Studies on the feasibility of targeting cytotoxics to melanoma

Qarawi, Mousa Adel

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STUDIES ON THE FEASIBILITY OF TARGETING CYTOTOXICS TO MELANOMA

Submitted by Mousa Adel.Qarawi

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

There is clear evidence from previous work carried out at Bath, on B16 murine melanoma cells, that selective receptor-mediated endocytosis using α-Melanocyte stimulating hormone (α-MSH) analogues can be achieved. However, the number of MSH receptors available is limited to 5000-20000 per B16 murine melanoma cell. Recycling does occur but it is expected that the number of molecules delivered will be measured in tens of thousands. This project further investigates the feasibility of delivering a cytotoxic agent linked to α-MSH analogue in sufficient quantity to obtain a selective cytotoxic action for treating melanoma.

In part I of this work the MTT microtitre growth inhibitory assay was optimised for B16 murine melanoma cells, and then used to assess the growth inhibition of various classes of cytotoxic agents on B16 mouse melanoma cells. A range of cytotoxics was investigated including existing clinical anti-cancer agents such as Methotrexate (MTX), as well as cytotoxics which are still in clinical trials such as the cyclopropylpyrroloindole analogues (CPI analogues) adozelesin and bizelesin. Bizelesin was the most toxic agent with an EC_{50} of 6.7 pM that is approximately three orders of magnitude more potent than daunorubicin (EC_{50} of 4 nM).

In addition to the above agents, small molecules based on the naturally occurring polyamine, spermine were designed and synthesised in collaboration with the medicinal chemistry group at Bath. A series of analogues were tested (using the optimised MTT assay), comprising of a polyamine conjugated to the 9 position of the polyaromatic acridine or anthracene, either through an amide linkage or directly by a covalent bond. It was anticipated that these conjugates would show bifunctional modes of DNA binding and hence enhanced cytotoxicity. All the conjugates tested were more potent than either spermine or acridine. The most toxic conjugate (**7**) had an EC_{50} of 0.27 μM that is approximately three orders of magnitude more potent than a 1/1 molar mixture of the conjugate's spermine and acridine based constituents, the EC_{50} was 400μM, as determined under similar incubation conditions.

In part II the most toxic compounds from part I were further investigated in studies which aimed to relate the mass of drug taken into the cell with the observed toxicity. The objective here was to evaluate which drugs could be delivered in sufficient mass by receptor-mediated endocytosis of α-MSH analogues to melanoma. Cytotoxics studied included the anti-metabolite MTX, the anthracyclines (doxorubicin & daunorubicin) and the CPI analogues (adozelesin & bizelesin). A biological assay was

devised and used to relate the amount of cellular uptake of the anthracycline antibiotic (daunorubicin), and the CPI analogues (adozelesin & bizelesin) to their toxicity on B16 cells. The estimates of cellular uptake from this assay have been compared for (daunorubicin and doxorubicin) by a fluorescence assay relating the toxicity of the anthracyclines to their cellular uptake, using the natural fluorescence property of the anthracyclines.

In Part III 3T3 fibroblast cells were transfected with plasmids carrying the MSH receptor subtype (MC1) and stable clones were generated. It was hoped that cell lines expressing high levels of transgene could be used as models to test the specficity of novel cytotoxic-MSH analogues, but unfortunately it was not possible to detect any MSH receptors using a radioligand binding assay although the presence of the plasmid in the transfected clones was demonstrated by the use of the geneticin resistance marker. The activity of a novel MTX-NLDP conjugate was investigated. The toxicity of this conjugate on cells expressing melanocortin receptors (MC1 on B16 cells and MC3 on transformed 293 /cDNA cells) and cell lines lacking the MSH receptor (Cos 7, 3T3 fibroblast and 293-vector) was assessed. The conjugate had non-specific toxicity (albeit reduced when compared to free MTX) against both cells which expressed melanocortin receptors and cells which lacked receptors. This correlated well with studies carried out at Bath by Whelan (1995) which suggested that the conjugate would not have cell-specific cytotoxicity on B16 cells because of the large difference between the number of MTX molecules required to kill a B16 cell (measured in millions) and the number of MTX-NLDP molecules which would be internalised by a B16 through receptor mediated endocytosis (tens of thousands). The mechanism of non selective toxicity was investigated by considering the stability of the conjugate. After HPLC analysis of the MTX-NLDP conjugate, it was shown to contain less than 1% of MTX impurities, these MTX impurities could account for the observed non selective toxicity of this conjugate.

Acknowledgements

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Abbreviations

ACTH	Adrenocorticotropic hormone
ADEPT	Antibody-directed enzyme prodrug therapy
α-MSH	α-Melanocyte stimulating hormone
ATP	Adenosine triphosphate
Boc	t-butoxycarbonyl
BSA	Bovine serum albumin
But	t-butyl
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CPI	Cyclopropylpyrroloindole
DCC	Dicyclohexylcarbodiimide
DDD water	Double deionised distilled water
DHFR	Dihydrofolate reductase
DMF	N,N-Dimethyl formamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DTIC	Dacarbazine
DTMP	Deoxythymidylate monophosphate
DUMP	Deoxyuridylate monophosphate
EC_{50}	The extracellular concentration of a compound, which inhibits the growth of cells, *in vitro* to 50% of their normal growth.
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
$F(glu)_n$	Folic acid polyglutamate
FA	Folinic acid
FAB-MS	Fast Atom Bombardment-Mass Spectroscopy
FACS	Fluorescence activated cell sorter
FCS	Foetal Calf Serum
FH_2	7,8-Dihydrofolate
FH_4	5,6,7,8-Tetrahydrofolate

$FH_2 (glu)_n$	Dihydrofolate polyglutamate
$FH_4 (glu)_n$	Tetrahydrofolate polyglutamate
Fmoc	Fluorenylmethoxycarbonyl
H_2 (g)	Hydrogen gas
HCl	Hydrochloric acid
HCG	Gonadotrophin
HEK	Human Embryonic kidney
HEPES	N-(2-hydroxyethyl) piperazine-N-2'-ethane sulphonic acid
^{3}H-MTX	Tritiated Methotrexate
HOBT	N-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
I.P	Intraperitoneal
IVIAD	Intravascular inactivation of active drug
LDL	Low density lipoprotein
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionisation-Time of Flight-Mass Spectroscopy
MC Receptor	Melanocortin Receptor
MeCN	Acetonitrile
MeOH	Methanol
MTIC	5-(3-Methyl-1-triazino)imidazole carboxamide
Mtr	Methoxytrimethylbenzenesulphonyl
MTT	3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazoliumbromide
MTT formazan	1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenyl formazan
MTX	Methotrexate
MTX-NLDP	MTX-[Nle^4,D-Phe^7]α-MSH
NaOH	Sodium hydroxide
NEAA	Non-essential amino acids
NLDP α-MSH	[Nle^4,D-Phe^7]α-MSH
OBut	t-butoxy
PBS	Phosphate buffered saline
Pd/c	Palladium on carbon
POMC	Pro-opiomelanocortin
RME	Receptor-mediated endocytosis

RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
S.E	Standard error
SFM	Serum Free Medium
TFA	Trifluoroacetic acid
U.V	Ultraviolet light
Z	Carbobenzyloxy

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Chapter 1

Introduction

1.1. Tumour biology and chemotherapy

Cancer is a disease process which may affect multicellular organisms and which is characterised by the seemingly uncontrolled multiplication and spread within such organisms of abnormal forms of their own cells. It is not a single disease, but a group of disease entities related by the manner in which they grow, spread and behave. Consequently, individual tumours, even those from the same organ, may vary in their biological activity (Elise and Liotta, 1995). The incidence, geographic distribution, and behaviour of specific types of cancer are related to multiple factors including sex, age, race, genetic predisposition, and exposure to environmental carcinogens.

Classification of tumours

Tumours can be classified into two main groups.

1-Benign neoplasms are tumours whose cells have been so altered (transformed) as to produce an abnormal accumulation of such cells. Benign lesions are often encapsulated and the cells comprising the tumour do not spread or invade. They cause damage by local pressure or obstruction, e.g. adenoma which is a benign tumour of epithelial origin.

2-Malignant neoplasms are composed of abnormal cells, these abnormal cells grow, invade locally and disseminate, giving rise to separate tumours at distant sites. This process is known as metastasis and the tumours formed are described as metastatic or secondary tumours, e.g. adenocarcinoma which is a malignant adenoma. When

tumours metastasise they disseminate from the primary focus via pre-existing channels and cavities of the body (Carter, 1975). Patients who die of cancer generally do so as a consequence of disseminated disease. Benign or malignant lesions may arise in any organ and almost all cell types.

Malignant tumours are also classified according to their histological origin. Those derived from epithelial tissue are called carcinomas, e.g. colonic carcinoma; those arising from connective muscle or osseous tissue are sarcomas e.g. fibrosarcoma; and those from lymphatic or haematopoitic tissue are lymphomas, leukaemias, or myelomas.

Cancer cells differ from normal cells in behaviour in that they can manifest three characteristics not seen in normal cells.

1-Uncontrolled proliferation

2-Invasiveness

3-The ability to metastasise

1.2. Cancer chemotherapy

The optimal way for dealing with virtually all types of cancer is prevention (Wattenberg, 1993). This means avoiding all kinds of occupational and environmental carcinogens. The second goal is to have early diagnosis and treatment of the disease. It is much easier to treat a malignant tumour in its early stages before it metastasises and spreads throughout the body. The detection of tumours in their early stage by the patient can be achieved by encouraging people to seek medical examination of any early symptoms of suspected tumours.

Tumour	Symptoms
breast cancer	painless lump
carcinoma of the lung	bronchial narrowing, breathlessness, cough and haemoptysis
colonic carcinoma	disturbed bowel habit, obstruction and chronic blood loss
brain tumours	may cause raised intracranial pressure or focal neurological signs.

Table 1.1. Clinical features of different tumours at their primary stage. From Peckham *et al.*, 1994.

In general benign tumours are readily treated by surgical removal and if this has been achieved completely, they should not recur. In contrast, malignant tumours contain cells that are capable of invading, spreading and seeding as secondary tumours or metastases, throughout the body. The widespread location and number of these secondary tumours renders conventional therapy, with surgery and radiation, virtually useless, thereby leaving chemical therapy practically the only option.

When considering chemotherapy for any type of tumour, the most desirable properties of any pharmaceutical preparation must surely be selectivity and potency (Double, 1992).

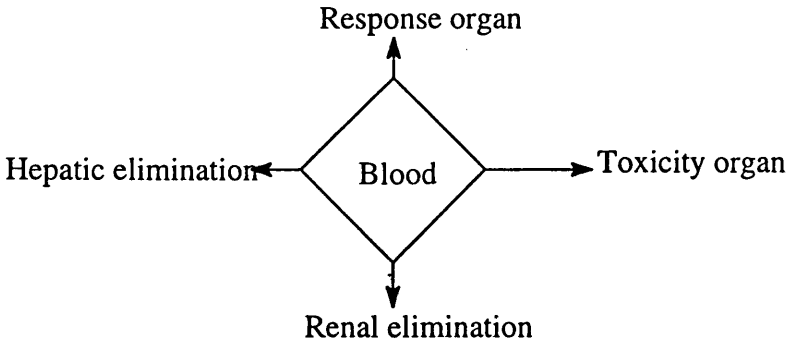


Fig. 1.1. Schematic diagram illustrating the drug disposition in the body. After administration into the blood compartment, the drug molecules are eliminated by eliminating organs (such as liver and kidney). The drug molecules are also transferred to target and toxicity organs. From Suzuki *et al.*, 1996.

It is known that cells in some normal tissues can divide at similar rate as cells in solid tumours, e.g. cells in the bone marrow and the epithelium of the gastrointenstinal tract. It is therefore obvious that the selective elimination of cancer cells cannot be achieved by treating a patient with a drug which indiscriminately damages all dividing cells. Chemotherapeutic agents that are effective also have detrimental effects on normal cells, particularly the rapidly proliferating marrow, and so current cancer chemotherapy is ultimately limited by its toxicity to these normal tissues.

The major problem is to find agents selective against cancer. That this is a difficulty is hardly surprising because neoplastic diseases, whatever their cause arise form normal tissues and remain very much like them in many ways. None of the drugs developed so far are selective enough to allow doses to be increased to the level required to kill tumour cells without being toxic at the same time. In order to have effective chemotherapy against the various types of malignant tumours it is important

to increase the selectivity of the toxicity of cytotoxic agents for tumour tissues or organs.

There are three main principles by which a selective agent can exert its favourable effect:

A- It can be accumulated principally by the target tissue or organ.

B- Or utilising comparative biochemistry, it may interfere or inhibit a chemical system important for the target tissue or organ, but not for non-target tissue

C- Or it may react exclusively with a cytological feature that exists only in the target tissue or cell. This approach may be described as cell specific targeting.

1.3. Cell specific targeting

Cell specific targeting is one approach which would help to alleviate the problems associated with non-selective therapeutic agents. There is an obvious appeal in attempting to localise cytotoxic drugs at tumour cells to minimise their systemic ill-effects. Paul Ehrlich was the first to suggest that molecules with an affinity for certain tissues might be able to serve as carriers of cytotoxic agents on appropriate target cells *in vivo*, since then many types of macromolecular carriers have been used to achieve selective toxicity of anti cancer agents on tumour tissues and organs.

For all macromolecular conjugates, persistent retention in the circulation, localisation near or at the tumour area and the enhanced uptake by malignant cells are the major features to enhance in order to improve the antitumour activity of these systems (Sezaki *et al.*, 1989).

Types of macromolecular carriers and/or targeting agents

Antibodies

Many investigators have sought to use antibodies to antigenic determinants expressed preferentially on tumour cells as carriers of cytotoxics. For this approach to succeed both the antibody and the toxic agent must retain their specificity when the two are linked together, or they could be linked in a manner allowing the release of the active agent after reaching the target cell.

Many antibody conjugates have been studied containing various cytotoxic elements (Pimm *et al.*, 1988), e.g. antibiotics (daunorubicin, doxorubicin), alkylating agents trenimon, p-phenylenediamine mustard), subclones derived from plants (gelonin, ricin, abrin) and bacterial toxins (diphtheria). Pimm *et al.*, 1988 demonstrated that a direct antibody-drug linked conjugate was able to deliver drug to subcutaneous human xenografts in mice, however the achievable tumour drug concentration was unlikely to be therapeutic. Immunotoxins (antibody conjugates to animal or plant toxins) possess a great advantage due to their high potency (only one toxin molecule generally required to cause cell death). The highly toxic component of these conjugates may prove undesirable if there are low levels of cross-reactivity, i.e. low level of antigen expression on normal tissue. *In vivo* they are rapidly cleared from the blood by the liver (probably due to mannose and fructose residues present in the toxin) which causes further problems due to release of free antibody (Blakey *et al.*, 1987). This may then saturate the antigen binding sites preventing any intact immunotoxin from binding.

Other novel approaches include antibody-directed pro drug therapy (ADEPT). In ADEPT the distinction between a pro drug and its active component provides a further opportunity to improve selectivity, by using a second enzyme system which must be retained within the vascular compartment to inactivate any active drug which is generated or otherwise enters the vascular compartment.

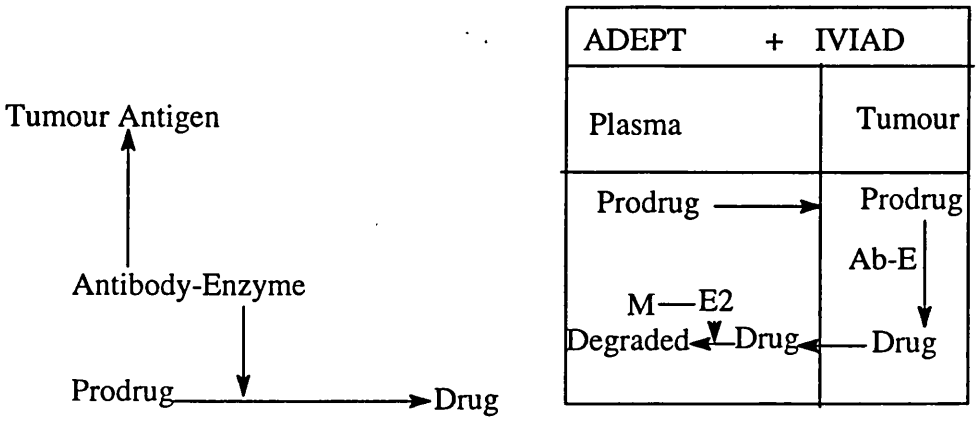


Fig 1.2. a. Antibody-directed enzyme prodrug therapy (ADEPT). b. The development of the ADEPT principle with intravascular inactivation of active drug (IVIAD). Active drug generated in the tumours or by residual enzymes at other sites can enter the vascular compartment and reach haemopoietic and other cell renewal systems and be dose limiting. An enzyme (E2) which degrades the active drug but not the prodrug is confined compartment by attachement to a macromolecule (M). From Bagshawe, 1994.

Pre-targeting antibody for imaging and therapy of cancer

In the pre targeting approach cold non-radiolabelled antibody is administered first to target the tumour cells, then a clearing step allows removal of circulating antibody.

Finally, a small molecule bearing the radioactivity is delivered using a molecular capture mechanism such as avidin-biotin (Fritzberg *et al.*, 1994).

Polyethylene Glycol

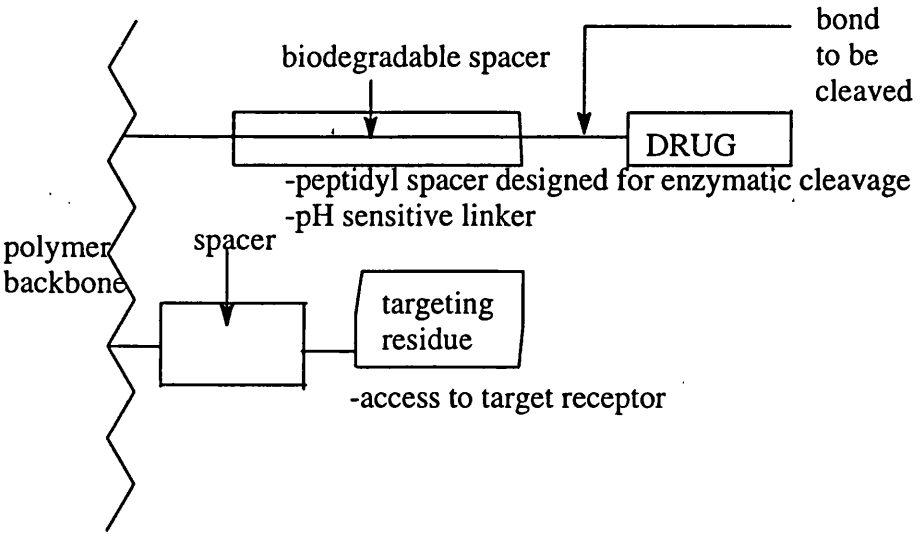


Fig 1.3. Schematic diagram showing the composition of a typical polymer-drug conjugate. From Duncan and Spreafico, 1994.

Polyethylene glycol has been used widely for protein conjugation (Fuertges and Abuchowski, 1990). Variation in the molecular weight of the PEG used (molecular weights (200-20000 Da) are available, the chemistry employed for conjugation, and the extent of surface modification of the drug provide the means to tailor-make specific PEG-drug products (Duncan and Spreafico, 1994). Thus the polymer drug conjugate can be designed to allow site-specific enzymatic or hydrolytic cleavage, which means both the rate and the site of drug delivery conjugate can be controlled (Kopecek, 1984).

Several PEG conjugates have been evaluated clinically (Fuertges and Abuchowski, 1990) including a PEG-conjugate of adenosine deaminase known as Pegademase (Hershfield *et al.*, 1987), which received market approval in 1990 in the US for the treatment of severe combined immunodeficiency associated with adenosine deaminase deficiency. PEG-asparaginase (L-asparaginase) has been used in the treatment of acute lymphoblastic leukaemia in patients hypersensitive to the native enzyme (Ho *et al.*, 1986).

Albumin and glycoproteins

The albumin itself lacks intrinsic site-selectivity but specificity has been provided when used as a carrier with antibodies(Balboni, *et al.*, 1976), e.g. Methotrexate was conjugated with both of murine monoclonal antibody (specific to an antigen on ascite mouse mammary tumour mm 46 cells) and human serum albumin (Fisher *et al.*, 1981).

The use of glycoproteins as drug carriers has been investigated (Dean, 1979), although as such they display no site selectivity. However, when the sialic acid moieties are removed from the terminal sugar branches, the resulting asialoglycoproteins are rapidly recognised and cleared by certain cells of the liver (depending on the sugar group(s) exposed). Therefore, glycoproteins can be modified to deliver drugs in a site-specific manner e.g. hepatocytes possess receptors for galactosyl-terminated gly-conjugates (Ashwell and Morrell, 1974). Other plasma proteins in addition to albumin have been suggested as carriers of anti cancer agents including fibrinogen and globulin (Szekerke *et al.*, 1972).

Dextran

Dextran is a synthetic polymer of linear chains of alpha-D glucose molecules. The polysaccharide dextran has been used for many years as a plasma expander but has more recently generated interest as a drug carrier. The varying rates of clearance of dextrans according to their molecular weights have led to the prospect of using dextrans as a drug (carrier) with the purpose of conferring greater chemical and biological stability to dextran-associated drugs.

The antitumour antibiotic mitomycin C was conjugated to dextran of varying molecular weights (10,000, 70,000 and 500,000) (Kato *et al.*, 1982). The resulting polycationic conjugates were almost as effective as free drug in growth inhibition of L1210 mouse leukemia cells after continuous exposure. It was concluded that the conjugate acted as a pro drug of mitomycin C, exhibiting their activity after release of drug probably by chemical liberation and not by the lysosomal enzymes. Dextran has also been used as drug-carrier for linkage to antibodies (Aron and Hurwitz, 1983) allowing a greater drug to antibody ratio.

Polypeptides

Synthetic polypeptides such as poly-L-lysine, polyaspartic acid and polyglutamic acid have been proposed as drug carriers, in particular the polycationic polypeptide poly-L-lysine. This compound in itself has some affinity for specific tumour cells and is capable of arresting their growth (Ryser, 1974). Furthermore its cellular uptake by endocytosis and its susceptibility to degradation by trypsin, enhances its potential use as a drug carrier. Ryser and Shen in 1978 conjugated MTX to poly-L-lysine and

tested it against a MTX-uptake resistant Chinese hamster ovary cell line. They discovered enhanced uptake and increased cytotoxicity compared to the free drug or the carrier and drug administered separately. These effects were not totally reflected *in vivo*, demonstrating that *in vitro* situations can be misleading. Feijen and co-workers covalently coupled adriamycin via an amide bond onto poly (α-L-glutamic acid). In contrast to the conjugates in which the drug was attached directly onto the carrier, conjugates with oligo peptide spacer arms readily yielded adriamycin upon digestion with a relatively specific protease such as papain (Heeswisk *et al.*, 1985).

Deoxyribonucleic acid

Site specificity of deoxyribonucleic acid (DNA) is based on the concept of lysosomotropic chemotherapy (De Duve *et al.*, 1974), the drug carrier complex enters the cell by endocytosis and is transported to the lysosomal compartment. The basis for the site-specificity of DNA is that certain tumour cells exhibit higher endocytic activity than normal cells, plus DNA is a potent inducer of pinocytosis and easily degraded by lysosomal hydrolases (Cohen and Parks, 1967).

Relatively stable drug-carrier conjugates have been formed between DNA and daunorubicin or doxorubicin (Atassi *et al.*, 1975). Both conjugates displayed equal or increased effectiveness over free drug in animal models. The conjugates were also associated with decreased toxicity as the bio-distribution of the drugs had been altered.

Hormones

Cancer cells frequently possess receptors for hormones the specific interaction between the hormones and these receptors could be used to direct drugs to cancer cells. As hormones generally exert their effects after binding to specific receptors on their target cells, they have potential for selective drug-delivery. The minimum compromise we have to make by using hormones as carriers is to sacrifice the normal target cells of the hormone. Clearly if killing normal target cells is lethal to the host, the hormone cannot be considered as a drug carrier. Also if the spectrum of target cells is too broad (such as insulin), no therapeutic advantage over the free toxin can be expected. Some attempts have been made to deliver drugs to specific cells via hormone carriers. Hormones that have been investigated as drug or toxin carriers include human placental lactogen, human chronionic gonadotrophin (hCG), epidermal growth factor (EGF) and melanotropin. Although these attempts were unsuccessful, knowledge of a hormones receptor characteristics, such as cell-type specificity, and whether or not the drug-hormone conjugate is internalised, may improve the use of hormones in site specific drug delivery. The very high affinity demonstrated by some hormone receptors may permit the advantageous use of hormone conjugates at low concentrations. One such hormone that has received particular attentions is MSH (α-melanocyte stimulating hormone).

When considering the use of hormones as targeting agents the interaction of the hormone with other cell types expressing the target receptor or a receptor for a structurally related hormone has to be taken into account (e.g. receptors for ACTH in the case of α-MSH). An ideal peptide-drug conjugate would be rapidly and specifically taken up by the target cell; this requires a high affinity of the conjugate for

the receptor, and a high rate of endocytosis/internalisation. Ideally, the receptor would be rapidly recycled and would participate in multiple rounds of delivery (Basu, 1990). Once internalised, the conjugate would have to be broken down into the carrier and the active drug. With internalised ligand-receptor complexes, the site of this process would be the lysosome, and it has to be insured that the active drug molecule reaches the target compartment without being degraded

1.4. Factors affecting targeting via membrane bound receptors.

Targeting to particular cells and tissues can be achieved by using ligand containing carriers which interact with specific receptors on the cell surface, e.g. EGF (Cawley *et al.*, 1980), transferrin (Bergamaschi *et al.*, 1988). However from the general overview of the endocytic pathway, many factors of targeting via a membrane bound receptor have to be considered.

1-The number of binding sites per cell.

2-The distribution of binding sites on other (non-target) cells.

3-Does ligand binding induce receptor-mediated internalisation?

4-If the receptor is recycled does the ligand dissociate within the cell and if so, is it transported further along the endocytic pathway or is it returned to the cell surface intact with the receptor?

5-How long before re-expression of the receptor on the plasma membrane after internalisation?

Choice of macromolecular carriers

The choice of carriers will depend on many factors including any known pharmacokinetic distribution of the macromolecule. Other important properties a carrier should possess are:

1. Lack of intrinsic toxicity and antigenicity by the carrier and its metabolic degradation products.
2. The carrier must have adequate functional groups for chemical fixation.
3. The carrier-drug conjugate must retain the desirable specificity of the original carrier compound.

The choice of drug will also depend on a number of factors:

1. It must have adequate groups in its molecular structure for conjugation.
2. It must be chemically stable in the conjugated form up until the point at which release may be required.
3. It must display sufficient toxicity at relatively low doses.

Optimisation of drug release and / or carrier degradation

The major sites for enzymatic metabolism of an endocytosed conjugate will be in the lysosome, although some proteases are present in earlier organelles e.g. early endosomes (Diment and Stahl, 1985). Lysosomal sensitive spacer linkages have been employed between drug and carrier in order to enhance the release of active drug from the conjugate. Most drug conjugates which release their cytotoxic component once selectively delivered to the lysosome can be termed as lysosomotropic agents as defined by De Duve *et al.*, 1974.

1.5. Important cellular uptake mechanisms for macromolecular drug delivery

It can be argued that the most important transport process with respect to macromolecular drug -delivery is endocytosis. Here plasma membrane invaginates and pinches off, internalising membrane proteins, lipids and extra cellular solutes. These newly-formed vesicles and their contents are then processed through various intracellular organelles before ligands or receptors generally reach the lysosomes, or are recycled back to the plasma membrane.

Two main types of endocytosis have been described. Firstly, fluid phase endocytosis (pinocytosis, non-specific endocytosis) is a constitutive uptake of soluble molecules which is concentration dependent, non-saturable, with relatively slow uptake and which is linear over a long time period. Secondly, adsorptive endocytosis (specific or non-specific), by which macromolecules bind to the cell membrane before their incorporation into endocytic vesicles. Receptor-mediated endocytosis is a particular case of adsorptive endocytosis, where specific uptake of macromolecules occurs via coated pits (Jarlozinska *et al.*, 1983).

Receptor-Mediated Endocytosis

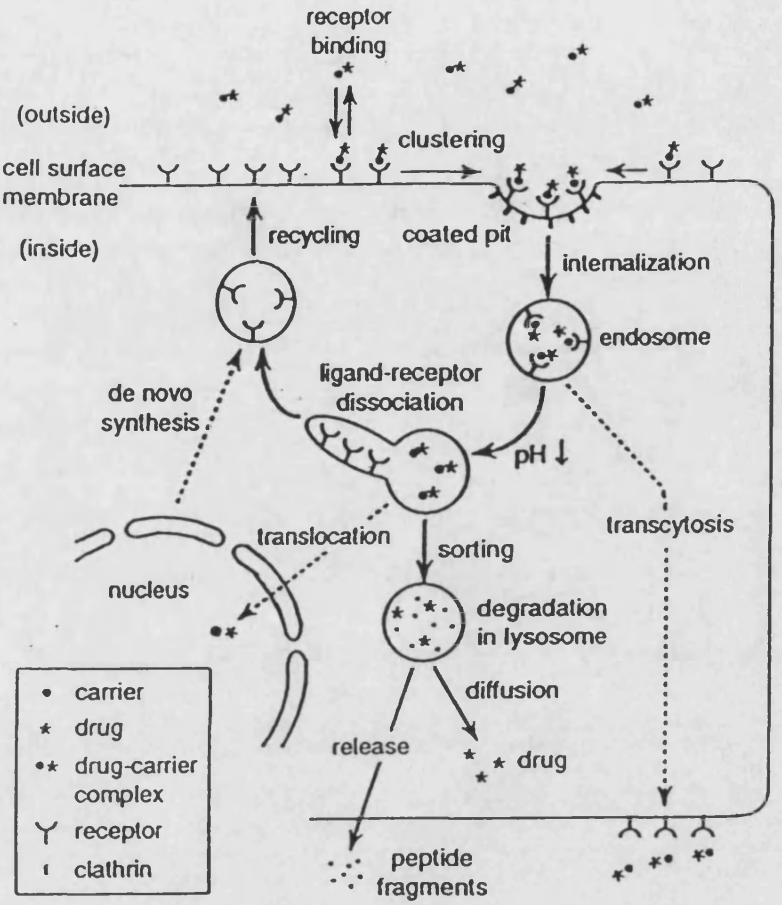


Fig 1.4. A schematic diagram for receptor-mediated endocytosis (RME) of drug-polypeptide complex. From Sato *et al.*, 1996.

Receptor-mediated endocytosis is the process whereby binding of a ligand to a cell-surface receptor is followed by internalisation of the receptor ligand complex. After reaching an acidic intracellular endosomal compartment, receptors and ligands are sorted along different pathways for delivery to lysosomes, transport across the cell, or return to the cell surface. Many physiological ligands are internalised via this pathway e.g. LDL, insulin, EGF (Deurs *et al.*, 1989) but binding of the ligand is not always a

prerequisite for triggering this process. Transferrin receptors can concentrate in coated pits in the absence of ligand and become internalised unoccupied (Watts, 1985). Coated pits are coated regions of the plasma membrane, composed of clathrin (fig 1.4). Other receptors (e.g. for EGF and insulin) can be randomly distributed on the plasma membrane in the absence of ligand and will only concentrate in coated-pits and be endocytosed upon binding (Dickson *et al.*, 1983). Different ligands can be internalised within the same coated pit but divergence of the ligands and/or receptors may occur through intracellular sorting.

In some cases endocytosis may not be required for the physiologic function of ligands e.g. EGF or insulin (Glenny *et al.*, 1988), however internalisation of the ligand-receptor complex may serve to regulate and control the membrane associated signal. Conversely endocytosis of receptor-bound ligands such as transferrin or LDL (Schneider, 1989) is essential for their physiological function.

In receptor-mediated endocytosis, the binding of molecules to specific membrane receptors is followed very shortly after, by concentration of the ligand-receptor complex into specialised clathrin-coated pit regions of the plasma membrane. These coated pits apparently pinch off to form coated vesicles within the cytoplasm. The coats are then rapidly lost, and the ligands are seen in a system of smooth vesicles and tubules (endosomes) close to, but not in continuity with, the cell membrane (Hand *et al.*, 1983).

1.6. Drug targeting to melanoma using α-MSH analogue as a carrier

Epidemiolgy of malignant melanoma

Melanoma is known to have a familial component and blue eyes, fair or red hair and a pale complexion have been demonstrated to increase risk. Furthermore, individuals who sunburn easily are at an increased risk, the association being particularly strong for sunburn in childhood. Freckles, either in childhood or as an adult are also associated with increased risk.

The incidence of malignant melanoma in the United Kingdom and Germany is now approximately 10 per 100,000 per annum giving an approximate life time risk of 1 in 200. The epidemiology of malignant melanoma is reviewed by Boyle *et al.*, 1996. Melanoma is commonest on the back and face in men and on the legs in women (Crombie, 1981).

Subdivisions of malignant melanoma

Cutaneous malignant melanoma is generally classified into four major histological groups (Buxton, 1993).

1-*Superficial spreading melanoma*; melanoma cells spread superficially in the epidermis becoming invasive after months or years.

2-*Nodular melanoma*; presents as a dark nodule from the start without a preceding *in situ* epidermal phase.

3-*Lentigo maligna melanoma*; initially there is a slowly growing pigmented macule that is present for many years befor a melanoma develops.

4-*Acral lentiginous melanoma*; initially, the lesion may present as banal-looking pigmented macular area.

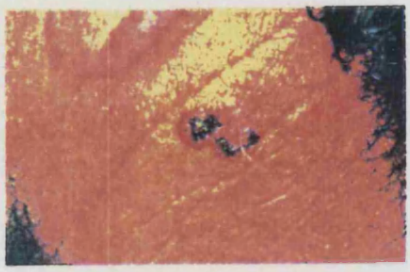


Fig 1.5. Superficial spreading malignant melanoma

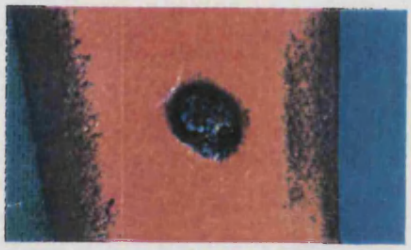


Fig 1.6. Nodular malignant melanoma

Fig 1.7. Lentigo maligna

Fig 1.8. Acral lentiginous malignant melanoma

Melanoma prevention and early detection

In the case of melanoma, primary prevention is usually centred around efforts to avoid excessive sun exposure, while secondary prevention concentrates on public education concerning features of early melanoma, such as development of pigmented lesions which may be early melanoma, and encouragement of the public to self-examine their skin and attend for surgical treatment, when any possible melanoma is at an early curable stage (Mackie, 1995).

Progression of melanoma

The critical progression predictors of the disease remain extent of radial growth, measuring tumour thickness and determining level of invasion, in addition to other factors such as mitotic rate, number of infiltrating lymphocytes (Slominski *et al.*, 1995). Also a variety of markers have been cited for prediction of disease recurrence and metastasis such as levels of intermediates of melanogenesis (Jimbow *et al.*, 1993).

Surgery of primary malignant melanoma

Biopsy remains the standard way of diagnosing malignant melanoma, providing the histopathologist with a complete specimen for micro staging. Tumours less than 1 mm thick require only 1 cm excision margins and they are usually completely cured (Slominski *et al.*, 1995), while those 1-4 mm thick need only 2 cm margins (Ball and Thomas, 1995). Survival is directly related to the measured depth of invasion. The spread of the tumour occurs to skin, subcutaneous tissues, distant lymph nodes, lungs, liver, bone and brain (Slominski *et al.*, 1995).

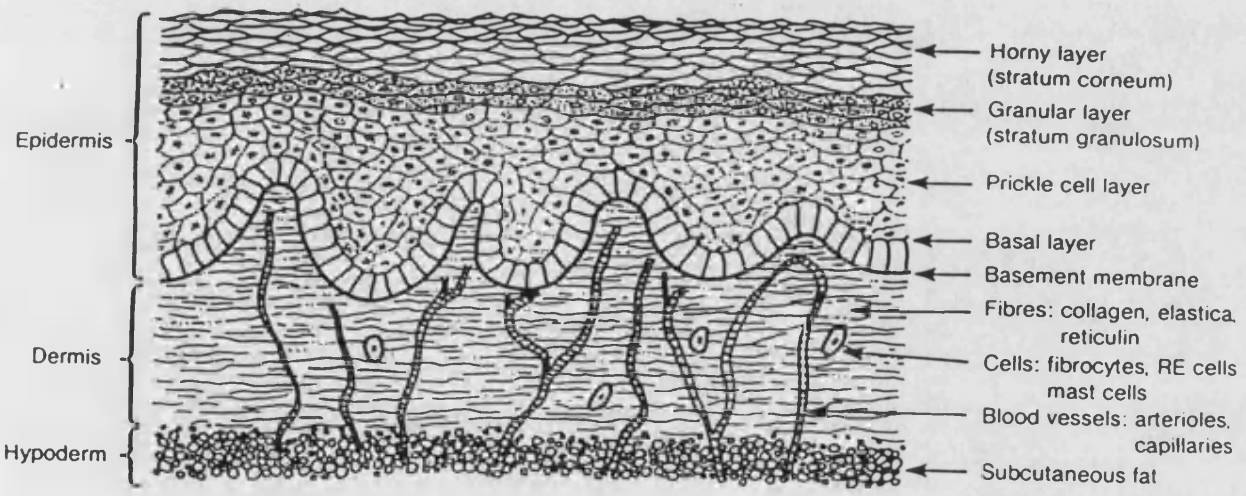


Fig 1.9. Anatomy of the skin. The epidermis is about 0.1mm thick, although the thickness is greater (0.8-1.4mm) on the palms and sole. Dermis varies in thickness, being thin (0.6mm) on the eye lids and thicker (3mm or more) on the back, palms and soles. Melanocytes are found in the basal layer.

Treatment of systemic melanoma

Treatment of patients with systemic melanoma should include careful evaluation for the potential role of surgery, radiotherapy and systemic therapy, however the main use of chemotherapy remains palliative, and Dacarbazine (DTIC) remains the most active agent used for systemic melanoma. The chemotherapeutic options available for the treatment of malignant melanoma include multi-agent therapy, high dose chemotherapy with autologous bone marrow rescue, adjuvant chemotherapy and regional perfusion (Lee *et al.*, 1995). Other approaches include active specific immunotherapy of melanoma, this approach attempts to stimulate the patient to reject his or her own tumour (Mitchell, 1995), targeted gene therapy (Hart and Vile, 1995) the use of biological response modifiers such as cytokines (Bridgewater and Gore, 1995) and finally drug targeted delivery to melanoma which is the subject of this project.

α-MSH secretion and its physiological effects

α-MSH is a pituitary tridecapeptide, and is one of several chemically and biologically related peptides which are derived from a large molecular weight precursor, pro-opiomelanocortin in the vertebrate pituitary and hypothalamus (Sawer *et al.*, 1980). Secretion of the hormone by the pituitary is under the control of the hypothalamus, various peripheral tissues are MSH sensitive, primarily the skin, where melanocytes are sited. The peptide is also synthesised and secreted within the brain, (including the pituitary and hypothalamus), it is considered a neuropeptide with various CNS related effects on development, adaptive behaviour, learning, neurotransmission and nerve regeneration (Eberle, 1988)

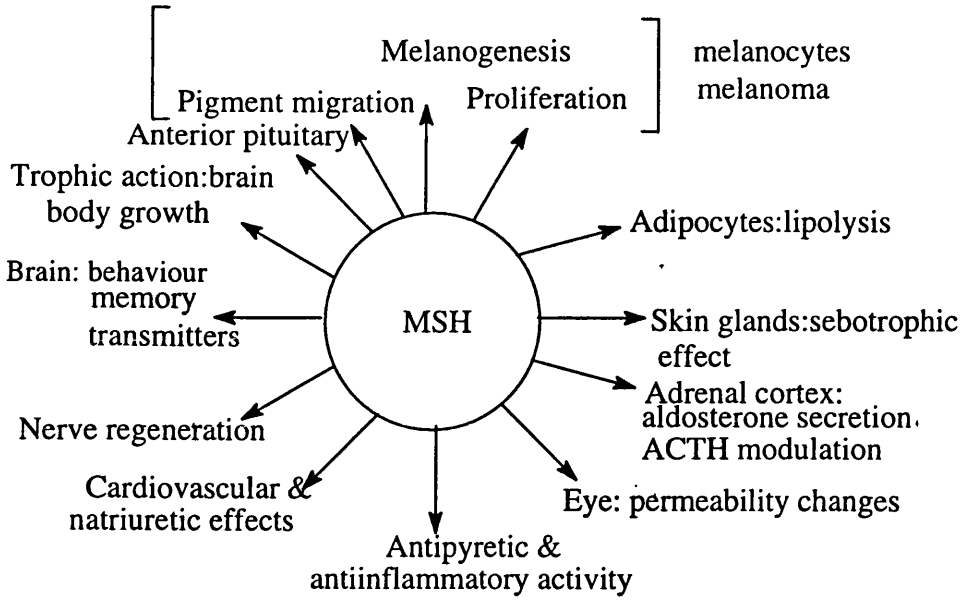


Fig 1.10. Summary of the various physiological effects of MSH peptides. From Eberle 1988).

Effect of melanotropins on pigment cells

Lerner and McGuire (1961) were the first to show that MSH peptides increase skin darkening in human subjects, and this has been confirmed more recently with NLDP-α-MSH, a potent analogue of α-MSH (Levine *et al.*, 1991). The hormone α-MSH stimulates melanogenesis in mammalian melanocytes and melanoma cells by activating tyrosinase, the rate limiting enzyme for melanin formation. Prota (1980) have shown that mammalian melanocytes produce two types of melanin, the brownish black eumelanin and the reddish yellow phaeomelanin. The initial steps in the synthesis of the two pigments are similar and are under the control of the enzyme tyrosinase, but higher levels of tyrosinase are required for the synthesis of eumelanin than for phaeomelanin (Burchill *et al.*, 1986).

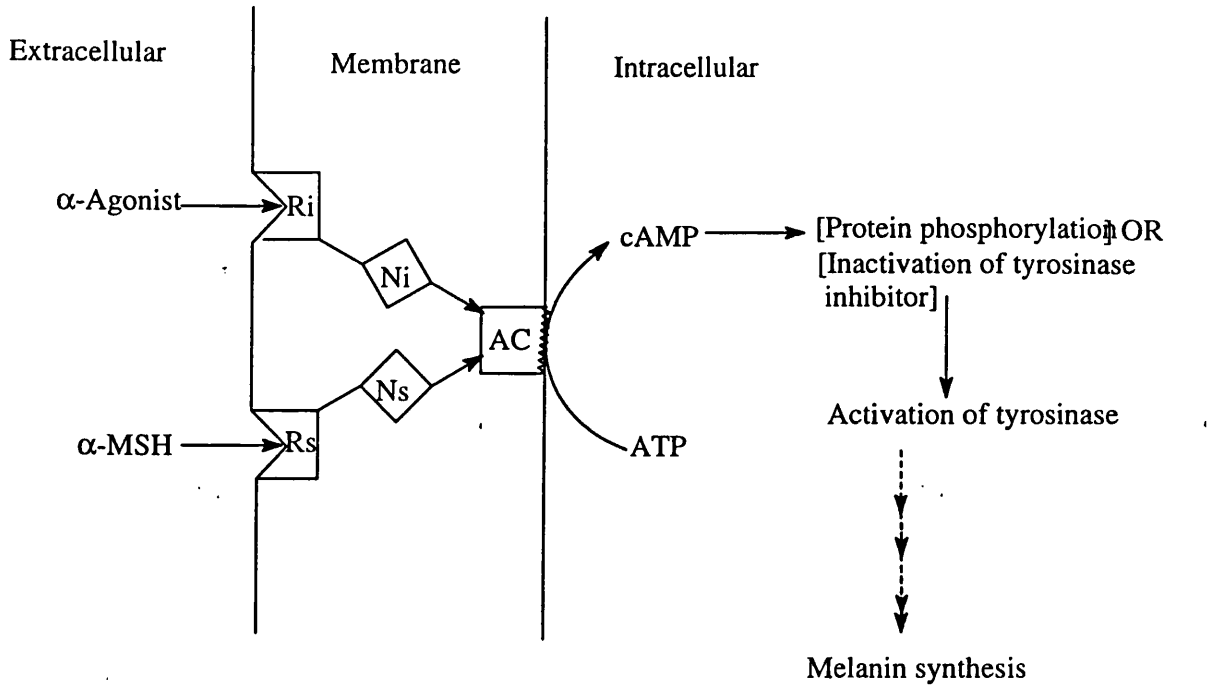


Fig 1.11. Scheme of the interaction of MSH receptor agonists with melanocortin receptors. Rs; MSH receptor stimulatory, Ri; MSH receptor inhibitory, Ns; stimulatory coupling protein, Ni; inhibitory coupling protein, AC; catalytic unit of the adenylate cyclase. (Adapted from Eberle, 1988).

It can be demonstrated that the hormone elicits its biological effect by binding to an extra cellular receptor since intracellular administration of MSH displayed no response. MSH binding stimulates adenylate cyclase causing intracellular levels of cAMP to rise, this in turn activates protein kinase (s) resulting in protein phosphorylation. It is unknown how the signal caused by MSH binding is terminated, but one or more of three possibilities is thought to occur:

1. Dissociation of MSH from the receptor.
2. Internalisation of the receptor/ligand complex.
3. Inactivation of MSH.

Sawer and his colleagues have developed a super potent and enzymatically resistant α-MSH analogue, namely [Nle^4,D-Phe^7]α-MSH (Sawer *et al.*, 1980) which has provided significant improvements over earlier systems for radioligand binding. They demonstrated unique biological properties including prolonged biological activity and enhanced potency relative to α-MSH in a number of biological systems (Tatro *et al.*, 1990).

Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH2

Figure 1.7. primary structure of [Nle^4,D-Phe^7]α-MSH

When used as drug carrier the hormone α-MSH and its analogue [Nle^4-D-Phe^7]α-MSH have several advantages in addition to their targeting potential. They are relatively easy to obtain in a pure form, their small size avoids the problems associated with larger conjugates and permits the study of chemical manipulations in the peptide sequence. Low immunogenicity would be expected due to their natural structural similarity but this may pose the potential problem of cross-recognition of the hormone or its analogue by different receptors. The sequence homology with other hormones is very close, ACTH has an identical central sequence to α-MSH. In this approach we would have to be prepared to lose the normal target cells with hormone receptors in addition to the tumour cells. Specifically the loss of normal pigmentation cells appears to be tolerable whereas melanoma is not. The effect of loss of other MSH receptors by cells, for example in the CNS, is unknown. α-MSH-related compounds could ultimately be used as pharmaceuticals for clinical application in treatment of pigment disorder, treatment of certain types of dementia, enhancement of nerve-regeneration and protection from nerve damage, control of fever and inflammation and diagnosis and therapy of melanoma Eberle 1988.

To envisage the use of MSH for selective drug delivery the pertinent characteristics for receptor-mediated endocytosis have to be elucidated. This therefore involves the determination of receptor binding characteristics, kinetics and the subsequent fates of ligand and receptor.

1.7. Scope and aims of this project

α-MSH derivatives either alone, or conjugated to a toxin or probe are potential agents for specifically targeting melanoma during diagnosis and therapy. In order to achieve selective drug delivery and targeting of α-MSH derivatives or their conjugates to melanoma cells. Targeted molecules should leave the blood circulation, ideally in close proximity of intended site of action. Also targeted molecules must interact specifically with and be internalised by the target cell, and sufficient number of molecules must be delivered per target cell (through receptor-mediated endocytosis) in order to produce the intended therapeutic effects.

The second requirement means that the MSH peptide must be conjugated to a very potent cytotoxic agent. In the work presented here we carried out *in vitro* evaluations of the cytotoxicity of a number of cytotoxics based on their extra cellular concentrations. These cytotoxics were a selection of existing clinical anti cancer agents and novel cytotoxics designed in conjunction with the medicinal chemistry group at this department. The relationship between cellular uptake and toxicity was investigated for the most toxic compounds. The toxicity of the MTX-NLDP conjugate on cells which were shown to possess MSH receptors and cells with no MSH receptors has been investigated.

Chapter 2

Materials and methods

2.1. CELL CULTURE

2.1.1. Reagents and buffers

Water

All water used for the preparation of cell culture media and solutions was freshly double glass distilled by a bi-distillation Fistreem still (Fisons Ltd) fitted with a Fistreem predeionizer (Fisons Ltd)

Phosphate buffered saline

Phosphate buffered saline without calcium and magnesium (PBS) was obtained from Oxoid Ltd in tablet form. One tablet was dissolved in 100 ml of freshly double distilled water before steam sterilisation in an autoclave (British Steriliser Co.Ltd, Swingclave Type SFT-LAB) at 121°C for 15 minutes and stored at 4°C for a maximum of six months.

