STUDIES OF THE MECHANISM OF ANTITUMOUR ACTIVITY OF N-METHYLFORMAMIDE

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NMF induces the terminal differentiation or acquisition of more benign characteristics in certain malignant cells *in vitro* and has good antitumour activity against murine tumours *in vivo*. This study was concerned with a comparison of the mechanism of antitumour activity of NMF *in vitro* and *in vivo* against the murine TLX5 lymphoma, which is sensitive to NMF *in vivo*.

TLX5 cells incubated continuously with NMF *in vitro* showed a concentration and time dependent decrease in cell growth rate, which was associated with an increase in membrane permeability, a decrease in cell size and at the higher NMF concentrations, cell death. Analysis of the cell cycle after incubation with NMF indicated an early G_1 phase arrest.

TLX5 cells were incubated with NMF and washed free of the drug. Analysis of clonogenicity and tumourigenicity showed that all viable cells retained their proliferative potential and malignancy. Therefore, TLX5 cells exposed to NMF *in vitro* are not terminally differentiated, but reside in a quiescent substate which was reversed on drug removal.

The intracellular GSH levels of TLX5 cells was decreased in a concentration and time dependent fashion by NMF. GSH depletion of TLX5 cells was not, however, a prerequisite for growth arrest, unlike the reported data for human colon carcinoma cell lines.

A single administration of NMF caused a dose dependent regression of the TLX5 lymphoma in tumour bearing mice. Cell death occurred by apoptosis and necrosis. The antitumour activity of NMF was dependent on formyl C-H bond fission, with the parent drug or metabolites reaching all parts of the tumour 4h after dosing. There was a non-dose dependent increase in the S phase population, which was due to an increase in DNA synthesis, 24h after administration of NMF.

NMF administration caused a decrease in GSH levels of the TLX5 lymphoma, which did not correlate with the antitumour response. However, the GSH depleting agent, BSO, marginally increased the antitumour activity of NMF.

Keywords: N-methylformamide, murine TLX5 lymphoma, glutathione, cell cycle, differentiation.

TO MY PARENTS

Experience is the name everyone gives to their mistakes.

Oscar Wilde (1854-1900)

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ABBREVIATIONS

A	adenosine
ADP	adenosine diphosphate
AO	acridine orange
AP_4A	diadenosine 5',5'''-P^1,P^4-tetraphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
BSO	DL-buthionine-[S,R]-sulphoximine
$^{14}CH_3NMF$	[^{14}C]-methyl labelled N-methylformamide
[^{14}C]NMF	[^{14}C]-labelled N-methylformamide
CRC	Cancer Research Campaign
dH_2O	double distilled water
DLD-1 clone A	human DLD-1 clone A colon carcinoma cells
DMF	N,N-dimethylformamide
DMSO	dimethylsulphoxide
DNAase type I	deoxyribonuclease type I
d-NMF	formyl deuterated N-methylformamide
DPM	disintegrations per minute
DTNB	5,5'-dithiobis (2-nitrobenzoic)acid
EB	ethidium bromide
E.coli	Escherichia coli
F	formamide
FACS	fluorescence activated cell sorter
FCM	flow cytometry
FDA	fluorescein diacetate
G	guanine

g	acceleration due to gravity
GSH	reduced glutathione
GSSG	oxidized glutathione
γ-GT	γ-glutamyl transpeptidase
h	hours
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HL-60	human promyelocytic HL-60 leukaemia cells
HMBA	hexamethylene bisacetamide
HMF	N-hydroxymethylformamide
[^{3}H]thymidine	methyl labelled tritiated thymidine
ip	intraperitoneal
min	minutes
MOPS	N-morpholinepropane-sulphonic acid
MRC	Medical Research Council
n	number of experimemts
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Na_2EDTA	sodium ethyleneaminetetra-acetic acid
NCI	National Cancer Institute
NEF	N-ethylformamide
NEM	N-ethylmaleimide
NMA	N-methylacetamide
NMF	N-methylformamide
p	probability
PBS	phosphate buffered saline
PI	propidium iodide
psi	pounds per square inch

RNAase type 1A	ribonuclease type 1A
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium 1640
s	seconds
sc	subcutaneous
SD	standard deviation
SE	standard error
T_L	lower threshold
TLC	thin layer chromatography
TLI	thymidine labelling index
TPA	12-O-tetradecanoylphorbol-13-acetate
T_U	upper threshold

SECTION 1: INTRODUCTION

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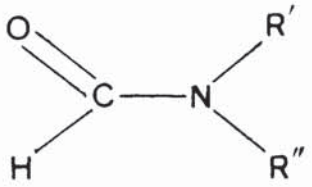
1.I General introduction

This thesis is concerned with studies of the mechanism of antitumour activity of N-methylformamide (NMF), *in vitro* and *in vivo*.

The structure of NMF and some related formamides which demonstrate antitumour activity are shown in table 1. The formamides illustrated are classified as polar solvents. In particular N,N-dimethylformamide (DMF) is used widely as an industrial solvent (Eberling, 1980); NMF is rarely used industrially. It was as a consequence of their solvent properties that the antitumour activity of the formamides was discovered in the early 1950's. A series of formamides were evaluated as potential solvents for the parenteral administration of antineoplastic drugs against the sarcoma 180. Formamide (F) and NMF were found to inhibit growth of the tumour and DMF had marginal activity (Clarke *et al*, 1953).

In a larger study of the biological activity of formamides and related compounds, against the Ehrlich ascites tumour, NMF was found to have the most potent antitumour activity (Furst *et al*, 1955). Slight alterations in chemical structure produced a dramatic decrease in the antitumour response.

As a consequence of its good activity in the murine models, NMF was entered into a small clinical trial of five patients, (Myers *et al*, 1956; see section 1.IV), no



Formamide	R′	R″
Formamide ; F	H	H
N-methylformamide ; NMF	CH_3	H
N,N-dimethylformamide ; DMF	CH_3	CH_3
N-ethylformamide ; NEF	CH_2CH_3	H

Table 1 : Structures of formamides discussed in this thesis.

tumour inhibition was reported but liver damage was evident. At the time this was considered an unacceptable side effect and interest in the drug was curtailed. Interest was rekindled, however, when NMF showed good activity against human tumour xenografts in the National Cancer Institute (NCI) screen (see section 1.IV) and because NMF was an inducer of differentiation *in vitro* (see section 1.II).

The following sections of this introduction will consider the activity and possible mechanisms of action of NMF and other polar solvents against tumour cells *in vitro*, and the activity of NMF as an antitumour agent *in vivo*.

The polar solvents are good inducers of differentiation of a number of tumour cell lines *in vitro* (see section 1.II). Differentiation is often associated with growth arrest and accumulation of cells in the G_1 phase of the cell cycle (see section 1.III). NMF possesses antitumour activity *in vivo* against animal and human tumours (see section 1.IV). In order to exert its antitumour activity and toxicity *in vivo*, NMF is thought to require metabolism to an active species (see section 1.V).

1.II Polar solvents as inducers of differentiation

The majority of antitumour drugs exert their activity, at least in part, via the production of cytotoxic lesions. Unfortunately these cytotoxic lesions are not restricted to the malignant cells and toxicity is often imparted on the normal host tissue. New concepts for therapy were sought which did not involve cytotoxicity. Conversion of malignant cells to differentiated mature cell types which do not proliferate may provide such a new approach. (Sartorelli, 1985 ; Bloch, 1984; Reiss et al, 1986; Sachs, 1978).

It has been suggested that the fundamental lesion in a neoplastic cell is its incapacity to differentiate terminally (reviewed by Freshney, 1985). Studies on murine tumour systems and clinical reports, indicated that the progeny of some malignant cells have the ability to spontaneously differentiate to mature cell types which have lost the capacity for self renewal (Pierce, 1970; Brinster, 1974). Thus for some cells at least, malignancy is a reversible state.

Under normal conditions, proliferation and maturation are regulated by protein factors (reviewed by Sachs, 1987). These growth and differentiation factors induce their respective target cells to either proliferate or differentiate to a more mature cell type. Many compounds have been found which have the capacity to induce differentiation, besides the natural differentiation factors. Physiological agents such as hormones (Lotem

and Sachs, 1975), vitamins (Abe et al, 1981), retinoids (Breitman et al, 1980; Takenaga et al, 1980)and calcium (Chapman, 1980; Hennings et al, 1980) can induce various cells to differentiate. Several exogenous agents can also induce differentiation; for example tumour promoters (Huberman and Callahan, 1979), mitomycin c, anthracyclines and ethionine (reviewed by Freshney, 1985), thioguanine, actinomycin D , butyric acid or its sodium salt (Rovera and Surrey, 1978; Terada et al, 1978; Leder and Leder, 1975) and polar solvents (Spremulli and Dexter, 1984). Starvation of essential substances can also cause differentiation; for example starvation of a single amino acid led to the maturation of human HL-60 promyelocytic leukaemic (HL-60) cells (Pilz et al, 1987).

NMF, like several other polar solvents is a good inducer of terminal differentiation in a number of leukaemic cell lines, including HL-60 and murine Friend erythroleukaemia (reviewed by Langdon and Hickman, 1987b; Spremulli and Dexter, 1984). NMF can induce the differentiation of some non-leukaemic cell lines, for example human DLD-1 clone A colon carcinoma (DLD-1 clone A), murine B16 melanoma and murine AKR-MCA fibroblasts, to a more benign phenotype (reviewed by Spremulli and Dexter, 1984; Langdon and Hickman, 1987b). The optimal concentrations of NMF required to induce maturation or terminal differentiation was 150mM to 170mM (Tanaka et al, 1975; Collins et al, 1978; Hager et al, 1980; Langdon and Hickman, 1987b).

Several leukaemic cell lines undergo terminal differentiation when incubated with polar solvents (see above). Markers of this terminal differentiation process can often be readily identified; for example NMF induced haemoglobin synthesis in Friend erythroleukaemia cells (Tanaka et al, 1975). Terminal differentiation is also associated with a loss in the ability of cells to proliferate, which ultimately leads to cell death. Since terminal differentiation of some leukaemic cell lines could be induced by polar solvents, it was suggested that leukaemias might be successfully treated by differentiation therapy in vivo (Honma et al, 1979; Sachs, 1987).

In contrast to the terminal differentiation of leukaemic cell lines, polar solvents induce a more benign phenotype in some non-leukaemic cell types (see above). Unlike terminal differentiation the more benign phenotype is unfortunately readily reversed when cells are incubated in drug free medium (reviewed by Spremulli and Dexter, 1984). Benign characteristics include altered morphology, increase in doubling time, loss of clonogenicity in soft agar and decrease in saturation density (Dexter et al, 1979). DMF has been the most extensively studied of the formamide analogues. DMF (0.8% $^{v}/_{v}$) caused complete loss of clonogenicity, a reduction of tumourigenicity and altered expression of carcinoembryonic antigen in DLD-1 clone A cells (Dexter et al, 1979) and altered the activity of seven purine metabolizing enzymes (Dexter et

al, 1981). DMF (1% $^{v}/_{v}$) altered cell surface protein antigens (Marks et al, 1986), increased growth factor levels (Levine et al, 1985) and restored normal membrane antigens (Chakrabarty et al, 1984) in murine AKR-MCA fibroblast cells. In another study NMF (1% $^{v}/_{v}$) increased the tyrosinase activity and melanin content of cultured B16 melanoma cells and caused changes similar to those elicited by DMF in the DLD-1 clone A cell line (Dexter et al, 1982b). NMF also decreased the expression of c-myc, c-fos, c-ras^{Ha} and c-ras^{Ki} oncogenes by DLD-1 clone A cells (Chatterjee et al, 1986). In each case the changes observed were considered to be representative of a more mature cell type.

The commitment of cells to undergo differentiation has been studied extensively in the HL-60 cell line. The HL-60 cells are bipotent in their differentiative capacity and will undergo myeloid/granulocytic or monocytic maturation in response to different inducers (Fontana et al, 1981). This cell line follows the granulocytic pathway when induced to differentiate by the alkylformamides (Langdon and Hickman, 1985). There is conflicting evidence as to whether cell proliferation is required for HL-60 cells to become committed to terminal differentiation. Ferrero et al, (1982) reported that terminal differentiation of HL-60 cells to granulocytes caused by the polar solvent, dimethylsulphoxide (DMSO), did not require cell division. However, Fibach et al, (1982) indicated that cell proliferation was coupled to

granulocytic differentiation, although cells lost their ability for self-renewal after 2-3 cell divisions. HL-60 cells required a minimum exposure period of 12h to DMSO (Tarella et al, 1982) or two division cycles to retinoic acid for significant granulocytic differentiation (Yen et al, 1984). The cellular regulatory events initiating a program of terminal differentiation of HL-60 cells in response to DMSO was S phase specific (Yen, 1985) and was associated with DNA replication and possibly involved the induction of gene amplification (Yen et al, 1987b). A two step model for induction of terminal differentiation was postulated with early precommitment events, that is events occurring before cells are committed to differentiate along a particular lineage, controlling the cell growth arrest and later events which regulate the choice of maturation pathway (Yen et al, 1987a).

The mode of action of differentiation inducers is largely unknown. The cellular response to hormonal signals has been well documented (Alberts et al, 1983), but this is not the case for other physiological and non-physiological inducers, which show no obvious structural requirements. Tanaka et al, (1975) have reported that highly polar compounds induce differentiation of Friend erythroleukaemia cells. However, a high dipole moment was not in itself a sufficient criterion to cause induction. Interestingly, a correlation between molecular weight of a compound and the logarithm of its optimal concentration to bring about

differentiation was noted (Langdon and Hickman, 1987a). This suggested that for the alkylformamides, acetamides, ureas and related compounds tested, no special structure was required to induce terminal differentiation of HL-60 cells to granulocytes. Also, maturation was induced at drug concentrations marginally below those which are cytotoxic, which suggests that a toxic threat to the cells could induce differentiation.

The cell membrane has been implicated as a locus of action of the polar solvents, and perhaps other inducers of differentiation. Polar solvents can penetrate cells and act as cryoprotectants (Preisler et al, 1976). Increased membrane viscosity associated with cell differentiation of Friend erythroleukaemia cells was observed for DMSO (Lyman et al, 1976) and hexamethylene bisacetamide (HMBA) (Tapeiro et al, 1980). No change in membrane microviscosity was seen with Friend cells resistant to differentiation by these agents. NMF (1% $^{v}/_{v}$) caused a significant increase in the membrane viscosity of DLD-1 clone A cells after 2 days of exposure, maximum changes occurred after 11 days. NMF (0.5% or 1.5% $^{v}/_{v}$) had little or no effect on membrane diffusion. These membrane changes correlated with the extent of cellular acquisition of a more benign phenotype (Dibner et al, 1985). DMF increased the expression of cell surface antigens in AKR fibroblasts (Chakrabarty et al, 1984) and increased the number of epidermal growth factor receptors (Levine et al, 1985). Also, the rate of nucleoside

membrane transport decreased profoundly in HL-60 cells after maturation induction by DMF (Chen et al, 1986). Ip and Cooper, (1980) used the HL-60 cell line to demonstrate that myeloid differentiation caused by DMSO occurred concomitantly with a progressive decrease in membrane fluidity, an effect not observed with 12-O-tetradecanoylphorbol-13-acetate (TPA), which caused cells to precede along the monocytic lineage.

Changes in ion flux across the plasma membrane may also have a role in the initiation of differentiation. The proliferation and differentiation of some cell types has been related to the activity of the Na^+-K^+-adenosine triphosphatase enzyme. Inhibition of this enzyme by ouabain promoted the erythroid lineage pathway of differentiation of Friend erythroleukaemia cells (Bernstein et al, 1976) and a pre-B lymphocyte cell line (Rossoff and Cantley, 1983). Terminal differentiation of HL-60 cells induced by DMSO caused an early rise in the activity of Na^+-K^+-adenosine triphosphatase (Ladoux et al, 1984) followed by a decrease in the pump activity.

The divalent cation Ca^{2+} is known to have an important role in cell growth and differentiation. A specific intra and extracellular Ca^{2+} ratio was required for erythroid differentiation of Friend erythroleukaemia cells (Chapman, 1980). Epidermal cells were induced to terminally differentiate by addition of Ca^{2+} (Hennings et al, 1980), in contrast, Ca^{2+} deprivation enhanced HL-60 cell maturation caused by retinoic acid (Okazaki et al,

1986).

The metabolism of phosphatidyl-inositols plays an important role in the response of cells to external stimuli (Berridge, 1987). DMSO caused a significant decrease in the levels of phosphatidyl-inositol metabolites within 2h of addition to culture (Faletto et al, 1985). The activation of protein kinase C is associated with the proliferative response to a number of mitogens (Faletto et al, 1985). Diacylglycerols, phospholipase C (Pincus et al, 1984) and the tumour promoting phorbol ester TPA (Yamasaki et al, 1977), all of which activate protein kinase C have been reported to inhibit DMSO induced differentiation.

Direct interaction with the genome has also been proposed as a mechanism by which antineoplastic agents can cause the acquisition of more benign characteristics (Scher and Friend, 1978; Terada et al, 1978). Yen et al, (1984) reported that there was a transient reduction in the size of HL-60 cell nuclei, which occurred as an early event during myeloid terminal differentiation induced by retinoic acid. The DNA may be altered by crosslinking, intercalation or the degree of methylation (Razin and Riggs, 1980; Simpkins et al, 1984). Scher and Friend, (1978) examined the DNA of Friend erythroleukaemia cells induced to differentiate by DMSO. The degree of DNA damage rose with the increase in DMSO concentration. Breaks in the DNA were detected prior to stimulation of globin messenger RNA and haemoglobin synthesis which are

markers of maturation in these cells. In epidermal cells, TPA induced single strand breaks in the DNA, but this was thought to be an indirect consequence of the induction of terminal differentiation (Hartley et al, 1985).

There is a relationship between the expression of cellular oncogenes and the control of cell growth and differentiation (Weinberg, 1984). NMF induced the maturation of DLD-1 clone A cells, which resulted in the reduced expression of c-myc, c-fos, c-ras^{Ha} and c-ras^{Ki} oncogenes. The expression of these oncogenes remained at control levels, however, when the maturation process was prevented by the co-addition of l-cysteine (Chatterjee et al, 1986).

Particular attention has been directed towards the role of the cellular oncogene c-myc in cell growth and differentiation. The expression of c-myc leads to the formation of a protein located in the cell nucleus, at present the function of this protein is uncertain (Alitalo et al, 1987). In general levels of c-myc mRNA parallel the rate of cell division. Expression of c-myc has often been associated with continuous proliferation, while cessation of expression occurs during terminal differentiation in several cell lines (Coppola and Cole, 1986). The expression of c-myc has been studied extensively in the HL-60 cell line, these cells have an elevated level of c-myc expression compared with other haemopoietic cell lines (Graham et al, 1985). Both monocytic differentiation induced by TPA (Chou et al,

1986) and granulocytic differentiation of HL-60 cells induced by DMSO (Grosso and Pitot, 1985) were associated with a decreased expression of c-myc. The decreased c-myc expression paralleled the degree of maturation of HL-60 cells induced by DMSO, but no such correlation existed with the extent of proliferation (Filmus and Buick, 1985).

1.III The effects of polar solvents and other inducers of differentiation on the cell cycle

NMF or DMF induced the acquisition of more benign characteristics in some human colon carcinoma cells, including a substantial increase in the cell population doubling time. NMF or DMF did not, however, effect the distribution of cells in the cell cycle (Leith et al, 1985; Leith et al, 1982a). The non-phase specific cell cycle effects of NMF or DMF are different to those reported for other polar solvents; for example DMSO (Friend et al, 1971), HMBA (Marks et al, 1987) and ethanol (Higgins and Borenfreund, 1986) caused G_1 phase arrest.

G_1 phase arrest frequently occurs when cells are induced to differentiate by a number of agents; for example vitamin D (Abe et al, 1986), retinoic acid (Breitman et al, 1980), TPA (Rovera et al, 1979) and sodium butyrate (D'Anna et al, 1980). The cell arrest was further characterised as being in the early G_1 phase in the case of sodium butyrate (Xue and Rao, 1981; Darzynkiewicz et al, 1981), although in lymphoblastoid cells accumulation in late G_1 phase was reported (Smith et al, 1985). A good correlation was found between terminal differentiation of Friend erythroleukaemia cells by a number of differentiation inducers and arrest of cell growth in the G_1 phase, however, this correlation was not found with all differentiation inducing agents. Also, arrest of cells in the G_1 phase did not necessarily lead to differentiation of the Friend cells (Friedman and

Schildkraut, 1978).

Drugs such as methotrexate (Friedman and Skehan, 1979), 1-β-D-arabinofuranosyl cytosine (Takeda et al, 1982) and cis-platinum (Kopf-Maier et al, 1983) prolong G_1 phase under appropriate conditions. G_1 phase accumulation can also result from changes in physiological conditions, for example serum or nutrient deprivation (Scott et al, 1982) or reduction of cellular glutathione (Ishii et al, 1985).

The nomenclature used today to describe the various phases of the cell cycle was first proposed by Howard and Pelc, (1953).

They subdivided the cell cycle into four phases: G_1, S, G_2 and M, which corresponds to: preparation of cells for DNA synthesis, replication of genetic material, preparation of cells for mitosis and mitosis, respectively. Later, another phase termed G_0 was added. This corresponds to quiescent non-cycling cells (Smith and Martin, 1973; see figure 1).

Cells can either be; (a) continuously dividing (or cycling); (b) G_0 cells, non-dividing but capable of re-entering the cell cycle under appropriate conditions; (c) terminally differentiated cells, destined to die without dividing again (Baserga and Surmacz, 1987; see figure 1).

In most plant and animal cells the entire cell cycle is completed in a day or less; a typical cycle takes about 20h, with approximate phase times of G_1 (8h); S (6h); G_2

Figure 1 : The cell cycle (from Baserga and Surmacz, 1987).

(5h) and M (1h). Given optimal conditions the length of the cell cycle is constant for a certain cell type. The tempo is slowed only under unfavourable circumstances (Mazia, 1974). The periods of S and G_2 do not usually show much variation (Smith and Martin, 1973). However, the length of the G_1 phase varies between cell types (Prescott, 1968).

It has been postulated that the cell cycle of eukaryotes consists of two cycles, a growth cycle and a chromosome cycle (Mazia, 1974). The chromosome cycle consists of S, G_2, and M phases while the growth cycle precedes continuously in parallel with the chromosome cycle.

The control of the cell cycle is the subject of lively debate. The continuum model proposed by Cooper, (1979 and 1987) suggests that there are no events that are unique to the G_1 phase. The model proposed that control of the cell cycle is mediated by factors or a function which is synthesized and present in all phases of the cell cycle. This model of the cell cycle control is in contrast to that proposed by Epifanova and Polunovsky, (1986) in which control over cell growth is achieved predominantly by regular transition of cells from proliferation to rest and vice versa. The model of Epifanova and Polunovsky, (1986) suggests further that the rest states are an obligatory part of each cell cycle.

There are three major points of the cell cycle at which proliferation can be regulated. These are;(i) G_0 to

G_1;(ii) G_1 to S;(iii) G_2 to M (Lord, 1986; Melchers and Lernhardt, 1985), with the G_1 to S transition being the major point of cell cycle regulation in mammalian cells. Since polar solvents and other differentiation inducers often cause G_1 phase arrest of cells (see above) the G_0 to G_1 and the G_1 to S transitions are likely points of cell cycle arrest.

Whether G_0 is a separate phase or just a prolongation of G_1 phase was for some time a subject of conjecture. Today it is widely accepted that there are distinct differences between these two phases. A comparison between G_0 and G_1 has been reviewed by Baserga, (1976). (a) the prereplicative phase of stimulated G_0 cells is usually substantially longer than the G_1 phase of the same cells; (b) there are differences in the sensitivity to inhibitory drugs; (c) there are differences in the compliment of ribosomes, in the amount of cellular RNA, and the electrophoretic pattern of non-histone nuclear proteins and other proteins; (d) most virally transformed cells, cannot enter G_0.

G_0 cells stimulated to emerge from quiescence, enter a competent state, in which they remain for a number of hours before entering another substate which is equivalent to that of cycling post-mitotic cells and is growth factor dependent. Subsequently cells enter a growth factor independent state which is followed by exit of cells into S phase (Pardee et al, 1986; Zetterberg and Larsson, 1985). Once in G_0, unless restored to the cycle, a cell

will settle into deeper levels of quiescence and finally die (Rossini et al, 1976). In normal tissue, cells naturally retire from the cell cycle in early G_1 phase (Rao, 1986). In fission yeast the cdc2 protein was found to be a key regulator of cell cycle commitment in G_1 phase (Nurse and Bissett, 1981). Recently a human homologue of the cdc2 gene was discovered; this may act at the G_1 phase commitment point in mammalian cells (Murray, 1987). Baserga and Surmacz, (1987) have recently suggested that proliferation specific events occur in mid or late G_1. They postulated that early events, after stimulation of G_0 cells by growth factors, are part of the common response of cells to a variety of environmental stimuli, while events occurring in mid or late G_1 are specific for cell proliferation.

Arrest of proliferating cells selectively in any part of the cell cycle other than G_1 is not possible by physiological agents as opposed to inhibitory drugs (Pardee et al, 1986). Scott et al, (1982) suggested that cells arrest their growth at different stages in G_1 phase under at least three external conditions. Prior to differentiation, growth arrest occurs at a stage in the G_1 phase (G_D) that is distinct from G_0 and the G_N growth arrest states induced by serum starvation or by deprivation of nutrients.

1.IV The antitumour activity of NMF in vivo and possible modes of action

NMF was first shown to possess antitumour activity against murine tumours in the 1950's. The demonstration of NMF antitumour activity prompted a limited clinical trial, in which an unacceptable degree of hepatotoxicity was reported; this led to a loss of interest in the drug (see section 1.I). Interest was reawakened, however, when NMF was shown to have good activity against several human tumour xenografts (NCI brochure, 1982).

