STUDIES OF THE MECHANISMS OF RESISTANCE OF NON-CYCLING CELLS TO AMSACRINE AND RELATED ANTITUMOUR DRUGS

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ABSTRACT

Several studies have shown that non-cycling cells are resistant to the cytotoxic effects induced by amsacrine (*m*-AMSA; 4'-(9-acridinylamino)methanesulphon-*m*-anisidide). This resistance may limit the activity of *m*-AMSA and related 9-anilinoacridine antitumour agents against solid tumours. The biochemical mechanism(s) for this resistance have been investigated using spontaneously transformed Chinese hamster fibroblast (AA8 cells) in log- and plateau-phase spinner cultures. In early plateau phase most cells entered a growth-arrested state with a G_1-G_0 DNA content and showed a marked decrease in sensitivity to cytotoxicity after a 1-h exposure to *m*-AMSA or its solid tumour-active analogue, CI-921. Studies with radiolabelled *m*-AMSA demonstrated that changes in sensitivity to *m*-AMSA-induced cell killing were not due to a difference in uptake or retention of drug by log- and plateau-phase cells, and there was no significant metabolism of drug by either log- or plateau-phase cells. Thus, after a 1-h exposure to [^{3}H]-*m*-AMSA at 37°C, a small proportion (1%) of cell-associated radioactivity was covalently bound to macromolecules, but most of the cell-associated radioactivity represented unchanged *m*-AMSA. There was no evidence for any oxidative metabolism to reactive quinoidal species in these tumour cells. However, studies with a fluorescence assay for DNA unwinding indicated that plateau-phase cells were 3 to 4 times less sensitive to *m*-AMSA-induced DNA breakage than log-phase cells, and changes in sensitivity to *m*-AMSA-induced DNA breakage correlated with changes in sensitivity to cell killing by *m*-AMSA as cell progressed from log to plateau phase. Further studies showed that the decrease in sensitivity to *m*-AMSA-induced DNA strand breakage correlated with a decrease in sensitivity to covalent DNA-protein complex formation in plateau-phase cells after *m*-AMSA treatment. Combined with evidence that the DNA lesions rapidly disappeared from both log- and plateau-phase cells following the removal of *m*-AMSA (half-time approx. 4 min), this indicated that the lesions detected by the FADU assay probably arose from the stimulation of DNA-topoisomerase II (topo II) cleavable complex formation by *m*-AMSA. K^+/SDS precipitation assays with [^{32}P] 3'-end-labelled pBR322 DNA indicated that nuclear extracts containing topo II activity from plateau-phase cells were 3- to 4-fold less sensitive to stimulation of DNA-topo II complex formation by *m*-AMSA than nuclear extracts from log-phase cells. This change in sensitivity to *m*-AMSA-induced DNA-topo II complex formation was therefore similar to that observed with intact cells. However, P4 unknotting assays indicated that topo II activity in nuclear extracts from plateau-phase cells was only 2-fold lower than that in nuclear extracts from log-phase cells. Resistance to treatment with *m*-AMSA may therefore reflect a decrease in topo II activity and/or a decrease in sensitivity of topo II enzymes to stimulation of cleavable complex formation by *m*-AMSA in non-cycling cells.

PREFACE

Despite the availability of a steadily increasing number of drugs for the treatment of cancers, most common tumours remain refractory to treatment with existing chemotherapeutic agents (Tattersall, 1981). Although significant advances toward the cure of cancer by chemotherapy have been achieved with cytotoxic agents directed toward some haematopoietic tumours and some rapidly growing tumours, these relatively responsive tumours represent only a small proportion of human cancers. The majority of human cancers are relatively slow-growing solid tumours which respond poorly to existing chemotherapeutic agents (Kennedy *et al.*, 1980).

The present studies were performed under the supervision of Dr W.R. Wilson in the Section of Oncology, Department of Pathology, in association with the Auckland Cancer Research Laboratories, University of Auckland School of Medicine. These studies form part of a wider series of investigations intended to elucidate factors responsible for the resistance of solid tumours toward antitumour agents, particularly those related to the 9-anilinoacridine antitumour drug, amsacrine (*m*-AMSA), which was developed at the Auckland Cancer Research Laboratories.

The development of *m*-AMSA represented a significant achievement since this compound was the first synthetic DNA intercalating agent to be selected for clinical trial as an antitumour agent. However, while *m*-AMSA has proved to have useful therapeutic activity against leukaemias, early clinical trials with this compound indicated that it was ineffective or only marginally effective against a wide range of solid tumours (see section 1.3). A component of the research programme at the Auckland Cancer Research Laboratories is therefore now directed toward the search for analogues of *m*-AMSA with improved therapeutic activity against a broader spectrum of tumours, including both leukaemias and solid tumours. This search currently follows an essentially empirical strategy by seeking correlations between physicochemical properties and biological activity of drugs (Denny *et al.*, 1982). However, it is intended that investigations providing further information on factors limiting the activity of these drugs against solid tumours may provide a more clearly defined basis for the rational design of *m*-AMSA analogues with broad spectrum antitumour properties.