Preparation of $NaHCO_3$ (7.5%)

$NaHCO_3$ (75g) dissolved in DDD water and made up to 1000 ml with DDD water. Volumes of 100 ml were transferred to 100 ml tissue culture glass bottles and sterilised by autoclaving as described above for PBS. It was stored at 4°C for a maximum period of six months.

Ethylenediaminetetraacetic acid (0.02%)

The disodium salt (0.1g) and 5 PBS tablets were dissolved in DDD water and made up to 500ml with DDD Water, 100 ml aliquots were put into sterile universal volumetric containers and stored at -20°C until required for a maximum of 6 months.

Trypan blue

This stain was obtained from Sigma Ltd and stored at room temperature as a 0.4% solution in PBS.

Growth media and additives

RPMI 1640 (Flow and Imperial Laboratories) was obtained as 10X sterile liquid concentrates containing phenol red without L-glutamine or sodium bicarbonate. The media supplements below were obtained sterile from Flow or Imperial Laboratories and aseptically aliquoted into 20ml aliquots, L-glutamine (200mM), an antibiotic solution (pen/strep) of penicillin (5000IU/ml) and streptomycin (5000mg/ml) and non-essential amino acids (NEAA).

RPMI 1640 media and NEAA were stored at 4°C while L-glutamine and pen/strep were stored frozen at -20°C.

Foetal calf serum

Foetal calf serum was received in 500ml bottles from Gibco and was aliquoted into 100ml samples and stored at or below -20°C.

Table 2.1 Preparation of RPMI 1640 medium. The medium was prepared in an aseptic manner, stored at 4°C for up to one month, it was examined visually for microbial contamination prior to use.

Reagent	volume (ml)
RPMI 1640	50
Foetal calf serum	50
7.5% $NaHCO_3$	13.5
MEM NONESSENTIAL AMINO ACIDS	5
PENICILLIN/STREPTOMYCIN	5
L-GLUTAMINE	5
DDD Water	to 500

Table 2.1 Formula of RPMI 1640 + FCS medium.

Reagent	volume (ml)
RPMI1640	50
7.5% $NaHCO_3$	13.5
MEM NONESSENTIAL AMINO ACIDS	5
PENICILLIN/STREPTOMYCIN	5
L-GLUTAMINE	5
DDD Water	to 500

Table 2.2 Formula of RPMI 1640 serum free medium.

2.1.2. Preparation of reusable items .

All recycled items were rinsed in tap water immediately after use and then processed as follows. Glassware were soaked in 2% solution of RBS 25 (Fisons Ltd) at approximately 40°C for 30 minutes, then thoroughly cleaned using a nylon brush. Articles were then rinsed in three changes of tap water, left for 30 minutes in the last rinse. The process was then repeated using single distilled water.

Finally, all glassware was left to stand in a large volume of freshly collected double distilled water for no longer than 2 hours. After drying in a hot air oven over night (Gallenkamp), all items were capped with aluminium foil and sterilised by dry heat at 160°C (Gallenkamp Sterilising Oven) for a minimum of 1 hour.

Non-glass items, mainly tips for Gilson pipettes, bottle caps and syringes were rinsed immediately after use and then cleaned by boiling in three changes of fresh distilled water. Finally, they were rinsed and left for 1 hour in a large volume of freshly double distilled water, dried, sealed in autoclave bags (DRG Hospital supplies) and sterilised in an autoclave (Drayton Castle Laboratory Steriliser) at 121 °C for 15 minutes.

2.1.3. Disposable items and equipment

Sterile tissue culture polystyrene flasks ($175cm^2$) were regularly obtained from Imperial Laboratories. Ninety six well plates were from Nunc. Thirty ml screw capped universal containers were obtained from Sterilin Ltd. Polypropylene 2ml

ampoules with screw-caps were obtained sterile from J.Bibby Sciences and used for the storage of cells in liquid nitrogen.

All manipulations requiring a sterile environment were performed in a vertical recirculating laminar flow cabinet (MDH Ltd). Other experimental protocols were performed at the bench.

All cells were stored in 2ml ampoules, shelved in the vapour phase of a Union Carbide LR - 40 liquid nitrogen refrigerator at approximately -148°C. Cells were maintained in a LEEC PF2 anhydric incubator (Laboratory and Engineering Company) with forced air circulation and thermostatic controls adjusted to give a temperature of 37°C, it was regularly checked with a digital thermometer with a thermocouple probe (Jenway Ltd) in a beaker of sterile water. An inverted biological microscope WILD M40 (wild Heerbrugg Ltd) was used for the examination of growing cell cultures and counting cells. A standard double haemocytometer (Fisons Ltd) was used to count cell density.

2.1 4. Cell Culture methods

Cell line sub-culture

Sub-culture of B16 cells was undertaken every 3 days when the cells had reached confluence (approximately $1x10^7$ cells /175cm^2) the growth had almost ceased, and further growth was limited by contact inhibition and the availability of nutrients in the medium. After this point, the medium pH dropped below 7.0, (indicated by the

change of the medium colour from golden orange to yellow). The sub-culture of 3T3, Cos7 and 293/MC3 was undertaken every 4 days.

The culture was optically examined to ensure that the cells were healthy with no free-floating cellular debris or contamination in the growth medium. Aseptically, the old medium was drained off and the monolayer was rinsed gently twice each with 5ml of PBS (to remove traces of serum which would inhibit the action of EDTA). The flask was then incubated with 2ml of 0.02% EDTA at 37°C for approx. 5-15 minutes. B16 cells were incubated with EDTA for 15 minutes while 3T3, 293/MC3 and Cos7 cells were incubated with EDTA for a few minutes. Upon removal from incubation the flask was gently agitated to dislodge the cells and 8 ml of fresh medium was added, using a sterile plugged pasteur pipette, the cell suspension was gently aspirated until well mixed, centrifuged at 1000rpm for 10 minutes, resuspended in 10ml media, 0.4 ml was put into a sterile test tube to determine cell density. A new $175cm^2$ culture flask containing 50ml of fresh media was then inoculated with 10^6 cells and purged with 5% CO_2 in air (BOC special gases) for 30 seconds before the cap was tightly sealed. The flask was labelled by the cell line, passage number and fraction subculture and its date (e.g. B16, I15, 1/10 mean the cells in the flask were B16 cell line, of passage no 15 subcultured from confluent flask in the ratio of 1/10 on the specified date) then they were placed in an incubator and checked every day for health and contamination until they reached confluence.

Determination of cell density

Subcultured cells prepared in a suspension form were thoroughly mixed and 0.1ml of trypan blue was mixed in with 0.4ml of the suspension for 5 minutes while being gently agitated.Viable cells excluded the dye while non-viable cells stained a dark blue. A cell density count would only include the former. A drop of the cell-dye mixture was loaded into a haemocytometer chamber under a coverslip pressed down such that interference patterns appeared along its edges. Each chamber was divided into nine large squares by triple white lines, the four corner squares were further subdivided into 16 squares/corner, and the central square was subdivided into 25 smaller squares. A total count was made on the four corner and the central square of the haemocytometer grid with an inverted microscope.

Since each large square had an area of $1mm^2$ and a depth of 0.1mm with the coverslip on, the total volume for each square was 10^{-4} ml. Where N is the mean of the 5 large squares, the cell density of the cell suspension was $Nx10^4$ cells/ml. To account for the dilution of the cell suspension with the dye, the end equation was $5(N X10^4/4)$ cells/ml.

Cells counted in this manner had an imprecision (CV%) of 6% for 4 separate readings of a cell suspension. The imprecision (CV%) = standard deviation/average number of cells

Cell storage

All cells were routinely stored frozen in liquid nitrogen, or its overlying vapour, after exposure to 10% of the cryptoprotectant dimethyl sulphoxide (DMSO, BDH Chemicals grade 1). (The DMSO is stored at room temperature in a dark glass container). Cell suspensions were prepared from the monolayer state during routine sub-culture, and centrifuged at 1000 rpm for 10 minutes (Jouan B3-11 Bench centrifuge). The supernatant was removed and the cell pellet resuspended in a volume of filter -sterilised (0.2μm sterile filters, Gelman Sciences) growth medium containing 10% DMSO to give a final cell density of $2x10^6$cells/ml. Replicate volumes of 1ml were placed in 2ml polypropylene ampoules then immediately placed in nitrogen vapour overnight after which they were transferred to a liquid nitrogen freezer for long term storage.

Recovery of cells from storage

Immediately upon removing ampoules from storage they were placed in a 37°C water bath, ensuring that the water did not rise above the screw cap. When completely thawed the contents were aseptically transferred into 175cm^2 flask containing 50 ml fresh medium. After 4-5 days the cells formed a monolayer suitable for routine subculture.

2.2. MTT assay

2.2.1. MTT reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder (1g) was obtained from Sigma chemical company (product number M 2128) and stored at 0-5 °C. MTT was dissolved in PBS at 10mg/ml, filtered to sterilise and remove a small amount of insoluble residue present in some batches of MTT. The filter used was 0.2μm pore size (sartorius AG Germany)]. Stock solution of MTT was stored at 4°C for a maximum of one month.

Formazan

1g (1-(4,5-Dimethylthiazol-2yl)-3,5-diphenyl-formazan (MTT formazan) powder was obtained from Sigma chemical company and stored at room temperature.

Dimethyl Sulfoxide (DMSO)

Dimethyl sulfoxide (DMSO) , was obtained from Sigma Chemical Company and stored in the dark at room temperature.

Dacarbazine

Dacarbazine powder (1g) was obtained from Sigma Chemical Company and stored at -20°C. Dacarbazine was dissolved in RPMI medium as required and the appropriate dilutions prepared.

Mitozolomide & Temozolomide

Mitozolomide (5g) and temozolomide (5g) were supplied as powders by Cancer Research Laboratories, University of Nottingham and stored at room temperature. The required amounts were dissolved in DMSO and the required dilutions were prepared in medium for incubation with the cells (DMSO in the medium was less than 1% v/v).

Ethidium bromide

Ethidium bromide powder (5g) was obtained from Sigma Chemical Company and stored at room temperature. It was dissolved as required in medium and the required dilutions were prepared for incubation with cells under study.

Fluorouracil

Fluorouracil powder (1g) was obtained from Sigma Chemical Company and stored at room temperature. It was dissolved as required in medium and the required dilutions were prepared for incubation with the cells under study.

Methotrexate

50mg/2ml Methotrexate vials (50mg/ml) were obtained from Lederle and stored in the dark at room temperature. Dilutions were prepared as required for incubation with cells under study.

Daunorobucin

Daunorubicin powder (5mg) was obtained from Sigma Chemical Company and stored at 4°C. Daunorubicin was dissolved in absolute ethanol and stored at 4°C. Dilutions were prepared as required for incubation with cells under study.

Doxorubicin

Doxorubicin powder (10mg) was obtained from Sigma Chemical Company and stored at 4°C. Doxorubicin was dissolved in absolute ethanol and stored at 4°C. Dilutions were prepared as required for incubation with cells under study.

Vinblastine

Vinblastine powder (5mg) obtained from Sigma Chemical Company and stored at 4° C. It was dissolved in 1ml DDD water and stored at 4°C. Dilutions were prepared as required for incubation with cells under study.

Adozelesin

Adozelesin powder (22mg) obtained from Upjohn company USA. It was dissolved in 500 µl dimethylformamide and stored at -20 C. Ten µl of this stock solution were diluted in 1ml DMF and stored at -20 C for no more than 1 week. From the second stock solution various dilutions were prepared in RPMI media for incubation with B16 cells.

Bizelesin

Bizelesin powder (22mg) obtained from Upjohn company USA. It was dissolved in 500 µl dimethylformamide, and stored at -20 C. Ten µl of this stock solution were diluted in 1ml DMF and stored at -20 C for no more than 1 week. From the second stock solution various dilutions were prepared in RPMI media for incubation with B16 cells.

2.2.2 Methods for MTT (Tetrazolium) assay

Optimisation of the MTT assay

Correlation of formazan concentration with absorbance

Various dilutions of formazan were prepared in DMSO, the absorbance of these dilutions was read in a 96 well plate using the Elisa plate reader, also and in a lambda 3 UV/VIS spectrophotometer (Perkin-Elmer).

Correlation of cell number with absorbance by (measuring absorbance at λ 540nm and background λ of 690 nm) of metabolised MTT after various incubation periods.

Various dilutions of the cells under investigation were made in serum free medium containing MTT 1mg/1ml and were seeded in a 96 well plate. They were incubated for 1,2,3 and 4 hours, after this the MTT assay was performed to determine the correlation between cell number and absorbance of MTT metabolised by the cells, and to determine the optimal incubation period for the cells to metabolise the MTT.

An estimation of the optimal growth conditions (cell density and incubation period)

Various dilutions of B16 cells were prepared in RPMI medium, seeded in 96 well plates and incubated at 37C for 1 hour, 24 hours, 48 hours, 72 hours and 96 hours. At the end of each incubation period the MTT assay was performed. This enabled us to estimate the approximate doubling time for B16 cells, and to determine optimal seeding density and incubation time. This procedure was repeated for 3T3, Cos7 and 293-MC3 cells.

Colorimetric MTT assay on B16 cells

The assay was performed in 96 well plates, each plate consists of 12 columns and each column has 8 wells. The first column was loaded with a blank solution and the sixth column with cells without the test compound. B16 cells, subcultured in 175cm flasks as described, were seeded at a density of 4000 cells/well. Serial dilutions of the test compound were prepared and added to the 96 well plate, they were then incubated at 37°C for 72 hours, the drug containing media was removed from the plate by flicking the plate. MTT solution (200 µl) in SFM 1mg/1ml was added and the cells were reincubated in the plate at 37°C for 3 hours. After this time the MTT solution was removed by flicking the plate and 200 µl of DMSO were added to each well and mixed thoroughly to dissolve the dark blue formazan crystals Plates were read within 30 minutes of adding the DMSO on a Dynatech MR 580 Micro Elisa reader, using a test wavelength of 540 nm, and a reference wave length of 690 nm.

2.3. Estimation of the cellular uptake of anthracyclines using fluorescence assay.

Estimation of the rate of cellular uptake of the anthracyclines (doxorubicin & daunorubicin) by B16 cells using FACS analysis.

Absorption spectra were determined for doxorubicin and daunorubicin to determine their absorption maxima, these were used as the excitation λ in order to get their emission spectra.

The B16 cells were incubated for 4 hours with each of the drugs (doxorubicin and daunorubicin) at 37°C at various drug concentrations in RPMI medium. Following drug exposure, the cells were washed twice with PBS, resuspended in PBS to a final concentration of $1\text{-}2x10^5$ cells/ml and used immediately for experiments with the fluorescence-activated cell sorter. For each concentration 5000 B16 cells were analysed. To measure the fluorescence intensity, drug-exposed cells were analysed with excitation λ of 488 nm and emission λ of 550 nm. The data obtained were displayed in the form of a histogram of cell number analysed by the various channels (y) versus log fluorescence of 5000 B16 cells (x)

Estimation of the fluorescence of anthracyclines internalised by B16 cells

The EC_{50} is defined as the extracellular concentration of a compound, which inhibits the growth of cells, *in vitro* to 50% of their normal growth.

The EC_{50} of the anthracyclines was determined , after four hours incubation with B16 cells at 37°C and 72 hours incubation in a drug free RPMI media, and the MTT assay was performed as described before.

B16 cells were incubated with the anthracycline (doxorubicin or daunorubicin) in RPMI medium at their EC_{50} concentrations (as determined following 4 hour continuous incubation of B16 cells with drug and 72 hours in drug free media for 4 hours at 37°C) in universal volumetrics on an orbit mixer (medium speed). After this the cells were centrifuged at 4000 rpm for 5 minutes, (the supernatant was kept for fluorescence measurements), the pellet (cells) were resuspended in 5 ml of ice cold PBS, they were centrifuged at 4000 rpm for 5 minutes (this was repeated twice) the supernatants were kept for further fluorescence measurements. The cell pellet was resuspended in acid-alcohol reagent containing 95% ethanol, 1N hydrochloric acid, and distilled water in 5:2.8:1.6 ratio for 30 minutes at 25°C, they were then homogenised by passing through an 18 gauge needle 10 times. Insoluble materials were removed by centrifugation at 4000 rpm for 20 minutes. The fluorescence of the extracted anthracycline (supernatant) at excitation λ of 488 nm emission λ of 550 nm was measured. The corresponding concentration was read off a calibration curve of fluorescence against anthracycline concentration.

2.4. Estimation of the relationship between toxicity and mass of cellular uptake of drugs using a biological assay

The EC_{50} of drug was determined, after 4 hours incubation with B16 cells and 72 hours incubation in a drug free RPMI medium, after this the MTT assay was

performed as described before. The same numbers of drug molecules was incubated with various number of B16 cells at 37°C for 4 hours, in 30ml universal container on an orbit mixer medium speed. The cells were centrifuged at 1500 rpm for 10 minutes. A toxicity assay of the supernatants on B16 cells was performed as described before (after incubating fresh samples of B16 cells with supernatant at 37°C for 4 hours and 72 hours in a drug free media). The cells (pellet) were resuspended in 5 ml ice cold PBS, and centrifuged at 1500 rpm for 10 minutes (this was repeated this twice). Each sample was diluted to a final concentration of 4000 cells /0.2 ml media and seeded in a 96 well plate. The plate was incubated at 37°C for 72 hours, after this the MTT assay was performed as described before.

2.5. Isolation of the plasmid pCDNAI/Neo

2.5.1. Buffers and reagents used in the isolation of the plasmid DNA.

Tryptone

Tryptone powder (500g) was obtained from Oxoid Ltd. and stored at room temperature until required.

Yeast extract

Yeast extract powder (500g) was obtained from Oxoid Ltd. and stored at room temperature until required.

Ampicillin

Ampicillin powder (5g) was obtained form Sigma Chemical Company and stored at 4 °C. It was dissolved in DDD water (100mg/ml), Sterilised by filtration and stored at -20°C. The stock solution was diluted in LB broth to 50µg/ml just before use.

1M Tris/HCl (pH 8.0)

Tris[hydroxymethyl]aminomethane powder (1kg) was obtained from Sigma Chemical Company, and stored at room temperature until required. Tris base (12.1g) was dissolved in 80ml DDD water, the pH was adjusted to 8.0 with 6M HCl, the solution was allowed to cool to room temperature then the pH was readjusted, and the solution made up to 100ml with DDD water. The solution was autoclaved at 121°C for 15 minutes. in on liquid cycle, and stored at 4°C for up to one month.

5M Potassium acetate

Potassium acetate powder (500g) was obtained from Sigma Chemical Company. Potassium acetate (49.1g) was dissolved in 80ml of pre warmed DDD water, the solution was then transferred to a measuring cylinder, and made up to 100ml with DDD water. The solution was autoclaved at 121°C for 15 minutes, and stored at room temperature.

0.5M EDTA

The disodium salt of EDTA (18.61g) was dissolved in 80 ml DDD water, the pH was adjusted to 8.0 with 6M HCl, then made up to 100ml with DDD water. The solution was autoclaved at 121°C for 15 minutes and stored at room temperature.

10 M NaOH

NaOH (40g) was dissolved in 100ml of DDD water, then autoclaved at 121°C for 15 minutes, and stored at room temperature until required.

Tryptone	10g
Yeast extract	5g
NaCl	10g
DDD Water	to 1000ml

Adjust pH to 7.5 with1M NaOH

Table 2.3. Formula of LB Broth.

The LB broth was autoclaved at 121°C for 15 minutes. It was discarded when it became cloudy.

5M Nacl	200µl
1M Tris.Cl (pH 8.0)	1000µl
0.5M EDTA (pH 8.0)	20µl
DDD water	to 100ml

Table 2.4. Formula of STE buffer.

The STE buffer was autoclaved at 121°C for 15 minutes and stored at 4°C

glucose	0.901g
1M Tris.Cl (pH 8.0)	2.5ml
0.5M EDTA (pH 8.0)	2ml
DDD water	to 100ml

Table 2.5. Formula of solution I

Solution I was autoclaved at 121°C for 15 minutes and stored at 4°C.

10M NaOH	20µl
10% SDS	100µl
DDD water	880µl

Table 2.6. Formula of solution II

Solution II was freshly prepared.

5M potassium acetate	60.0 ml
glacial acetic acid	11.5 ml
DDD water	28.5 ml

Table 2.7. Formula of solution III

Solution III was autoclaved at 121°C for 15 minutes and stored at 4°C

Tris.Cl (pH 8.0)	1.0 ml
EDTA (pH 8.0)	0.2 ml
DDD water	to 100ml

Table 2.8. Formula of TE buffer (pH 8.0)

The buffer was autoclaved at 121°C for 15 minutes and stored at room temperature.

2.5.2. Plasmid DNA isolation methods.

Cell storage

Bacteria were stored in media containing 10% glycerol at -80°C without significant loss of viability.

1-A culture flask containing 10 ml of LB broth (containing ampicillin at 50µg/ml) was inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking

2- 0.9ml of the overnight culture were transferred to a microfuge tube containing 0.1ml of sterile glycerol, then vortexed.

3-The glycerinated cultures were then stored at -80°C.

Viable bacteria were recovered by scratching the surface of the frozen stock with a sterile platinum loop. The frozen suspension was then returned to -80°C freezer. Several vials of each strain were stored.

Small scale preparation of plasmid DNA (Alkaline lysis method)

Harvesting and lysis of bacteria

Harvesting

1- 10ml of LB medium containing 50µg/ml of ampicillin in a loosely capped 30ml universal tube were inoculated with the bacteria from a frozen stock as described above. The culture was incubated overnight at 37°C with vigorous shaking.

2-The bacterial culture was centrifuged at 4000 rpm for 10 minutes

3-The medium was removed by aspiration, leaving the bacterial pellet as dry as possible.

Lysis by alkali

Lysis by alkali was carried out as described by Maniatis *et al.*, 1982.

1-The bacterial cell pellet obtained in step 3 above was resuspended in 1ml of STE and recentrifuged at 12000 rpm for 2 minutes. The supernatant was removed leaving the pellet as dry as possible

2-The bacterial pellet was resuspended in 200 µl of ice -cold solution I by vigorous vortexing.

3-A volume of 400µl of freshly prepared solution II was added.

The tube was closed tightly, and the contents mixed by inverting the tube rapidly five times. Making sure that the entire surface of the tube comes in contact with solution II. IT WAS NOT VORTEXED. The tube was stored on ice until the next step.

4-300µl of ice-cold solution III was added. The tube was closed and vortexed gently in an inverted position for 10 seconds to disperse solution III through the viscous bacterial lysate. The tube was stored on ice for 5 minutes.

5-Then centrifuged at 12,000 for 5 minutes at 4°C in a microfuge. The supernatant was transferred to a fresh tube.

6-An equal volume of phenol:chloroform was added and mixed by vortexing. After centrifuging at 12,000 for 2 minutes at 4°C in a microfuge, 600µl of the supernatant (top layer) was transferred to a fresh tube.

7-The double-stranded DNA was precipitated with 600µl of isopropanol (mixed by vortexing and allowed the mixture to stand for 5 minutes at room temperature).

8-Centrifuged at 12,000 for 10 minutes at 4°C in a microcentrifuge.

9-The supernatant was removed by gentle aspiration. The tube was left in an inverted position on a paper towel to allow all of the fluid to drain a way for 10 minutes. Any drops of fluid adhering to the walls of the tube was removed by aspiration.

10-The pellet of double-stranded DNA was rinsed with 1ml of 70% ethanol at 4°C. The supernatant was removed as described above. This step was repeated once more.

11-The nucleic acid was redissolved in 50µl of TE (pH 8.0) containing DNAase free pancreatic RNAase (20µg/ml), vortexed briefly and stored at -20°C until required.

Quantitation of DNA

Spectrophotometric determination of the amount of DNA

absorbance was read using spectrophotometer, Spectronic 601 (Milton Roy)

An OD_{260} of 1 corresponds to 50µg/ml for double stranded DNA.

The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid.

Pure preparations of DNA have OD_{260}/OD_{280} values of 1.8.

If there is contamination with protein or phenol, the ratio above will be significantly less than the value given above, and accurate quantitation of the amount of nucleic acid will not be possible using this method.

2.6. Generation of permanent transfectants of 3T3 cells

Geneticin powder was purchased from Gibco Laboratories, it was stored at room temperature (15°C to 30°C). Geneticin solution in water was stored at -20°C for no more than one month

MTT assay of geneticin on 3T3 cells

Geneticin was dissolved in RPMI media containing 10 % FCS without antibiotics at a concentration of 5 mg/ml and filtered using 0.22 micron filter. 96 well culture plate was prepared by adding geneticin to the growth medium to the desired concentrations. 4000 3T3 cells were added to each well and then incubated in a humidified CO_2 atmosphere at 37°C for 4 days, the supernatant was removed by flicking the plate and the MTT assay was performed as described before.

Electroporation of 3T3 cells

Electroporation cuvettes 0.4 cm were placed on ice at the beginning of the experiment. 1)- 3T3 cells were grown to 50-70% confluency in 175 cm^2 flasks, then washed and detached from the flask as described previously. 2)- To the cell suspension 7 ml of RPMI/10% FCS were added. The cells were pelleted at 1000 g for 8 minutes. 3)- They were then re suspended in 5 ml ice cold PBS and re-pelleted, this was repeated twice. 4)-The cells were resuspended to a conc. of approx. 1-2 x 10^7cells ml^{-1} in ice cold PBS, 0.5 ml of the cell suspension being placed in each of the 0.4 cm electroporation cuvettes. 5)- To each electroporation cuvette 20µg plasmid DNA was added and mixed well by flicking the tube. 6)- The cuvettes were then incubated on ice for approximately 5 mins. 7)- The cells were electroporated using the following electroporation parameters: 250 µF (using the capacitance extender) for time 4-5 ms. 8)- After pulse delivery, the cuvettes were incubated on ice for a further 5 minutes. 9)- The contents from each cuvette was placed into a 175 cm^2 flask containing fresh culture medium. 10)- The cells were gassed with CO_2 and incubated at 37°C. They were left for 3 days before incubating with geneticin.

Selection for geneticin resistant cells.

Seventy two hours after transfection of 3T3 cells with plasmids carrying the geneticin resistant genes, the cells were incubated with growth medium containing geneticin at 1 mg/1 ml. The medium was replaced every 24 hours, then every 3 days, for approx. 3 weeks. After this time individual colonies were transferred in to single wells of 24 well plate (Nunc) using an inoculation loop. Their medium was replaced with fresh

growth media containing geneticin at 1 mg/1 ml every 72 hours, until the cells were confluent in the wells, the contents of each well were then transferred to a single 25 cm culture flask (Nunc) and incubated with 5 ml growth media containing geneticin at 1 mg/1 ml. This medium was replaced every 3 days until the cells are confluent when they could be used for experiments or frozen down as described before.

2.7. Binding assay reagents and methods

component	volume
25 m M HEPES	5 ml
0.2% BSA	5 ml
SFM	to 50 ml

Table.2.9. Formula of binding buffer.

Binding Assay

Several clones of the geneticin resistant cells were seeded at a density of $5x10^5$ cells per well in 24 well plates, gassed and incubated overnight in the normal manner. On removal from incubator they were washed twice with SFM and allowed to cool to 4°C. Aliquots of binding buffer (0.5 ml) containing single concentration of the iodinated ligand was added to the cells. Eight of the sixteen replicate wells also received non-radiolabelled ligand at 1000 times the concentration of the radiolabelled ligand in order to assess non-specific binding. The cells were then re incubated at 4°C, on ice

for 8 hours, after which the residual ligand was removed by two washings of ice-cold SFM.

After this the cells were lysed and removed by the addition of 0.5 ml of 1M NaOH.

The radioactivity was then read on the gammacounter.

Calculation of radiotracer concentration

Ratio of ^{125}I : [Nle^4,D-Phe^7]-α-MSH, 1:1

1 mmole $^{125}I = 1x10^{-3}$ moles [Nle^4,D-Phe^7]-α-MSH

Specific activity of carrier free Na $^{125}I = 80.5X10^{12}$ Bq/m mole

1 mole [^{125}I-Tyr^2][Nle^4,D-Phe^7]-α-MSH = $80x10^{12}x10^3$ Bq

1 Bq = 1 disintegration per second

1 Bq = 60 disintegration per minute

The efficiency of the gammacounter was 70% (as reported by Sahm, 1994)

Therefore: 1 mole [^{125}I-Tyr^2]-[Nle^4,D-Phe^7]-α-MSH = 80.5 x 10^{12} x 10^3x 60 x 70/100 = 3.38 x 10^{18}cpm.

2.7. HPLC analysis of MTX-NLDP

MTX-NLDP NLDP and MTX samples were dissolved in 0.05M phosphate buffer pH 2.7 at concentration of $1.3x10^{-5}$M. 50 µl samples were analysed by A C18 column 25cm in length and 2.5cm in diameter (Vydac®) reverse phase HPLC using a linear gradient. The solvents were 80% phosphate buffer pH 2.7 / 20% acetonitrile (A) and 95% acetonitrile / 5% phosphate buffer pH 2.7 (B). A U.V detector was employed 307nm. Flow rate was 1.5ml/minute. All reagents employed were of analytical grade.

The table below shows the gradient of the two solvents A and B used for the HPLC analysis and the time intervals.

Time (minute)	% A	% B
0	80	20
15.0	0	100
30.0	0	100
40.0	80	20
80.0	80	20

Table 2.10. gradient of solvents A and B.

2.8. Synthesis of the polyamine conjugate)

The synthesis of the polyamine conjugates was carried out by Simon Carrington, School of pharmacy, Bath University, however the main points of the methods used will be described. A full description of the methodology is described by Carrington *et al.*, 1996, Carrington *et al.*, 1997 and Qarawi *et al.*, 1997.

General details

Amide bond formation by dicyclohexylcarbodiimide (DCC)/N-hydroxybenzotriazole (HOBt) condensation-General procedure; The carboxylic acid (1 equiv.), DCC (1.5 equiv.) and catalytic HOBt (0.05 equiv.) were dissolved in solvent and stirred at 25°C.

The precipitate was filtered off and the filtrate was evaporated *in vacuo*. The residue was purified by flash column chromatography on silica gel to yield the desired amide.

Removal of carbobenzyloxy (Z)-protecting groups by hydrogenolysis-General procedure

A solution of Z-protected polyamine in methanol (MeOH) was added to 10% palladium on carbon. The mixture was hydrogenated at atmospheric pressure for 24 h and then filtered through celite. The filter cake was washed with MeOH (3x20ml) and the combined filtrate was concentrated *in vacuo*. The residue was purified by flash column chromatography to yield the deprotected polyamine.

The general methodology for the synthesis of several of these conjugates is presented here as an example

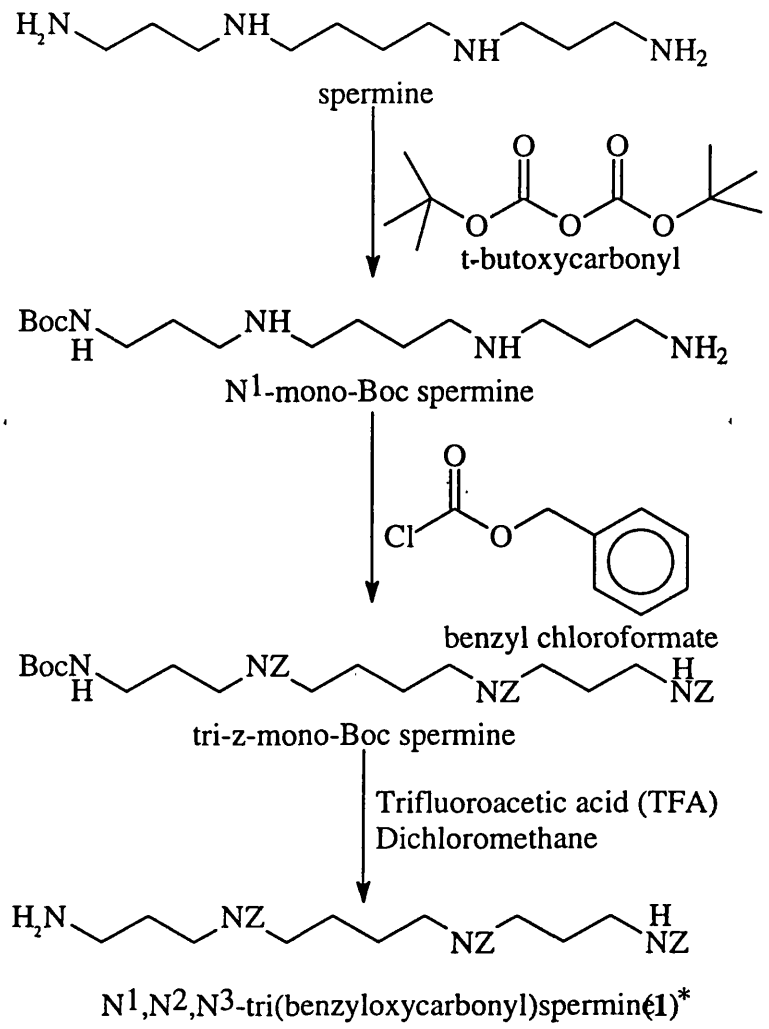


Fig 2.1. General methodology for the generation of spermine protected with benzyloxy carbonyl (Z) groups.

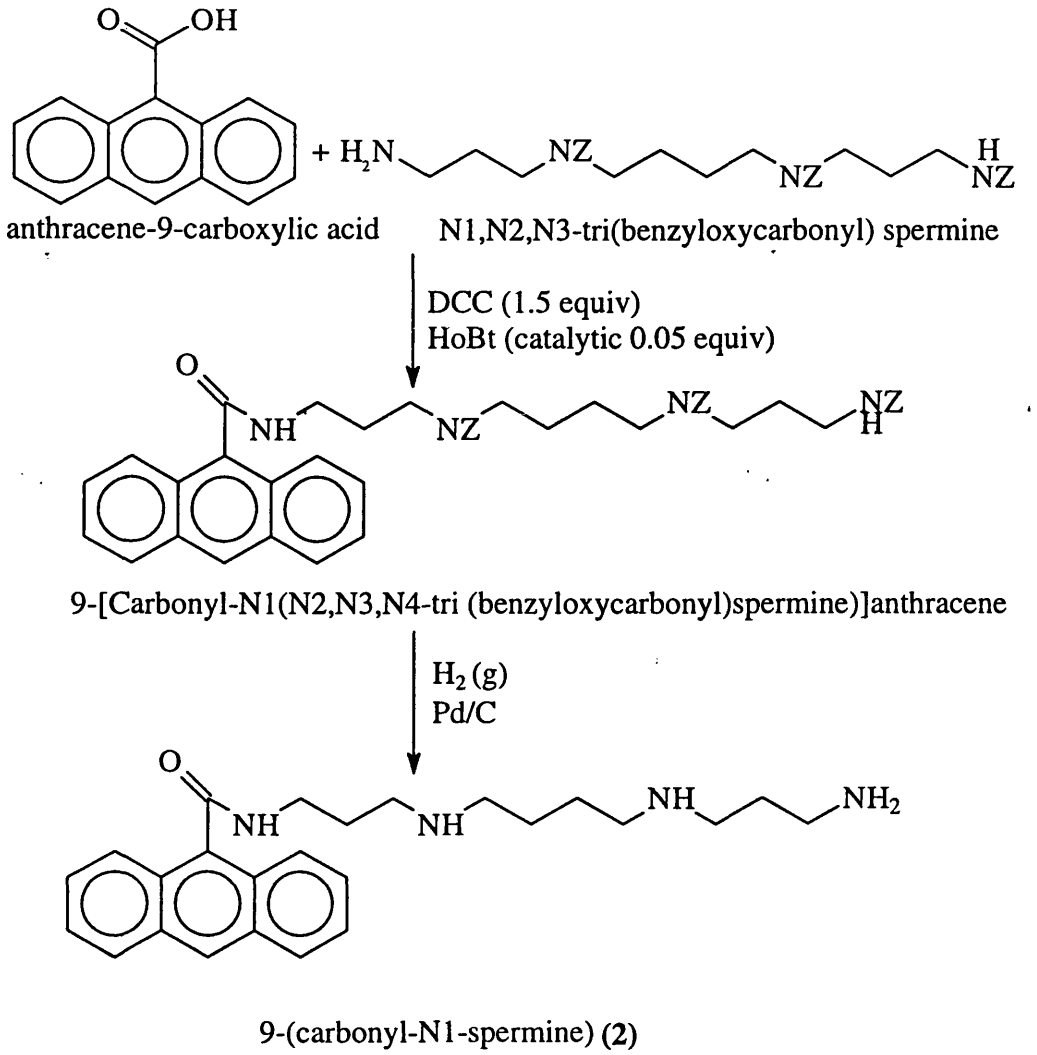


Fig 2.2. General methodology for the synthesis of 9-(carbonyl-N1-spermine)anthracene (2).

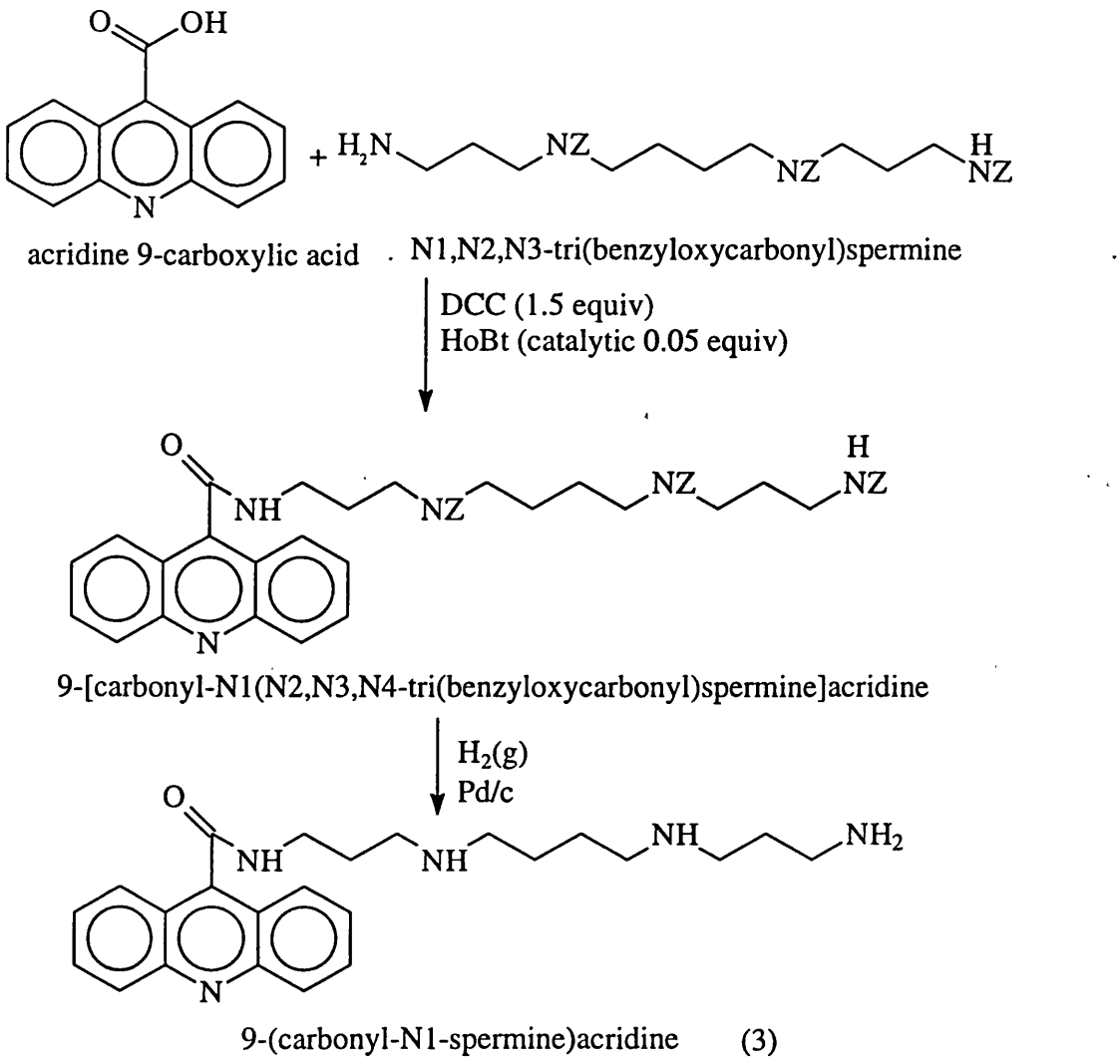


Fig 2.3. General methodology for the synthesis of 9-(carbonyl-N1-spermine) acridine (3)

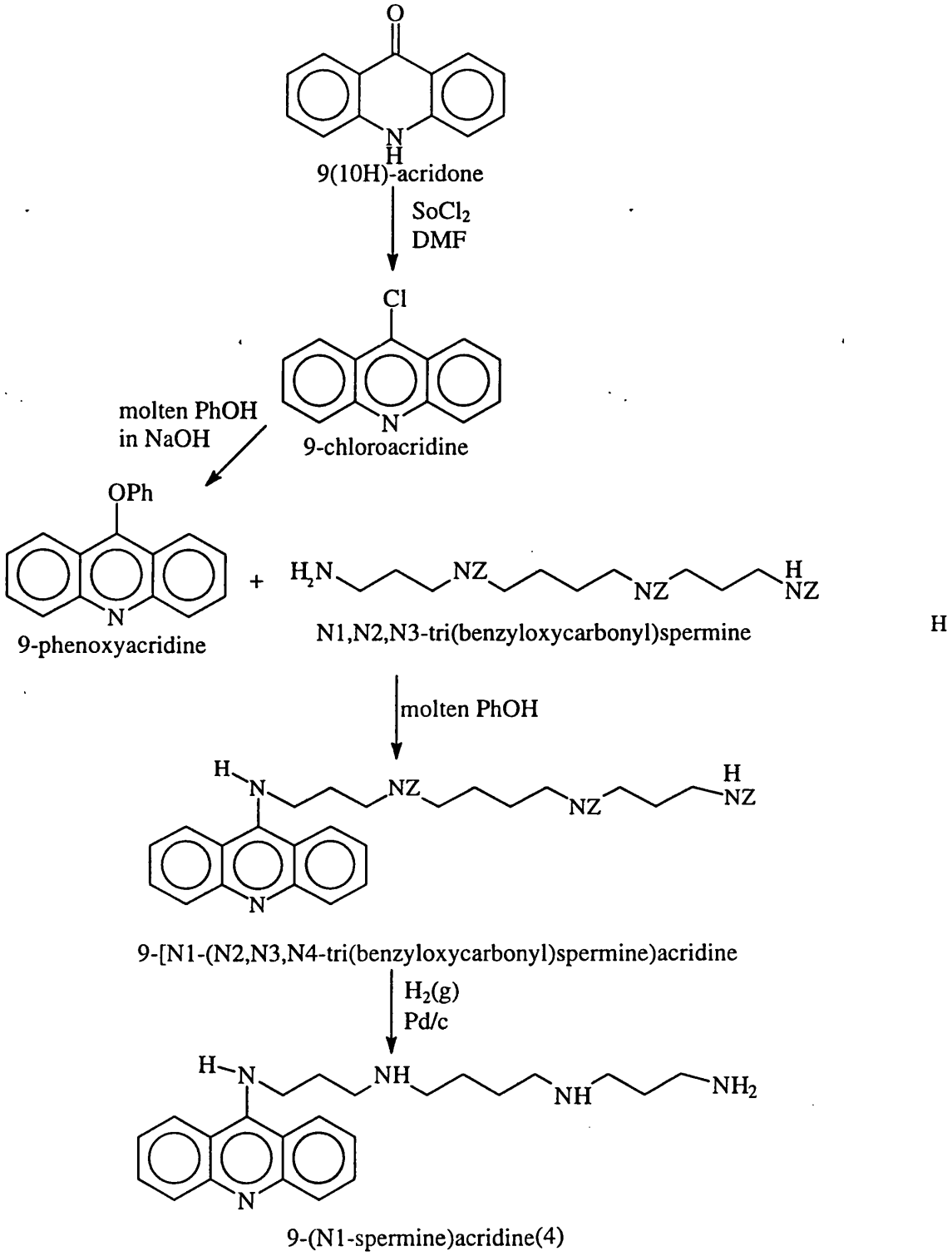


Fig 2.4. General methodology for the synthesis of 9-(N1-spermine)acridine (4)

2.9. Peptide synthesis

The synthesis of [Nle^4, D-Phe^7]α-MSH peptide and the peptide derivative of Methotrexate (MTX) was carried out by Dr. G.W.J. Olivier, School of Pharmacy, Bath University, however the main points of the methods used will be described.

2.9.1. Reagents

Amino acid derivatives, polydimethylacrylamide-kieselguhr resin (Pepsyn K) and p-[R,S-α-1(9H-fluoren-9-yl)methoxy-formamido-2,4-dimethoxybenzyl]phenoxy-acetic acid (AM-linker) were obtained from MilliGen. Hydroxybenzotriazole (HOBT), trifluoroacetic acid (TFA), diisopropylcarbodiimide (DIC), 1,2-ethanedithiol (EDT), anisol and phenol were purchased from Aldrich. Analytical and semi-preparative HPLC-columns were packed with Techosphere 10μm as stationary phase (HPLC Technology). Preparative HPLC was carried out using a C18 protein-peptide column 25cmx2.5cm i.d. All other reagents were of analytical grade.

2.9.2. [Nle^4, D-Phe^7]α-MSH Peptide synthesis:

Peptides were prepared by solid-phase using Fmoc strategy (Atherton and Sheppard, 1989). The carboxamide forms of the peptides were prepared using the AM-linker on Pepsyn k resin. All the amino acid reagents were employed as their pentafluorophenyl esters with the exceptions of serine, where the 3,4-dihydro-4-oxobenzotriazin-3-yl ester was used, and Fmoc-D-Phe-OH, which was treated with DIC and HOBT to form its HOBT ester in situ.

Side chain protecting groups were as follows:

Arginine; methoxytrimethylbenzenesulphonyl (Mtr)

Glutamic acid; t-butoxy (OBut)

Histidine; t-butoxycarbonyl (Boc)

Lysine; t-butoxycarbonyl (Boc)

Serine; t-butyl (But)

Tyrosine; t-butyl (But).

In each case a four fold molar excess of reagents was used.

Deprotection and cleavage was effected by the use of 2% EDTA, 2% anisole and 1% water in TFA for 12 hours, at room temperature. Peptide purification took place using semi-preparative HPLC with a gradient elution of 0.1% TFA in water and 0.1% TFA in acetonitrile water (70:30) at a flow rate of 3 ml per minute.

The eluent was monitored by U.V spectrophotometry at 217 nm. Fractions were collected at 30 second intervals and checked by analytical scale HPLC. Peptide fractions containing the peptide were then pooled and freeze-dried.

Confirmation of peptide purity was undertaken by co-chromatographic techniques with purchased [Nle^4, D-Phe^7]α-MSH (Sigma Chemical Co.), FAB-MS and MALDI-TOF MS: α-MSH M+H calculated; 1664.8, found 1664; [Nle^4, D-Phe^7]α-MSH , M+H calculated 1646.8, found 1647.

Stock solution of peptide (1 mg/ml) was made up in sterile 0.1 mM HCl and was stored at 4°C prior to usage.

2.9.3. Synthesis of MTX-[Nle4, DPhe7]α-MSH:

To prepare N-MTX-[Nle4, DPhe7]α-MSH, Fmoc-[Nle4, DPhe7]α-MSH on resin was deprotected, at the N terminus, using 20% piperidine in DMF. A mixture of MTX and diisopropylcarbodiimide (DIC) in DMF were added to form the N-MTX-[Nle^4, $DPhe^7$]α-MSH product. FAB-MS was used for confirmation of the identity. It is noteworthy that N-MTX-[Nle^4, $DPhe^7$]α-MSH was expected to be a mixture of two products coupled to the α or δ carboxyl groups of the glutamate residue of MTX. Also racemisation at this glutamate residue occurred making the product a mixture of 4 compounds. HPLC analysis suggested similar amounts of the four species.

Chapter 3

***In vitro* evaluation of the toxicity of a selection of anti cancer agents on B16 cells using an optimised microtitre MTT assay.**

3.1. Introduction

Many biological assays require the measurement of surviving and/or proliferating cells. This can be achieved by several methods e.g., counting cells that include/exclude dye, as an indicator of cell membrane integrity, chromium release, in which radioactive chromate bound to cellular protein is released as a function of cell lysis, measuring incorporation of radioactive DNA precursors such as [^{3}H] thymidine or [^{125}I] iodo-deoxyuridine) during cell proliferation, as an index correlating inhibition of DNA synthesis with cell death, *in vitro* colony formation techniques (Roper and Drewinko, 1976), and measuring the metabolism of tetrazolium bromide to formazan by the de-hydrogenase enzymes in the mitochondria of living cells, as in the MTT bioassay (Mosmann, 1983).