NMF, along with DMF was found to be active against two human colon cancer cell lines xenografted in nude immunosuppressed mice (Dexter et al, 1982a). Gescher et al, (1982) tested NMF, NEF and F against three murine tumours (Sarcoma 180, M5076 reticulum cell sarcoma and TLX5 lymphoma). NMF had significant activity, F had little or no activity and NEF had no significant activity. Langdon et al, (1984) confirmed that NMF was active against the M5076 reticulum cell sarcoma. Also, NMF treatment decreased the metastatic potential of HCA-1 murine hepatocarcinoma cells when treated either in vitro and transplanted into mice or when dosed in vivo (Tofilon et al, 1987). In further studies however, NMF showed no antineoplastic activity against the B16 melanoma, the CD 8F mammary tumour, the colon 38 tumour or Lewis lung carcinoma (NCI brochure, 1982).

On the basis of the antitumour data of NMF against human tumour xenografts, clinical trials were introduced.

In phase I clinical trials the dose limiting toxicity was hyperbilirubinaemia (McVie et al, 1984), other toxic manifestations observed were nausea and vomiting, hepatotoxicity, thrombocytopenia, fatigue, hyperphosphataemia, peripheral neuropathy and flushing as a consequence of interaction with alcohol (McVie et al, 1984; Wiemann et al, 1985; Ettinger et al, 1985; Spremulli et al, 1983). Partial responses were seen with colon, prostate and cervical carcinomas. The recommended dose for the phase II trials was $800mg/m^2/day$ x 5, repeated every 2 or 3 weeks (McVie et al, 1984). This dose was increased up to $1000mg/m^2$, but toxicity resulted in the lowering of the dose in some phase II trials. Toxicities observed in these phase II trials (Sternberg et al, 1986; Tauer et al, 1986; Eisenhauer et al, 1986; Vogel et al, 1986) were similar to those reported for phase I with hepatotoxicity being dose limiting, there were also some additional side effects of skin rashes and gastrointestinal disturbances. There was a large degree of individual variability in toxicity which could not be readily explained (Eisenhauer et al, 1986). Unfortunately, no disease regression was reported in any of these phase II trials.

Toxicological studies in mice indicated that NMF did not cause myelosuppression (Langdon et al, 1985a; Langdon et al, 1985c), a toxicity which is a common problem of most antitumour agents. Clinical trials (see above) confirmed the lack of leukopenia, with hepatotoxicity as

the major toxic manifestation. Due to the lack of myelosuppression it was hypothesized that NMF could be used clinically in combination with myelosuppressive agents. In studies on murine models a combination of NMF with either cyclophosphamide (Langdon et al, 1985c) or cis-platinum (Harpur et al, 1986) was used. There was additive antitumour activity with little or no augmentation of the respective toxicities. An enhanced chemotherapeutic activity of NMF occurred when it was combined with menadione sodium bisulphite or sodium ascorbate, although the mechanism of this synergism is unknown (Osswald et al, 1987).

Dexter et al, (1984) found that administration of NMF for 19 consecutive days enhanced the growth inhibitory action of x-irradiation in human colon carcinoma xenografts and in a murine tumour model. NMF increased the radiosensitivity of the tumour and its metastases with no concomitant increase in the radiation response of the normal tissue (Iwakawa et al, 1987). Despite the disappointing clinical results obtained with NMF, further trials have been proposed in which NMF will be used as a radiosensitizer. These proposed trials are based on the encouraging results obtained both in vitro and in vivo when NMF was combined with radiotherapy (see section 1.V; Dexter et al, 1984; Iwakawa et al, 1987).

There have been several studies to investigate the mechanism of antitumour activity of NMF in vivo. Although, NMF can induce the differentiation of a number

of cell types *in vitro* (see section 1.II), it is not known whether NMF acts as a maturation inducer, or as a cytotoxic/cytostatic agent *in vivo*.

Much of the work on the mechanism of the antitumour activity of NMF *in vivo* was performed when NMF was first shown in the 1950's to be an active antitumour agent. It was postulated that NMF may act as a metabolic antagonist for certain simple compounds in particular the one carbon intermediates, vital for some biosyntheses. Preliminary tests involved administration of formate, acetate and glycine in an attempt to block the NMF antitumour response in sarcoma 180, no such block was observed (Clarke *et al*, 1953). In this study it was also tried without success to reverse the cytotoxic effects of NMF with folic acid or folinic acid; these agents were employed since folic acid antagonists interfere with formate incorporation into nucleotides. Barclay *et al*, (1954) found that NMF stimulated incorporation of formate into the nucleic acids of the liver and in a later study (Barclay and Garfinkel, 1957) compared an NMF sensitive and non-sensitive tumour. The sensitive tumour showed marked inhibition of formate incorporation whereas the insensitive tumour exhibited only slight inhibition. Skipper *et al*, (1955) investigated the growth inhibitory effect of formamides on Escherichia coli (E.coli) and found that this inhibition could be prevented by 2,6-diamino-purine or 2,6-diamino-purine-riboside; they suggested that purine metabolism was affected. Furthermore, Morrison and

Higgins, (1956) suggested that NMF antagonised the metabolism of nucleic acids and cross resistance was observed when purine synthesis antagonists were compared with NMF (Sartorelli and LePage, 1958). However, Tarnowski and Stock, (1957) did not observe any synergistic therapeutic response when NMF was combined with known inhibitors of purine biosynthesis. In another study Eidinoff et al, (1961) suggested that NMF enhanced pyrimidine synthesis, possibly acting at the carbamoyl aspartic acid stage. However, NMF caused a marked decrease in pyrimidine biosynthesis in E.coli with a concomitant increase in the pools of ribonucleotide triphosphates (Tomisek, et al, 1969).

1.V The role of glutathione in the metabolism and toxicity of NMF

DLD-1 clone A cells acquire the characteristics of a more benign phenotype when they are incubated with NMF in culture (see section 1.II), this maturation was accompanied by a fall in the intracellular glutathione levels (Cordeiro and Savarese, 1984). NMF (170mM for 96h) inhibited cell growth, clonogenicity and elicited a fall in glutathione content to 12% of control levels. The growth and glutathione depletory effects of NMF against the DLD-1 clone A cells were restored to control values on co-incubation with 0.5mM l-cysteine (Cordeiro and Savarese, 1984). The γ-glutamyl cysteine synthetase inhibitor D,L-buthionine-[S,R]-sulphoximine (BSO) depleted DLD-1 clone A cellular glutathione and mimicked the ability of NMF to cause cytostasis and the induction of a more benign phenotype in these cells (Cordeiro and Savarese, 1986). BSO, also mimicked the ability of NMF to decrease the expression of the c-myc oncogene (see section 1.II). Thus, for the DLD-1 clone A cell line, NMF mediated depletion of intracellular glutathione was apparently responsible for the observed cytostasis and acquisition of more benign characteristics (Cordeiro and Savarese, 1986).

There have been a number of reviews on the metabolism and functions of glutathione (Kosower and Kosower, 1978; Meister and Anderson, 1983; Reed and Beatty, 1980; Larsson et al, 1983; Sakamoto et al, 1983).

Glutathione is the most important non-protein thiol in living systems and is found throughout the animal and plant kingdom. Glutathione content represents around 90% of the total non-protein low molecular weight thiols found in cells (Kosower and Kosower, 1978). The structure of the reduced form of glutathione was identified as γ-glutamylcysteinylglycine (γ-GLU-CYS-GLY; see figure 2).

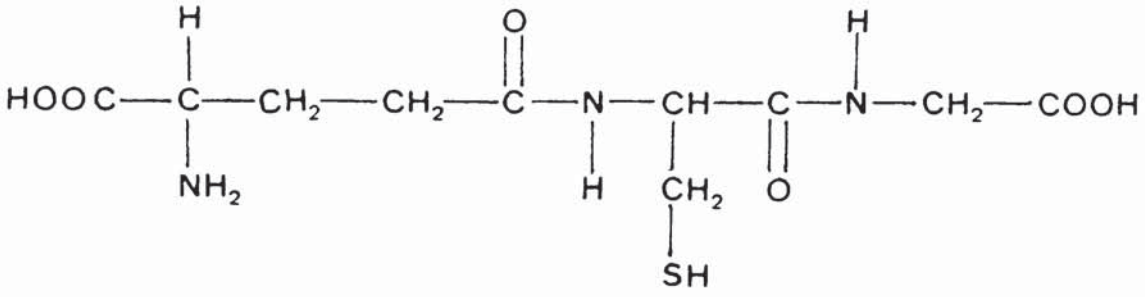


Figure 2 : The structure of glutathione.

The intracellular glutathione concentration is maintained in the range of 0.5 to 10mM; this is dependent on the cell type (Kosower and Kosower, 1978). Glutathione is present in all cultured mammalian cells (Bannai, 1983) and all tissues examined, with the highest concentrations found in the liver (Kosower and Kosower, 1978).

Under normal conditions glutathione is present in the reduced state (GSH) with less than 5% of the total present as the oxidized form, glutathione disulphide (GSSG). This

balance is maintained by intracellular glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (see figure 4).

There are two main compartments of intracellular GSH, the major pool being labile and found in the cytoplasm with a secondary pool in the mitochondria. Mitochondrial GSH consists of about 10% of the total GSH, with a half-life of 30h in rat hepatocytes, compared with a half-life of 1h in the cytoplasm. GSH is synthesized only in the cytoplasm and can then be transported to the mitochondria (Griffith and Meister, 1985).

$$\text{l-glutamate} + \text{l-cysteine} \xrightleftharpoons{\gamma\text{-glutamylcysteine synthetase}} \gamma\text{-l-glutamyl-l-cysteine} \quad \text{(reaction 1)}$$

$$\gamma\text{-l-glutamyl-l-cysteine} + \text{glycine} + \text{ATP} \xrightleftharpoons{\text{GSH synthetase}} \text{GSH} + \text{ADP} + \text{Pi} \quad \text{(reaction 2)}$$

Figure 3: The synthesis of GSH from constituent amino acids (from Bannai and Tateishi, 1986)

Reaction 1, which is inhibited in a feedback reaction by GSH, is considered to be the rate limiting step in the synthesis of GSH (Meister and Tate, 1976). Since cells are able to synthesize their own GSH, the availability of precursor amino acids is an important regulatory factor in this anabolic process. Glutamate and glycine are readily synthesized; glutamate from glutamine by deamination and glycine from serine *via* a serine hydroxymethyltransferase catalyzed reaction (Martin *et al*, 1983). Cysteine, can be synthesized by the cystathionine pathway from methionine. This pathway, however, is often unable to supply

sufficient quantities of the amino acid (Eagle et al, 1966) and cysteine is therefore an essential amino acid for several cultured cell lines.

The uptake and release of l-cysteine is primarily mediated by system ASC, a transport system for some neutral amino acids. In culture medium cysteine is readily autoxidised to cystine. Cystine is transported as the anion and is readily converted back to cysteine intracellularly (Bannai and Tateishi, 1986).

A number of thiol compounds including GSH have beneficial effects on the growth of some cultured mammalian cells (Ishii et al, 1981; Ishii et al, 1985; Cordeiro and Savarese, 1986). Enhancement of the intracellular GSH concentration induces DNA synthesis in lymphocytes (Fidelus and Tsan, 1986). The reduction of ribonucleoside diphosphates to the corresponding 2'-deoxyribose compounds by glutaredoxin depends on reduction of this enzyme by GSH (Holmgren, 1981). Alterations in intracellular GSH content can also modulate protein synthesis (Kosower and Kosower, 1978; Zehavi-Willner, 1973). GSH can serve as a reservoir of l-cysteine for protein synthesis (Takeishi et al, 1977). However, l-cysteine is considered to be an unlikely limiting amino acid for protein synthesis in most cells (Bannai, 1983). Undoubtedly GSH is important for protein synthesis and its depletion can result in the inhibition of thermotolerance and the synthesis of heat shock proteins (Russo et al, 1984; Shelton et al, 1986). This

may be linked to the cellular differentiation process, since it has been postulated that heat shock proteins may play a role in cellular maturation (Langdon and Hickman, 1987b) and DLD-1 clone A cells acquired more mature characteristics when the cellular GSH was depleted by incubation with NMF (Cordeiro and Savarese, 1986).

One of the major functions of GSH is to detoxify reactive species. The intermediates may be electrophiles, peroxides or free radicals. In these reactions GSH serves as either a nucleophile leading to conjugate formation or as a reductant. These reactions may occur both non-enzymatically or enzymatically (Larsson et al, 1983).

NMF and DMF enhanced the cytotoxicity of x-irradiation, cis-platinum, mitomycin c, 5-fluorouracil and bleomycin against human colon carcinoma cells. This increased sensitivity was thought to be a reflection of the decreased GSH levels and the corresponding reduction in cellular protection (Dexter et al, 1984; Dexter et al, 1983; Leith et al, 1985; Tofilon et al, 1986; Leith et al, 1982b; Arundel et al, 1985). Indeed, GSH depleted cells exhibited increased radiosensitivity compared to undepleted controls (Phillips et al, 1986), an effect which was reversed on GSH repletion (Jensen and Meister, 1983). Radiosensitization occurred with several, but not all differentiating agents (Leith et al, 1986) and indeed in the case of doxorubicin, NMF induced resistance to cell kill (Ferrari et al, 1986). Selective inhibition of GSH synthesis by BSO led to a decrease in cellular GSH levels

and an increase in cytotoxicity to melphalan, mitomycin c, cyclophosphamide (Ozols et al, 1987; Ono and Shrieve, 1986) and to the radiosensitizer, misonidazole (Ono et al, 1986).

A severe problem of antitumour chemotherapy is the fulminant toxicity to the host; NMF is no exception to this rule.

Morrison and Higgins, (1956) administered 0.5g/Kg NMF ip to mice; they observed no mortality, but the weights of the spleen and liver decreased. The same dose, however, in nude mice was equivalent to the LD_{50} (Dexter et al, 1982a).

The major side effect of NMF reported in the first clinical trial was hepatotoxicity (Myers et al, 1956; see section 1.IV), this was later confirmed in studies of mice (Langdon et al, 1985a; Whitby et al, 1984b; Dexter et al, 1982a; Lundberg et al, 1981) in which liver enzymes were elevated in the plasma. The hepatotoxicity of DMF was postulated to be due to the major urinary metabolite NMF (Lundberg et al, 1981); this was later found to be an artifact of the analysis procedure and N-(hydroxymethyl)-N-methylformamide is now thought to be the major metabolite of DMF (Kestell et al, 1986a).

NMF associated toxicities other than hepatotoxicity have been reported (see section 1.IV). NMF is a well known irritant and 0.1ml produced moderate damage to the rabbit eye after 24h which receded to slight damage after 48h (Conquet et al, 1977). NMF was embryotoxic when

applied to the skin of rats at doses of 0.6g/Kg (Stula and Krauss, 1977). The number of dead implants increased, foetal weight decreased and all offsprings were malformed when rabbits were given 50 mg/Kg NMF orally on days 6-18 after conception (Merkle and Zeller, 1980). Also a wide number of foetal abnormalities have been described after dermal or oral treatment with NMF (Kennedy, 1986).

NMF induced hepatotoxicity was associated with a decrease in the hepatic glutathione levels (Gescher et al, 1982; Pearson et al, 1987a). Depletion of hepatic GSH pools by BSO exacerbated the toxicity, while toxicity was reduced by co-addition of thiol compounds (Pearson et al, 1987a). Many chemicals are known to deplete liver GSH often after a single dose. Depletion to 20-25% of control GSH levels was the nadir for reparable damage (Reed and Beatty, 1980), provided rapid resynthesis occurred. Agents such as sulphydryl nucleophiles, for example N-acetylcysteine, increased GSH synthesis and decreased the level of reactive metabolite which alleviated or at least reduced hepatic injury caused by acetaminophen (Corcoran et al, 1985a; Corcoran, et al, 1985b).

Studies were conducted to elaborate the mechanism of NMF induced hepatotoxicity. The decrease in GSH levels suggested that an electrophilic metabolite was being formed, which caused the hepatotoxicity and perhaps the lipid peroxidation observed (Whitby et al, 1984b). The periacinar liver damage corresponded to the tissue distribution of cytochrome P450 (Whitby et al, 1984b);

however, metabolic activation by this route was thought to be unlikely (Pearson et al, 1987b).

A direct correlation between hepatic GSH levels, the severity of hepatic necrosis and the degree of covalent binding to liver macromolecules has been demonstrated previously for bromobenzene (Reed and Beatty, 1980). Studies which utilized [^{14}C]NMF revealed that radioactivity was covalently bound to liver macromolecules (Pearson et al, 1987b). This study also used different strains of mice with different susceptibilities to NMF induced hepatotoxicity; the results indicated that damage corresponded to the extent of NMF metabolism (Pearson et al, 1987b). The metabolic pathway which N-alkylformamides undergo is also an important criterion for hepatotoxicity. A recent study suggests that N-alkylformamides which undergo metabolic oxidation at the formyl moiety generate hepatic lesions, whereas hydroxylation at the α-carbon of the N-alkyl group constitutes a detoxification pathway (Kestell et al, 1987).

An important question in the study of the mechanism of action of the antitumour activity of NMF is whether the parent drug or a metabolite is the active species. Concentrations of about 150mM NMF are required to induce differentiation (Collins et al, 1978); this is greatly in excess of the plasma concentrations reported for the maximum tolerated doses, which are 7mM in mice (Brindley et al, 1982) and 1.7mM in man (McVie et al, 1984). The difference in the concentrations of NMF in vitro and in

vivo, and the fact that NMF is not concentrated in the TLX5 tumour or other organs (Barlow, 1982) suggests that metabolism is required for NMF to exert antitumour activity in vivo.

Investigations of the structure-activity relationships among formamides demonstrated that only NMF had substantial antitumour activity (Gescher et al, 1982; Gate et al, 1986), although all the formamide analogues can induce terminal differentiation of HL-60 cells (Langdon and Hickman, 1987b). It was suggested that the active antitumour species of NMF in vivo was a metabolite rather than the parent drug itself (Gescher et al, 1982; Cooksey et al, 1983). Furthermore, when the metabolism of NMF was inhibited antitumour activity was also decreased (Masuda et al, 1986).

Knowledge of the metabolic pathway of NMF would be useful for the elucidation of the mechanism by which the drug exerts its antitumour and toxic effects. Early pharmacological studies demonstrated that after intraperitoneal (ip) administration of 400mg/Kg [^{14}C] methyl labelled NMF ($^{14}CH_3NMF$), 73.6% of the dose was excreted in the urine after 24h, 26% of the excreted radioactivity was unchanged NMF and 3% of the radioactivity was excreted as carbon dioxide (Brindley et al, 1982). One urinary metabolite was tentatively identified as N-hydroxymethylformamide (HMF) and F was found as a metabolite in the plasma and urine (Gescher et al, 1982). Ross et al, (1981) found F to be a major

urinary metabolite following administration of NMF to mice.

NMF ([^{14}C] labelled) in the methyl or formyl moiety was used to analyse further the metabolic fate of NMF. The major route of elimination was *via* the kidneys, although 39% of the dose was excreted *via* the lungs as carbon dioxide, when the [^{14}C] formyl labelled NMF was used which compared with 15% for the [^{14}C] methyl labelled drug. One of the urinary metabolites was identified as methylamine (Kestell *et al*, 1985). In further studies in mice another metabolite was identified as a mercapturic acid, N-acetyl-S-(N-methylcarbamoyl)cysteine which amounted to 16% of the [^{14}C] NMF dose (Kestell *et al*, 1986b). This metabolite was also identified in the urine of rats and patients. Recently S-(N-methylcarbamoyl)glutathione was found as an NMF metabolite in the bile of mice (Threadgill *et al*, 1987). The formation of N-acetyl-S-(N-methylcarbamoyl)cysteine involves oxidative rupture of the formyl C-H bond of NMF and conjugation with GSH. Cleavage of the formyl C-H bond is the rate limiting step in the metabolic formation of methylamine *in vivo* (Threadgill *et al*, 1986).

The conjugation of GSH with electrophiles is the most widely known biological role of GSH (Reed and Beatty, 1980; see figure 4) and is catalyzed by a number of isozymes termed glutathione-S-transferases (Chasseaud, 1979). These enzymes serve as binding, scavenger or transfer proteins (Jakoby and Habig, 1980). GSH

Figure 4 : An outline of the metabolism of glutathione (from Meister and Anderson, 1983).

conjugates are usually less toxic than the parent compound. They are excreted preferentially into the bile by a carrier-mediated transport system (Inoue et al, 1984). GSH conjugates are generally converted to the N-acetyl-l-cysteine derivatives (mercapturic acids) by a series of enzymatic steps initiated by γ-glutamyl transpeptidase (γ-GT), enzymes which are located mainly on the external surface of renal tubules, in particular the proximal convoluted and straight segments (Meister and Anderson, 1983). The γ-glutamyl moiety of the conjugate is transferred to an acceptor (usually an amino acid); the resultant cysteinylglycine conjugate is converted by the action of dipeptidase to the corresponding cysteinyl conjugate which is then N-acetylated to form the mercapturic acid which is excreted in the urine (Meister and Anderson, 1983; Larsson et al, 1983; see figure 4). This pathway also has an important function in the metabolism of endogenous regulators such as prostaglandins and leukotrienes (Rouzer et al, 1982).

GSH conjugation can in some instances lead to the formation of species more toxic than the parent drug; for example 1,2-dibromoethane, which is itself mutagenic has markedly enhanced toxicity as a result of conjugation with GSH (Brem et al, 1974).

Another major function of glutathione is as a regulator of reductive processes (Reed, 1986). GSH is involved in three main types of oxidation-reduction reactions.

(a) one electron reactions, including free radicals which lead to formation of glutathione disulphide.

$$GSH + R^{\cdot} \longrightarrow RH + GS^{\cdot}$$

$$2GS^{\cdot} \longrightarrow GSSG$$

(b) two electron oxidation <u>via</u> the formation of an intermediate, followed by displacement by a thiol anion.

$$RS^{-} + I_2 \longrightarrow RSI + I^{-}$$

$$RS^{-} + RSI \longrightarrow RSSR + I^{-}$$

(c) thiol disulphide interchange, which is important in the regulation of protein disulphides and mixed disulphides.

$$R'S^{-} + RSSR \longrightarrow R'SSR + RS^{-}$$

(from Kosower and Kosower, 1978)

Several enzymes catalyze the oxidation of thiols to disulphides, in particular; thiol oxidase, GSH peroxidase and the flavin-containing monooxygenases (Larsson <u>et al</u>, 1983). Intracellular GSH is converted to GSSG by GSH peroxidase, which catalyses the reduction of hydrogen peroxide and other peroxides (Meister and Anderson, 1983; see figure 4). GSH peroxidase is highly specific for GSH as the sulphydryl substrate, but is relatively non-specific towards the peroxide substrate (Reed and Beatty, 1980). The GSSG formed in the oxidation reactions

is readily converted back to GSH by glutathione reductase. GSH can also act as a cofactor and operates as such for the glyoxalase enzymes which catalyzes the conversion of methylglyoxal into lactic acid (Douglas et al, 1985).

The efflux of GSH (Meister and Anderson, 1983) and GSSG (Sies and Akerboom, 1984), have been reported for a variety of cells and tissues. The transport of GSH is primarily a unidirectional efflux, although uptake can take place (Bannai and Tateishi, 1986). The major fraction of the GSH exported was found as mixed disulphides of GSH and cysteine in the culture medium of fibroblast cells. Since the enzymes that catabolize GSH are localized on the external surface of certain cells, efflux constitutes the initial step of GSH degradation (Bannai and Tsukeda, 1979).

Reversible loss of GSH may result from reactions which involve the formation of GSSG, mixed disulphides and thioesters. Irreversible loss of GSH may occur when endogenous compounds such as leukotriene C_4 are formed or exogenous compounds react with GSH (Igwe, 1986), for example the GSH conjugate formed from the oxidation product of NMF, which is ultimately excreted in the urine as N-acetyl-S-(N-methylcarbamoyl)cysteine (Kestell et al, 1987; Threadgill et al, 1987).

The structural integrity of cell membranes depends upon the GSH/GSSG ratio. The GSH/GSSG ratio regulates the thiol/disulphide ratio in proteins and protects cells from lipid peroxidation through detoxification of free radicals

and the decomposition of lipid hydroperoxides (Kosower and Kosower, 1978; Larsson et al, 1983). Membrane functions such as ion and sugar transport and mitochondrial function, are all dependent upon the thiol-disulphide balance of the various proteins (Kosower and Kosower, 1978).

Glutathione possesses a critical role in the regulation of calcium sequestration and transport (Larsson et al, 1983; Chavez et al, 1985; Thor et al, 1985). The ability of mitochondria to remain impermeable to calcium is thought to be controlled by the GSH/GSSG ratio (Beatrice et al, 1984).

It has been suggested that liver necrosis induced by some hepatotoxins is the consequence of interference with Ca^{2+} sequestration by the endoplasmic reticulum or mitochondria which results in a disturbance of intracellular Ca^{2+} homeostasis (Younes et al, 1983). Indeed disturbances of Ca^{2+} levels is an early event in the genesis of lethal cell injury produced by an acute oxidative stress (Bellomo et al, 1982). Chemical induced hepatic cell death is not caused by an increased total cellular Ca^{2+} resulting from the influx of extracellular Ca^{2+} (Fariss and Reed, 1985; Reed and Fariss, 1984). The administration of NMF to mice caused a dramatic decrease in the ability of the liver mitochondria to sequester Ca^{2+}. An effect which was dose dependent and not caused by formamide analogues which were not hepatotoxic (Whitby et al, 1984a).

It is not known whether NMF induced hepatotoxicity and antitumour activity are causally related, although the evidence suggests that NMF requires metabolism to exert both activities. Masuda et al, (1986) reported that diethyldithiocarbamate, a suppressor of microsomal drug metabolizing enzymes, delays NMF metabolism and reduces both hepatotoxicity and antitumour activity against Ehrlich ascites and sarcoma 180.