One of the major factors limiting the use of *m*-AMSA in the treatment of solid tumours may be its poor activity against non-cycling cells (Denny *et al.*, 1983c). There is now considerable evidence from *in vitro* studies to suggest that non-cycling cells are much

less sensitive to *m*-AMSA than cycling cells (see section 1.6.2). Evidence that non-cycling cells may also provide a limitation to successful treatment of tumour cells *in vivo* has recently been provided by the demonstration that a variant of the Lewis lung tumour with an elevated proportion of non-cycling cells shows enhanced resistance to *m*-AMSA analogues in mice (Baguley *et al.*, 1985).

This thesis examines possible biochemical mechanisms for resistance of non-cycling cells to *m*-AMSA and investigates whether the resistance of non-cycling cells is also observed with a related 9-anilinoacridine derivative, CI-921, which has demonstrated promising activity against experimental solid tumours. The thesis commences with a comprehensive literature review, which is intended to provide an appreciation of the many factors which may be important in determining the resistance of tumours to antitumour drugs. Since mechanisms of resistance of non-cycling cells to *m*-AMSA may be related to the mode of action of this compound, the review also provides a detailed discussion of the mechanism of cytotoxicity of *m*-AMSA and related 9-anilinoacridine antitumour drugs.

The experimental section of this thesis is presented in chapters 2 to 5. Chapter 2 describes studies performed to characterize an *in vitro* model for non-cycling tumour cells, which was produced by growing transformed Chinese hamster ovary fibroblasts (AA8 cells) to high density (plateau phase) in spinner culture. This chapter also contains results from investigations comparing the differences in sensitivity of cells from log- (cycling) and plateau-phase (non-cycling) cultures to both *m*-AMSA and CI-921. Chapter 3 reports investigations of the metabolism of *m*-AMSA in log- and plateau-phase AA8 cells. The metabolism of *m*-AMSA has not previously been studied in tumour cells. These investigations therefore required the development of an HPLC method which would be capable of detecting several different potential metabolites. Studies performed to characterize this HPLC system are described in detail. Chapter 4 reports studies of the uptake and retention of *m*-AMSA in log- and plateau-phase AA8 cells. Chapter 5 contains results from a series of studies performed to compare the amounts of *m*-AMSA-induced DNA damage in log- and plateau-phase cells and to investigate the mechanisms for the reduced amounts of DNA breakage observed in plateau-phase cells.

The thesis concludes with a general discussion which considers the significance of the material presented in this thesis and includes proposals for future research.

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Investigations of the role of topoisomerase II (topo II) enzymes in the resistance of plateau-phase cells to *m*-AMSA-induced cell killing were performed in collaboration with Professor Ray Ralph, Dr Erasmus Schneider and Ms Sandy Darkin from the Department of Cellular and Molecular Biology at the University of Auckland. Both the P4 unknotting assays for topo II strand-passing activity and the *in vitro* assays measuring the formation of covalent DNA-protein complexes in *m*-AMSA-treated cells were performed by Erasmus Schneider. The assays measuring the formation of covalent DNA-protein complexes in *m*-AMSA-treated cells were performed by Sandy Darkin. I wish to thank Erasmus and Sandy for their willing participation in this study and would like to thank Professor Ralph for supporting this collaboration.

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UPTAKE AND RETENTION OF *m*-AMSA BY LOG- AND PLATEAU-PHASE AA8 CELLS