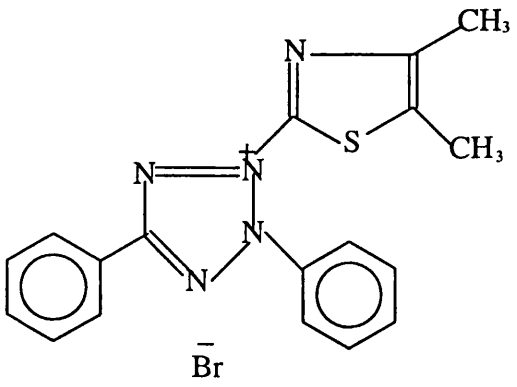


Fig 3.1. Chemical structure of 3-[4,5-dimethylthiaziol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

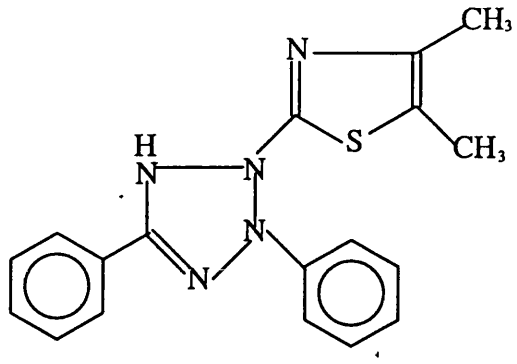


Fig 3.2. Chemical structure of (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan (MTT formazan)

MTT assay

For this study the MTT assay was chosen for the following reasons. It is a simplified cellular cytotoxicity assay. The assay appears suitable for initial stage *in vitro* drug screening (Alley *et al.,* 1988). This approach to *in vitro* screening has the potential to identify new agents with perhaps novel mechanisms for the following reasons time taken for the assay and to process results is relatively short, there are no radiolabelled substances involved, it could yield accurate results which would be easily understood as they could be directly related to cell viability, and finally the reagents used in the assay are relatively cheap to buy.

Vital parameters that may vary between different MTT assays; include inoculum density, culture conditions, duration of drug exposure, duration of the recovery period after drug exposure and the nature of the end point used to quantify drug effects (Alley *et al,* 1988)

Therefore inter assay comparisons, evaluations and interpretation of drug action on the basis of median inhibitory concentrations from data collected among different cell lines are extremely difficult.

Because of these difficulties before testing cytotoxicity of novel compounds on a particular cell line using a specific assay conditions, it is important to be confident that the assay conditions and the toxicity data acquired from such an assay are a true reflection of the toxicity of the compounds under study.

N.B. The term cytotoxic compound is used here to mean compounds that inhibit cell division and are potentially useful in cancer chemotherapy.

In this chapter, we have used B16 mouse melanoma cells, for the first stage of screening for the *in vitro* evaluation of a variety of anti neoplastic agents. These cytotoxics were chosen to represent the whole range of available anti cancer agents with regard to mechanism of action. They therefore include compounds such as the DNA alkylating agents temozolomide, the anti metabolite fluorouracil, the vinca alkaloids and the anthracycline antibiotics (doxorubicin, and daunorubicin).

Presented below here is a summary of the various classes of anti cancer agents that have been selected and a description of their reported mechanism of action.

Alkylating drugs

The alkylating agents used in chemotherapy encompass a diverse group of chemicals that have in common the capacity to contribute, under physiological conditions, alkyl groups to biologically vital macromolecules. DNA appears to be the most critical target for alkylation (McCormick *and* McElhinney, 1990)

The mechanism of action of alkylating agents may be due to antineoplastic action based on the alkylation power of the compounds. Alkylation may also interfere with synthesis or cross-linking in a number of places, for example an attachment at the 7 position of guanine may prevent H bonding between the chains of DNA, arresting proper replication.

Enzymes responsible for removal of DNA lesions act to protect cells from the cytotoxic effects of alkylating agents (Orren and Sancar, 1987), e.g. The ATase gene codes for the expression of O^6-alkyl guanine-DNA alkyl transferase, which protects cells against the effects of alkylation at the O^6 position of guanine (Pegg, 1990). It has been demonstrated that ATase-deficient cell lines are more sensitive to killing by simple methylating and chloroethylating agents than ATase proficient cells (D'Incalci *et al.*, 1988).

Dacarbazine (DTIC) is reported in the literature to have some activity against malignant melanoma. 'In the treatment of metastatic melanoma, DTIC is considered

the single most effective chemotherapeutic agent available' (Lee *et al.*, 1995) and it is the only drug indicated for treating malignant melanoma in the British National Formulary (BNF, 1997).

DTIC undergoes metabolic N-demethylation to give the cytotoxic metabolite mono methyl triazine, 5- (3-methyl-1-triazino) imidazole-carboxamide (MTIC), which methylates DNA, producing among 12 other DNA lesions, O^6- methyl guanine (Meer *et al.*, 1986). There is increasing evidence to suggest that O^6- methyl guanine is the principle cytotoxic event following DTIC administration and that ATase gene expression may be a major factor in cellular resistance to such agents (Lee *et al*, 1993).

It appears that there is a difference in the rate and extent of N-demethylation of dacarbazine between humans and rodents. For instance it has been observed that following administration of dacarbazine, plasma levels of MTIC are much higher in rodents than in humans. Also studies in the rat have shown that DNA methylation occurred to a broadly similar level in all tissues following administration of methylating agents, even those requiring metabolic activation (Kleihues *et al.*, 1976).

There is no evidence to suggest tumour activation of DTIC, thus a pro drug form of MTIC , which does not depend on host metabolic activation to unstable species, but relies instead on chemical transformation and which has good pharmacodynamics, presented advantages compared with DTIC (Stevens *et al.*, 1987).

Two pro drugs which do not require metabolic activation are mitozolomide and temozolomide. At physiological pH temozolomide undergoes chemical degradation to MTIC without the requirement of metabolic activation as in the case of DTIC (Tsang *et al.*, 1991). A flow cytometry study has shown that temozolomide induces a block in S (late)-G2-M both in vitro and in mice (Catapano *et al.*, 1987). This block occurs at least two cell divisions after drug treatment, in contrast to many DNA-interacting agents, including mitozolomide (Broggini *et al.*, 1986), which induces a pre-mitotic block a few hours after drug treatment (Baer *et al.*, 1993).

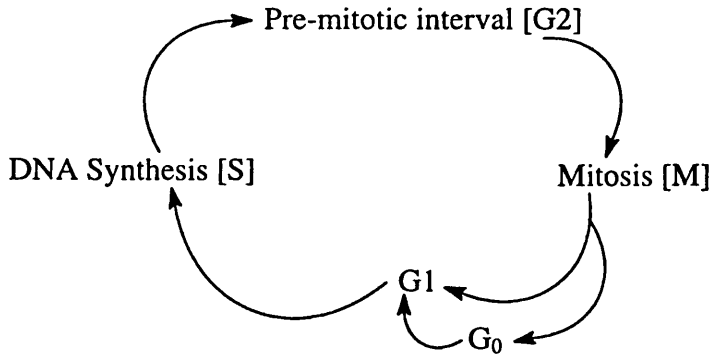
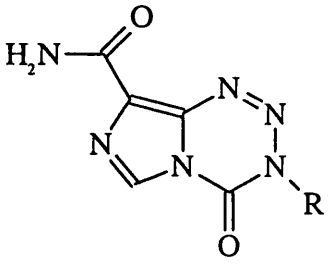


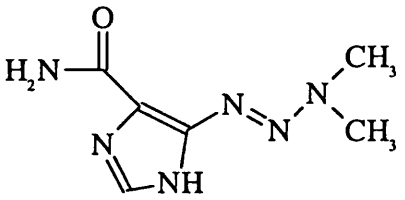
Fig 3.3. The cell cycle; G1 is the period between mitosis and the beginning of DNA synthesis. Resting cells (cells that are not preparing for cell division) are said to be in a subphase of G1, G_0. S is the period of DNA synthesis; G2 is the premitotic interval; and M is the period of mitosis.

Mitozolomide is a pro-drug of the cytotoxic triazine MCTIC (Stevens *et al.*, 1984). It is a chloroethylating agent (Gibson *et al.*, 1986). The mechanism of action of mitozolomide appears to involve chloroethylation of DNA (Gibson *et al.*, 1985). One postulated mechanism of the cytotoxic effect of chloroethylating agents is the formation of guanine-cytosine DNA inter strand cross-links which are produced in a two step reaction from the mono aduct O^6-chloroethyl guanine (Tong *et al.*, 1982). It is thought that ATase reduces the cytotoxicity of mitozolomide by removing the alkyl group from the 0^6 position of guanine before inter strand cross-links can be formed (D'Incalci *et al.*, 1988).



R = CH_3 (temozolomide)

R = $(CH_2)_2Cl$ (mitozolomide)



DTIC

Fig 3.4. Chemical structures of temozolomide, mitozolomide and Dacarbazine.

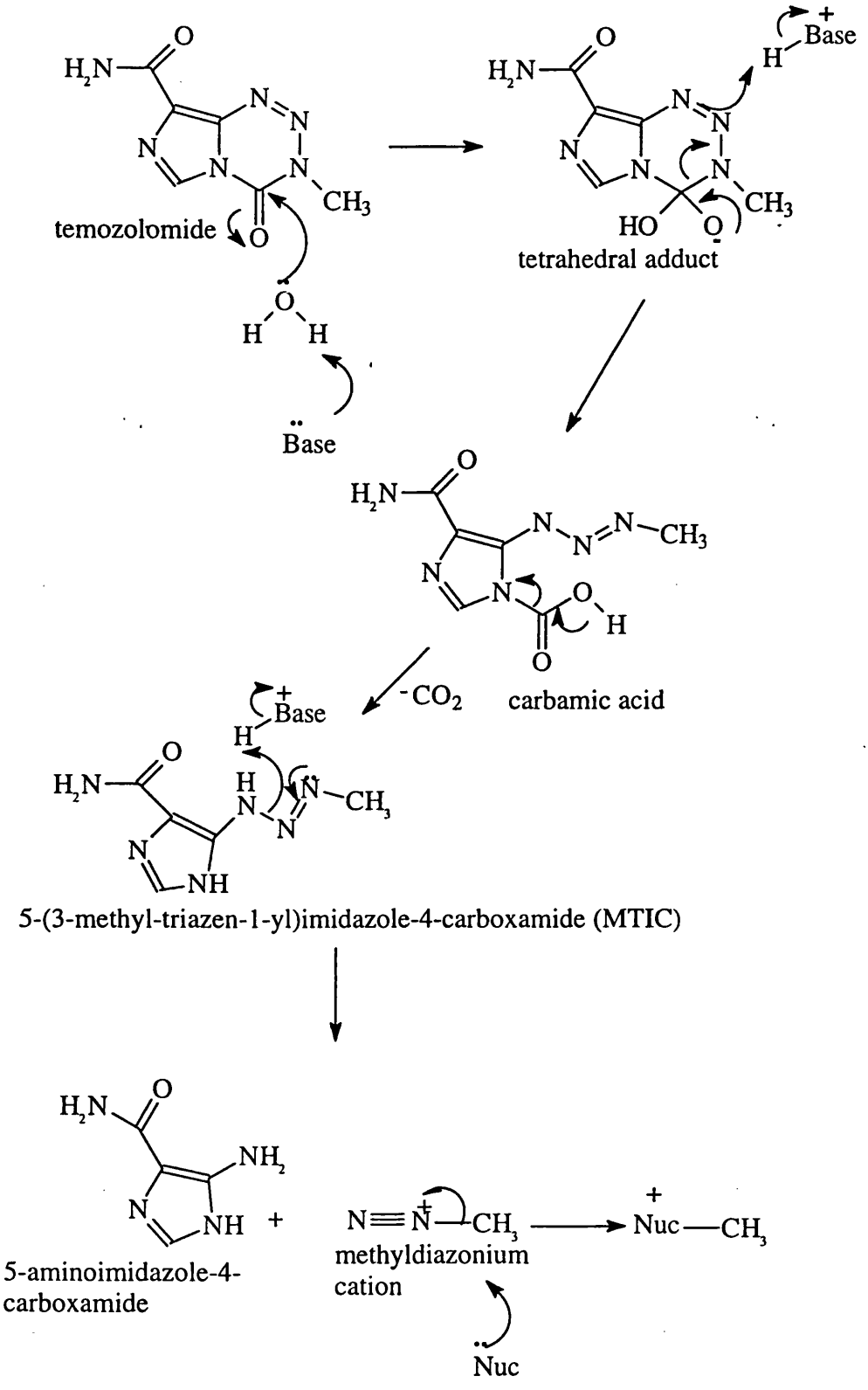
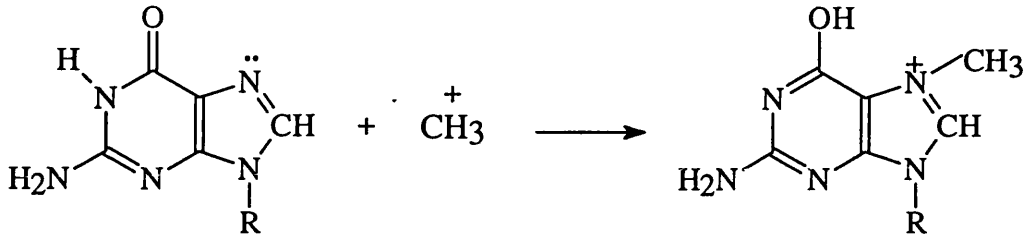


Fig 3.5. Reaction scheme for the base-catalysed decomposition of temozolomide to MTIC and the subsequent decomposition of MTIC to produce methyldiazonium cation. Adapted from Denny *et al.*, 1994.



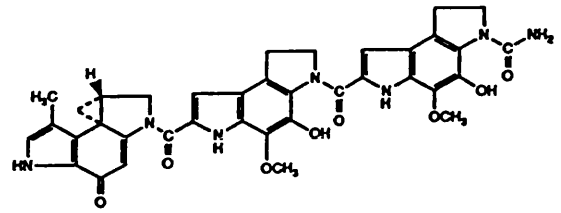
Guanine in DNA Carbonium ion Guanine alkylated at the 7-nitrogen

Fig 3.6. The reaction by which the carbonium ion becomes covalently bonded to the N-7 atom of guanine residue in DNA.

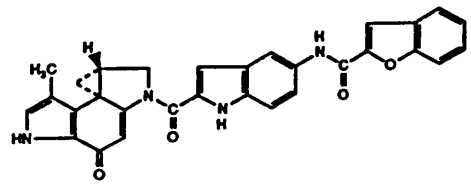
The phase I trial of mitozolomide was completed in 1985 (Newlands *et al.*, 1985) and a number of phase II studies were performed which showed minor anti tumour activity in small cell carcinoma of the lung and malignant melanoma, but severe mylosuppression precluded its further clinical development (Harding *et al.*, 1988).

Temozolomide was selected for further clinical development in view of its experimental anti tumour activity and much lower toxicity in the pre-clinical screen (Stevens *et al.*, 1987). It has shown some promising anti tumour activity against high-grade gliomas, melanoma and mycosis fungoides (Newlands *et al.*, 1992).

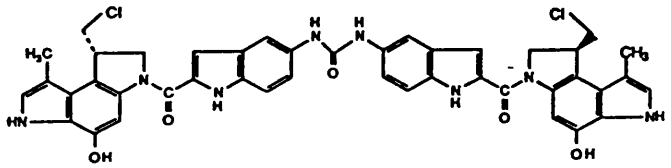
CC-1065 and it's analogues



CC-1065



ADOZELESIN



BIZELESIN

Fig 3.7. Chemical structures of CC-1065, adozelesin and bizelesin

CC-1065 is a very potent new antitumour antibiotic, this antibiotic is produced by *Streptomyces zelenius nov. sp.*, it was discovered at the Upjohn company (Hanka *et al.*, 1978).

Adozelesin and bizelesin are two synthetic second-generation analogues of CC-1065, they have excellent antitumour activity but are devoid of the delayed hepatotoxicity associated with CC-1065 (Lee & Gibson, 1993).

The mechanism of action of CC-1065 is via its activity as a DNA minor- groove binder. It contains a cyclopropylpyrroloindole (CPI) group, which mediates the formation of N^3-adenine covalent adducts in double-stranded DNA in a sequence - selective fashion.

Adozelesin is a DNA minor groove binding, sequence-selective, mono functional alkylating agent modelled on the potent cytotoxic antibiotic CC-1065. Bizelesin, a synthetic bifunctional analogue of CC-1065, contains two DNA-reactive cyclopropylpyrroloindole (CPI) subunits connected with a rigid bis (indolecarboxylic acid) linker, bizelesin features two chloromethyl groups capable of covalently bonding two adenine residues (Mitchell *et al.*, 1991). The covalent binding is between adenine residues six base pairs apart, also the binding is dependent upon the intervening sequence within A/T-rich DNA (Lee and Gibson, 1993).

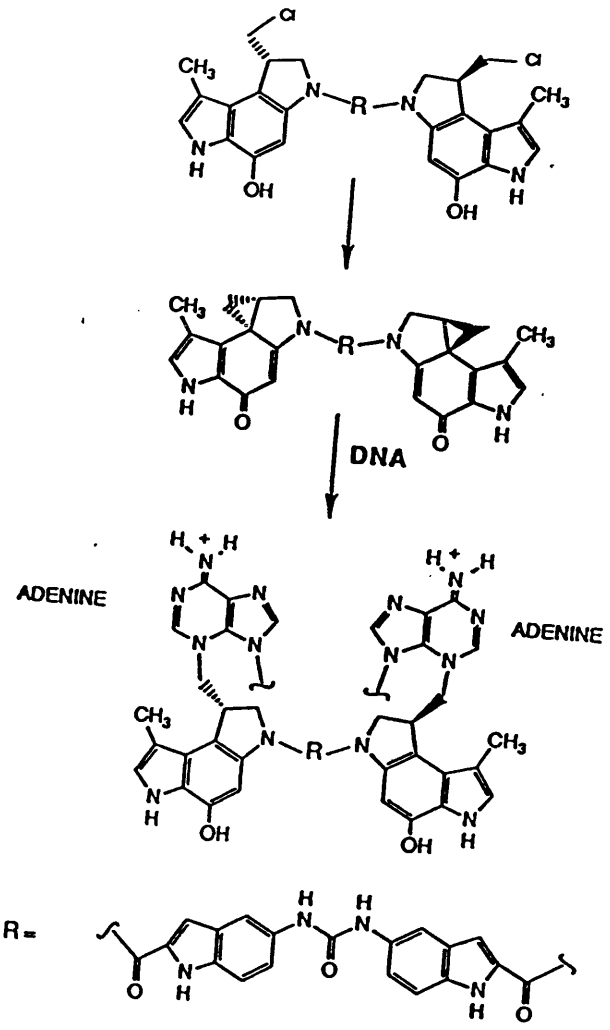


Fig 3.8. Cyclisation of the prodrug, bizelesin to give the cyclopropyl derivative followed by the reaction of adenines of opposite strands to form the cross-linked adduct. From Frederick and Hurley, 1993.

Bizelesin shows good antitumour efficacy both *in vitro* and *in vivo* and is generally 2-30 fold more potent than adozelesin (a monofunctional analogue) when tested against human carcinoma cells (Mitchell *et al.*, 1991). Adozelesin is currently in phase II

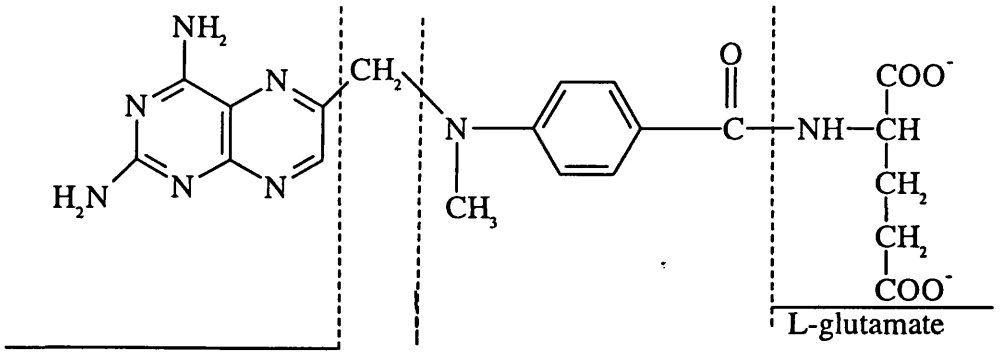
clinical trials, and bizelesin is currently being developed for phase I clinical trials in humans (Fleming *et al.*, 1992).

Anti metabolites

Antimetabolites are compounds that mimic the structures of normal metabolic constituents, including folic acid, pyrimidines or purines. Generally the pharmacokinetics of these agents resemble the natural substances with which they compete or replace and hence inhibit cell metabolism and growth (Balis *et al.*, 1983)

Methotrexate

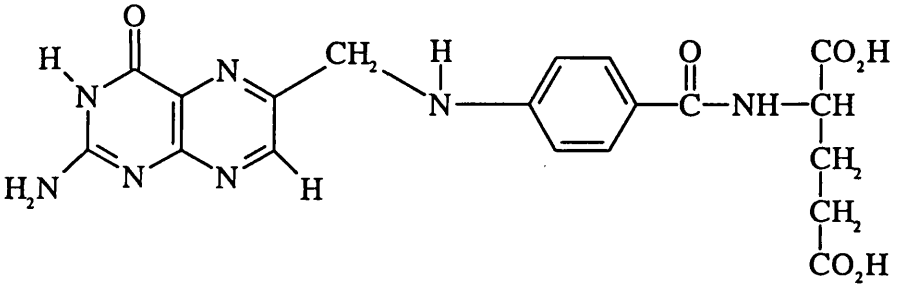
Folic acid is an essential dietary factor, from which is derived a series of tetrahydrofolate cofactors that provide single carbon groups for the synthesis of DNA precursors (thymidylate and purines) and RNA (purines). Inhibition of DHFR leads to partial depletion of these tetrahydrofolate cofactors and a vast accumulation of the toxic substrate, FH2 polyglutamates. Methotrexate is a folate analogue which binds more tightly to dihydrofolate reductase (DHFR) than does folate (Sirotnak, 1985). The conversion of dihydrofolate to tetrahydrofolate is thereby inhibited and the pool of reduced folates required for the synthesis of thymidylate and purines is depleted (Shen and Azarnoff, 1978).



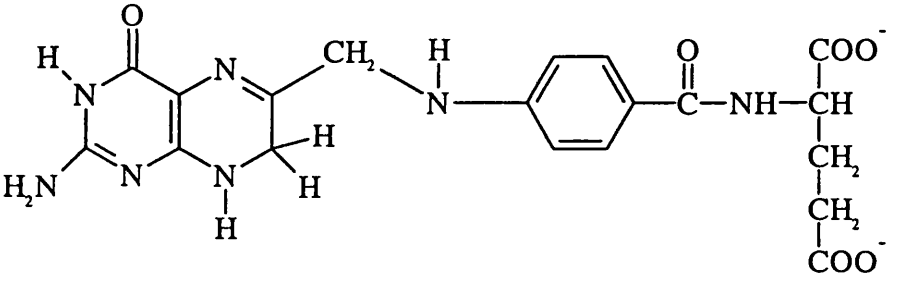
Pteridine ring

P-aminobenzoyl-L-glutamine

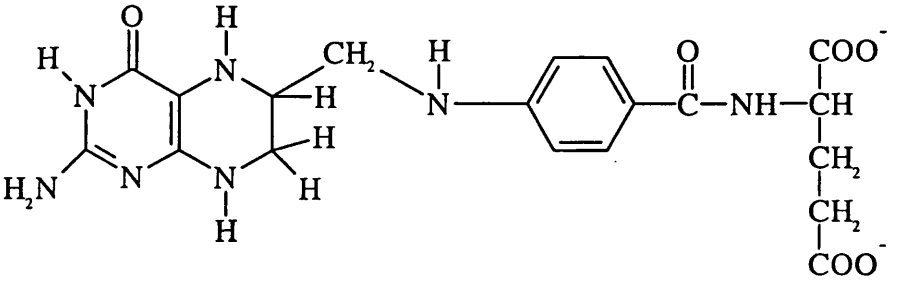
Methotrexate



Folic acid



7,8-Dihydrofolate



5,6,7,8-Tetrahydrofolate

Fig 3.9. The chemically related structures of MTX, Folic acid, 7,8-dihydrofolate and 5,6,7,8-tetrahydrofolate.

Fluorouracil

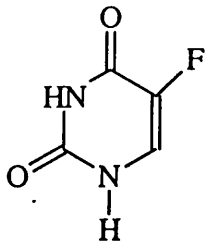
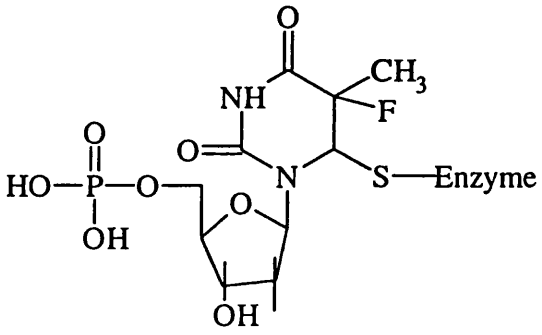


Fig 3.11. Chemical structure of Fluorouracil



5-Fluoro-2'-deoxyuridine 5'-monophosphate (FDUMP)

Fig 3.12. Chemical structure of fluorodeoxyuridine monophosphate. From Coulson, 1988.

Fluorouracil interferes with thymidylate synthesis and therefore with synthesis of DNA. It is converted into a fraudulent nucleotide fluorodeoxyuridine monophosphate (FDUMP). This interacts with thymidylate synthetase and the folate co factors, but cannot be converted into thymidylate because, in FDUMP, fluorine has replaced hydrogen at C5 where methylation would take place, and this carbon-fluorine bond is less susceptible to enzymatic cleavage than the carbon-hydrogen bond. The result is inhibition of DNA synthesis but not RNA or protein.

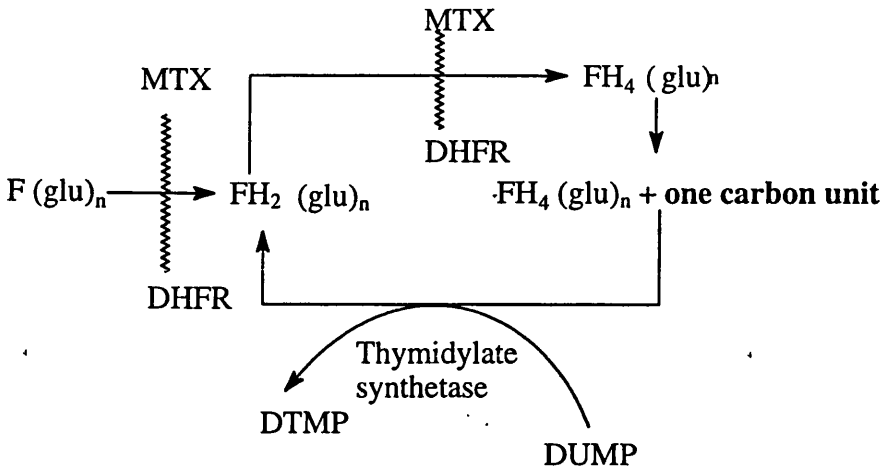


Fig 3.10. Action of Methotrexate on thymidylate synthesis. Tetrahydrofolate polyglutamate [FH_4 (glu)n] function as a carrier of one carbon unit, providing the methyl group necessary for the conversion of deoxyuridylate monophosphate (DUMP) to deoxythymidylate monophosphate (DTMP) by thymidylate synthetase. This is one-carbon transfer results in oxidation of [FH_4 (glu)n] to [FH_2 (glu)n] (Rang *et al.*, 1995).

Methotrexate is transported into cells via the carrier system present for the naturally occurring reduced folates (Reviewed by Sirotnak, 1985). The tetrahydrofolates act more efficiently as enzyme co factors when present as polymers with glutamate than as monomers. MTX is transported in the blood as a monomer but undergoes enzyme-catalysed polymerisation within cells and becomes trapped intracellularly as a polymer.

The blockade of the thymidylate synthetase reaction inhibits DNA synthesis, while cellular production of both RNA and protein continues. An imbalance in growth occurs that is not compatible with cell survival

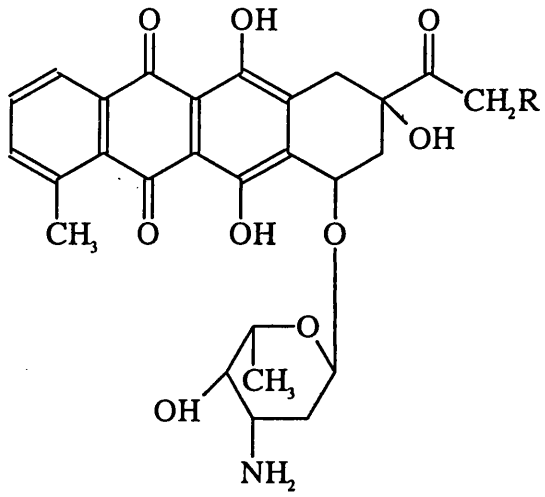
It has been shown that 5-FU is much more lethal to logarithmically growing cells than to stationary cells, however there is no clearly demonstrated effect at a definite stage of the cell cycle.

Vinca alkaloids

The vinca alkaloids are naturally occurring, dimeric indole derivatives. Isolated from the periwinkle plant, *Vinca rosea* (Balis *et al.*, 1983).

Mechanism of action; although the antitumour effect of the vinca alkaloids has been largely attributed to their ability to arrest mitosis by dissolution of microtubular mitotic spindles, vinca alkaloids also inhibit a variety of biosynthetic pathways which possibly contributes to their toxicity (Hickman and Tritton, 1992).

Daunorubicin and doxorubicin



R = H (daunorubicin)
R = OH (doxorubicin)

Fig 3.13. Chemical structures of daunorubicin and doxorubicin.

Daunorubicin and doxorubicin are glycosidic antibiotics, they consist of a planar tetracyclic ring linked by a glycosidic bond to the amino sugar. They are produced by the bacterium *Streptomyces peucetius* var. *caesius*. Doxorubicin was isolated by Arcamone *et al.*, 1969. These anthracycline antibiotics and their derivatives are a among the most important of the newer anti tumour agents.

Mechanism of action

They bind preferentially between the bases in double stranded DNA via strong intercalative bonds and weak ionic bonds thus inhibiting biosynthesis of DNA and RNA, targeting to specific enzymes like topoisomerase II . Doxorubicin interferes with DNA breakage reunion by the enzyme, stabilising an enzyme-DNA intermediate in which the two DNA strands are broken and covalently attached at their 5' termini, one to each subunit of the enzyme dimer. These "cleavable complexes" are the principal lesion by which topoisomerase II inhibitors exert their cytotoxic effects (Marsh *et al.*, 1996).

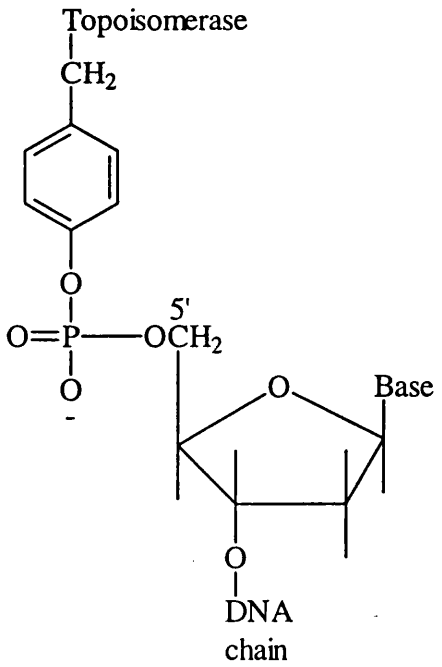


Fig 3.14. Covalent enzyme-substrate intermediate in the action of topoisomerases. The 5'-phosphate end of the cleaved DNA strand is covalently linked to the hydroxyl group of a specific tyrosine residue of the enzyme. From Marsh *et al.*, 1996.

Amsacrine

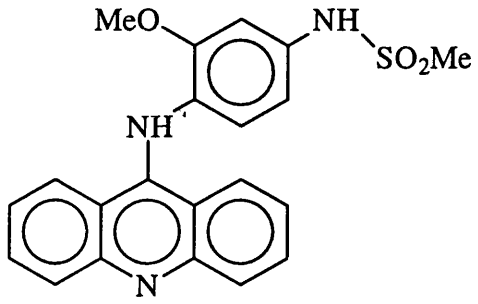


Fig 3.15. Chemical structure of Amsacrine

Amsacrine is a 9-anilinoacridine which displays good anti-anti cancer activity and has found use in the treatment of leukaemia (Llamma *et al.*, 1990). This drug resulted from a structure-activity program in the late 1960's led by Cain investigating substituted heterocycles which have the ability to intercalate the heterocyclic base pairs of DNA.

Mechanism of action of Amsacrine

Amsacrine binds tightly to double-stranded DNA by intercalation of the acridine chromophore between the base pairs. Topoisomerase II is suggested to be the primary target for Amsacrine, which have been shown to induce protein-associated DNA strand breaks by stabilising a "cleavable complex" between topoisomerase II and DNA (Llamma *et al.*, 1990).

Such intercalators have potential as anti-cancer agents. Amongst the analogues tested, a series of acridinium compounds were shown to have good anti tumour activity (Cain *et al.*, 1971). Initial results with a series of 9-anilinoacridine established that having a methanesulfonamide group at the 1'position gave a compound with good activity, water solubility, and stability (Atwell *et al.*, 1972). Studies on Amsacrine and related analogues confirmed its ability to intercalate DNA and also established that the strength of intercalation closely correlates to the observed activity as an anti-cancer agent (Ferguson and Baguley, 1981). Polyamines, such as spermine are known to groove-bind to DNA from either the major or the minor groove (Adlam *et al.*, 1994).

In order to obtain compounds with an enhanced strength of binding and therefore potentially more cytotoxic, we identified target compounds which retained 9-acridine of Amsacrine, but also incorporated a cytotoxic polyamine moiety. The toxicity of these compounds are tested on B16 cells in chapter 4.

3.2. Results

Prior to using the MTT assay to assess the cytotoxicity of anti neoplastic agents on B16 cells control experiments were performed to ascertain the relation between B16 cell number and amount of tetrazolium metabolised to formazan, as well as the optimal time required for the metabolism of tetrazolium (Fig 3.1). This graph confirms the linear relation between amount of formazan produced and number of viable B16 murine melanoma cells. It also shows the optimal incubation time of cells with MTT to be 3 h as beyond this time no further significant amount of formazan is generated.

The optimal seeding density was determined over several time periods (Fig. 3.2) allowing us to estimate the doubling time for B16 cells to be approximately 24 h. From this it was determined that for experiments over 48 h and 72 h the optimal seeding density is 4000 cells per well. These initial seeding densities give enough cell growth to allow conveniently large amounts of formazan metabolite to be generated on addition of MTT, without limiting the growth of the cells as they become confluent.

Optimisation of the MTT assay

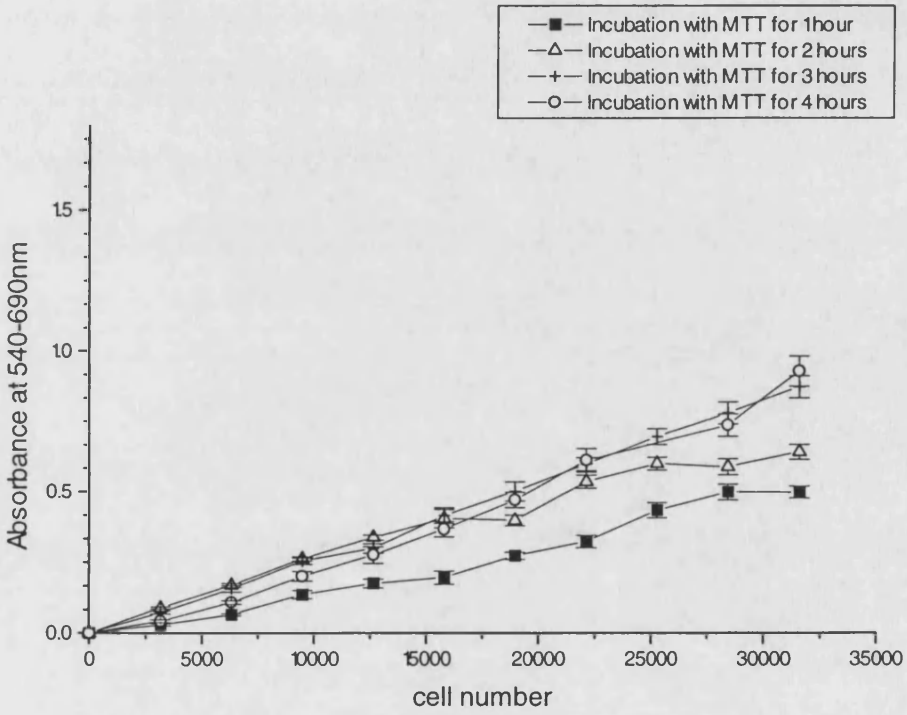


Fig 3.16. Plot of viable B16 murine melanoma cell number with the absorbance of their metabolised MTT at 540nm (test wavelength) and 690nm (background)), after incubation of cells with MTT for various time periods. Each point represents the mean value for 8 independent determinations performed in one experiment.

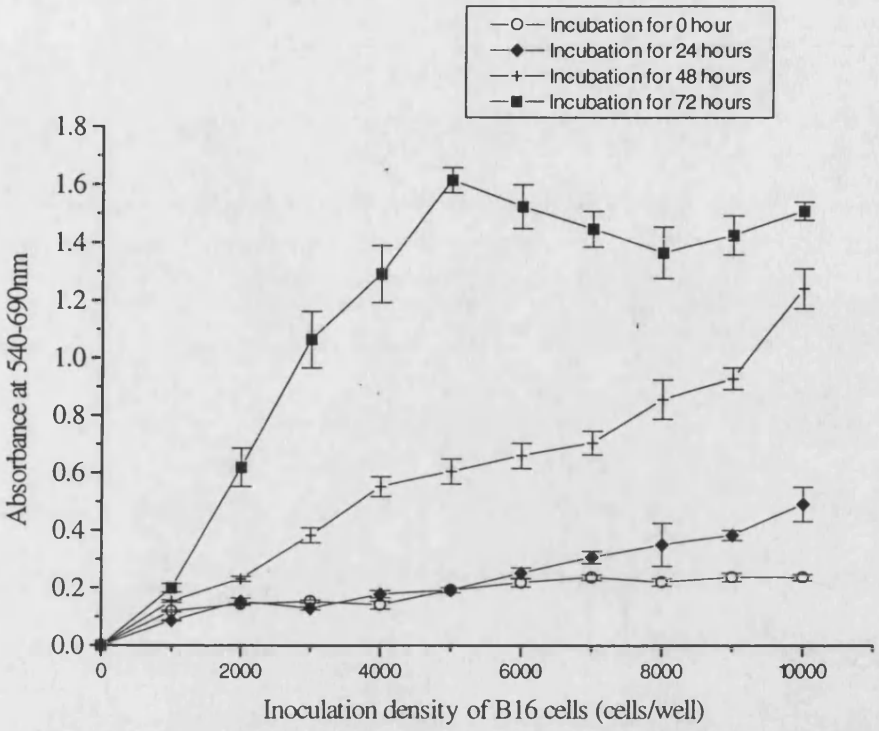


Fig 3.17. Relation between absorbance of metabolised MTT by B16 cells and their inoculation density, after incubation in RPMI media for various time periods, followed by incubation with serum-free RPMI media containing 1mg/ml MTT. Each point represents the mean value for 8 independent determinations performed in one experiment.

The cell doubling time is defined here as the time taken for a certain number of cells to double once. Two cell doubling times is the time taken for a certain number of cells to double twice. So if we start with 10 cells, the number of cells after two doubling times is 10 x 2 x 2 = 40 cells, and the number of cells after three cell doubling times is 10 x 2 x 2 x 2 = 80 cells.

A rough estimation of the doubling time for each cell line investigated enabled us to design a protocol for the MTT assay, appropriate for each cell line. e.g. The cell doubling time for B16 cells was determined as follows; First B16 cells were seeded in a 96 well plate at various densities and the MTT assay was performed immediately after wards, from this a correlation between absorbance and cell number was obtained (fig 3.16). So by incubating B16 cells at lower densities for various time periods and then performing the MTT assay aferwards (fig 3.17) we were able to predict the cell doubling time.

Here is an example of how the doubling time for B16 cells is calculated from figs 3.15 and 3.16. The absorbance of formazan metabolised by 10000 B16 cells is approx. 0.3 (fig 3.15). From figure (3.16) incubating approx. 1200 B16 cells for 72 hours then performing the MTT assay, the absorbance was 0.3. This means the number of doublings the 1200 had made to become 10000 cells is approx. 3 doublings (1200 x 2 x 2 x 2 = 9600). This calculation can be repeated for other cell densities..

Toxicity assays on various classes of anti cancer agents using B16 mouse melanoma cells

The EC_{50} of the toxic compounds examined here have been determined using the non-linear least squares regression analysis using a MINSQ software. The results were normalised with respect to a net of values obtained for ethidium bromide whose in figures (3.18) and (3.19).

Here is a brief description of the non-linear least squares regression analysis; It is a technique used to quantify the relationship between a dependent variable (in this

case the dependent variable is the absorbance of the metabolised MTT) and an independent variable (in this case the cytotoxic compound concentration) resulting in a mathematical model of the relationship.

After a model is fitted the observed points will not necessarily lie on the fitted line. The scatter about the line will depend on how good the model is. If we draw a vertical line from each observed point to the fitted line, square all these distances and add them up we obtain the residual sum of squares. The expression "fitting a line to the observed points" means the process of finding estimates of the slope and intercept that results in a calculated line which fits the observation "best" by "best" we mean the estimates which minimise the residual sum of squares. Hence this method is called the method of least squares. Data were always plotted and examined visually before doing a regression. In order to be certain that the relationship between absorbance and concentration fits our model.

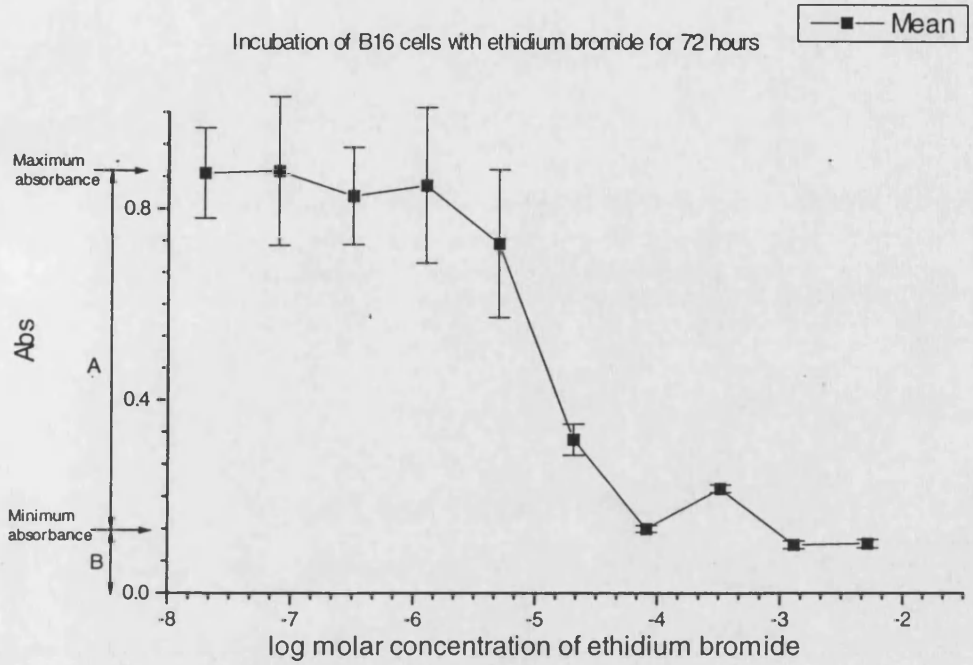


Fig 3.18. Dose dependent growth inhibitory effect of ethidium bromide on B16 cells. Each point represents the mean value for 8 independent determinations performed in one experiment. S.E of less than 20%.

In the toxicity assay above

Abs = A/(1+C/EC) + B

Where is

C: molar concentration of Ethidium bromide

B: minimum absorbance

A: maximum absorbance - minimum absorbance

EC_{50}: extra cellular concentration which give 50% drop in absorbance (that is 50% drop in the value of A)

For the above toxicity assay A=0.786, B=0.103, and the EC_{50}=1.12E-5 M.

The absorbance values of the above assay were normalised by subtracting B from the absorbance values obtained and divide by A then multiply by 100'

The normalised toxicity assay results are presented below

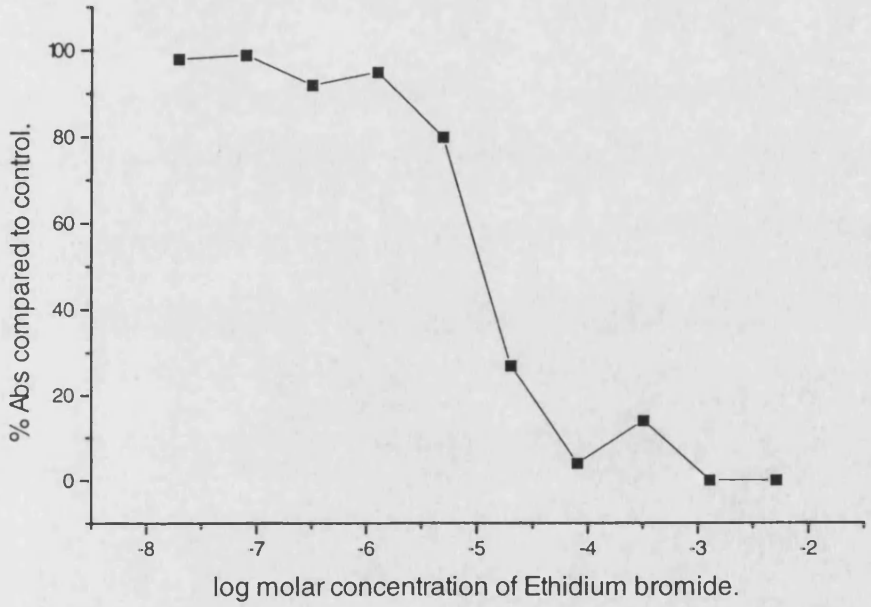


Fig 3.19. A normalised toxicity assay of ethidium bromide on B16 cells.

Cytotoxic compound	Mean EC_{50} (M)	SE [±]	N
Mitozolomide	1.00E-04	3.00E-05	3
Dacarbazine	3.00E-04	1.10E-04	3
Ethidium bromide	1.00E-05	6.33E-07	3
Amsacrine	1.84E-06	1.77E-7	3
Fluorouracil	5.06E-07	1.99E-07	3
Methotrexate	5.60E-08	2.50E-08	3
Vinblastine	5.27E-09	2.90E-09	3
Daunorubicin	4.10E-09	1.29E-09	3
Doxorubicin	1.55E-08	5.65E-09	3
Adozelesin	7.63E-11	1.93E-11	3
Bizelesin	6.72E-12	1.09E-12	3

Table 3.1. EC_{50} values on B16 cells, as determined by the MTT assay, after 72 hours continuous incubation of compounds with B16 cells.

3.3. Discussion of results

In 1983 Mosmann demonstrated that, under appropriate conditions, MTT reduction to formazan is proportional to the number of metabolically viable cells in culture. *In vitro* drug sensitivity measurements utilising tetrazolium reduction have been reported to correlate with cellular protein, dye exclusion and clonogenic assay methodologies under a variety of conditions (Carmichael *et al.*, 1987)

The protocol used here involved use of continuous test compound exposure, following low density cell inoculation and 72 hours culture duration. These conditions were selected for several reasons, for B16 mouse melanoma cells a 72 hours growth interval was required to achieve optimal growth and a minimum of 3 cell doubling times. This is necessary to generate levels of formazan suitable for test compound assays, as demonstrated at the beginning of this chapter. Continuous test compound exposure insures that agents with minimal growth inhibitory activity due to limited solubility in culture medium and / or which require extended contact with cells are detected.

To avoid false-negative endpoints in test compound evaluation, each culture plate contains a standard configuration of test compound blank wells (lacking cells) which permit visual as well as spectrophotometric detection of chemical MTT reduction as well as a means to measure absorbance contributions from chromogenic drug solutions (Alley *et al.*, 1988).

