Treatments are as indicated.
the slight stimulation of complex formation usually observed in isolated nuclei may be due to residual heparin-sensitive PDC enhancing activity.

The PDC enhancing activity in an active DEAE column fraction and the 70 kDa renatured protein were also sensitive to heparin. Heparin (0.2 μg / ml) reduced PDC formation in these fractions approximately 4.5-fold (Figure 8.8).

8.3.5.2 Sensitivity of the PDC enhancing activity to anti-rat liver casein kinase II antiserum.

The sensitivity of the PDC enhancing activity to anti-rat liver CKII antiserum was tested. The antibody used was highly specific for cytosolic CKII and the nuclear NKII, but not reactive against cAMP-dependent protein kinase, casein kinase I and nuclear protein kinase-N (Goueli and Ahmed, 1988).

PDC enhancing factor (1 unit) at three stages of purification, namely, in crude extracts, active column fractions and the renatured 70 kDa protein, were incubated with 50 μg of CKII antibody in standard reaction conditions (Section 5.2.5) and PDCs quantitated by SDS/K+ precipitation. As shown in Figure 8.8, the antibody to CKII reduced activity approximately 9-10 fold in all three fractions tested.

8.3.5.3 Protein kinase activity of the 70 kDa protein.

The ability of the renatured 70 kDa protein to phosphorylate a synthetic peptide substrate, ser.glu.glu.glu.glu.glu, specific for casein kinase II was tested. As shown in Figure 8.9, the 70 kDa protein phosphorylated the synthetic substrate for CKII which incorporated approximately 10 pmol 32P/min/mg protein. Moreover, phosphorylation of the substrate was sensitive to 0.2 μg/ml heparin and 50 μg of anti-CKII
FIGURE 8.9: Protein kinase activity of the renatured 70 kDa protein. Phosphorylation of a synthetic peptide substrate for casein kinase II by the renatured 70 kDa protein was tested as described in Section 8.2.6. Reactions were carried out with 2 μl aliquots of 70 kDa protein and 0.5 mg/ml peptide in a total volume of 50 μl, for 30 min at 30°C. Sensitivity of the reaction to 0.2 μg/ml heparin (H), 50 μg anti-CKII antibody (Ab) or 1 mM Spermine (S) was also tested. Treatments are as indicated.
antiserum which reduced incorporation of phosphate to 4.6 pmol/min/mg protein and 0.7 pmol/min/mg protein, respectively.

The polyamine spermine (1 mM), is known to stimulate CKII phosphorylation 2-3 fold (Singh, 1989). However, under conditions used in this study only a 1.3 fold stimulation of kinase activity was observed (Figure 8.9). Similarly, spermine had little detectable stimulatory effect on the PDC enhancing activity of the 70 kDa protein as measured by SDS/K+ precipitation (data not shown).

Taken together the results suggested that the 70kDa PDC enhancing factor is a heparin-sensitive protein kinase with properties similar to casein kinase II.

8.4 DISCUSSION

I have shown that a protein factor, distinct from the known topoisomerases I and II, present in tumour cell extracts is required for topoisomerase II-DNA complex formation in amsacrine-treated isolated cell nuclei (Chapters 5-7). Fractionation of these cell extracts by ammonium sulphate precipitation followed by DEAE-cellulose chromatography and excision and renaturation from SDS-PAGE gels of a predominant protein band, resulted in a 3571-fold purification of PDC enhancing activity (Table 8.1).

An approximately 70 kDa protein was shown by SDS-PAGE to increase in fractions from successive purification steps (Figure 8.4). When this protein was recovered from the gels and renatured it showed heparin-sensitive protein-kinase activity with the specific CKII substrate, it stimulated formation of topoisomerase II-DNA complexes and was inhibited by antibody to rat liver casein kinase II.
These observations suggest that the 70 kDa PDC enhancing protein bears some relationship to casein kinase II or nuclear kinase II.

Casein kinase II belongs to a family of, at present, three kinases, CKI, CKII and CKIII (Hathaway and Traugh, 1982; Schneider et al., 1986). CKII appears to be a growth related enzyme (Prowald et al., 1984; Schneider et al., 1986) which preferentially phosphorylates acidic protein substrates such as casein and phosvitin on serine or threonine residues (Hathaway and Traugh, 1982). CKII is found in the cytoplasm and nucleus of cells consistent with its wide actions and variety of substrates, including eukaryote initiation factors, RNA polymerase II protein phosphatase modulators, cyclic AMP-dependent protein kinase regulatory subunit RII, nucleolin, the steroid receptor-binding heat shock protein hsp 90, non-histone chromosomal proteins, and topoisomeraseases I and II. A variety of evidence, such as substrate specificity, co-factor requirements, heparin- and antibody sensitivity and polyamine stimulation suggests that another protein kinase, nuclear kinase II (NKII) is closely related to CKII. Rat liver NKII is composed of two 72 kDa subunits whereas cytosolic CKII is composed of 2 (α and α'; Mr 35-44 kDa) and 2 β (Mr 24-26 kDa) subunits. It is interesting that the NKII 72 kDa subunits are readily cleaved by proteases to fragments of similar sizes to those found in CKII suggesting that proteolysis of NKII might produce CKII (Delpech et al., 1986). However, the exact relationship between CKII and NKII is not yet resolved. Possibly proteolysis plays a role in the formation, translocation or localization of CKII.

From the above data, I conclude tentatively that the PDC enhancing protein discovered in this study is a protein kinase sensitive to heparin and anti-CKII antibody which possesses specificity for a known substrate for CKII, has a molecular weight of 70 kDa and modulates drug-induced
topoisomerase II-DNA complex formation. It is possible that this 70 kDa protein is a non-proteolysed form of NKII or an as yet unidentified casein kinase with similar properties.

My results indicate that in isolated nuclei and presumably in intact cells, amsacrine-induced topoisomerase II-DNA complex formation is dependent on the presence of a 70 kDa protein kinase with casein kinase-like properties. Models attempting to explain the apparent involvement of the PDC enhancing factor are discussed in the concluding chapter of the thesis.
A major goal of cancer therapy research is to identify critical biochemical targets in tumour cells in order to develop effective cancer chemotherapy that kills tumour cells without affecting normal cell function. A large body of evidence has accumulated which suggests that DNA breakage by DNA topoisomerase II might provide such a target for anticancer drugs (reviewed in Ralph and Schneider, 1987; Hancock et al., 1988; Liu, 1989). However, the mechanism linking drug-induced topoisomerase II-associated DNA breaks with drug cytotoxicity is poorly understood and this problem was investigated in the preceding chapters.

Recent studies have indicated that non-cycling cells are much more resistant to drugs affecting topoisomerase II. Therefore, the other major objective of my research was to elucidate at the molecular level possible reasons for the resistance of G1(G0) phase cells to amsacrine, with particular reference to the role of topoisomerase II in resistance.

During the course of the research, a cytoplasmic factor was discovered that enhanced the formation of amsacrine-induced topoisomerase-DNA lesions. Because of its possible relationship to topoisomerase II-mediated drug action and resistance, the nature of this factor was determined.

The pertinent findings are discussed below in relation to cytotoxicity, drug resistance and implications for cancer chemotherapy.

9.1 The question of cytotoxicity

The unusual properties of DNA lesions induced by anticancer drugs
targeted at topoisomerase II have led to the suggestion that the amounts of cleavable complexes should parallel drug cytotoxicity (Nelson et al., 1984; Tewey et al., 1984a, b). However, at the outset of my study, the precise mechanisms relating formation of these complexes to cell killing were obscure. Indeed, a number of studies had been reported that both supported (Glisson et al., 1986a; Pommier et al., 1986b) and disputed (Ross et al., 1979; Zwelling et al., 1981; Zwelling et al., 1982a, b) a causal relationship between topoisomerase II-mediated damage to DNA and drug cytotoxicity.

A variety of experimental approaches were used by different workers. One such approach involved comparisons of cytotoxic potential and inhibition of topoisomerase II activity by drugs from different chemical classes. However, as discussed in Chapter 3, some drugs may simultaneously activate several cytotoxic mechanisms and therefore such an approach can lead to difficulty with interpretation of results. To eliminate some of the variability inherent in comparing structurally dissimilar drugs, the cytotoxicity and topoisomerase II reactivity of drugs within a single chemical class, synthetic acridines, were compared (Chapter 5). Using this approach, with various 9-aminoacridine derivatives, it was demonstrated that topoisomerase II-DNA breaks correlate well with drug cytotoxicity. A similar conclusion was reached by Rowe et al. (1986a) who utilised a series of acridines having the same site specificity on DNA to examine the level of protein linked breaks in the DNA of L1210 cells. Furthermore, in agreement with Glisson et al. (1986a) it was concluded that drug induced formation and stabilization of the topoisomerase II-DNA complex, rather than inhibition of its formation or of strand-passing functions appears to be related to DNA strand breakage and cell death.
However, it appears that neither the capacity to induce DNA breaks or the qualitative nature of the topoisomerase II-DNA lesions, in terms of cell killing potential, is uniform even amongst the acridine derivatives (or cell lines, See Section 9.2) and the apparent differences in interactions between acridine carboxamide, C-6 bisacridine and amsacrine with topoisomerase II may indicate that other factors are involved (see Chapter 3).

Recently it has been suggested that secondary events beyond formation of the cleavable complex may be important for the expression of lethality (Pommier et al., 1986b; Rowe et al., 1986a; Pommier et al., 1988). The possibility that additional factors are involved in converting the initial cleavable complex into a lethal lesion has been implied from a number of experiments that were discussed in Chapter 1 (Section 1.5). Taken together, these experiments suggest that the production or level of cleavable complexes alone is not sufficient to predict cytotoxicity.

In conclusion, it appears that the formation of the cleavable complex and DNA breakage are important steps in drug action, which do not however, necessarily lead to drug-induced cell killing and that ongoing events, possibly mediated by additional factors are necessary for maximal cytotoxicity.

9.2 The role of topoisomerase II in amsacrine resistance.

Effective treatment of solid tumours by drugs is proving one of the most challenging and difficult aspects of cancer therapy research today. It has been suggested that the low numbers of actively cycling cells in solid tumours may limit the tumour-killing activity of topoisomerase II-specific drugs, since non-cycling cells per se appear to be less
sensitive to these drugs. Therefore the resistance of non-cycling cells to amsacrine, with particular reference to the involvement of topoisomerase II, was investigated using two tissue culture model systems for non-cycling cells, namely plateau-phase CHO-AA8 cells and temperature sensitive mouse mastocytoma 21-Fb cells.

CHO-AA8 cells when grown to plateau-phase were shown by Robbie et al. (1988) to resemble populations of non-cycling cells in solid tumours, with approximately 82% of the cells entering a state of growth arrest with G1-G0 DNA content. The 21-Fb cold sensitive cell cycle mutants also arrest in G1 upon incubation at the non-permissive temperature, but in contrast to the CHO-AA8 system, the 21-Fb cells develop a more differentiated, normal mast cell phenotype (Zimmerman et al., 1981; 1983; Laeng et al., 1985). This qualitative difference in the state of growth arrest was reflected in the sensitivity and response of the different cells to amsacrine (Chapter 4). For example, there was a close relationship between cell proliferative state, amsacrine-induced cytotoxicity, DNA breaks and topoisomerase II activity in CHO-AA8 cells. In contrast, there was no correlation between growth state, amsacrine sensitivity and topoisomerase II mediated DNA breakage, protein-DNA complex formation or strand-passing activities with 21-Fb cells. In cell survival experiments, the sensitivity of arrested 21-Fb cells to amsacrine decreased less than 2-fold, whereas there was a 4- to 8-fold decrease in drug sensitivity of arrested CHO-AA8 cells relative to their proliferating counterparts. However, in both cell lines it was found that when arrested in G1, intact cells, nuclei and nuclear extracts were much less susceptible to amsacrine-induced effects such as DNA breakage and formation of protein-DNA complexes than proliferating cells, nuclei or extracts. Furthermore, in both cell lines the changes in sensitivity to
amsacrine-induced protein-DNA complex formation were similar in cells and nuclei compared to extracts using an exogenous DNA substrate suggesting that reduction in sensitivity to drug-induced lesions was not related to growth-related changes in chromatin conformation. Consequently, quantitative or qualitative alterations of topoisomerase II may have influenced the sensitivity of cells to drug-stimulated cleavable complex formation and as a consequence, drug cytotoxicity. Indeed, a 16-fold reduction in DNA topoisomerase II catalytic activity in arrested 21-Fb cells was observed, however this was much greater than anticipated from the marginal 2-fold decrease in amsacrine sensitivity of these cells. Quite the reverse was found with CHO-AA8 cells. In plateau-phase CHO-AA8 cells up to an 8-fold decrease in sensitivity to amsacrine was seen whereas there was only a 2-fold reduction in topoisomerase II activity. In agreement with the results in Chapter 3, there was a positive correlation between the inhibition of the unknotting reaction of topoisomerase II by amsacrine and the cytotoxic action of the drug on growing- or plateau-phase cells.

The data taken together suggested that qualitatively altered drug-enzyme or enzyme-DNA interactions in plateau-phase CHO-AA8 cells contributed to the observed resistance of this population of G1 phase cells to amsacrine. In contrast, it is likely that the reduction in amsacrine-induced DNA breakage in arrested 21-Fb cells was due to quantitatively lower enzyme activity, although a qualitative modification of topoisomerase II, for example by phosphorylation, cannot be excluded. However, the exact reasons for the difference in amsacrine sensitivity of growth arrested CHO and 21-Fb cells are still unclear although a number of possible explanations were considered in Section 4.10.3.

In mixing experiments, neither CHO-AA8 or 21-Fb cells arrested in G1
phase appeared to contain inhibitory factors which reduced the sensitivity of topoisomerase II to amsacrine (Dr E. Schneider, pers. comm.). However, it is possible that the absence, reduction or alteration of a protein-factor(s) required for the formation, stabilization (Chapter 7) or processing of cleavable complexes (Chow et al., 1988; Schneider et al., 1988d) may contribute to differential levels of drug resistance.

It appears that there is an inherent difference in the nature or phenotype of the DNA lesions observed in each of the populations of G1 phase cells studied. For example, the lesions in arrested 21-Fb cells, though fewer in number than in proliferating cells, still translated into lethal events, whereas those in plateau-phase CHO cells did not. This in turn may reflect differences in the quality or phenotype of the growth arrest. For example, if 21-Fb cells arrest at or near the G1/S border they may have a low sensitivity to amsacrine-induced breaks similar to G0/G1 phase cells but have an enhanced sensitivity to amsacrine cytotoxicity not evident in cells arrested with a true G0/G1 DNA content in agreement with the results of Chow and Ross (1987) and Estey et al. (1987a) (see Section 4.10.3). Low levels of drug-induced DNA lesions may equate to significant drug cytotoxicity if they occur in crucial actively expressed genes or in their regulatory signals and prolonged persistence of the lesions causes downstream processing which translates the lesions into lethal events (Pommier et al., 1986). Alternatively, 21-Fb cells may contain greater amounts of secondary factors which facilitate the formation or processing of cleavable complexes. These situations may not prevail in cells arrested with a true G0/G1 DNA content such as the plateau-phase CHO-AA8 cells used in this study or the mouse mastocytoma cells arrested by serum deprivation or DBcAMP described by Hutchins (1986) (see Section 4.10.3).
From my results and those of others, I can only reiterate that a very complex relationship exists between amsacrine-induced DNA breakage, topoisomerase II-DNA complex formation, topoisomerase II activity and drug cytotoxicity. Moreover, a complex set of parameters can influence formation of amsacrine-induced topoisomerase II-mediated lesions and cytotoxicity in different cells or even a single type of cell under different growth conditions.

It is clear that further biochemical and genetic experiments are required to resolve the relationship between cell proliferative state, amsacrine sensitivity and topoisomerase II activity. The studies described in this thesis, for example using the P4-unknotting assay, only provide a measure of functional activity of extracted topoisomerase II enzymes in an in vitro assay system and do not provide a direct measure of the topoisomerase II content of cells. Furthermore, quantitative comparisons of changes in topoisomerase II content (determined by immunoblot analysis) and activity (measured by decatenation assays) (Pommier et al., 1986b; Sullivan et al., 1987) have shown a discrepancy between results obtained by these methods. Therefore the importance of measuring topoisomerase II content and topoisomerase II messenger RNA production or stability in addition to enzyme activity in CHO-AA8 and 21-Fb cell systems studied in this thesis is emphasized and warrants further investigation.