Since metabolism of NMF was required to exert the antitumour response, suitable metabolic candidates have been considered. Cooksey et al, (1983) found that although HMF was more cytotoxic than NMF in vitro, it had little or no activity against tumours which are sensitive to NMF in vivo. Methylamine is an endogenous amine and is thought to be an unlikely antitumour agent, although reactive species such as nitrosamines and azoxymethane can be formed from methylamine (Kestell et al, 1985).

The mercapturic acid characterized in urine after treatment with NMF resulted from the corresponding S-(N-methylcarbamoyl)glutathione conjugate (Threadgill et al, 1987), this may act as a carbamoylating agent (Pearson et al, 1987b). Since NMF does not react chemically with glutathione, there must first of all be an oxidation step at the formyl carbon of NMF (Kestell et al, 1986b). The mechanism of this step is unknown, although possible formation of the highly reactive methyl isocyanate has been postulated (Kestell et al, 1986b; see figure 5).

The metabolism of the formamide series NEF, DMF, and

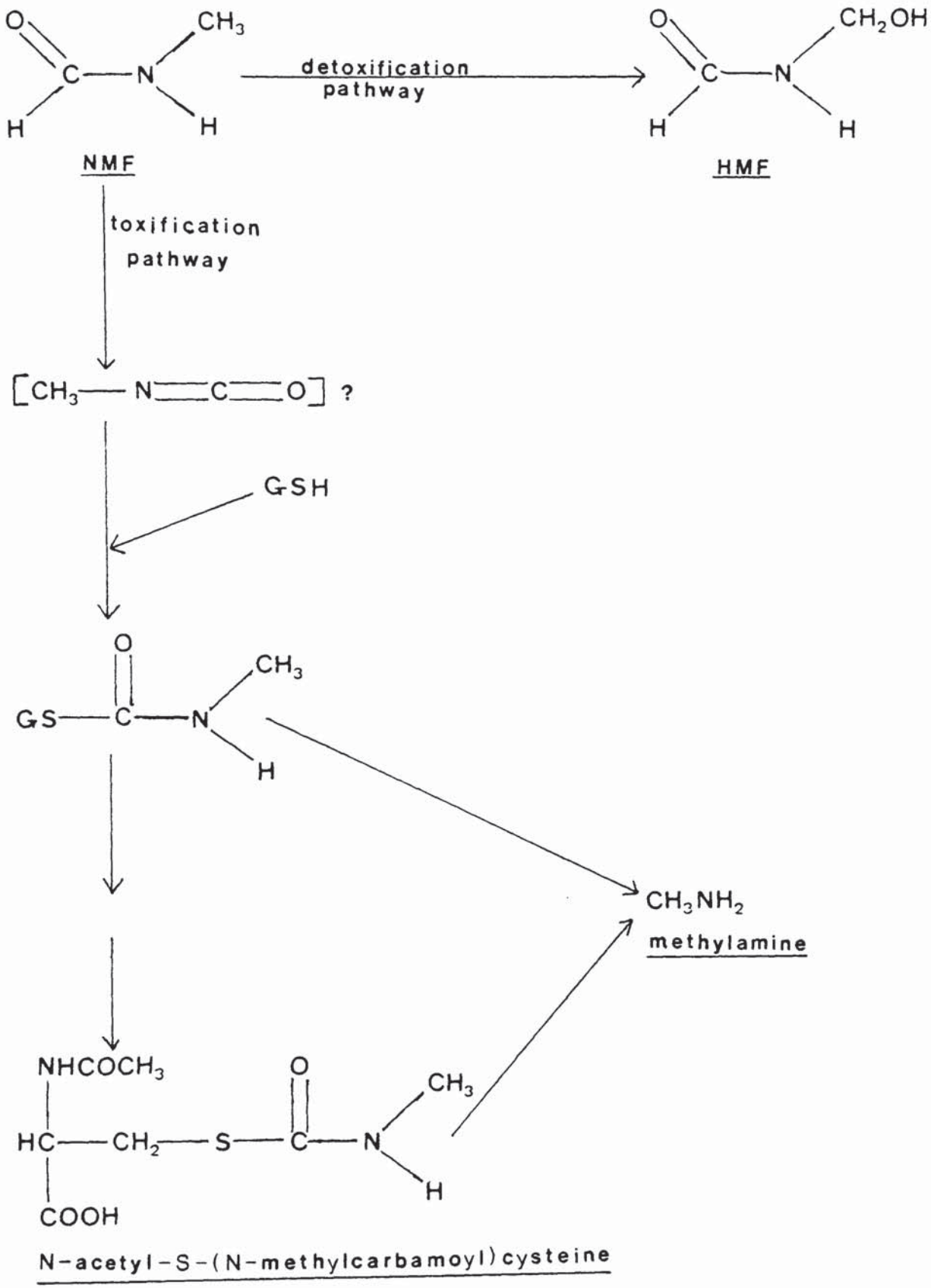


Figure 5 : An outline of the NMF metabolic pathway.

N-methylacetamide (NMA) were investigated and the metabolic pathways for the N-alkylformamides were established, namely hydroxylation at the α-carbon of the N-alkyl group and oxidation of the formyl moiety (Kestell et al, 1987). NEF was the only agent metabolized by the same pathway as NMF, but NEF is only marginally active as an antitumour agent against the TLX5 lymphoma (Gescher et al, 1982), even though it is hepatotoxic (Kestell et al, 1987). The active antitumour species may be one or more of the metabolites resulting from the GSH conjugation pathway as S-(N-alkylcarbamoyl)cysteines have demonstrated antitumour activity (Nemeth et al, 1978), as have some related S-carbamoyl-amino acid analogues (Skinner et al, 1958).

1.VI Aims

The aim of the present studies was to investigate the mechanisms of the antitumour activity of NMF.

The TLX5 lymphoma was used both *in vitro* and *in vivo* throughout these studies. The TLX5 lymphoma was induced in the thymus of x-irradiated CBA mice (Connors and Jones, 1970). The tumour is sensitive to NMF *in vivo* (Gescher *et al*, 1982) and is routinely used in the Pharmaceutical Sciences Institute, Aston University, UK, to screen potential antitumour agents.

This thesis is in two parts. In part 1, the effects of NMF on tumour cells *in vitro* will be discussed and in part 2, a comparative report on studies of the antitumour activity of NMF *in vivo* will be presented.

The specific questions that this thesis addresses are as follows:

(a) What effect does NMF have on the growth, morphology and viability of TLX5 cells?

(b) Are the antitumour effects of NMF associated with changes in the cell cycle kinetics?

(c) Does glutathione have a role in the antineoplastic activity of NMF?

(d) Is metabolism of NMF required for the antitumour activity?

(e) How do the effects of NMF on the growth and cell cycle of TLX5 cells compare with the formamide analogues, DMF and NEF?

(f) Does NMF induce the terminal differentiation of TLX5

cells *in vitro*?

SECTION 2: MATERIALS

SECTION 2: MATERIALS

2.I Chemicals

2.I.i Purchased from Sigma Chemical Company Limited, Poole, Dorset, England

Bovine serum albumin (BSA), DL-buthionine-[S,R]-sulphoximine, collagenase type I, l-cysteine, deoxyribonuclease type I (DNAase type I), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), ethidium bromide (EB), fluorescein diacetate (FDA), reduced glutathione, oxidized glutathione, glutathione reductase type III, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), N-morpholinepropane-sulphonic acid (MOPS), nicotinamide adenine dinucleotide phosphate reduced form type I, N-ethylmaleimide (NEM), propidium iodide (PI), QAE-25-120 sephadex, ribonuclease type 1A (RNAase type 1A), saponin, tetramethylurea, toluidine blue, triton X-100, trypan blue.

2.I.ii Purchased from BDH Chemicals Limited, Poole, Dorset, England

Acetone, dimethylformamide, ethanol, 25% electron microscopy grade gluteraldehyde, metaphosphoric acid, methanol, propan-2-ol, sodium chloride, sodium ethylenediaminetetra-actic acid (Na_2EDTA), toluene, trichloroacetic acid.

2.I.iii Purchased from Fisons PLC, Loughborough, Leicestershire, England

Folin-Ciocalteau reagent, glacial acetic acid, hydrochloric acid, isopentane, luma gel scintillant, potassium chloride, sodium cacodylate, sodium hydroxide, trisodium phosphate.

2.I.iv Purchased from Gibco Limited, Paisley, Glasgow, Scotland

Horse serum, Roswell Park Memorial Institute medium 1640 (RPMI 1640 medium) with 25mM HEPES buffer and l-glutamine.

2.I.v Purchased from Fluka Chemicals Limited, Glossop, Derbyshire, England.

N-ethylformamide.

2.I.vi Purchased from Emscope Laboratories Limited, Ashford, Kent, England

2% osmium tetroxide.

2.I.vii Other chemicals

All other chemicals were purchased as described in the methods or were of analytical grade.

2.I.viii Tissue culture materials

50ml and 250ml culture flasks, 35mm petri dishes (Nunclon, Kamstrup, Roskilde, Denmark), class II Gelaire

BSB 3 microbiological safety cabinet with unidirectional laminar downflow, gassing incubator (Flow Laboratories, Irvine, Scotland), 30ml sterile universals, 7ml bijou bottles, sterile pipettes, polyethylene test-tubes (Sterilin Limited, Feltham, England).

2.II Buffers and solutions

2.II.i Cell lysis buffer

tris (trizma base) 2.06g

ammonium chloride 7.50g

dH_2O to 1l

2.II.ii Phosphate buffered saline (PBS)

KCl 0.2g

KH_2PO_4 0.2g

NaCl 8.0g

$Na_2HPO_4.2H_2O$ 1.15g

Na_2EDTA 0.372g

dH_2O to 1l

2.II.iii Electron microscopy

stock cacodylate buffer

sodium cacodylate 4.28g

dH_2O to 100ml

adjusted to pH 7.2 with 0.2M hydrochloric acid.

Fix 1: gluteraldehyde 2.5% in 0.1M cacodylate buffer

stock cacodylate buffer 20ml

25% EM grade gluteraldehyde 4ml

dH_2O to 40ml

Fix 2: osmium tetroxide 1% in 0.1M cacodylate buffer

2% osmium tetroxide 5ml

stock cacodylate buffer 5ml

2.II.iv HEPES buffer

NaCl 8.3g

KCl 0.5g

HEPES 2.4g

NaOH 1M 5.5ml

dH_2O to 1l

adjusted to pH 7.4 with concentrated hydrochloric acid. 20ml of a $CaCl_2.2H_2O$ solution (170 mg/ml) was added to the buffer just prior to use.

2.II.v Flow cytometry

RNAase solution

RNAase type I 1mg

PBS to 1ml

propidium iodide staining solution

propidium iodide 200 ug/ml

sodium citrate 0.1% $^{w}/_{v}$

triton X-100 0.1% $^{w}/_{v}$

sodium chloride 0.9% $^{w}/_{v}$

dissolved in dH_2O

solution A

triton X-100 0.1% $^{v}/_{v}$

sucrose 0.2M

Na_2EDTA $10^{-3}M$

dissolved in citrate-phosphate buffer ($2 \times 10^{-2}M$) at pH 3.0

solution B

acridine orange 22.1uM

NaCl 0.1M

dissolved in citrate-phosphate buffer ($10^{-2}M$) at pH 3.8

2.II.vi Total glutathione assay

phosphate-EDTA buffer

$Na_2HPO_4.2H_2O$ 125mM

Na_2EDTA 6.3mM

dissolved in dH_2O and adjusted to pH 7.5

saturated trisodium phosphate solution

Trisodium phosphate 12.5g was dissolved in dH_2O using heat and constant stirring. When dissolved, the volume was made up to 25ml. Constant stirring was required for the solid to remain in solution.

DTNB solution

DTNB 23.8mg

phosphate-EDTA buffer, pH 7.5 to 10ml

the solution was stored at -20 ^{0}C in the dark until used.

NADPH solution

NADPH 6.3mg

phosphate-EDTA buffer, pH 7.5 to 25ml

the solution was prepared just before use and was kept on ice.

glutathione reductase solution

glutathione reductase (500 units in 2.5ml) 90ul

phosphate-EDTA buffer, pH 7.5 250ul

the solution was prepared just before use and was kept on ice.

2.II.vii Oxidized glutathione assay

potassium phosphate buffer (0.01M)

KH_2PO_4 680mg

dH_2O to 500ml

adjusted to pH 6.2

potasssium phosphate buffer (0.25M)

KH_2PO_4 17.011g

Na_2EDTA 0.186g

adjusted to pH 6.2 for NEM and GSSG separation procedure or adjusted to pH 7.2 for GSSG assay.

NADPH solution

NADPH 6.3mg

0.25M potassium phosphate buffer, pH 7.2 to 5ml

the solution was prepared just before use and was kept on ice.

2.II.viii Protein assay

reagent A: 2% ($^{w}/_{v}$) Na_2CO_3 in 0.1M NaOH

reagent B: 0.5% ($^{w}/_{v}$) $CuSO_4.5H_2O$ in 1% ($^{w}/_{v}$) sodium potassium tartrate

reagent C: mix 50ml of reagent A with 1ml of reagent B. Use within one day.

Folin-Ciocalteau reagent diluted 1:1 with dH_2O

SECTION 3: METHODS

SECTION 3: METHODS

3.I IN VITRO

3.I.i Cell culture

The TLX5 lymphoma was routinely passaged in female CBA/CA mice as described in section 3.II.ii. Peritoneal ascites fluid was removed aseptically from mice and washed in cell lysis buffer (Boyle, 1968) to remove erythrocyte contamination. An *in vitro* cell line was established by transferring cells to RPMI 1640 medium supplemented with 17% horse serum. Cells were maintained in the logarithmic phase of growth at 37^{0}C in an atmosphere of 10% carbon dioxide and 90% air.

Preliminary studies revealed that a constant doubling time of between 14 and 16h was obtained by the 10^{th} subculture. The malignancy of the TLX5 cells was lost when cells were injected into CBA/CA mice after the 25^{th} subculture. Therefore, all experiments were performed on TLX5 cells in the 10^{th} to 20^{th} subculture (approximately the 50^{th} to 100^{th} cell generation); during this period a constant doubling time was maintained and cells retained their malignancy.

3.1.ii Cell counts

A model ZM coulter counter (Coulter electronics Limited, Luton, Bedfordshire, England) was used routinely to count TLX5 cells. An aliquot of tumour cell suspension was diluted with isoton (Coulter electronics Limited, Luton, Bedfordshire, England) and counted at the

predetermined settings of current: 200, attenuation: 8, lower threshold (T_L): 10, upper threshold (T_U): 99.9. Cells were occasionally counted by introduction of a cell suspension into a haemocytometer (Weber Scientific International Limited, Lansing, Sussex, England) and analysed under a CK light microscope (Olympus, Tokyo, Japan).

3.I.iii Trypan blue exclusion

A cell suspension (approximately 100ul) was mixed with 50ul of filtered 0.5% trypan blue in 0.9% sodium chloride solution. After 1 min the suspension was introduced into a haemocytometer and examined under a light microscope. The number of cells which excluded trypan blue were counted and values expressed as a percentage of the total number of cells.

3.I.iv Cell size

A model ZM coulter counter was used to measure cell diameter and volume. The settings used were current: 200, full scale: 10, attenuation: 8. The cell diameter was calculated by measuring the total number of cells (x) and assuming a normal population size distribution, T_L was altered to find the cell diameter at which 0.5x number of cells was recorded. Mean cell volume was calculated from the mean cell diameter assuming cells were spherical in shape.

3.I.v Cell centrifugation

Routinely cell suspensions were transferred to 30ml sterile universal tubes or polyethylene test-tubes and

centrifuged in an Heraeus Christ labofuge 6000 centrifuge (LKB Limited, Croydon, Surrey, England) at a speed of 1,500 rpm for 5 min. Cell suspensions were also centrifuged at 11,600g on a Beckman microfuge B for 30s (Beckman-RIIC Limited, High Wycombe, Buckinghamshire, England).

3.I.vi Electron microscopy

TLX5 cell suspensions were harvested from culture flasks. The cells were centrifuged to form a pellet in polyethylene test tubes. Cells were washed in PBS and re-centrifuged. Fix 1 was added to the cell pellet for 1 to 2h. The fixative was removed with a Pasteur pipette, making sure that the cell pellet was always covered by liquid. Fix 2 was then added to the test tubes for 1h, before being drained off and replaced with 70% ethanol. The samples were then processed by Stuart Townsend at the MRC Radiobiology Unit, Harwell, Didcot, Oxon, England. Samples were dehydrated through graded alcohols to propylene oxide and embedded using Spurr's resin. Ultra-thin sections were cut onto M50 copper grids and examined under a CORA transmission electron microscope after staining with uranyl acetate and lead citrate.

3.I.vii Flow cytometry

The technique of flow cytometry (FCM) was first described by van Dilla et al, (1969). FCM provides for the simultaneous analysis of multiple parameters and the rapid observation of individual cells in aqueous suspension at the rate of several thousand per second.

A number of parameters can be analysed by FCM. For these studies cell fluorescence emission after excitation of intracellular fluorescence probes by a monochromatic laser beam were measured. This technique allowed for the analysis of DNA, RNA and cellular retention of fluorescein. The utility of the DNA distribution in cytokinetics is that it shows the distribution of cells throughout the cell cycle. Cells in G_1 phase have a constant (2n) DNA content, cells in G_2M phase have twice the G_1 phase DNA content (4n). S phase cells have intermediate DNA contents and form a continuum between the peaks for the G_1 and the G_2M phase cells.

3.I.viii Fluorescein and ethidium bromide markers of membrane integrity

For the analysis of membrane integrity, dual parameter fluorescein diacetate (FDA) and ethidium bromide (EB) staining was used according to the method of Aeschbacher et al, (1986). FDA freely enters intact cells where it is hydrolysed by esterases to form its fluorochrome, fluorescein, which is retained by the cells due to its polarity. EB is excluded from intact cells and stains only the nucleic acids of membrane-damaged cells. The combination of both fluorochromes results in counter staining in which intact cells fluoresce green (cytoplasm) and membrane-damaged cells fluoresce red (nucleus and RNA), while green fluorescence intensity decreases due to leakage of fluorescein from the cells.

FDA was dissolved in acetone to give a concentration

of 10mg/ml, which was stored as a stock solution at -20^{0}C. EB (3mg) was dissolved in 50ml of culture medium and 50ul of FDA stock solution was added. The assay was started by addition of 1ml of dye solution (at 37^{0}C) to 1ml of cell suspension. This gave final dye concentrations of 30ug/ml EB and 5ug/ml FDA. The cell suspension was allowed to equilibrate for 20 min at room temperature prior to FCM analysis on a FACS 440 flow cytometer (Becton Dickinson, Cowley, Oxon, UK) using an argon laser operating at an excitation wavelength of 488nm. Green fluorescence (fluorescein) and red fluorescence (EB) were measured at fluorescence emission wavelengths of 515 to 545nm and >590nm respectively using appropriate filters, which prevented overlap of fluorescence from the green and red channels. The data for 2 x 10^4 cells was accumulated and processed on a Consort 40 computer (Becton Dickinson, Cowley, Oxon, UK) attached to a Tektronix 4612 printer.

3.I.ix Cell cycle analysis

For cell cycle analysis, cells were fixed and stained essentially using the methods of Gray and Coffino, (1979). 1 x 10^6 cells were harvested from suspension cultures, the medium was removed and cells were washed in 10ml of PBS at 4^{0}C. Pelleted cells were then fixed by the dropwise addition of 3ml of 70% ethanol under constant agitation and stored in the dark, for not more than two weeks at 4^{0}C prior to analysis.

Fixed cells were resuspended in 1ml of RNAase solution and incubated at 37^{0}C for 20 min. This procedure

was used to degrade the RNA, so that only DNA interaction with the fluorochrome was recorded. The RNAase solution was removed and cells were stained by the addition of 1ml of propidium iodide (PI) staining solution. Samples were processed on a FACS 440 flow cytometer using an argon laser operating at an excitation wavelength of 488nm. PI emission fluorescence was recorded for 2 x 10^4 cells per sample and the data was accumulated and processed on a Consort 40 computer. The proportion of cells in each phase of the cell cycle was calculated from the area under the curve assuming a normal population distribution.

3.I.x DNA and RNA dual parameter flow cytometry

The method used to measure cellular DNA and RNA was described by Darzynkiewicz et al, (1980) which was a modified version of the original method (Darzynkiewicz et al, 1976). The procedure involved the treatment of unfixed cells with the chelating agents, citrate and EDTA in the presence of a non-ionic detergent, triton X-100 at pH 3.0. The chelating agents cause unfolding of the ribosomes (Tal, 1969) and the denaturation of double stranded RNA. The detergent treatment renders the cells permeable to the fluorochrome. Cell staining was performed at pH 3.8 which is the optimal pH for the discrimination of DNA and RNA staining by the fluorochrome acridine orange (AO) (electro pure, Polysciences Incorporated, Northhampton, England).

A 0.2ml aliquot (2 x 10^5 to 5 x 10^5 cells) was withdrawn from suspension culture and pipetted into 0.5ml of solution A. 1 min later cells were stained by the addition of 1ml of solution B. After 5 min equilibration

at room temperature the fluorescence intensity of individual cells was measured on a FACS 440 flow cytometer operating at an excitation wavelength of 488nm. Green fluorescence (515 - 575nm) and red fluorescence (600 - 650nm) was recorded for each cell and stored on a Consort 40 computer. AO was present at a final dye concentration of 13uM in the presence of 1mM Na_2EDTA. Under these conditions interaction of the dye with DNA results in green fluorescence with maximal emission at 530nm and interaction with RNA gives red fluorescence at a maximal emission wavelength of 640nm (Darzynkiewicz et al, 1980).

3.I.xi Total intracellular glutathione assay

The total intracellular glutathione content (GSH + GSSG) of the cells was measured essentially by the method of Griffith, (1980) as modified by Whitby et al, (1984b). 1 x 10^7 cells were removed from suspension culture and centrifuged at 1,500 rpm for 5 min. The cell pellet was deproteinized with 1ml of 10% ($^{w}/_{v}$) metaphosphoric acid. The acidic supernatant (300ul) was neutralised to pH 7.0 - 7.5 with 120ul of saturated sodium phosphate solution. A 140ul aliquot was assayed for glutathione content in a quartz cuvette (light path 10mm, Hellmann Limited, West Cliffe on Sea, Essex, England) which contained 700ul NADPH solution, 100ul DTNB solution and 10ul glutathione reductase solution. The conversion of DTNB to 2-nitro-5-thiobenzoic acid was monitored spectrophotometrically at 412nm and at a temperature of 30 ^{0}C using a Beckman DU-7 spectrophotometer (Beckman-RIIC

Limited, High Wycombe, Buckinghamshire, England). A cuvette which contained 700ul NADPH solution, 100ul DTNB solution 140ul of 10% metaphosphoric acid/saturated sodium phosphate solution at pH 7.0 - 7.5 and 10ul glutathione reductase solution was used as a reference.

In order to produce calibration values, standard solutions of GSH were prepared in 10% ($^{w}/_{v}$) metaphosphoric acid and assayed as described for the cell samples. A calibration curve was performed for each individual experiment and sample values of total glutathione content were calculated from the linear portion of the calibration curve.

3.I.xii Oxidized glutathione assay

The preparation of cell samples for the analysis of oxidized glutathione (GSSG) content was described by Akerboom and Sies, (1981). A major problem in measuring GSSG levels in biological samples is the presence of GSH. Firstly there is generally a high GSH/GSSG ratio and secondly GSH is prone to undergo autoxidation to GSSG. Chemical interaction of GSH can prevent autoxidation. For this purpose N-ethylmaleimide (NEM) was used, excess NEM must however be removed before analysis of GSSG, because it inhibits glutathione reductase (Meister and Anderson, 1983).

1×10^{7} cells from suspension culture were pelleted and deproteinized with 1.2ml of 10% ($^{w}/_{v}$) metaphosphoric acid. 400ul of 0.2M NEM was added to give a final NEM concentration of 50mM. 1.1ml of the supernatant was

carefully neutralized to pH 6.2 with a solution of 2M potassium hydroxide and 0.3M N-morpholinepropane-sulphonic acid (MOPS). 1ml of the neutralized supernatant was then applied to a sephadex column as described below.

To separate NEM from GSSG the following procedure was employed at 4 ^{0}C: QAE-25 Sephadex was swollen in 0.01M potassium phosphate buffer at pH 6.2 and then degassed using a Buckner filter attached to a vacuum pump. 5ml of the gel was packed into a chromatography column with a 70um filter (Amicon Wright, Stonehouse, Gloucestershire, England), plugged with glass wool and attached to a two way tap (Palmer Bioscience, Sheerness, Kent, England). The gel was equilibrated with 10ml of 0.01M potassium phosphate buffer at pH 6.2. A 1ml aliquot of the neutralized sample was applied to the column and NEM was eluted with 12ml of 0.01M potassium phosphate buffer, pH 6.2. GSSG was subsequently eluted with 4ml of 0.25M potassium phosphate buffer at pH 6.2. The 4ml eluate was adjusted to pH 7.2 with 150ul of a solution of 2M potassium hydroxide and 0.3M MOPS.

The GSSG content of the samples was assayed using the procedure described in section 3.I.xi with the following modifications: the buffer used was 0.25M potassium phosphate at pH 7.2 and the sample cuvette contained 700ul of eluate, 140ul NADPH solution, 100ul DTNB solution and 10ul glutathione reductase solution. In the reference cuvette the sample was replaced by 700ul of 0.25M potassium phosphate buffer at pH 7.2. Standard solutions

of GSSG and GSSG + GSH were assayed by the same procedure used for the cell samples. The sample values of GSSG were calculated from the standard calibration curve. Values for GSSG obtained agreed to within ± 4% when GSSG alone or in combination with 40 fold excess GSH were compared.