The MTT assay was used to examine the toxicity of various anti cancer agents. These toxicity data are based on the growth inhibitory effect of the extra cellular concentration of test compound under study, on B16 mouse melanoma cells.
The toxicity of three triazines (DTIC, temozolomide and mitozolomide) was looked at. Of these temozolomide did not show any growth inhibitory effect at concentrations $< 1x10^{-4}$ M (assay result not shown). The MTT toxicity assay involves looking at its inhibitory effect after three cell doubling times but temozolomide has been shown, to cause block in S (late)-G2-M at least two cell

divisions after drug treatment (Catapano *et al.*, 1987), even incubating temozolomide with B16 cells for 6 days, that is approx. six cell doubling times did not cause any significant growth inhibition at the above concentration (results not shown). The other two triazines were found to be the least toxic compared to several classes of anti cancer agents (table above) against B16 mouse melanoma. However DTIC is the only anti melanoma agent available for therapeutic use (BNF, number 33, 1997), this serves to inforce the idea that many other factors must be considered when assessing the potential importance of any novel anti cancer agent, such as tissue distribution, time to release active species and biochemical selectivity in addition to toxicity data. The toxicity of ethidium bromide was investigated with a view to using it as a tool because of its fluorescence properties (EC_{50} of 10 μM). The antimetabolites fluorouracil (EC_{50} 0.5μM) and MTX (60nM) were approx. 2 and 3 orders of magnitude more potent than mitozolomide

Vinblastine and daunorubicin had EC_{50} values 5nM and 4nM, respectively. Doxorubicin was approx. three fold less potent than daunorubicin.
The cyclopropylpyrroloindole (CPI) analogues were by far the most toxic compounds looked at with the EC_{50} for adozelesin approx. 80 pM, while that for bizelesin 7 pM. Thus bizelesin is approximately three orders of magnitude more potent than daunorubicin or vinblastine and approx. eight orders of magnitude more potent than mitozolomide. This illustrates the huge potency of bizelesin compared to other cytotoxics. The EC_{50} for adozelesin and bizelesin on leukemia L1210 cells was in the pM range after incubation with the cells for 1-5 hours (Upjon Chemical Company, data sheet). The toxicity of the cytotoxic compounds looked at here seem to be similar to the literature values which were determined using similar MTT assay

conditions e.g. EC_{50} of MTX as reported by Whelan, 1995 (6E-8), EC_{50} of Vinblastine on the human gastrointestinal carcinoma cells (HT-29), was 3nM, the EC_{50} of doxorubicin on a small cell lung carcinoma (NC1-H249) was 100nM (Carmichael *et al.*, 1987). The MTT assay was used by Alley *et al.*, in 1988 to investigate the toxicity of doxorubicin on 54 different cancer cells and found that all the cell types looked at had EC_{50} values within one order of magnitude of 10 nM which is the same order of magnitude of our estimated EC_{50} for doxorubicin. The above comparisons may serve to illustrate that the MTT assay used here does not under or over estimate the growth inhibitory effects of compounds under study.

Although the above EC_{50} values give an indication of the relative potency of various cytotoxics on B16 cells, they are not enough on their own, when assessing the potential use of any of them in forming the toxic component in a drug delivery system for B16 mouse melanoma cells. The mass of drug internalised, which is responsible for such toxicity data, also needs to be measured.

Chapter 4

The Growth inhibitory effects of novel spermine analogues on B16 murine melanoma cells.

4.1. Introduction

Polyamines are generally structurally simple aliphatic compounds consisting of two or three flexible carbon chains that are connected by basic nitrogen atoms. They are often substituted with primary amino functional groups on each end of the chain, but they may also contain basic secondary amines within the chain. The aliphatic polyamines spermidine and spermine are natural constituents of most living organisms (Heston, 1991). At physiological pH, most naturally occurring polyamines are fully protonated, the pKa values for spermine are 11.50, 10.95, 9.79 and 8.90 (Takeda *et al,* 1983; Usherwood and Blagbrough, 1989), therefore the structure of spermine at physiological pH is polycationic: $NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_2^+(CH_2)_3NH_3^+$.

Function of polyamines

One facet of the polycationic nature of polyamines is that they are able to interact with anions. Electrostatic interactions of polyamines with anionic sites of macromolecules (nucleic acids, proteins, anionic sites of lipid membranes) is the mechanistic basis for the majority of biological polyamine function (Heston, 1991). Polyamines are increasingly recognized as having an important role in many cellular processes, including cell growth and replication (Heby and Persson, 1990). They are known to have anti-tumor activity which is attributed to general depletion of polyamine pools, down-regulation of enzymes such as ornithine decarboxylase and

spermine/spermidine-N^1-acetyltransferase (Porter *et al.*, 1991), or DNA binding and interference with DNA transcription (Feuerstein *et al.*, 1990).

Interaction of polyamines with DNA

Evidence for DNA interactions, obtained from experiments conducted in cell-free systems, includes the ability of polyamines to precipitate DNA and to raise the melting temperature of natural DNA (Heston, 1991). The positive charges of polyamines interact with the negative charges on the sugar-phosphate backbone of DNA causing them to bind from either the major or minor groove (Rodger *et al.*, 1995). This electrostatic bonding can be further supplemented by hydrophobic van der Waals' interactions between methylene groups in the polyamine chain and methyl groups on thymidine (Adlam *et al.*, 1994).

Cellular uptake of polyamines

Translocation of charged polyamines across the cell membrane requires a transporter protein, this is an energy requiring active transport process (Rinehart and Chen, 1984). Seiler and co-workers (1990) showed that, in most cells, the polyamine uptake process is saturable, carrier mediated and energy dependent, and that the rate of uptake is reduced at 4 °C compared with that at 37°C. They have also shown that the antibiotics valinomycin and gramicidin curtail polyamine transport in almost all cells studied (Seiler *et al.*, 1990).

The use of other polyamine sources in addition to *de novo* synthesis has been demonstrated for tumor growth (Seiler *et al.*, 1990), growing solid tumors (Moulinoux *et al.*, 1989), and for normal rapidly growing cells (Jann *et al.*, 1978) or organs like the prostate (Heston, 1991). In neoplasia, the level of polyamine biosynthesis activity is significantly greater than that of the surrounding normal tissue, even when the latter is itself rapidly proliferating such as in the case of intestinal mucosa (Luk and Baylin, 1984). Therefore, polyamine active transport systems may be exploited both in drug delivery and tissue targeting (Cohen and Smith, 1990).

The use of spermine to target drugs whose site of action is the DNA

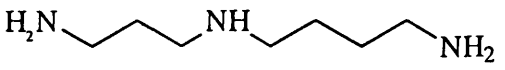
Spermine is an attractive candidate for the targeting of drugs whose site of action is nuclear DNA, because of two important features. The nature of its interaction with DNA, polyammonium salts bind to DNA through an electrostatic interaction, with relatively high affinities (Cohen and Smith, 1990). Also of significance, it has been shown that whilst constrained to remain close to DNA, the polyammonium cations retain a high degree of freedom or motion within the polycation-DNA complex (Wemmer and Scrivenugopal, 1985). Thus, conjugation of a drug to a polyammonium cation may confer significantly enhanced affinity for DNA, but the high mobility of polyamines will allow drugs to locate at their appropriate, specific sites on DNA. The existence of an active uptake system for polyamines in a variety of cell types, especially in tumor cells, allows the design of a polyamine based conjugate which is potentially selective for tumour cells.

DNA intercalators incorporate planar aromatic ring systems which bind between the heterocyclic base pairs, perpendicular to the axis of the double helix (Wilson, 1990).

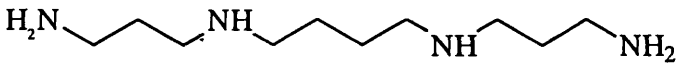
Such intercalation is mediated by interaction between the π-orbitals in the ligand and those in the DNA bases. The 9-aminoacridine derivative amsacrine is an intercalator which has been shown to have anti-cancer activity, currently finding use in the clinic to treat leukemia (Baguley, 1991). Structure-activity relationship studies of a series of analogues have shown that the strength of DNA intercalation closely correlates with the anti-tumor activity (Ferguson and Baguley, 1981).

In order to obtain compounds with enhanced cytotoxicity, conjugates with bifunctional modes of binding to DNA were designed. Studies of amsacrine and other ligands with substituents on the intercalating aromatic ring system have shown that such substituents can protrude into one of the DNA grooves. If this substituent is a known groove-binder, such as spermine, then spectroscopy and computer-aided molecular modelling have shown that it is possible to obtain molecules simultaneously displaying both modes of binding (Adlam *et al.*, 1994; Rodger *et al.*, 1994; 1995). This dual interaction should strengthen the association between ligand and DNA, and therefore increase the cytotoxicity compared to that shown by either the groove binder or the intercalator alone. The objective here was to synthesize compounds consisting either of an anthracene or an acridine unit linked to spermine via an amide or aniline bond at position 9. These conjugates were synthesised and purified by S.Carrington, as described by Carrington *et al.*, 1996; and Qarawi *et al.*, 1997. Their toxicity was determined as described in chapter 2 using B16 murine melanoma cells in the MTT-formazan assay (Mosmann 1983) modified for use with this specific murine cell line. The only drug indicated in the BNF (1997, number 33) for the treatment of melanoma is dacarbazine **7**, a 5-(aminodiazo)-4-carboxamideimidazole. A recent attempt to improve the efficacy of this basic

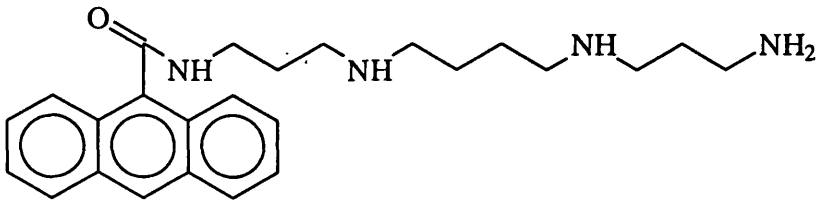
heterocycle for the treatment of melanoma is the development of mitozolomide which incorporates an N-(2-chloroethyl)urea, within a constrained bicyclic analogue of dacarbazine. This analogue of mitozolomide, a potential aziridinium ion containing nitrogen mustard was the subject of a phase I trial which was completed in 1985 (Newlands *et al.*, 1992) and subsequently a number of phase II studies were performed which showed minor antitumour activity in small cell carcinoma of the lung and malignant melanoma. However severe mylosuppression precluded its further clinical development (Harding *et al.*, 1988) . Nevertheless, it is of interest that both the existing treatment for melanoma and its latest analog contain at least two basic nitrogen atoms capable of protonation at physiological pH.



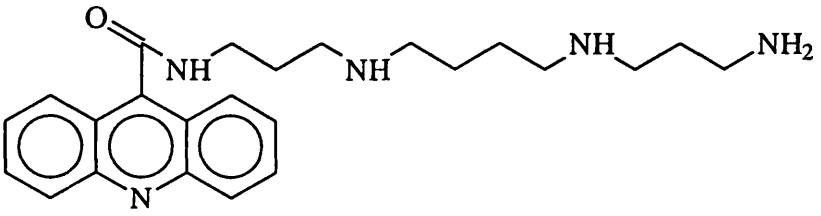
spermidine



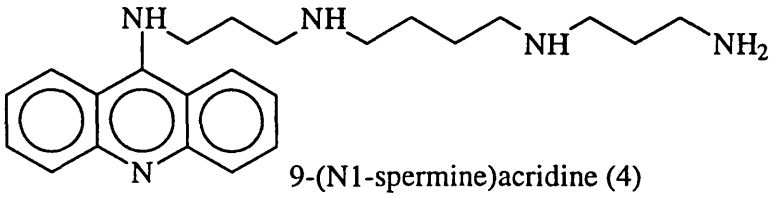
spermine (1)



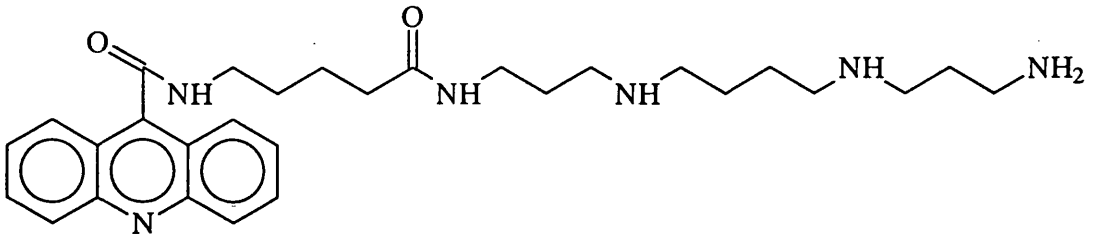
9-(carbonyl-N1-spermine)anthracene (2)



9-(carbonyl-N1-spermine)acridine (3)

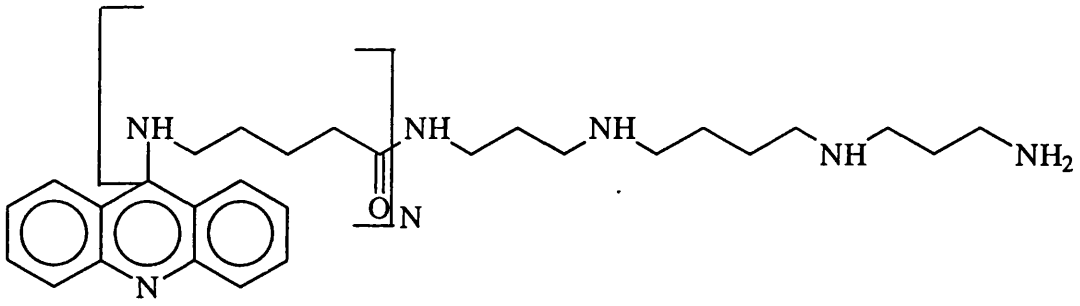


9-(N1-spermine)acridine (4)



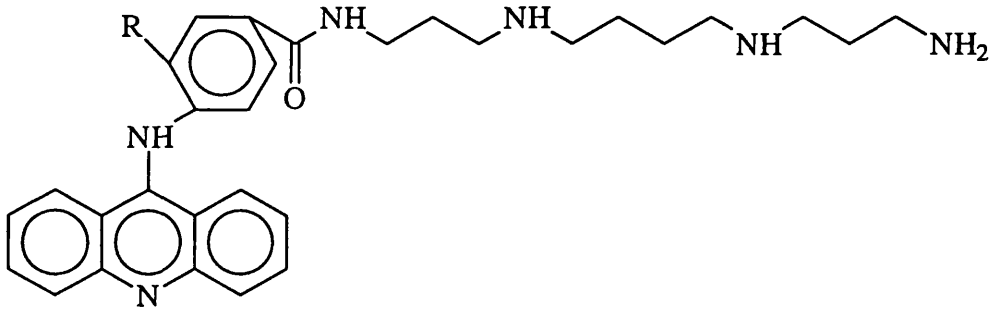
N1-(acridine carbonyl-5-aminopentonyl)spermine (5)

Fig 4.1. Chemical structure for spermidine , spermine and the polyamine conjugates 2 to 5.



[N=1]; N1-(9-acridinyl-5-aminopentonyl)spermine (6)

[N=2]; N1-(acridinyl-9-aminobutylaminobutyl carbonyl)spermine (7)



[R=OH]; N1-[4'-(acridinyl-9-amino)-3-hydroxybenzoyl]spermine (8)

[R=H]; N1-[4'-(acridinyl-9-amino)benzoyl]spermine (9)

[R=OMe]; N1-[4'-(acridinyl-9-amino)-3-methoxybenzoyl]spermine (10)

Fig 4.2. Chemical structure for polyamine conjugates referred to in this chapter

4.2. Results

Conjugates of spermine with either 9-anthracene or 9-acridine carboxylic acids have been designed in order to achieve a more specific interaction with DNA than the

tricyclic aromatic intercalator or the polyamine groove-binder alone, as described earlier. A comparison of the ability of these conjugates to control the growth of B16 murine melanoma cells was undertaken using the MTT assay (Mosmann, 1983) and their EC_{50} values have been determined using the least squares estimation method table (4.1).

Compound	Mean EC_{50}(M)	SE [±]
9-Anthracene carboxylic acid	>1.2E-3	
9-Acridine carboxylic acid hydrate	>8.0E-4	
Spermine (**1**)	4.5E-4	1.8E-4
1/1 Molar mixture of spermine and 9-anthracene carboxylic acid	2.6E-4	8.0E-5
1/1 Molar mixture of spermine and acridine carboxylic acid hydrate	3.9E-4	8.0E-5
9-(carbonyl-N1-spermine)anthracene (**2**)	2.0E-5	6.99E-6
9-(carbonyl-N1-spermine)acridine (**3**)	5.40E-6	1.10E-6
9-(N1-spermine)acridine (**4**)	1.02E-6	5.57E-7
N1-(acridine carbonyl-5-aminopentonyl)spermine (**5**)	1.93E-6	2.78E-7
N1-(9-acridinyl-5-aminopentonyl)spermine (**6**)	3.0E-5	2.0E-5
N1-(acridinyl-9-aminobutylaminobutylcarbonyl)spermine (**7**)	2.67E-7	1.20E-8
N1[4'-(acridinyl-9-amino)-3-hydroxybenzoyl]spermine (**8**)	9.0E-5	3.0E-5
N1-[4'-(acridinyl-9-amino)benzoyl]spermine (**9**)	9.20E-6	2.30E-6
N1-[4'-(acridinyl-9-amino)-3-methoxybenzoyl]spermine (**10**)	4.92E-6	5.70E-7

Table 4.1. EC_{50} values from 48 h MTT assays of polyamine conjugates on B16 murine melanoma cells. Each EC_{50} value represents the average of 3 independent assays with 6 replicate samples for each data point.

The results from the MTT assays are shown in Table 4.1. Based on the average EC_{50} values at 48 hours, the 9-anthracene carboxylic acid and 9-acridine carboxylic acid both had no detected effect at concentrations below 1.2E-3 M and 8.0E-4 M, respectively. Spermine inhibited cell growth with an EC_{50} of 4.5E-4M and simply mixing spermine with (starting material) aromatic acids showed no increase in potency over spermine alone. This experiment acts as a control for any amide bond

hydrolysis, in order to ascertain if the conjugate is simply acting as a pro-drug for spermine.

The conjugate containing the anthracene unit showed inhibition with an EC_{50} of 2.0E-5 M, an increase in potency of an order of magnitude over spermine whilst **7**, the conjugate containing the acridine analogue had an EC_{50} of 2.67E-7 M, an increase in potency by three orders of magnitude over spermine.

These results show a significant increase in the toxicity of some of the conjugates compared to a mixture of their components. Questions as to whether the observed toxicity of the conjugates is due, in part, to differences in their cellular uptake or caused entirely by a more specific interaction with the DNA need to be addressed. Neither, in these studies, have we addressed the question of whether the conjugates use polyamine transporters in order to gain access to their intracellular sites of action. However, the fluorescence of 9-aminoacridines, e.g. conjugate **7**, will be a useful spectroscopic property in developing convenient, accurate and sensitive assays for this polyamine conjugate in studies of its distribution in biological tissues and fluids.

We examined the toxicity of (**1**), (**4**), (**7**) and (**10**) on B16 cells over various time periods in order to determine whether the observed toxicity could be altered by changing the incubation period.

compound	Incubation period (days)	Mean EC_{50} (M)	SE [±]
1	2	4.50E-4	1.8E-4
1	3	4.30E-4	1.50E-4
4	2	1.02E-6	5.57E-7
4	3	1.16E-6	7.83E-7
4	6	6.83E-7	2.53E-7
7	2	2.67E-7	1.20E-8
7	3	1.06E-7	8.95E-9
7	6	5.77E-8	1.17E-8
10	2	4.92E-6	5.7E-7
10	3	1.65E-5	1.75E-6

Table 4.2. EC_{50} values from MTT assays of **1**, **4**, **7** and **10** following incubation with B16 cells over various time periods. Each EC_{50} value represents the average of 3 independent assays with 6 replicate samples for each data point.

Table 4.2 shows the toxicity of spermine (**1**), 9-acridine spermine (**4**), (**7**) and (**10**) on B16 cells over 2, 3 and 6 days. There is a small difference in the toxicity of each compound on B16 cells over these periods. From these results, we conclude that there is little significant metabolic influence on the observed toxicity data obtained with these compounds.

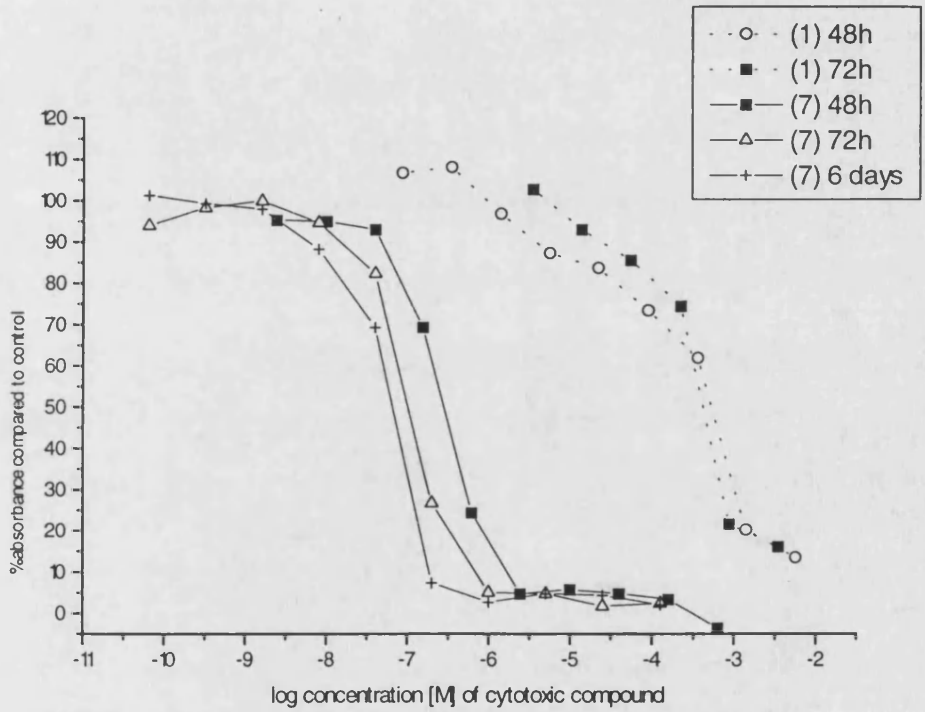


Fig. 4.3. Dose-dependent growth inhibitory effect of **1** (broken line) and **7** (solid line) following exposure of B16 cells to polyamines for various time periods as estimated by the MTT assay. Each curve represents the average of 3 independent assays with 6 replicate samples for each data point. Error bars have been omitted for clarity (SE of all values <20%).

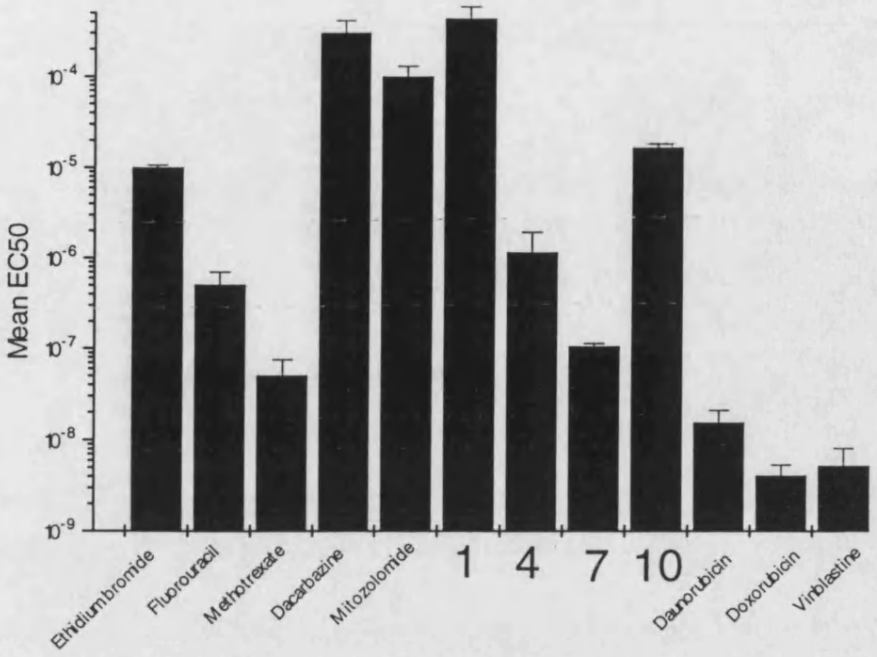


Fig. 4.4. Mean EC_{50} values of a selection of cytotoxic agents compared to spermine (**1**), acridine monospermine (**4**) and (**7**) and (**10**) as determined using the 72 h MTT assay on B16 murine melanoma cells. Each mean EC_{50} value represents the average of 3 independent assays with 6 replicate samples for each data point.

4.3. Discussion of results

Rationale behind the design and synthesis of polyamine conjugates.

Polyamines, such as the naturally occurring tetra-amine spermine, are known to exhibit anti-tumour activity through general depletion of polyamine pools, by down-regulation of key enzymes (Bernacki *et al.*, 1992), or by DNA binding and the resulting interference with transcription. At physiological pH, the amine functional groups are all protonated. These four positive charges are able to interact with negative charges on the sugar-phosphate backbone of DNA with binding from major

and/or minor grooves. Anthracene and acridine containing compounds are known to bind to DNA through intercalation. Studies on acridine analogues have shown a correlation between their efficiency as intercalators and their cytotoxicity (Baguley, 1991). It is therefore postulated that conjugates containing spermine covalently linked to anthracene (**2**) or acridine (**3**) via an amide bond would display bifunctional modes of binding and enhanced cytotoxicity (Adlam *et al.*, 1994). In another acridine analogue (**5**) one 5-aminovaleric acid spacer was incorporated between the spermine and acridine components of the conjugate in order to introduce a region of flexibility between acridine and spermine, allowing the two binding regions to optimise their interactions. Spermine exhibited an EC_{50} value of 450μM and co-administration of the anthracene or acridine carboxylic acids with spermine showed no improvement in potency over spermine alone. The anthracene and acridine conjugates **2** and **3** showed EC_{50} values of 20μM and 5μM, respectively. The synthetic compounds **2** and **3** are more potent than spermine, the potency of the acridine conjugate (**3**) being approximately two orders of magnitude higher than spermine alone. The compound **5** which contain the 5-aminovaleric acid spacer between the acridine and spermine units, did show a significant increase in activity compared to the acridine conjugate **3** with EC_{50} of 2μM. The growth inhibitory effect of novel acridine conjugates which were synthesised by N-alkylation at position-9 by spermine (**4**) or by spermine linked to one or two molecules of 5-aminovaleric acid **7** and **6,** respectively, was also examined. These 5-aminovaleric acid units were incorporated in the conjugates in order to introduce a region of flexibility between the acridine and spermine, allowing the two binding regions to optimise their interactions. Compound **4** had an EC_{50} of 1μM which makes this compound more active inhibitor of B16 cell growth than any compound tested so far, the incorporation of a 5-aminovaleric acid unit (**7**) resulted in a compound which is approx. five fold more potent than **4**, however the incorporation of another 5-aminovaleric acid unit (**6**) resulted in a large drop in potency with EC_{50} of 30μM.

By linking polyamines to 9-anilinoacridines, it is envisaged that the resulting conjugates would display bifunctional modes of DNA binding, the strength of binding being greater than that of the intercalator or polyamine alone. Two compounds were proposed, para- disubstituted benzamide linking spermine to 9-anilinoacridine through an amide bond at the 1'-position **9** and trisubstituted aromatic incorborating the potentially important 3'-methoxy group **10**. The synthetic compound **9** had an EC_{50} of 9μM while **10** had an EC_{50} of 5μM.

The most potent compound in this series was **7** with EC_{50} of 0.27μM. This compound is approximately three orders of magnitude more potent than spermine alone and five-fold more potent than **4**. The growth inhibitory effect of these compounds was not significantly altered by increasing the incubation time with B16 cells in contrast to results seen with other polyamine conjugates reported by Porter *et al.*, 1991. This supports the belief that the main effect of these compounds is due to binding to the DNA rather than metabolic influence. Whether or not the analogue interaction with DNA is solely responsible for the EC_{50} properties is still not at all clear. These are novel spermine analogues which offer a new lead in the design of cytotoxic polyamines with anti-cancer activity. However for the purpose of drug targeting to melanoma these compounds were not considered toxic enough for further evaluation of the relationship between their cellular uptake and toxicity on B16 murine melanoma cells. In the absence of any stability studies on these polyamine conjugates. They were assumed unstable thus the toxicity assay was performed on all of them as soon as they were synthesised. The next step could be a full assessment of the stability of these conjugates, and determination of the biological activity of their degradation products.

Chapter 5

Relationship between cellular uptake and toxicity for the anthracycline antibiotics (doxorubicin & daunorubicin) and the CC-1065 analogues (adozelesin & bizelesin) on B16 cells

5.1. Introduction

In this project we are interested in identifying cytotoxic compounds which are potent enough, in order to be considered as possible candidates for targeted drug delivery to melanoma cells. Having screened a selection of available cytotoxic agents and a series of novel polyamine conjugates, the most toxic compounds were the anthracycline antibiotics (doxorubicin and daunorubicin) and the CC-1065 analogues (adozelesin and bizelesin). In order to assess the potential for using any cytotoxic compound in forming the toxic component in a drug -NLDP conjugate for targeting to melanoma cells, it is necessary to relate the toxicity to the amount of cellular uptake by B16 cells of the cytotoxic compound under investigation. Here we attempt to determine the relation between cellular uptake and toxicity for these compounds. None of the polyamine conjugates was considered sufficiently toxic for further study, due to their relatively high EC_{50}.

Fluorescence of anthracycline antibiotics is one of the most striking physicochemical features of this class of antineoplastic agents. It has been shown that argon laser excitation in a flow cytometer (cell sorter) can be used for rapid detection and quantitation of cellular doxorubicin and daunorubicin fluorescence in a heterogeneous cell population (Krishan *et al.*, 1986). This property has been utilised in developing convenient, accurate and sensitive assays for anthracyclines in tissues and biological fluids, and it is used here to relate cellular uptake of the anthracycline antibiotics to their toxicity on B16 cells. This assay is based on incubating a certain number B16 cells with e.g. EC_{50} concentration of the anthracycline for a period of time then extracting the internalised anthracycline molecules, measuring their fluorescence and reading the corresponding anthracycline concentration from a calibration curve of fluorescence against concentration. Using this procedure the number of anthracycline molecules required to cause 50% inhibition of B16 cell growth can be determined.

FACS analysis of the uptake of daunorubicin and doxorubicin by B16 cells provides a direct qualitative comparison of their cellular uptake. However, FACS analysis is not suitable for a quantitative determination of the concentration of the anthracycline antibiotics producing a particular fluorescence. This is due to the fact that FACS measures the fluorescence emitted from a particular cell or bead, so it is not possible to get a calibration curve of fluorescence against concentration of the anthracycline antibiotics.

FACS provides a very sensitive comparison between the pattern of cellular uptake of each of the anthracycline antibiotics in relation to extracellular concentration, while spectrofluorometer measurements provide a quantitative determination of cellular concentration.

A biological assay was devised to assess the relationship between the mass of cytotoxic agents internalised by B16 cells and the observed toxicity of the cytotoxic agent being examined. The assay was performed on the CPI analogues (adozelesin and bizelesin) and daunorubicin. This biological assay is designed to test the growth inhibitory effect of the same number of molecules of a cytotoxic agent on increasing numbers of B16 cells. This means that if the fraction of molecules removed by a certain number of B16 cells is very small e.g. 1 or 2% of the total molecules available, then doubling the number of B16 cells means that 2 or 4% of the total molecules available will be removed, with minimal (undetectable) effect on the growth inhibition data, and a minute reduction in the number of molecules in the supernatant thus the toxicity of the supernatant remains unchanged. On the other hand if the fraction of cytotoxic molecules removed by a certain number of B16 cells is high e.g. 40% of the initial number of molecules, then doubling the number of B16 cells mean that 80% of total drug molecules are removed by B16 cells, leaving 20% of the initial number of molecules in the supernatant. The reduction in the amount of drug remaining in the supernatant was determined using a second cytotoxicity assay. The concentration of the cytotoxic agent in the supernatant which produces the observed reduction in cell growth

compared to control was read off a standard growth inhibitory curve for the compound being investigated. From the above the total number of molecules available, and the number of molecules remaining in the supernatant after incubation with a certain number of B16 cells can be estimated, from this the number of cytotoxic molecules required to produce a reduction in the growth of B16 cells compared to control cells can be elucidated. Control experiments were carried out to assess the stability of cytotoxic agent in the supernatant. One problem with this assay is its inability to assess the number of molecules remaining on the cell surface which may subsequently be washed away. However this difficulty also exists with other assays, such as the fluorescence assay, designed to estimate the cellular uptake of the anthracycline antibiotics.

5.2. Results

In this chapter the toxicity of methotrexate, doxorubicin, daunorubicin, adozelesin and bizelesin on B16 melanoma cells have been determined following different periods of continuous incubation with B16 cells (fig 5.3). The graph in (fig 5.4) shows a dose-dependent growth inhibitory effect of adozelesin and bizelesin following continuous exposure to B16 cells for 4 hours and 72 hours as determined from the MTT assay.

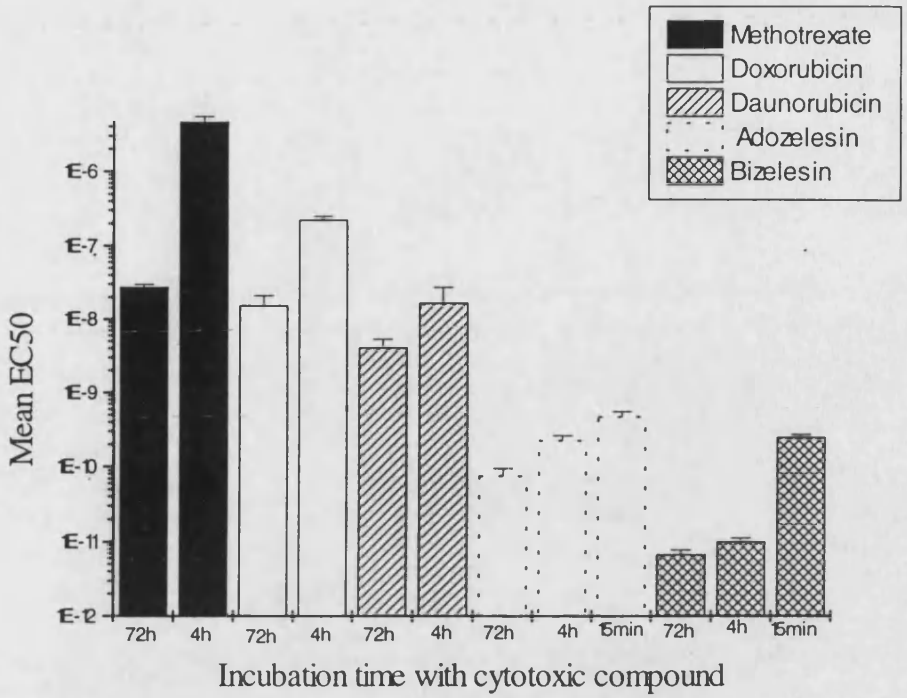


Fig.5.3. Mean EC_{50} values of methotrexate, doxorubicin, daunorubicin, adozelesin and bizelesin as determined using the mtt assay after incubation with B16 murine melanoma cells for various time periods. Each mean EC_{50} value represents the average of 3 independent assays with 6 replicate samples for each data point.

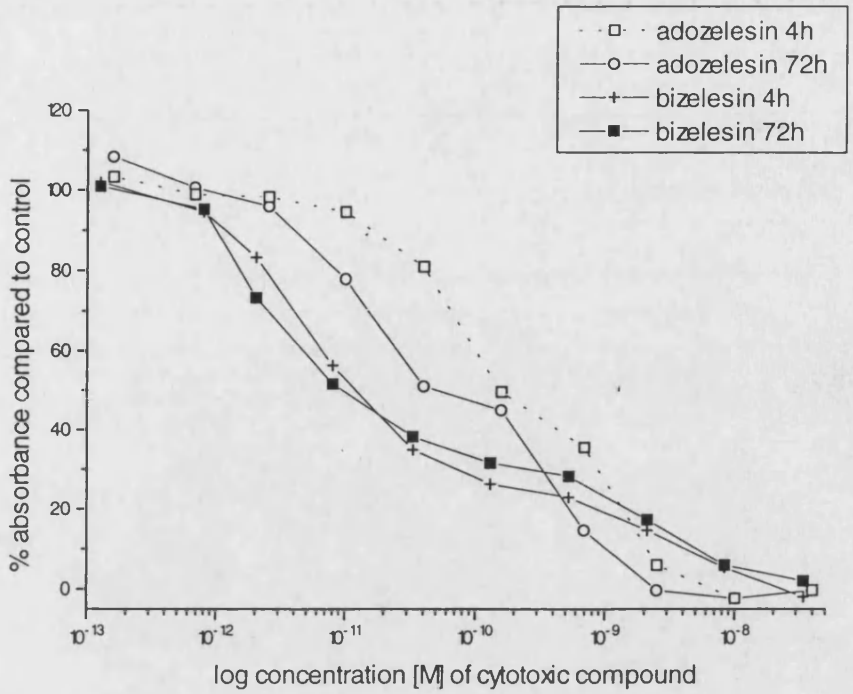


Fig.5 4. Dose-dependent growth inhibitory effect of adozelesin and bizelesin following continuous exposure to B16 cells for 4 hours and 72 hours as estimated by the MTT assay. Each curve represents the average of 3 independent assays with 6 replicate samples for each data point. Error bars have been omitted for clarity (SE of all values < 20%).

In order to measure the fluorescence of the anthracycline antibiotics (doxorubicin and daunorubicin), it was necessary to determine the absorption maxima and use this as the excitation wavelength when determining the emission maxima for doxorubicin and daunorubicin figs 5.5 and 5.6, respectively.

The graph in fig 5.9 shows the fluorescence of the anthracycline antibiotics internalised by 5000 B16 cells, as measured using FACS. Figs 5.10 and 5.11 are calibration curves of fluorescence against the concentration of the anthracycline antibiotics doxorubicin, daunorubicin, respectively, as measured using spectrofluorometer.

The results of the biological assays on daunorubicin, adozelesin and bizelesin are shown in figs 5.12, 5.13 and 5.14, respectively.

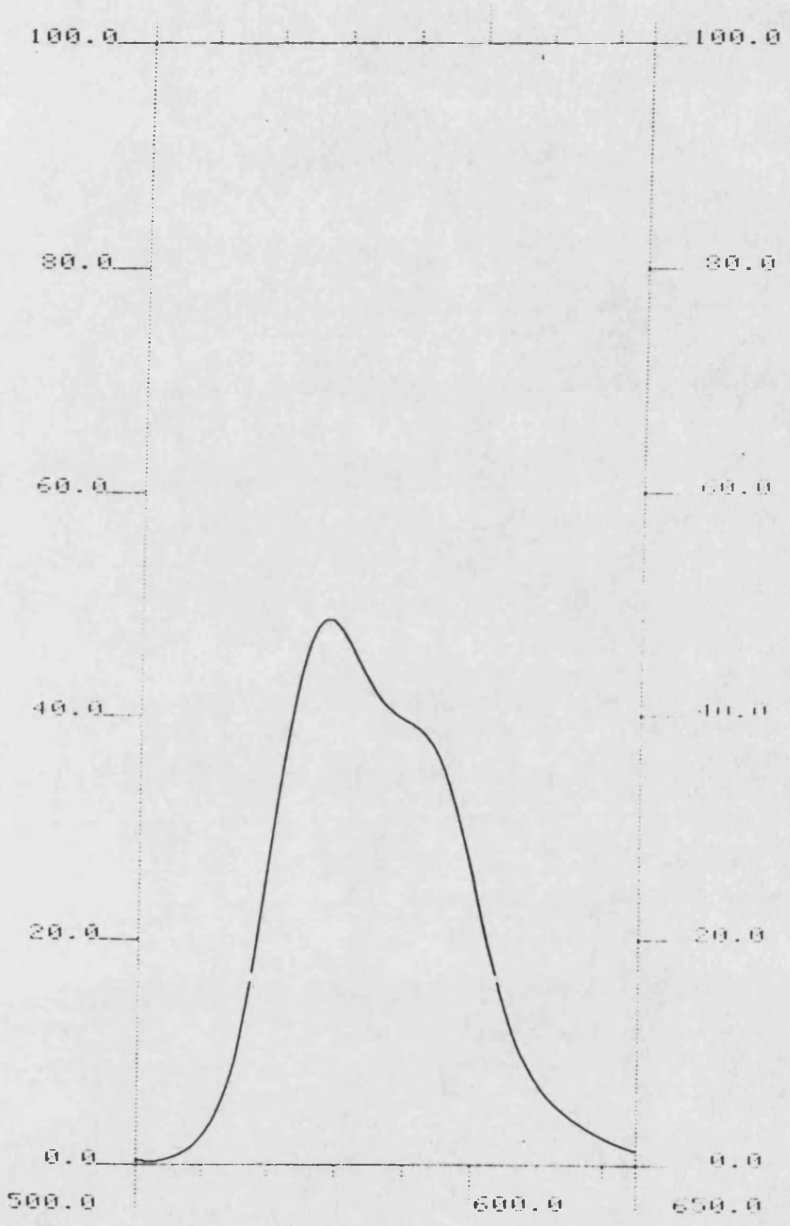


Fig 5.5. Emission spectra of doxorubicin at excitation λ of 488nm

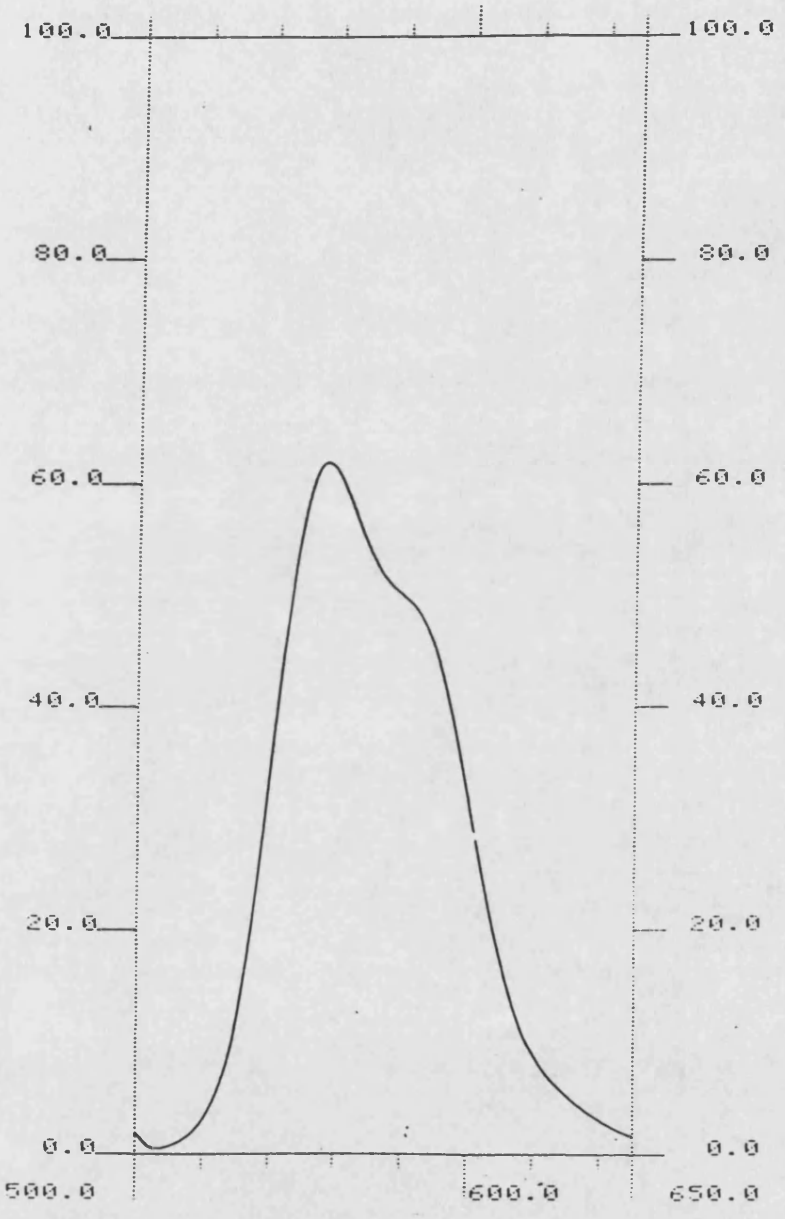


Fig 5 6. Emission spectra of daunorubicin at excitation λ of 488nm

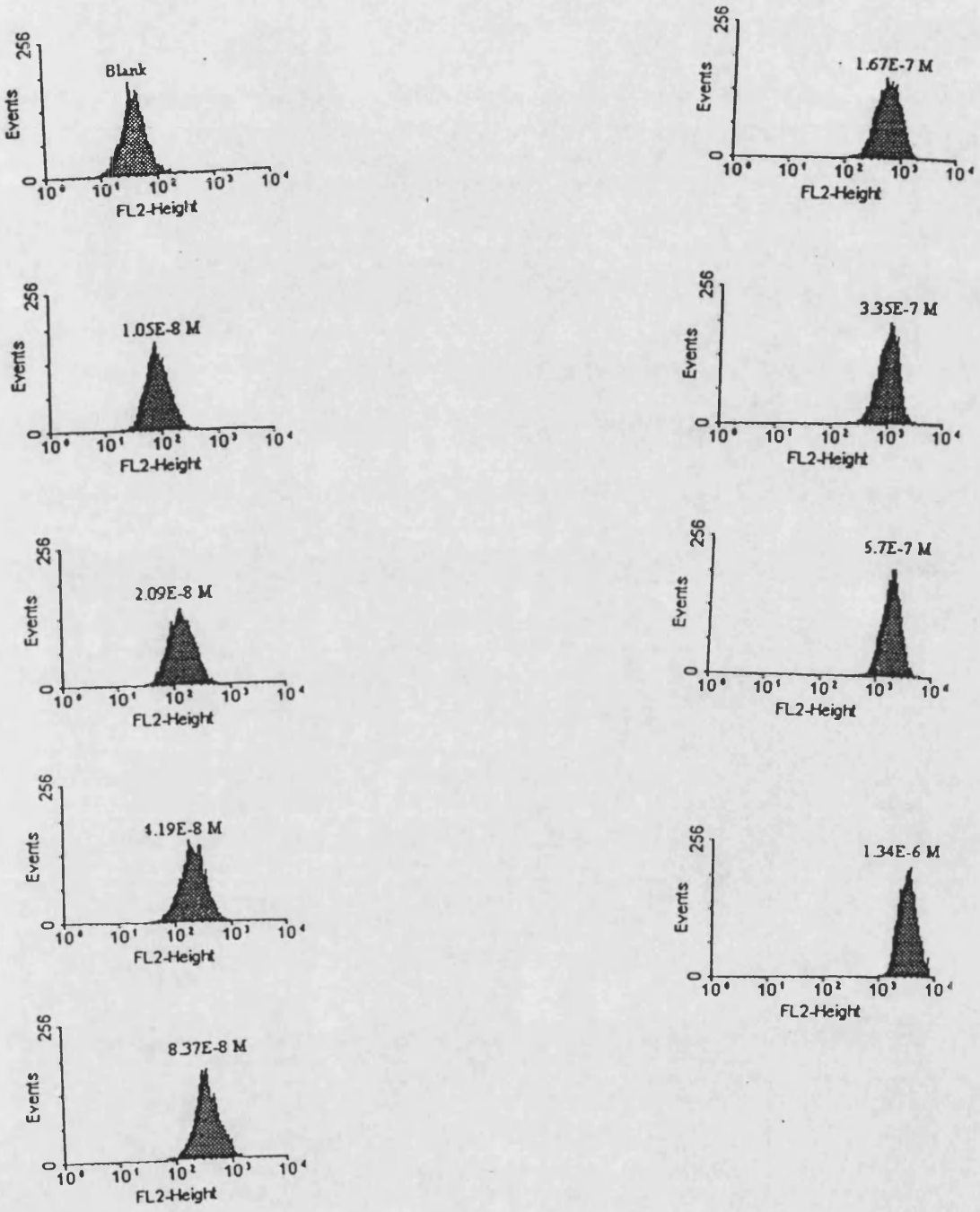


Fig.5.8. FACS analysis showing fluorescence of daunorubicin internalised by B16 cells after incubating various concentrations of daunorubicin with B16 cells for 4 hours at 37°C (excitation λ of 488nm and emission λ of 575 nm). The fluorescence of 5000 B16 cells was measured for each concentration.