In addition, it will be of interest to pursue possible differential effects of qualitative modifications of topoisomerase II on drug action in drug-sensitive and drug-resistant CHO-AA8 cells. Hopefully some light may be shed on the involvement of phosphorylation in drug action by studies stemming from the discovery of a protein kinase required for drug-stimulated topoisomerase II-DNA complex formation as discussed in
Section 9.3.

9.3 A protein factor that enhances amsacrine mediated formation of topoisomerase II-DNA complexes: Speculations on its role in drug action, drug resistance and cancer cell growth.

Previously, I and collaborators used cell free systems and isolated nuclei to investigate relationships between the cytotoxic effects of topoisomerase II (Chapter 4; Schneider et al., 1988b, c). However, initial attempts to quantitate amsacrine-induced topoisomerase II-DNA complexes in isolated K21 cell nuclei were unsuccessful and despite extensive efforts to improve their detection, only low levels of PDC (up to 30% of the levels of PDC in whole cells treated with equivalent drug concentrations) could be detected (Chapter 5). Indeed, it was puzzling that drug-induced protein-DNA complexes could not be detected in my hands by SDS/k+ precipitation whereas others (Pommier et al., 1982; 1984b; Markovits et al., 1987b; Covey et al., 1988; Pierson et al., 1988) could detect PDC's in nuclei by alkaline elution. However, it has been reported previously that although detectable, the amounts of drug-induced PDC's produced in nuclei are significantly lower than in intact cells measured by alkaline elution (Pommier et al., 1982; Pierson et al., 1988). In fact, closer examination of the results presented in Pierson et al. (1988), Covey et al. (1988) and Markovits et al. (1987b), showed that at equivalent drug concentrations, the levels of PDC produced in nuclei were approximately 18 - 50% of the PDC levels induced in intact cells. These results are comparable to the levels of PDC routinely achieved in K21 or L1210 nuclei reported in this thesis. Despite alkaline elution being a more sensitive technique than SDS/k+ precipitation for
quantitation of drug-induced DNA lesions, the disparity between data obtained with whole cells compared to isolated nuclei was of great interest.

Preservance with attempts to resolve the problems associated with detecting PDC's in isolated murine mastocytoma cell nuclei eventually led to the discovery of a cytoplasmic factor that restored the formation of amsacrine-induced topoisomerase II-DNA complexes in isolated nuclei to levels routinely achieved in intact cells. This factor appeared to be a labile protein distinct from the two classes of topoisomerase and it was rapidly lost from cells grown with cycloheximide or cordycepin, indicating that continuous RNA and protein synthesis were required to maintain its activity. A preliminary assessment of the generality of the PDC enhancing factor indicates that it is reasonably widespread with similar activity being found in four murine and one human cell line. Moreover, a degree of functional conservation, at least in mammalian cell systems, was also indicated. Possible involvement of the factor in drug resistance is suggested by two lines of evidence: factor activity was depleted in murine mastocytoma cells induced to arrest in G1 phase and also in human Jurkat cells selected for resistance to amsacrine.

Fractionation of mastocytoma cell extracts by salt precipitation, DEAE-cellulose chromatography and SDS-PAGE showed the PDC enhancing activity to reside in a 70 kDa protein and a preliminary identification suggests that the 70 kDa protein is a heparin-sensitive protein related to casein kinase II or nuclear kinase II.

Consideration of the in vivo role of the PDC enhancing factor must take into account its implied association with topoisomerase II. Specific antibodies have revealed that topoisomerase II is a prominent component of mitotic chromosome scaffolds and the interphase nuclear matrix (Berrios
et al., 1985; Earnshaw et al., 1985; Gasser et al., 1986). The enzyme is localized at the base of chromatin loops within metaphase chromatids (Earnshaw and Heck, 1985) and recent studies have defined a very specific class of DNA sequence that mediates loop anchorage. Moreover, these sequences contain consensus topoisomerase II binding sites and they reside close to known enhancers (Cockerill and Garrard, 1986; Jarman and Higgs, 1988), therefore topoisomerase II may be involved in DNA replication, transcription or recombination and drug-induced DNA cleavage at such sites could have potentially lethal consequences for cells.

Various workers have suggested that there are two pools of topoisomerase II, representing functional states or isoforms (Chapter 4, Part II; Earnshaw et al., 1985; Minford et al., 1986; Drake et al., 1987; Schneider et al., 1988c) which may have different sensitivities to amsacrine. It is conceivable that modification of topoisomerase II or its isoforms by phosphorylation may have implications for the efficacy of drugs targeted at the enzyme. My results indicate that in isolated nuclei amsacrine-induced topoisomerase II-DNA complex formation is dependent on the presence of a 70 kDa protein kinase with casein kinase-like properties. Furthermore, it is known that phosphorylation of topoisomerase II by casein kinase II in vitro stimulates enzyme activity.

One possible model for the involvement of the PDC enhancing factor in modulation of topoisomerase II activity and drug action is presented in Figure 9.1 A. Phosphorylation of topoisomerase II (TII) by casein kinase II (CKII) or a related enzyme(s) converts the enzyme to a drug sensitive PDC forming state in vivo, possibly by affecting its action on, or association with, the nuclear matrix or scaffold. Indeed, it has been proposed (Drake et al., 1987) that one form of topoisomerase II, the
FIGURE 9.1: Models for the involvement of the PDC enhancing factor in topoisomerase II- and amsacrine-action.
matrix or scaffold associated enzyme bound tightly to DNA loses its DNA strand passing catalytic function, whereas a second non-matrix bound or "soluble" form (which may be phosphorylated), produces topoisomerization and forms readily reversible complexes with DNA. This model is supported by the release of PDC forming activity when normally inactive K21 nuclei are extracted with strong salt (Chapter 4, Part II: Schneider et al., 1988c). As a consequence of factor depletion during nuclei isolation only a fraction of the potential drug-induced PDCs in whole cells would be expected in isolated nuclei, as seen with K21 cell nuclei (Figures 5.3, 6.1) and in L1210 cell nuclei when measured by alkaline elution (Pommier et al., 1982, 1984b; Covey et al., 1988; Pierson et al., 1988).

Therefore, the fraction of PDCs produced in isolated nuclei may reflect the residual factor that activates matrix associated topoisomerase II and the different proportions of PDC formation obtained with nuclei isolated from different cells may reflect the amount of factor retained using different nuclei isolation methods (Chapter 4, Part I: Schneider et al., 1988b; Bakic et al., 1986. c.f. Pommier et al., 1982, 1984b; Covey et al., 1988; Pierson et al., 1988).

Alternatively, phosphorylation of topoisomerase II at specific sites may cause a structural change which converts the enzyme to a form which binds drugs such as amsacrine preferentially or more tightly resulting in prolonged stabilization of the cleavable complex which permits further processing of the cleavable complex leading to cell death.

From such a model one can envisage a role for the PDC enhancing factor in the phenomenon of drug resistance. For example, reduced or modified kinase activity or an altered phosphorylation site on the topoisomerase II enzyme may directly affect the action or association of topoisomerase II with DNA or indeed the affinity of topoisomerase II-targeted drugs for the
enzyme shifting the equilibrium between cleavable and non-cleavable complexes to the latter. In support of this idea, it has been reported (Kaiserman, 1988) that dephosphorylation of topoisomerase I prevents the formation of camptothecin-induced topoisomerase I-DNA complexes.

What is not explained by this model is the ability of purified topoisomerase II to form drug-induced complexes with DNA, although the state of phosphorylation of the purified enzyme is not usually known and protein kinase activity has previously been shown to co-purify with topoisomerase II (Sander et al., 1985).

A second and perhaps more plausible model to explain the PDC enhancing factor modulation of topoisomerase II activity and drug action is presented in Figure 9.1 B. In this model, it is proposed that topoisomerase II is prevented from forming drug-induced protein-DNA complexes by an auxiliary protein inhibitor. Phosphorylation of the inhibitor protein (I) by the casein kinase II-like factor (CKII) inactivates the inhibitor and frees topoisomerase II (TII) allowing the enzyme to react with DNA. This model is equivalent to the "bound" versus "free" topoisomerase II forms described in the first model, whereby inhibited topoisomerase II represents the non-functional matrix-bound form and the non-inhibited topoisomerase II represents the functional "soluble" form of the enzyme. The release of active topoisomerase II from nuclei after salt extraction may be facilitated by the inactivation or dissociation of the inhibitor by salt or detergents used for nuclei isolation. One possible candidate for the inhibitor protein may be the scaffold-associated protein SC-2 which appears to be an integral component of nuclear scaffolds, but as yet has had no in vivo function assigned. Nuclear scaffolds are stabilized by Cu$^{2+}$ and Ca$^{2+}$ (Lebkowski and Laemmli, 1982a, b; Lewis and Laemmli, 1982) and I was unable to extract
topoisomerase II from nuclei prepared in buffers containing either \( \text{Ca}^{2+} \) or \( \text{Cu}^{2+} \) (see Chapter 5). It is possible that under such conditions a scaffold-associated protein forms an extremely stable complex with topoisomerase II that prevents its release.

Model B would also explain the ability of topoisomerase II, purified away from an inhibitory factor, to form drug-induced complexes with exogenous DNA. In fact, Ishida et al. (1988) found no significant difference in drug-induced DNA cleavage activities of topoisomerase II isolated from cells sensitive (BHK) or resistant (Nov^r A2) to topoisomerase II-targeted drugs, although significant resistance to the drugs was conferred at the level of the nucleus in Nov^r A2 cells and which was independent of drug transport phenomena. These results and those of others (e.g. Pommier et al., 1986b) suggest that there is in fact an inhibitor of topoisomerase II in nuclei whose activity is not controlled under conditions conferring drug resistance. Reduced sensitivity to drugs may then be explained not only by cell-cycle or other associated depletions in the casein kinase-like PDC enhancing protein, but also by greater synthesis or accumulation of the inhibitor in, for example, non-cycling cells. If true, the inhibitor would present a novel target for new anticancer agents with specificity for non-proliferating cells otherwise resistant to drugs.

I have described only two of several possible models for the involvement of the PDC enhancing activity with topoisomerase II activity, drug action and resistance. Of the models presented, I favour the latter as it appears to satisfy most of the inconsistencies between the data presented here and that reported in the literature. Obviously both models should be tested and the addition of purified PDC enhancing factor to nuclei from drug-resistant cells would be a logical starting point.
However, the possibility that topoisomerase II must be phosphorylated to facilitate drug binding and protein-DNA complex formation is also intriguing. Further studies comparing the state of phosphorylation of the enzyme with drug efficacy would be interesting.

Whatever the mechanism of action of the PDC enhancing factor may be, without doubt it suggests new approaches for overcoming the resistance of non-cycling cells to amsacrine and related antitumour compounds.

9.4 General Consideration

It has been shown in this thesis and by others that topoisomerase II is involved in conferring drug resistance to some populations of non-cycling cells. However, no clear explanation or mechanism by which these events are achieved or how cell death occurs has been resolved.

What is clear is that altered topoisomerase II activity is likely to play a major role in the resistance of some cells to intercalators, and it seems that more than one mechanism may be responsible for modulating drug sensitivity of topoisomerase II. The results presented here and in the literature favour qualitative alterations of topoisomerase II, either by covalent modification or specific inhibitors of topoisomerase II activity.

One of the most encouraging aspects of my research has been the discovery of a protein factor that directly influences drug-induced topoisomerase II action. Elucidation of this interaction and of the mechanisms which control it will undoubtedly increase further understanding of the molecular basis of drug selectivity and hopefully it will ultimately lead to the synthesis of drugs with greater specificity and broad spectrum activity.
REFERENCES.


Relationship between sensitivity to

4’-(9-acridinylamino)methanesulfon-m-anisidine and DNA topoisomerase II
in a cold-sensitive cell-cycle mutant of a murine mastocytoma cell line

Erasmus Schneider, Anne-Marie Hutchins *, Sandra J. Darkin, Penelope A. Lawson and Raymond K. Ralph

Department of Cellular and Molecular Biology, University of Auckland, Auckland (New Zealand)

(Received 30 May 1988)

Key words: Amsacrine; DNA topoisomerase II; DNA damage; Cytotoxicity; (Murine mastocytoma cell)

The cold-sensitive (proliferating at 39.5 °C, reversibly arrested in G1-phase at 33 °C) cell-cycle mutant 21-Fb of the murine mastocytoma cell line P815 was used to study the effect of amsacrine on non-cycling cells. The sensitivity of arrested 21-Fb cells decreased less than 2-fold in cell survival experiments when compared to proliferating cells. In contrast, DNA breakage and stimulation of protein-DNA complex formation in intact or lysed cells was reduced approx. 10-fold in arrested cells and DNA topoisomerase II activity in arrested cells was only 5% of the activity in proliferating cells. Thus, there was no correlation between cell survival and DNA damage or DNA topoisomerase II activity in drug-treated cells.

Introduction

Several anticancer drugs, including amsacrine (4’-(9-acridinylamino)-methanesulfon-m-anisidine, mAMSA), adriamycin, the ellipticines and epipodophyllotoxins have been shown to act on the enzyme DNA topoisomerase II (EC 5.99.1.3) [1–4]. Cells treated with these drugs exhibit an increased number of DNA breaks presumably due to drug-mediated inhibition of the DNA rescaling mechanism after the strand-passing reaction of topoisomerase II. Consequently, the enzyme is stabilised in a covalent protein-DNA complex. This complex can be isolated, and the amount of drug-induced complex formation serves as a measure for drug activity [5].

Despite topoisomerase II being ubiquitous, there are marked differences in the response of different tumours towards the cytotoxic action of the topoisomerase II-specific anticancer drug amsacrine [6–8]. Amsacrine is a synthetic DNA-intercalating agent with useful clinical activity against leukaemias and lymphomas [9,10] but little activity against solid tumours [11]. While the reasons for the resistance of solid tumours are not known, it has been suggested that the low number of actively cycling cells in solid tumours may limit the tumour-killing activity of topoisomerase II-specific drugs, since non-cycling cells per se appear to be less sensitive to these drugs [12–14].

In a number of reports, the activity of topoisomerase II has been examined in relation to changes in the proliferative activity of cells. During terminal differentiation of erythroblasts into erythrocytes and of myoblasts into myotubes, a total loss of topoisomerase II antigen was observed [15]. On the other hand, when peripheral
Blood lymphocytes (which lack detectable topoisomerase II) were stimulated to proliferate, de novo enzyme synthesis occurred and exactly paralleled the onset of DNA synthesis [15,16]. Considerable increases in topoisomerase II activity were also observed in regenerating rat liver after partial hepatectomy [17]. Recently, several studies investigating the relationship between the state of cell proliferation or the phase of the cell cycle, DNA topoisomerase II activity and drug sensitivity, found that topoisomerase II activity and drug sensitivity both vary as a function of cell proliferation [18-24]. In general, maximal drug sensitivity was observed in actively proliferating or S-phase cells, concomitant with a high topoisomerase II activity, although there was some variation between different cell lines. For instance, when Sullivan et al. [18,19] studied drug–enzyme interactions in relation to the state of cell proliferation, they found little difference in drug sensitivity between log- and plateau-phase HeLa or L1210 cells, but a marked decrease in sensitivity of plateau-phase CHO cells towards amsacrine or etoposide compared with log-phase cells. The decreased drug sensitivity in plateau-phase cells was accompanied by decreased topoisomerase II activity and a reduced capacity to form cleavable complexes. Similarly, Markovits et al. [20] reported higher frequency of amsacrine- or etoposide-induced protein-DNA complex formation in nuclei from exponentially growing than in those from quiescent 3T3 or L1210 cells, and maximum protein DNA complex formation coincided with the peak of DNA synthesis and topoisomerase II strand-passing activity. On the other hand, when we compared amsacrine sensitivity, topoisomerase II activity and protein-DNA complex formation in log- and plateau-phase CHO-A8 cells, plateau-phase cells exhibited a marked reduction in amsacrine sensitivity and in drug-induced protein-DNA complex formation, whereas topoisomerase II activity was only slightly reduced [24]. A lack of correlation between maximal sensitivity to drug cytotoxicity and maximal DNA breakage was reported for HeLa and Balb/c 3T3 cells [22,23]. Together, these data suggest a complex relationship between the cytotoxic effects of topoisomerase II-directed anticancer drugs, topoisomerase II and drug-induced DNA breakage.