3.I.xiii Clonogenic analysis

For clonogenic analysis cells from suspension culture were washed twice with fresh medium and plated at a density of 10^3 to 10^6 cells per plate, on 35mm petri dishes in growth medium containing 0.36% Noble agar (Difco Laboratories Limited, East Molesley, Surrey, UK). In preliminary studies this concentration of agar was found to be optimal for the formation of cell colonies. Plates were incubated at 37^0C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cloning efficiencies were determined after 6 days incubation, by counting colonies of >50 cells under a light microscope.

3.I.xiv Studies of tumourigenicity

TLX5 cells were incubated in vitro with various concentrations of NMF for 48h. Cells were then washed twice with 0.9% sterile saline and 3×10^5 cells in 0.1ml saline were injected sc into 20g female CBA/CA mice. The survival times of animals which received NMF treated cells was compared with the untreated controls.

3.I.xv Studies of the metabolism of NMF

TLX5 cells were incubated with 106mM [^{14}C] methyl labelled NMF ($^{14}CH_3NMF$) (5uCi), which was synthesized by Dr M.D. Threadgill according to the method of Threadgill

and Gate, (1983). In another flask, 106mM $^{14}CH_3NMF$ (5uCi) was added to medium alone, both flasks were incubated for 48h at 37^0C. 40ug/ml saponin solution was added to disrupt the cell membrane. Medium was then acidified with hydrochloric acid to pH 1 - 2. Acidic media would stabilize any S-(N-carbamoyl) mercapturates that may be formed (Kestell et al, 1985). The medium was freeze dried overnight to remove water and any unreacted $^{14}CH_3NMF$, using a freeze drier (Edwards High Vacuum, Crawley, Sussex, England).

The residue was reconstituted in 4ml of methanol. 20ul of each sample was spotted onto a silica gel 60 F_{254} pre-coated plate of 0.2mm thickness (20 x 20cm) (E Merck, Darmstadt, West Germany). Plates were developed for 6h in butan-1-ol: water: methanol (8: 2: 1 $^v/_v$) according to the method of Kestell et al, (1985). Radioactive metabolites were located by autoradiographic analysis of the TLC plate using an X-ray film (Singul-XRP, Ceaversken AB, Strangas, Sweden). A 20ul aliquot of methanol sample was counted on a Packard Tricarb CA 2000 scintillation counter (United Technologies Packard, Pangbourne, Buckinghamshire, UK) using luma gel as scintillant, to give an indication of the required time for autoradiographic exposure of the TLC plate.

The X-ray film was developed in a darkroom fitted with a dark red Wratten number 2 filter using Kodak D19 developer (Kodak, Chalon-sur-Saone, France) for 5 min followed by a 10s rinse in distilled water, 5 min in

Kodafix (Kodak Limited, Manchester, England) and 15 min in tap water before the film was allowed to air dry.

3.II IN VIVO

3.II.i Animals

CBA/CA female mice (18 - 23g) were supplied by Bantim and Kingman (Hull, UK). Mice were housed for at least one week before they were used for experiments. Animals were fed on a 41B modified breeding diet (Pilsbury, Birmingham, UK) and water ad libitum.

3.II.ii Tumour source and maintenance

The TLX5 lymphoma is a fast growing invasive tumour which was originally induced in the thymus of CBA mice by x-irradiation (Connors and Jones, 1970). The tumour is routinely passaged at 7 day intervals by ip injection into CBA/CA mice of 2×10^5 cells in sterile saline. The cells are obtained from the peritoneal ascitic fluid of mice from the previous passage, after mice were killed by cervical dislocation.

3.II.iii Measurement of NMF antitumour activity

A regimen of dosing tumour bearing animals which has previously been employed for antitumour drug testing against the TLX5 lymphoma in vivo has the following protocol: 2×10^5 TLX5 cells are implanted sc in the inguinal region of female CBA/CA mice, drugs were then administered daily for 5 days commencing 72h after tumour implantation. Antitumour activity was then quantified by comparing the survival times of treated animals (T) to the untreated control mice (C) (Gate et al, 1986). To assess the effects of NMF on the tumour volume of the TLX5 lymphoma, a palpable tumour which could be measured by

calipers was required, before and after administration of drug. Tumour volume measurements during this period cannot be achieved with the above protocol. Therefore a modified regimen was used: 1×10^7 cells were implanted sc and mice were injected with NMF on day 5 or days 5 and 6 after tumour implantation. Antitumour activity was assessed by measuring tumour volumes and animal survival times.

3.II.iv Tumour volume measurements

The volume of the TLX5 lymphoma grown sc in the inguinal region of mice was measured by calipers. The tumour volume was calculated by the method of Geran et al, (1972) using the formula:

$$\text{volume} = \frac{l \times w^2}{2}$$

where l represents the longest tumour diameter and w the diameter perpendicular to this axis.

3.II.v Mice survival times

Antitumour activity was also expressed as a percentage increase in survival time using the formula:

$$\% \text{ increase in survival time} = \frac{T}{C} \times 100$$

where T is the mean day of death of treated animals and C is the mean day of death of the untreated control animals.

3.II.vi Drug administration to mice

All drugs except DL-buthionine-[S,R]-sulphoximine, were dissolved in 0.9% sterile saline and administered by the intraperitoneal (ip) route. For ip administration, the drug was injected into the peritoneal cavity of the animals. The control mice were injected with an

equivalent volume of 0.9% sterile saline.

3.II.vii Electron microscopy of the TLX5 lymphoma

The TLX5 lymphoma was excised from sacrificed mice and cut by a scalpel into $1mm^3$ cubes in PBS, taking care to avoid crushing or tearing the tumour. The tumour samples were then fixed and stained for electron microscopy as described in section 3.I.vi.

3.II.viii Tumour disaggregation to single cell suspension

Mice which were recipients of 1×10^7 TLX5 cells sc in the left inguinal region were sacrificed. The lymphoma was carefully excised and added to PBS or HEPES buffer. The tumour was sliced into $1mm^3$ fragments with a scalpel blade, care was taken to avoid crushing or tearing the tissue. Fragments were incubated with 12ml of digesting medium (see below) for 45 min in a water bath (The Mickle Laboratory Engineering Company, Gomshall, Surrey, England) at 37^0C with constant agitation. After incubation fragments were allowed to settle and an aliquot of the cell suspension was washed with buffer. The viability of the cells was measured by trypan blue exclusion and the degree of cell clumping by light microscopy.

Preliminary experiments were conducted to establish the most appropriate digesting medium. The following enzyme mixtures were used:

(1) 0.025% pronase in HEPES buffer (Anderson et al, 1983).

(2) 0.1% DNAase type I, 0.14% collagenase type I in medium (Ota and Drewinko, 1985).

(3) 0.025% collagenase type I, 0.05% pronase, 0.04% DNAase

type I in PBS (Allalunis-Turner and Siemann, 1986).
(4) 0.15% collagenase type I in HEPES buffer (Whitby et al, 1984b).

Digesting media 2 and 4 gave the best results in terms of cell separation and viability. Since the addition of DNAase did not improve the disaggregation of cells, digesting medium 4 was used in experiments to prepare single cell suspensions from the solid tumour.

3.II.ix Bone marrow disaggregation to single cell suspension

Both femurs were exposed from sacrificed mice and the muscle was teased away from the bone. The ends of the femurs were cut perpendicular to the plane of the bone and the marrow was removed by inserting a needle and flushing through with 0.1ml PBS. A single cell suspension was obtained by continual uptake and expulsion of bone marrow suspension through a 0.45 x 10mm needle (Becton Dickinson, Dun Laoghaire, Ireland).

3.II.x Analysis of tumour or bone marrow cell cycle

A single cell suspension of TLX5 cells was prepared as described in section 3.II.viii. Erythrocytes were removed by cell lysis buffer and cells were resuspended in HEPES buffer at 4^0C. A single cell suspension of bone marrow cells was prepared as described in section 3.II.ix. 1 x 10^6 TLX5 or bone marrow cells were then fixed in 70% ethanol and analysed as described in section 3.I.ix.

3.II.xi Total glutathione assay of the TLX5 tumour

Mice were sacrificed and the TLX5 tumour was excised. A portion of the tumour was added to 50ml/g of 10% ($^{w}/_{v}$) metaphosphoric acid and homogenized at speed 5 using a model S63C homogenizer fitted with a teflon pestle. The resultant suspension was centrifuged at speed 7 (3000g) for 5 min on a labofuge 6000. 300ul of the acidic supernatant was then assayed for total glutathione content as described in section 3.I.xi.

3.II.xii DL-buthionine-[S,R]-sulphoximine dose

The dose of BSO administered was based on that used by Drew and Miners, (1984). BSO was dissolved in 0.1M sodium hydroxide, final pH 8.5 and 0.4ml was administered ip to give a dose of 1,600 mg/Kg.

3.II.xiii Incorporation of tritiated thymidine

To measure the extent of DNA synthesis in the TLX5 lymphoma, methyl-labelled tritiated thymidine incorporation ([^{3}H] thymidine) was used. An *in vitro* method of cell labelling with [^{3}H] thymidine was employed rather than an *in vivo* method; this was because of the reported variability of cell labelling *in vivo*, which depended on the distance of the cell from the blood supply. This variability in cell labelling led to a large degree of variation in [^{3}H] thymidine incorporation between samples from the same tumour (Denekamp and Kallman, 1973). The use of cell suspensions allows uniform incorporation of label and random counting of labelled cells (Ota and Drewinko, 1985).

Two methods of [^{3}H] thymidine incorporation into DNA are commonly used; these involve measurement of label present in the acid insoluble cell fraction or autoradiography of cell samples assessed by measuring the number of labelled cells. Both methods were used for comparison purposes in these experiments. The amount of thymidine incorporated into a cell depends upon factors such as intracellular pools, activity of thymidine transport mechanisms and the activities of a number of enzymes including thymidine kinase (Pardee et al, 1986).

A single cell suspension of TLX5 cells was prepared from the solid tumour as described in section 3.II.viii and cells were resuspended in RPMI 1640 medium. Cells were labelled with [^{3}H] thymidine according to the method of Ota and Drewinko, (1985). 3ml of the cell suspension was incubated with 7.5uCi of [^{3}H] thymidine (specific activity 5.0 Ci/mmol)(Amersham International PLC, Amersham, UK) for 30 min in a water bath at 37^{0}C with continuous agitation. The cell suspension was then chilled on ice for 4 min to inhibit further uptake of label. The medium was removed and cells were washed once in fresh RPMI 1640 medium and once in HEPES buffer. At this stage samples were either prepared for autoradiography (see below) or for incorporation of radioactivity into the acid insoluble fraction.

Whatman GF/C 2-5cm glass microfibre filters (Whatman Limited, Maidstone, England) were soaked in absolute ethanol prior to vacuum filtration using a Buckner filter.

3×10^5 cells were pipetted onto the filter and 5ml of ice cold 5% ($^{w}/_{v}$) trichloroacetic acid was added and filtered. The resultant precipitate was washed with ethanol to remove cell debris. The filters were air dried and the number of disintegrations per minute (DPM) of the acid insoluble fraction was measured on a Tri-Carb 2000CA liquid scintillation analyzer using 0.9% 2,5-diphenyloxazole (Koch-light Ltd, Haverhill, England) in toluene as scintillant.

For autoradiography slides were cleaned by the method of Stein and Yanishevsky (1979). Micro slides (76 x 26 mm, thickness 1.0/1.2mm) with ground edges frosted at one end (Chance Propper Limited, Smethwick, England) were cleaned using the procedure (a) 95% ethanol for 5 min (b) 1M hydrochloric acid for 5 min (c) dH_2O for 5 min, followed by four short rinses in dH_2O. Cell suspensions were spread onto the slides and allowed to air dry, before being fixed in methanol containing 10% ($^{v}/_{v}$) acetic acid for 15 - 30 min and air dried (Ota and Drewinko, 1985).

Ilford K5 gel (Ilford Limited, Mobberley, Cheshire, England) was used as the photographic emulsion. All procedures were performed in a darkroom using a dark red Wratten number 2 filter. A 1:1 dilution of the emulsion gel with dH_2O was heated in a waterbath at 40 - 43^0C for 30 min with gentle stirring to avoid overheating but prevent frothing.

Slides were dipped one at a time into the emulsion with an even slow motion, excess emulsion was allowed to

drain from the slides which were then dried vertically for 1h. Slides were exposed in a light - tight box for 7 days at 4^0C. Slides were developed in kodak D19 developer for 5 min, rinsed briefly in tap water and fixed for 5 min in kodafix. Slides were finally rinsed in tap water for at least 5 min and allowed to air dry.

Cells were stained for 30 min with 1% ($^{w}/_{v}$) toluidine blue in propan-2-ol after dehydrating the slides through graded alcohol. Slides were then blotted on filter paper and excess stain removed by washing in propan-2-ol for 1 min. Slides were air dried and the thymidine labelling index (TLI) was determined by scoring at least 400 cells per slide which contained >6 grains per cell.

3.II.xiv Study of NMF and metabolites distribution

Mice bearing the TLX5 lymphoma were injected with 400 mg/Kg $^{14}CH_3NMF$ ip (5uCi). 4h later the mice were sacrificed and the procedures described in 3.II.xv to 3.II.xviii were performed.

3.II.xv Distribution of radioactivity throughout the TLX5 lymphoma

The TLX5 lymphoma was excised and cut in half, one half was placed in a plastic beaker and covered with isopentane (a cryoprotectant). The beaker was plunged into liquid nitrogen for about 30s. The frozen tumour sample was stored on dry ice prior to sectioning. Sections (20um thick) were cut on a Brights QR cryostat from all parts of the tumour and placed on slides. The slides were covered with an X-ray film, in a darkroom and

exposed in a light-tight box for 7 days at 4^0C. The X-ray film was then developed as described in section 3.I.xv.

3.II.xvi Measurement of radioactivity of the TLX5 lymphoma and other organs

The TLX5 lymphoma, kidney and liver were removed from mice. The organs were homogenized in dH_2O (20% $^{w}/_{v}$) at speed 5 on a model S63C homogenizer. The bone marrow was removed and homogenized as described in section 3.II.ix. 0.1ml of the homogenate was added to 1ml of the tissue solubilizer soluene 350 (United Technologies Packard, Pangbourne, Buckinghamshire, England). Samples were left for 1 week at room temperature in an airtight container and radioactivity was counted on a Tri-Carb 2000CA liquid scintillation counter using luma gel as scintillant.

3.II.xvii NMF assay by gas chromatography

A 0.1ml aliquot of sample homogenate was added to 0.1ml of internal standard (27 ug/ml tetramethylurea in acetone). The resultant precipitate was pelleted in a Beckman microfuge B and 1.5 to 2ul of supernatant was injected onto the gas chromatograph using a 5ul glass pipette (Scientific Glass Engineering Pty Limited, Ringwood, Australia). The chromatograph used was a Pye Unicam GC 204 instrument, fitted with a nitrogen detector and a glass 0.25 inch diameter, 2m long column consisting of 8% carbawax 20M + 2% potassium hydroxide on chromosorb W-AWDMCS 100 - 120 mesh (Phase Separations Limited, Clwyd, Wales). Data was recorded on a euroscribe recorder. The following temperatures were used:

injector = 200^0C

column = 170^0C

detector = 200^0C

The gas flow rates were:

nitrogen = 40 ml/min

hydrogen = 15 psi

air = 15 psi

Sample concentrations of NMF were calculated from a standard curve of NMF/internal standard peak height ratio versus NMF concentration. The limits of sensitivity for NMF were about 2 ug/ml. The recovery of NMF was >98% when samples of control liver, kidney, TLX5 lymphoma or bone marrow were spiked with 100 ug/ml NMF.

3.II.xviii Protein assay

The protein content of the organ homogenates was assayed by the method of Lowry et al, (1951). Various dilutions of bovine serum albumin (BSA) protein standard or organ homogenates were added to a test tube to give a total volume of 0.4ml. 2ml of reagent C was added, the solution was mixed and allowed to stand for 10 min. 0.2ml of diluted Folin-Ciocalteau reagent was added and the mixture left for 30 min. The absorbance of standards or samples was measured at a wavelength of 750nm and temperature of 25^0C on a Beckman DU 7 spectrophotometer against a blank which contained 0.4ml of dH_2O instead of BSA or sample. Sample protein values were calculated from a standard curve.

PART ONE Studies of the effects of NMF on TLX5 lymphoma cells in vitro.

SECTION 4: RESULTS AND DISCUSSION

SECTION 4: RESULTS AND DISCUSSION

4.I The effects of NMF on the growth, viability, cell size and ultrastructure of TLX5 cells

4.I.i Introduction

NMF induces the terminal differentiation of leukaemia cells or the reversible maturation of some cell lines derived from solid tumours *in vitro*. The acquisition of more benign characteristics is associated with a decreased growth rate (see section 1.II). The maturation of DLD-1 clone A cells induced by NMF or DMF was accompanied by an increase in cell size (Leith *et al*, 1982a). Cell size changes in HL-60 cells depended on the differentiation lineage. Retinoic acid or DMSO-induced granulocytic maturation produced a decrease in cell volume (Ladoux *et al*, 1987; Stendahl *et al*, 1982), whereas monocytic maturation brought about by 1-β-D-arabinofuranosylcytosine caused an increase in cell size (Ross, 1985).

The plasma membrane structure can be altered by polar solvents. NMF induced maturation of DLD-1 clone A cells corresponded to an increase in the membrane viscosity (Dibner *et al*, 1985) and DMSO caused a progressive decrease in the membrane fluidity of HL-60 cells (Ip and Cooper, 1980). Cellular maturation caused by NMF occurs at concentrations which are marginally below those which produce cytotoxicity (Langdon and Hickman, 1987a). At cytotoxic drug concentrations cell membrane damage can

occur.

In view of the published work the hypothesis was tested that NMF may effect the cell growth, cell volume and membrane integrity of TLX5 cells. Transmission electron microscopy was also used to study the ultrastructural changes in TLX5 cells brought about by exposure of cells to NMF.

4.I.ii Results

The growth and cell viability (as measured by trypan blue exclusion) of TLX5 cells incubated continuously in the presence of NMF are shown in figure 6. NMF concentrations of less than 43mM had no significant effect on cell growth or viability, whereas higher concentrations (upto 170mM) caused a progressive decrease in growth rate and viability. Incubation of cells with 106mM NMF for 48h produced a 37% growth inhibition, while viability was >80%.

Figure 7 shows that the mean cell diameter and mean cell volume decreased as NMF concentrations increased, when cells were exposed to the drug for 48h. The mean cell diameter of controls was 10.71 ± 0.09 um and the mean cell volume of controls was 642 ± 17 um^3.

In an experiment in which membrane integrity was assessed by measurement of trypan blue exclusion or fluorescein and ethidium bromide dual staining, measured by flow cytometry, it was seen that the most sensitive index of membrane leakage, fluorescein fluorescence,

decreased prior to the intrusion into the cell of either trypan blue or ethidium bromide (figures 8, 9a and 9b). This occurred at an NMF concentration of 43mM after an exposure time of 48h. Higher NMF concentrations (106 or 170mM) resulted in further evidence of membrane damage as indicated by all three markers of membrane integrity.

For the preparation of electron micrographs of NMF treated cells (figure 10), TLX5 cells were incubated with NMF for 48h, before being fixed and stained. The samples were then sectioned and analysed for morphological changes at the MRC radiobiology unit at Didcot, Oxon, by Stuart Townsend.

The following observations were made: Control samples and TLX5 cells exposed to 43mM NMF showed similar characteristics. The nuclei were large and pleomorphic with distinct nucleoli. There were nuclear pockets and clefts present which are typical of lymphomas (McKenna et al, 1979). The rough endoplasmic reticulum was seen as short tubular formations and the cytoplasm contained many polyribosomes with a few lysosomes and some lipid droplets. The mitochondria varied in number, shape and size.

The TLX5 cells exposed to 106mM and 170mM NMF were grouped together as they were similar in appearance. A number of cells showed examples of pyknosis (nuclear condensation), karyolysis (nuclear dissolution) and karyorrhexis (nuclear fragmentation). Many of the intact cells had an increased number of cytolysosomes, with

little or no change in other organelles. Few cells were seen in mitosis.

4.I.iii Discussion

NMF brought about a decrease in the growth rate of TLX5 cells (figure 6). This might be the result of differentiation to non-proliferating cells or alternatively it may be due to a perturbation of proliferation which is not related to maturation.

Membrane integrity can be directly assessed either by detecting penetration of external substances into the cell, such as trypan blue or ethidium bromide or by measuring the ability of the cell to retain intracellular substances, such as fluorescein. Measurement of fluorescein fluorescence was the most sensitive indicator of membrane integrity, which is in accordance with previous observations (Aeschbacher et al, 1986). Exposure of cells to NMF (43mM) for 48h caused membrane leakage as measured by the decreased fluorescence of fluorescein, there was no cellular uptake of trypan blue or ethidium bromide under these conditions (figures 6, 8, 9a and 9b). The contrast in the results observed with these three markers of cell membrane integrity suggests that NMF initially produces minor membrane damage. The deleterious effect on the cell membrane is enhanced as the concentration of NMF or time of exposure to the drug is increased.

A number of physiological and biochemical parameters

may be altered by changes in the membrane structure, these include the expression of membrane bound molecules such as receptors, enzymes and antigens (Dibner et al, 1985). Indeed, DMF increased the expression of cell surface antigens in AKR fibroblasts (Chakrabarty et al, 1984). DMF also increased the number of epidermal growth factor receptors in this same cell line (Levine et al, 1985).

NMF caused a decrease in the cell volume of TLX5 cells. This is different to the increase observed for the DLD-1 clone A cells but similar to the decreased cell volume reported for granulocytic maturation of HL-60 cells (see section 4.I.i).

The electron micrographs of TLX5 cells which had been incubated with 106mM or 170mM NMF for 48h showed evidence of cell death (figure 10). There are two kinds of pathological mechanisms by which eukaryotic cells die, necrosis and apoptosis (reviewed by: Wyllie et al, 1980; Duvall and Wyllie, 1986; Searle et al, 1982). Necrosis occurs as the result of a progressive breakdown of ordered structure and function after irreversible cell injury, which is preceded by reversible changes. Apoptosis is an active cellular destruction, which characteristically affects single scattered cells in solid tumours.

Early changes of necrosis are associated with an increase in plasma membrane permeability. Na^+, K^+, Ca^{2+} and Mg^{2+} move down concentration gradients which leads to cellular uptake and redistribution of water, followed by an increase in the cell volume. Initially this uptake of

water causes reversible cell swelling and dilation of organelles; the nuclear chromatin flocculates, but the chromatin pattern is conserved. These early alterations are followed by irreversible swelling of organelles due to the changes in ion and water fluxes. The permeability of the plasma membrane increased when the TLX5 cells were incubated with NMF (figures 8, 9a and 9b), but this effect on membrane integrity was associated with a decrease in the cell volume (figure 7) and maintenance of organelle structures (figure 10). These characteristics are more indicative of apoptosis than necrosis, since apoptosis is accompanied by condensation of the cytoplasm and organelle morphology is unchanged. However, the chromatin pattern of the TLX5 cells exposed to NMF was maintained during the early stages of interaction with NMF which led to cell death and also apoptotic bodies were not observed. The later stages of pyknosis, karyorrhexis and karyolysis are also typical of necrosis. The cytolysosomes observed in the TLX5 cells treated with NMF suggest sub-lethal damage. The release of lysosomal enzymes is thought to be a consequence rather than a cause of cell death.

It is therefore evident that NMF causes a decrease in the growth of TLX5 cells and is also cytotoxic at concentrations of 106 or 170mM which results in an increase in membrane permeability and decrease in cell size, these early events can ultimately lead to cell death.

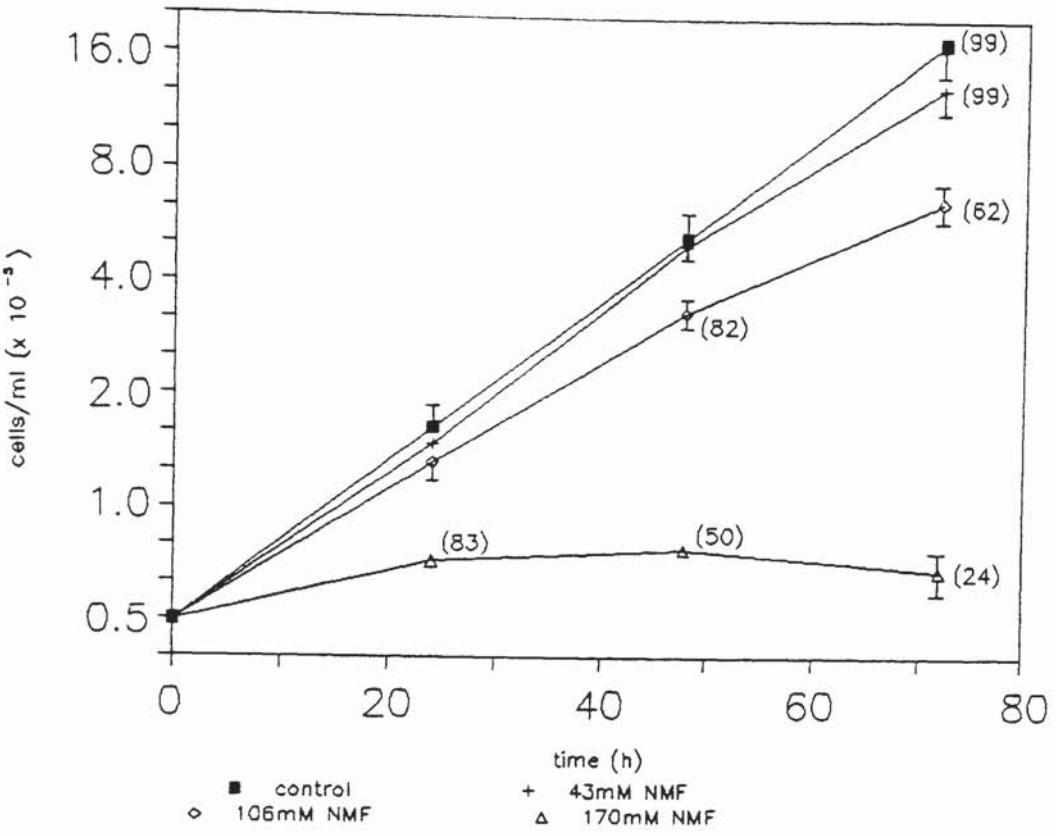


Figure 6 : Concentration dependent effects of continuous incubation with NMF on the growth and viability of TLX5 cells over a 72h exposure period. Figures in parenthesis indicate % viability as measured by the exclusion of trypan blue (n=6, ±SD).