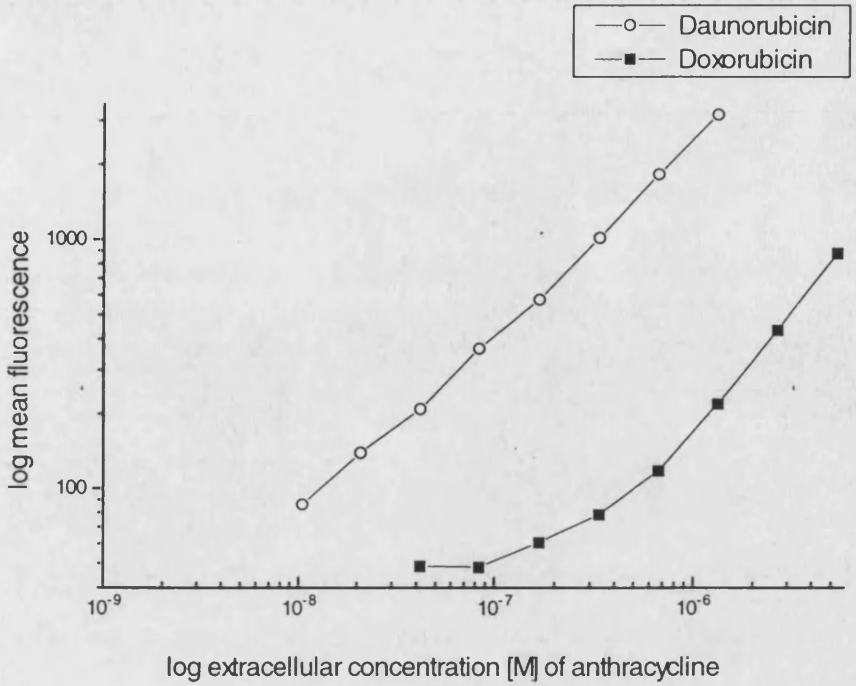


Fig 5.9. Fluorescence of the anthracycline antibiotics internalised by B16 cells, after incubating various concentrations of the antibiotics with B16 cells for four hours at 37°C (excitation λ of 488 nm and emission λ of 575 nm). The fluorescence of 5000 B16 cells was measured for each concentration.

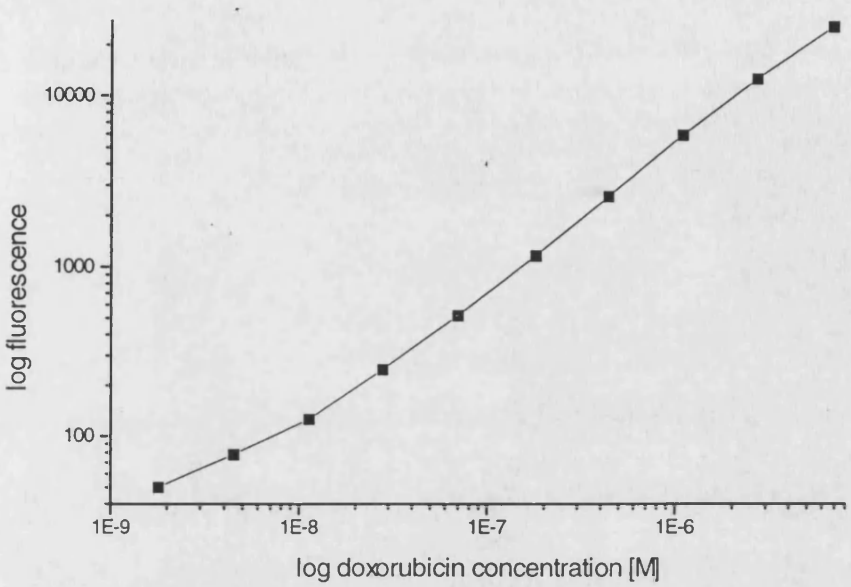


Fig 5.10. A calibration curve of fluorescence (at excitation λ of 488 nm and emission λ of 575 nm) and doxorubicin concentration as determined using a spectrofluorometer.

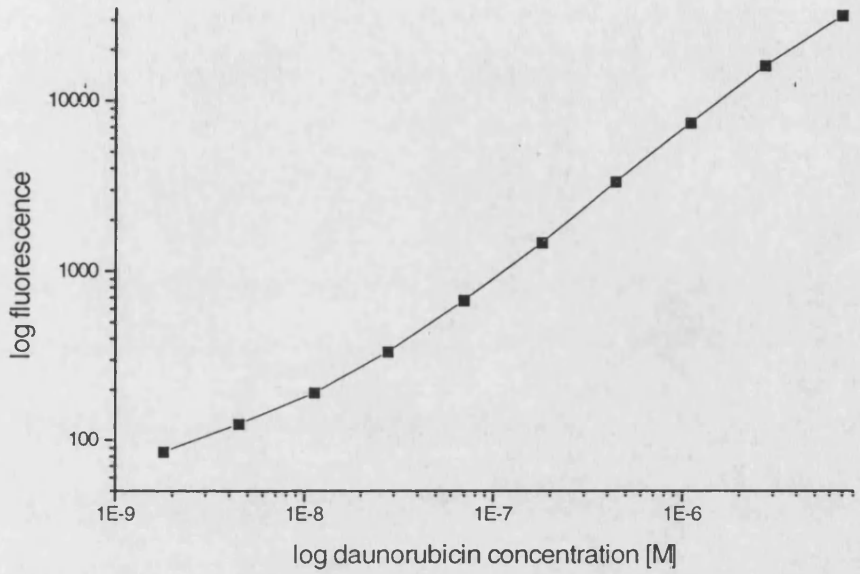


Fig 5.11. A calibration curve of fluorescence (at excitation λ of 488 nm and emission λ of 575 nm) and daunorubicin concentration as determined using a spectrofluorometer.

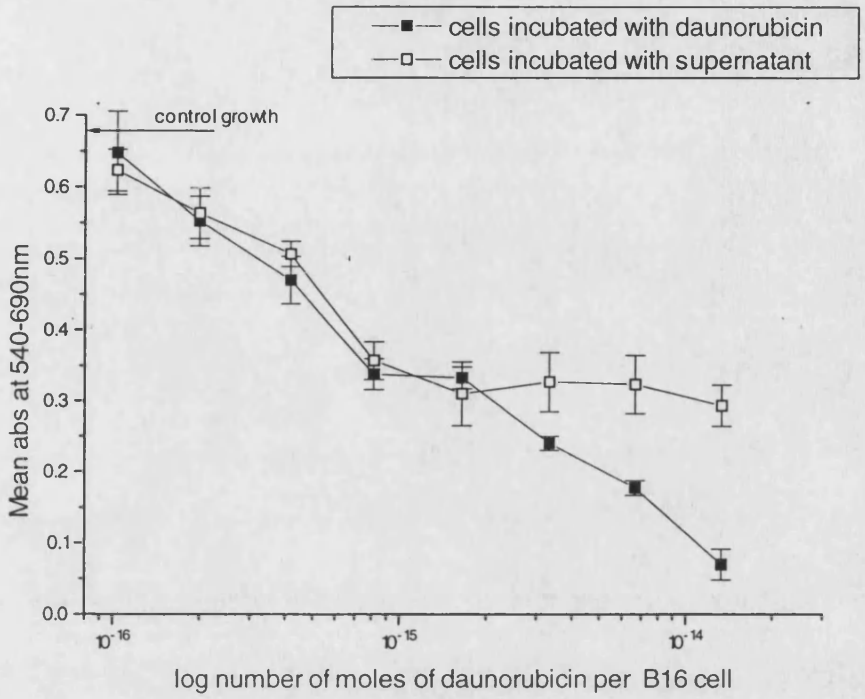


Fig.5.12. Growth inhibitory effect of a constant number of moles of daunorubicin on various numbers of B16 cells, after incubation at 37°C for four hours and incubation in a drug free media for 72 hours. A growth inhibitory effect of the supernatant above (after incubation with B16 cells for four hours) was determined by incubation with a fresh sample of B16 cells for four hours and incubating the cells in a drug free medium for 72 hours. Each curve represents one experiment with four replicate samples for each data point used. The above experiment was repeated twice giving similar results.

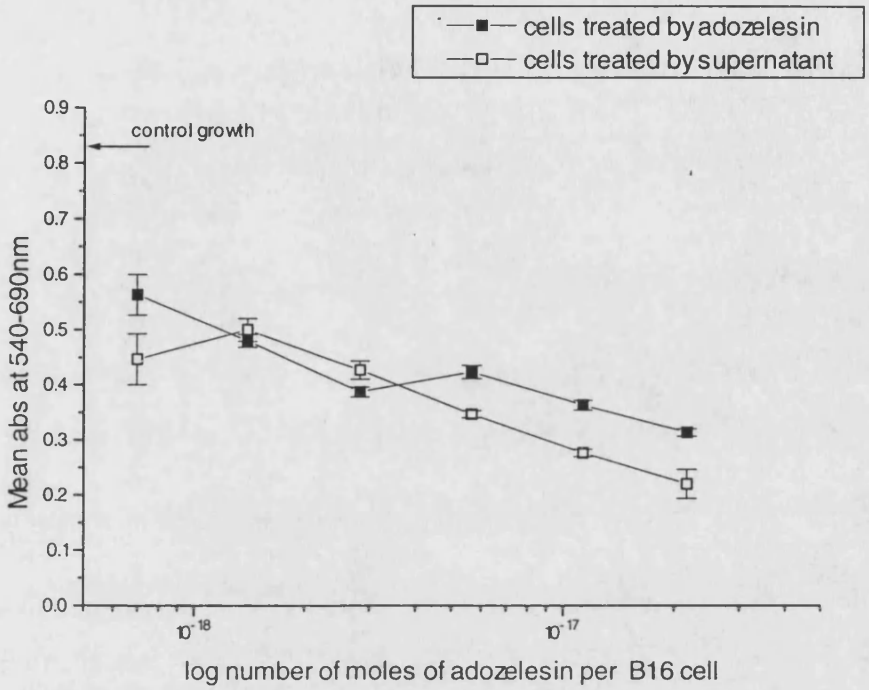


Fig.5.13. Growth inhibitory effect of a constant number of moles of adozelesin on various numbers of B16 cells, after incubation at 37°C for four hours and incubation in a drug free medium for 72 hours. A growth inhibitory effect of the supernatant above (after incubation with B16 cells for four hours) was determined by incubation with a fresh sample of B16 cells for four hours and incubating the cells in a drug free media for 72 hours. Each curve represents one experiment with four replicate samples for each data point used. The above experiment was repeated twice giving similar results.

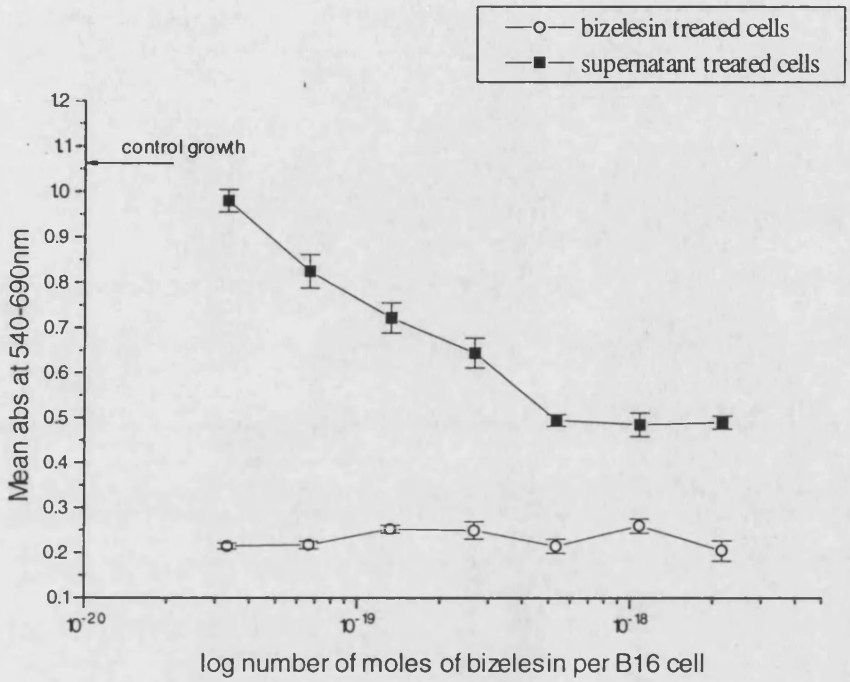


Fig.5.14. Growth inhibitory effect of a constant number of moles of bizelesin on various numbers of B16 cells, after incubation at 37°C for four hours and incubation in a drug free medium for 72 hours. A growth inhibitory effect of the supernatant above (after incubation with B16 cells for four hours) was determined by incubation with a fresh sample of B16 cells for four hours and incubating the cells in a drug free media for 72 hours. Each curve represents one experiment with four replicate samples for each data point used. The above experiment was repeated twice giving similar results.

Discussion

Estimation of doxorubicin and daunorubicin uptake by B16 cells

In the experiments described in this chapter the cytotoxicity of the two antibiotics daunorubicin and doxorubicin have been determined after 4 hours continuous incubation with B16 cells, at which point the amount of anthracyclines responsible for the observed toxicity was estimated.

Following 72 hours continuous incubation of the anthracycline antibiotics (doxorubicin and daunorubicin) with B16 cells they appear to have similar EC_{50} values (chapter 3). There was a slight change in the EC_{50} of daunorubicin following only 4 hours incubation with B16 cells, and a further incubation of the cells in a drug free media for a further 72 hours. The concentration of doxorubicin which resulted in 50% cell death after incubation with B16 cells for four hours was 2.27E-7M as determined by the MTT assay, compared to 1.55E-8M following 72 hours continuous incubation (fig 5.3). That is the EC_{50} of doxorubicin approximately one order of magnitude higher when determined after 4 hours incubation with B16 cells compared to 72 hours incubation period.

These results are supported by FACS analysis of B16 cells after incubating them with either doxorubicin or daunorubicin for 4 hours (figs 5.9). It shows higher cellular fluorescence of B16 cells (after incubating them with

daunorubicin) than B16 cells incubated with similar concentrations of doxorubicin. There are several possible reasons for this. The plasma membrane has been suggested as an important target in the cytotoxic effect of doxorubicin (Tokes *et al.*, 1982).

Cells exposed to daunorubicin were 2 to 4 times more fluorescent than were cells similarly exposed to doxorubicin and the intracellular appearance of daunorubicin fluorescence was much more rapid (Krishan and Ganapathi, 1980).

Chemical structures of daunorubicin and doxorubicin differ only by a single hydroxyl group on C14 (fig 5.1). The extra hydroxyl group on doxorubicin means doxorubicin is more polar (more lipophobic / more hydrophilic) than daunorubicin which means doxorubicin is less able to cross biological lipid membranes than daunorubicin . This may explain why cellular uptake of daunorubicin markedly exceeds that of doxorubicin. Another possible explanation for the differences in cellular uptake of these anthracyclines is that the active efflux of doxorubicin may be more rapid than that of daunorubicin.

Estimation of doxorubicin uptake by B16 cells

In order to get an approximate estimation of the number of moles of doxorubicin which are internalised in order to cause on average 50% growth inhibition of B16 cell, we incubated 5 x 10^5 B16 cells in 25 ml medium (v1) containing 2.27E-7 M doxorubicin (c1). These B16 cells were then washed

and the internalised doxorubicin was extracted in 4 ml of extraction solution (v2) (as described in methods section) and its fluorescence was measured and the equivalent concentration (c2) was read of a calibration curve of doxorubicin fluorescence against its concentration (fig 5.10).

Number of moles of anthracycline internalised by one B16 cell (N)

= c2 x v2 / 1000 x n = N

Where is

c2: Concentration of anthracycline in the extraction solution

v2: Volume of the extraction solution

n: Number of B16 cells from which the anthracycline was extracted

Fluorescence of internalised doxorubicin after incubating 2.27E-7 M (c1) with B16 cells for four hours was (250.00±15.70; N = 6). This is equivalent to 1.5E-8 M (c2) (fig 5.10). From this, the number of moles of doxorubicin internalised per one B16 cell is (1.5E-8 X 4 /1000 x 5E5) ≡ 1.2E-16 moles of doxorubicin are required to inhibit on average the growth of B16 cell by 50%, compared to control growth of B16 cell incubated under the same conditions but without the presence of doxorubicin.

Fraction of the EC_{50} of anthracycline antibiotic which is internalised to cause the 50% growth inhibition = [c2 x v2 / c1 x v1] x 100%

Where is

c1: Concentration of the anthracycline in the incubation medium

v1: Volume of the incubation medium

c2: Concentration of the anthracycline in the extraction solution

v2: Volume of the extraction solution

Fraction of the $EC_{50}$of doxorubicin which is internalised by B16 cells to cause the 50% growth inhibition = [1.5E-8 x 4 / 2.27E-7 x 25] x 100% ≅ 1% of the $EC_{50}$of doxorubicin is internalised by B16 cells to cause the 50% growth inhibition.

Estimation of daunorubicin uptake by B16 cells

In order to get a rough estimation of the number of moles of daunorubicin which are internalised in order to inhibit on average the growth of B16 cell to 50% compared to control. We incubated 5 x 10^5 B16 cells in 25 ml media (v1) containing 1.67E-8 M daunorubicin (c1) for 4 hours. These B16 cells were washed and the internalised daunorubicin was extracted in 4 ml of extraction solution (v2) (as described in methods section) and its fluorescence was measured and the equivalent concentration was read of a calibration curve of daunorubicin fluorescence against its concentration (c2) (fig 5.11). Fluorescence of internalised daunorubicin after incubating 1.67E-8 M with B16 cells for 4 hours was (166-67±9.10; N = 6). This is equivalent to 8.0xE-9 M (fig 5.11). From this the number of moles of daunorubicin internalised by a

B16 cell is (8xE-9 X 4/ 1000 x 5xE5) = 6.4xE-17 moles of daunorubicin are required on average to inhibit the growth of B16 cell to 50% compared to control.

Fraction of the $EC_{50}$of daunorubicin which is internalised by B16 cells to cause the 50% growth inhibition = [8E-9 x 4 / 1.67E-8 x 25] x 100% ≅ 8 % of the $EC_{50}$of daunorubicin is internalised by B16 cells to cause the 50% growth inhibition.

From above daunorubicin and doxorubicin appear to have similar potency on B16 cells in terms of the number of moles of each of them which causes 50% growth inhibition. Due to differences in the ability of these two anthracycline antibiotics to be internalised by B16 cells, about one order of magnitude more molecules of doxorubicin are required in the extracellular cellular environment in order to internalise similar number of molecules as daunorubicin, this is in agreement with the FACS analysis for the cellular uptake of these two anthracycline antibiotics (fig 5.9).

The cellular uptake of adozelesin and bizelesin by B16 cells

After only 15 minutes of continuous incubation with B16 cells the toxicity of adozelesin (EC_{50} 4.8E-10 M) (fig 5.3) was not significantly different from the four hour incubation period (EC_{50} 2.28E-10M).(fig 5.3). The change was much greater for bizelesin which had EC_{50} of 1.01E-11 M (fig 5.3) after 4 hours of continuous incubation with B16 cells, this was reduced to EC_{50} of

2.5E-10M (fig 5.3) after only 15 minutes of continuous incubation with B16 cells, that is approximately 25 fold reduction in the potency of bizelesin.

From the above results it appears that adozelesin needs a shorter time to be internalised by B16 cells than bizelesin although bizelesin is more potent than adozelesin once its internalised by B16 cells.

The concentration of bizelesin which reduces the growth of B16 cells to 80% of the control after 4 hours incubation with the compound was 2.5E-12M (fig 5.4), and that for adozelesin was 4E-11 M (Fig 5.4) this means that bizelesin is approximately 16 fold more potent than adozelesin under these conditions. The concentration of bizelesin which inhibits the growth to 20% of the control cells was 1E-9 M and this is the same concentration of adozelesin which inhibits the growth of B16 cells to 20% of the control growth, adozelesin go on to be more toxic than bizelesin at concentrations higher than 1E-9M (Fig 5.4) under similar incubation conditions.

The solubility of adozelesin and bizelesin in water is approximately 1μg/ml (Upjon Chemical Company data sheet). The EC_{50} for adozelesin after incubation with B16 cells for four hours was 2.28xE-10M. Molecular weight for adozelesin is 502.23. This means the amount of adozelesin dissolved in the medium at the EC_{50} value is 1.14E-4μg/ml which is 8.8E3 fold less than its solubility in water.

The EC_{50} of bizelesin is 1E-11M after 4hours incubation with B16 cells. Molecular weight for bizelesin is 815-7. This means the amount of bizelesin dissolved in the medium at the EC_{50} value is 8.15E-6μg/ml which is 1E5 fold less than its solubility in water. This rules out any influence of the solubility of either of the compounds on the EC_{50} data and on the observed results of the biological assays for both compounds

Adozelesin appears to enter the cell much more freely than bizelesin. Thus increasing the extracellular concentration of adozelesin means a rapid increase in the mass of drug internalised by B16 cells compared to bizelesin which appears to be more restricted in entering the cell, it follows that increasing the extracellular concentration of bizelesin increases the mass of drug crossing the cell membrane but this increase is much less than that for adozelesin.

In the biological assay described here, a constant concentration of drug (10x of the EC_{50}) was incubated with increasing number of B16 cells. This assay explores what happen at the EC_{50} of the cytotoxic agents looked at here. Does the cytotoxic agent become internalised totally from the media, or is the fraction responsible for for the EC_{50} too small compared to the amount of cytotoxic agent remaining in the medium.

An equation for the novel biological assay is derived here

If we define the following

C: Initial amount of compound in the incubation medium

A: Amount of compound internalised by one cell

T: Number of cells in the incubation medium

R: Amount of compound remaining in the incubation medium

Then;

$R = C-[A \times T]$ 1

$[A \times T] = C-R$ 2

$A = C-R / T$ 3

C is constant; T is dependent variable; R can be determined indirectly by performing a cytotoxicity assay on the supernatant and reading the concentration from a calibration curve of dose dependent growth inhibitory effect of the same compound. Amount of compound internalised by one cell (A) is the only unknown in equation 3, and so can be determined.

When the amount of compound removed by cells in the incubation medium is too small ($A \times T \cong 0$), then $C \cong R$ 4

When the amount of compound removed by cells in the incubation medium is too large ($A \times T \cong C$), then $R \cong 0$ 5

From (figure 5.12) it appears that at less than 1E-15 moles of daunorubicin per a B16 cell, the amount of daunorubicin internalised by a B16 cell is very small compared to the amount of daunorubicin remaining in the media $C \cong R$. As the number of moles of daunorubicin per a B16 cell in the media is greater

than 2E-15 moles, the larger the amount of daunorubicin per a B16 cell resulted in increase in the amount of daunorubicin being internalised by a B16 cell (this is illustrated by an increase in the growth inhibitory activity fig 5.12) while the amount of daunorubicin remaining in the supernatant appears to change very little (this is illustrated by a constant growth inhibitory activity of the supernatant). This gives the impression that daunorubicin enters the B16 cell rapidly and raising the amount of daunorubicin in the media above certain threshold will result in increase in the amount of daunorubicin entering the cell leaving a threshold level of daunorubicin in the media. These results appear to be in agreement with the FACS analysis described here which shows that internalisation of daunorubicin occurs very quickly at much lower concentrations than doxorubicin (fig 5.9). Also from figure 5.12 it appears that less than 8E-16 moles of daunorubicin are required to inhibit on average the growth of a B16 cell by 50%, the amount of daunorubicin remaining in the media appears to have similar growth inhibitory effect on a fresh sample of B16 cells.

It was determined from the fluorescence assay that 8 % of the $EC_{50}$of daunorubicin is internalised by B16 cells to cause the 50% growth inhibition, this explains the apparent unchange in the toxicity of the supernatant on a fresh sample of B16 cells. The number of moles of daunorubicin which are required on average to inhibit the growth of B16 cell to 50% compared to control is 6.4xE-17 as determined from the fluorescence assay. This is only one order of magnitude different from the value predicted by the biological assay.

From the biological assay of adozelesin on B16 cells (fig 5.13), it appears that less than 3E-18 moles of adozelesin are required to inhibit on average the growth of a B16 cell by 50% compared to control cell. The supernatant appears to have very similar growth inhibitory effect on a fresh sample of B16 cells indicating that the amount of adozelesin removed from the media to cause the EC_{50} is too small compared to the number of moles of adozelesin remaining in the supernatant.

From the biological assay of bizelesin on B16 cells (fig 5.14) it appears that less than 2E-20 moles of bizelesin are required to inhibit on average the growth of a B16 cell by 50% compared to control cell, with the amount of bizelesin remaining in the media exerting minor inhibitory effect, indicating that either most of the drug is internalised into B16 cells or fallen below threshold at which the amount of bizelesin in the supernatant is too dilute to inter the cell. Increasing the number of moles of bizelesin per a B16 cell to 3E-19 moles resulted in a very similar growth inhibitory effect on a B16 cell, while the supernatant appears to have enough bizelesin to inhibit the growth of a fresh sample of B16 cells by 50% compared to control. This may indicate that bizelesin is extremely potent but it is more restricted in entering the cell thus the EC_{50} value for this compound will underestimate its true potency. These results are in agreement with the results obtained following toxicity assay of bizelesin on B16 cells after various periods of incubation with bizelesin (fig 5.3), and that bizelesin appears to be extremely potent but it's cellular uptake appears to be restricted (figure 5.4).

It was observed that a 90% reduction in the growth of BSC-1 cells was observed, with 1E2 bizelesin lesions per cell compared to 1E4 lesions induced by the parent analogue CC-1065 (Woynarowski et al., 1995). The same authors have demonstrated a reduced lesion formation to DNA in whole cells compared to purified DNA, they have attributed the reduced lesion formation in whole cells to "limited entry of bizelesin into the cell". These observations are in a complete agreement with our findings here.

The above results illustrate the importance of distinguishing between the EC_{50} value of a cytotoxic compound and the potency of that toxic compound inside the cell. They also show that daunorubicin and doxorubicin are approximately equipotent in terms of the number of moles required to cause 50% growth inhibition of B16 cells, they also show that adozelesin and bizelesin are more potent than daunorubicin in terms of the number of moles required to cause 50% growth inhibition by at least 20 and 3200-fold respectively. When considering candidates for drug targeting to MSH receptors we are concerned with the number of moles which inhibit the growth of a melanoma cell rather than the EC_{50} of that compound the above results indicate the importance between distinguishing between these two indicators.

Chapter 6

Targeting of Methotrexate to melanoma by way of melanocyte stimulating hormone

6.1. Introduction

MSH receptors

Receptors identified in several types of murine melanoma appear to be comparable in terms of binding properties, the dissociation constant (kd) of α-MSH ranged from 1.31-2.6E-9 M in B16 and Cloudman melanoma cells (Siegrist *et al.*, 1988). There was more variability in the kd values obtained in human lines at 0.92-2.2E-10M (Chhajlani and Wikberg, 1992). Receptors of human melanoma appeared to have approximately a 10 fold greater affinity for α-MSH than murine lines.

The nomenclature of the cloned receptors is as follows. There are four melanocortin receptors from human origin they are called (MC-1 to MC-4) MC-1 being the first to be cloned and MC-4 the last. There is one melanocortin receptor from rat hypothalamus and it is called MC3-R which is an analogue of the human MC-3, and finally there is a melanocortin receptor from Cloudman mouse and it is called MSH-R (Table 6.1).

R. subtype.	R. origin	Tissues expressed in	Affinity for the natural melanotropins	Reference
MC-1	Human	Melanoma cells, but not other tissues	α-MSH >ACTH (1-39) >β-MSH >δ-MSH >ACTH (4-10)	Chhajlani and Wikberg, 1992
MC-2	Human	Brain tissues not in melanoma cells	α-MSH >ACTH (1-39) >β-MSH >δ-MSH	Chhajlani *et al*, 1993
MC-3	Human	Brain, placenta and gut	no data could be found	Gantz *et al*, 1993
MC-4	Human	Brain notably absent in adrenal cortex, melanocytes and placenta	α-MSH=β-MSH=δ-MSH=ACTH	Gantz *et al*, 1993
MC3-R	Rat	present primarily in the hypothalamus, but in smaller amounts in other brain regions	δ2-MSH >δ1-MSH=α-MSH=ACTH (1-39)>>> ACTH (4-10)	R.Cone, personal communicatio n
MSH-R	Mouse	Cloudman melanoma cell	α-MSH=β-MSH, δ-MSH had little or no affinity	Solca *et al*, 1989

Table 6.1. Melanocortin receptor subtypes.

Generation of 3T3 cells permanently transfected with PCDNAI/Neo+MC1)

When examining the selective toxicity of a melanotropin analogue linked to a cytotoxic agent on cell lines carrying MSH receptors, it is much easier to observe and interpret any selective toxicity due to the interaction of the conjugate with MSH receptors, if we have two cell cultures generated from the same clone, one carrying the MSH receptor and one without . In this chapter we attempt to transfect 3T3 cells with (pCDNAI/Neo + MC1) using electroporation and thereby generate stable clones of 3T3 cells with plasmids carrying the genetic code for expression of MC1 receptors and another culture of cells with the same palsmids lacking the genetic code for

expression of MC1 receptors. Electroporation is a process whereby cells in suspension are mixed with the DNA to be transfected. This cell/DNA mixture is subsequently exposed to a high-voltage electric field. This creates pores in the membrane of treated cells that are large enough to allow the passage of macromolecules such as DNA into the cells. Such DNA molecules are ultimately transported to the nucleus, and a subset of these molecules are integrated into the host chromosomes. Here we tried to select the clones of 3T3 cells which are permanently transfected with the plasmid.

The observation that electroporation yields a high frequency of permanent transfectants, has a high efficiency of transient gene expression, and is subsequently easier to carry out than alternative techniques has resulted in its increasing use in many applications (Potter, 1988) The amount of DNA that can be introduced into the nucleoli of electroporated mammalian cells is in the range of 0.5 pg, corresponding to 10^4 DNA molecule or 8% of total endogenous host DNA. The maximum size of DNA molecule that can be introduced by electroporation is at least 150 kb (Potter, 1988).

To facilitate the isolation of cells stably transfected with the DNA of interest, a gene encoding a dominant selectable marker is usually included in the transfection protocol (in this case the plasmid containing a gene coding for resistance to geneticin). It is important to keep subculturing the transfected cells in the selection media (a media

which contains geneticin), because transfected DNA can be relatively unstable in the host genome and therefore the reversion rates of transfected cells can be quite high.

Specific growth inhibitory effect of MTX-NLDP conjugate on cells expressing the MSH receptors.

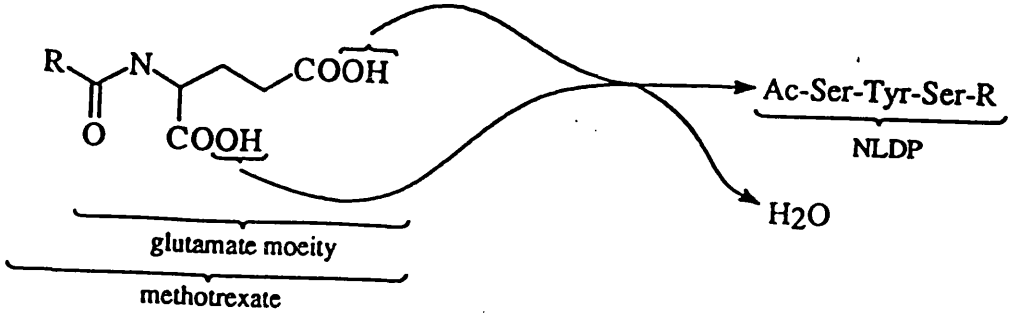


Fig 6.1. Formation of the MTX-[Nle^4,D-Phe^7]α-MSH. The above reaction results in 4 separate isomers, depending on which carboxyl group reacts with NLDP and because of possible racemisation of the α-carbon of the MTX-glutamate moiety.

The antifolate Methotrexate (MTX) enters cells through the reduced folate carrier, present on almost all human cells due to the dependence on extracellular folate sources. The drug exhibits very low specificity against tumour cells due to an increased folate requirement, but it still has low selectivity for some tumour tissues. The peptide analogue of MSH, NLDP-MSH, interacts with high affinity to specific membrane receptors on some cells and will therefore have a more defined mode of cellular binding. In order to increase the site specificity with which MTX exerted its inhibitory effects on melanoma cells, the molecule was chemically coupled to an analogue of the naturally occurring hormone α-MSH namely [Nle^4,D-phe^7]α-MSH) on an equimolar basis forming the hormone drug conjugate (MTX-[Nle^4,D-phe^7]α-

MSH). The hypothesis was that if MTX-NLDP was not taken up by its normal route that is the folic acid pathway, but rather by a cell-specific receptor-mediated process (in this case utilising the α-MSH receptor). Then the drug would be targeted to cells which possess the MSH receptor. (rational for using the different cell lines) .

The growth inhibitory effect of the MTX-NLDP and MTX was assessed on the following cell lines .

B16 cells are melanoma cells expressing the MSH receptor subtype MC1 (Qarawi, 1994) the number of receptors expressed varies between 5000-20000 (Sahm, 1994), because our MTX-NLDP conjugate is designed specifically to interact selectively on cells expressing the MC1 receptors on melanoma cells. Melanoma cells appear to be the only site where the expression of MC1 subtype is reported (Chhajlani and Wikberg, 1992). B16 cells provide a suitable *in vitro* model for melanoma cells.

293-MC3 cells, these are 293 epithelial cells transfected with the genetic code for the MSH receptor subtype MC3, and they were shown to express MC3 receptors (Qarawi, 1994). Due to the distribution of this receptor subtype to other parts of the body such as brain, placenta and gut (Gantz *et al.*, 1993). Thus it was important to determine whether the MTX-NLDP conjugate has any selective toxicity against other MSH receptor subtypes such as MC3.

Cos 7 and 293 cells express very few if any MSH receptors (Doherty, personal communication) and the same applies to 3T3 cells this study, so they provide three models for detection of non-selective toxicity of the conjugate.

There are several important questions which must be asked when considering the use of the above hormone in a drug delivery system. 1-Is there a distribution of binding sites on other (non-target) cells ? MC1 is the only melanocortin receptor subtype known to date to be expressed only in melanoma cells (table 6.1). 2-What is the number of binding sites per cell ? For B16 mouse melanoma cells there is inter experimental differences in receptor number expressed by the cells which can vary between 5,000 and 20,000 (Sahm, 1994). From binding studies on human melanoma cell lines, the number of binding sites is generally lower than murine melanomas, e.g. from undetectable to 2000 sites/cell (Eberle, 1988). 3-Does ligand binding induce receptor-mediated internalisation ? The conjugate MTX-[Nle^4,D-phe^7]α-MSH demonstrated similar profiles of surface binding to the [Nle^4,D-phe^7]α-MSH , the dissociation constant of the MTX-NLDP conjugate for MSH receptors on B16 cells was 6nM, compared to 0.48nM for NLDPα-MSH thus the conjugate has a ten-fold lower affinity for the MSH receptor on B16 cells (Richards, 1992). It has also been shown that the conjugate is internalised via receptor-mediated endocytosis (Whelan, 1995). If the receptor is recycled does the ligand dissociate within the cell and if so is it transported further along the endocytic pathway or is it returned to the cell surface intact with the receptor ? This question still has not been answered but Whelan (1995) has estimated that approx. 40,000 radiolabelled NLDPα-MSH molecules are internalised by receptor mediated endocytosis by a single B16 cell.

It has also been demonstrated that the lysosomal degradation products of MTX-NLDP had an equivalent inhibitory activity on DHFR as free MTX (Richards, 1992).

6.2. Results

MTT assay of geneticin on 3T3 cells

The MTT assay of geneticin on 3T3 cells was performed in order to determine the sensitivity of 3T3 cells to geneticin prior to transfection with a plasmid which carries the geneticin resistant gene.

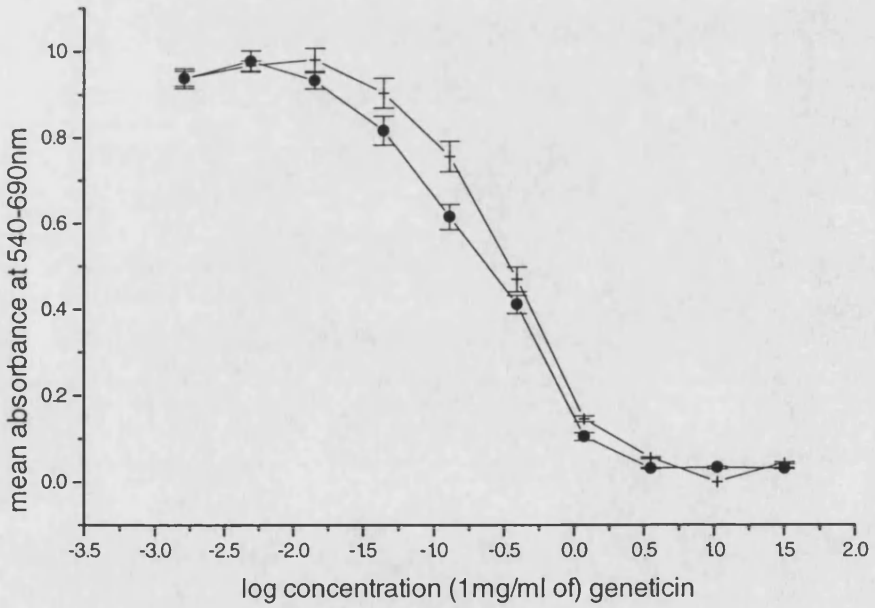


Fig 6.2. Dose-dependent growth inhibitory effect of geneticin on 3T3 cells (prior to transfection) following exposure of 3T3 cells to geneticin for 96 hours as determined using the MTT assay. Each curve represents one independent experiment with 6

replicate samples for each data point used. A total of two independent experiments were performed.

In order to determine the level of expression of MC1 receptors on transfected 3T3 cells, a binding assay was performed on 3T3 cells transfected with the genetic code for MC1 receptor and control 3T3 cells.

3T3 clone	Total binding CPM	Non specific binding CPM
pCDNA/neo	327±16	272±10
clone 1	363±11	372±29
clone 2	316±22	324±29
clone 3	324±26	341±53

Table.6.1 The specific binding activity of three clones of 3T3 cells transfected with plasmid containing MC1 receptor and cells transfected with plasmid without the genetic code for the receptor after incubation with 2.28E-10 M of radioiodinated NLDPα-MSH(H) and 2.28E-7M non iodinated ligand (H+C). Each value represents the average of three independent binding assays with 3 replicate samples for each data point.

3T3 clone	Total binding CPM (H)	Non specific binding CPM (H+C)
pCDNA/neo	630±20	690±26
clone 1	911±37	806±30
clone 2	650±26	594±30

clone 3	642±26	624±37

Table.6.2 The specific binding activity of three clones of 3T3 cells transfected with plasmid containing MC1 receptor and cells transfected with plasmid without the genetic code for the MC1 receptor after incubation with 4.56E-10 M of radioiodinated NLDPα-MSH (H) and 4.56E-7M non iodinated ligand (H+C). Each value represents the average of three independent binding assays with 3 replicate samples for each data point.

Using the MTT assay, growth inhibitory effects of MTX and the hormone drug conjugate (N^{α}MTX-[Nle^{4},D-phe^{7}]α-MSH) were examined against a variety of cell lines in order to see whether the N^{α}MTX-[Nle^{4},D-phe^{7}]α-MSH has selective toxicity against cells with MSH receptors as compared with cells without the MSH receptor. These cell lines included

B16; Murine melanoma cells which have surface MSH receptors.

293-MC3; Transformed human epethelial 293 cells expressing MC3 receptors .

Cos 7; Transformed cells from the kidney of green African monkey.

3T3; Murine embryonal fibroblast.

The MTT assay was optimised for each cell line, prior to carrying out the MTT assay on these cell lines, in order to assess the growth inhibitory effect of MTX and MTX-NLDP conjugate on them. The time required for viable cells to metablise detectable amounts of tetrazolium to formazan was determined as 4 hours for 3T3, Cos 7 and 6 hours for 293 cells. Incubation time of 96 hours of each cell line was required to achieve between 2 to 3 cell doublings.

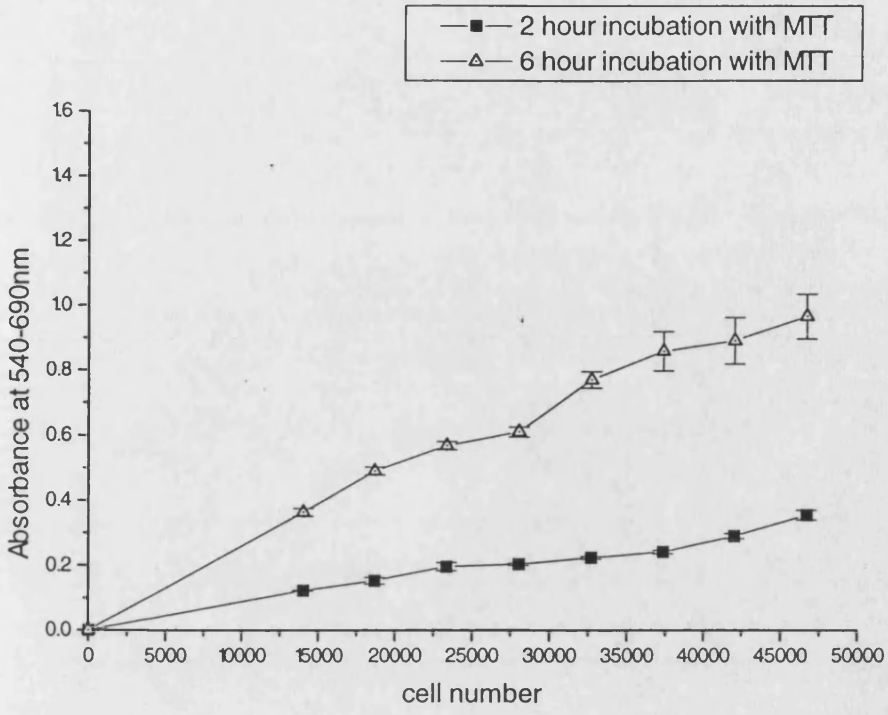


Fig 6.3. Correlation of viable 293 cell number with the absorbance of their metabolised MTT at 540nm (test wavelength) and 690nm (background), after incubation of cells with MTT for various time periods. Each point represents the mean value for 8 independent determinations performed in one experiment.

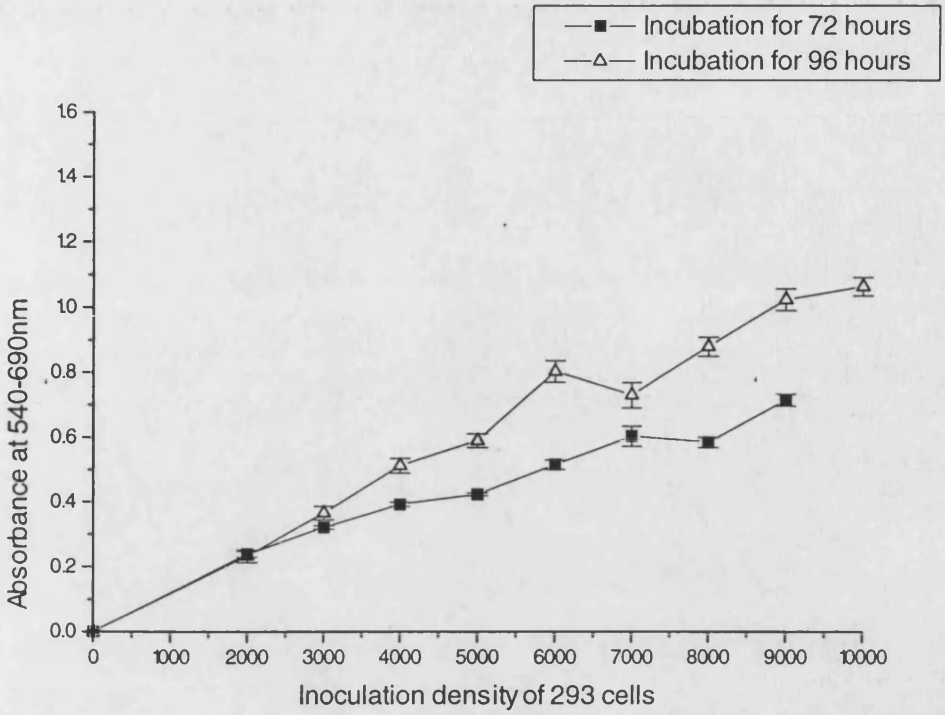


Fig 6.4. Relation between absorbance of metabolised MTT by 293 cells and their inoculation density, after incubation in RPMI media for various time periods, followed by incubation with serum-free RPMI media containing 1mg/ml MTT for 6 hours. Each point represents the mean value for 8 independent determinations performed in one experiment.

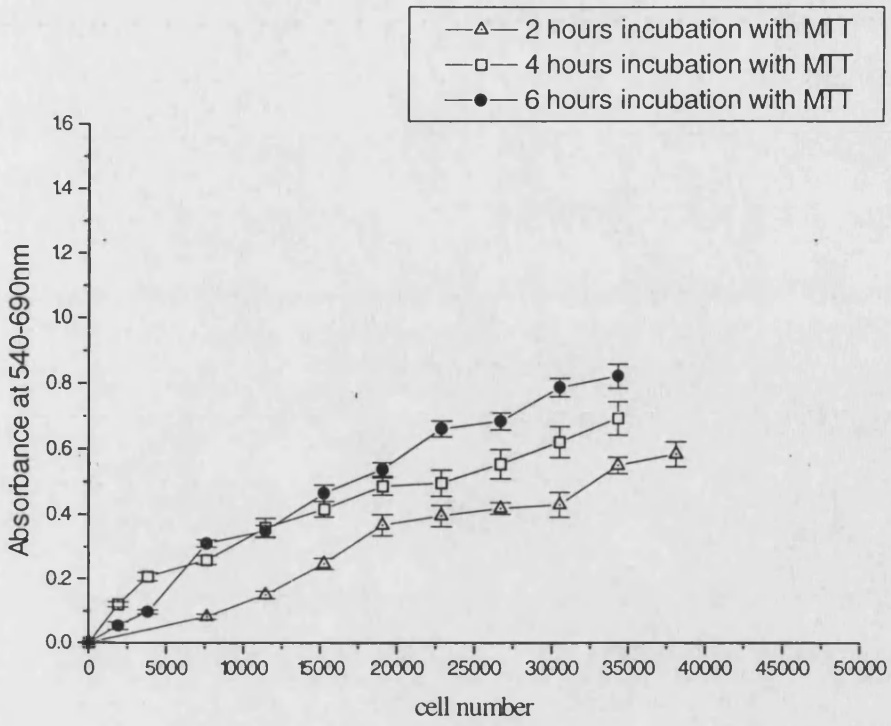


Fig 6.5. Correlation of viable 3T3 cell number with the absorbance of their metabolised MTT at 540nm (test wavelength) and 690nm (background), after incubation of cells with MTT for various time periods. Each point represents the mean value for 8 independent determinations performed in one experiment.

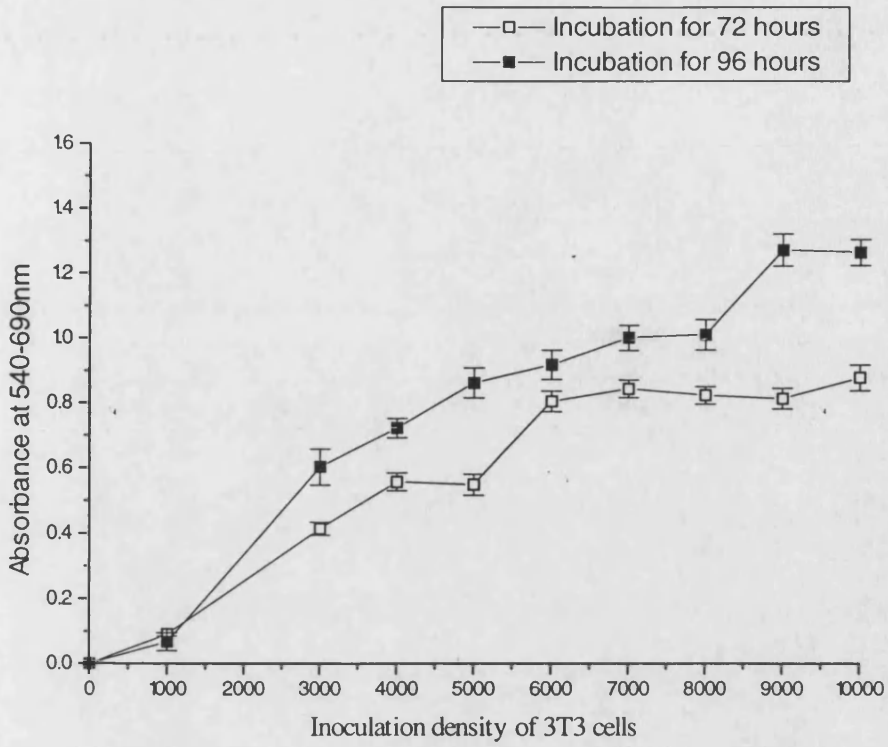


Fig 6.6. Relation between absorbance of metabolised MTT by 3T3 cells and their inoculation density, after incubation in RPMI media for various time periods, followed by incubation with serum-free RPMI media containing 1mg/ml MTT for four hours. Each point represents the mean value for 8 independent determinations performed in one experiment.