To investigate further these interactions, we have used 21-Fb cells, a cold-sensitive (proliferating at 39.5°C, reversibly arrested in G1-phase at 33°C) cell-cycle mutant of the P815-X2 murine mastocytoma cell line. After cell-cycle arrest, the 21-Fb cells have been shown to exhibit a dominant phenotype in cell-fusion experiments, to resemble normal mast cells and to differentiate [25-27]. We compared the sensitivity of proliferating and arrested cells to amsacrine with cell survival, DNA breakage and protein-DNA complex formation assays and we also measured topoisomerase II activities. Our results showed a lack of correlation between cell survival and DNA damage.

Materials and Methods

Materials. [Methyl-3H]Thymidine (70-90 Ci/mmol) was from Amersham and (α-labelled 35S) dATP (500 Ci/mmol) was from New England Nuclear. Phage P4 was a generous gift from Dr. R. Calendar, University of California, Berkeley. PM2 bacteriophage and host bacteria Alteromonas espejiana were kind gifts from Drs. M.J. Waring, University of Cambridge, U.K., and R. Morgan, University of Alberta, Edmonton, Canada. Phage PM2 was grown and purified essentially as described by Espejo and Canelo [28]. The restriction enzyme EcoRI was from Promega Biotec and DNA Pol I Klenow fragment and proteinase K were from Boehringer Mannheim. Amsacrine (mAMSA), provided as the isethionate salt by Dr. R.C. Baguley, University of Auckland medical school, was stored as a 1 mM stock solution in water at -20°C and working dilutions were made immediately prior to use. Dextran grade B 150-200 was from BDH, Hoechst 33258 was from Calbiochem Biochemicals and p-iodonitrotetrazolium violet was from Serva, Heidelberg, F.R.G. The proteinase inhibitors apronin, leupeptin, α2-macroglobulin, phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate were from Sigma. RPMI 1640 and horse serum were from Gibco, New Zealand.

Cell Culture. A clonal subline (K21, wild-type) of the P815-X2 mouse mastocytoma cell line and the clonal cell-cycle mutant 21-Fb (cold-sensitive, multiplying at 39.5°C, arrested at 33°C) [25] were
kindly provided by Dr. R. Schindler, University of Berne, Switzerland. The cells were grown in RPMI 1640 medium supplemented with 10% horse serum in a 5% CO₂/95% air atmosphere and subcultured as recommended [25]. Growth arrest of 21-Fb cells was induced by growing the cells at their non-permissive temperature of 33°C for 4 days.

**Drug toxicity assays.** mAMSA sensitivity of log-phase and arrested 21-Fb cells was determined by colony formation in soft agarose. Cultures were treated with 0–10 μM mAMSA for 60 min, washed twice with medium, resuspended and serially diluted with fresh medium to between 250 cells/ml and 400,000 cells/ml and aliquots (0.25 ml) mixed with 5 ml 0.2% agarose in medium were poured onto a layer of 0.3% agarose in medium in 60 mm plastic petri dishes. The soft agarose was supplemented with 10% horse serum, 0.1 mg/ml folic acid, 20 μM sodium pyruvate and 20 μM each L-alanine, L-aspartic acid, L-glutamic acid and L-proline. The plates were incubated at 39.5°C for 10–12 days. Colonies were stained overnight with 4-iodonitrotetrazolium violet (1 mg/ml; 1 ml/plate) and the relative survival of drug-treated cultures compared to untreated controls was determined. Cloning efficiency of arrested 21-Fb cells was improved by incorporating 0.2 mM cysteine in the cloning medium.

**Extraction of DNA topoisomerase II activity. (1)** For catenation assays: aliquots of approx. 3·10⁷ cells were collected by centrifugation, washed once in Tris-buffered saline (25 mM Tris-HCl (pH 7.5)/135 mM NaCl/5 mM KCl) and resuspended in 1 ml 10 mM Tris-HCl (pH 7.5)/10 mM KCl/1.5 mM MgCl₂. After swelling for 15 min on ice, the cells were disrupted with a Dounce homogeniser and the nuclei were recovered by centrifugation at 1200 x g for 10 min at 4°C. After resuspension in 0.5 ml 20 mM Tris-HCl (pH 7.5)/800 mM KCl/5 mM MgCl₂/10 mM 2-mercaptoethanol, the nuclei were sonicated briefly, then slowly mixed with 1/2 volume of 18% (w/v) poly(ethylene glycol)6000/1 M NaCl/1 mM dithiothreitol to precipitate nucleic acids. After 30 min at 0°C, the extract was centrifuged at 30,000 x g for 30 min at 4°C to obtain a clear supernatant containing topoisomerase II activity and 0.4 mg/ml protein, which was stored at −70°C. (2) For P4-unknotting and protein-DNA complex-formation assays: in this procedure, the following proteinase inhibitors were added at the indicated final concentrations to all buffers immediately prior to use: aprotinin, 1% (v/v); leupeptin, 0.1 mg/ml; α₂-macroglobulin, 0.01 mg/ml; phenylmethylsulfonyl fluoride, 1 mM; diisopropyl fluorophosphate, 0.1 mg/ml. This effectively inhibited a strong proteolytic activity observed mainly in arrested 21-Fb cells, but did not adversely affect topoisomerase II activities per se. Aliquots of 3·10⁷ cells were collected by centrifugation, washed once in ice-cold Tris-buffered saline and resuspended in 0.4 ml buffer A (20 mM Tris-HCl (pH 7.2)/150 mM KCl/2 mM MgCl₂/2 mM CaCl₂/0.1 mM diithiothreitol/10 mM Na₃S₂O₈/2% (w/v) dextran grade B) to a cell density of (4–5)·10⁷ cells/ml. Triton X-100 was added to a final concentration of 0.1% and the cells were lysed for 10 min at 0°C with occasional gentle mixing, followed by centrifugation at 1200 x g for 10 min at 4°C. The supernatant was retained for extraction of the soluble topoisomerase II activity (see below) and the nuclei pellet was resuspended in 0.4 ml buffer B (buffer A with 5 mM MgCl₂ and without CaCl₂), centrifuged at 1200 x g for 10 min at 4°C and resuspended in 0.4 ml buffer C (20 mM Tris-HCl (pH 8.0)/150 mM KCl/5 mM MgCl₂/0.1 mM dithiothreitol/10 mM Na₃S₂O₈). The supernatant from the last nuclei wash did not contain any detectable P4-unknotting activity. For topoisomerase II extraction, the nuclei and the cytoplasmic supernatant were treated separately with 20 mM 2-mercaptoethanol and 20 mM EDTA for 30 min at 4°C, followed by 1 x 15 s burst of sonication before 1/3 volume of 24% (w/v) poly(ethylene glycol)6000/2 M KCl/10 mM Na₃S₂O₈ was added and the mixtures were held for 30 min on ice. The resulting precipitates were removed by centrifugation at 30,000 x g for 30 min at 4°C to obtain clear supernatants containing the nuclear or soluble (from the cytoplasmic fraction) topoisomerase II activities, respectively. Extracts from proliferating and arrested cells were diluted to equal protein concentrations of approx. 0.1 mg/ml (nuclear) and 3 mg/ml (soluble) and stored at −70°C.
The phage PM2 catenation assay. To measure topoiso- 
"merase II activity, the standard catenation assay 
(50 μl) contained 20 mM Tris-HCl (pH 7.5)/6 mM 
MgCl2/0.5 mM EGTA/2 mM dithiothreitol/1 mM 
ATP/18 μg/ml bovine serum albumin/8% (w/v) 
poly(ethylene glycol)6000/25 mM KCl and 10 μl extract. The reaction 
was initiated by adding 0.2 μg PM2 DNA and in-
cubated for 60 min at 30°C. The reactions 
were terminated by adding 1% SDS and 0.4 mg/ml 
proteinase K for 30 min at 37°C and reaction 
products were electrophoresed in 0.7% agarose 
gels in TBE buffer (89 mM Tris-borate (pH 7.5)/89 
mM boric acid/2 mM EDTA) for 16 h at 50 V. After 
staining with Ethidium bromide, the gels 
were photographed under ultraviolet illumination. 
Photographic negatives were analysed by scanning 
on a microdensitometer (Joyce, Loebl and Co, 
Model MK III C). 1 unit of topoiso- 
"merase II activity was defined as the minimum amount of 
extract (in μg protein) that produced catenanes 
from 0.2 μg PM2 DNA in 60 min at 30°C.

The phage P4 DNA unknotting assay. Knotted 
phage P4 DNA was prepared as described by Liu 
et al. [29]. Assay mixtures (20 μl) contained 50 
mM Tris-HCl (pH 7.5)/10 mM MgCl2/0.5 mM 
EGTA/0.5 mM dithiothreitol/30 μg/ml BSA/1 
mM ATP and 0.24 μg P4 DNA. After adding 4 μl 
of extract (which provided 120 mM KCl), the 
assays were incubated for 30 min at 37°C. To 
remove proteins, 1 μl 10% SDS and 1 μl 10 
mg/ml proteinase K were added and the mixture 
was incubated for a further 30 min at 37°C. 
Samples were electrophoresed on 0.7% agarose 
gels in TBE buffer for 4 h at 100 V, and the gels 
were stained with ethidium bromide and photo-
graphed under ultraviolet illumination. Photo-
geraphic negatives were then analysed by scanning 
on a microdensitometer. 1 unit of topoiso- 
"merase II activity was defined as the amount of extract 
in (μg protein) that completely unknotted 0.24 μg 
phage P4 DNA.

Quantitation of mAMSA-stimulated covalent 
protein-DNA complex formation. The SDS/K+ 
precipitation assay described by Liu et al. [30] and 
Rowe et al. [5] was used to measure the drug-induced 
formation of protein-DNA complexes in intact and 
lysed 21-Fb cells and nuclear extracts 
from proliferating and arrested 21-Fb cells. For 

studies with cells, the DNA of proliferating cells 
was labelled with 3 μCi/ml [methyl-3H]thymidine 
(70–90 Ci/mmol) in AHTG medium (medium 
supplemented with 0.01 mM amethopterin/0.03 
mM hypoxanthine/0.01 mM cold thymidine/0.1 
mM glycine) for 24 h at 39.5°C. The DNA of 
cells to be arrested was similarly labelled for the 
last 24 h of culture at 39.5°C and for the first 24 
h at 33°C. The cells were collected by centri- 
fugation and washed once with Tris-buffered saline. 
One half of the cells was kept on ice until required 
for cell lysate preparation (see below). The rest 
were resuspended in fresh growth medium to a 
final density of 2·10⁶ cells/ml, distributed in 49 
μl aliquots (10⁵ cells) into 96-well microtiter plates 
and treated with 0–20 μM mAMSA for 10 min at 
37°C. To overcome the plasma membrane barrier, 
cells were resuspended in nuclei buffer (20 mM 
Tris-HCl (pH 7.2)/150 mM KCl/5 mM 
MgCl2/10 mM Na2HPO4/2% (w/v) dextran 
150–200) at 5·10⁷ cells/ml and lysed with 0.05% 
Triton X-100 for 10 min at 0°C. To initiate 
drug-stimulated protein-DNA complex formation, 
cell lysates containing 10⁵ nuclei were added to 
the following reaction mixture in 96-well plates in 
a total volume of 50 μl/well: 50 mM Tris-HCl 
(pH 7.5)/10 mM MgCl2/120 mM KCl/5 mM 
EGTA/2.25 mM EDTA/0.5 mM dithiothreitol/ 
30 μg/ml bovine serum albumin/2 mM ATP/0– 
20 μM mAMSA and additional cytoplasmic ex-
tract. The cytoplasmic extract was prepared by digi- 
onin extraction of corresponding proliferating 
or arrested 21-Fb cells, as described by Blank-Liss 
and Schindler [31], but containing proteinase 
hibitors, and added in predetermined optimal 
amounts of 30 μg and 40 μg protein (extracted 
from 2.4·10⁶ and 3.2·10⁶ cells, respectively) of 
extract prepared from proliferating or arrested 
cells, respectively. No protein-DNA complex-form-
ing activity was detected in this cytoplasmic 
extract. Incubation was for 10 min at 37°C. Follow-
ing drug treatment, the cells or nuclei were 
collected by centrifugation at 12000 x g for 10 min 
at room temperature. The supernatants were dis-
carded and the cells or nuclei were lysed with 0.2 
ml of prewarmed (65°C) lysis solution (1.25% 
SDS/5 mM EDTA/0.4 mg/ml salmon sperm 
DNA) and transferred to Eppendorf tubes. Each 
well was rinsed with a further 0.2 ml lysis solution,
which was then combined with the corresponding original lysate; 0.1 ml of prewarmed (37°C) 325 mM KCl solution was added and the mixture was immediately vortexed for 10 s at the highest setting. The tubes were held for 30 min on ice and the resulting precipitates were then pelleted by centrifugation in a microfuge for 10 min at 4°C. After the supernatants had been carefully removed, the pellets containing protein-DNA complexes were resuspended in 1 ml of ice-cold wash solution (10 mM Tris-HCl (pH 7.5)/100 mM KCl/2 mM EDTA) and collected on GF/C filters (Whatman) under gravity. The filters were washed five times with wash solution under vacuum and dried, and the radioactivity on the filters was measured in a liquid scintillation spectrometer. The total acid-precipitable radioactivity per assay (i.e., per 10^3 cells or nuclei) was routinely found to be approx. 5 - 10^4 cpm.

To determine protein-DNA complex formation in nuclear or soluble extracts, 35S-3'-end-labelled EcoRI digested pBR322 DNA was prepared according to Maniatis et al. [32] and used as a substrate. Protein-DNA binding reaction mixtures (50 µl) contained 50 mM Tris-HCl (pH 7.5)/10 mM MgCl2/5 mM EGTA/0.5 mM dithiothreitol/30 µg/ml bovine serum albumin/2 mM ATP/50 ng 35S-3'-end labelled pBR322 DNA and various amounts of drug. The reactions were initiated by adding 10 µl of nuclear or soluble extract containing approx. 0.3 µg or 10 µg protein, respectively (which provided 120 mM KCl) and incubation was for 30 min at 37°C. The reaction was stopped by adding 100 µl of a prewarmed (37°C) solution containing 0.2 M NaOH/5 mM EDTA/2% SDS/0.5 mg/ml herring DNA for 10 min at 37°C, followed by addition of 50 µl of a solution containing 0.4 M Tris-HCl (pH 7.9)/0.4 M HCl/0.25 M KCl to precipitate covalent protein-DNA complexes for 10 min at 4°C. The precipitates were collected on GF/C filters under gravity and washed several times with wash solution under vacuum. Radioactivity retained on the filters was measured in a liquid scintillation spectrometer.

DNA breakage. DNA breakage induced by mAMSA in proliferating or arrested 21-Fb cells was determined by the fluorescence enhancement assay for DNA unwinding described by Kanter and Schwartz [33]. In this technique, DNA breaks are detected through the enhancement of the rate of alkaline denaturation of DNA, using the bisbenzamide fluorophore H33258 as a probe for residual double-stranded DNA after a fixed denaturation time. Cells were drug-treated for 60 min, centrifuged and resuspended to 10^6 cells/ml in ice-cold phosphate-buffered saline. Aliquots of this suspension were assayed in triplicate for residual double-stranded DNA after adding an equal volume of 0.1 M NaOH and allowing DNA to denature for 30 min at room temperature (group B samples). Three samples were treated similarly under non-denaturing conditions (group A) and three were sonicated for 5 s before denaturation to ensure complete unwinding (group C). Fluorescence intensities were determined using a Shimadzu RF-540 spectrofluorophotometer (excitation 351 nm, emission 451 nm). The fraction of residual double-stranded DNA, F, in group B samples after the fixed unwinding period was calculated using the relationship F = (B - C)/(A - C) where A, B, and C are the mean relative fluorescence intensities in group A, B, and C respectively.

Protein determination. Protein concentrations were determined according to Bradford [34].