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9-AA	9-aminoacridine
AAMP	4'-(9-acridinylamino)-3'-methoxyphenol
ADP-ribose	adenosine(5')diphospho(5')-β-D-ribose
ALL	acute lymphoblastic leukaemia
Alpha MEM	Alpha modified minimal essential medium
AML	acute myelogenous leukaemia
AMSA	4'-(9-acridinylamino)methanesulphonanilide
9-AO	9(10H)-acridone
[α-^{32}P]-dATP	deoxyadenosine 5'-[α-^{32}P]triphosphate
aprt	adenine phosphoribosyl transferase
ara-C	1-β-D-arabinofuranosyl cytosine
AT	ataxia-telangiectasia
ATP	adenosine 5'-triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BSA	bovine serum albumin
cDNA	complementary DNA
CHO	Chinese hamster ovary (cells)
CI-921	*N*,5-dimethyl-9-[(2-methoxy-4-methyl-sulphonylamino)phenyl-amino]-4-acridinecarboxamide
[^{14}C]-inulin	[*carboxyl*-^{14}C]-carboxyl inulin
[^{14}C]-*m*-AMSA	[9-*acridinyl*-^{14}C]-*m*-AMSA
C.V.	coefficient of variation
D_0	mean lethal dose
DAPI	4',6'-diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
d_6-DMSO	hexadeuteriodimethylsulphoxide
DFMO	α-difluoromethylornithine
dGTP	2'-deoxyguanosine 5'-triphosphate
DMF	dimethylformamide
DMSO	dimethyl sulphoxide
DNAase	deoxyribonuclease
DNP	deoxynucleoprotein
DOC	deoxycholate
DPC	DNA-protein cross-link
DSB	(DNA) double-strand break

DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	1,4-dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
ECG	electrocardiogram
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid
FADU	fluorescence assay for DNA unwinding
FCS	foetal calf serum
FD	field desorption (mass spectrometry)
g.i.	gastrointestinal
9-GS-A	*S*-(9-acridinyl)-glutathione thioether
6'-GS-AAMP	4'-(9-acridinylamino)-6'-(*S*-glutathionyl)-3'-methoxyphenol
GSH	reduced glutathione
5'-GS-*m*-AMSA	4'-(9-acridinylamino)-5'-(*S*-glutathionyl)-3'-methoxymethane-sulphonanilide
6'-GS-*m*-AMSA	4'-(9-acridinylamino)-6'-(*S*-glutathionyl)-3'-methoxymethane-sulphonanilide
GS-MSA	*S*-[4-amino-3-methoxymethanesulphonanilid-5-yl]-glutathione
GSSG	oxidized glutathione
[^{3}H]-*m*-AMSA	[*acridinyl-G-*^{3}H]-*m*-AMSA
[^{3}H]-*m*-AQDI	[*acridinyl-G-*^{3}H]-*m*-AQDI
HMG	high mobility group (protein)
^{1}H-NMR	proton nuclear magnetic resonance
$^3H_20$	tritiated water
HPLC	high performance liquid chromatography
hprt	hypoxanthine phosphoribosyl transferase
hsp	heat shock protein
[^{3}H]-TdR	[5-*methyl-*^{3}H] thymidine
HU	hydroxyurea
5-ID	5-iminodaunorubicin
i.p.	intraperitoneal
i.v.	intravenous
LDS	lithium dodecyl sulphate
m-AMSA	4'-(9-acridinylamino)methanesulphon-*m*-anisidide
m-AQDI	*N*1'-methanesulphonyl-*N*4'-(9-acridinyl)-3'-methoxy-2',5'-cyclodexadiene-1',4'-diimine
m-AQI	3'-methoxy-4'-(9-acridinylamino)-2',5'-cyclohexadien-1'-one

MeCN	acetonitrile
2-Me-9-OH-E^+	2-methyl-9-hydroxyellipticinium
MI	mitotic index
MSA	4-amino-3-methoxymethanesulphonanilide
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NPSH	non-protein sulphydryl
o-AMSA	4'-(9-acridinylamino)methanesulphon-*o*-anisidide
PALA	*N*-(phosphonoacetyl)-L-aspartic acid
PBS	phosphate-buffered saline
PCSII/AA	PCSII containing 1% acetic acid
PDR	pleiotropic drug resistance
PEG	polyethylene glycol
Pha	phytohaemagglutinin
pH*e*	extracellular pH
pH*i*	intracellular pH
PLD	potential lethal damage
PLDR	potential lethal damage repair
PLM	percent labelled mitoses
poly (ADP-ribose)	polymer of ADP-ribose
POPOP	1,4-bis-[2-(5-phenyloxazolyl)]benzene
PPO	2,5-diphenyloxazole
NCI	National Cancer Institute
NMR	nuclear magnetic resonance
QSAR	quantitative structure-activity relationship
SAR	structure-activity relationship
s.c.	subcutaneous
SCE	sister chromatid exchange
S.D.	standard deviation
SDS	sodium dodecyl sulphate
S.E.	standard error
SSB	(DNA) single-strand break
TAF	tumour angiogenesis factor
TCA	trichloroacetic acid
TdR	thymidine
TEAP	triethylammonium phosphate
TLC	thin layer chromatography

TMS	tetramethylsilane
topo I	DNA topoisomerase I (EC 5.99.1.2)
topo II	DNA topoisomerase II (EC 5.99.1.3)
Tris	tris (hydroxymethyl)aminomethane
XP	xeroderma pigmentosum