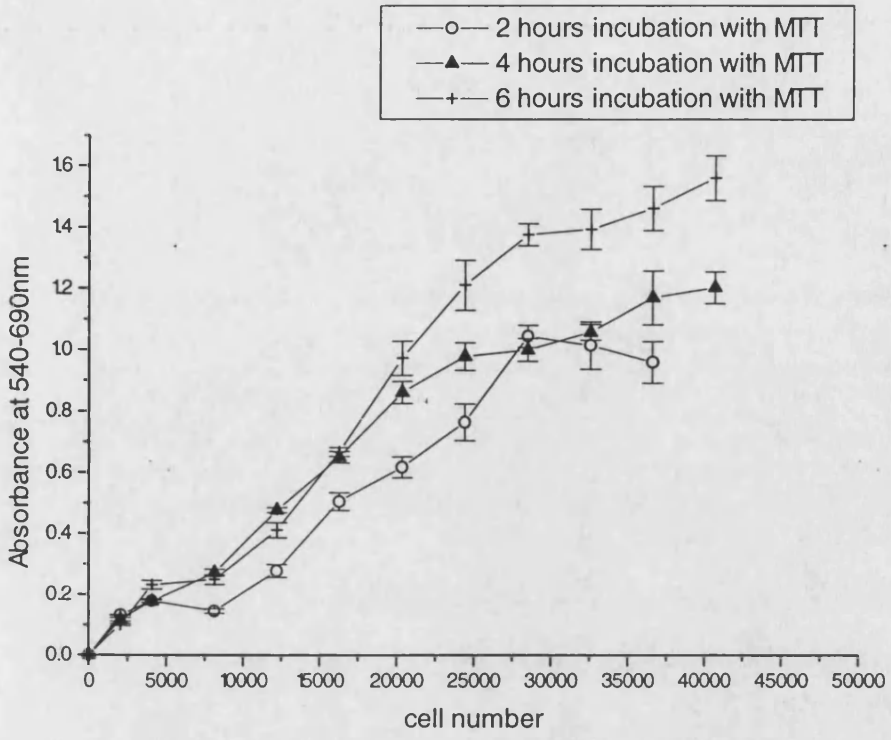


Fig 6.7. Correlation of viable Cos 7 cell number with the absorbance of their metabolised MTT at 540nm (test wavelength) and 690nm (background), after incubation of cells with MTT for various time periods. Each point represents the mean value for 8 independent determinations performed in one experiment.

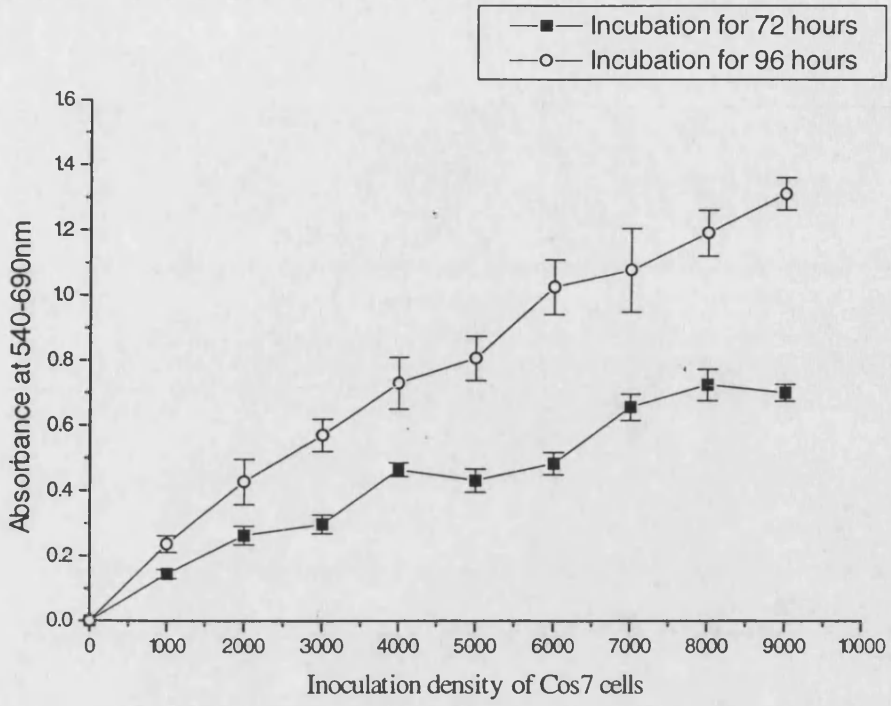


Fig 6.8. Relation between absorbance of metabolised MTT by Cos 7 cells and their inoculation density, after incubation in RPMI media for various time periods, followed by incubation with serum-free RPMI media containing 1mg/ml MTT for four hours. Each point represents the mean value for 8 independent determinations performed in one experiment.

Cell line	EC_{50} (M) a	EC_{50} (M) b	EC_{50} (M) c	mean EC_{50}	SE [±]	N
B16	1.00E-07	3.0E-8	2.20E-8	5.0E-8	2.5E-8	3
293-MC3	3.00E-08	5.90E-08	4.50E-08	4.47E-08	8.37E-09	3
Cos 7	2.80E-07	3.50E-07	1.60E-07	2.63E-07	5.55E-08	3
3T3	3.70E-08	2.50E-07	3.20E-08	1.07E-07	7.25E-08	3

Table 6.3. EC_{50} values from MTT assay of MTX on several cell lines, after incubation with B16 murine melanoma cells for 72 hours, with 293-MC3 cells and 3T3 for 96 hours. Each mean EC_{50} value represents 1 independent assay with 6 replicate samples for each data point.

Cell line	EC_{50} (M) a	EC_{50} (M) b	EC_{50} (M) c	mean EC_{50}	SE [±]	N
B16	6.60E-04	2.10E-04	7.30E-05	3.10E-4	1.80E-04	3
293-MC3	5.00E-06	2.12E-05	2.80E-05	2.0E-05	6.82E-06	3
293-Vector	7.40E-06			7.40E-06		1
Cos 7	2.70E-05	3.50E-05		3.0E-05	2.83E-06	2
3T3	4.20E-06	6.12E-06	1.50E-06	3.94E-06	1.34E-06	3

Table 6.4. EC_{50} values from MTT assay of MTX-NLDP on several cell lines, after incubation with B16 murine melanoma cells for 72 hours, with 293-MC3 cells, 293-vector and 3T3 for 96 hours. Each mean EC_{50} value represents 1 independent assay with 6 replicate samples for each data point.

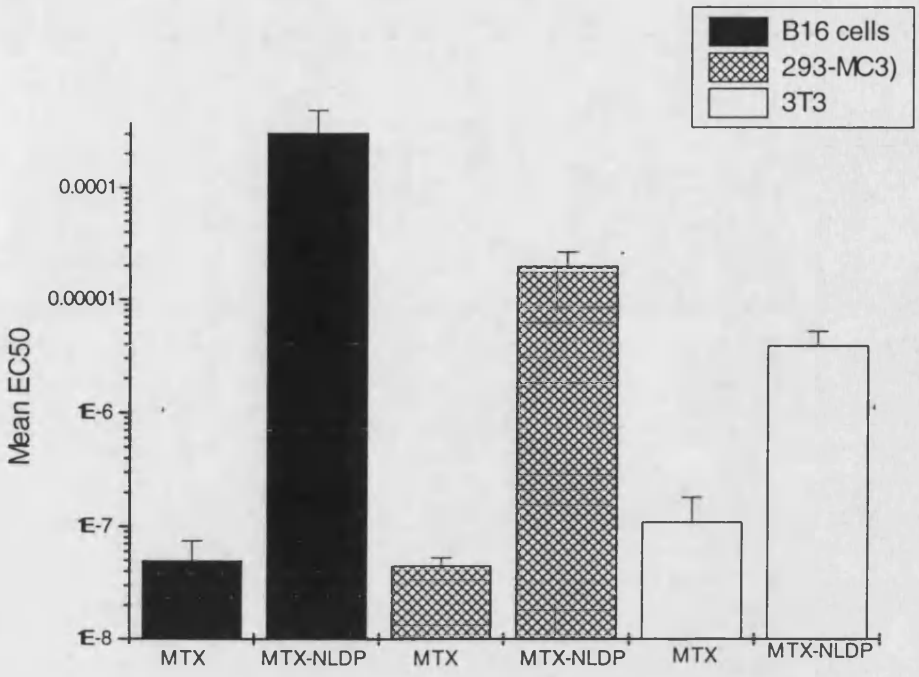


Fig. 6.9. Mean EC_{50} values of Methotrexate and MTX-NLDP as determined using the MTT assay after incubation with B16 murine melanoma cells for 72 hours, with 293-MC3 cells and 3T3 for 96 hours. Each mean EC_{50} value represents the average of 3 independent assays with 6 replicate samples for each data point.

HPLC analysis of the MTX-NLDP conjugate was performed at 307nm (the absorption maxima for MTX). The analysis was carried out in order to detect any MTX impurities in the conjugate.

Each of figs 6.10, 6.11, and 6.12 are elution profiles of 1.3E-5 M MTX, MTX-NLDP and NLDP, at different detection sensitivity to illustrate the fact that the MTX-NLDP conjugate contains only traces of MTX impurities and can only be detected at an extremely sensitive setting.

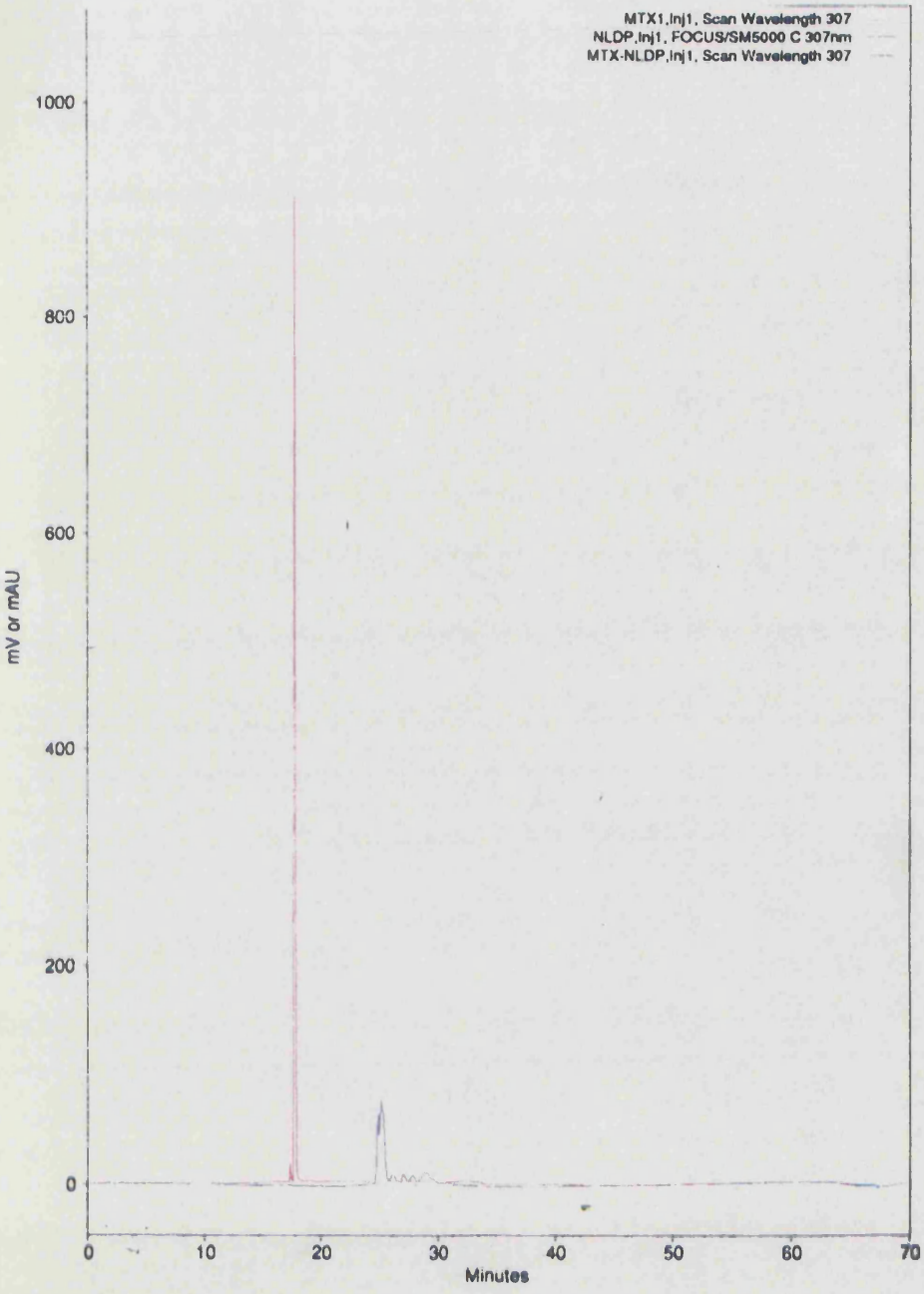


Fig 6.10. This figure shows the elution profile of the following samples at 307nm: [Nle^4,D-Phe^7]α-MSH, N$^\alpha$MTX[Nle^4,D-Phe^7]α-MSH, MTX. (see text for details).

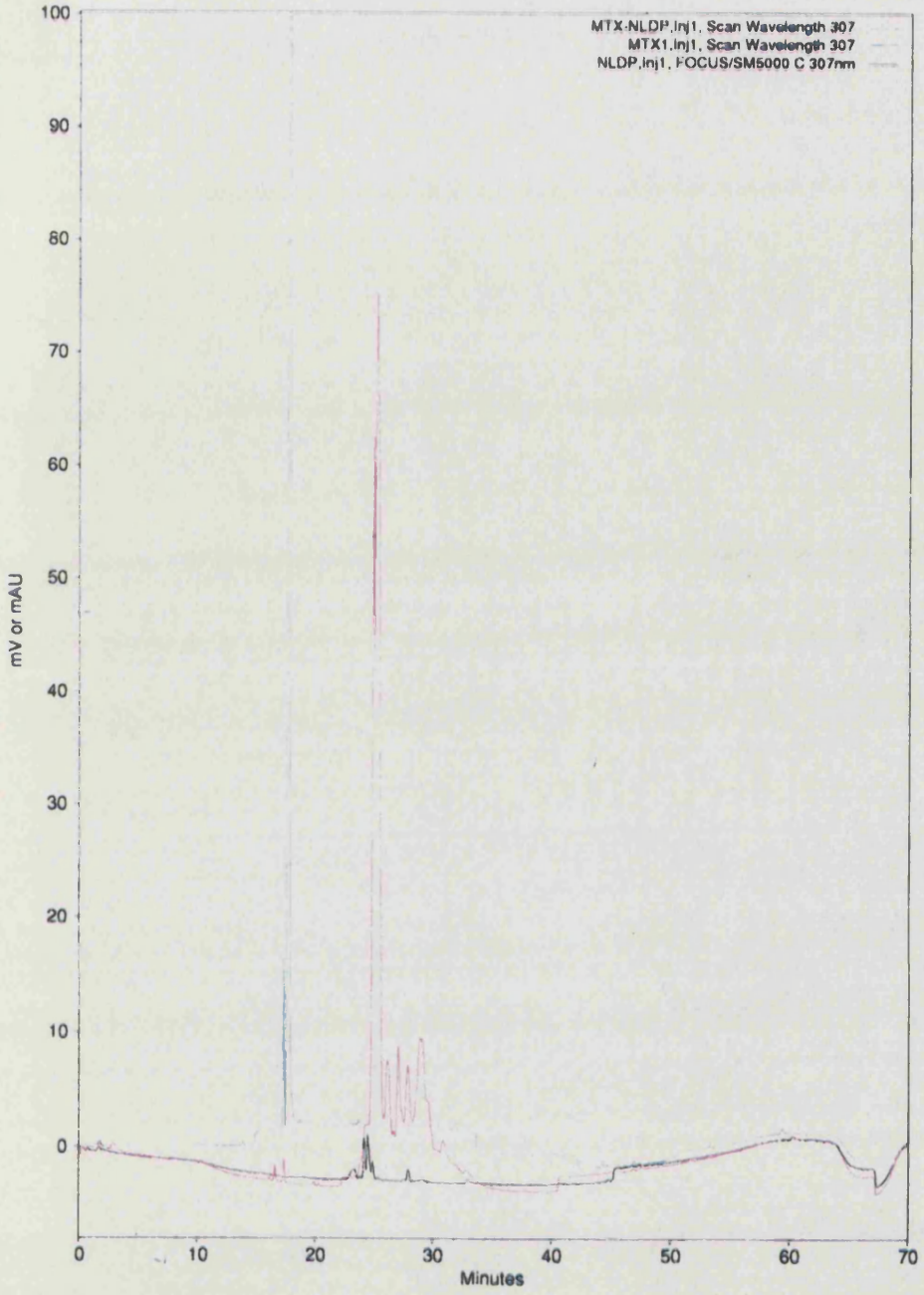


Fig 6.11. This figure shows the elution profile of the following samples at 307nm: [Nle^4,D-Phe^7]α-MSH, N$^{\alpha}$MTX[Nle^4,D-Phe^7]α-MSH, MTX. (see text for details).

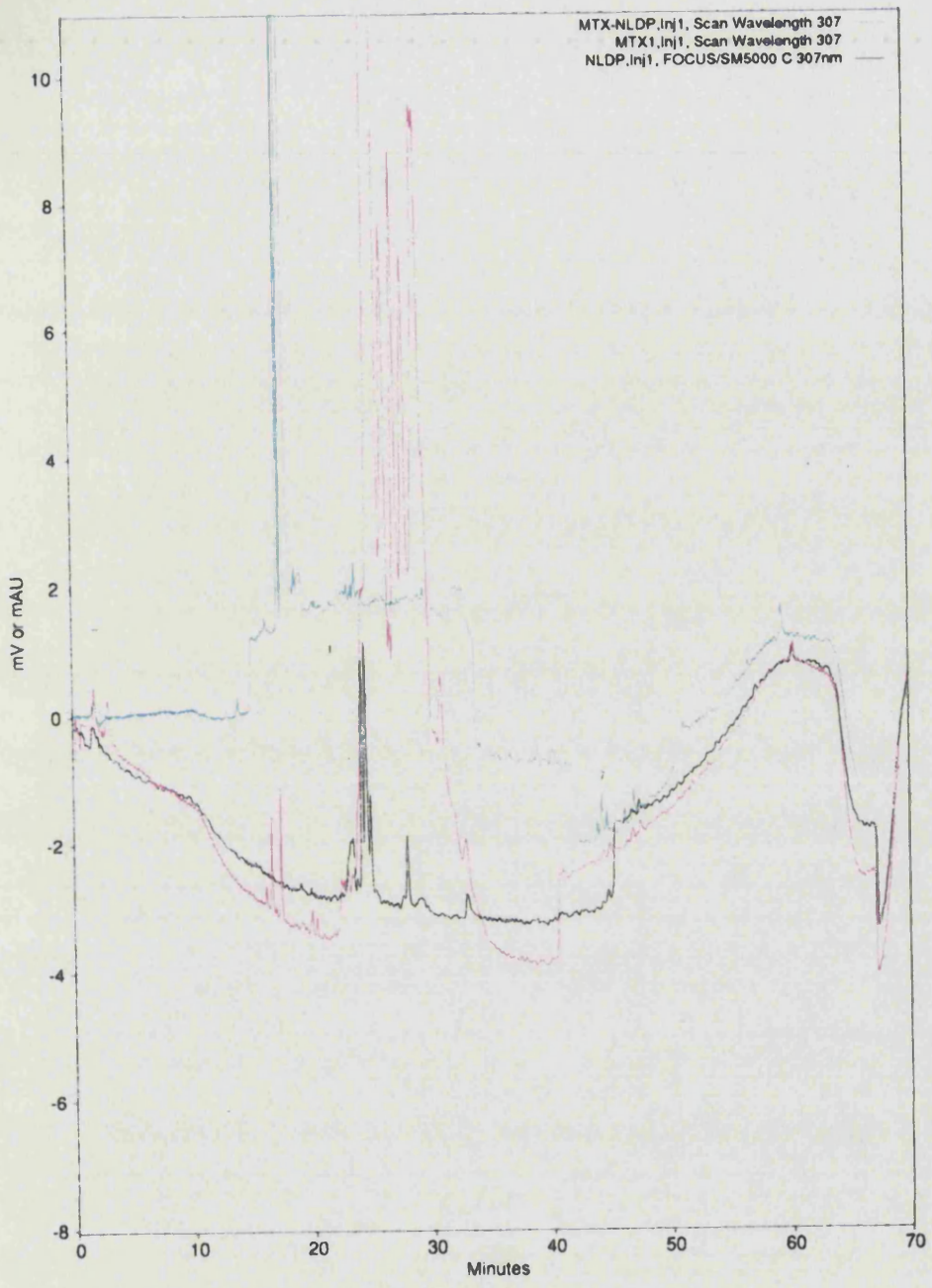


Fig 6.12. This figure shows the elution profile of the following samples at 307nm: [Nle^4,D-Phe^7]α-MSH, N$^\alpha$MTX[Nle^4,D-Phe^7]α-MSH, MTX. (see text for details).

The stability of the MTX-NLDP conjugate was then assessed and a complete analysis report is presented in appendix E. An elution profile for a peak with retention time of 2.53 minutes was observed when pure MTX sample was injected. Another peak was observed when a MTX-NLDP sample was injected into the HPLC with retention time of 2.60 minutes. The area under this peak was (1157389) compared to a peak at retention time of 2.65 minutes when the same MTX-NLDP sample was incubated at 37°C for 96 hours with an area of (8532496). This presents more than seven folds increase in the level of apparent MTX impurities in the conjugate after 96 hours incubation compared to a freshly dissolved conjugate in sterile PBS.

6.3. Discussion

The expression of MC1 receptors on the stably transfected cells was assessed using a binding assay, the transfected cells have not exhibited specific binding activity when compared to 3T3 cell transfected with vector. It is possible that the number of MC1 receptors expressed on 3T3 cells was not high enough to be detected using this binding experiment.

The results of growth inhibitory assays of MTX and MTX-NLDP conjugate on several cell lines are summarised in tables 6.3 and 6.4, these results show that the conjugate is several orders of magnitude less potent than that of MTX alone. The conjugate appears to have similar toxicity against 293-MC3 cells which have on average 50,000 MSH receptors (Sahm, 1994) and (293-vector, 3T3 Fibroblast and Cos 7 cells which have no MSH receptors). B16 murine melanoma cells (which have between 5,000

and 20,000 MSH receptors/cell (Sahm, 1994) were at least 10 times more resistant to the conjugate.

One explanation of these observations is that some of the conjugate is internalised into B16 and 293-MC3 cells, by receptor-mediated endocytosis, but the number of molecules being taken up through this specific route was not sufficient to have any growth inhibitory effects. Since we need approx. 5.4E-5 molecules of MTX in order to achieve on average growth inhibition of half the B16 cells (Whelan, 1995)

The MTX-NLDP conjugate had a forty fold lower affinity than MTX for DHFR (Richards, 1992). If we consider the different types of endocytosis discussed in chapter one, pinocytosis and adsorptive endocytosis will be responsible for greater share of the cellular uptake of the conjugate when the extracellular concentration of the conjugate is increased by a 6000-fold. Thus it is possible that a contribution to the toxicity data of the conjugate is due simply to pinocytosis and adsorptive endocytosis of the MTX-NLDP conjugate.

Also due to the differences between the potency of the conjugate and that of MTX as determined using the MTT assay, with MTX being several orders of magnitude more potent than MTX-NLDP it was important to find out if the conjugate contains traces of free MTX, and to be sure that the observed toxicity of the conjugate has not been caused mainly by free MTX. HPLC analysis of the MTX-NLDP conjugate (fig 6.11) has shown that the conjugate contains traces of MTX impurities.

It would appear that most of the observed toxicity of the conjugate can be accounted for by the presence of free MTX, hence the increased toxicity of the MTX-NLDP conjugate on 3T3 and 293 cells which were incubated with the conjugate for 96 hours compared to it's toxicity on B16 cells which were incubated with the conjugate for 72 hours might be explained by the minor degradation of the MTX-NLDP.

From above it is clear that the use of the MTX-NLDP conjugate as a specific drug delivery system for melanoma was not successful for several reasons outlined in the discussion above. It is hoped to consider the practical difficulties observed with this conjugate, and our interpretation for its lack of specificity for melanoma and try to circumvent them in the design of a future drug targeted melanotropin analogue specific for melanoma cells.

Concluding discussion

The feasibility of active targeting of cytotoxics to melanoma cells by conjugation to MSH analogues, has been investigated here.

Before initiation of this study, it had already been established in our laboratory, that [^{125}I-Tyr^{2}-Nle^{4},D-Phe^{7}]α-MSH displayed specific binding to MSH receptors expressed by B16 cells of Kd 0.37-.87 nM. It was also known that between 5000 and 20000 binding sites per B16 cell were available, the NLDPα-MSH has been shown to elicit biological action through binding to the MSH-membrane receptor (Sahm, 1994). In the work carried out by Adam, 1993 it was shown that [^{125}I-Tyr^{2}-Nle^{4},D-Phe^{7}]α-MSH was bound to the MSH receptor on the surface of B16 cells and was subsequently internalised, and that once within the cells, the ligand made its way to the lysosome, were the degradation of the ligands occur. Work done by Sahm, 1994 has shown that coupling of drugs to the N-terminus of α-MSH has very little effect on the affinity of α-MSH to the MSH receptor on B16 cells. By adding larger moieties to the [NLDP]α-MSH such as biotin [^{125}I-Tyr^{2}-Nle^{4},D-Phe^{7}]α-MSH, internalisation occurred but at a reduced rate (Adam, 1993). Whelan, 1995 has shown that approximately 4E4 [NLDP]α-MSH molecules are internalised by a B16 cell through receptor mediated endocytosis.

From above we need a potent drug to conjugate with the N-terminus of a melanotropin analogue in order to get enough molecules to be internalised via receptor mediated endocytosis to cause a B16 cell death. Ideally we are looking at a cytotoxic agent

which is potent enough if internalised by melanoma cells through receptor mediated endocytosis, this mean it needs tens of thousands of molecules to kill a melanoma cell.

In vitro screening was carried out to determine the EC_{50} values of a selection of existing cytotoxic agents and novel polyamine conjugates. Work carried out here demonstrated that the EC_{50} value of a cytotoxic agent is not the only indicator of potency, it should be looked at in conjunction with further analysis of the cellular uptake, in order to be able to establish what fraction of the extracellular concentration of the cytotoxic agent is responsible for the observed EC_{50} value. This is important here because we are interested in potency in terms of the amount of drug which needs to be internalised through receptor mediated endocytosis rather than the amount of drug in the extracellular environment which causes growth inhibition.

Thus for small lipid soluble compounds, which are internalised rapidly by B16 cells through passive diffusion of the drug from the incubation media, such as daunorubicin which crosses the cell membrane rapidly through passive diffusion (Tarasiuk *et al.*, 1989). The EC_{50} values are generally a good indication of their potency.

On the other hand for a compound which is very polar, and /or transported into cells via active transport process, such as MTX which is transported into cells via the carrier system present for the naturally occurring reduced folates. The EC_{50} values might be an underestimation of the potency of these compounds. It is possible that only a minute fraction of the extracellular drug is internalised into the cell, before an equilibrium is reached. This internalised minute fraction could be responsible for the

observed toxicity, provided that the drug does not act on cell membrane to cause toxicity.

The selectivity of an existing N^{α}MTX-[Nle^4,D-Phe^7]α-MSH was investigated The conjugate employed in this study consisted of two components, both capable of binding to cell membranes. The antifolate MTX enters cells through the reduced folate carrier, present on almost all human cells due to dependence on extracellular reduced folate sources. The affinities usually encountered for MTX and reduced folates are in the micro-molar range (Ritchards, 1992), while the peptide analogue of MSH (NLDPα-MSH) interacts with high affinity, kd values in nano-molar range , and will thus have a more defined mode of cellular binding.

Therefore if the effects of the conjugate were mediated exclusively through MSH receptors it would be very logical to assume that a very high level of N^{α}MTX-[Nle^4,D-Phe^7]α-MSH must be present before any growth inhibitory effect is seen on 293-vector and 3T3 cells if we were to claim any selectivity of N^{α}MTX-[Nle^4,D-Phe^7]α-MSH for cells expressing the MSH receptor. Given that the mass of MTX which needs to be internalised by a B16 cell in order to inhibit its growth is approximately 1E6 molecules (1.7E-18 moles of methotrexate) (Whelan, 1995), and the number of NLDP molecules internalised by a B16 cell through receptor mediated endocytosis (4E4 molecules) we would have expected N^{α}MTX-[Nle^4,D-Phe^7]α-MSH to be less potent than MTX due to the reduced number of conjugate molecules internalised by a B16 cell but more selective for cells expressing the MSH receptors than free MTX.

The MTX-NLDP conjugate did not show any selective toxicity on cells expressing the MSH receptor *in vitro*. The lack of selectivity of the conjugate *in vitro* was attributed to the presence of traces of MTX impurities, these MTX impurities would have masked any selective toxicity of the conjugate. From this it is believed that the *in vitro* comparison of cytotoxicity of MTX and MTX-NLDP conjugate is less than ideal and can be misleading. This is due to the fact that MTX-NLDP was designed in order to have different pharmacokinetics from free MTX. The *in vitro* assay does not permit MTX-NLDP to demonstrate fully its altered pharmacokinetics, and despite the fact that the MTX-NLDP had traces of free MTX their effect *in vivo* could be minute. The conjugate is designed to be selectively retained by cells expressing the melanotropin receptors and free MTX would be washed away with the flow from the receptor site, thus it is possible that the MTX-NLDP conjugate has selective toxicity against melanoma cells *in vivo*.

The *in vitro* system for assessing the growth inhibitory effect, which is described here has the potential to rapidly identify new agents with perhaps novel mechanisms, such as the novel polyamine conjugates. It was further modified to obtain a relation between the growth inhibitory effect and cellular uptake, but the *in vitro* system may not be adequate for assessing alterations in the pharmacokinetics of a drug-polymer conjugate compared to a free drug. The *in vitro* screening system should be complemented by a parallel *in vivo* models where data obtained from *in vitro* models is examined.

Undoubtedly the study of drug-targeting via MSH (or other hormones) involves many different aspects with apparently insurmountable problems, e.g. the establishment of a suitable model for the study of transcytosis in the endothelial vasculature, or a successful biodistribution of the conjugate *in vivo*. However I hope this study has formed part of the platform for the advancement of further work.

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Appendix A

Experimental data for chapter 3

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.49713	0.02215	31600
B	0.49725	0.02835	28440
C	0.43263	0.02785	25280
D	0.32262	0.02258	22120
E	0.27238	0.01324	18960
F	0.1955	0.02403	15800
G	0.1745	0.01561	12640
H	0.13563	0.01448	9480
I	0.06463	0.00338	6320
J	0.029	0.00398	3160

Table 3.1a. Incubation of B16 cells with mtt for 1 hour.

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.63875	0.02669	31600
B	0.58725	0.02869	28440
C	0.59875	0.02259	25280
D	0.53388	0.02338	22120
E	0.397	0.01862	18960
F	0.4035	0.03267	15800
G	0.33688	0.00863	12640
H	0.26138	0.00753	9480
I	0.16838	0.00672	6320
J	0.08888	0.00396	3160
k	0		0

Table 3.1. b Incubation of B16 cells with mtt for 2 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.87175	0.03954	31600
B	0.77816	0.03944	28440
C	0.694	0.02823	25280
D	0.59737	0.0237	22120
M	0.50038	0.03446	18960
E	0.41425	0.02809	15800
F	0.29838	0.01666	12640
G	0.25363	0.01167	9480
H	0.15588	0.01145	6320
I	0.07243	0.00548	3160
J	0		0

Table 3.1. c. Incubation of B16 cells with mtt for 3 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.92875	0.05382	31600
B	0.73575	0.03983	28440
C	161.72863	105.46	25280
D	0.61075	0.04272	22120
E	0.46938	0.02711	18960
F	0.36675	0.02692	15800
G	0.275	0.03045	12640
H	0.20025	0.01697	9480
I	0.10863	0.00671	6320
J	0.0425	0.00508	3160

Table 3.1. d Incubation of B16 cells with mtt for 4 hours

column	y (mean Abs)	se`[yEr±]	x (cell number)
A	0.23738	0.01086	10000
B	0.23775	0.01425	9000
C	0.221	0.01326	8000
D	0.23725	0.00957	7000
E	0.21913	0.01414	6000
F	0.19263	0.00917	5000
G	0.14113	0.01366	4000
H	0.15375	0.00478	3000
I	0.14325	0.00409	2000
J	0.11975	0.00313	1000

Table 3.2. a Incubation of B16 cells for 0 hour, and with mtt for 3 hours

column	y (mean Abs)	se`[yEr±]	x (cell number)
A	0.4918	0.06002	10000
B	0.3838	0.01732	9000
C	0.3518	0.0749	8000
D	0.3082	0.02149	7000
E	0.253	0.01746	6000
F	0.1928	0.00882	5000
G	0.1788	0.01274	4000
H	0.1294	0.00523	3000
I	0.1532	0.01201	2000
J	0.0874	0.00206	1000
k	0		0

Table 3.2. b Incubation of B16 cells at 37°C in RPMI media for 24 hours, and with mtt for 3 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	1.24338	0.06916	10000
B	0.92963	0.03777	9000
C	0.85725	0.06859	8000
D	0.70562	0.04124	7000
E	0.66013	0.04406	6000
F	0.60513	0.04337	5000
G	0.5525	0.03461	4000
H	0.38375	0.02606	3000
I	0.23175	0.00865	2000
J	0.15438	0.00425	1000

Table 3.2. c Incubation of B16 cells at 37°C in RPMI media for 48 hours, and with mtt for 3 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	1.51038	0.03116	10000
B	1.42725	0.06744	9000
C	1.36729	0.08871	8000
D	1.4485	0.06129	7000
E	1.525	0.07584	6000
F	1.61625	0.04373	5000
G	1.29263	0.09855	4000
H	1.06488	0.09832	3000
I	0.61943	0.06694	2000
J	0.20075	0.01513	1000

Table 3.2. d Incubation of B16 cells at 37°C in RPMI media for 72 hours, and with mtt for 3 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log mitozolomide) cocentration
A	0.67014	0.0501	76	-8.701
B	0.88771	0.1145	107	-8.0996
C	0.78557	0.10144	93	-7.497
D	0.769	0.11043	90	-6.896
E	0.843	0.11243	101	-6.293
F	1.00443	0.1672	123	-5.691
G	0.842	0.07238	101	-5.089
H	0.71157	0.06111	58	-4.488
I	0.39386	0.02762	37	-3.886
J	0.28029	0.00786	21	-3.283

Table 3.3. a Incubation of B16 cells with mitozolomide for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log mitozolomide) concentration
A	0.813	0.10309	120	-8.701
B	0.70583	0.12461	104	-8.0996
C	0.80067	0.08425	118	-7.497
D	0.61333	0.05237	90	-6.896
E	0.552	0.05308	81	-6.293
F	0.54317	0.07984	80	-5.691
G	0.67617	0.08419	100	-5.089
H	0.6295	0.10293	93	-4.488
I	0.338	0.02229	50	-3.886
J	0.174	0.0192	25	-3.283

Table 3.3. b Incubation of B16 cells with Mitozolomide for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log mitozolomide concentration)
A	0.76443	0.12487	91	-7.39
B	0.77814	0.12172	93	-6.39
C	0.872	0.15798	107	-5.39
D	0.68686	0.09675	80	-4.789
E	0.40914	0.0325	39	-4.488
F	0.34757	0.03622	30	-4.187
G	0.311	0.01913	25	-3.89
H	0.317	0.01951	26	-3.58
I	0.13729	0.0029	-1	-2.982
J	0.13243	0.00535	-1	-2.68

Table 3.3 c. Incubation of B16 cells with mitozolomide for 72 hours

column	y (mean Abs)	se[yEr±]	y (% Abs compared to control)	x (log dacarbazine concentration)
A	1.267	0.09921	101	-8.22
B	1.26714	0.14004	101	-7.22
C	1.30117	0.10259	110	-6.22
D	1.18329	0.13168	95	-5.22
E	1.06	0.10376	87	-4.22
F	0.48257	0.07322	48	-3.22
G	0.18313	0.0173	28	-2.90

Table 3.4 a Incubation of B16 cells with dacarbazine for 72 hours

column	y (mean Abs)	se[yEr±]	y (% Abs compared to control)	x (log dacarbazine concentration)
A	0.782	0.04727	93	-6.83
B	0.85167	0.03177	101	-6.22
C	0.79	0.04546	94	-5.62
D	0.79833	0.03027	95	-5.02
E	0.71667	0.03853	86	-4.42
F	0.36167	0.0212	42	-3.82
G	0.25833	0.01249	30	-3.21
H	-0.02117	0.00751	-3	-2.61
I	0.11183	0.00508	12	-2.01
J	-0.07833	0.00859	-10	-1.41

Table 3.4 c. Incubation of B16 cells with dacarbazine for 72 hours

column	y (meanAbs)	se [yEr±]	y (% Abs compared to control)	x (log ethidium bromide concentration)
A	0.87414	0.03538	98	-7.705
B	0.87871	0.05835	99	-7.1
C	0.827	0.03817	92	-6.5
D	0.84857	0.06108	95	-5.9
E	0.72757	0.0582	80	-5.3
F	0.318	0.01217	27	-4.69
G	0.13371	0.00257	4	-4.09
H	0.21686	0.00299	14	-3.49
I	0.10043	0.00303	0	-2.889
J	0.10314	0.00339	0	-2.287

Table 3.5 a. Incubation of B16 cells with Ethidium bromide for 72 hours, ,

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log ethidium bromide concentration)
A	0.86714	0.04281	85	-7.705
B	0.89186	0.04936	88	-7.1
C	0.99843	0.03338	98	-6.5
D	0.91729	0.05909	90	-5.9
E	0.81243	0.05086	80	-5.3
F	0.16614	0.0047	16	-4.69
G	0	0	0	-4.09
H	0	0	0	-3.49
I	0	0	0	-2.889
J	0	0	0	-2.287

Table 3.5. b Incubation of B16 cells with Ethidium bromide for 72 hours

column	y (mean Abs)	se [yEr±]	% Abs compared to control	x (log ethidium bromide concentration)
A	1.064	0.06418	108	-7.705
B	0.90271	0.06367	90	-7.1
C	0.91429	0.03617	92	-6.5
D	0.899	0.06133	90	-5.9
F	0.78257	0.07034	77	-5.3
G	0.25143	0.01981	21	-4.69
H	0.076	0.00267	2	-4.09
I	0.07986	0.00211	3	-3.49
J	0.08271	0.00269	3	-2.889
K	0.11814	0.00198	7	-2.287

Table 3.5 c. Incubation of B16 cells with ethidium bromide for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log fluorouracil concentration)
A	1.37771	0.10002	101	-8.59176
B	1.27286	0.16026	92	-7.89279
C	1.29843	0.10908	94	-7.19382
D	1.18171	0.14898	84	-6.49485
E	0.474	0.0156	23	-5.79588
F	0.35443	0.01304	13	-5.09691
G	0.34714	0.0246	12	-4.39794
H	0.24629	0.00992	3	-3.69897
I	0.15629	0.00778	0	-3
J	0.12729	0.03266	0	-2.30103

Table 3.6.a Incubation of B16 cells with fluorouracil for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log fluorouracil concentration)
A	1.48838	0.15308	105	-8.59176
B	1.3445	0.10179	94	-7.89279
C	1.16538	0.11822	80	-7.19382
D	1.05271	0.09402	72	-6.49485
E	0.31971	0.01756	15	-5.79588
F	0.20357	0.01687	6	-5.09691
G	0.18471	0.00725	5	-4.39794
H	0.15671	0.00462	2	-3.69897
I	0.116	0.00469	0	-3
J	0.10329	0.0038	0	-2.30103

Table 3.6 b. Incubation of B16 cells with flourouracil for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log fluorouracil concentration)
A	1.74571	0.09616	84	-8.1
B	1.93143	0.04067	93	-7.4
C	0.80429	0.06785	39	-6.7
D	0.05743	0.00273	3	-6
E	0.072	0.00544	4	-5.3
F	0	0	0	-4.61
G	0	0	0	-3.91
H	0	0	0	-3.21

Table 3.6. c. Incubation of B16 cells with flourouracil for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control	x (log Methotrexate concentration)
A	1.13667	0.02591	84	-9.1
B	1.355	0.02907	110	-8.69
C	1.34333	0.04432	108	-8.29
D	1.115	0.02553	81	-7.89
E	1.045	0.00922	73	-7.5
F	0.915	0.02094	58	-7.1
G	0.74	0.02206	38	-6.7
H	0.54833	0.02023	15	-6.3
I	0.425	0.01688	1	-4.3

Table 3.7. a. Incubation of B16 cells with methotrexate for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log methotrexate concentration)
B	1.21833	0.0199	89	-9.1
C	1.25167	0.01108	92	-8.69
D	1.34	0.01033	101	-8.29
E	1.3	0.06693	97	-7.89
F	1.32333	0.04738	99	-7.5
G	1.11667	0.06168	79	-7.1
H	0.69833	0.09119	37	-6.7
I	0.56	0.04919	23	-6.3
J	0.37167	0.01424	4	-4.3

Table 3.7 .b. Incubation of B16 cells with Methotrexate for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log Methotrexate concentration)
A	1.28	0.01983	108	-9.7
B	1.07667	0.02076	81	-9.1
C	1.39167	0.03646	123	-8.69
D	1.09333	0.01961	83	-8.29
E	1.10833	0.03146	85	-7.89
F	1.26167	0.034	106	-7.5
G	1.01167	0.01558	72	-7.1
H	0.87667	0.02704	54	-6.7
I	0.74333	0.01994	36	-6.3
J	0.48	0.01506	0	-4.3

Table 3.7 .c.- Incubation of B16 cells with Methotrexate for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control0	x (log vinblastine concentration)
A	1.50667	0.08239	99	-10.377
B	1.4875	0.14983	97	-9.775
C	1.34567	0.16578	84	-9.173
D	1.5792	0.16339	106	-8.57
E	0.67333	0.05821	22	-7.97
F	0.83883	0.10341	37	-7.365
G	0.71117	0.07667	26	-6.76
H	0.40817	0.02916	-2	-6.16
I	0.3495	0.01622	-8	-5.56
J	0.33267	0.01749	-7	-4.958

Table 3.8. a Incubation of B16 cells with vinblastine for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log vinblastine concentration)
A	1.79129	0.07759	89	-9.77
B	1.37	0.08058	68	-9.17
C	0.85143	0.12481	42	-8.57
D	0.07317	0.00241	3	-7.97
E	0.09071	0.00127	4	-7.37
F	0.045	0.00179	1	-6.16

Table 3.8.b. Incubation of B16 cells with Vinblastine for 72 hours

column	Y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log vinblastine concentration)
A	1.8005	0.06894	101	-10.377
B	1.79167	0.06322	93	-9.775
C	1.51067	0.05507	73	-9.173
D	1.34583	0.08837	57	-8.57
E	0.89367	0.03514	13	-7.97
F	1.0705	0.06202	31	-7.365
G	0.99783	0.05296	24	-6.76
H	0.60867	0.02377	-13	-6.16
I	0.6175	0.0426	-13	-5.56
J	0.623	0.02218	-12	-4.958

Table 3.8. c Incubation of B16 cells with vinblastine for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	log daunorubicin concentration
A	1.61533	0.07971	86	-10.47
B	1.92833	0.09843	103	-9.87
C	1.50433	0.09435	80	-9.27
D	0.762	0.05499	41	-8.66
E	0.13417	0.04878	8	-8.06
F	0.0685	0.00417	4	-7.46
G	0.06183	0.0037	4	-6.86
H	0.04017	0.00087	3	-6.26

Table 3.9.a. Incubation of B16 cells with daunorubicin for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	log daunorubicin concentration
A	1.00667	0.04485	100	-9.78
B	0.89167	0.06019	88	-9.17
C	0.71667	0.10098	70	-8.57
D	0.36	0.01751	33	-7.97
E	0.24833	0.02056	22	-7.37
F	-0.00767	0.01463	3	-6.76
G	0.05567	0.00414	2	-6.16
H	0.04567	0.00415	1	-4.96

Table 3.9.b. Incubation of B16 cells with daunorubicin for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	log daunorubicin concentration
A	0.86667	0.0206	91	-9.78
B	0.92	0.03578	96	-9.17
C	0.6	0.02769	61	-8.57
D	0.29333	0.02124	27	-7.97
E	0.17667	0.00667	15	-7.37
F	0.10567	0.00488	7	-6.76
G	0.0195	0.0162	-3	-6.16
H	0.01667	0.00123	-3	-4.96
I	0.03733	0.00294	-1	-4.36

Table 3.9.c. Incubation of B16 cells with daunorubicin for 72 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log doxorubicin concentration)
A	1.06167	0.01682	85	-9.78
B	1.255	0.09066	100	-9.18
C	1.13	0.06598	90	-8.58
D	0.43833	0.04854	33	-8.00
E	0.18667	0.03007	13	-7.37
F	0.01833	0.01327	-1	-6.77
G	0.06467	0.00877	3	-4.96
H	0.11033	0.01415	7	-4.36

Table 3.10. a. Incubation of B16 cells with doxorubicin for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log doxorubicin concentration)
A	1.625	0.10049	89	-9.78
B	1.86167	0.07391	103	-9.18
C	1.59333	0.13698	86	-8.58
D	0.96667	0.02642	47	-8.00
E	0.625	0.04342	26	-7.37
F	0.29167	0.01276	5	-6.77
G	0.17833	0.01249	-2	-6.17
H	0.23933	0.02037	2	-5.56
I	0.22167	0.01579	1	-4.96
J	0.22333	0.01382	1	-4.36

Table 3.10. b Incubation of B16 cells with doxorubicin for 72 hours

column	y (mean Abs)	se [yEr±)	y (% Abs compared to control)	x (log doxorubicin concentration)
A	1.44333	0.10935	89	-10.47
B	1.46333	0.08617	91	-9.87
C	1.525	0.07343	95	-9.27
D	0.8272	0.07868	51	-8.66
E	0.15217	0.01455	9	-8.06
F	0.14717	0.01535	9	-7.46
G	0.01767	0.00247	11	-6.26

Table 3.10. c. Incubation of B16 cells with doxorubicin for 72 hours

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log sibromycin concentration)
A	1.23667	0.02871	95	-10.8
B	1.32833	0.02056	102	-10.22
C	1.31167	0.01352	101	-9.62
D	1.22167	0.01621	94	-9.02
E	1.10333	0.05129	85	-8.42
F	0.58	0.01713	45	-7.82
G	0.395	0.02012	30	-7.21
H	0.0115	0.00356	1	-6.61
I	0.01167	0.00477	1	-6.01
J	-0.00167	0.00167	-1	-5.41

Table 3.11. a. Incubation of B16 cells with sibromycin for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log sibromycin concentration)
A	1.145	0.05182	98	-10.8
B	1.17167	0.05805	99	-10.22
C	1.15833	0.04672	100	-9.62
D	1.09833	0.0585	95	-9.02
E	1.01	0.04435	87	-8.42
F	0.56	0.02852	48	-7.82
G	0.38167	0.02626	32	-7.21
H	0.03017	0.00065	2	-6.61
I	0.03667	0.00333	3	-6.01
J	-0.00217	0.00217	0	-5.41

Table 3.11.b. Incubation of B16 cells with sibromycin for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log sibromycin concentration)
A	1.22833	0.01249	101	-10.8
B	1.20333	0.01308	98	-10.22
C	1.21333	0.01406	99	-9.62
D	1.17167	0.01493	96	-9.02
E	1.08167	0.00749	88	-8.42
F	0.59	0.00775	47	-7.82
G	0.46167	0.01682	36	-7.21
H	0.05667	0.00211	2	-6.61
I	0.05333	0.00211	1	-6.01
J	0.05167	0.00307	1	-5.41

Table 3.11.c. Incubation of B16 cells with sibromycin for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log adozelesin concentration)
A	1.115	0.01875	109	-12.8
B	1.02	0.02145	100	-12.17
C	0.985	0.02306	95	-11.6
D	0.78833	0.04003	75	-11
E	0.54	0.0139	49	-10.4
F	0.525	0.01875	46	-9.8
G	0.215	0.00342	13	-9.16
H	0.03533	0.00353	0	-8.6
I	0.079	0.00184	0	-8
J	0.04833	0.00307	0	-7.4

Table 3.12.a. Incubation of B16 cells with adozelesin for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log adozelesin concentration)
A	1.10667	0.02765	104	-12.8
B	1.03167	0.02738	96	-12.17
C	1.03167	0.02926	96	-11.6
D	0.86833	0.03156	78	-11
E	0.58667	0.02216	47	-10.4
F	0.475	0.01522	34	-9.8
G	0.23667	0.00494	9	-9.16
H	0.15167	0.00167	-1	-8.6
I	0.135	0.00224	-3	-8
J	0.15333	0.00333	-1	-7.4

Table 3.12.b. Incubation of B16 cells with adozelesin for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log adozelesin concentration)
A	0.99	0.0313	112	-12.8
B	0.93667	0.03303	106	-12.17
C	0.86667	0.02883	97	-11.6
D	0.732	0.05722	81	-11
E	0.535	0.02975	57	-10.4
F	0.51667	0.01085	55	-9.8
G	0.24167	0.00477	22	-9.16
H	0.06	0	0	-8.6
I	0.03333	0.00211	-4	-8
J	0.06	0.00258	0	-7.4

Table 3.12.c. Incubation of B16 cells with adozelesin for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log bizelesin concentration)
A	1.14833	0.03763	100	-12.9
B	1.09667	0.01892	96	-12.1
C	0.828	0.02853	72	-11.7
D	0.55167	0.02414	48	-11.1
E	0.385	0.02363	33	-10.48
F	0.32	0.01483	28	-9.88
G	0.27	0.01693	23	-9.28
H	0.15667	0.01085	14	-8.68
I	0.00833	0.00401	1	-8.08
J	0	0	0	-7.47

Table 3.13.a. Incubation of B16 cells with bizelesin for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log bizelesin concentration)
A	1.30667	0.02591	102	-12.9
B	1.232	0.0481	96	-12.1
C	0.815	0.0715	63	-11.7
D	0.70667	0.02974	55	-11.1
E	0.51	0.00683	40	-10.48
F	0.42167	0.02301	33	-9.88
G	0.40667	0.02459	32	-9.28
H	0.22	0.02066	17	-8.68
I	0.14667	0.00211	12	-8.08
J	0.04333	0.00333	3	-7.47

Table 3.13.b. Incubation of B16 cells with bizelesin for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log bizelesin concentration)
A	1.09	0.01633	101	-12.9
B	1.02	0.0188	94	-12.1
C	0.92333	0.03106	85	-11.7
D	0.562	0.01241	52	-11.1
E	0.44667	0.01542	42	-10.48
F	0.37333	0.02011	34	-9.88
G	0.335	0.02172	30	-9.28
H	0.23333	0.01476	21	-8.68
I	0.05667	0.00333	5	-8.08
J	0.03	0.00365	3	-7.47

Table 3.13.c. Incubation of B16 cells with bizelesin for 72 hours.