Results

mAMSA cytotoxicity in proliferating and arrested 21-Fb cells

To determine whether arrested 21-Fb cells were less sensitive than their proliferating counterparts to mAMSA cytotoxicity, proliferating (at 39.5°C) and arrested (4 days at 33°C) cells were exposed to various drug concentrations for 60 min at their respective culture temperature and cell survival was assessed by colony formation in soft agarose. As can be seen in Fig. 1, arresting 21-Fb cells at 33°C decreased their sensitivity to mAMSA only slightly, increasing the C_{50} (the drug concentration needed to produce 90% cell killing) by 50% from 1.2 µM to 1.8 µM mAMSA. To confirm that the difference in drug sensitivity between proliferating and arrested 21-Fb cells was not due to the different incubation temperatures, we used wild-type K21 cells as a control. Both the proliferation characteristics and the mAMSA sensitivity
of residual duplex DNA to half the control value under the conditions of the assay. $C_{1/2}$ values for proliferating and arrested 21-Fb cells were 0.12 and 0.78 $\mu$M, respectively, indicating a more than 6-fold increase in the resistance of arrested cells. This value was in contrast to the 1.5-fold increase in drug resistance found in cell survival experiments.

**Stimulation of protein-DNA complex formation**

The mAMSA-induced formation of protein-DNA complexes with cellular DNA in intact and lysed cells was measured by the SDS/K+ precipitation assay (Fig. 3). A marked mAMSA dose-dependent stimulation of protein-DNA complex formation up to approx. 9-fold stimulation with 10 $\mu$M mAMSA was observed in proliferating cells, with little difference between intact and lysed cells. In contrast, stimulation of complex formation in arrested cells, after an initial steep increase paralleling that in non-arrested cells, reached saturation at approx. half the amount of stimulation found in proliferating cells with 10 $\mu$M mAMSA. This was followed by a slight drop in complex formation at higher drug concentrations in both arrested and
soluble extracts
tially
most 10-fold
presented
extracts
nuclear extracts
formation
further stimulation at
parable
between
stimulation was
stimulating proliferating cells. In contrast to the results with intact cells, lysed arrested cells exhibited a less than 2-fold stimulation of protein-DNA complex formation at 10 μM mAMSA, and little additional stimulation was observed at higher drug concentrations. Thus, the differences in mAMSA stimulation of protein-DNA complex formation between proliferating and arrested cells were comparable to the differences in DNA breakage, indicating a 6-fold increase in resistance to drug-mediated DNA damage in arrested cells.

Results of a similar experiment with nuclear extracts from proliferating and arrested cells using [35S] 3'-end-labelled DNA as substrate are presented in Fig. 4. With 10 μM mAMSA, stimulation of protein-DNA complex formation with nuclear extracts from proliferating cells was almost 10-fold greater than that obtained with nuclear extracts from arrested cells, with little further stimulation at higher drug concentrations. When the same experiment was performed with soluble extracts (see below), the result was essentially the same as with nuclear extracts. However, some stimulation was apparent in soluble extracts from arrested cells at and above 5 μM mAMSA. Thus, the differences in protein-DNA complex formation between soluble and nuclear extracts from arrested cells seemed to be similar to those between intact and lysed arrested cells.

Quantitation of DNA topoisomerase II activity

To determine whether there was any relationship between the state of proliferation and the sensitivity to mAMSA and DNA topoisomerase II activity in 21-Fb cells, we compared the strand-passing activities of nuclear extracts from proliferating and arrested cells. After preparing serial 2-fold dilutions of nuclear extracts from proliferating and arrested cells, a PM2 catenation assay was performed with each dilution to determine the amount of extract that contained 1 unit of topoisomerase II activity. The results of a typical experiment, presented in Fig. 5, show at least a 16-fold reduction in topoisomerase II activity in extracts from arrested cells (compare lanes 4 and 15). Essentially the same result was obtained in P4-unknotting assays (Fig. 6, compare lanes 5 and 8). The same differences in activity were also reflected in the specific activities of the nuclear

Fig. 3. Stimulation of protein-DNA complex formation in intact and lysed cells. mAMSA-stimulated formation of protein-DNA complexes was measured in intact and lysed 21-Fb cells that had been continuously grown at 39.5 °C or that had been arrested for 4 days at 33°C. Cellular DNA was pre-labelled with [3H]thymidine and protein-DNA complexes formed were precipitated by SDS/K+. •, intact cells grown at 39.5 ºC; ●, lysed cells grown at 39.5 ºC; ▲, intact cells arrested for 4 days at 33°C; ▼, lysed cells arrested for 4 days at 33°C. Values given are means ± S.E. from three independent experiments.

Fig. 4. Stimulation of topoisomerase II-DNA complex formation in nuclear and soluble extracts from proliferating or arrested 21-Fb cells. Stimulation by mAMSA of the topoisomerase II-DNA complex was determined using 50 ng 3'-end-labelled pBR322 DNA as substrate. ■, nuclear extract from cells grown at 39.5 °C; ●, nuclear extract from cells arrested for 4 days at 33°C; ●, soluble extract from cells grown at 39.5 °C; ▲, soluble extract from cells arrested for 4 days at 33°C. Values given are means ± S.E. from four experiments.
Fig. 5. Quantitation of nuclear DNA topoisomerase II activities by PM2 DNA catenation in nuclear extracts from proliferating or arrested 21-Fb cells. Nuclear extracts from proliferating and arrested cells were serially 2-fold diluted and with each dilution, a PM2 DNA catenation assay was performed. Reaction products were separated on 0.7% agarose gels, stained with ethidium bromide and photographed under ultraviolet light. Lane 1: PM2 DNA; (A) lanes 2–8: 2-fold-increasing amounts, as indicated, of nuclear extract from cells grown at 39.5°C; (B) lanes 9–15: 2-fold-increasing amounts, as indicated, of nuclear extracts from cells arrested for 4 days at 33°C. C, catenated network; OC, open circular; L, linear; S, supercoiled PM2 DNA.

Fig. 6. Quantitation of nuclear DNA topoisomerase II activities by P4 DNA-unknotting assays in nuclear extracts from proliferating or arrested 21-Fb cells. Nuclear extracts were serially 2-fold diluted and with each dilution, a P4 DNA-unknotting assay was performed. Reaction products were analysed as in Fig. 5. Lanes 1–7, 2-fold-decreasing amounts, as indicated, of nuclear extract from cells grown at 39.5°C; lanes 8–14, 2-fold-decreasing amounts, as indicated, of nuclear extract from cells arrested for 4 days at 33°C. Thus, the nuclear topoisomerase II activity in arrested 21-Fb cells was only approx. 5% of the activity in proliferating cells. Furthermore, the fact that the two different extracts used to measure catenation activity of 8.1 and 0.5 units/μg protein and to measure unknotting activity of 20 and approx. 1.25 units/μg protein for extracts from proliferating and arrested cells, respectively.
methods gave the same results, makes it unlikely that the observed reduction in activity was due to different extract composition (e.g., salt concentration), which might alter the assay specificity (e.g., catenation in low salt vs. decatenation in high salt).

When we lysed cells and separated the nuclei by centrifugation, the remaining soluble fraction contained ATP-dependent P4-unknotted and mAMSA-stimulated protein-DNA complex-forming activity with 3'-end-labelled DNA as substrate. Therefore, we concluded that this activity was a type II topoisomerase (5,29). To determine whether there was any difference in the relative amounts of soluble and nuclear topoisomerase II activities before and after cell-cycle arrest, we also assayed the P4-unknotted activity in the soluble fraction of proliferating and arrested cells. Soluble extracts from proliferating or arrested cells were serially 2-fold diluted and a P4-unknotted assay was performed with each dilution. As shown in Fig. 7, unknotted activity was clearly detected in soluble extracts from both proliferating and arrested cells. One unit of activity was obtained with 0.8 and 3.5 μg protein of extracts from proliferating and arrested cells, respectively (lanes 5 and 10), which corresponded to an approx. 4- to 8-fold decrease in soluble topoisomerase II activity in cell-cycle-arrested 21-Fb cells. This decrease was less than the reduction in enzyme activity in nuclear extracts, which raises the question of whether the two activities were due to different enzymes. Recently, Drake et al. [38] reported two distinct forms of topoisomerase II in mouse leukaemia cells and suggested that they had different sensitivities to mAMSA and were present in different relative amounts in sensitive and drug-resistant cells. Also, Heller et al. [39] found multiple forms of type II topoisomerase in Drosophila. At present, we do not know whether the soluble and nuclear activities in P815 cells are different. However, in initial attempts to isolate both enzymes by DEAE column chromatography, the two activities were eluted at different salt concentrations, suggesting that they might be different (B. Gallaher, unpublished results).

To determine whether the reduction in enzyme activity was caused by an inhibitor, we performed mixing experiments with extracts prepared from proliferating and arrested cells. When increasing amounts of extracts from arrested cells were added to the corresponding extracts from proliferating
cells and P4-unknotting and protein-DNA complex formation assays were performed, there was no evidence for the presence of an inhibitor in either nuclear or soluble extracts from arrested 21-Fb cells (data not shown). Therefore, it is likely that the reduction in topoisomerase II activity was due to a decrease in enzyme content, although inactivation due to enzyme modification cannot be excluded.

Discussion

The question of resistance of mammalian cells to mAMSA and other topoisomerase II-specific anticancer drugs has attracted considerable attention over recent years. Almost simultaneously, several authors suggested that the stabilisation of the topoisomerase II-DNA-cleaveable complex rather than the inhibition of the strand-passing reaction might be responsible for the cytotoxic action of these drugs [2-4] and that the pharmacological effects of the drugs are not due to the inactivation of topoisomerase II but are the consequence of converting the enzyme into a DNA-damaging agent [40]. Generally, a positive correlation between cytotoxicity, DNA damage and protein-DNA complex formation has been found [18-20,24,36,41], although some exceptions have been reported [21,23,42]. For example, Rowe et al. [5] found a direct correlation between in vitro cytotoxicity of a series of amsacrine derivatives and their potential to stimulate protein-DNA complex formation. Also, in a number of studies, only the drug-stimulated protein-DNA complex-forming activity and not the strand-passing activity of topoisomerase II was altered in drug-resistant cells [43-46]. Thus, there is considerable evidence to suggest an important role for the protein (topoisomerase II)-DNA complex in mAMSA-induced cell killing, although the exact mechanisms of drug action are still obscure.

To investigate further the role of DNA topoisomerase II and DNA damage in mAMSA-mediated cell killing, we used a cold-sensitive cell-cycle mutant of the P815 murine mastocytoma cell line, which upon incubation at the nonpermissive temperature, arrests in G1 and develops a differentiated, normal mast-cell-like phenotype [25-27]. In cell survival experiments, only a marginal decrease in mAMSA sensitivity was observed in arrested cells, which was similar to results of others with log- and plateau-phase L1210 cells [19], but contrasts with results in CHO cells [18,36]. While the exact reasons for these differences are not known, it is possible that differences between cell lines, e.g., their degree of malignancy, are important in determining sensitivity to anticancer drugs. The few comparative studies reported so far tend to suggest that less malignant cells become more resistant to anti-cancer drugs when entering quiescence or plateau-phase [18-21]. In agreement with this possibility, we found little change in drug sensitivity after cell-cycle arrest in our 21-Fb cells, a mutant of the highly malignant P815 cell line. On the other hand, it appears that the method used to arrest cells and the arrested phenotype do not influence drug sensitivity. Other studies in our laboratory have shown that arresting P815 cells with N\textsuperscript{6},O\textsuperscript{2}-dibutyryl cyclic AMP, by serum deprivation or using heat- or cold-sensitive cell-cycle mutants did not significantly affect the difference in drug sensitivity between proliferating and arrested cells (Hutchins, A.M., unpublished results), despite the fact that N\textsuperscript{6},O\textsuperscript{2}-dibutyryl cyclic AMP arrested and cold-sensitive arrested cells differentiated, while the others did not.

To determine whether the response of cells to mAMSA was reflected at the molecular level, we examined DNA breakage and cleavable complex formation. The marked reduction in DNA breakage in arrested cells after mAMSA treatment was paralleled by a similar reduction in mAMSA-stimulated protein-DNA complex formation. Since it was possible that the availability of mAMSA at its site of action, i.e., the topoisomerase II molecule, was altered in arrested cells, we also measured protein-DNA complex formation by lysed cells to circumvent possible plasma membrane-related side effects, such as altered drug uptake or efflux. These results confirmed the decrease in drug-stimulation of protein-DNA complex formation detected with intact cells after cell-cycle arrest. Therefore, we concluded that (i) altered intracellular drug concentrations were not the cause of the observed responses of arrested 21-Fb cells to mAMSA and (ii) that intact cells were not necessary for the formation of the protein-DNA complex. However, the fact that we were unable to
detect drug-induced protein-DNA complex formation in nuclei from P815 cells without adding a 30-fold excess of cytoplasmic extract, prepared by digitonin extraction of cells (see Materials and Methods) and which itself did not contain any detectable topoisomerase II activity (Darkin, S.J., unpublished results), suggests that some additional factor(s) might be involved in protein-DNA complex formation and possibly cell killing. We are currently investigating this observation further.

Recently, Zwelling et al. [21] suggested that the chromatin conformation might influence intercalator-induced protein-associated DNA cleavage, and Riou et al. [47,48] have shown that mAMSA stimulates DNA breakage preferentially in transcriptionally active, DNAase I-hypersensitive sites. Therefore, it is conceivable that in arrested cells, there are less potentially cleavable DNA sites for topoisomerase II to act. To eliminate the possibility that altered DNA influenced the results of our protein-DNA complex formation studies, we also used nuclear and soluble extracts with 3'-end labelled DNA as a uniform substrate for the complex-formation reaction. Again, the differences in mAMSA-stimulated protein-DNA complex formation between extracts from proliferating and arrested cells were similar to those with intact and lysed cells. Since the use of 3'-end labelled DNA as a substrate for the complex-formation reaction specifically detects topoisomerase II-DNA complexes [5], these results suggest that differences in the amounts of protein-DNA complex formed were due a reduction or alteration in topoisomerase II. Therefore, we measured the strand-passing activity in nuclear extracts by PM2 catenation and P4-unknotted assays. Both methods showed that there was an approx. 20-fold reduction in topoisomerase II activity when the cells became arrested, similar to the reduction in protein-DNA complex formation. Consequently, it is likely that the reduction of mAMSA-induced DNA breakage was due to lower enzyme activity present in arrested cells, although an alteration of topoisomerase II activity by enzyme modification, such as phosphorylation [49,50] or ADP-ribosylation [51,523] cannot be excluded. Alternatively, as yet unidentified additional factor(s) that modify the topoisomerase II-mAMSA interactions in arrested 21-Fb cells could be involved.

With the 21-Fb cells used in this study, there was a lack of correlation between the small increase in resistance to mAMSA in cell survival and the large decreases in sensitivity to DNA breakage and protein-DNA complex formation and in topoisomerase II activity when cells entered quiescence. Similarly, Chow and Ross [22] and Estey et al. [23] found no correlation between mAMSA-induced DNA cleavage and mAMSA-induced cell killing using synchronised Balb/c 3T3 or HeLa cells. They observed the lowest DNA cleavage frequency in late G1- and S-phase cells, but maximal cytotoxicity of mAMSA in early S-phase cells. Furthermore, there was only a small increase in cell survival of G1-phase cells as compared to asynchronously proliferating cells. It is possible that our 21-Fb cells arrest in G1 at or near the G1/S-phase border, with a concomitant low sensitivity to mAMSA-induced DNA breakage but significant sensitivity to mAMSA cytotoxicity, which might explain the poor correlation between cytotoxicity and DNA cleavage induced by mAMSA. It has recently been suggested [53,54] that the formation of cleavable complexes is an early step in a cascade of events eventually leading to cell death, and that the DNA breaks must be modified or elicit secondary lethal events to kill cells. If so, it is conceivable that the breaks in arrested 21-Fb cells, though fewer in number, are of a different quality, being more susceptible to modification by additional factors or events. For instance, it is possible that in arrested cells, topoisomerase II predominantly binds to actively expressed genes whose cleavage is lethal to the cell, while in proliferating cells, cleavable complex formation occurs also at many other nonessential sites. From this reasoning, it follows that the location of the cryptic DNA breaks formed in the cleavable complex may be more important than their actual formation. Alternatively, it is possible that the topoisomerase-mediated DNA breaks in arrested cells persist longer due to a delayed resealing, thus being more susceptible to secondary events, which turn them into lethal lesions. However, although we cannot completely exclude this possibility, given the fact that we do not know the precise nature of the cold-sensitive mutation, we consider this to be unlikely, because no difference in resealing of DNA breaks after drug removal was observed between log- and plateau-phase
CHO-AA8 cells [36] and in different phases of the cell cycle in HeLa cells [23]. In conclusion, our results confirm further the complexity of the mechanisms of drug-induced cell killing.