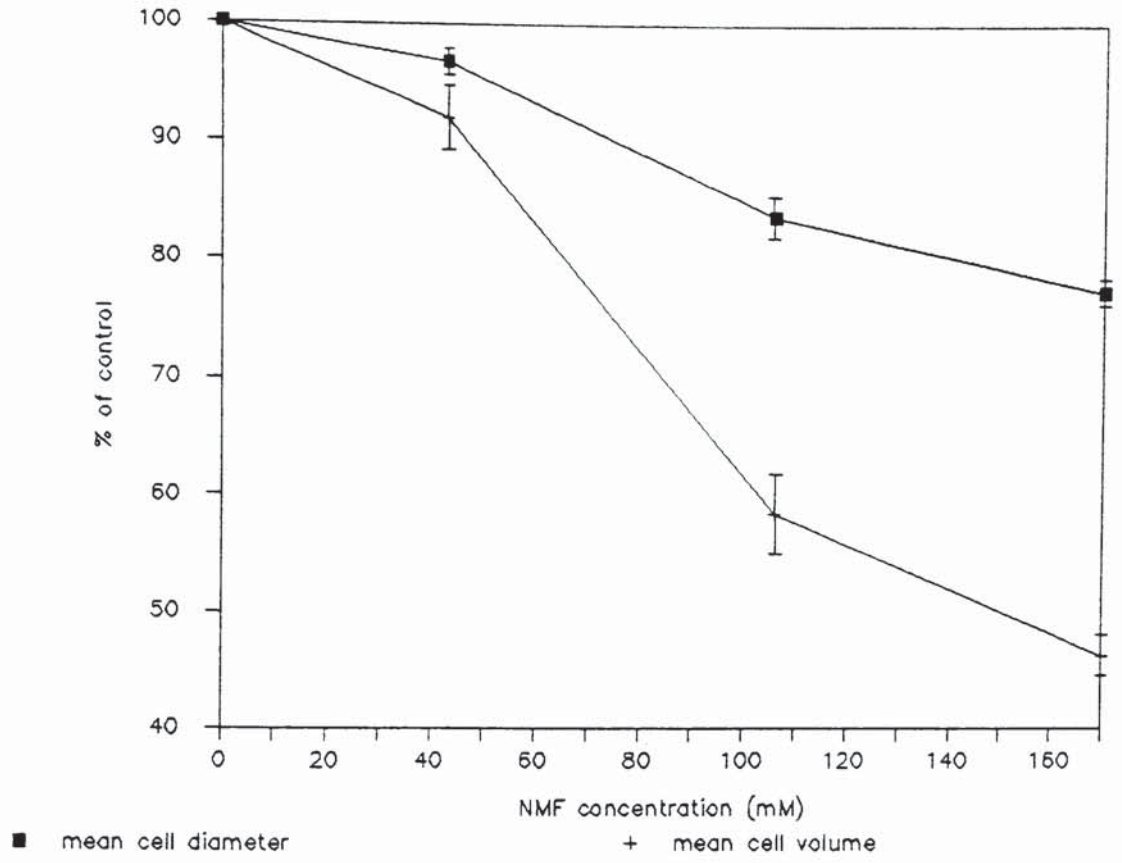


Figure 7 : Mean cell diameter and mean cell volume after incubation with NMF for 48h (n=3, ±SD).

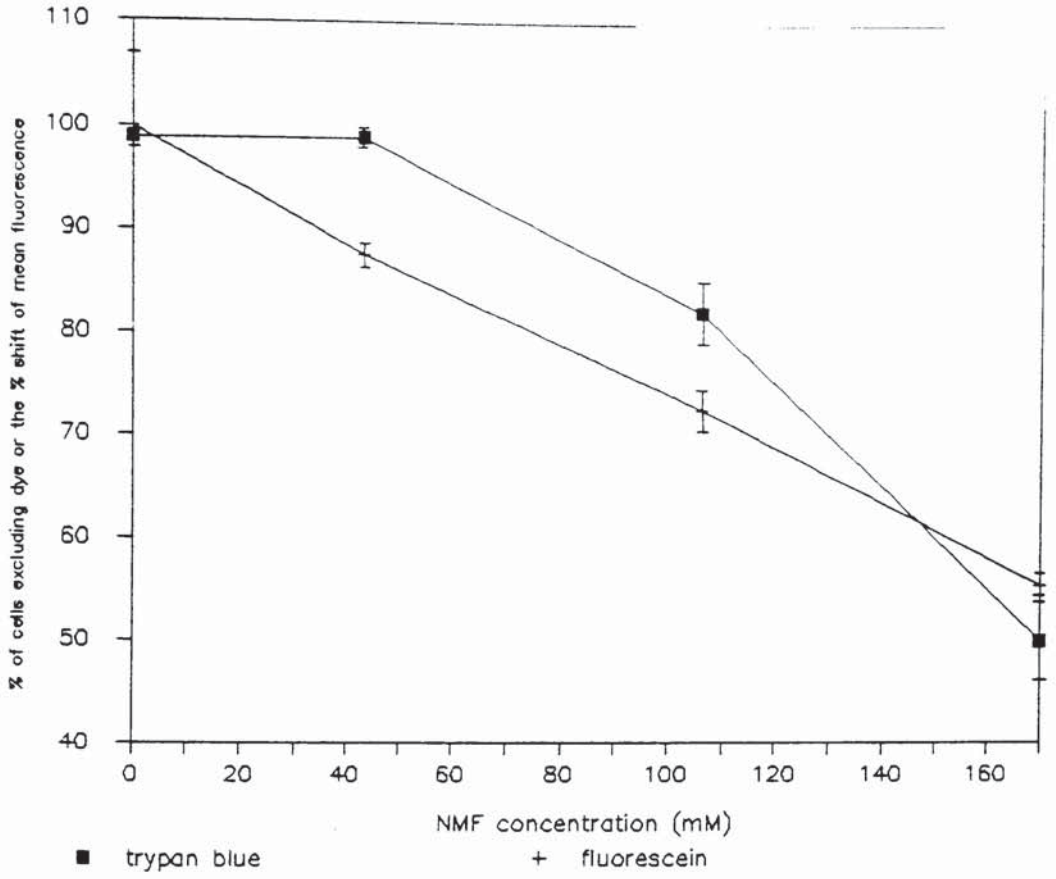


Figure 8 : Comparison between trypan blue exclusion and cellular retention of fluorescein as indicators of membrane integrity after incubation with NMF for 48h (n=3, ±SD).

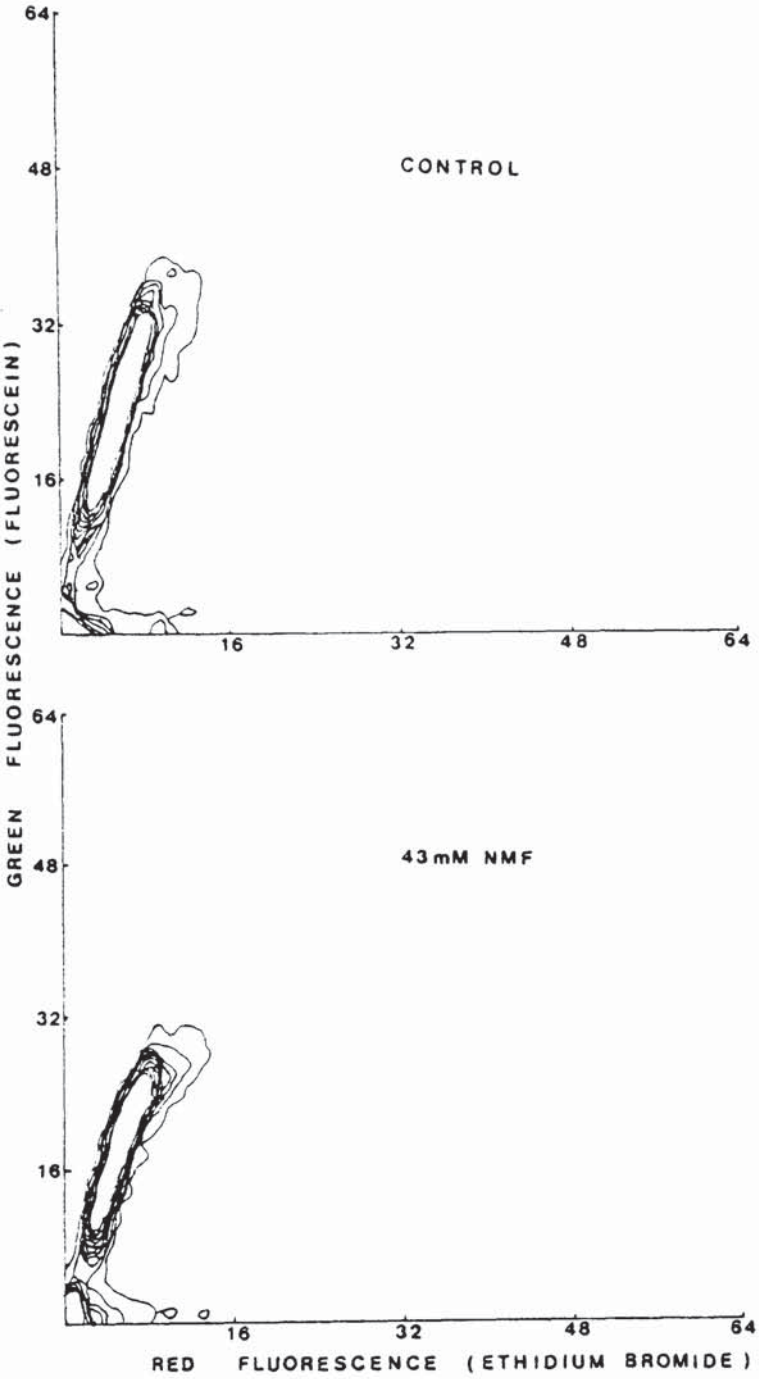


Figure 9a : Contour plots of the changes in fluorescein and ethidium bromide fluorescence intensity after incubation of cells with NMF for 48h.

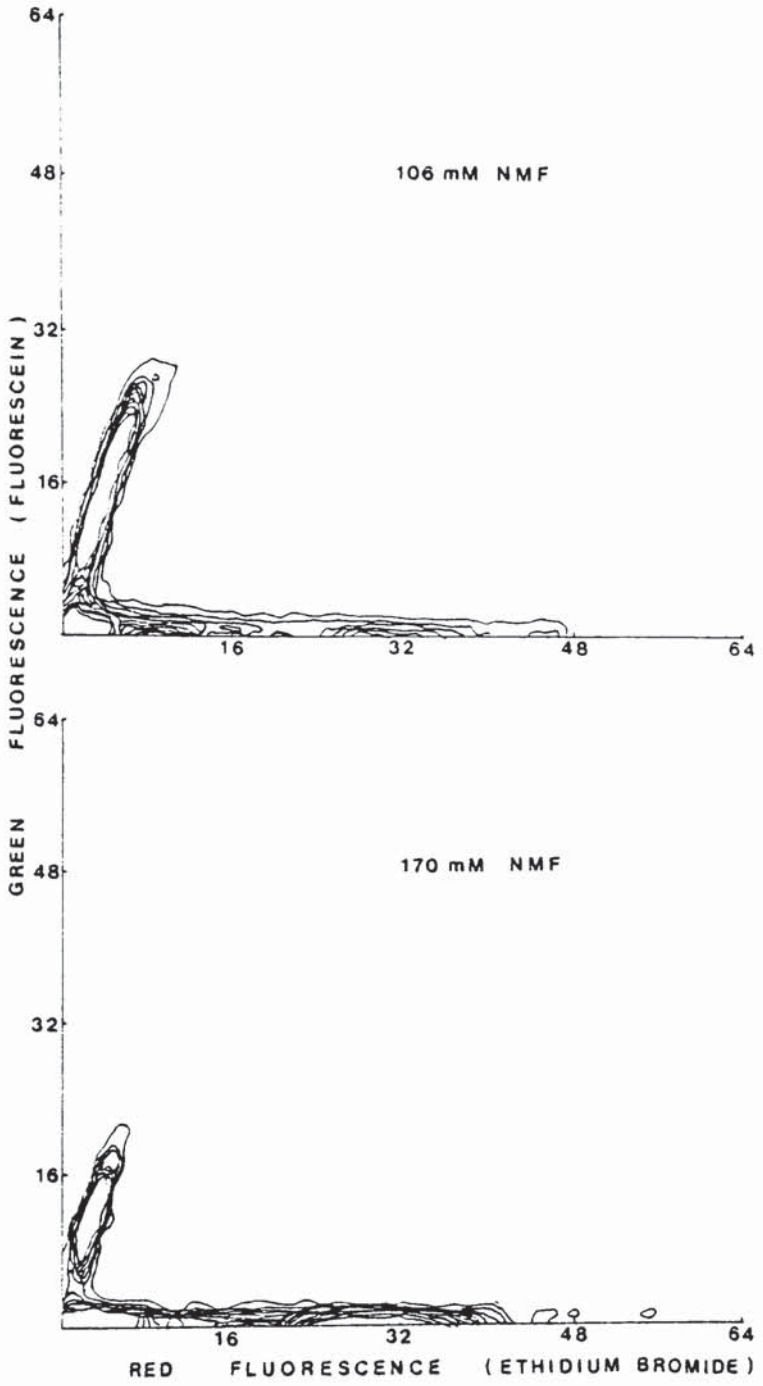
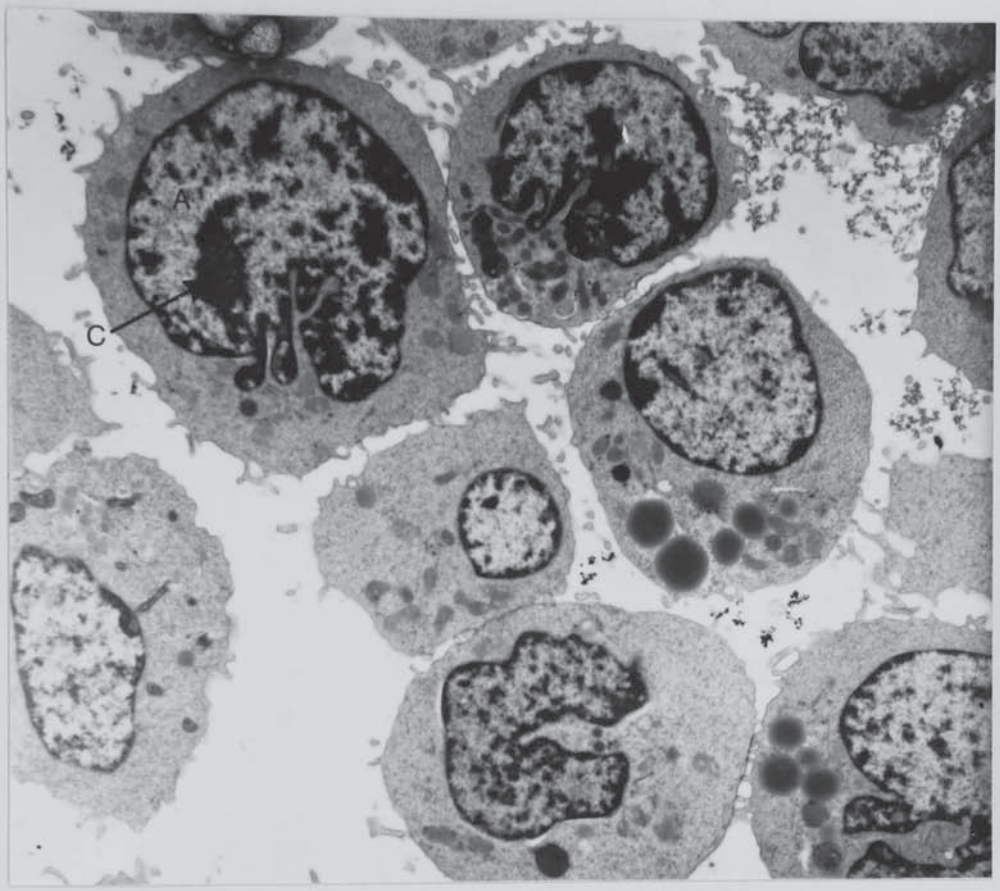


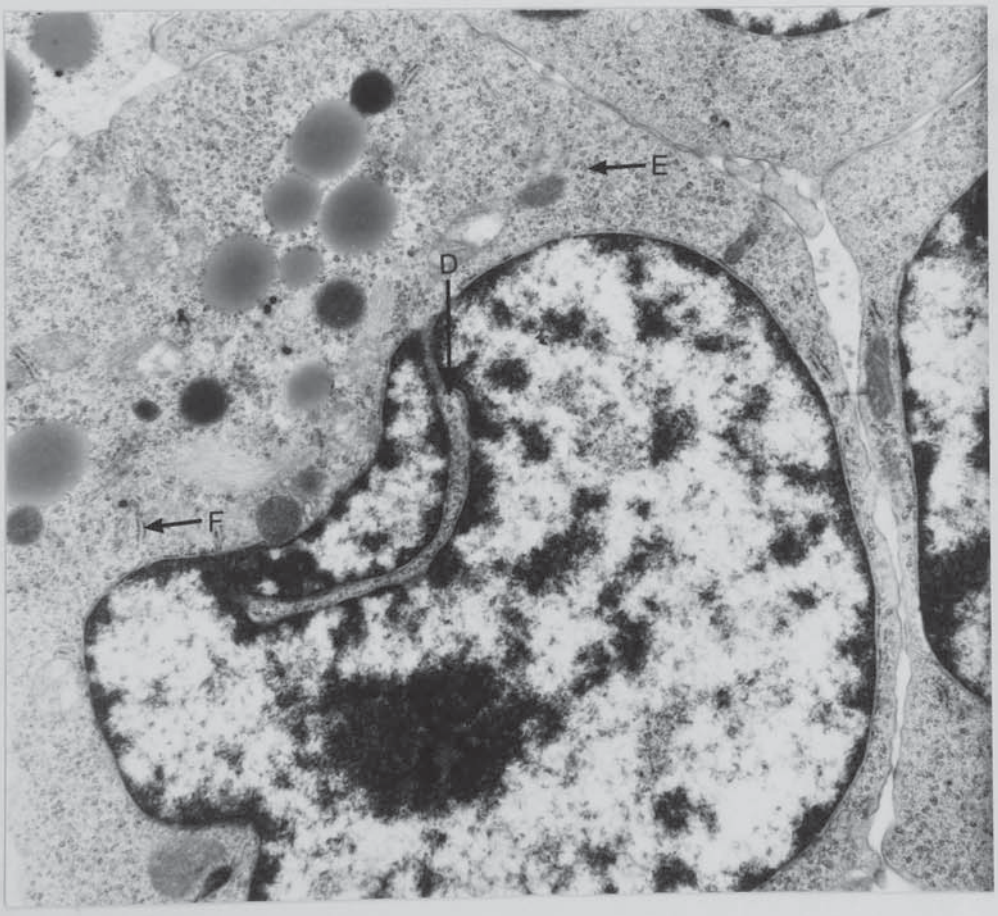
Figure 9b : Contour plots of the changes in fluorescein and ethidium bromide fluorescence intensity after incubation of cells with NMF for 48h.

Figure 10 : Transmission electron micrographs of cells after incubation with (a) control or 43mM NMF (magnification x4,600), (b) control or 43mM NMF (magnification x11,500), (c) 106mM or 170mM NMF (magnification x4,600), (d) 106mM or 170mM NMF (magnification x11,500), for 48h. A=large pleomorphic nucleus; B=lipid droplets; C=nucleolus; D=nuclear cleft; E=polyribosomes; F=rough endoplasmic reticulum; G=pyknosis; H=cell lysis; I=cytolysosomes; J=mitochondria.

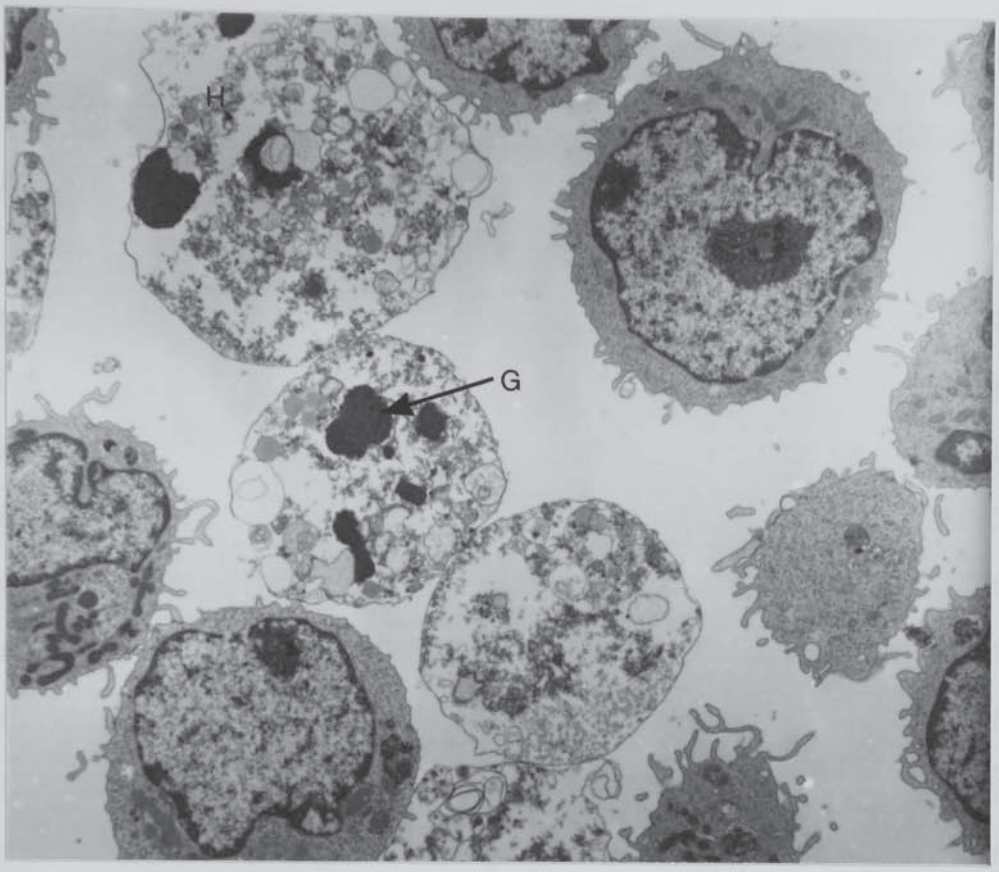
(a)



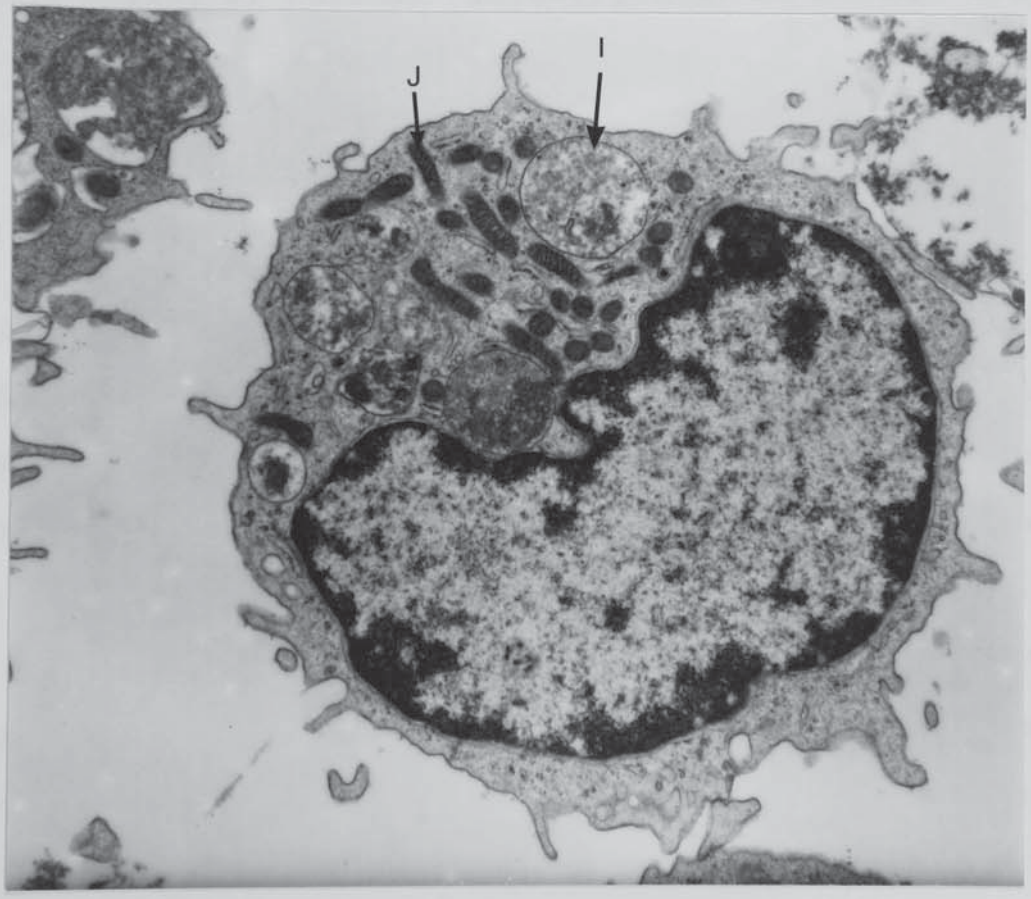
(b)



(c)



(d)



4.II The effects of NMF on the cell cycle of TLX5 cells

4.II.i Introduction

In a study of the mechanism of antiproliferative action of a drug, investigation of the cell cycle kinetics is a useful guide to possible cellular loci of activity. Polar solvents can cause G_1 phase arrest of some cell lines in culture (see section 1.III). Previous reports of the effects of DMF and NMF on the cell cycle, state that prolongation occurred in each phase and this effect on the cell cycle accounted for the increased doubling time of the colon carcinoma cells (Leith et al, 1982a; Leith et al, 1985). The fact that each phase of the cell cycle was affected equally is unexpected for polar solvents and therefore worthy of further investigation in another cell line. The studies described here used FCM to investigate the effects of NMF on the cell cycle of cultured TLX5 cells

4.II.ii Results

TLX5 cells were incubated with various concentrations of NMF for 48h, the cells were then analysed by FCM (figure 11). There was a concentration dependent increase in the proportion of cells in the G_1 phase of the cell cycle with a concomitant decrease in the population of S and G_2M phase cells. Representative DNA histograms of the TLX5 cell samples are shown in figure 12. In each case the non-viable cells at lower DNA fluorescence were

excluded. At the highest NMF concentration (170mM), 99% of the viable cell population was in G_1 phase with no cells found in the S phase and 1% in G_2M.