Appendix B

Experimental data for chapter 4

column	y (mean Abs)	se [yEr±]	x (log 9-anthracene carboxylic acid)
A	0.35783	0.01219	-7.726
B	0.4425	0.04639	-7.123
C	0.44733	0.03161	-6.52
D	0.37217	0.03721	-5.919
E'	0.42883	0.03868	-5.317
F	0.459	0.0338	-4.71
G	0.45	0.04317	-4.113
H	0.40867	0.05007	-3.511
I	0.51283	0.03257	-2.909

Table 4.1.a. Incubation of B16 cells with 9-anthracene carboxylic acid for 48 hours

column	y (mean Abs)	se [yEr±]	x (log 9-anthracene carboxylic acid)
A	0.74671	0.07178	-7.7
B	0.77529	0.1381	-7.1
C	0.784	0.07505	-6.49
D	0.81243	0.10154	-5.89
E	0.89214	0.06141	-5.29
F	0.969	0.05013	-4.69
G	0.908	0.05228	-4.09
H	0.79043	0.08859	-3.48
J	1.02875	0.0506	-2.279

Table 4.1.b. Incubation of B16 cells with 9-anthracene carboxylic acid for 48 hours

column	y (mean Abs)	se [yEr±]	x (log acridine carboxylic acid hydrate)
A	0.60243	0.02122	-8.418
B	0.51629	0.03148	-7.81
C	0.54029	0.01685	-7.214
D	0.47957	0.0201	-6.61
E	0.53443	0.02791	-6.01
F	0.54436	0.04108	-5.408
G	0.55714	0.04283	-4.806
H	0.466	0.03602	-4.204
I	0.49343	0.01776	-3.602

Table 4.2.a. Incubation of B16 cells with acridine carboxylic acid hydrate for 48 hours

column	y (mean Abs)	se [yEr±]	x (log acridine carboxylic acid hydrate)
A	0.60086	0.02141	-8.418
B	0.62543	0.01299	-7.81
C	0.71771	0.02547	-7.214
D	0.668	0.01088	-6.61
E	0.67529	0.01487	-6.01
F	0.68114	0.02717	-5.408
G	0.67571	0.01424	-4.806
H	0.68843	0.03623	-4.0204
I	0.69743	0.02676	-3.602

Table 4.2.b. Incubation of B16 cells with acridine carboxylic acid hydrate for 48 hours

column	y (mean Abs)	se [yEr±]	x (log butrescine)
A	0.665	0.00645	-6.66
B	0.7775	0.02358	-6.1
C	0.7125	0.03966	-5.5
D	0.775	0.03884	-4.9
E	0.785	0.02754	-4.2
F	0.7375	0.05706	-3.65
G	0.6	0.05612	-3.05
H	0.5325	0.05202	-2.45

Table 4.3.a. Incubation of B16 cells with butrescine for 48 hours

column	y (mean Abs)	se [yEr±]	x (log butrescine)
A	0.57	0.03416	-6.66
B	0.775	0.00957	-6.1
C	0.6975	0.05437	-5.5
D	0.705	0.01258	-4.9
E	0.7075	0.0281	-4.2
F	0.6475	0.02016	-3.65
G	0.6225	0.01702	-3.05
H	0.4925	0.01493	-2.45

Table 4.3.b. Incubation of B16 cells with butrescine for 48 hours

column	y (mean Abs)	se [yEr±]	x (log butrescine)
A	0.48	0.03136	-6.66
B	0.585	0.05867	-6.1
C	0.7075	0.01652	-5.5
D	0.5175	0.0075	-4.9
E	0.5175	0.02689	-4.2
F	0.4975	0.04131	-3.65
G	0.4225	0.05138	-3.05
H	0.385	0.01323	-2.45

Table 4.3.c. Incubation of B16 cells with butrescine for 48 hours

column	y (mean Abs)	se [yEr±]	x (log spermidine)
A	0.72	0.0272	-7.4
B	0.65	0.01703	-6.8
C	0.588	0.03231	-6.2
D	0.544	0.03544	-5.6
E	0.592	0.01594	-5
F	0.488	0.01393	-4.4
G	0.468	0.0102	-3.8
H	0.504	0.01288	-3.2
I	0.41	0.01265	-2.6

Table 4.4.a. Incubation of B16 cells with spermidine for 48 hours

column	y (mean Abs)	se [yEr±]	x (log spermidine)
A	0.752	0.02131	-7.4
B	0.628	0.05093	-6.8
C	0.574	0.02293	-6.2
D	0.51	0.03899	-5.6
E	0.452	0.02311	-5
F	0.412	0.01934	-4.4
G	0.35	0.01789	-3.8
H	0.334	0.01435	-3.2
I	0.27	0.00548	-2.6

Table 4.4.b. Incubation of B16 cells with spermidine for 48 hours

column	y (mean Abs)	se [yEr±]	x (log spermidine)
A	0.42	0.02074	-7.4
B	0.45	0.0228	-6.8
C	0.416	0.01536	-5.6
D	0.39	0.02569	-5
E	0.386	0.02205	-4.4
F	0.312	0.0398	-3.8
G	0.346	0.02015	-3.2
H	0.172	0.00374	-2.6

Table 4.4.c. Incubation of B16 cells with spermidine for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log spermine concentration)
B	0.65729	0.02983	109	-7.068
C	0.71214	0.05835	119	-6.465
D	0.61029	0.06121	100	-5.86
E	0.46314	0.02676	72	-5.26
F	0.44729	0.02554	69	-4.66
G	0.34783	0.02801	50	-4.055
H	0.333	0.02432	47	-3.45
I	0.07986	0.00345	-1	-2.85
J	0.0518	0.0058	-6	-2.248

Table 4.5.a. Incubation of B16 cells with spermine for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log spermine concentration)
A	0.70667	0.0152	116	-7.71
B	0.68	0.03256	112	-7.12
C	0.63	0.06039	105	-6.51
D	0.53833	0.05307	93	-5.91
E	0.54667	0.01892	95	-5.31
F	0.48333	0.02171	85	-4.71
G	0.39	0.00316	73	-4.11
H	0.43667	0.01838	80	-3.5
I	0.15	0.00577	40	-2.9
J	0.05467	0.00446	27	-2.3

Table 4.5.b. Incubation of B16 cells with spermine for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log spermine concentration)
A	0.86143	0.02087	93	-7.88
B	0.92286	0.04057	98	-7.28
C	0.97286	0.07322	103	-6.67
D	0.91857	0.05671	98	-6.07
E	0.903	0.05013	96	-5.47
F	0.88429	0.03308	95	-4.87
G	0.97714	0.04034	104	-4.27
H	0.75714	0.072	83	-3.66
I	0.109	0.00069	23	-3.06
J	0.08029	0.00944	21	-2.46

Table 4.5.c. Incubation of B16 cells with spermine for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 1/1 molar mixture of spermine and 9-anthracene carboxylic acid)
A	0.8705	0.0331	86	-7.66
B	1.0225	0.06553	100	-7.06
C	1.2682	0.08201	124	-6.46
D	0.88067	0.03174	89	-5.858
E	0.92383	0.06278	91	-5.25
F	0.99067	0.11166	97	-4.65
G	0.91683	0.04636	91	-4.052
H	0.43383	0.03342	44	-3.45
I	0.1095	0.00891	13	-2.848
J	0.08233	0.01043	11	-2.246

Table 4.6.a.. Incubation of B16 cells with 1/1 molar mixture of spermine and 9-anthracene carboxylic acid for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 1/1 molar mixture of spermine and 9-anthracene carboxylic acid)
A	0.8208	0.07579	74	-7.66
B	1.02983	0.06437	94	-7.06
C	1.22633	0.07595	113	-6.46
D	0.86617	0.03478	79	-5.858
E	1.03017	0.1052	94	-5.25
F	0.9695	0.10885	88	-4.65
G	0.9095	0.0466	83	-4.052
H	0.44383	0.03459	38	-3.45
I	0.095	0.00866	5	-2.848
J	0.07817	0.00679	4	-2.246

Table 4.6.b.. Incubation of B16 cells with 1/1 molar mixture of spermine and 9-anthracene carboxylic acid for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 1/1 molar mixture of spermine and 9-anthracene carboxylic acid)
A	0.6895	0.02946	83	-7.85
B	0.90825	0.0141	109	-7.25
C	0.72967	0.03287	88	-6.645
D	0.74867	0.02481	90	-6.045
E	0.89167	0.02363	107	-5.44
F	0.95167	0.04626	114	-4.84
G	0.52867	0.02152	64	-4.238
H	0.1635	0.00177	19	-3.636
I	0.15175	0.00165	18	-3.034

Table 4.6.c.. Incubation of B16 cells with 1/1 molar mixture of spermine and 9-anthracene carboxylic acid for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 1/1 molar mixture of spermine and acridine carboxylic acid hydrate)
A	0.43	0.01155	110	-7.07
B	0.42333	0.01726	107	-6.6
C	0.395	0.01118	100	-6.12
D	0.37	0.0177	92	-5.6
E	0.385	0.00847	97	-5.2
F	0.35167	0.01621	87	-4.69
G	0.36167	0.0204	90	-4.21
H	0.27833	0.01195	66	-3.73
I	0.21	0.01065	46	-3.26
J	0.11167	0.00167	17	-2.79

Table 4.7.a. .. Incubation of B16 cells with 1/1 molar mixture of spermine and acridine carboxylic acid hydrate

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 1/1 molar mixture of spermine and acridine carboxylic acid hydrate)
A	1.114	0.05862	104	-7.07
B	1.104	0.00678	103	-6.6
C	1.078	0.02596	101	-6.12
D	1.126	0.03027	106	-5.6
E	1.018	0.06406	95	-5.2
F	0.914	0.02839	85	-4.69
G	0.722	0.01772	65	-4.21
H	0.762	0.01356	69	-3.73
I	0.42	0.01049	35	-3.26
J	0.164	0.0186	10	-2.79

Table 4.7.b. .. Incubation of B16 cells with 1/1 molar mixture of spermine and acridine carboxylic acid hydrate

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 1/1 molar mixture of spermine and acridine carboxylic acid hydrate)
A	0.888	0.03513		-7.07
B	0.924	0.0186		-6.6
C	0.868	0.02871		-6.12
D	0.688	0.01985		-5.6
E	0.59	0.05206		-5.2
F	0.484	0.01568		-4.69
G	0.406	0.00748		-4.21
H	0.334	0.01208		-3.73
I	0.224	0.00812		-3.26
J	0.0444	0.00246		-2.79

Table 4.7.c. .. Incubation of B16 cells with 1/1 molar mixture of spermine and acridine carboxylic acid hydrate

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of mono-anthracenyl spermine)
A	1.27571	0.099	114	-9.16
B	1.14483	0.04063	101	-8.56
C	1.05471	0.05519	93	-7.96
D	1.21971	0.06352	109	-7.36
E	0.9974	0.05277	87	-6.75
F	1.02733	0.06316	91	-6.15
G	0.95543	0.03567	83	-5.55
H	0.89829	0.0476	78	-4.947
I	0.35829	0.03501	26	-4.34
J	0.24243	0.00893	15	-3.74

Table 4.8.a. .. Incubation of B16 cells with Mono-anthracenyl spermine for 48 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of mono-anthracenyl spermine)
A	0.726	0.08352	100	-7.725
B	0.77386	0.10442	108	-7.12
C	0.64043	0.08433	88	-6.52
D	0.61071	0.08789	83	-5.92
E	0.48014	0.06106	64	-5.32
F	0.25983	0.03546	32	-4.71
G	0.06614	0.00492	3	-4.11
H	0.06229	0.00246	2	-3.51
I	0.05229	0.00241	0	-2.909
J	0.0685	0.00511	3	-2.307

Table 4.8.b. Incubation of B16 cells with Mono-anthracenyl spermine for 48 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of mono-anthracenyl spermine)
A	0.43833	0.04086	123	-7.62
B	0.48	0.01966	132	-7.09
C	0.37333	0.02777	107	-6.6
D	0.40167	0.02626	114	-5.52
E	0.23167	0.02926	75	-5
F	0.305	0.01258	91	-4.48
G	0.03967	0.01425	31	-3.95
H	0.03683	0.00145	31	-3.43
I	0	0	21	-2.9

Table 4.8.c. .. Incubation of B16 cells with Mono-anthracenyl spermine for 48 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 9-amidospermine acridine)
A	0.61486	0.03806	91	-8.418
B	0.58171	0.01642	86	-7.81
C	0.79543	0.06844	118	-7.214
D	0.63767	0.07576	94	-6.61
E	0.60457	0.03577	89	-6.01
F	0.5224	0.02779	77	-5.408
G	0.07933	0.04166	11	-4.806
H	0.06857	0.0295	9	-4.204
I	0.078	0.01249	10	-3.602

Table 4.9.a. Incubation of B16 cells with 9-amidospermine acridine for 48 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 9-amidospermine acridine)
A	0.61486	0.03615	95	-8.418
B	0.76057	0.04838	125	-7.81
C	0.55343	0.01003	81	-7.214
D	0.53257	0.02497	77	-6.61
E	0.53	0.04125	77	-6.01
F	0.58733	0.02791	89	-5.408
G	0.20514	0.02316	9	-4.806
H	0.21114	0.01396	11	-4.204
I	0.24314	0.01665	17	-3.602
J	0.14257	0.0039	0	-3

Table 4.9.b. Incubation of B16 cells with 9-amidospermine acridine for 48 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 9-amidospermine acridine)
A	0.69	0.01747	111	-8.418
B	0.66057	0.0068	105	-7.81
C	0.57457	0.01945	86	-7.214
D	0.55171	0.02356	82	-6.61
E	0.54771	0.0173	82	-6.01
F	0.38029	0.02316	48	-5.408
G	0.19243	0.01573	10	-4.806
H	0.18629	0.00983	9	-4.204

Table 4.9.c. Incubation of B16 cells with 9-amidospermine acridine for 48 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
A	0.62029	0.01641	100	-8.418
B	0.56971	0.02101	90	-7.81
C	0.58029	0.01738	92	-7.214
D	0.46714	0.01267	70	-6.61
E	0.21671	0.00545	22	-6.01
F	0.182	0.01023	15	-5.408
G	0.117	0.01008	2	-4.806

Table 4.10.a. Incubation of B16 cells with acridine monospermine for 48 hours for 48 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
A	0.53029	0.01149	91	-8.418
B	0.54143	0.02764	94	-7.81
C	0.53771	0.01436	94	-7.214
D	0.50171	0.00811	83	-6.61
E	0.228	0.00592	12	-6.01
F	0.216	0.00373	10	-5.408
G	0.20829	0.00348	7	-4.806
H	0.20029	0.00331	5	-3.602

Table 4.10.b. Incubation of B16 cells with acridine monospermine for 48 hours for 48 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
A	0.68557	0.0224	125	-9
B	0.58143	0.03569	104	-8.39
C	0.566	0.0345	101	-7.79
D	0.47457	0.0264	82	-7.19
E	0.3705	0.03926	61	-6.58
F	0.425	0.03245	73	-6
G	0.31071	0.03255	49	-5.38
H	0.05217	0.0177	-3	-4.78
I	0.08329	0.00174	3	-4.18
J	0.091	0.00115	5	-3.58

Table 4.10.c. Incubation of B16 cells with acridine monospermine for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 5b)
A	0.50833	0.00792	112	-9.12
B	0.41667	0.01542	91	-8.52
C	0.45833	0.01195	100	-7.9
D	0.42333	0.01229	93	-7.32
E	0.37833	0.01327	82	-6.72
F	0.38667	0.0143	84	-6.11
G	0.13	0.01461	25	-5.51
H	0.08667	0.00211	15	-4.91
I	0.047	0.00342	5	-4.31

Table 4.11.a. Incubation of B16 cells with 5b for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 5b)
A	0.278	0.01114	80	-9.12
B	0.3	0.02	87	-8.52
C	0.366	0.014	107	-7.9
D	0.36	0.01703	104	-7.32
E	0.372	0.0153	107	-6.72
F	0.244	0.01965	70	-6.11
G	0.082	0.01855	25	-5.51
H	0.016	0.004	6	-4.91
I	0.037	0.0049	12	-4.31

Table 4.11.b. Incubation of B16 cells with 5b for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log 5b)
A	0.4	0.02915	94	-9.12
B	0.4225	0.02955	98	-8.52
C	0.42	0.03391	98	-7.9
D	0.4275	0.02358	100	-7.32
E	0.3925	0.0272	92	-6.72
F	0.3675	0.02358	87	-6.11
G	0.13	0.00913	37	-5.51
H	0	0	10	-4.91
I	0	0	10	-4.31

Table 4.12.c. Incubation of B16 cells with 5b for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 6b)
A	0.485	0.01848	95	-9.06
B	0.51	0.01	100	-8.46
C	0.5075	0.01436	100	-7.85
D	0.55	0.0178	106	-7.26
E	0.535	0.02598	103	-6.65
F	0.555	0.00866	106	-6
G	0.445	0.04941	89	-5.4
H	0.3225	0.00629	69	-4.8
I	0.27	0.00577	61	-4.2
J	0	0	18	-3.6

Table 4.13.a. Incubation of B16 cells with (6b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 6b)
A	0.504	0.01778	111	-9.06
B	0.498	0.01685	111	-8.46
C	0.408	0.01715	89	-7.85
D	0.458	0.01828	101	-7.26
E	0.368	0.022	79	-6.65
F	0.452	0.02267	99	-6
G	0.37	0.02258	79	-5.4
H	0.3	0.01761	61	-4.8
I	0.138	0.01114	21	-4.24
J	0.0914	0.00299	9	-3.6

Table 4.13.b. Incubation of B16 cells with (6b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 6b)
A	0.5525	0.06486	85	-8.62
B	0.395	0.01936	73	-8.01
C	0.505	0.02217	99	-7.42
L	0.61	0.04021	125	-6.81
D	0.4125	0.02175	77	-6.21
E	0.4125	0.04922	77	-5.6
F	0.2325	0.01109	35	-5
G	0.14	0.01354	14	-4.4
H	0.1	0.0169	4	-3.8

Table 4.13.c. Incubation of B16 cells with (6b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	0.3025	0.05793	80	-8.62
B	0.37	0.0745	107	-8
C	0.3525	0.02955	100	-7.4
D	0.2675	0.03376	69	-6.81
E	0.15	0.00408	23	-6.21
F	0.1225	0.0025	11	-5.61
G	0.1125	0.0025	7	-5
H	0.1225	0.0025	11	-4.4
I	0.09975	0.00025	3	-3.8
J	0.05625	0.00075	-13	-3.2

Table 4.14.a. Incubation of B16 cells with (7b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	0.564	0.02249	102	-8.62
B	0.516	0.02088	93	-8
C	0.472	0.03072	82	-7.4
D	0.424	0.02421	70	-6.81
E	0.222	0.00663	25	-6.21
F	0.144	0.00245	7	-5.61
G	0.116	0.00245	2	-5
H	0.122	0.002	2	-4.4
I	0.134	0.00245	4	-3.8
J	0.104	0.00245	-2	-3.2

Table 4.14.b. Incubation of B16 cells with (7b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	0.476	0.04468	104	-8.62
B	0.402	0.04352	85	-8
C	0.454	0.044	97	-7.4
D	0.328	0.04329	69	-6.81
E	0.15	0.02098	25	-6.21
F	0.024	0.00245	-4	-5.61
G	0.0782	0.00111	8	-5
H	0.048	0.002	1	-4.4
I	0.054	0.00245	3	-3.8
J	0.062	0.002	4	-3.2

Table 4.14.c. Incubation of B16 cells with (7b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 8b)
A	0.67	0.00837	95	-9.17
B	0.662	0.0153	94	-8.51
C	0.704	0.02272	99	-7.9
D	0.668	0.02267	95	-7.31
E	0.734	0.02182	102	-6.71
F	0.766	0.02839	107	-6.11
G	0.76	0.02646	105	-5.5
H	0.6375	0.0275	91	-4.9
I	0.415	0.03884	64	-4.3
J	0.165	0.0119	34	-3.7

Table 4.15.a. Incubation of B16 cells with (8b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 8b)
A	0.53	0.01304	103	-9.17
B	0.504	0.02227	98	-8.51
C	0.532	0.0153	103	-7.9
D	0.508	0.06312	100	-7.31
E	0.452	0.03813	91	-6.71
F	0.53	0.03808	103	-6.11
G	0.544	0.05066	104	-5.5
H	0.39	0.0501	83	-4.9
I	0.338	0.01934	75	-4.3
J	0.0875	0.01548	39	-3.7

Table 4.15.b. Incubation of B16 cells with (8b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 8b)
A	0.61333	0.01978	100	-9.17
B	0.61667	0.02789	102	-8.51
C	0.57833	0.03911	96	-7.9
D	0.59667	0.04072	99	-7.31
E	0.61833	0.02626	102	-6.71
F	0.60167	0.02167	99	-6.11
G	0.57833	0.03544	96	-5.5
H	0.47167	0.02358	79	-4.9
I	0.22167	0.01046	41	-4.3
J	0.06583	0.01172	18	-3.7

Table 4.15.c. Incubation of B16 cells with (8b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 9b)
A	0.714	0.02482	93	-8.5
B	0.826	0.05391	107	-7.9
C	0.812	0.07946	105	-7.3
D	0.75	0.05788	97	-6.7
E	0.61	0.01924	81	-6.1
F	0.566	0.01965	76	-5.5
G	0.26	0.02145	38	-4.9
H	0.038	0.002	12	-4.3
I	-0.0222	0.00196	5	-3.6
J	-0.056	0.00245	0	-3.1

Table 4.16.a. Incubation of B16 cells with (9b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 9b)
A	0.578	0.03308	106	-8.5
B	0.536	0.01965	98	-7.9
C	0.518	0.05044	94	-7.3
D	0.48	0.03017	86	-6.7
E	0.492	0.06367	88	-6.1
F	0.588	0.0586	108	-5.5
G	0.232	0.03007	38	-4.9
H	0.0576	0.00216	6	-4.3
I	0.13	0.0108	19	-3.6
J	0.034	0.00245	0	-3.1

Table 4.16.b. Incubation of B16 cells with (9b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 9b)
A	0.816	0.03501	111	-8.5
B	0.772	0.03904	105	-7.9
C	0.658	0.02417	92	-7.3
D	0.61	0.03633	85	-6.7
E	0.566	0.02542	80	-6.1
F	0.514	0.01661	73	-5.5
G	0.12	0.04278	24	-4.9
H	-0.076	0.006	-1	-4.3
I	-0.018	0.0049	7	-3.6
J	-0.031	0.00458	5	-3.1

Table 4.16.c. Incubation of B16 cells with (9b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 10b)
A	0.83	0.04062	101	-8.6
B	0.89	0.04021	109	-8
C	0.7625	0.00854	92	-7.4
D	0.835	0.0433	101	-6.8
E	0.875	0.03403	106	-6.2
F	0.6875	0.03065	83	-5.6
G	0.2875	0.02175	32	-5
H	0.0975	0.0025	8	-4.4
I	0.1275	0.0025	11	-3.8
J	0.1175	0.0025	10	-3.2

Table 4.17.a. Incubation of B16 cells with (10b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 10b)
A	0.802	0.05826	96	-8.6
B	0.824	0.09201	98	-8
C	0.782	0.10047	94	-7.4
D	0.89	0.03647	106	-6.8
E	0.7	0.08142	85	-6.2
F	0.57	0.06535	71	-5.6
G	0.08	0.02864	18	-5
H	-0.05	0	3	-4.4
I	-0.05	0	3	-3.8
J	-0.046	0.004	3	-3.2

Table 4.17.b. Incubation of B16 cells with (10b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 10b)
A	0.60667	0.01856	113	-8.6
B	0.73667	0.02404	101	-8
C	0.76	0.01155	104	-7.4
D	0.77	0.01732	105	-6.8
E	0.71333	0.05175	97	-6.25
F	0.60667	0.02848	85	-5.6
G	0.54667	0.04096	78	-5
H	0	0	10	-4.4
I	0.03	0	13	-3.8
J	0.03333	0.00333	13	-3.23

Table 4.17.c. Incubation of B16 cells with (10b) for 48 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
A	0.857	0.02018	98	-9.87
B	0.904	0.01208	104	-9.17
C	0.894	0.01661	102	-8.47
D	0.816	0.02482	93	-7.77
E	0.676	0.02713	76	-7.07
F	0.472	0.01655	50	-6.37
G	0.168	0.00583	13	-5.67
H	0.0928	0.00132	4	-5
I	0.064	0.00122	1	-4.28
J	0.0682	0.00153	1	-3.58

Table 4.18.a. Incubation of B16 cells with acridine monospermine for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
A	1.33357	0.00934	101	-8.11
B	1.34571	0.04577	102	-7.508
C	1.209	0.05242	91	-6.913
D	1.13229	0.06396	85	-6.309
E	0.72614	0.061	54	-5.707
F	0.41743	0.01884	29	-5.105
G	0.24886	0.01918	16	-4.504
I	0.02957	0.00413	-1	-3.3

Table 4.18.b. Incubation of B16 cells with acridine monospermine for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
B	0.835	0.14666	88	-9.17
C	0.9725	0.14551	106	-8.47
D	0.92	0.13626	100	-7.77
E	0.755	0.03014	78	-7.07
F	0.5525	0.04308	52	-6.37
G	0.245	0.00866	12	-5.67
H	0.16	0	1	-5
I	0.18	0.00408	4	-4.28
J	0.1475	0.0025	0	-3.58

Table 4.18.c. Incubation of B16 cells with acridine monospermine for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
A	1.12917	0.14537	89	-9.87
B	1.18667	0.0754	94	-9.17
C	1.24667	0.11248	98	-8.47
D	1.225	0.13552	96	-7.77
E	1.145	0.12798	90	-7.07
F	0.58833	0.06316	46	-6.37
G	0	0	0	-5.67
H	0.10183	0.00295	8	-5
I	0.0495	0.00085	4	-4.28
J	0.0355	0.00022	3	-3.58

Table 4.19.a. Incubation of B16 cells with acridine monospermine for 6 days

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
A	1.325	0.03973	109	-9.87
B	1.175	0.03603	104	-9.17
C	1.125	0.0303	104	-8.47
D	1.07	0.05164	89	-7.77
E	1.135	0.0559	94	-7.07
F	1.22833	0.06819	101	-6.37
G	0.14183	0.01131	15	-5.67
H	0.018	0.00063	5	-5
I	0.1	0.00258	11	-4.28
J	-0.01767	0.0119	2	-3.58

Table 4.19.b. Incubation of B16 cells with acridine monospermine for 6 days

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of acridine monospermine)
A	1.945	0.04249	111	-9.87
B	1.66	0.06947	95	-9.17
C	1.62833	0.03701	94	-8.47
D	1.745	0.05359	100	-7.77
E	1.33333	0.05678	78	-7.07
F	1.12667	0.12325	67	-6.37
G	0.04383	0.00199	8	-5.67
H	-0.036	0.00306	8	-5
I	0.01167	0.0033	6	-3.58

Table 4.19.c. Incubation of B16 cells with acridine monospermine for 6 days

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	1.10083	0.04352	98	-10.19
B	1.09833	0.0341	98	-9.5
C	1.105	0.01607	99	-8.8
D	1.02667	0.01145	92	-8.1
E	0.90167	0.01579	81	-7.4
F	0.23167	0.00792	22	-6.7
G	0.0085	0.00134	2	-6
H	0.04017	0.00031	5	-5.3
I	-0.02233	0.00189	-1	-4.6
J	0.04	0.00063	5	-3.9

Table 4.20.a. Incubation of B16 cells with 7b for 72 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	1.11	0.03795	93	-10.19
B	1.18667	0.03528	100	-9.5
C	1.17667	0.01606	99	-8.8
D	1.13833	0.01701	96	-8.1
E	1.00167	0.02574	84	-7.4
F	0.35167	0.02344	31	-6.7
G	0.08067	0.00244	8	-6
H	0.0565	0.00115	6	-5.3
I	0.002	0.00144	0	-4.6
J	-0.02933	0.00269	-1	-3.9

Table 4.20.b. Incubation of B16 cells with 7b for 72 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	0.74333	0.01706	91	-10.19
B	0.795	0.00563	97	-9.5
C	0.82833	0.02509	102	-8.8
D	0.78333	0.02028	96	-8.1
E	0.66	0.02556	82	-7.4
F	0.19167	0.00946	27	-6.7
G	0.0035	0.00081	5	-6
H	-0.008	0.00203	3	-5.3
I	-0.012	0.00246	6	-4.6
J	-0.00583	0.00168	3	-3.9

Table 4.20.c. Incubation of B16 cells with 7b for 72 hours.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	0.95333	0.05011	106	-10.19
B	1.06467	0.06785	99	-9.5
C	1.08333	0.03518	101	-8.8
D	1.025	0.06092	96	-8.1
E	0.89	0.05317	84	-7.4
F	0.0505	0.00262	9	-6.7
G	-0.02367	0.00088	3	-6
H	0.02033	0.00076	7	-5.3
I	0.041	0.00126	9	-4.6
J	-0.0725	0.00145	-1	-3.9

Table 4.21.a. Incubation of B16 cells with 7b for 6 days.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	1.48833	0.03928	97	-10.19
B	1.515	0.03243	98	-9.5
C	1.50167	0.02971	98	-8.8
D	1.33833	0.03728	87	-8.1
E	0.93833	0.0622	62	-7.4
F	0.06367	0.00824	6	-6.7
G	0.0066	0.00267	3	-6
H	0.02583	0.00101	4	-5.3
I	-0.00317	0.00108	2	-4.6
J	0.02417	0.00119	4	-3.9

Table 4.21.b. Incubation of B16 cells with 7b for 6 days.

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log concentration of 7b)
A	1.18167	0.03027	101	-10.19
B	1.18	0.05073	101	-9.5
C	1.09833	0.03487	95	-8.8
D	0.95167	0.04385	82	-8.1
E	0.70667	0.03721	62	-7.4
F	0.06083	0.00536	7	-6.7
G	0.012	0.00181	2	-6
H	0.01867	0.01019	3	-5.3
I	0.00933	0.00115	2	-4.6
J	0.01717	0.00789	3	-3.9

Table 4.21.c. Incubation of B16 cells with 7b for 6 days.

Appendix C

Data for experiments in chapter 5

column	y (mean Abs).	se [yEr±]	y (% Abs compared to control)	x(log methotrexate concentration
A	0.6775	0.04328	93	-8.6
B	0.6525	0.01548	100	-8
C	0.5775	0.0525	92	-7.4
D	0.575	0.06513	90	-6.8
E	0.5125	0.05105	81	-6.2
F	0.4375	0.05879	70	-5.6
G	0.025	0.01041	3	-5
H	0.075	0.00289	11	-4.4
I	0.025	0.00289	3	-3.8
J	0.0375	0.0025	6	-3.2

Table 5.1.a Incubation of B16 cells with methotrexate for 4hours, then without methotrexate for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x(log methotrexate concentration
A	0.702	0.03513	98	-8.6
B	0.688	0.02818	97	-8
C	0.694	0.03957	97	-7.4
D	0.672	0.04224	94	-6.8
E	0.718	0.02905	101	-6.2
F	0.592	0.07081	82	-5.6
G	0.166	0.01939	20	-5
H	0.116	0.004	13	-4.4
I	0.068	0.00583	6	-3.8
J	0.05	0.00548	3	-3.2

Table 5.1.b Incubation of B16 cells with methotrexate for 4hours, then without methotrexate for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x(log methotrexate concentration
A	0.5775	0.02626	94	-8.6
B	0.6225	0.0225	100	-8
C	0.6125	0.02496	98	-7.4
D	0.5875	0.05836	95	-6.8
E	0.5075	0.03092	83	-6.2
F	0.51	0.0505	83	-5.6
G	0.045	0.00866	11	-5
H	0.0325	0.0025	9	-4.4
I	0.0225	0.0025	8	-3.8
J	-0.02	0.00408	1	-3.2

Table 5.1.c Incubation of B16 cells with methotrexate for 4hours, then without methotrexate for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x(log doxorubicin concentration
A	1.02667	0.09054	98	-8.9
B	1.10667	0.06586	107	-8.3
C	0.935	0.05812	91	-7.7
D	0.73	0.02817	69	-7.1
E	0.51833	0.01352	48	-6.5
F	0.26833	0.00946	24	-5.9
G	0.042	0.00129	2	-5.3
H	0.06867	0.00099	4	-4.69
I	0	0	0	-4.09
J	0	0	0	-3.49

Table 5.2.a. Incubation of B16 cells with doxorubicin for 4hours, then without doxorubicin for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x(log doxorubicin concentration)
A	1.24667	0.06302	113	-8.9
B	1.06	0.06501	95	-8.3
C	0.915	0.05321	81	-7.7
D	0.83333	0.06302	73	-7.1
E	0.504	0.0425	42	-6.5
F	0.31	0.0228	24	-5.9
G	0.14333	0.00211	8	-5.3
H	0.08	0.00516	2	-4.69
I	0	0	0	-4.09
J	0	0	0	-3.49

Table 5.2.b. Incubation of B16 cells with doxorubicin for 4hours then without doxorubicin for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x(log doxorubicin concentration)
A	0.81	0.04143	125	-9.78
B	0.58	0.03189	73	-9.18
C	0.665	0.04093	99	-8.58
D	0.64	0.03873	95	-7.98
E	0.46	0.04491	63	-7.38
F	0.4025	0.0125	52	-6.77
G	0.3125	0.00479	36	-6.2
H	0.135	0.00645	4	-5.57
I	0.0775	0.00479	-5	-4.97
J	0.0965	0.00176	-2	-4.37

Table 5.2. c Incubation of B16 cells with doxorubicin for 4 hours then withou it for 72 hours

column	y(mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log Daunorubicin concentration)
A	0.99167	0.04854	100	-9.77
B	0.88667	0.03073	88	-9.2
C	0.84167	0.04191	82	-8.57
D	0.55	0.01183	47	-8
E	0.29667	0.00615	16	-7.4
F	0.20333	0.00422	4	-6.8
G	0.17667	0.00211	1	-6.15
H	0.145	0.00224	-4	-5.56
I	0.14167	0.00401	-4	-4.95
J	0.12167	0.00307	-6	-4.35

Table 5.3.a. Incubation of B16 cells with Daunorubicin for 4 hours, then without it for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log Daunorubicin concentration)
A	0.86833	0.0324	95	-9.77
B	0.79	0.02781	85	-9.2
C	0.475	0.05536	45	-8.57
D	0.32333	0.0408	26	-8
E	0.30667	0.00882	25	-7.4
F	0.175	0.00671	7	-6.8
G	0.11167	0.00307	0	-6.15
H	0.05167	0.00307	-7	-5.56
I	0.07	0.00258	-5	-4.95
J	0.05	0.00365	-7	-4.35

Table 5.3. b. Incubation of B16 cells with Daunorubicin for 4 hours, then without it for 72 hours

column	y (mean Abs)	se [yEr±]	y (% Abs compared to control)	x (log Daunorubicin concentration)
A	0.566	0.03501	109	-9.77
B	0.48	0.02324	89	-9.17
C	0.49	0.04827	91	-8.57
D	0.456	0.04654	83	-7.97
E	0.258	0.01744	39	-7.37
F	0.194	0.00678	25	-6.76
G	0.132	0.0086	11	-6.16
H	0.0768	0.0024	-1	-5.56
I	0.114	0.00245	7	-4.96
J	0.0264	0.0016	-11	-4.36

Table 5.3. c. incubation of B16 cells with daunorubicin for 4 hours, then without it for 72 hours.

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log adozelesin concentration)
K	0.94167	0.03229	108	-12.8
A	0.865	0.03686	98	-12.17
B	0.84667	0.03073	97	-11.6
C	0.84	0.02745	96	-11
D	0.685	0.02643	76	-10.4
E	0.415	0.03212	44	-9.8
F	0.33167	0.01249	34	-9.16
G	0.135	0.005	10	-8.6
H	-0.0375	0.00171	-10	-8
I	0.06167	0.00307	2	-7.4

Table 5.4.a. Incubation of B16 cells with adozelesin for 4 hours then without adozelesin for 72 hours

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log adozelesin concentration)
A	1.01167	0.04813	105	-12.8
B	0.95333	0.03955	99	-12.17
C	0.91333	0.02642	94	-11.6
D	0.88833	0.03798	92	-11
E	0.755	0.02335	77	-10.4
F	0.50667	0.02171	51	-9.8
G	0.33333	0.01909	31	-9.16
H	0.07333	0.00422	3	-8.6
I	0.04333	0.00558	0	-8
J	0.015	0.00671	-3	-7.4

Table 5.4.b. Incubation of B16 cells with adozelesin for 4 hours then without adozelesin for 72 hours

column	y(mean Abs)	se (yEr±)	y(% abs compared to control)	x(log adozelesin concentration)
A	1.26333	0.03201	97	-12.8
B	1.3	0.03337	100	-12.17
C	1.35	0.02966	104	-11.6
D	1.25333	0.04794	96	-11
E	1.15667	0.05175	90	-10.4
F	0.66833	0.02587	54	-9.8
G	0.51	0.01528	42	-9.16
H	0.011	0.00771	5	-8.6
I	-0.02	0.00258	3	-8
J	-0.06667	0.00333	0	-7.4

Table 5.4.c. Incubation of B16 cells with adozelesin for 4 hours then without adozelesin for 72 hours

doxorubicin concentration [M]	Fluorescence
6.90E-06	25344
2.70E-06	12544
1.10E-06	5888
4.40E-07	2582
1.80E-07	1168
7.00E-08	520
2.80E-08	248
1.13E-08	126
4.50E-09	78
1.80E-09	50

Table 5.8. Fluorescence of various concentrations of doxorubicin at (excitation λ of 488nm, and emission λ of 560nm0.

daunorubicin concentration [M]	fluorescence
6.90E-06	31744
2.70E-06	16128
1.10E-06	7424
4.40E-07	3347
1.80E-07	1472
7.00E-08	672
2.80E-08	336
1.13E-08	192
4.50E-09	125
1.80E-09	85

Table 5.9. Fluorescence of various concentrations of daunorubicin (excitation λ of 488nm, and emission λ of 560nm).

sample tested	fluorescence (a)	fluorescence (b)	fluorescence (c)	fluorescence (d)	mean fluo	Se [±]
wash 1	2532	2404	2756	2596	2572	73.2
wash 2	888	792	696	792	792	39.2
wash 3	581	653	589	573	599	18.3
extracted doxorubicin	15564	15360	16384	15360	15667	243.8

Table 5.10. After incubating 9.6×10^{-6} M Doxorubicin in 4ml RPMI media with 5×10^{5} B16 cells for 4 hours. Fluorescence at (excitation λ of 488nm, and emission λ of 560nm).of samples from three consecutive washing steps compared to fluorescence of sample containing extracted doxorubicin from B16 cells.

sample tested	fluorescence (a)	fluorescence (b)	fluorescence (c)	fluorescence (d)	mean fluo	Se [±]
wash 1	56	40	48	112	64	16.3
wash 2	72	56		64	64	4.6
wash 3	120	96	72	66	88.5	12.3
extracted daunorubicin	3568	3632	3376	1840	3104	424.8

Table 5.11. After incubating 7×10^{-7} M Daunorubicin in 4ml RPMI media with 5×10^{5} B16 cells for 4 hours. Fluorescence at (excitation λ of 488nm, and emission λ of 560nm).of samples from three consecutive washing steps compared to fluorescence of sample containing extracted daunorubicin from B16 cells.

column	y(mean Abs)	se (yEr±)	x (daunorubicin molecules per single B1cell)
A	1.26	0.07778	31000000
B	0.6475	0.05822	62000000
C	0.5525	0.03449	1.2E+08
D	0.47	0.03317	2.5E+08
E	0.3375	0.02175	4.9E+08
F	0.3325	0.01548	9.9E+08
G	0.24	0.01	2E+09
H	0.1775	0.01031	4E+09
I	0.07	0.0216	8E+09
J	0.03575	0.02065	1.6E+10

Table 5.12.a. MTT assay of daunorubicin on B16 cells after incubating the same number of daunorubicin molecules with various number of B16 cells for 4 hours, then incubation in a drug free media for 72 hours.

column	y(mean Abs)	se (yEr±)	x (daunorubicin molecules per single B16 cell)
A	0.70333	0.09905	31000000
B	0.61667	0.05207	62000000
C	0.58333	0.10333	1.2E+08
D	0.42	0.01732	2.5E+08
E	0.35	0.04509	4.9E+08
F	0.27667	0.02186	9.9E+08
G	0.29333	0.03383	2E+09
H	0.26667	0.01667	4E+09
I	0.27333	0.01856	8E+09
J	0.34333	0.00882	1.6E+10

Table 5.12.b. MTT assay of the supernatant containing daunorubicin molecules on B16 cells after only 4 hours incubation with B16 cells and 72 hours incubation in drug free media. This supernatant contained daunorubicin remaining after incubating the same number of daunorubicin molecules with various number of B16 cells.

column	y(mean Abs)	se (yEr±)	x (Adozelesin molecules/a single B16 cell)
A	0.56333	0.03667	420000
B	0.48	0.01155	840000
C	0.38667	0.00882	1700000
D	0.42333	0.01202	3400000
E	0.36333	0.00882	6800000
F	0.31333	0.00667	13000000
G	0.21	0	54000000
H	0.22	0.00577	1.08E+08
I	0.135	0.00354	2.2E+08

Table 5.13.a. MTT assay of adozelesin on B16 cells after incubating the same number of adozelesin molecules with various number of B16 cells for 4 hours, then incubation in a drug free media for 72 hours.

column	y(mean Abs)	se (yEr±)	x (Adozelesin molecules per a single B16 cell)
A	0.72	0.01414	420000
B	0.63	0.03536	840000
C	0.5	0.00707	1700000
D	0.49	0	3400000
E	0.38	0.00707	6800000
F	0.425	0.01061	13000000
H	0.425	0.01061	54000000
I	0.315	0.01061	1.08E+08
J	0.335	0.02475	2.2E+08

Table 5.13.b. MTT assay of the supernatant containing adozelesin molecules on B16 cells after only 4 hours incubation with B16 cells and 72 hours incubation in drug free media. This supernatant contained adozelesin remaining after incubating the same number of adozelesin molecules with various number of B16 cells.

column	y(mean Abs)	se (yEr±)	x (bizelesin molecules per a single B16 cell)
A	0.215	0.00645	20000
B	0.2175	0.00854	40000
C	0.2525	0.00854	80000
D	0.25	0.01915	160000
E	0.215	0.01555	320000
F	0.26	0.01581	650000
G	0.205	0.02255	1300000
H	0.255	0.01893	2600000
I	0.2725	0.01974	5200000
J	0.28	0.01155	10300000

Table 5.14.a. MTT assay of bizelesin on B16 cells after incubating the same number of bizelesin molecules with various number of B16 cells for 4 hours then incubation in a drug free media for 72 hours.

column	y(mean Abs)	se (yEr±)	x (bizelesin molecules per a single B16 cell)
A	0.98	0.02483	20000
B	0.825	0.03663	40000
C	0.7225	0.03351	80000
D	0.645	0.03279	160000
E	0.495	0.01323	320000
F	0.485	0.0263	650000
G	0.49	0.01472	1300000
H	0.4375	0.0272	2600000
I	0.5325	0.01887	5200000
J	0.4625	0.03038	10300000

Table 5.14.b. MTT assay of the supernatant containing bizelesin molecules on B16 cells after only 4 hours incubation with B16 cells and 72 hours incubation in drug free media. This supernatant contained bizelesin remaining after incubating the same number of bizelesin molecules with various number of B16 cells.

Appendix D

Data for experiments in chapter 6

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.08183	0.00788	7620
B	0.15	0.01125	11430
C	0.24667	0.01726	15240
D	0.36667	0.03283	19050
E	0.395	0.03304	22860
F	0.41833	0.01778	26670
G	0.43	0.03795	30480
H	0.55	0.02517	34290
I	0.585	0.03797	38100

Table 6.1.a. Incubation of 3T3 cells with mtt for 2 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.12	0.00816	1900
B	0.20667	0.01308	3810
C	0.25833	0.01327	7620
D	0.358	0.02956	11430
E	0.416	0.02441	15240
F	0.486	0.02694	19050
G	0.496	0.04007	22860
H	0.554	0.04456	26670
I	0.622	0.04727	30480
J	0.694	0.05115	34290

Table 6.1.b. Incubation of 3T3 cells with mtt for 4 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.05333	0.01174	1900
B	0.09667	0.00667	3810
C	0.31	0.01125	7620
D	0.34833	0.01701	11430
E	0.465	0.02527	15240
F	0.53833	0.02056	19050
G	0.66167	0.02428	22860
H	0.685	0.02604	26670
I	0.78833	0.02833	30480
J	0.82333	0.03712	34290

Table 6.1.c. Incubation of 3T3 cells with mtt for 6 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.09057	0.00524	1000
B	0.41429	0.01974	3000
C	0.55714	0.02714	4000
D	0.54857	0.0315	5000
E	0.80571	0.03221	6000
F	0.84429	0.0268	7000
M	0.82429	0.02698	8000
G	0.81429	0.03191	9000
H	0.87857	0.03991	10000

Table 6.2.a. Incubation of 3T3 cells at 37°C in RPMI media for 72 hours, and with mtt for 4 hours.

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.06729	0.02739	1000
B	0.60286	0.05541	3000
C	0.72286	0.03006	4000
D	0.86286	0.04617	5000
E	0.91857	0.04501	6000
F	1.00143	0.03888	7000
G	1.01143	0.04728	8000
H	1.27286	0.04965	9000
I	1.26571	0.04052	10000

Table 6.2.b. Incubation of 3T3 cells at 37°C in RPMI media for 96 hours, and with mtt for 4 hours.