Acknowledgments

This work was supported by the Medical Research Council of New Zealand, the Cancer Society of New Zealand, the Auckland University Grants Committee and fellowships to E.S. from the NZUGC and the Swiss Cancer League and to S.J.D. from the Auckland Medical Research Foundation. We would also like to thank Dr. R. Schindler for kindly donating the temperature-sensitive P815 cell mutants, Drs. M.J. Waring and R. Morgan for providing the phage PM2 and Dr. R. Calendar for phage P4.

References


A protein factor that enhances amsacrine-mediated formation of topoisomerase II-DNA complexes in murine mastocytoma cell nuclei

Sandra J. Darkin and Raymond K. Ralph

Department of Cellular and Molecular Biology, University of Auckland, Auckland (New Zealand)

(Received 27 September 1988)

Key words: Amsacrine; Topoisomerase II-DNA complex; Topoisomerase II-DNA complex formation enhancing factor; Nucleus;
(Murine mastocytoma cell)

Extracts of K21 murine mastocytoma cells contain a factor that enhances formation of amsacrine-induced topoisomerase II-DNA complexes (PDCs) when added to isolated K21 nuclei. The PDC-enhancing activity is reduced in extracts from 2 or 6 h cycloheximide or cordycepin-treated cells, implying that continuous protein synthesis is required to maintain the factor. The factor is heat-labile, proteinase-sensitive and has other properties that distinguish it from the two known classes of topoisomerases. The data suggest that the factor is a labile protein with a molecular weight in excess of 50,000. This appears to be the first direct evidence of a protein factor that modulates drug-induced topoisomerase II action.

Introduction

DNA topoisomerase II (EC 5.99.1.3.) is the nuclear target of several anticancer drugs, including amsacrine (4′-(9-acridinylamino)methanesulphon-m-anisidide (mAMSA)), adriamycin, ellipticines and epipodophyllotoxins [1]. The drugs interfere with DNA breakage-rejoining by stabilising a cleavable complex between DNA and topoisomerase II [1,2]. The amount of drug-induced complex serves as a measure for drug activity [3] and the 'frozen' topoisomerase II-DNA complex is thought to be a key factor in drug-mediated cytotoxicity. Recently, a correlation between sister chromatid exchange and cytotoxicity was reported and attributed to the double-strand DNA breaks induced by anticancer drugs [4]. However, others have found no correlation between DNA breakage and cytotoxicity [5-8]. For example, in studies on amsacrine or etoposide-mediated cytotoxicity and DNA breakage in relation to cell cycle stage, Estey et al. [6] and Chow and Ross [5] found maximal cytotoxicity in S phase cells, whereas maximal DNA breakage occurred in G2/M phase cells concomitant with maximal topoisomerase II activity. These and other studies have led to the conjecture that stabilisation of the cleavable complex is an essential initial step in a cascade of events eventually leading to cell death, and Rowe et al. [3] have proposed that cleavable complexes are disrupted by additional factors which reveal cryptic DNA breaks and generate open double-strand breaks that are lethal and/or re-combinogenic. In support of the need for other factors, recent studies have shown that inhibiting protein synthesis with cycloheximide in Balb/C 3T3, CCRF-CEM and L1210 cells [7] and K21 mouse mastocytoma cells [9] diminishes the cytotoxic action of etoposide or amsacrine. Moreover, the cytoprotection conferred by cycloheximide was not a result of decreased topoisomerase II. Furthermore, prolonged treatment of proliferating splenocytes by etoposide and other topoisomerase II inhibitors induces DNA fragmentation by a mechanism which does not directly involve topoisomerase II [10].

Previously, we successfully used isolated CHO cell nuclei to examine the relationship between the cytotoxicity of anticancer drugs and topoisomerase II, with particular reference to drug resistance [11] and K21 murine mastocytoma cells to establish a cell-free system to study the relationship between the cytotoxic effects of topoisomerase II-directed anticancer drugs, topoi-
somerase II and drug-induced DNA breakage [8]. In contrast to results with CHO cells, we were unable to detect amssacrine-induced topoisomerase II-DNA complexes in the nuclei from K21 cells. We now report that a cytoplastic factor facilitates formation of amssacrine-induced topoisomerase II-DNA complexes (PDCs) in isolated K21 cells, and evidence is provided that the ‘PDC-enhancing activity’ resides in a protein distinct from topoisomerases I or II. Possible in vivo roles for such a factor and its relationship to amssacrine-mediated cytotoxicity are discussed.

Materials and Methods

Materials. Digitonin, Mops and dextran grade B 150-200 were from BDH. Enzymes immobilised on beaded agarose (bacterial protease type XXIV-A, 2 units per 2 ml packed gel; trypsin, from bovine pancreas, 78 units per ml packed gel, and papain, 825 units per g lyophilised solid), EGTA and proteinase inhibitors aprotinin, leupeptin, $\alpha_1$-macroglobulin, phenylmethylsulfonylfuoride (PMSF) and diisopropylfluorophosphate (DFP) were from Sigma Chemical Co., as was the protein synthesis inhibitor, cycloheximide. Dialysis membranes Spectropor 6 ($50000 M$, cut off) and standard Visking ($10000 M$, cut off) were from Spectrum Medical Industries Inc. and Serva, Heidelberg, F.R.G., respectively. RPMI 1640 and horse serum were from Gibco, N.Z. and [methyl-$^3$H]thymidine (70–90 Ci/mmol) was from Amersham. Amsacrine, provided as the isethionate salt by Dr. B.C. Baguley, University of Auckland Medical School, was stored as a 1 M stock solution in water at $-20^\circ$C from which working dilutions were made immediately prior to use.

Cell culture A clonal subline (K21, wild type) of the P815-X2 mouse mastocytoma line was kindly provided by Dr. R. Schindler, University of Berne, Switzerland. The cells were grown in RPMI 1640 medium, supplemented with 10% horse serum in a 5% CO$_2$/95% air atmosphere and subcultured as recommended [12].

Preparation of cytoplasmic extracts. Cytoplasmic extracts were prepared by digitonin extraction of log phase K21 cells as described by Blank-Liss and Schindler [13], but including the proteinase inhibitors aprotinin, 400 units/ml; leupeptin, 0.1 mg/ml; $\alpha_1$-macroglobulin, 0.01 mg/ml; PMSF, 1 mM and DFP, 0.1 mg/ml. Resulting extracts were diluted to 2.5 mg protein/ml and stored in 100 $\mu$m aliquots at $-90^\circ$C.

Quantitation of amssacrine-stimulated covalent protein-DNA complex formation. The SDS/K$^+$ precipitation assay described by Rowe et al. [3] was used to measure the drug-induced factor-enhanced formation of protein-DNA complexes (PDC) in isolated nuclei. Radioactive labelling of the DNA of proliferating K21 cells and conditions for cell lysis were essentially as described by Schneider et al. [8]. However, nuclei were collected by centrifugation at 1200 $\times$ g for 10 min at 4°C then resuspended in nuclei buffer at $5 \times 10^7$/ml. An aliquot of nuclei was examined with a microscope and the number of nuclei was measured using a haemocytometer. To induce drug-stimulated factor-enhanced PDC formation, 10$^5$ nuclei were added to the following reaction mixture in 96-well plates in a total volume of 50 $\mu$l/well: 50 mM Tris-HCl (pH 7.5); 10 mM MgCl$_2$; 120 mM KCl; 5 mM EGTA; 2.25 mM EDTA; 0.5 M dithiothreitol; 30 $\mu$g/ml bovine serum albumin; 2 mM ATP with or without 10 $\mu$m amssacrine; 0–100 $\mu$m of cytoplasmic extract, followed by incubation for 10 min at 37°C. The nuclei were then collected by centrifugation at 1200 $\times$ g for 10 min at room temperature. Protein-DNA complexes were precipitated with SDS and K$^+$ and quantitated as previously described [8]. The total acid-precipitable radioactivity per assay (i.e., 10$^5$ nuclei) was routinely found to be approx. 5 $\times$ 10$^4$ cpm.

It was necessary to determine optimal amounts of each cytoplasmic extract ($\mu$m protein); therefore, a concentration range (0–100 $\mu$m protein) was tested for the ability to enhance mAMSA-stimulated PDC formation. The amount of extract ($\mu$m protein) that produced maximal protein-DNA complex formation was defined as 1 unit.

In each experiment, the stimulation of PDC formation in whole cells by 10 $\mu$m amssacrine was determined as an internal control. This was also performed in a 96-well plate; however, cells were distributed in 49 $\mu$l aliquots (10$^4$ cells) in fresh growth medium. Conditions of drug incubation and the precipitation of PDCs were as described for isolated nuclei. A 10–12-fold stimulation of PDCs in whole cells was routinely achieved with 10 $\mu$m amssacrine.

Dialysis of cytoplasmic extracts through selective membranes. In one series of experiments, K21 cytoplasmic extracts were dialysed against 2000 vol. of 1.1-fold concentrated reaction buffer for 6 h at 4°C using selective membranes with 10 and 50 kDa molecular mass cut-offs. Dialysed extracts were then tested for PDC formation-enhancing activity.

Heat inactivation of cytoplasmic extracts. The thermal stability of the PDC-enhancing component of K21 cytoplasmic extracts was tested by heating extracts at 65 or 95°C for 0–20 min. Heat-treated extracts were subsequently tested for ability to enhance formation of drug-stimulated PDC in isolated nuclei.

Treatment of cytoplasmic extracts with immobilised enzymes. The gels with immobilised bacterial proteinase, trypsin or papain were washed several times with water and then with extract buffer (without digitonin and proteinase inhibitors) according to the manufacturer’s instructions. For enzymatic digestion, undiluted cytoplasmic extracts (freshly prepared without proteinase inhibitors) were incubated with the enzyme gels at 30°C for 2 h with constant gentle agitation. As con-
controls, extract buffer was also incubated with the gels. The extracts or extract buffer were then separated from the gels by centrifugation. The protein concentration of each extract was determined and the extracts were diluted to 2.5 mg/ml protein with non-enzymatically treated extract buffer containing the proteinase inhibitor cocktail described above. Proteinase inhibitors were also added to the extract buffer that had been incubated with the enzyme gels. Both the extracts and the control buffers (enzyme-treated) were stored at -90 °C prior to assay.

**Determination of protein concentration** Protein concentrations were determined according to Bradford [14].

**Results**

**Facilitation of amscarcine-induced PDC formation in isolated nuclei by cytoplasmic extracts**

When protein-DNA complexes (PDCs) in K21 cells and nuclei were measured by the SDS/K+ precipitation assay, there was routinely an approx. 10-fold stimulation of PDC formation in whole cells with 10 μM amscarcine, whereas there was less than or equal to 3-fold stimulation of PDCs in isolated nuclei at equivalent or higher drug concentrations (Fig. 1). Attempts to increase PDC formation in nuclei by altering the assay conditions (e.g., pH, ion composition of buffers, period of drug incubation, nuclei density and cell lysis conditions) were unsuccessful, although it was observed that fully complex-forming activity was lost im-

![Fig. 1](image1.png)

**Fig. 1.** The effect of cytoplasmic extracts on amscarcine-induced protein-DNA complex formation in isolated nuclei. Protein-DNA complexes induced by 10 μM amscarcine in isolated K21 nuclei were measured by SDS/K+ precipitation. Cytoplasmic extracts and reaction conditions are described in Materials and Methods. Cellular DNA was prelabelled with [3H]thymidine. K21 nuclei with 0–100 μg cytoplasmic protein. K21 nuclei with 0–100 μg cytoplasmic protein. K21 nuclei with 0–100 μg cytoplasmic protein. K21 nuclei with 0–100 μg cytoplasmic protein. The amount of each extract (μg protein) that produced maximal PDC formation was defined as 1 unit.

![Fig. 2](image2.png)

**Fig. 2.** The effect of inhibiting protein synthesis on PDC-enhancing activity. Cytoplasmic extracts were prepared from K21 cells grown for 2 or 6 h with or without 10 μg/ml cycloheximide. The enhancement of amscarcine induced topoisomerase II-DNA complexes in K21 nuclei by extracts (0–40 μg protein) from untreated and cycloheximide-treated cells was determined by SDS/K+ precipitation. a, nuclei with untreated cell extract; b, nuclei with extract from cells grown for 2 h with cycloheximide; d, nuclei with extract from cells grown for 6 h with cycloheximide.

mediately after disrupting the plasma membrane with detergents or by freeze-thawing. This suggested that a cytoplasmic or membrane component might be necessary for nuclei to achieve the levels of PDC formation obtained with intact K21 cells. Fig. 1 shows that the addition of cytoplasmic extract to isolated K21 nuclei restored the level of PDC formation induced by 10 μM amscarcine to that of whole cells. A peak of PDC-enhancing activity was consistently obtained with 20–30 μg of cytoplasmic protein (e.g., Figs. 1 and 2) and the amount of each extract (μg protein) that produced maximal PDC formation was defined as 1 unit.

To exclude the possibility that the PDC-enhancing activity was an alternative form of topoisomerase II such as described by Schneider et al. [8] or Drake et al. [15], cytoplasmic extracts were tested for topoisomerase II DNA-binding activity using [3H]-3'-end labelled pBR322 DNA as substrate as described by Liu et al. [16] and topoisomerase II strand-passing activity using the P4 unknotted assay [17]. No topoisomerase II activity was detected in any cytoplasmic extract prepared with cytoplasmic digitonin, although these assays readily detected topoisomerase II activity in more dilute extracts prepared with buffers containing higher salt concentrations and Triton X-100 [8].

Topoisomerase I was not responsible for the PDC-enhancing activity in cell extracts because topoisomerase I activity is not increased by amscarcine [18]. Furthermore, topoisomerase I produces protein com-
plexes with nicked DNA in the absence of drugs and nicks in DNA produced during nuclei isolation [19] would have provided the appropriate substrates. However, there was no stimulation of PDC formation by cytoplasmic extracts added to isolated nuclei in the absence of amascarine and the radioactive DNA precipitated by SDS/K⁺ with non-drug treated nuclei in the presence or absence of cytoplasmic extract was routinely approx. 1% of the total radioactive DNA in the nuclei, which was the same as the background obtained with untreated whole cells.

The PDC-enhancing factor showed a striking decrease in activity with increasing concentrations of the cytoplasmic extract. Because cytoplasmic extracts prepared with or without proteinase inhibitors showed identical activity profiles, this effect did not appear to be due to proteolysis of topoisomerase II unless the proteinase was not affected by the potent inhibitor cocktail employed. Moreover, a corresponding decrease in PDC formation in non-drug-treated controls did not occur at high extract concentrations (data not shown). However, competition between proteins at high extract concentrations might explain the decrease in PDC-enhancing activity. For example, the factor could modulate topoisomerase II by phosphorylation, which is negated by phosphatases in high concentrations of cytoplasmic extract.

The inclusion of proteinase inhibitors during nuclei isolation and in the assay reaction mixture did not affect the amascarine-induced increase in PDC formation with or without cytoplasmic extract (data not shown). Therefore, reconstruction of full PDC formation in K21 nuclei by cytoplasmic extracts was not due to the proteinase inhibitors in the added extracts.

The effect of inhibiting protein synthesis on cytoplasmic PDC-enhancing activity

To determine whether inhibiting protein synthesis affected the PDC-enhancing activity, cytoplasmic extracts were prepared from K21 cells before and after treating the cells for 3 or 6 h with 10 μg/ml cycloheximide. The extracts were then tested for ability to enhance amascarine-induced PDC formation in isolated K21 nuclei. As shown in Fig. 2, inhibition of protein synthesis substantially reduced PDC-enhancing activity, with reductions in PDC formation to approx. 40 or 20% that of the untreated extract after 2 and 6 h cycloheximide treatment, respectively. Since cycloheximide has previously been shown not to affect the assay of topoisomerase II [9], these results suggested that the PDC-stimulating activity was associated with a labile protein, with a half-life of 2 h or less.

Physicochemical characterization of the cytoplasmic PDC-enhancing activity

The properties of the PDC-enhancing activity in cytoplasmic extracts from proliferating K21 cells was investigated in a preliminary characterization.