The results described in 4.I indicate that 106mM NMF caused growth inhibition without adversely affecting viability. This concentration was used to study the time dependent effects of NMF on the cell cycle. Cells were exposed to 106mM NMF for upto 72h. The proportion of G_1 phase cells increased in a time dependent fashion (figure 13).

Further studies were conducted to ascertain the subcompartment of the G_1 phase in which TLX5 cells were arrested after exposure to NMF. Simultaneous dual parameter analysis of DNA and RNA was used after staining with acridine orange (AO)(Darzynkiewicz et al, 1980) (figure 14). The upper contour plot shows the control, with the display focused on the G_1 phase only, S and G_2M phase cells are off scale. The G_1 phase was classified into G_{1A}, G_{1B}, G_{1C} and G_{1D} according to the RNA content of the cells, G_{1A} represented the lowest RNA levels. The middle contour plot shows that when TLX5 cells were incubated with 170mM NMF for 48h, they were in the G_{1A} substate. The lower contour plot reveals that TLX5 cells which were confluent for 3 days also occupied the G_1 phase subcompartments with low RNA content. The proportion of cells in the various G_1 substates after 48h incubation with NMF are shown in figure 15. As the NMF concentration increased there was a shift of the G_1 population to a

substate of lower RNA content.

There was a proportion of cells with a very low RNA content and DNA content below that of G_1 phase cells (figure 14), these are indicated by the box and termed G_{0Q}. The G_{0Q} population could be due to dead cells. However, table 2 shows a comparison of the % of the population in G_{0Q} with cell viability, as measured by trypan blue exclusion. These parameters did not correlate and the results suggest that G_{0Q} contained a number of viable cells. For example, TLX5 cells incubated with 106mM NMF for 48h had an 18% decrease in viability, as measured by exclusion of trypan blue, but 47.4% of the cell population were present in G_{0Q}.

4.II.iii Discussion

NMF caused a concentration dependent increase in the proportion of cells in the G_1 phase of the cell cycle (figures 11, 12 and 13). This is in contrast to the cell cycle kinetics reported for the DLD-1 colon carcinoma cells on incubation with NMF or DMF, but similar to the results for other polar solvents in some cell types (see section 1.III). The G_1 phase is therefore the major stage of the cell cycle at which NMF affects TLX5 cells, although minor perturbations in other phases may occur. At the highest NMF concentration (170mM for 48h) 1% of cells were apparently in the G_2M phase. This may be the case but alternatively it is possible that these cells represent G_1 phase cells which are tetraploid.

The G_1 phase has been divided into distinct sub-compartments based on a number of criteria; for example measurement of the time taken for cells to enter S phase or observation of interphase chromatin by premature chromosome condensation (Hittelman and Rao, 1978). Darzynkiewicz et al, (1980) based their classification on the differences in the cellular RNA content. After mitosis cells reside in a compartment with a low RNA content, an increase in RNA above a threshold level is then required for G_1 cells to initiate DNA synthesis. Total RNA content increased during G_1 to G_2 by a factor of about 3 in Ehrlich ascites tumour cells (Skog and Tribukait, 1985).

NMF caused a concentration dependent accumulation of TLX5 cells in the G_1 phase sub-compartments with low RNA content (figure 14), this is indicative of cell arrest in early G_1 or G_0 phase. Isolated nuclei which are devoid of cytoplasmic RNA will have a low RNA content. However the low RNA content of the TLX5 cells exposed to NMF was not due to contamination with nuclei, since incubation of TLX5 cells with DNAase prior to addition of AO did not effect the distribution of cells in the G_1 phase (data not shown). Also, the procedure used in this study effectively eliminated the staining of nuclei, due to the detergent instability of the unfixed intracellular structures.

Differentiation inducers other than NMF, such as sodium butyrate (Xue and Rao, 1981; Darzynkiewicz et al ,

1981) and vitamin D_3 (Abe et al, 1986) brought about accumulation of cultured cells in early G_1 phase which was associated with maturation. The arrest of cells in early G_1 phase may be due to a number of mechanisms. For example; sodium butyrate inhibited histone deacetylase, which resulted in the accumulation of acetylated histones in chromatin (Candido et al, 1978). The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3, inhibited the production of interleukin-3 by WEHI-3 myelomonocytic leukaemic cells which corresponded with differentiation to macrophages (Abe et al, 1986).

Ethanol also caused the growth arrest of rat hepatoma cells in early G_1 phase, as measured by a cellular reduction of RNA content (Higgins and Borenfreund, 1986). In another study, Higgins, (1986) reported that hepatoma cells were growth arrested in the G_1 phase by DMSO. The G_1 arrested cells had a low cellular RNA content, but returned to exponential growth when DMSO was removed from the medium. Friend erythroleukaemia cells incubated with retinoic acid resided in G_1 phase. These quiescent cells had a low cellular RNA content, similar to Friend cells induced to terminally differentiate by DMSO. The retinoic acid treated cells, however, were not considered to have terminally differentiated (Traganos et al, 1984).

TLX5 cells which had been confluent for 3 days also resided in early G_1 phase. This is at variance with a report by Hittelman and Rao, (1978) who indicated that benign cell populations which were not proliferating,

arrest their growth in early G_1 phase, while malignant cells accumulate in late G_1. Senescent cells also arrest cell cycle progression in late G_1 phase (Gorman and Cristofalo, 1986). The position of growth arrest in G_1 phase can vary according to the causative process; for example differentiation of 3T3 T proadipocytes preceded growth arrest induced by serum deprivation, which came before G_1 arrest by nutrient deprivation (Wille and Scott, 1982; Scott et al, 1982).

It is not yet clear by what mechanism NMF causes the low cellular RNA content of TLX5 cells. The major form of RNA is rRNA which comprises >80% of the total RNA (Darzynkiewicz et al, 1979). Another polar solvent, HMBA promoted the differentiation of murine erythroleukaemia cells, which corresponded with a progressive decrease in the synthesis of rRNA (Marks et al, 1987). NMF may act by inhibiting DNA transcription and thereby reducing RNA synthesis. A similar mechanism was proposed to explain the early G_1 phase arrest of rat hepatoma cells by ethanol (Higgins and Borenfreund, 1986).

Accumulation of a labile protein has been proposed as the mechanism by which cells pass the restriction point in G_1 and traverse into S phase (Rossow et al, 1979). A relationship between RNA content and protein synthesis was established in Ehrlich ascites tumour cells (Skog and Tribukait, 1985), therefore the shift of TLX5 cells to a lower RNA content when exposed to NMF possibly indicates that there is a low rate of protein synthesis. If TLX5

cells treated with NMF had a low rate of protein synthesis, they may not be able to synthesize the necessary protein to traverse from G_1 to S phase.

Receptor binding may also be affected by NMF. The solvent alters membrane morphology (see section 4.I) and cells which were arrested in G_1 possessed a reduced number of epidermal growth factor receptors (Robinson et al, 1981), although DMF increased the number of epidermal growth factor receptors when it induced the maturation of AKR fibroblasts (Levine et al, 1985). Early G_1 phase cells are growth factor dependent, this precedes a growth factor independent pre-DNA synthetic phase in late G_1 (Zetterberg and Larsson, 1985).

The subpopulation of TLX5 cells designated G_{0Q} (table 2), could be the result of cell damage. However, the G_{0Q} population did not correlate with a concomitant loss of cell viability, as measured by trypan blue exclusion. Therefore at least a proportion of the G_{0Q} fraction is comprised of intact, viable cells. The low AO dye binding capacity of the G_{0Q} cells may reflect on the degree of chromatin condensation. The level of AO fluorescence depends on the accessibility of the dye to sites of DNA intercalation and this dye interaction is dependent on the degree of chromatin condensation (Walker et al, 1986). Thus for the TLX5 cells exposed to NMF, the apparent low DNA content may be due to the presence of highly condensed chromatin, which is representative of a quiescent cell type. Non-cycling cells have previously been shown to

bind less AO (Smets, 1973) or EB (Nicolini et al, 1977) than cycling cells. Furthermore, a decrease in the accessibility of DNA to several fluorescent dyes was observed on cell differentiation to non-proliferating cells (Darzynkiewicz et al, 1984). An increase in chromatin decondensation occurs in the early pre-replicative phase of G_0 cells stimulated to proliferate (Baserga and Nicolini, 1976).

The electron micrographs of TLX5 cells incubated with NMF in vitro (see section 4.I) did not show clear evidence of chromatin condensation of the viable cells, although chromatin condensation does occur as a result of pyknosis in the early stages of cell death, but it is likely that membrane integrity would be affected at this stage and therefore these cells would take up trypan blue. The depressed RNA synthesis of TLX5 cells after incubation with NMF may contribute to cell death, since this is thought to be an initiating factor in cell necrosis (Bridges et al, 1983).

Therefore it can be concluded that TLX5 cells incubated with NMF reside in early G_1 phase. The phase specific arrest is typical of quiescent cells.

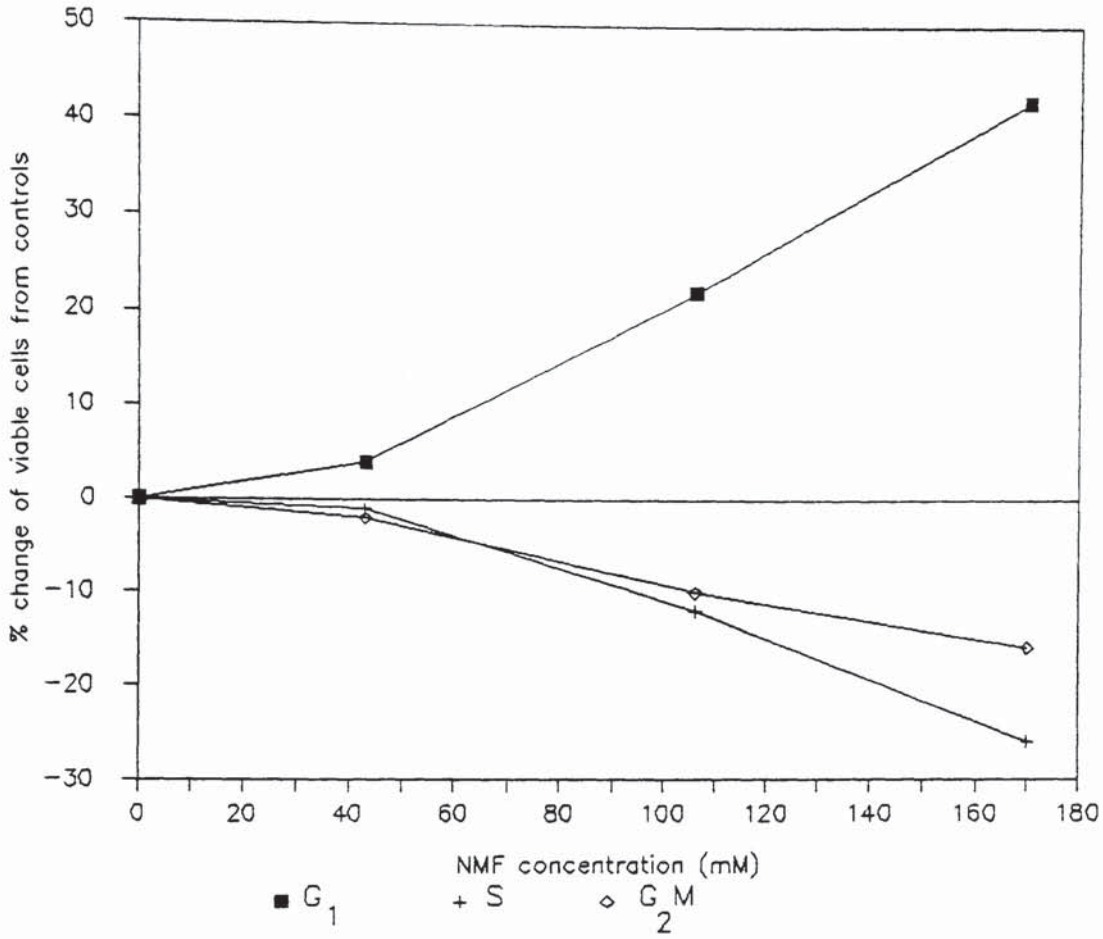


Figure 11 : Percentage of viable cells in different phases of the cell cycle, after incubation with NMF for 48h (means of two independent experiments).

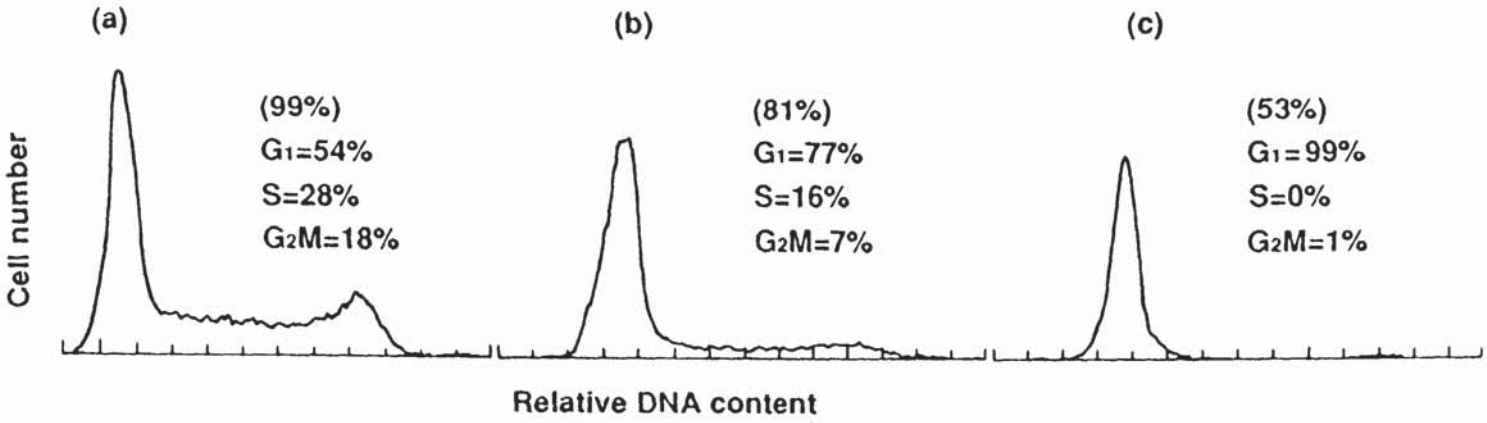


Figure 12 : Representative DNA histograms of cells after incubation with NMF for 48h (a) controls (b) 106mM NMF (c) 170mM NMF. Figures in parenthesis indicate % viability.

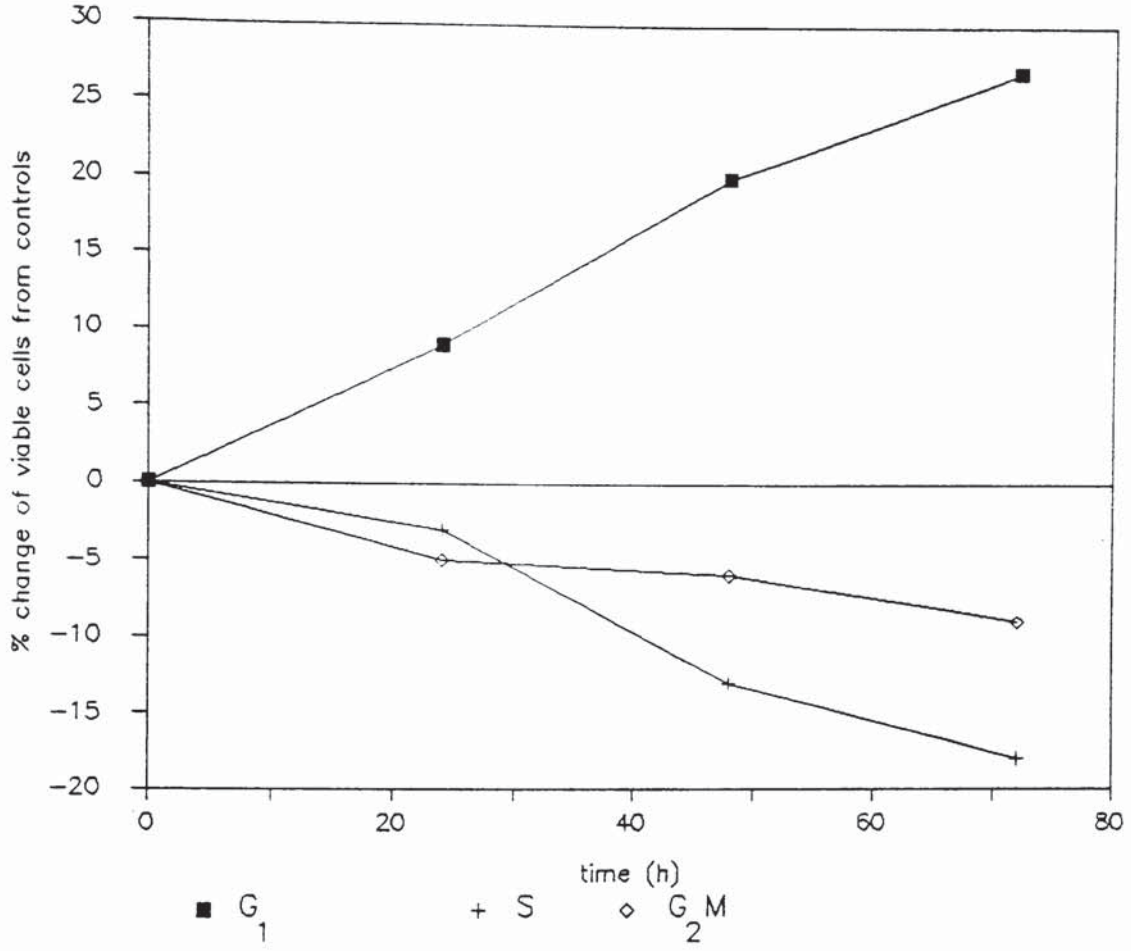


Figure 13 : Percentage of viable cells in different phases of the cell cycle after incubation with 106mM NMF for various times (means of two independent experiments).

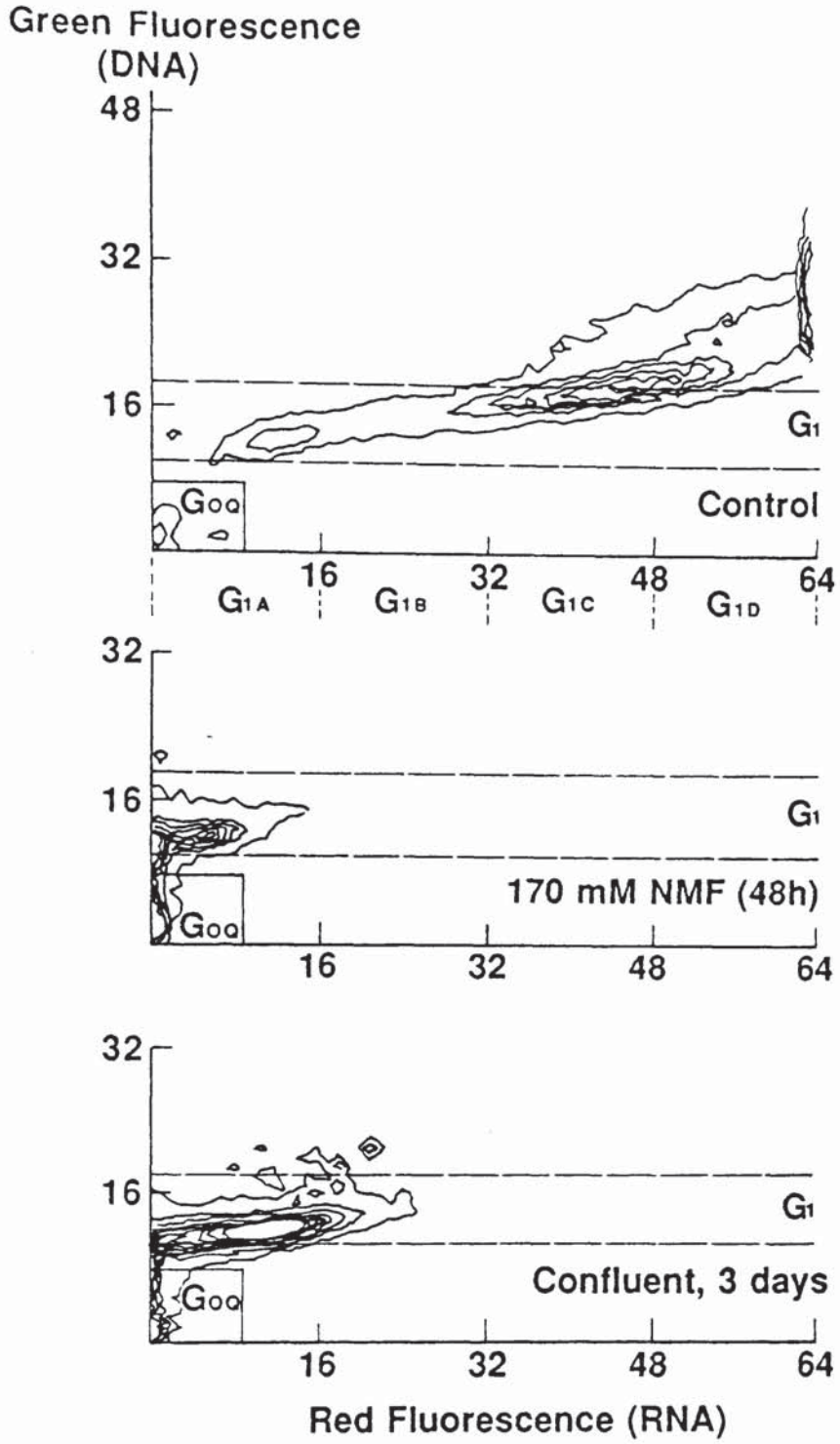


Figure 14 : Sub-compartments of the G_1 phase as measured by acridine orange staining of the DNA and RNA.

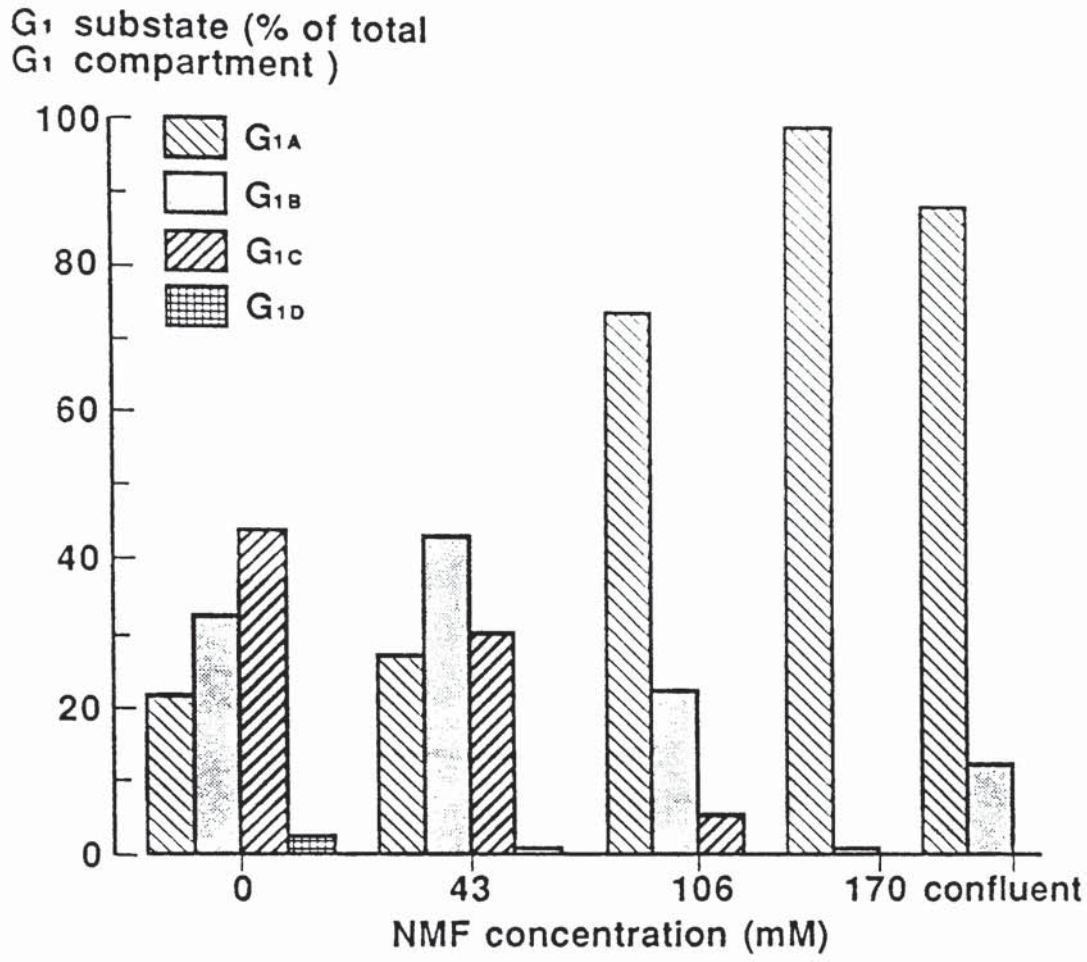


Figure 15: Change in the proportion of cells in the G_1 phase sub-states after incubation with NMF for 48h or for confluent cells (means of two independent experiments).