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.1224	0.00178	14000
B	0.1524	0.01018	18666
C	0.1952	0.01256	23333
D	0.2028	0.00309	28000
E	0.2226	0.00371	32666
F	0.2406	0.00527	37333
G	0.2892	0.00796	42000
H	0.3534	0.01517	46666

Table 6.3.a. Incubation of 293 cells with mtt for 2 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.3625	0.01181	14000
B	0.49	0.01225	18666
C	0.5675	0.0125	23333
D	0.61	0.01472	28000
E	0.77	0.02483	32666
F	0.8575	0.06005	37333
G	0.89	0.0713	42000
H	0.965	0.06886	46666

Table 6.3.b. Incubation of 293 cells with mtt for 6 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.23875	0.0091	2000
B	0.32075	0.00504	3000
C	0.39175	0.0067	4000
D	0.4235	0.00437	5000
E	0.51575	0.01426	6000
F	0.60375	0.03083	7000
G	0.58475	0.01582	8000
H	0.7145	0.0184	9000

Table 6.4.a. Incubation of 293 cells at 37°C in RPMI media for 96 hours, and with mtt for 6 hours.

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.2325	0.01931	2000
B	0.365	0.02102	3000
C	0.5125	0.02287	4000
D	0.59	0.02121	5000
E	0.80333	0.03333	6000
F	0.73	0.03894	7000
G	0.88	0.02972	8000
H	1.025	0.03329	9000
I	1.065	0.02843	10000

Table 6.4.b. Incubation of 293 cells at 37°C in RPMI media for 96 hours, and with mtt for 6 hours.

column	y (mean Abs)	se [yEr±]	x (cell number)
K	0.13143	0.00143	2033
A	0.17857	0.0067	4066
B	0.14429	0.00782	8133
C	0.27714	0.0209	12199
D	0.50429	0.02935	16265
E	0.61714	0.03435	20331
F	0.76429	0.06027	24397
G	1.04429	0.03637	28463
H	1.01429	0.07718	32529
I	0.96	0.06777	36595

Table 6.5.a. Incubation of Cos 7 cells with mtt for 2 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.11714	0.00565	2033
B	0.17714	0.0036	4066
C	0.27143	0.01164	8133
D	0.47571	0.00812	12199
E	0.65	0.01877	16265
F	0.86143	0.03582	20331
G	0.97857	0.04533	24397
H	1	0.03658	28463
I	1.05714	0.0257	32529
J	1.17	0.08816	36595
K	1.20286	0.05195	40661

Table 6.5.b. Incubation of Cos 7 cells with mtt for 4 hours

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.14457	0.01385	1000
B	0.26286	0.02876	2000
C	0.29714	0.0286	3000
D	0.46429	0.02125	4000
E	0.43143	0.03582	5000
F	0.48429	0.03463	6000
G	0.65714	0.04069	7000
H	0.72571	0.04815	8000
I	0.70186	0.02579	9000

6.6.a. Incubation of Cos 7 cells at 37°C in RPMI media for 72 hours, and with mtt for 4 hours.

column	y (mean Abs)	se [yEr±]	x (cell number)
A	0.23667	0.02525	1000
B	0.42667	0.06975	2000
C	0.57	0.04906	3000
D	0.73	0.07979	4000
E	0.807	0.06884	5000
F	1.02667	0.08413	6000
G	1.07833	0.1281	7000
H	1.1925	0.07021	8000
I	1.31333	0.04971	9000

Table 6.6.b. Incubation of Cos 7 cells at 37°C in RPMI media for 96 hours, and with mtt for 4 hours.

col	y (mean Abs)	se [yEr±]	x (log NLDP concentration)
A	1.25833	0.01249	-6.72
B	1.205	0.0303	-6.29
C	1.205	0.00847	-5.86
D	1.55	0.07132	-5.44
E	1.355	0.02172	-5.01
F	1.345	0.02187	-4.58
G	1.325	0.02202	-4.16
H	1.365	0.01522	-3.73
I	1.28333	0.0152	-3.3
J	1.055	0.02432	-2.88

Table 6.7.a. Incubation of B16 cells with NLDP for 72 hours.

col	y (mean abs)	se [yEr±]	x (log NLDP concentration)
A	0.91167	0.02822	-6.72
B	0.96	0.01592	-6.29
C	0.97167	0.01662	-5.86
D	1.23667	0.01542	-5.44
E	1.49167	0.04629	-5.01
F	1.41833	0.07432	-4.58
G	1.32667	0.05194	-4.16
H	1.155	0.02062	-3.73
I	1.09167	0.06177	-3.3
J	1.21667	0.01202	-2.88

Table 6.7.b. Incubation of B16 cells with NLDP for 72 hours.

col	y (mean Abs)	Se [Er±]	x (log NLDP concentration)
A	0.985	0.01522	-6.72
B	1.105	0.01147	-6.29
C	1.17	0.01862	-5.86
D	1.14333	0.01706	-5.44
E	1.24	0.02852	-5.01
F	1.1775	0.02863	-4.58
G	1.09167	0.02868	-4.16
H	1.19333	0.02155	-3.73
I	1.08167	0.03978	-3.3
J	0.89333	0.02654	-2.88

Table 6.7.c. Incubation of B16 cells with NLDP for 72 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log Methotrexate concentration)
A	1.13667	0.02591	84	-9.1
B	1.355	0.02907	110	-8.69
C	1.34333	0.04432	108	-8.29
D	1.115	0.02553	81	-7.89
E	1.045	0.00922	73	-7.5
F	0.915	0.02094	58	-7.1
G	0.74	0.02206	38	-6.7
H	0.54833	0.02023	15	-6.3
I	0.425	0.01688	1	-4.3

Table 6.8.a. Incubation of B16 cells with Methotrexate for 72 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log Methotrexate concentration)
B	1.21833	0.0199	89	-9.1
C	1.25167	0.01108	92	-8.69
D	1.34	0.01033	101	-8.29
E	1.3	0.06693	97	-7.89
F	1.32333	0.04738	99	-7.5
G	1.11667	0.06168	79	-7.1
H	0.69833	0.09119	37	-6.7
I	0.56	0.04919	23	-6.3
J	0.37167	0.01424	4	-4.3

Table 6.8.b. Incubation of B16 cells with Methotrexate for 72 hours.

col	y (mean Abs)	Se [yEr±]	y (%Abs compared to control)	x (log Methotrexate concentration)
A	1.28	0.01983	108	-9.7
B	1.07667	0.02076	81	-9.1
C	1.39167	0.03646	123	-8.69
D	1.09333	0.01961	83	-8.29
E	1.10833	0.03146	85	-7.89
F	1.26167	0.034	106	-7.5
G	1.01167	0.01558	72	-7.1
H	0.87667	0.02704	54	-6.7
I	0.74333	0.01994	36	-6.3
J	0.48	0.01506	0	-4.3

Table 6.8.c. Incubation of B16 cells with Methotrexate for 72 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	1.275	0.0317	107	-6.86
B	1.27333	0.03201	107	-6.44
C	1.22167	0.04453	104	-6.01
D	0.99333	0.01382	91	-5.58
E	0.96917	0.03298	90	-5.16
F	1.105	0.05566	97	-4.73
G	0.98167	0.03049	90	-4.31
H	0.86	0.04683	83	-3.88
I	0.59	0.05983	68	-3.46
J	0.12833	0.0233	41	-3.03

Table 6.9.a. Incubation of B16 cells with MTX-NLDP for 72 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	1.19167	0.01579	97	-6.86
B	1.04	0.02828	84	-6.44
C	1.155	0.03452	93	-6.01
D	1.00667	0.06173	81	-5.58
E	0.99667	0.04835	80	-5.16
F	1.04667	0.05018	84	-4.73
G	0.825	0.01727	65	-4.31
H	0.68667	0.04787	53	-3.88
I	0.36167	0.01851	25	-3.46
J	0.14583	0.03073	6	-3.03

Table 6.9.b.Incubation of B16 cells with MTX-NLDP for 72 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	1.14167	0.06405	97	-6.44
B	1.22283	0.11343	105	-6.01
C	1.04667	0.09069	88	-5.58
D	1.10333	0.14961	93	-5.16
E	1.04833	0.09243	88	-4.73
F	0.68833	0.07661	54	-4.31
G	0.51167	0.05816	37	-3.88
H	0.31833	0.02651	18	-3.46

Table 6.9.c. Incubation of B16 cells with MTX-NLDP for 72 hours.

col	y (mean	Se [yEr±]	y (% Abs compared to control)	x (log Methotrexate concentration)
A	0.846	0.03203	105	-9.5
B	0.712	0.04352	80	-8.9
C	0.808	0.04329	98	-8.2
D	0.504	0.02713	73	-7.5
E	0.374	0.02159	16	-6.8
F	0.364	0.00927	14	-6.1
G	0.272	0.01594	-3	-5.4
H	0.3	0.01183	2	-4.7
I	0.258	0.0097	-6	-4

Table 6.10.a. Incubation of 293-MC3 cells with Methotrexate for 96 hours

col	y (mean)	Se [yEr±]	y (% Abs compared to control)	x (log Methotrexate concentration)
A	0.59167	0.03683	103	-9.5
B	0.55	0.01713	93	-8.9
C	0.57	0.02206	98	-8.2
D	0.41333	0.01054	57	-7.5
E	0.29667	0.00843	27	-6.8
F	0.315	0.00619	32	-6.1
G	0.15483	0.01925	-9	-5.4
H	0.23167	0.01621	10	-4.7
I	0.133	0.01436	-15	-4
J	0.17333	0.01358	-5	-3.3

Table 6.10.b. Incubation of 293-MC3 cells with Methotrexate for 96 hours

col	y (mean)	Se [yEr±]	y (% Abs compared to control)	x (log Methotrexate concentration)
A	0.53667	0.03997	94	-9.5
B	0.52667	0.01476	92	-8.9
C	0.57833	0.01078	105	-8.2
D	0.365	0.01025	49	-7.5
E	0.27	0.01211	24	-6.8
F	0.22333	0.00667	11	-6.1
G	0.19333	0.01202	3	-5.4
H	0.19667	0.01892	4	-4.7
I	0.15833	0.00543	-6	-4
J	0.13667	0.00955	-11	-3.3

Table 6.10.c. Incubation of 293-MC3 cells with Methotrexate for 96 hours

col	y (mean	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	0.82857	0.02029	88	-9.45
B	0.86	0.02478	92	-8.85
C	0.89714	0.02958	98	-8.24
D	0.93286	0.0475	103	-7.64
E	0.94571	0.0127	105	-7.04
F	0.93857	0.02773	104	-6.44
G	0.79333	0.06417	82	-5.83
H	0.465	0.0131	32	-5.23
I	0.45167	0.01249	30	-4.63
J	0.24667	0.00803	0	-4.03

Table 6.11.a. Incubation of 293-MC3 cells with MTX-NLDP for 96 hours

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	0.673	0.03767	71	-9.55
B	0.84333	0.02716	96	-8.95
C	0.79	0.01693	88	-8.35
D	0.82833	0.04061	94	-7.7
E	1.00167	0.06353	120	-7.1
F	0.935	0.02825	110	-6.5
G	0.935	0.06526	110	-5.9
H	0.87667	0.06854	101	-5.3
I	0.39833	0.01195	30	-4.7
J	0.4	0.01065	30	-4.14

Table 6.11.b. Incubation of 293-MC3 cells with MTX-NLDP for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	0.68	0.00816	95	-9.55
B	0.584	0.0199	80	-8.95
C	0.625	0.0461	86	-8.35
D	0.62333	0.06591	86	-7.7
E	0.73333	0.05931	103	-7.1
F	0.69833	0.04942	97	-6.5
G	0.97333	0.07654	140	-5.9
H	0.75833	0.0204	107	-5.3
I	0.27667	0.00558	32	-4.7
J	0.30333	0.00989		-4.14

Table 6.11.c. Incubation of 293-MC3 cells with MTX-NLDP for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	1.288	0.04769	105	-9.5
B	1.152	0.02973	84	-8.9
C	1.17	0.04159	87	-8.3
D	1.356	0.02839	115	-7.7
E	1.226	0.02293	95	-7.1
F	1.244	0.03789	98	-6.5
G	1.216	0.05192	94	-5.9
H	1.034	0.1353	66	-5.3
I	0.668	0.05407	9	-4.7
J	0.726	0.034	18	-4.1

Table 6.12.a. Incubation of 293-vector cells with MTX-NLDP for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	1.4625	0.05573	127	-9.59
B	0.925	0.02398	70	-8.99
C	1.545	0.06357	135	-8.38
D	1.0175	0.03568	80	-7.78
E	0.935	0.10169	71	-7.18
F	0.955	0.17188	73	-6.6
G	0.66	0.04708	42	-6
H	0.5	0.10108	25	-5.37
I	0.395	0.05236	14	-4.77
J	0.095	0.03279	-17	-4.17

Table 6.12. b. Incubation of 293-vector cells with MTX-NLDP for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (%Abs compared to control)	x (log Methotrexate concentration)
A	0.82714	0.04534	88	-9.09
B	0.79143	0.06486	81	-8.67
C	0.95571	0.03747	111	-8
D	0.85871	0.04418	93	-7.59
E	0.94286	0.06225	109	-7.19
F	0.76286	0.07599	70	-6.8
G	0.45157	0.04315	19	-6.4
H	0.43429	0.02724	15	-6
I	0.45	0.01272	18	-5.3

Table 6.13.a. Incubation of cos 7 cells with Methotrexate for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log Methotrexate concentration)
A	0.93714	0.03871	89	-9.09
B	0.90714	0.04518	84	-8.67
C	0.95	0.0234	91	-8
D	1.05429	0.03199	107	-7.59
E	1.11286	0.05112	116	-7.19
F	0.83143	0.04611	72	-6.8
G	0.53	0.02449	25	-6.4
H	0.51143	0.04085	22	-6
I	0.45857	0.01668	14	-5.3
J	0.50571	0.01043	21	-3.3

Table 6.13.b. Incubation of cos 7 cells with Methotrexate for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log Methotrexate concentration)
A	1.14	0.03402	101	-9.09
B	1.12143	0.03446	99	-8.67
C	1.02286	0.03198	85	-8.00
D	0.98	0.04353	80	-7.59
E	1.12	0.05542	99	-7.19
F	0.66857	0.03391	38	-6.80
G	0.58286	0.03053	26	-6.40
H	0.46714	0.02427	10	-6.0
I	0.43857	0.01752	7	-5.30
J	0.40429	0.00972	2	-3.3

Table 6.13.c. Incubation of cos 7 cells with Methotrexate for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	1.20429	0.03909	98	-7.56
B	1.14143	0.02577	91	-7.13
C	1.17857	0.02492	95	-6.7
D	1.13286	0.05209	90	-6.28
E	1.24714	0.08624	103	-5.86
F	1.27143	0.07268	105	-5.43
G	1.06143	0.05302	83	-5
H	0.65143	0.01223	37	-4.58
I	0.51571	0.02136	23	-4.15
J	0.48571	0.01811	19	-3.73

Table 6.14.a. Incubation of cos 7 cells with MTX-NLDP for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
C	1.07857	0.0346	90	-6.7
D	1.09571	0.02768	92	-6.28
E	1.13571	0.04076	97	-5.86
F	1.18571	0.03897	97	-5.43
G	1.13714	0.03676	97	-5
H	0.72143	0.02577	44	-4.58
I	0.58714	0.01267	26	-4.15
J	0.55571	0.01192	22	-3.73

Table 6.14.c. Incubation of cos 7 cells with MTX-NLDP for 96 hours.

COL	y (mean Abs)	Se [y Er±]	y (% Abs compared to control)	x (log Methotrexate concentration)
A	0.84667	0.07982	100	-9.09
B	0.81	0.10328	94	-8.67
C	0.68167	0.06374	73	-8
D	0.61	0.09832	61	-7.59
E	0.47167	0.026	38	-7.19
F	0.36167	0.03692	20	-6.8
G	0.255	0.00719	3	-6.4
H	0.27	0.01211	5	-6
I	0.25333	0.01476	2	-5.3

Table 6.15.a. Incubation of 3T3 cells with Methotrexate for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	X (log Methotrexate concentration)
A	0.47333	0.02472	83	-9.09
B	0.50333	0.01229	91	-8.67
C	0.56667	0.02431	107	-8
D	0.58	0.0531	111	-7.59
E	0.455	0.02778	80	-7.19
F	0.36667	0.01606	57	-6.8
G	0.265	0.01432	32	-6.4
H	0.23667	0.01453	25	-6
I	0.23333	0.00558	24	-5.3
J	0.25333	0.00803	29	-3.3

Table 6.15.b. Incubation of 3T3 cells with Methotrexate for 96 hours.

	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log Metotrexate concentration)
A	0.57167	0.03439	88	-9.09
B	0.645	0.06114	106	-8.67
C	0.51167	0.063	74	-8
D	0.44	0.07243	56	-7.59
E	0.33667	0.04709	39	-7.1
F	0.295	0.04249	21	-6.8
G	0.23	0.03022	6	-6
H	0.24	0.02082	8	-5.3
I	0.22667	0.00955	5	-3.3

Table 6.15.c. Incubation of 3T3 cells with Methotrexate for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	0.55	0.02059	83	-6.28
B	0.54714	0.0219	83	-5.86
C	0.46429	0.01798	61	-5.43
D	0.29429	0.00429	17	-5
E	0.26286	0.00421	9	-4.58
F	0.28	0.00724	14	-4.15
G	0.26857	0.00459	11	-3.73

Table 6.16.a. Incubation of 3T3 cells with MTX-NLDP for 96 hours

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
C	0.54286	0.02801	88	-6.7
D	0.53571	0.01212	87	-6.28
E	0.59286	0.02244	101	-5.86
F	0.46714	0.02135	69	-5.43
G	0.30286	0.01409	26	-5
H	0.23143	0.00404	8	-4.58
I	0.24286	0.0119	11	-4.15
J	0.24286	0.01248	11	-3.73

Table 6.16.b. Incubation of 3T3 cells with MTX-NLDP for 96 hours.

col	y (mean Abs)	Se [yEr±]	y (% Abs compared to control)	x (log MTX-NLDP concentration)
A	0.50571	0.01571	80	-6.7
B	0.50286	0.02784	79	-6.28
D	0.45429	0.01702	67	-5.86
E	0.24	0.02469	10	-5.43
F	0.23	0.00724	8	-5
H	0.20286	0.00837	1	-4.15
I	0.25286	0.00474	14	-3.73

Table 6.16.c. Incubation of 3T3 cells with MTX-NLDP for 96 hours

Appendix E

HPLC analysis report on the stability of the MTX-NLDP conjugate.

The table below shows the gradient of the two solvents A and B used for the HPLC analysis of the stability of MTX-NLDP conjugate, and the time intervals.

Time (minute)	% A	% B
0	80	20
2.0	0	100
5.0	0	100
8.0	80	20
10.0	80	20

Table 6.17. gradient of solvents A and B.

In order to assess the stability of the MTX-NLDP, 0.22mg of MTX-NLDP powder was dissolved in 1ml PBS and the samples incubated at 37°C for 24h, 48h, 72h and 96h. HPLC analysis of 10µl samples of $1.3x10^{-5}$M MTX-NLDP was undertaken to determine the stability of the conjugate under physiological conditions for various time periods, in addition to the HPLC analysis of a 10µl sample of $8.5x10^{-10}$M MTX. The HPLC procedure is described in chapter 2 the U.V detection wavelength was 217nm.

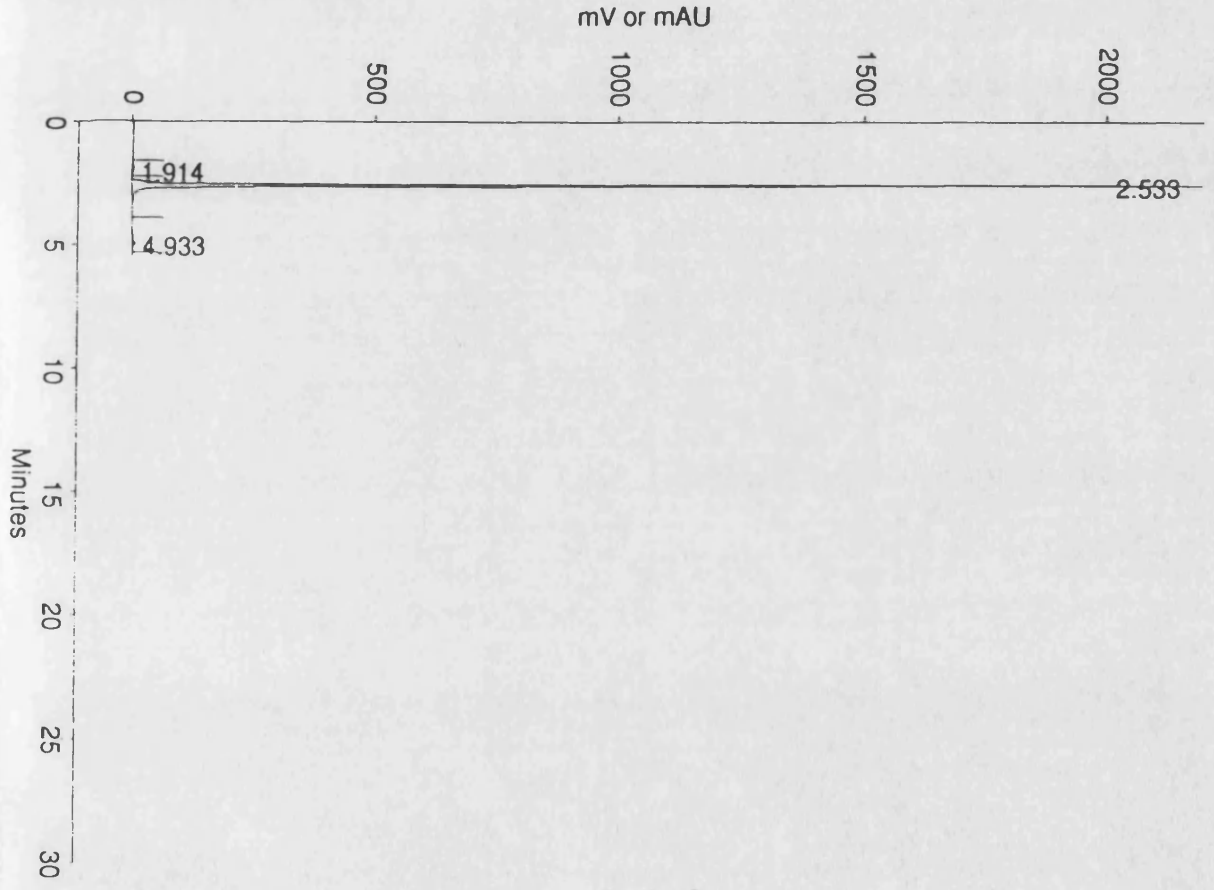
Mode: Acquired Data
Reported On: 20-06-96 17:48:47
Original Results: D:\TSP\SYSTEM1\Data\mtx,I.RES
Notes:

Analysis Report

Name: mtx,I
Vial: A32
Injection: 1 of 1
Description: 8.5E-10
Type: Sample
Injected On: 19-06-96 18:06:42
Injection Volume: 10.0 uL

Acquisition Log
Column Pressure (PSI): 2724
Column Temperature (C): N/A
Pump Flow Stability: N/A
Noise (microAU): 1e+002
Drift (microAU/min): 4e+002
Run-Time Messages: None

Signal 1: Scan Wavelength 217
Calculation Type: Area Percent



Component	RT(min)	Area	Height	Area%	Peak Type
Unident0001	1.914	81239	4010	0.79	Fused
Unident0002	2.533	10148162	1950768	98.33	Fused
Unident0003	4.933	91323	6980	0.88	Last Fused
Totals		10320724	1961758	100.00	

Mode: Acquired Data
Original Results: D:\TSP\SYSTEM1\[RES
Notes:

Reported On: 21-06-96 09:10:23

Analysis Report

Name: MTX - M.LDR 0 HR.
Description: 24 HOURS
Type: Sample
Injection Volume: 10.0 uL

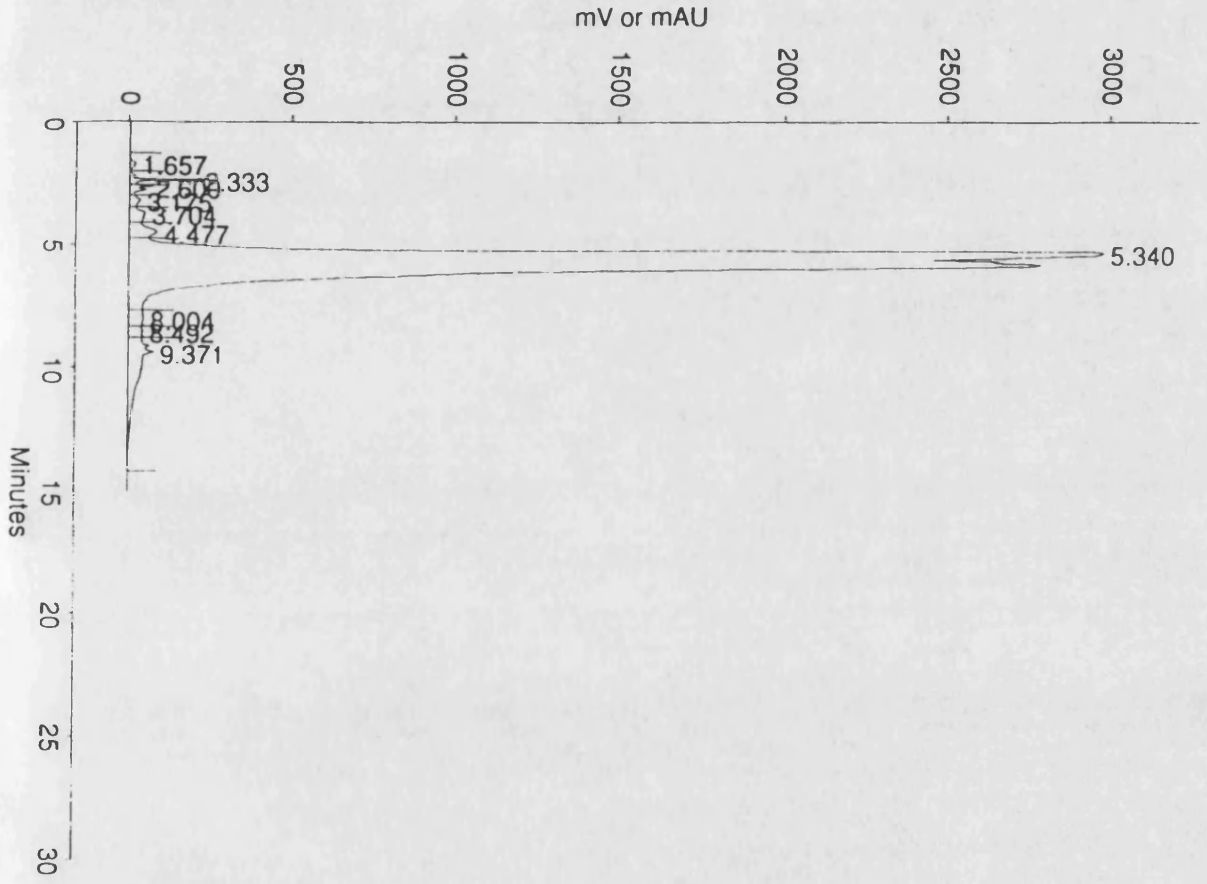
Vial: A26

Injection: 1 of 1

Injected On: 19-06-96 15:52:04

Acquisition Log
Column Pressure (PSI): 2674
Column Temperature (C): N/A
Pump Flow Stability: N/A
Noise (microAU): 4e+001
Drift (microAU/min): 8e+001
Run-Time Messages: None

Signal 1: Scan Wavelength 217
Calculation Type: Area Percent



Component	RT(min)	Area	Height	Area%	Peak Type
Unident0001	1.657	322246	16908	0.17	Fused
Unident0002	2.333	1252944	206516	0.68	Fused
Unident0003	2.600	837944	72158	0.45	Fused
Unident0004	3.175	705170	33880	0.38	Fused
Unident0005	3.704	1474741	49104	0.80	Fused
Unident0006	4.477	2417877	86262	1.31	Fused
Unident0007	5.340	167679192	2985896	90.77	Fused
Unident0008	8.004	1789740	47060	0.97	Fused

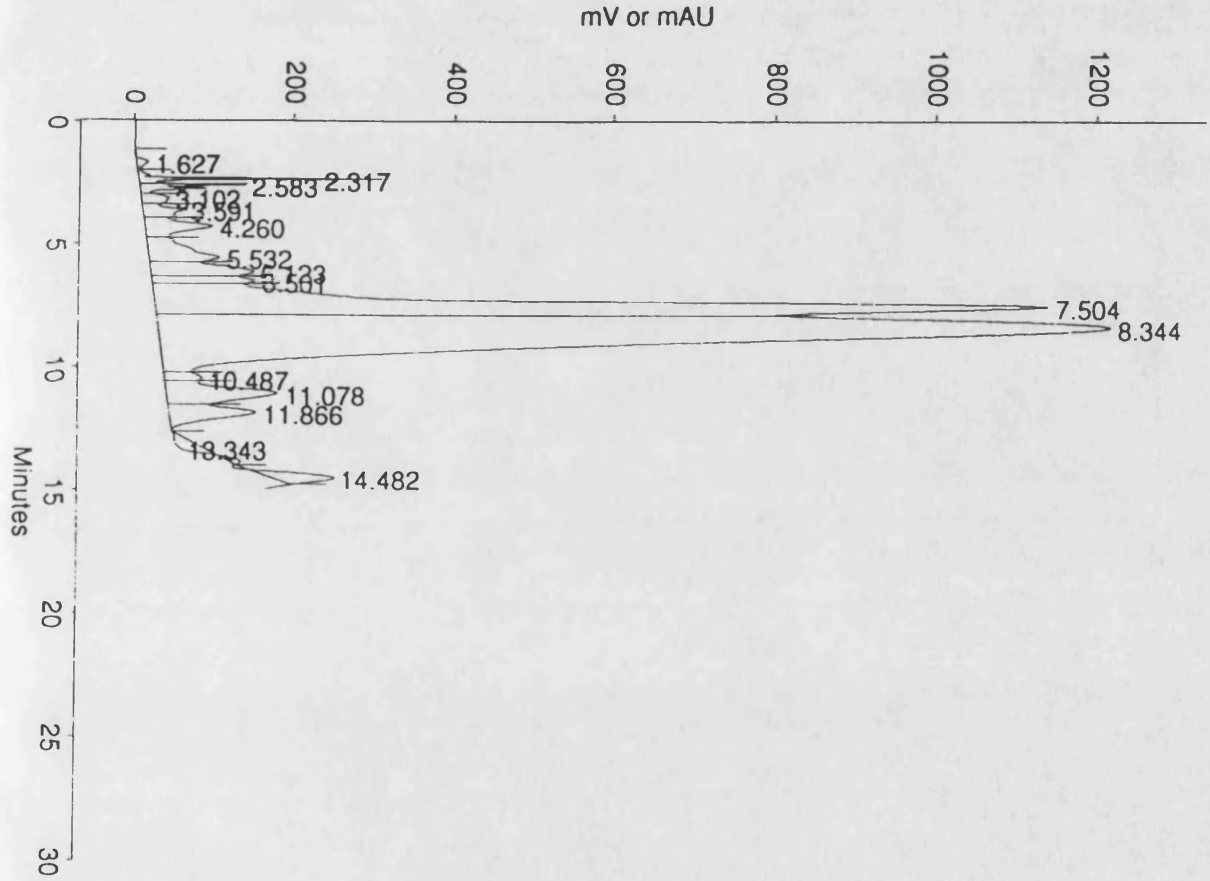
Mode: Acquired Data
Reported On: 20-06-96 18:04:22
Original Results: D:\TSP\SYSTEM1\Data\MTX-NLDP_24HR.RES
Notes:

Analysis Report

Name: MTX-NLDP_24HR
Vial: A24
Injection: 1 of 1
Description: 24 HOURS
Type: Sample
Injected On: 19-06-96 15:16:54
Injection Volume: 10.0 uL

Acquisition Log
Column Pressure (PSI): 2620
Column Temperature (C): N/A
Pump Flow Stability: N/A
Noise (microAU): 5e+001
Drift (microAU/min): -4
Run-Time Messages: None

Signal 1: Scan Wavelength 217
Calculation Type: Area Percent



Component	RT(min)	Area	Height	Area%	Peak Type
Unident0001	1.627	225666	14688	0.15	Fused
Unident0002	2.317	1515796	264072	0.99	Fused
Unident0003	2.583	1157389	132160	0.75	Fused
Unident0004	3.102	702393	35340	0.46	Fused
Unident0005	3.591	1256394	54330	0.82	Fused
Unident0006	4.260	2447128	84474	1.59	Fused
Unident0007	5.532	3422213	90352	2.23	Fused
Unident0008	6.123	3810393	130600	2.48	Fused

Mode: Acquired Data
Reported On: 20-06-96 18:04:22
Original Results: D:\TSP\SYSTEM1\Data\MTX-NLDP_24HR.RES

Unident0009	6.501	2202378	129646	1.43	Fused
Unident0010	7.504	40253115	1111314	26.22	Fused
Unident0011	8.344	85207052	1184522	55.51	Fused
Unident0012	10.487	944454	47350	0.62	Fused
Unident0013	11.078	5186878	138776	3.38	Fused
Unident0014	11.866	3465019	107790	2.26	Fused
Unident0016	14.482	1699125	89586	1.11	Last Fused
Totals		153495393	3615000	100.00	

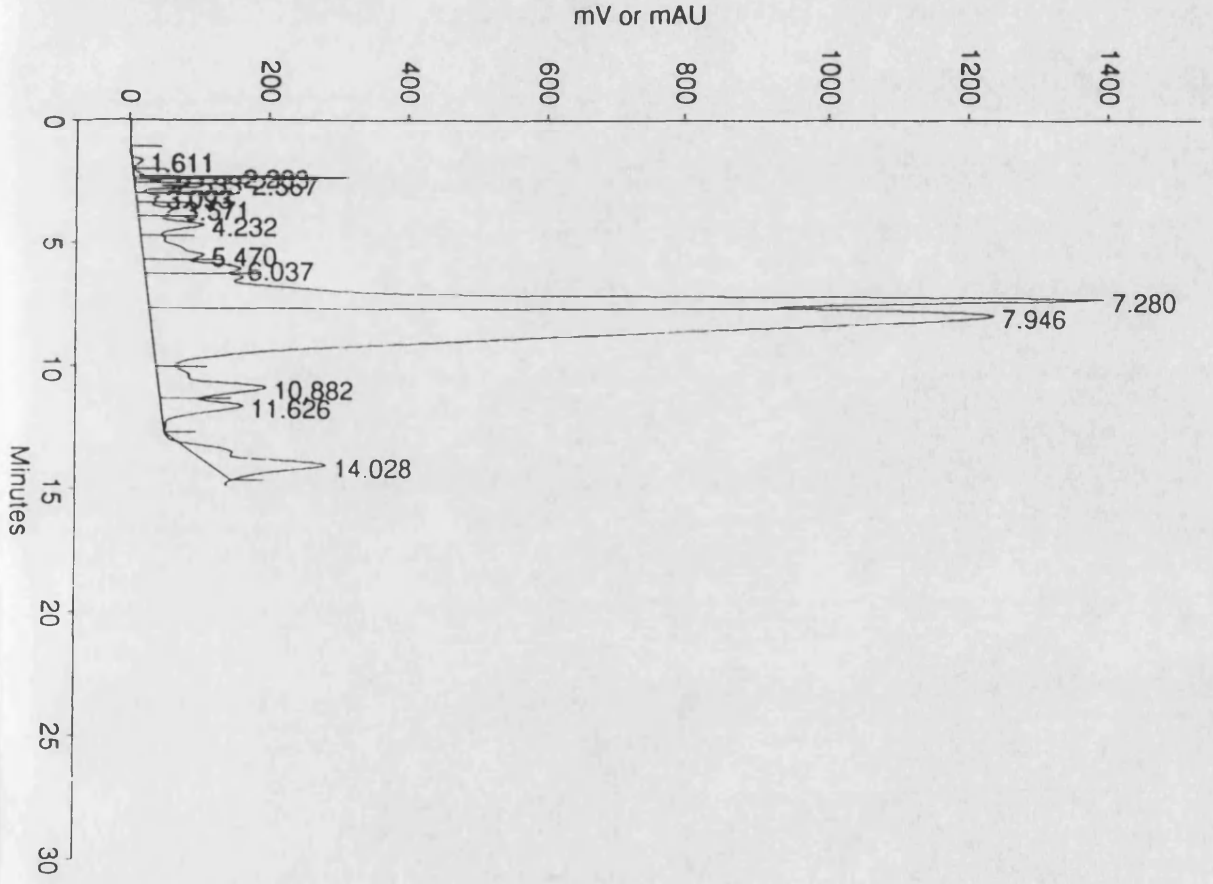
Mode: Acquired Data
Reported On: 20-06-96 18:06:21
Original Results: D:\TSP\SYSTEM1\Data\MTX-NLDP_48HR.RES
Notes:

Analysis Report

Name: MTX-NLDP_48HR
Vial: A22
Injection: 1 of 1
Description: 48 HOUR
Type: Sample
Injected On: 19-06-96 14:44:35
Injection Volume: 10.0 uL

Acquisition Log
Column Pressure (PSI): 2665
Column Temperature (C): N/A
Pump Flow Stability: N/A
Noise (microAU): 6e+001
Drift (microAU/min): -7e+002
Run-Time Messages: None

Signal 1: Scan Wavelength 217
Calculation Type: Area Percent



Component	RT(min)	Area	Height	Area%	Peak Type
Unident0001	1.611	234694	16260	0.15	Fused
Unident0002	2.283	1187886	228036	0.74	Fused
Unident0003	2.533	317616	39306	0.20	Fused
Unident0004	2.567	1448176	147570	0.90	Fused
Unident0005	3.093	595387	33578	0.37	Fused
Unident0006	3.571	1383217	60766	0.86	Fused
Unident0007	4.232	2658352	93402	1.65	Fused
Unident0008	5.470	3363962	89892	2.09	Fused

Mode: Acquired Data
Reported On: 20-06-96 18:06:21
Original Results: D:\TSP\SYSTEM1\Data\MTX-NLDP_48HR.RES

Unident0009	6.037	3875864	138784	2.41	Fused
Unident0010	7.280	45595289	1369190	28.36	Fused
Unident0011	7.946	83663819	1202924	52.03	Fused
Unident0012	10.882	6236641	154286	3.88	Fused
Unident0013	11.626	3719124	115796	2.31	Fused
Unident0014	14.028	6509982	182308	4.05	Last Fused
Totals		160790009	3872098	100.00	

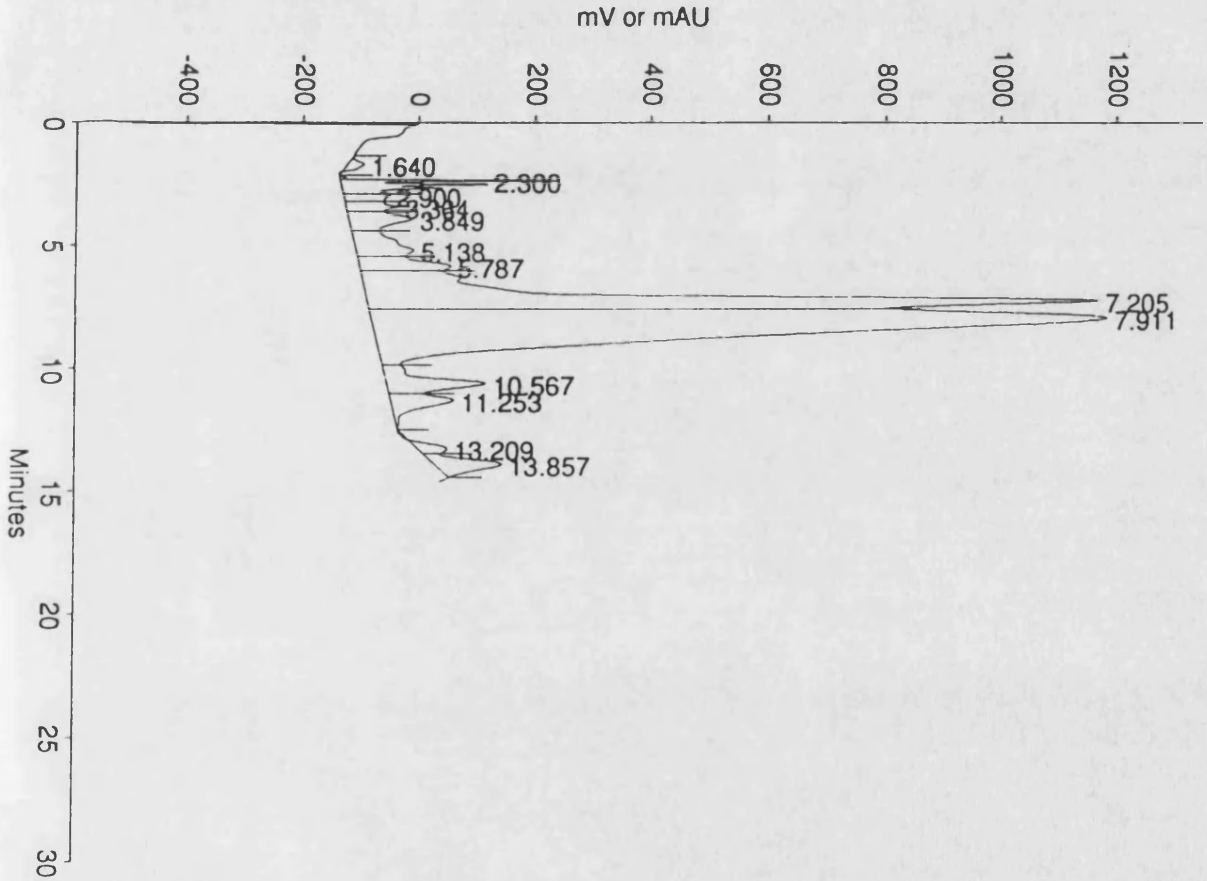
Mode: Acquired Data
Reported On: 20-06-96 18:08:20
Original Results: D:\TSP\SYSTEM1\Data\MTX-NLDP_72HR.RES
Notes:

Analysis Report

Name: MTX-NLDP_72HR
Vial: A21
Injection: 1 of 1
Description: 72 HOUR
Type: Sample
Injected On: 19-06-96 14:05:49
Injection Volume: 10.0 uL

Acquisition Log
Column Pressure (PSI): 2690
Column Temperature (C): N/A
Pump Flow Stability: N/A
Noise (microAU): 1e+005
Drift (microAU/min): 3e+005
Run-Time Messages: None

Signal 1: Scan Wavelength 217
Calculation Type: Area Percent



Component	RT(min)	Area	Height	Area%	Peak Type
Unident0001	1.640	567286	29670	0.34	Fused
Unident0002	2.300	4806691	316742	2.85	Fused
Unident0003	2.900	1324800	79512	0.79	Fused
Unident0004	3.364	1767760	90528	1.05	Fused
Unident0005	3.849	3726584	110482	2.21	Fused
Unident0006	5.138	4665463	103146	2.77	Fused
Unident0007	5.787	4656617	160332	2.77	Fused
Unident0008	7.205	45797622	1260316	27.20	Fused

Mode: Acquired Data
Reported On: 20-06-96 18:08:20

Original Results: D:\TSP\SYSTEM1\Data\MTX-NLDP_72HR.RES

Unident0009	7.911	86635975	1259212	51.45	Fused
Unident0010	10.567	5966125	173150	3.54	Fused
Unident0011	11.253	3705605	105572	2.20	Fused
Unident0012	13.209	1106968	64828	0.66	Fused
Unident0013	13.857	3648089	113366	2.17	Last Fused
Totals		168375585	3866856	100.00	

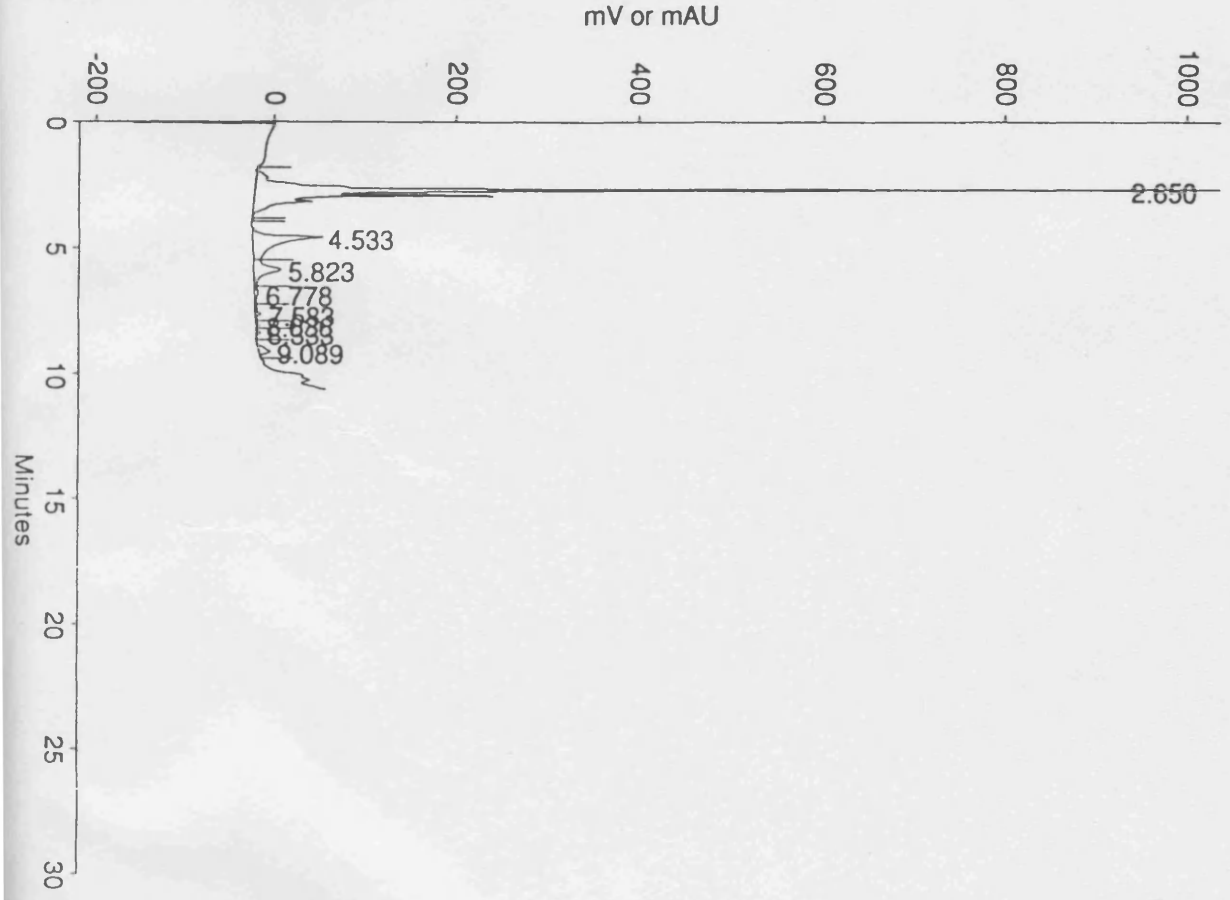
Mode: Acquired Data
Reported On: 20-06-96 18:01:12
Original Results: D:\TSP\SYSTEM1\Data\MTX-NLDP_STAB1.RES
Notes:

Analysis Report

Name: MTX-NLDP_STAB1
Vial: A20
Injection: 1 of 1
Description: 96HOUR
Type: Sample
Injected On: 19-06-96 13:36:48
Injection Volume: 10.0 uL

Acquisition Log
Column Pressure (PSI): 2658
Column Temperature (C): N/A
Pump Flow Stability: N/A
Noise (microAU): 3e+004
Drift (microAU/min): 1e+005
Run-Time Messages: None

Signal 1: Scan Wavelength 217
Calculation Type: Area Percent



Component	RT(min)	Area	Height	Area%	Peak Type
Unident0001	2.650	8532496	1047242	75.06	Resolved
Unident0002	4.533	1664262	74066	14.64	Fused
Unident0003	5.823	716849	28842	6.31	Fused
Unident0004	6.778	109398	3762	0.96	Fused
Unident0005	7.583	87686	5548	0.77	Fused
Unident0006	8.036	37323	3422	0.33	Fused
Unident0007	8.333	42239	3588	0.37	Fused
Unident0008	9.089	177771	12780	1.56	Last Fused

Mode: Acquired Data
Reported On: 20-06-96 18:01:12

Original Results: D:\TSP\SYSTEM1\Data\MTX-NLDP_STAB1.RES

Totals	11368024	1179250	100.00