The cytoplasmic PDC-enhancing activity was rapidly lost at 65 and 95°C. At 65°C, only 40% of the activity...
remained after 30 min, and all activity was lost within 2 h, whereas incubation at 95 °C caused a more rapid loss of activity, with less than 50% remaining after 1 min (Fig. 3). The activity was retained by size-selective dialysis membranes with molecular mass cut-offs of 10 and 50 kDa, indicating that it was associated with material above 50,000 molecular weight (data not shown).

Treatment of extracts with immobilised proteinase destroyed the PDC-enhancing activity. Fig. 4 shows that proteolytic digestion of cytoplasmic extracts with trypsin or papain reduced the activity to the basal 2-fold increase seen with nuclei alone. The bacterial proteinase XXV-A was less effective, reducing the activity by 54%. Control experiments showed that the loss of PDC-enhancing activity from enzymatically treated extracts was not due to digestion of topoisomerase II per se in the assays by contaminating proteinase leached from the immobilised enzymes (Fig. 4). These properties all suggest that the PDC-enhancing activity is a protein.

Discussion

We have used cell-free systems and isolated nuclei to examine the relationship between the cytotoxic effects of topoisomerase II-directed anticancer drugs on cells and topoisomerase II [8,11]. However, only a low level of amascrine-induced topoisomerase II-DNA complexes was detected in isolated K21 cell nuclei [8]. As shown above, a factor present in cell extracts with diglottonin restored drug-induced PDC formation in K21 nuclei to the level induced in whole cells. Since diglottonin extraction of cells releases cytosolic proteins without releasing lysosomal or mitochondrial enzymes [20], the PDC-enhancing activity is presumably located in the cytoplasm, although leakage from nuclei cannot be excluded. Treating cells with cycloheximide prior to preparing extracts substantially reduced the PDC-enhancing activity. Moreover, qualitatively similar results were achieved with 10 μM corycycin (data not shown), indicating that continuous RNA and protein synthesis was required to maintain the enhancing factor. In view of its properties, the possibility that the factor was a type I or II topoisomerase was eliminated. A preliminary physicochemical characterisation of the factor showed that it was sensitive to heat, non-dialysable and destroyed by proteinases. Together, these results indicate that the PDC-enhancing activity resides in a labile cytoplasmic protein in excess of 50,000 molecular weight that is distinct from the two known classes of eukaryotic topoisomerases.

Consideration of the in vivo role of the PDC-enhancing factor must take into account its implied association with topoisomerase II. Specific antibodies have revealed that topoisomerase II is a prominent component of mitotic chromosome scaffolds and the interphase nuclear matrix [21–23]. The enzyme is located at the base of chromatin loops within metaphase chromatids [24] and recent studies have defined a very specific class of DNA sequence that may mediate loop anchorage. Moreover, these sequences contain consensus topoisomerase II binding sites [25] and they reside close to known enhancers [26]; therefore, topoisomerase II may be involved in DNA replication, transcription or recombination, and drug-induced DNA cleavage at such sites could have potentially lethal consequences for cells.

Various workers have suggested that there are two pools of topoisomerase II, representing different functional states or isoforms [8,15,21,27], and which may have different sensitivities to amascrine. It has been proposed that one form, the matrix- or scaffold-associated enzyme, when bound tightly to DNA loses its DNA strand-passing catalytic function, whereas the second non-matrix bound or 'soluble' form produces topoisomerase and forms readily reversible complexes with DNA. We propose a topoisomerase II-modulating function for the factor in K21 cells that converts topoisomerase II to a drug-sensitive PDC-forming state in vivo, possibly by affecting its action on, or loosening its association with, the nuclear matrix or scaffold. This is supported by the release of PDC-forming activity when normally inactive K21 nuclei are extracted with salt [8]. As a consequence of factor depletion during nuclei isolation, only a fraction of the potential drug-induced PDCs in whole cells would be expected in isolated nuclei, as seen with K21 cell nuclei (Fig. 1) and in L1210 cell nuclei when measured by alkaline elution [8,19,28–30]. Therefore, the fraction of PDCs produced in isolated nuclei may reflect the residual factor that activates matrix-associated topoisomerase II and the different proportions of PDC formation obtained with nuclei isolated from different cells may reflect the amount of factor retained using different isolation methods [11,31 cf. 8,19,28–30]. The topoisomerase II modulating factor may normally play a role in DNA replication as part of a replisome complex [32] or in transcription or recombination.

Topoisomerase II has been shown to be modified by protein kinase C [33], casein kinase II [34] or ADP-ribosylation [35]; however, ours is the first direct evidence of a protein that stimulates drug-induced topoisomerase II action. Further elucidation of the nature of the factor and its association with topoisomerase II should facilitate greater understanding of its in vivo function and the control of topoisomerase II. Purification of the factor is in progress.

Acknowledgments

This work was supported by the Medical Research Council of New Zealand, the Cancer Society of New Zealand and a senior scholarship to S.J.D. from Auck-
land Medical Research Foundation. We thank Dr E. Schneider for helpful discussions.

References


Cell Line Selectivity and DNA Breakage Properties of the Antitumour Agent N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide: Role of DNA Topoisomerase II

ERASMUS SCHNEIDER,* SANDRA J. DARKIN,** PENEOLOPE A. LAWSON,† LAI-MING CHING, RAYMOND K. RALPH† and BRUCE C. BAGULEY‡

†Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand and Cancer Research Laboratory, University of Auckland School of Medicine, Auckland, New Zealand

Abstract—N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide (NSC 601316) is a DNA intercalating experimental antitumour agent which is curative against the Lewis lung carcinoma in mice. Its action has been compared with ansacrine, its inactive isomer oAMSA, the solid tumour active derivative CI-921 (NSC 343499), a C-6 methylene chain-linked bisacridine (NSC 210733), 9-aminoacridine and quinacline. All compounds inhibited the unknotting of phase P4 DNA by topoisomerase II in nuclear extracts prepared from L1210 cells. NSC 601316 inhibited growth of cultured L1210, P388, P/AMSA (P388 resistant to ansacrine) and P/ACTD (resistant to actinomycin D) cell lines at concentrations of 87, 150, 2020 and 150 nM respectively. A 1 h drug exposure to 0.85 μM NSC 601316 killed 50% of L1210 cells. L1210 cells treated for 1 h with NSC 601316 accumulated DNA breaks and protein-DNA cross-links. There was a good correlation between DNA breakage and cytotoxicity, but the relationship between drug concentration and number of protein-DNA cross-links was non-linear and differed from that of ansacrine and CI-921. There was also a positive correlation between the degree of cross-resistance of P/AMSA cells (which have altered topoisomerase II function) and ability to induce DNA breakage or protein-DNA complexes. The results suggest that topoisomerase II is the target of action of NSC 601316.

INTRODUCTION

ACRIDINECARBOXAMIDE (N-[2-(dimethylamino)ethyl]acridine-4-carboxamide; NSC 601316; see Fig. 1 for structure), was recently synthesized in the Cancer Research Laboratory [1]. It differs from 9-anilinoacridine derivatives such as mAMSA [2] and its derivative CI-921 [3] in several important respects. While mAMSA has only marginal antitumour activity in vivo against the Lewis lung carcinoma growing as lung nodules in mice [3], acridinecarboxamide cures approx. 90% of mice when treatment is commenced 5 days after inoculation of the tumour [1]. It is even more active against this tumour than CI-921, which has been selected for clinical trial on the basis of its solid tumour activity.

Acridinecarboxamide is more lipophilic than mAMSA, and in contrast to mAMSA the ionization of its acridine nitrogen is almost completely suppressed [1]. It binds to double-stranded DNA by intercalation with an association constant which is similar to that of CI-921 [1]. The side-chains of acridinecarboxamide and mAMSA project in opposite directions from the acridine nucleus and may thus occupy different grooves when bound to DNA. Kinetic studies using stopped flow techniques have shown that the rate of dissociation of acridinecarboxamide from DNA intercalation sites is much slower than that of mAMSA ([4] and Dr W.A. Denny, personal communication).

As a further step towards understanding the basis for the high activity of acridinecarboxamide towards solid tumours, its mode of action and cell line selectivity have been compared with those of several other acridine derivatives (see Fig. 1 for structures). mAMSA is in clinical use for the treatment of acute
leukaemia [5] and is a potent and specific inhibitor of the enzyme DNA topoisomerase II [6]. oAMSA, an isomer of mAMSA, lacks antitumour activity and is correspondingly less active in vitro against topoisomerase II [6] while CI-921 is a potent inhibitor of this enzyme [7]. C-6-bisacridine was selected as a bifunctional intercalator with experimental antileukaemia activity [8]. 9-aminoacridine (an antibacterial) and quinacrine (an antimalarial) were also included for comparison.

**MATERIALS AND METHODS**

**Materials**

Methyl[^3]H]thymidine (70-90 Ci/mmol) was obtained from Amersham and [32P]dATP (800 Ci/mmol) from New England Nuclear. Phage P4 was a generous gift from Dr. R. Calendar, Department of Molecular Biology, Berkeley, California. The restriction enzyme EcoRI was obtained from Promega Biotec and DNA Pol I Klenow fragment from Boehringer Mannheim. mAMSA (isethionate salt) was provided by the Warner-Lambert Company, and 9-aminoacridine hydrochloride and quinacrine hydrochloride were obtained from the Sigma Chemical Co. oAMSA methanesulfonate, CI-921 isethionate, and C-6-bisacridine dihydrochloride were synthesized in the Cancer Research Laboratory, Auckland. Drug solutions for DNA studies were stored as 1 mM stock solutions in water at -20°C, and working dilutions were made immediately prior to use. Dextran grade B (MW 150-200 K) was obtained from BDH, proteinase K from Sigma Chemical Co., and Hoechst 33258 from Calbiochem Biochemicals.

**Cell lines**

P388 mouse leukaemia cells were from the National Cancer Institute, U.S.A. P/ACTD (resistant to actinomycin D) cells were from Mason Research Inc., Worcester, U.S.A. The P/AMSA line originated in Dr. R. Johnson’s laboratory [9] and was provided by Dr. R.C. Jackson, Warner-Lambert Co., Michigan, U.S.A. L1210 cells were from Arthur D. Little Inc., U.S.A. Lines were stored in liquid nitrogen and propagated in carrier DBA/2J mice before establishing in culture.

**Growth inhibition assays**

These were performed in 24-well trays. Leukaemia cells were grown in RPMI 1640 medium supplemented with 50 μM 2-mercaptoethanol, FBS (10%) and antibiotics (streptomycin 100 μg/ml; penicillin 100 U/ml). Drugs were added after 2 h, the cultures harvested after a further 3 days [10]. In experiments with mAMSA and CI-921, the RPMI 1640 medium was supplemented with 50 μM ascorbate to prevent aerial oxidation of the drug [10]. Other compounds were resistant to oxidation. The IC₅₀ was defined as the drug concentration required to reduce the number of cells in a culture by 50% with respect to control cultures.

**Clonogenicity assays**

L1210 cells were cultured at 5 × 10⁴ cells/ml in αMEM supplemented with FBS (10%) and 50 μM 2-mercaptoethanol. The cells were used after 24 h of culture, when the density was 1.5-2.0 × 10⁵ cells/ml. Cells were incubated with drug in growth medium (10 ml) at 37°C for 1 h. Cells were then centrifuged, washed once, counted and plated in 1% methylcellulose in growth medium. Colony growth was determined after incubation at 37°C for 5 days [11]. The IC₅₀ was defined as the drug concentration required to reduce the number of surviving clonogenic cells in a culture by 50% with respect to control cells.

**Phage P4 DNA unknotting assay**

Logarithmic-phase L1210 cells were used for the preparation of nuclei essentially as previously described [12] but using 0.1% Triton X for cell lysis and omitting Ca²⁺ from all nuclei isolation buffers. Topoisomerase II activity was recovered from the nuclei after destabilization of the scaffold with 2-mercaptoethanol and EDTA, sonication, precipitation of nucleic acids with polyethylene glycol and centrifugation at 30,000 g for 30 min.
[12]. Knotted phase P4 DNA was prepared as described [13]. Assay mixtures (20 μl) contained 50 mM Tris–HCl, pH 7.5; 10 mM MgCl₂; 0.5 mM EDTA; 0.5 mM dithiothreitol; 30 μg/ml bovine serum albumin; 1 mM ATP; 0.24 μg P4 DNA and varying amounts of drugs. After addition of 4 μl of nucleofect extract (approx. 1 unit of topoisomerase activity, 20–40 ng protein and 120 mM KCl), tubes were incubated for 30 min at 37°C. To remove proteins, 1 μl 10% sodium dodecyl sulphate (SDS) and 1 μl 10 mg/ml protease K were added and the mixture was incubated for a further 30 min at 37°C. Samples were electrophoresed in 0.7% agarose gels in 89 mM Tris–borate, pH 7.5; 89 mM boric acid; 2 mM EDTA. Gels were stained with ethidium bromide and photographed under u.v. illumination. Photographic negatives were analysed by scanning on a microdensitometer (Joyce, Loebi and Co.). One unit of topoisomerase II activity was defined as the amount of extract that completely unknotted 0.24 μg phase P4 DNA in the absence of drug. Protein concentrations were determined according to Bradford [14].

Quantitation of mAMSA-stimulated covalent protein–DNA complex formation

The SDS/K⁺ precipitation assay [15, 16] was used to measure drug-induced formation of protein–DNA complexes in whole cells and nuclear extracts. For studies with whole cells, log-phase L1210 cells were labelled with 0.5 μCi/ml [methyl-³H]thymidine (70–90 Ci/mmol) overnight, collected by centrifugation and washed once with PBS (137 mM NaCl; 2.6 mM KCl; 8 mM Na₂HPO₄; 1.4 mM KH₂PO₄). Cells were resuspended in fresh growth medium at 1 × 10⁶ cells/ml, distributed in 1 ml aliquots in 24-well multiwell plates and incubated for 1 h at 37°C. They were then centrifuged at 1200 g for 10 min at room temperature, the supernatants discarded, the cells lysed with SDS and protein–DNA complexes collected as previously described [12]. Protein–DNA complexes were resuspended in 1 ml ice-cold 10 mM Tris–HCl, pH 7.5; 100 mM KCl; 2 mM EDTA and collected on Whatman GF/C filters under gravity. Filters were washed five times with wash solution under vacuum, dried and the retained radioactivity measured in a liquid scintillation spectrometer. The total acid-precipitable radioactivity per assay (10⁵ cells) was routinely 5–7 × 10⁶ cpm.

DNA breakage

DNA breakage induced by the acridine drugs in L1210 cells was determined by the fluorimetric assay of DNA unwinding (FADU) method [17]. Cells were drug treated for 60 min, centrifuged and resuspended to 10⁶ cells/ml in ice-cold PBS. Aliquots of this suspension were assayed in triplicate for residual double-stranded DNA after adding an equal volume of 0.1 M NaOH and allowing DNA to denature for 30 min at room temperature (group B samples). Three samples were treated similarly under non-denaturing conditions (group A) and three were sonicated for 5 s before denaturation to ensure complete unwinding (group C). Fluorescence intensities were determined using a Shimadzu RF-540 spectrofluorimeter (excitation 351 nm, emission 451 nm). The fraction of residual double-stranded DNA, F, in group B samples after the fixed unwinding period was calculated using the relationship \( F = (B-C)/(A-C) \) where A, B and C are the mean relative fluorescence intensities in groups A, B and C respectively.

RESULTS

In vitro cytotoxicity of acridine derivatives in survival assays

Growth inhibition tests using a panel of cultured mouse cell lines are shown in Table 1. Acridine carboxamide was considerably less toxic potent than mAMSA and CI-921 against L1210 and P388 lines. P/AMSA cells, were highly cross-resistant to acridinecarboxamide and CI-921, slightly cross-resistant to oAMSA, and slightly collaterally sensitive to C6-bisacridine, 9-aminoacridine and quinacrine. P/ACTD cells were cross resistant to C-

![Table 1. In vitro growth inhibitory activity and cytotoxicity of acridine derivatives](image-url)
6 bisacridine but not to acridine carboxamide, mAMSA, oAMSA or CI-921 (Table 1).

The cytotoxicity of antitumour acridines was also compared following a short term incubation. Logarithmic phase L1210 cells were exposed to drug for 1 h, then washed free of drug and assayed for ability to form colonies in vitro. D_{50} values are shown in Table 1. D_{10} values (10% survival) were approx. 3-fold higher than D_{50} values, which were in turn 5-10-fold higher than the D_{50} values determined for continuous exposure.