NMF concentration (mM)	Non-viable population (% of total cells)	G_{0Q} (% of total cells)
0	1	2.9
43	1	5.7
106	18	47.4
170	50	68.2
confluent	4	22.6

Table 2 : Comparison of the percentage of non-viable cells (as measured by trypan blue exclusion) and the percentage of cells in G_{0Q} after incubation with NMF for 48h or cells which were confluent for 3 days (means of two independent experiments).

4.III The effects of NMF on the glutathione status of TLX5 cells

4.III.i Introduction

Incubation of DLD-1 clone A cells with NMF led to the acquisition of more benign characteristics (Dexter et al, 1979), these changes were associated with a decrease in the total intracellular glutathione levels (Cordeiro and Savarese, 1984). The growth and glutathione levels were restored to normal values when DLD-1 clone A cells were exposed to 0.5mM l-cysteine in the presence of NMF (170mM for 96h) (Cordeiro and Savarese, 1984). In a later study Cordeiro and Savarese, (1986) showed that the fall in the glutathione levels was due to a decrease in GSH, the GSSG levels were not altered by NMF. The extent of growth inhibition elicited by NMF was directly proportional to the level of intracellular GSH. This study also reported that the effects produced by NMF were mimicked by the depletion of intracellular GSH caused by BSO. Interestingly, a recent report by Arundel and Tofilon (1987) did not observe glutathione depletion of the DLD-1 clone A cells when the same exposure conditions to NMF as those used by Cordeiro and Savarese (1984 and 1986) were employed.

Murine L1210 leukaemia cells were arrested in the G_1 phase when their cellular glutathione levels were decreased to 10% of control cells (Ishii et al, 1985). It has been outlined in section 4.II that TLX5 cells

incubated with NMF were arrested in the G_1 phase of the cell cycle. Here the hypothesis has been tested that the G_1 phase arrest of TLX5 cells is related to the changes in the glutathione levels and therefore the effects of NMF on the glutathione status of TLX5 cells was investigated.

4.III.ii Results

TLX5 cells were incubated with various concentrations of NMF for 48h. There was an NMF concentration dependent decrease in the total intracellular glutathione levels (figure 16). TLX5 cells incubated with 106mM NMF for 48h, a concentration which caused a 37% growth inhibition, possessed total intracellular glutathione levels of 19.7 ± 6.4% of controls, while viability was maintained at 80% as measured by trypan blue exclusion. When TLX5 cells were incubated with 106mM NMF over a 72h exposure period, a time-dependent decrease in the intracellular glutathione levels was observed (figure 17).

The assay method used for the above experiments measured total intracellular glutathione levels, that is both the oxidized (GSSG) and reduced (GSH) forms. GSSG was measured in TLX5 cells after incubation with NMF for 48h (table 3). No significant change in the GSSG levels in comparison with the total glutathione levels was observed. Therefore the intracellular depletion of glutathione was not the consequence of the oxidation of GSH.

When TLX5 cells were incubated with 106mM NMF for 48h

in the presence of 0.05 to 5mM l-cysteine, the NMF-induced depletion of intracellular GSH levels was not reversed (table 4).

To test whether the GSH-depletory and growth inhibitory effects of NMF on TLX5 cells were related, TLX5 cells were incubated with various concentrations of BSO over a 48h exposure period. BSO caused a decrease in the intracellular glutathione levels, but at cellular glutathione concentrations comparable to treatment with 106mM NMF for 48h there was no significant effect on cell growth or viability (table 5).

The relationship between cell density and glutathione status of TLX5 cells was investigated to find out whether confluent G_1 arrested cells (see section 4.II) had altered glutathione levels. Figure 18 indicates that total intracellular glutathione levels fell as cells reached confluency. The glutathione levels of 3 $x10^6$ cells/ml was 7.5 ± 4.8% of that of cells in logarithmic growth phase (2.6 $x10^5$ cells/ml).

4.III.iii Discussion

NMF caused a concentration and time dependent decrease in the intracellular GSH levels of TLX5 cells (figures 16 and 17). The results are similar to those of the DLD-1 clone A cells (Cordeiro and Savarese, 1984; Cordeiro and Savarese, 1986). In that study, 170mM NMF depleted the glutathione pools of DLD-1 clone A cells by 88% of controls after 96h incubation. This compares with

a >80% decrease in glutathione levels of TLX5 cells incubated with 106mM NMF (48h) (figures 16 and 17). In another study, incubation of HL-60 cells for 96h with 180mM NMF caused granulocytic maturation with no accompanying change in glutathione levels (Lazenby et al, 1987). Speier and Newburger, (1986) also investigated the effect of a formamide on HL-60 intracellular glutathione levels. 60mM DMF caused granulocytic maturation of the HL-60 cells which was paralleled by a fall in both the GSH and GSSG contents to a minimum of 63% and 76% of controls respectively after 7 days exposure to DMF. In another cell line, the A549 lung adenocarcinoma, the cytostatic effect of 180mM NMF was associated with a 45% decrease in the intracellular glutathione levels (Lazenby et al, 1987), but there was no evidence of induction of differentiation.

Intracellular glutathione can protect cells from reactive drugs or their metabolites (see section 1.IV). The depletion of glutathione mediated by NMF, can enhance cell cytotoxicity caused by other drugs or x-irradiation. When DLD-1 clone A cells were incubated with NMF (1% $^{v}/_{v}$) for 3 passages they were more susceptible to x-irradiation than control cells. The authors speculated that free radical damage was enhanced due to the decreased cellular glutathione levels and consequently reduced detoxification (Leith et al, 1985). This mechanism was also thought to explain why bleomycin toxicity was enhanced after DLD-1 clone A cells were incubated with NMF and exposed to x-irradiation and bleomycin (Leith et al, 1986).

Depletion of glutathione, other than by NMF, can also increase the cytotoxicity of xenobiotics or x-irradiation. BSO (0.5 to 1mM) sensitized A549 cells to x-irradiation (Phillips et al, 1986) and increased melphalan induced cytotoxicity of a human ovarian cancer cell line (Ozols et al, 1987). Jensen and Meister, (1983) reported that cells which were depleted of cytoplasmic glutathione exhibited increased radiosensitivity compared to control cells. Partial repletion of glutathione prior to irradiation led to radiosensitivity comparable to that of non-depleted control cells.

NMF did not alter the intracellular GSSG levels of TLX5 cells (table 3) which is in accordance with the results of Cordeiro and Savarese, (1986) and suggests that species which lead to the oxidation of GSH are not formed by NMF in culture.

The rate limiting amino acid for GSH synthesis is l-cysteine (see section 1.III). The GSH depletory effect of NMF on DLD-1 clone A cells was reversed by addition of 0.5mM l-cysteine (Cordeiro and Savarese, 1984). For L1210 leukaemia cells, depletion of GSH brought about by vitamin K was responsible for growth inhibition, this was abrogated by co-addition of 1mM cysteine (Akman et al, 1985). l-Cysteine (0.05 to 5mM) had no effect on the depletion of intracellular GSH of TLX5 cells caused by NMF (table 4). Transport of l-cysteine into the TLX5 cells may be affected by alterations of the plasma membrane initiated by NMF (see section 4.I) or GSH synthesis may

not be rate limiting in the NMF induced depletion process.

Intracellular GSH was not an essential entity for TLX5 cell growth. GSH depletion caused by BSO had no significant effect on the growth or viability of TLX5 cells over a 48h exposure period (table 5), unlike the DLD-1 clone A and L1210 leukaemia cells (see introduction). Cell lines such as the DLD-1 clone A and L1210 leukaemia cells are dependent for their growth on substances in the medium which maintain intracellular l-cysteine levels; for example l-cysteine or precursors (Cordeiro and Savarese, 1986), 2-mercaptoethanol (Ishii et al, 1981) or L-2-oxo-thiazolidine-4-carboxylate (Fidelus and Tsan, 1986).

Depletion of cellular GSH does not effect cell viability provided rapid resynthesis can take place. GSH levels of hepatocytes fell to 5 to 10% of control cells without enhanced toxicity (Reed and Fariss, 1984). Viability of TLX5 cells exposed to BSO for 48h remained at 96% or greater. However, longer exposure times resulted in a fall of viability (data not shown). Although the NMF mediated depletion of TLX5 cellular GSH for 48h in itself may not be detrimental to cell survival, it might however reduce the ability of the cells to react with toxic species which may ultimately result in cell death.

The concentrations of BSO used to cause GSH depletion of 80% or more in TLX5 cells were 3 to 5uM (table 5), these concentrations are considerably lower than the 7.5mM (Cordeiro and Savarese, 1986) or 0.05mM BSO (Dethlefsen et

al, 1986) used in other studies. Higher concentrations of BSO (upto 100uM) did not enhance the depletion of intracellular GSH in TLX5 cells (data not shown). There are two main pools of cellular glutathione, these are found in the cytoplasm and mitochondria. The mitochondrial pool consists of about 10% of total glutathione and has a half life of about 30h in rat hepatocytes, while the half-life of the cytoplasmic pool is 2h (Reed and Farris, 1984). When TLX5 cells were incubated with NMF (170mM for 48h or 106mM for 72h) a small amount of intracellular GSH remained. The GSH present under these conditions of NMF exposure may be due to the stable mitochondrial pool.

When TLX5 cells became confluent and reached a plateau of growth, the cellular glutathione levels decreased (figure 18). Similar results were reported for 3T3 fibroblasts when the cells became quiescent due to a high cell density (Shaw and Chou, 1986). The decreased glutathione levels could be due to nutrient deprivation and subsequent inability of the cell to synthesize glutathione. The decreased GSH levels of confluent TLX5 cells could also be the consequence of the G_1/G_0 phase arrest (see section 4.II). Mitogenic stimulation of quiescent 3T3 fibroblasts led to a rapid 2-3 fold elevation of cellular GSH followed by a gradual increase as the cells entered S phase. Indeed, cell cycle dependent variations in intracellular glutathione concentrations have been cited in other cell lines.

Chinese hamster ovary cells accumulated in G_1/G_0 when the cells reached plateau phase, these cells contained only 25% of the glutathione levels of cycling G_1 cells. The concentration of glutathione remained constant for the remainder of the cell cycle (Harris and Teng, 1973). The fact that BSO mediated depletion of TLX5 cell glutathione does not effect cell growth rate (table 3), render it unlikely that glutathione alone is responsible for the growth arrest and G_1 phase accumulation of confluent TLX5 cells.

The mechanism involved in the fall of cellular glutathione of TLX5 cells induced by NMF is still unclear. Glutathione may be present in the medium. Efflux of GSH (Bannai and Tsukeda, 1979) was reported for human fibroblast cells in culture and GSSG release has been shown for a number of cell types (Sies and Akerboom, 1984). Loss of GSH from TLX5 cells into the medium is feasible since NMF decreased the membrane integrity of TLX5 cells in culture (see section 4.I). NMF may also act as a competitive inhibitor of an enzyme(s) involved in GSH synthesis or the drug could be metabolized *via* GSH conjugation and result in GSH depletion. The role of GSH conjugation in the metabolism of NMF *in vitro* will be discussed in section 4.VI.

In conclusion, NMF caused the depletion of cellular GSH in TLX5 cells *in vitro*, however, this was not responsible for the growth inhibition and G_1 phase arrest of these cells. Other reports demonstrate that some cell

lines are dependent on GSH for growth and maturation, such a relationship therefore appears to be cell type dependent.

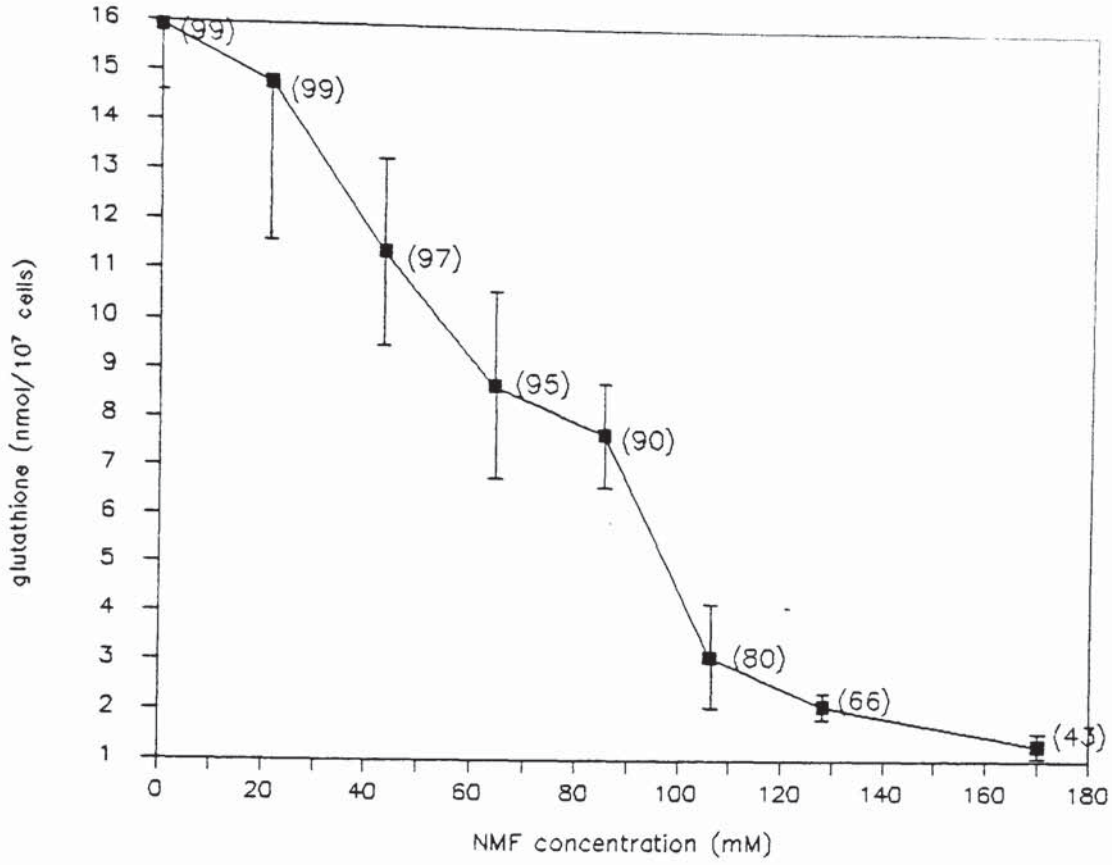


Figure 16 : Concentration dependent effects of NMF on the total intracellular glutathione levels after incubation for 48h. Figures in parenthesis indicate % viability (n=4, ±SD).

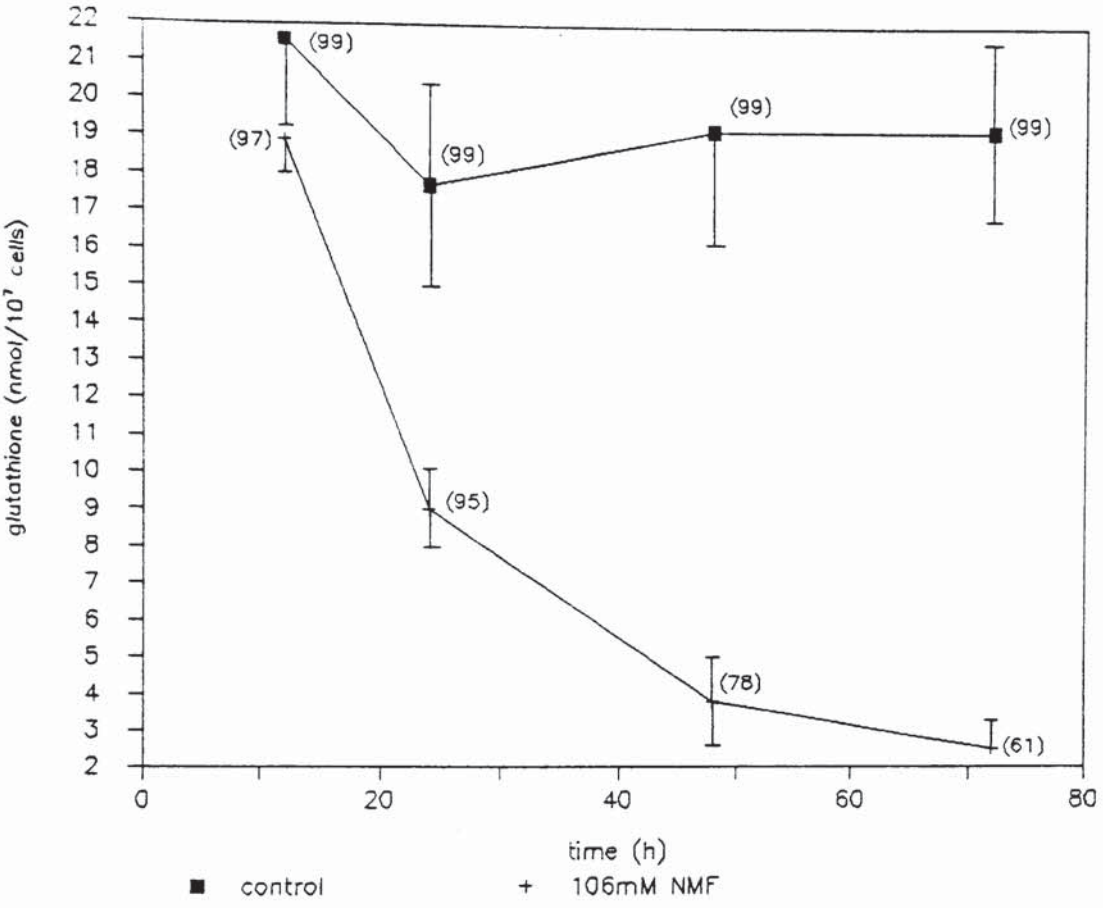


Figure 17 : Time course of the fall in total intracellular glutathione induced by 106mM NMF. Figures in parenthesis indicate % viability (n=4, ±SD).

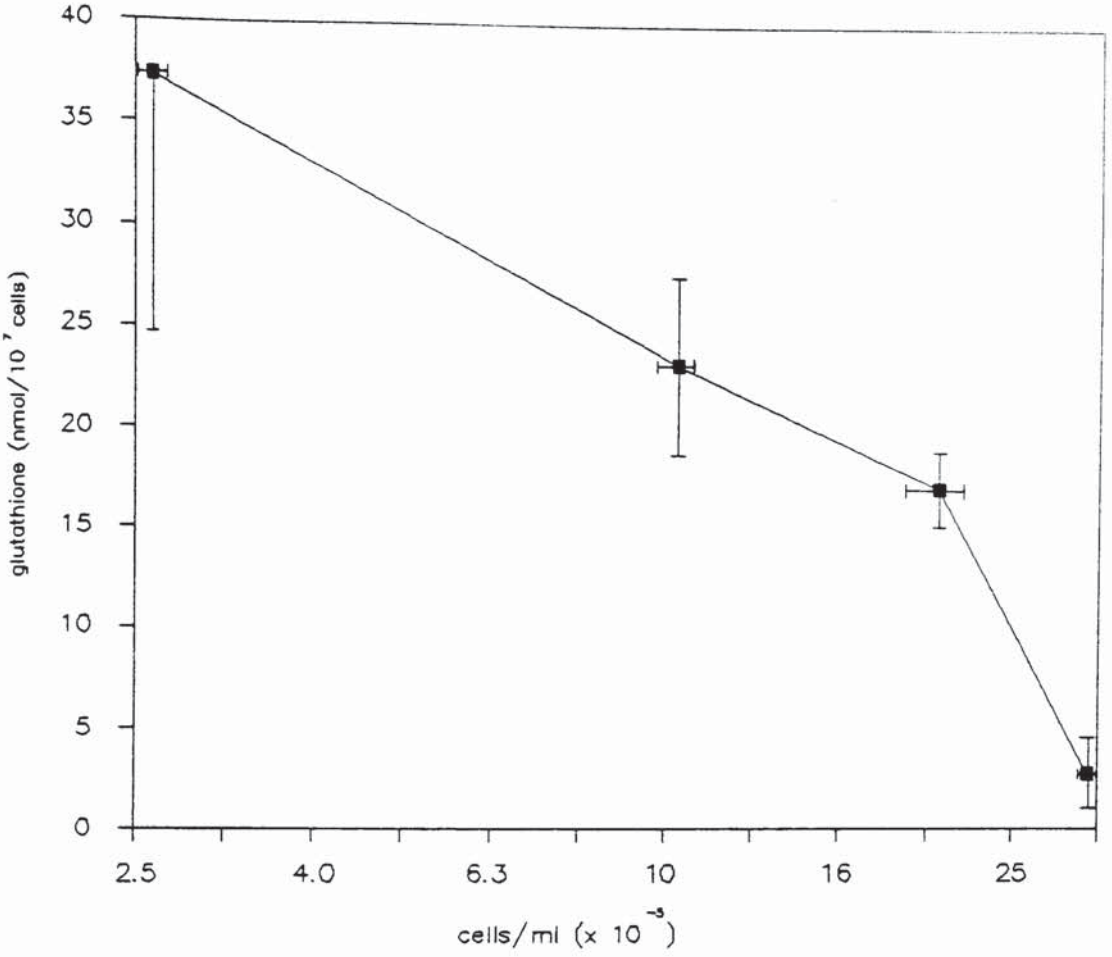


Figure 18 : Cell density dependent changes in the total intracellular glutathione levels (n=3, ±SD).

NMF concentration (mM)	total intracellular GSSG levels (nmol/10^7 cells)
0	0.7
43	1.0
106	0.7

Table 3 : Total intracellular GSSG levels after incubation with NMF for 48h (means of two independent experiments).

l-cysteine (mM)	NMF present	total intracellular glutathione (nmol/10^7 cells)
0	-	16.6
0.05	-	16.7
0.5	-	16.0
5.0	-	12.4
0	+	4.3
0.05	+	4.7
0.5	+	5.4
5.0	+	2.9

Table 4 : Effects of l-cysteine on the depletion of total intracellular glutathione caused by 106mM NMF for 48h (means of two independent experiments).

BSO (uM)	total intracellular glutathione content (nmol/10^7 cells)[a]		cell growth (% of controls)[b]	
	incubated for 24h	incubated for 48h	incubated for 24h	incubated for 48h
0	19.5 ± 0.8	20.2 ± 2.4	- (99)	- (99)
1	12.1 ± 0.8	13.3 ± 2.0	102(99)	100(99)
2	7.6 ± 1.3	8.3 ± 1.5	101(99)	98(98)
3	3.9 ± 0.7	5.8 ± 1.4	100(99)	92(97)
4	3.2[c] ± 0.4	4.2[c] ± 0.2	100(98)	94(97)
5	3.3[c]	3.7[c]	106(98)	92(96)

Table 5 : Changes in total intracellular glutathione levels and cell growth induced by BSO. Figures in parenthesis indicate % viability. [a] n=4, ±SD; [b] n=4; [c] n=2.

4.IV Studies on the effects of the removal of NMF from the medium on TLX5 cell growth, viability and cell cycle kinetics

4.IV.i Introduction

As discussed previously, NMF is one of a number of polar solvents which can induce the maturation of certain cell types. Maturation of leukaemia cell lines may lead to the terminal differentiation, for example Friend erythroleukaemia (Tanaka et al, 1975) and HL-60 cells (Collins et al, 1978; Langdon and Hickman, 1987b) can be induced to terminally differentiate by NMF.

However, cell lines derived from solid tumours do not usually undergo terminal differentiation; instead cells take on a more mature phenotype but regain their malignant state when replated in medium free from the agent which induced maturation. For example NMF or DMF induced benign characteristics in DLD-1 clone A carcinoma cells (Cordeiro and Savarese, 1986; Dexter et al, 1979) or fibroblast cells (Chakrabarty et al, 1984).

This study investigated the effect of NMF removal from the medium on the TLX5 cell growth, viability and cell cycle kinetics to elucidate whether terminal differentiation had resulted from drug exposure.

4.IV.ii Results

TLX5 cells were incubated with 106mM NMF for 48h. The cells were then washed twice and replated in drug free

medium. The growth of untreated cells was not affected by this procedure (figure 19). Cells which had been exposed to NMF displayed an initial fall in viability from 81 ± 4% to 45 ± 4% (data not shown) during the first 24h in fresh medium, this was followed by a recovery of cell growth and viability during the next 72h (figure 19).