**Effect of acridinecarboxamide and related compounds on DNA strand breakage**

In order to determine the relative capacity of acridine derivatives to cause DNA breakage, a FADU assay was used which has recently been shown to give the same information as alkaline elution techniques [18, 19]. DNA breaks were detected in L1210 cells following a 1 h drug exposure by enhancement of the rate of alkaline denaturation of DNA, using the biotbenzamide fluorophore Hoechst 33258 as a probe for residual double-stranded DNA after a fixed denaturation time. Acridine carboxamide was less potent than mAMSA or CI-921 at high drug concentrations, consistent with the D_{50} results (Fig. 2). However, at low drug concentrations acridinecarboxamide produced almost as much DNA breakage as did mAMSA and CI-921. oAMSA had a slight effect and the other compounds produced little or no DNA breakage.

![Graph](image)

**Stimulation of protein–DNA complex formation**

Acridine derivatives were compared in the extent to which they induced protein–DNA complex formation in whole L1210 cells in response to varying amounts of drug after a 1 h treatment (Fig. 3). As in the DNA breakage assay, only acridinecarboxamide, mAMSA, CI-921 and to a small extent oAMSA induced dose-dependent protein–DNA complex formation. Acridinecarboxamide was unusual in that it induced a higher rate of protein–DNA complex formation than mAMSA at low drug concentrations (1 μM induced as much complex as 5 μM mAMSA) but there was a gradual decline in complex formation at higher drug concentrations and no stimulation at all at 20 μM. An experiment using nuclear extracts from L1210 cells and [32P]3'-end-labelled pBR322 DNA [12] produced the same overall pattern as that observed in whole cells except that the stimulation of protein–DNA complex formation by acridinecarboxamide occurred at higher drug concentrations and over a wider concentration range than with whole cells (results not shown).

**Inhibition of topoisomerase II**

To test the effect of the compounds on topoisomerase II activity, drugs were incubated with nuclear extract in a P4 unknotting assay and analysed by gel electrophoresis (Fig. 4). The minimal concentrations of drug inhibiting topoisomerase II P4 unknotting activity were: acridinecarboxamide, 50 μM; mAMSA, 20 μM; CI-921, 20 μM; oAMSA, 100 μM; C6-bisacridine, 20 μM; 9-aminoacridine, 20 μM; quinacrine, 50 μM.
Fig. 4. Drug effects on the P4 unknotting activity in nuclear extracts. The minimal amount of nuclear extract from L1210 cells required for complete unknotting of 0.24 μg th P4 DNA (1 unit) was used in an unknotting assay with varying amounts of drug (0–100 μM) added as indicated. Reaction products were separated on a 0.7% agarose gel, stained with 0.5 μg/ml ethidium bromide and photographed under u.v. illumination.
DISCUSSION

The effects of acridinecarboxamide on DNA breakage (Fig. 2) and on the formation of protein–DNA complexes (Fig. 3), together with the cross-resistance of acridinecarboxamide to P/AMSA (Table 1), a cell line with altered topoisomerase II activity [9], support the proposal that topoisomerase II is the likely cytotoxic target of acridinecarboxamide. A clear relationship is apparent for the acridine derivatives in Fig. 1 between the degree of cross-resistance to the P/AMSA cell line (as shown by the ratio of IC₅₀ values) and the induction of DNA breakage as measured by the FADU assay. Acridinecarboxamide, mAMSA and CI-921 show good activity and oAMSA shows a small effect while C6-bisacridine, 9-aminoacridine and quinacrine are inactive. In contrast to the P/AMSA results, acridinecarboxamide shows no cross-resistance to P/ACTD which is cross-resistant to vincristine and has the characteristics of a multidrug resistant line [20]. It is possible this property, in addition to factors such as drug distribution [10], explains the high activity of acridinecarboxamide against solid tumours.

There is an obvious difference between acridinecarboxamide and mAMSA in the dose dependence of formation of DNA–protein complexes (Fig. 3). The IC₅₀ concentration (0.85 μM) of acridinecarboxamide induces an 11-fold increase in complex formation, while equitoxic concentrations of mAMSA (0.046 μM) or CI-921 have no significant effect. In contrast, these concentrations cause similar DNA breakage (30–40% reduction in fluorescence) for all three drugs. The reason for the inhibition of drug-stimulated formation of protein–DNA complexes by high concentrations of acridinecarboxamide (Fig. 3), which is similar to that reported for 2-methyl-9-hydroxyellipticine [21], has not been elucidated.

Our results extend those of Rowe et al. [16] who showed that the capacity of mAMSA and other acridine-derived topoisomerase II directed anticancer drugs to cause DNA breakage was related to cytotoxicity as measured by IC₅₀ assays. It is clear that the correlation between cytotoxicity and the capacity to introduce DNA breaks is not a universal feature of acridine derivatives, since C6-bisacridine has been reported to have biological activity [8]. Markovits et al. [22] have suggested a different mechanism of action for the anticancer agent ditercalium, which is also a bifunctional intercalator, and this mechanism may also apply to C-6 bisacridine.

In agreement with others [6] we find no correlation between the drug concentrations needed to inhibit DNA strand-passing activity (Fig. 4) and IC₅₀ or IC₉₀ values. For instance, 9-aminoacridine has a much lower cytotoxic activity but shows the same inhibition of topoisomerase II as mAMSA in the phase P4 unknotting assay. A qualitatively similar result has also been obtained using a cationisation assay for the measurement of topoisomerase II activity (A.M. Hutchins and R.K. Ralph, unpublished). Formation and stabilization of the cleavable complex by topoisomerase II, rather than inhibition of its formation, appears to be related to DNA strand breakage and cell death.

In conclusion, although acridinecarboxamide has been demonstrated to target the enzyme topoisomerase II, it differs from 9-anilinoacridine derivatives typified by CI-921 and mAMSA in being less potent both in vitro (Table 1) and in vivo [1]. This is surprising since acridinecarboxamide has a similar DNA binding constant to that of CI-921 [1, 3] and slightly higher potency than CI-921 or mAMSA in stimulating the formation of protein–DNA complexes (Fig. 3). It is notable that acridinecarboxamide, like its 9-amino derivative [4], lacks the presence of easily and reversibly oxidizable functions such as the phenolic or anilino groups which are found in mAMSA, CI-921 [23, 24], the anthracyclines, ellipticines, epipodophyllotoxins and anthracyclines. It is possible that the chemical reactivity of these topoisomerase II-directed cytotoxic drugs contributes an additional mechanism of toxicity, not shown by acridinecarboxamide, by which DNA breakage is induced at drug concentrations which cause only low amounts of protein–DNA complex formation. If this is the case, then acridine carboxamide, by not having this second mechanism, may belong to a distinct class of topoisomerase II-directed cytotoxic agent.

Acknowledgements—The authors are grateful to Linley Fray for IC₅₀ determinations, to Margaret Snos for help with the manuscript.

REFERENCES


Mechanism of resistance of non-cycling mammalian cells to 4'-[9-acridinylamino]methanesulphon-m-anisidide: role of DNA topoisomerase II in log- and plateau-phase CHO cells

Erasmus Schneider a, Sandra J. Darkin a, Maxine A. Robbie b, William R. Wilson b and Raymond K. Ralph a

* Department of Cellular and Molecular Biology and b Section of Oncology, Department of Pathology, University of Auckland, Auckland (New Zealand)

(Received 15 September 1987)

Key words: DNA topoisomerase II; Amsacrine; Drug resistance; (Noncycling cells)

CHO-AA8 cells were used as a model system to study the role of DNA topoisomerase II in the resistance of non-cycling cells to amsacrine. Plateau-phase AA8 cells have previously been shown to be resistant to amsacrine and to contain fewer DNA breaks than log-phase cells after drug treatment (Robbie, M.A., Baguley, B.C., Denny, W.A., Gavin, J.R. and Wilson, W.R. (1988) Cancer Res., in press). The phage P4-unknotting activity of nuclear extracts decreased 2-fold when AA8 cells entered into the non-cycling state, but there was no difference in sensitivity to amsacrine between log- and plateau-phase nuclear extracts. Drug stimulation of protein-DNA complex formation was similar in whole cells, isolated nuclei and nuclear extracts from either log- or plateau-phase cells. However, stimulation of complex formation in cells, nuclei or nuclear extracts was approx. 4-fold lower in plateau-phase than in log-phase. The data presented suggested that drug–enzyme interaction was altered in plateau-phase cells.

Introduction

Several anti-cancer drugs, including the DNA intercalator, amsacrine (4'-[9-acridinylamino]methanesulphon-m-anisidide, mAMSA), adriamycin or the ellipticines, as well as the non-intercalating epipodophyllotoxins, have been shown to act on the enzyme DNA topoisomerase II (EC 5.99.1.3) [1–4]. Cells treated with these drugs exhibit an increased number of DNA breaks, presumably due to drug-mediated inhibition of the DNA resealing mechanism after the strand passing reaction of topoisomerase II. Consequently, the enzyme is stabilised in a covalent protein-DNA complex. This complex can be isolated, and the amount of drug-induced complex formation serves as a measure for drug activity [5].

Despite topoisomerase II being ubiquitous, there are marked differences in the response of different tumours towards the cytotoxic action of the topoisomerase II-specific anticancer drug, amsacrine [6–8]. Amsacrine is a synthetic DNA-intercalating agent with useful clinical activity against leukaemias and lymphomas [9,10] but little activity against solid tumours [11]. While the reasons for the resistance of solid tumours are not known, it has been suggested that the low number of actively cycling cells in solid tumours may limit the tumour-killing activity of topoisomerase II.

Abbreviations: CHO, Chinese hamster ovary; mAMSA, 4'-[9-acridinylamino]methanesulphon-m-anisidide.

Correspondence: E. Schneider, Department of Cellular and Molecular Biology, University of Auckland, Private Bag, Auckland, New Zealand.
specific drugs, since non-cycling cells per se appear to be less sensitive to these drugs [12–14]. In a number of reports, the activity of topoisomerase II has been examined in relation to changes in the proliferative activity of non-transformed cells. Considerable increases in topoisomerase II activity were observed in both regenerating rat liver following partial hepatectomy and in concanavalin A-stimulated guinea-pig lymphocytes [15,16]. In each case, enzyme activity and DNA synthesis both increased dramatically when resting cells resumed exponential growth. In contrast, Tricoli et al. [17] found no difference in topoisomerase II activity between log-phase and confluent mouse embryo fibroblasts, and no change in enzyme activity as a function of the cell-cycle phase was observed. When Sullivan et al. [18] studied drug–enzyme interactions in relation to the state of cell proliferation, they found little difference in drug sensitivity between log- and plateau-phase HeLa or L1210 cells, but a marked decrease in sensitivity of plateau-phase CHO cells towards amsacrine or the epipodophyllotoxin VP-16 compared with log-phase cells. The decreased drug sensitivity in plateau-phase cells was concomitant with decreased topoisomerase II activity and a reduced capacity to form the cleavable complex. Recently, Markovits et al. [19] reported higher frequencies of amsacrine- or VP-16-induced protein-DNA complex formation in nuclei from exponentially growing than from quiescent 3T3 or L1210 cells, and maximum protein-DNA complex formation coincided with the peak of DNA synthesis and topoisomerase II strand passing activity. Together, these results suggested that there exists a close relationship between the proliferative state of cells, the sensitivity to the cytotoxic activity of amsacrine, and topoisomerase II activity.

In a recent report, Robbie et al. [20] demonstrated that CHO-AA8 cells gradually lost their sensitivity towards amsacrine upon entry into plateau-phase. Their studies showed that this drug resistance was not due to reduced drug uptake or differences in intracellular drug concentrations. Furthermore, no evidence for drug metabolism or conversion into more active compounds was found in either log- or plateau-phase cells. However, there was a close relationship between amsacrine-induced cytotoxicity and DNA breaks, suggesting that the differences in drug-induced cell killing of log- and plateau-phase cells may have been due to differences in drug-induced DNA breakage. Therefore, we extended these investigations to study the role of DNA topoisomerase II in the differential sensitivity of log- and plateau-phase AA8 cells to amsacrine. We compared the topoisomerase II activities in nuclear extracts from log- and plateau-phase cells as well as the formation of protein-DNA complexes to identify possible reasons for differences in cell killing by amsacrine.

Materials and Methods

Materials

Methyl[3H]thymidine (20 Ci/mmol) was from Amersham and [α-32P]dATP (800 Ci/mmol) was from New England Nuclear. Phage P4 was a generous gift from Dr. R. Calendar, University of California. The restriction enzyme EcoRI was from Promega Biotec and DNA Pol I Klenow fragment from Boehringer-Mannheim. Amsacrine, (mAMSA), provided as the isothionate salt by Dr. B. Baguley, University of Auckland, was stored as a 1 mM stock solution in water at −20°C. Working dilutions were made immediately prior to use. Dextran grade B 150–200 was from BDH and proteinase K was from Sigma.

Cell culture

AA8 cells, a subline of the CHO cell line, were grown in a spinner culture, as described by Robbie et al. [20]. The cell kinetics and drug sensitivity of the log- and plateau-phase suspension cultures of CHO-AA8 cells used in this study have been described in detail by Robbie et al. [20]. Briefly, the growth rate declined when the cell density exceeded approx. 8·10^5 cells/ml, with a concomitant decrease in thymidine incorporation and accumulation of cells in G1 phase. Maximum cell density was reached at 2·10^6 cells/ml. Dose-response survival curves for cell killing by mAMSA exhibited a continuous increase in drug resistance up to 4- to 8-fold during growth from log-phase (2·10^5 cells/ml) into plateau-phase (1.4·10^6 cells/ml). For all experiments described in this report, spinner cultures with the above cell densities were used as a source of log- and plateau-phase cells, respectively.
Extraction of DNA topoisomerase II activity

Aliquots of 2·10^7 log- or plateau-phase cells were collected by centrifugation, washed once in ice-cold Tris-buffered saline (25 mM Tris-HCl (pH 7.4)/137 mM NaCl/5 mM KCl) and resuspended in 0.4 ml buffer A (20 mM Tris-HCl (pH 7.2)/150 mM KCl/5 mM MgCl_2/1 mM EGTA/10 mM Na_2S_2O_3/2% (w/v) dextran grade B) to a cell density of (4–5)·10^7 cells/ml. Triton X-100 was added to a final concentration of 0.2% and the cells were lysed for 20 min at 0°C with occasional gentle mixing, followed by centrifugation at 1200×g for 10 min at 4°C. The supernatant was discarded and the nuclei pellet was resuspended in 0.4 ml buffer A, layered on 3 ml 30% (w/v) sucrose in buffer A and sedimented at 1200×g for 10 min at 4°C. The nuclei were resuspended in 0.4 ml buffer A, centrifuged at 1200×g for 10 min at 4°C and resuspended in 0.4 ml buffer B (20 mM Tris-HCl (pH 8.0)/150 mM KCl/1 mM EGTA/10 mM Na_2S_2O_3). The nuclei were treated with 20 mM 2-mercaptoethanol/20 mM EDTA for 30 min at 4°C, followed by 3×15 s bursts of sonication before 1/3 vol. of 24% poly(ethylene glycol) 6000/2 M KCl/10 mM Na_2S_2O_3 was added, and the mixtures were held for 30 min on ice. The resulting precipitates were removed by centrifugation at 30,000×g for 30 min at 4°C to obtain clear supernatants with topoisomerase II activity. Nuclear extracts from log- and plateau-phase cells were diluted to equal protein concentrations of approx. 100 µg/ml, and stored at −70°C.

The phage P4 DNA-unknotting assay

Kotted phage P4 DNA was prepared as described by Liu et al. [22]. Assay mixtures (20 µl) comprised 50 mM Tris-HCl (pH 7.5)/10 mM MgCl_2/0.5 mM EDTA/0.5 mM dithiothreitol/30 µg/ml bovine serum albumin/1 mM ATP and 0.24 µg P4 DNA. After adding 4 µl of nuclear extract (which provided 120 mM KCl), the assays were incubated for 30 min at 37°C. To remove proteins, 1 µl 10% SDS and 1 µl 10 mg/ml proteinase K were added and the mixture was incubated for a further 30 min at 37°C. Samples were electrophoresed on 0.7% agarose gels in Tris-borate/EDTA buffer (89 mM Tris-borate (pH 7.5)/89 mM boric acid/2 mM EDTA), and the gels were stained with ethidium bromide and photographed under ultraviolet illumination. Photographic negatives were then analysed by scanning on a microdensitometer (Joyce, Loeb & Co., model MK III C). 1 unit of activity was defined as the amount of extract (in µg protein) that completely unknotted 0.24 µg phage P4 DNA.

Quantitation of mAMSA-stimulated covalent protein-DNA complex formation

The SDS/K^+ precipitation assay described by Liu et al. [22] and Rowe et al. [5] was used to measure the drug-induced formation of protein-DNA complexes in whole cells, isolated nuclei and nuclear extracts. For studies with whole cells and nuclei, the DNA of log- and plateau-phase AA8 cells was labelled with 0.1 µCi/ml [methyl-^3H]-thymidine (20 Ci/mmol) and 10^-6 M nonradioactive thymidine for 24 h. The cells were collected by centrifugation and washed once with phosphate-buffered saline (137 mM NaCl/2.6 mM KCl/8 mM Na_3HPO_4/1.4 mM KH_2PO_4). One-half of the cells from each population was held on ice until required for nuclei isolation (see below). The remaining cells were resuspended in fresh growth medium to a final density of 1·10^7 cells/ml, distributed in 1 ml aliquots into 24-well microtiter plates and incubated for 1 h at 37°C. For nuclei isolation, cells were resuspended in nuclei buffer 1 (20 mM Tris-HCl (pH 7.2)/150 mM KCl/5 mM MgCl_2/0.1 mM dithiothreitol/10 mM Na_2S_2O_3/2% (w/v) dextran grade B) at 2·10^7 cells/ml and lysed with 0.1% Triton X-100 for 10 min at 0°C. Nuclei were collected by centrifugation at 1200×g for 10 min at 4°C, resuspended in the same volume of buffer 1, and overlaid onto 3 ml 30% (w/v) sucrose in buffer 1. Following centrifugation (1200×g, 10 min, 4°C), the nuclei were resuspended in 1 ml buffer 1, recentrifuged and resuspended in 1 ml buffer 2 (buffer 1 with 5 mM EGTA and no dextran). An aliquot of nuclei from each population was examined under a microscope and the nuclei were counted using a haemocytometer. The nuclei were then diluted to 1·10^3 nuclei/ml with buffer 2 and incubated for 1 h at 37°C in 1 ml aliquots. After 1 h incubation, log- and plateau-phase cells or nuclei were treated with various concentrations of mAMSA for 60 min at 37°C. Following drug
treatment, cells or nuclei were collected by centrifugation at 1200 x g for 10 min at room temperature. The supernatants were discarded and the cells or nuclei lysed with 1 ml of prewarmed (65°C) lysis solution (1.25% SDS/5 mM EDTA/0.4 mg/ml salmon sperm DNA). The lysates were transferred to Eppendorf tubes and after addition of 0.25 ml of prewarmed (37°C) 325 mM KCl solution, were immediately vortexed for 10 s at the highest setting. The tubes were held for 30 min on ice and the resulting precipitates were then pelleted by centrifugation in a microfuge for 10 min at 4°C. After the supernatants had been carefully removed, the pellets containing protein-DNA complexes were resuspended in 1 ml of ice-cold wash solution (10 mM Tris-HCl (pH 7.5)/100 mM KCl/2 mM EDTA) and collected on GF/C filters (Whatman) under gravity. The filters were then washed five times with wash solution under vacuum, dried, and the radioactivity on the filters was measured in a liquid scintillation spectrometer. The total acid-precipitable radioactivity per assay (i.e., per 10^3 cells or 10^5 nuclei) was routinely found to be approx. 5 x 10^4 cpm.

To determine protein-DNA complex formation in nuclear extracts, 32-P-3’-end labelled EcoRI digested pBR322 DNA was prepared according to Maniatis et al. [23] and used as a substrate. Protein-DNA-binding reaction mixtures (50 ml) contained 50 mM Tris-HCl (pH 7.5)/10 mM MgCl_2/0.5 mM EDTA/5 mM EGTA/0.5 mM dithiothreitol/60 mM KCl/30 mg/ml bovine serum albumin/2 mM ATP/50 ng 32-P-3’-end labelled pBR322 DNA and varying amounts of mAMSA. The reactions were initiated by adding 5 ml of extract containing approx. 0.2 mg protein (which provided an additional 60 mM KCl to make the final KCl concentration 120 mM) and incubation was for 30 min at 37°C. The reaction was stopped by adding 100 ml of a prewarmed (37°C) solution containing 0.2 M NaOH/5 mM EDTA/2% SDS/0.5 mg per ml herring DNA for 10 min at 37°C, followed by addition of 50 ml of a solution comprising 0.4 M Tris-HCl (pH 7.9)/0.4 M HCl/0.25 M KCl to precipitate covalent protein-DNA complexes for 10 min at 4°C. The precipitates were collected on GF/C filters under gravity and washed several times with wash solution under vacuum. Radioactivity retained on the filters was measured in a liquid scintillation spectrometer.

**Protein determination**

Protein concentrations were determined according to Bradford [24].

**Results**

**Quantitation of DNA topoisomerase II activities**

In order to determine whether the increased resistance of plateau-phase cells to the action of mAMSA was due to a decrease in topoisomerase II activity, we compared the P4-unknotting activity of nuclear extracts from log- and plateau-phase cells. After preparing serial 2-fold dilutions of both extracts, a P4-unknotting assay was performed with each dilution to determine the amount of extract that contained one unit of topoisomerase II activity. A typical experiment is presented in Fig. 1, showing a slight reduction of ATP-dependent (lanes 2 and 9) topoisomerase II activity in plateau-phase nuclear extracts (compare lanes 5 and 12). This was also reflected in the specific activities of log- and plateau-phase nuclear extracts of 15.5 ± 1.4 and 10.9 ± 1.9 units/µg protein (+S.E., six independent experiments), respectively. Together with the consistently observed reduction in protein content of plateau-phase nuclei of approx. 30% (data not shown), this amounted to an overall decrease in nuclear topoisomerase II activity of 50% in plateau-phase cells. However, this difference in strand-passing activity was unlikely to account solely for the 4- to 8-fold [20] decrease in mAMSA-induced cell killing.

**Inhibition of unknotting activity by mAMSA**

To test whether the reduced drug sensitivity of plateau-phase cells was due to a decreased sensitivity of the topoisomerase II strand-passing activity by inhibition by mAMSA, P4-unknotting assays were performed with different mAMSA concentrations and 1 unit of log- or plateau-phase nuclear extract. As shown in Fig. 2, inhibition of unknotting was observed with concentrations of 10 µM mAMSA or more, and there was no apparent difference in sensitivity between log- and plateau-phase nuclear extracts. Therefore, we concluded that the inhibition of the unknotting reac-
Fig. 1. Quantitation of DNA topoisomerase II activities. Nuclear extracts from log- and plateau-phase cells were serially 2-fold diluted and with each dilution, a P4 DNA-unknotting assay was performed. Reaction products were separated on a 0.7% agarose gel, stained with 0.5 μg/ml Ethidium bromide, and photographed under ultraviolet light. Lane 1, P4 DNA alone; lane 2, undiluted nuclear extract from log-phase cells, without ATP; lanes 3–8, serially 2-fold diluted extracts as indicated from log-phase cells; lane 9, undiluted nuclear extract from plateau-phase cells, without ATP; lanes 10–15, serially 2-fold diluted extracts as indicated from plateau-phase cells.

Fig. 2. Inhibition of the unknotting reaction by mAMSA. The minimal amount of each extract required for complete unknotting of 0.24 μg P4 DNA (1 unit) was used in an unknotting assay with varying amounts of mAMSA (0–100 μM) added. Reaction products were separated on a 0.7% agarose gel, stained with 0.5 μg/ml Ethidium bromide and photographed under ultraviolet illumination. Lane 1, P4 DNA alone; lanes 2–8, 1 unit of nuclear extract from log-phase cells with 0, 2, 5, 10, 20, 50, 100 μM mAMSA respectively; lanes 9–15, 1 unit of nuclear extract from plateau-phase cells with 0, 2, 5, 10, 20, 50, 100 μM mAMSA, respectively.
II-specific anticancer drugs [2–4]. Instead, it has been suggested that the formation and stabilisation of the cleavable complex by mAMSA and other drugs was directly related to DNA-strand breaks and thus to cell death.

**Stimulation of protein-DNA complex formation**

The mAMSA-induced formation of the protein-DNA complexes with cellular DNA in whole cells and isolated nuclei was measured by the SDS/K⁺ precipitation assay (Fig. 3). A marked dose-dependent stimulation of protein-DNA complex formation up to approx. 14-fold stimulation with 20 μM mAMSA was observed in log-phase cells, with little difference between whole cells and isolated nuclei. On the other hand, complex formation in plateau-phase cells and nuclei gradually increased to 4-fold stimulation with 20 μM mAMSA, and only a slight increase was obtained with higher drug concentrations. Again, there were only minor differences between whole cells and isolated nuclei. When the initial slopes of the graphs between 0 μM and 2 μM mAMSA were compared, it could be calculated that in plateau-phase cells, 12-fold greater mAMSA concentrations were needed to produce the same stimulation of complex formation than in log-phase cells. This value is comparable to the 4- to 8-fold increase in mAMSA concentration needed to produce the same amount of cell killing in plateau-phase cultures as in log-phase cultures [20].

Results of a similar experiment with nuclear extracts from log- and plateau-phase cells using [³²P]³'-end labelled DNA as substrate for complex formation are presented in Fig. 4. mAMSA-induced stimulation of

---

![Fig. 3. Stimulation of protein-DNA complex formation in whole cells and isolated nuclei.](image1)

![Fig. 4. Stimulation of topoisomerase II-DNA complex formation in nuclear extracts.](image2)
protein-DNA complex formation with nuclear extracts from log-phase cells was 3-fold that obtained with nuclear extracts from plateau-phase cells with 20 \( \mu \)M \( m\)AMSA. Although the initial stimulation with low \( m\)AMSA concentrations is similar in both extracts, saturation of stimulation was attained at a considerably lower drug concentration in plateau-phase extracts, and only a little further stimulation was observed with higher drug concentrations.

**Discussion**

The question of resistance of mammalian cells to \( m\)AMSA and other DNA topoisomerase II-specific anti-cancer drugs has attracted considerable attention over recent years. Almost simultaneously, several authors suggested that the stabilisation of the topoisomerase II-DNA-cleavable complex rather than the inhibition of the strand-passing reaction might be responsible for the cytotoxic action of these drugs [2-4]. Using a drug-resistant cell line, Glisson et al. [25] showed that the topoisomerase II strand-passing activity was identical and equally sensitive to inhibition by etoposide (VP-16) in the sensitive parent and the resistant mutant cell lines. In contrast, only nuclear extracts from the resistant cell line were refractory to drug-stimulated formation of the cleavable complex. Similar results were also reported by other authors [26-28]. Further support for the cleavable-complex hypothesis came from Rowe et al. [5] who found a direct correlation between the in vitro cytotoxicity of a series of amsacrine derivatives and their potential to stimulate protein-DNA complex formation. Finally, while our work was in progress, Sullivan et al. [18] and Markovits et al. [19] reported a decrease in topoisomerase II strand-passing activity and a decreased formation of drug-stimulated protein-DNA complexes in cell-cycle arrested cells. Thus, there is considerable evidence to suggest an important role for the covalent protein (topoisomerase II)-DNA complex in \( m\)AMSA-induced cell killing.

To investigate a possible role of DNA topoisomerase II in drug resistance, we used AA8 cells which, upon entry into plateau-phase, developed resistance to \( m\)AMSA-induced cell killing which was accompanied by a gradual decrease in sensitivity to drug-induced DNA breakage [20]. In agreement with other studies, we found an approx. 2-fold-reduced DNA topoisomerase II catalytic activity in plateau-phase cells. This decrease in activity is smaller than that reported by Sullivan et al. [18] in a different CHO sub-line or in arrested temperature-sensitive P815 mouse mastocytoma cells (Hutchins, A.M., personal communication). Although we do not know the reasons for this difference, it is unlikely that our relatively high residual activity in plateau-phase cells was due to contamination of the nuclei with cytoplasmic topoisomerase II activity, because the last supernatant during nuclei isolation contained no detectable DNA-unknotted activity. Although the different methods used in the individual studies may contribute to the divergence in the published results, it is also possible that the nature of the cells used e.g., the degree of malignancy or transformed vs. nontransformed cells, determines the quality of the arrested state and hence the decrease in enzyme activity when cells enter stationary phase. Despite these differences, our results further confirmed that drug resistance of arrested cells is unlikely to be explained simply by a drop in topoisomerase II activity, as the degree of resistance (4- to 8-fold [20]) was significantly higher than expected from a 50% reduction in topoisomerase II activity. Furthermore, we did not detect any decreased sensitivity towards inhibition of the unknotted reaction by \( m\)AMSA in nuclear extracts from plateau-phase cells. Therefore, it is unlikely that alterations in the topoisomerase II strand-passing catalytic activity contributed to the observed drug resistance in plateau-phase AA8 cells.

To determine whether the decreased DNA breakage in plateau-phase cells [20] was in fact due to a modified topoisomerase II action, we examined the formation of protein-DNA complexes in whole cells from log- and plateau-phase cultures. These results clearly showed that the plateau-phase cells were much less susceptible to \( m\)AMSA-stimulated formation of protein-DNA complexes than cells in log-phase. Although Robbie et al. [20] found no difference in drug uptake by log- or plateau-phase cells, we could not completely exclude the possibility that the activity of \( m\)AMSA against plateau-phase cells might be
limited by its availability at its site of action, i.e., the topoisomerase II molecule within the nucleus. Therefore, we measured the mAMSA-stimulated formation of protein-DNA complexes in isolated nuclei to circumvent possible plasma membrane- or cytoplasm-related side effects. These results confirmed the difference in drug-stimulated protein-DNA complex formation detected with whole cells. Furthermore, no significant differences between whole cells and isolated nuclei from either log- or plateau-phase were observed. This clearly indicated that (i) the cells did not have to be intact for the formation of the protein-DNA complex and (ii), reduced drug sensitivity of plateau-phase cells was unlikely to be due to altered intracellular drug concentrations.

Recently, Zwelling et al. [30] suggested that chromatin conformation might influence intercalator-induced protein-associated DNA cleavage, and Riou et al. [31,32] have shown that mAMSA stimulates DNA breakage preferentially in transcriptionally active, DNAase-I-hypersensitive sites. Therefore, it is conceivable that in arrested cells, there are less potentially cleavable DNA sites for topoisomerase II to act. To eliminate the possibility that altered DNA influenced the results of our protein-DNA complex formation studies, we also used nuclear extracts with 3'-end labelled DNA as a uniform substrate for the complex-formation reaction. Again, similar differences in mAMSA-stimulated protein-DNA complex formation between log- and plateau-phase extracts were obtained as with whole cells and isolated nuclei, and no significant differences between whole cells, isolated nuclei or nuclear extracts from either log- or plateau-phase were observed. These results suggest that differences in the amounts of the protein-DNA complex formed were due to alterations in topoisomerase II. This is further supported by the fact that when using 3'-end labelled DNA, which specifically detects topoisomerase II-DNA complexes [5], as a substrate in the complex-formation reaction with nuclear extracts, essentially the same results were obtained as with whole cells and isolated nuclei.

It is interesting to note that in plateau-phase cells, although some drug-stimulated protein-DNA complex formation occurred, this remained limited even at high drug concentrations. It is tempting to speculate that alterations in drug–enzyme or in enzyme–DNA interactions are involved, which limit the amount of protein-DNA complex formed. Possibilities include posttranslational enzyme modifications [33–36] as a result of entry of cells into stationary phase or additional factors that modify mAMSA binding to topoisomerase II [25,27,28]. Preliminary results from our laboratory (unpublished), in which the addition of log-phase nuclear extract to extract from plateau-phase nuclei did not restore the protein-DNA complex formation activity, may indicate that an additional factor is present. The presence of such a factor could well open new perspectives in the treatment of resistant tumours.

Acknowledgements

We wish to thank Dr. R. Calendar for providing us with phage P4. This work was supported by the Medical Research Council of New Zealand, the Auckland Division, Cancer Society of New Zealand, the University Grants Committee, the Auckland Medical Research Foundation and the Swiss Cancer League.

References

272