The results demonstrated in figure 19 were obtained for the whole population of TLX5 cells and the outgrowth could be either due to the growth of all cells or only an NMF resistant population. In order to study the consequence of exposure to NMF for the proliferating stem cell population, cells were incubated with various concentrations of NMF for 48h, they were then washed free of the drug and plated in soft agar. The NMF concentration-dependent effect on the cloning potential of these cells is shown in figure 20. The clonogenic efficiency of the control cells was 30.1 ± 1.7%. NMF at 106mM reduced the clonogenic efficiency to 54.5 ± 4.3% of control cells, which when compared to the overall viability of the cell population (45 ± 4%) indicates that all the stem cells in the viable cell population formed colonies. No colonies were formed in the soft agar after the six day incubation period with 170mM NMF for 48h, of these cells 50% were viable (see section 4.I) as measured by trypan blue exclusion, immediately after NMF was removed from the medium. The lack of formation of colonies under these conditions and the fact that no intact cells were observed under the light microscope,

indicates that the viable cells eventually died.

In order to investigate the tumourigenicity of the TLX5 cells after exposure to NMF, TLX5 cells were incubated with 170mM NMF for 48h and then washed free of the drug and implanted sc into CBA/CA mice. There was a significant increase in the survival times of the animals compared to the untreated controls (table 6). Gibson and Hickman, (1982) have shown that for TLX5 cells there is a direct relationship between the cell number injected into the mice and their survival times. The survival times of the mice which were recipients of cells treated with 170mM NMF corresponds to a 1000 fold decrease in viable cells compared with control cells. Therefore, although these cells produced no clones when plated in soft agar, some cells did survive and proliferate under favourable conditions as indicated by these tumourigenicity studies. When TLX5 cells were incubated with 106mM NMF for 48h and injected into the CBA/CA mice, survival times were comparable with those of control mice which indicates further that the viable cells retained their proliferative potential and malignancy.

The cell cycle phase distribution of TLX5 cells was measured after cells were exposed to 106mM NMF for 48h followed by drug removal (figure 21). After an initial increase in the G_1 phase population cells returned to a normal cell cycle distribution 72h later.

4.IV.iii Discussion

Terminal differentiation of a malignant cell is characterized by the transformation to a cell type which is viable but has no proliferative capacity. The terminally differentiated cell enters the G_0 phase of the cell cycle, from which it ultimately dies (Baserga and Surmacz, 1987).

Cell maturation or acquisition of a more benign phenotype occurs when the malignant cells adopt the morphology of the normal cell counterpart or the characteristics of a cell further along the normal lineage pathway. Also, parameters used to demonstrate the acquisition of a more benign phenotype include a reduction of tumourigenicity and clonogenic potential, increase in doubling time (Dexter et al, 1979; Spremulli and Dexter, 1984) and accumulation of cells in the G_1 phase of the cell cycle (see section 1.III). These effects occur without an adverse change in cell viability.

NMF brought about a concentration dependent decrease in the growth rate of TLX5 cells (see section 4.I) and cells were arrested in the G_1 phase (see section 4.II). These observations can be interpreted has being consistent with differentiation. However, when NMF was removed from the medium cell numbers increased (figure 19) and all viable cells retained their proliferative potential and formed clones in soft agar (figure 20). The viable cells were also tumourigenic (table 6) and the cells returned to a normal cell cycle phase distribution (figure 21). The

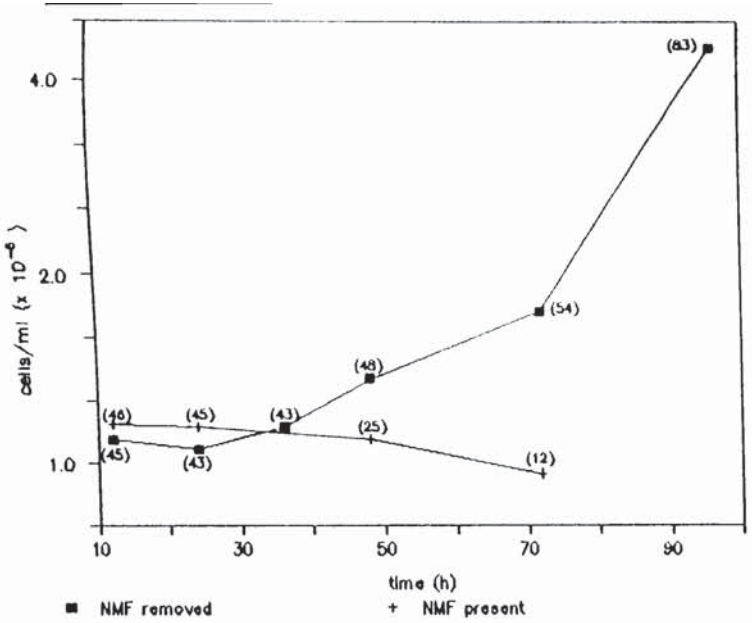
results indicate that the cells which retain their viability are not terminally differentiated by NMF, but are arrested in G_1 phase, which can be reversed on drug removal for a number of cells.

There was a fall in viability after TLX5 cells were exposed to NMF (106mM for 48h) and then replated in drug free medium (figure 19), which was associated with an increase in the G_1 phase population (figure 21). These observations suggest that some cells are destined to die after incubation with NMF and that the remaining cells initially take on a more quiescent state. This interpretation agrees with observations made on electron micrographs of TLX5 cells after incubation for 48h with NMF (106 or 170mM) (see section 4.I). The cells enter a pathway leading to cell death from which some cells are able to revert back to the normal malignant phenotype on drug removal. Some cells which are perhaps further along the pathway reach an irreversible stage from which they cannot escape death.

It cannot be discounted that the TLX5 cells which ultimately die after exposure to NMF may have terminally differentiated. This possibility could only be established if markers representative of the developmental lineage of these cells were known and could be identified prior to cell death. A similar arguement could be applied to the viable cells. One could argue that they take on a more benign phenotype in response to NMF which is reversed in the absence of NMF, but again this could only be

established by suitable markers.

In conclusion, the results suggest that NMF does not cause TLX5 cells to terminally differentiate to viable cells which have lost the capacity for self renewal. Instead, TLX5 cells reside in a quiescent state, from which on NMF removal some cells die while others regain their normal growth characteristics.



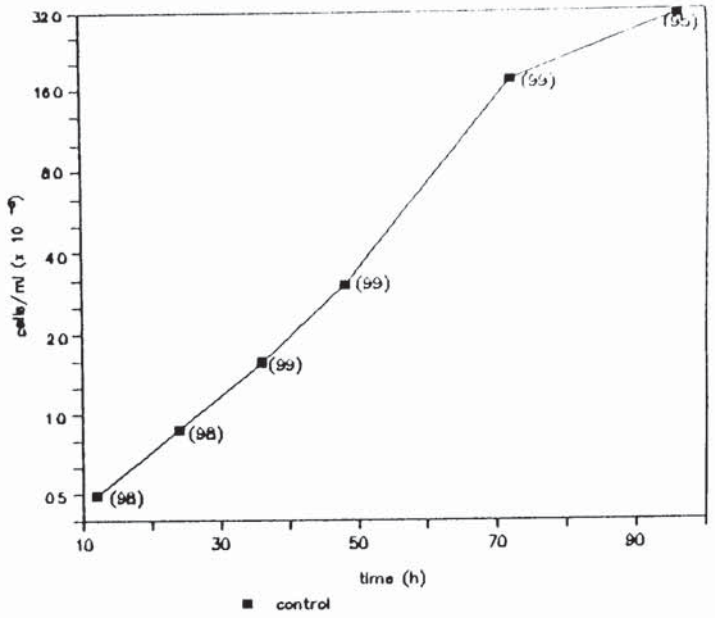


Figure 19 : Effect of the removal of NMF from the medium on cell growth and viability (figures in parenthesis) after incubation with 106mM NMF for 48h (n=2).

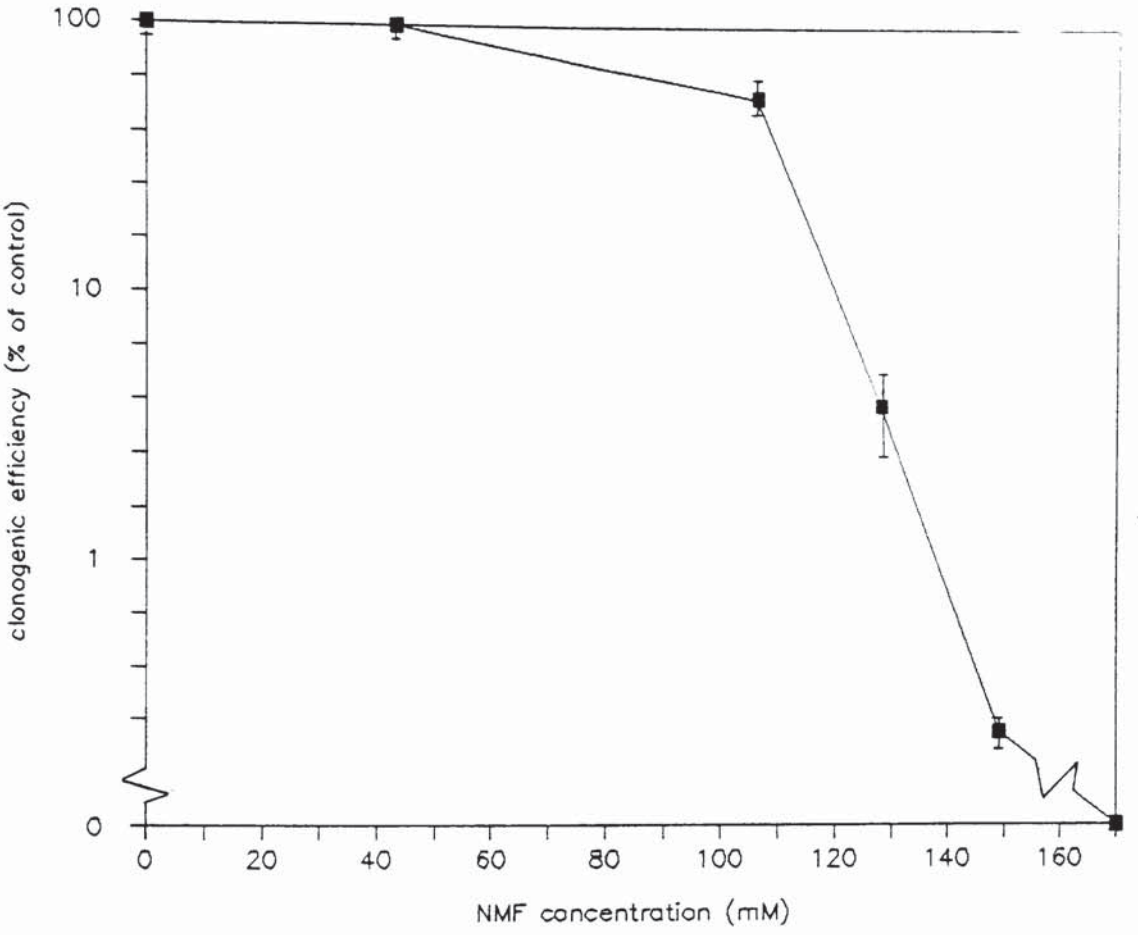


Figure 20 : Clonogenic efficiency of cells after incubation with NMF for 48h (n=4, ±SD).

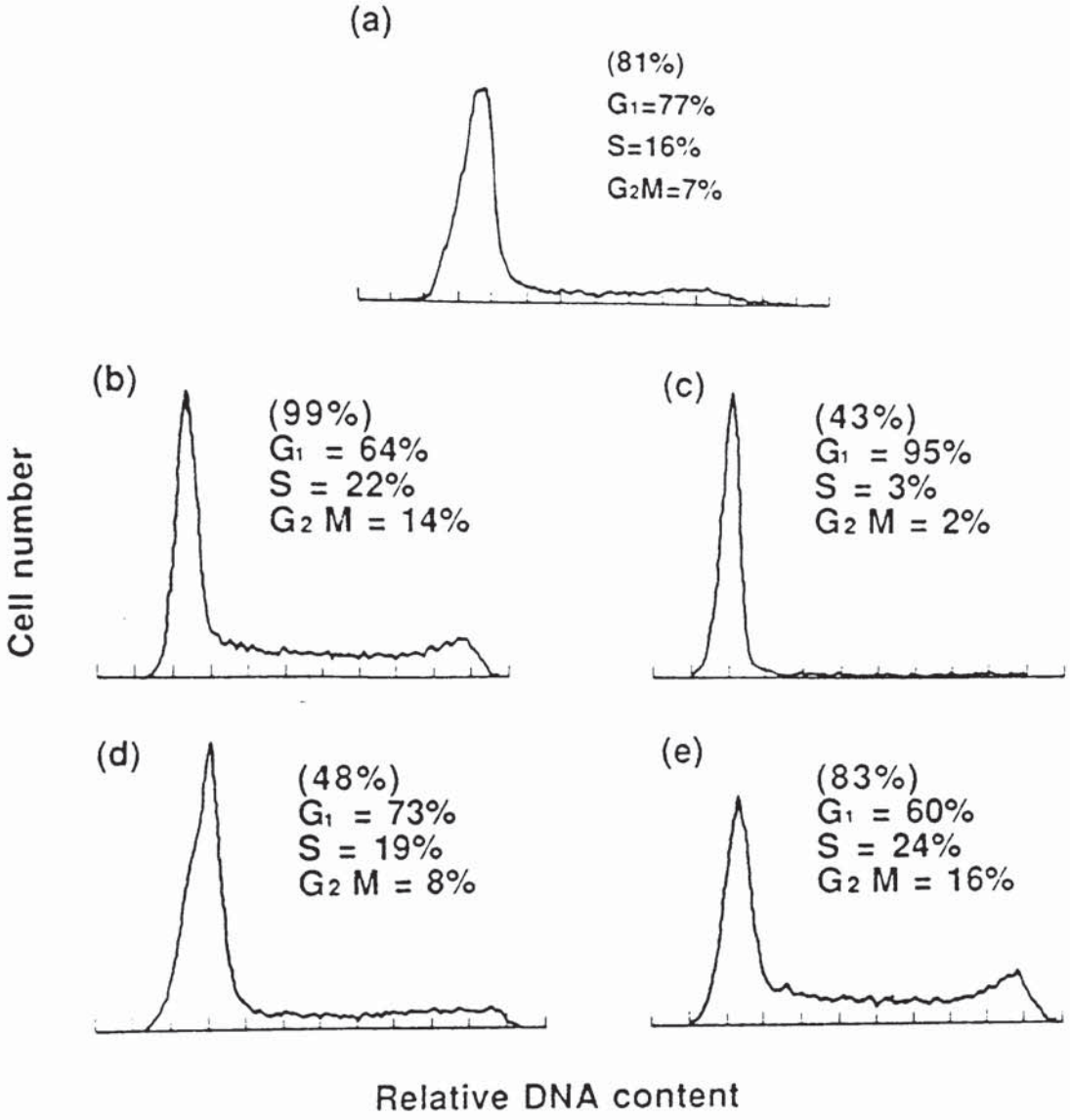


Figure 21 : Representative DNA histograms of cells which had been incubated with 106mM NMF for 48h (a) washed free of the drug and analysed at time intervals; (b) untreated cells; (c) 24h; (d) 48h; (e) 72h after the removal of NMF. Figures in parenthesis indicate % viability.

NMF concentration (mM)	death days	mean
0	9, 10, 10, 10, 10	9.8
43	9, 10, 10, 11, 11	10.2
106	10, 10, 10, 11, 11	10.4
0	10, 10, 10, 11, 11	10.4
170	11, 12, 12, 14, 15	12.8

Table 6 : Survival times of CBA/CA mice inoculated with cells incubated with or without NMF for 48h.

4.V A comparison of the effects of NMF, DMF and NEF on TLX5 cell growth and cell cycle kinetics and the role of tonicity in the growth inhibition and glutathione depletory action of NMF

4.V.i Introduction

The formamide analogues NMF, DMF and NEF are good inducers of terminal differentiation in the HL-60 cell line (Langdon and Hickman, 1987a; Langdon and Hickman, 1987b). NMF and DMF in particular have been shown to induce maturation of a number of cell types (reviewed by Spremulli and Dexter, 1984).

Exposure of cells to polar solvents can cause growth inhibition with cell arrest in G_1 phase (see section 1.III). NMF and DMF did not cause phase specific cell cycle arrest of DLD-1 clone A cells (Leith et al, 1982a). However, NMF did cause G_1 phase accumulation of TLX5 cells in vitro (see section 4.II). A study was conducted to compare the effects of NMF with DMF and NEF on the growth and cell cycle kinetics of TLX5 cells in culture, prior to a similar study in vivo. Although the formamide analogues can induce differentiation of a number of cell lines, the response of tumours to the formamides is different in vivo. NMF has good antitumour activity against the TLX5 lymphoma in vivo, whereas DMF and NEF are not active or marginally active respectively against this tumour (Gescher et al, 1982).

The concentration of NMF required to induce

differentiation in most cell lines is about 170mM or 1% $^{v}/_{v}$ (see section 1.II). These high concentrations may alter cell growth, survival and perhaps glutathione levels due to a change in the osmolarity of the medium. Therefore, the effects of D-glucose on the growth and glutathione status of TLX5 cells was compared with those of NMF.

4.V.ii Results

TLX5 cells were incubated with NMF, DMF or NEF over a 72h exposure period. There was a concentration and time dependent decrease in the growth rate for each of the formamide analogues. For a given drug concentration the order of growth inhibitory activity was NEF > DMF > NMF. A similar order was seen for the fall in viability caused by these solvents (table 7).

TLX5 cells were incubated with each of the formamides (106mM) for 48h, cell cycle kinetics were then analysed by FCM. For each of these analogues there was an increase in the proportion of cells in G_1 . The percentage of cells in the G_1 phase was 95% for DMF, 68% for NEF and 77% for NMF which compared with 63% for the control cells (figure 22).

To investigate the consequence of tonicity alterations on TLX5 cell growth, the effect of NMF was compared with that of D-glucose. TLX5 cells were incubated with drug for 48h and cell growth measured as a percentage of control. For drug concentrations of 43 or 106mM there was no significant difference in the growth

inhibition caused by these compounds (figure 23), there was however a significant decrease in cell growth caused by NMF compared with D-glucose at a concentration of 170mM, but the viability was 50% compared with 98% for the D-glucose treated cells at this concentration.

A further study was conducted to establish whether the similarity in effect caused by NMF and D-glucose also extended to the glutathione status of TLX5 cells. Cells were incubated with drug at concentrations of upto 170mM for 48h. There was a concentration dependent fall in the intracellular glutathione levels after incubation with NMF, similar to to those recorded previously (see section 4.III). D-glucose, however, had no effect on the intracellular glutathione levels (figure 24).

4.V.iii Discussion

The formamides NMF, DMF and NEF decreased the growth rate of TLX5 cells (table 7). NEF caused the most potent growth inhibition and the greatest cytotoxicity. The effects of DMF and NEF on the growth and viability of the TLX5 cells are different to those reported for the HL-60 cell line, for which DMF and NEF were equitoxic at a concentration of 100mM, although NEF caused a marginally greater growth inhibition (Langdon and Hickman, 1987b). In the study using HL-60 cells, a concentration of 180mM NMF was required to elicit similar changes in growth and viability to 100mM DMF or NEF.

The three formamides also caused G_1 arrest of the

TLX5 cells (figure 22), and DMF treated cells had the highest proportion of G_1 phase arrested cells. This phase specific accumulation is therefore a common action of these agents on the TLX5 cells. As discussed earlier the formamides can induce cell differentiation. There is a good correlation between molecular weight and the drug concentration required for optimal differentiation (see section 1.III). Although DMF and NEF have the same molecular weight they were not equitoxic or equicytostatic against the TLX5 cells. Nevertheless, the overall similarity of the effects of these formamides against the TLX5 cells and those reported in other cell lines (reviewed by Spremulii and Dexter, 1984) suggest that a common mechanism is responsible for their activity.

A high osmolarity of the medium reduced the cellular ATP content of Ehrlich ascites cells, which resulted in a fall of the cell growth rate (Pohlit and Heyder, 1981). The optimal concentration of NMF employed to cause maturation of cells is around 170mM (1% $^{V}/_{V}$). At this concentration it is possible that the change in osmolarity of the medium could effect cellular functions including growth and glutathione status, since glutathione synthesis requires ATP. This seems to be the case as far as growth is concerned. For 43 and 106mM of either NMF or D-glucose there was a direct relationship between cell growth and drug concentration, NMF however was a more potent growth inhibitor at 170mM than D-glucose (figure 23). This may well be a consequence of the greater cytotoxicity of NMF

at this concentration (50% to 98% viability for NMF and D-glucose respectively). No similarity between D-glucose and NMF was observed for the effects on glutathione status in TLX5 cells. NMF induced intracellular depletion of glutathione was not due to the osmolarity of the medium since TLX5 cells incubated with D-glucose (upto 170mM for 48h) displayed no loss of glutathione content (figure 24); this agrees with Cordeiro and Savarese, (1984) for the DLD-1 clone A cells. Glutathione is not rate limiting for growth of TLX5 cells in culture (see section 4.III), therefore it is not surprising that D-glucose induced growth inhibition of TLX5 cells was not related to intracellular glutathione levels.

Overall, the results indicate that the change in the tonicity of the medium, caused by NMF, DMF, NEF or D-glucose is important for TLX5 cell growth in culture. Hypertonic medium prevented mouse myeloma (MPC 11) cell protein synthesis at the level of polypeptide chain initiation (Kruppa and Clemens, 1984). Ribosomal protein S6 dephosphorylation occurred when the tonicity of the growth medium was raised by 120mM sodium chloride. Ribosomal protein S6 phosphorylation occurs prior to protein synthesis and is thought to play a crucial role in the regulation of cell division (Chambard and Pouyssegur, 1986). Furthermore, ribosomal protein S6 phosphorylation is controlled by the intracellular pH. Cellular alkalinization caused by activation of the Na^+/H^+ antiporter led to ribosomal protein S6 phosphorylation,

protein synthesis and progression through G_1 phase of the cell cycle (Chambard and Pouyssegur, 1986).

The hypertonic medium caused by the addition of NMF, DMF, NEF or D-glucose at the concentrations studied to TLX5 cells may affect the intracellular pH or ribosomal protein S6 phosphorylation and ultimately prevent protein synthesis and cell traverse of the G_1 phase.

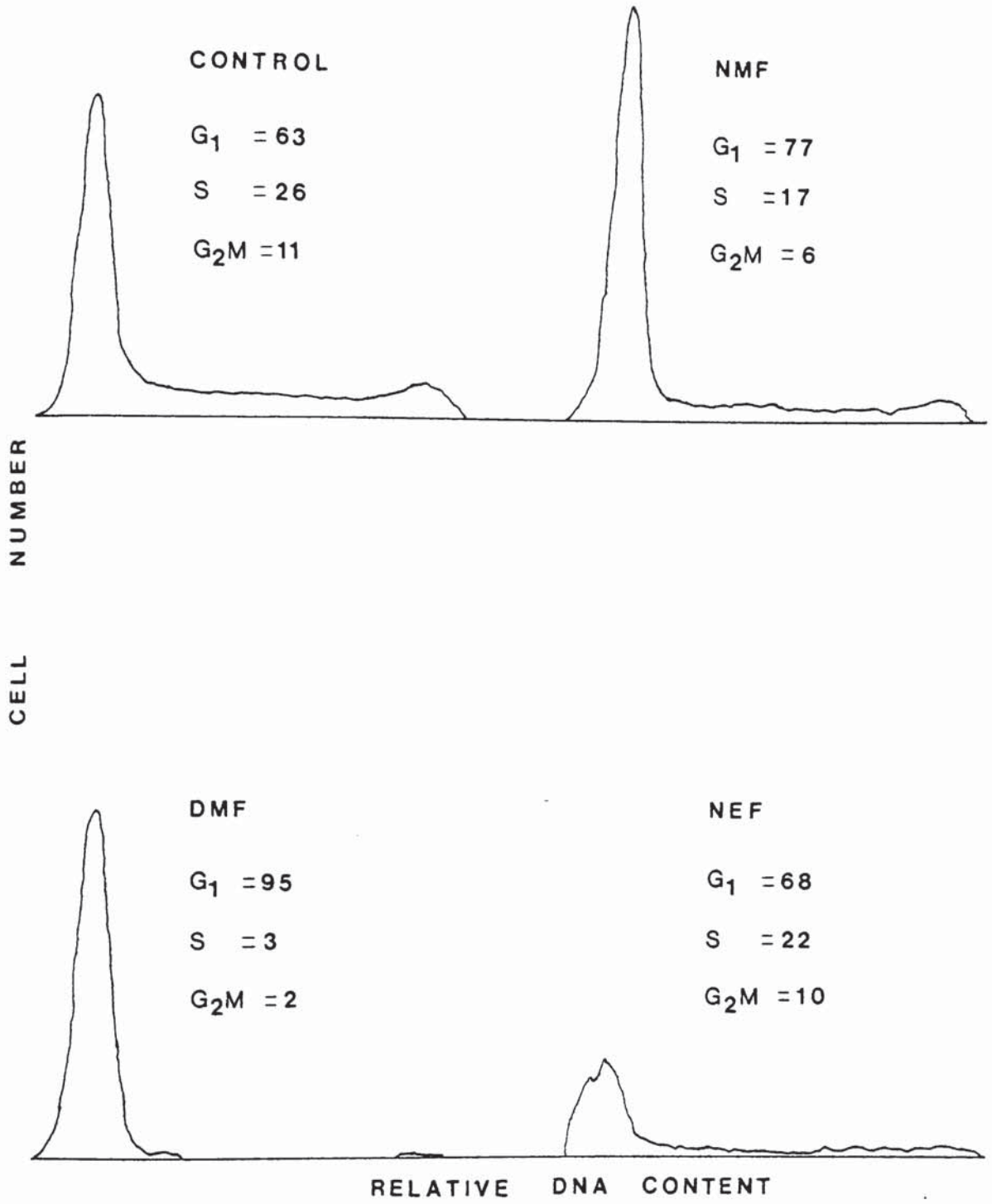


Figure 22 : Representative DNA histograms of cells which had been incubated with NMF, DMF or NEF (106mM) for 48h. Values are expressed as the % of viable cells.

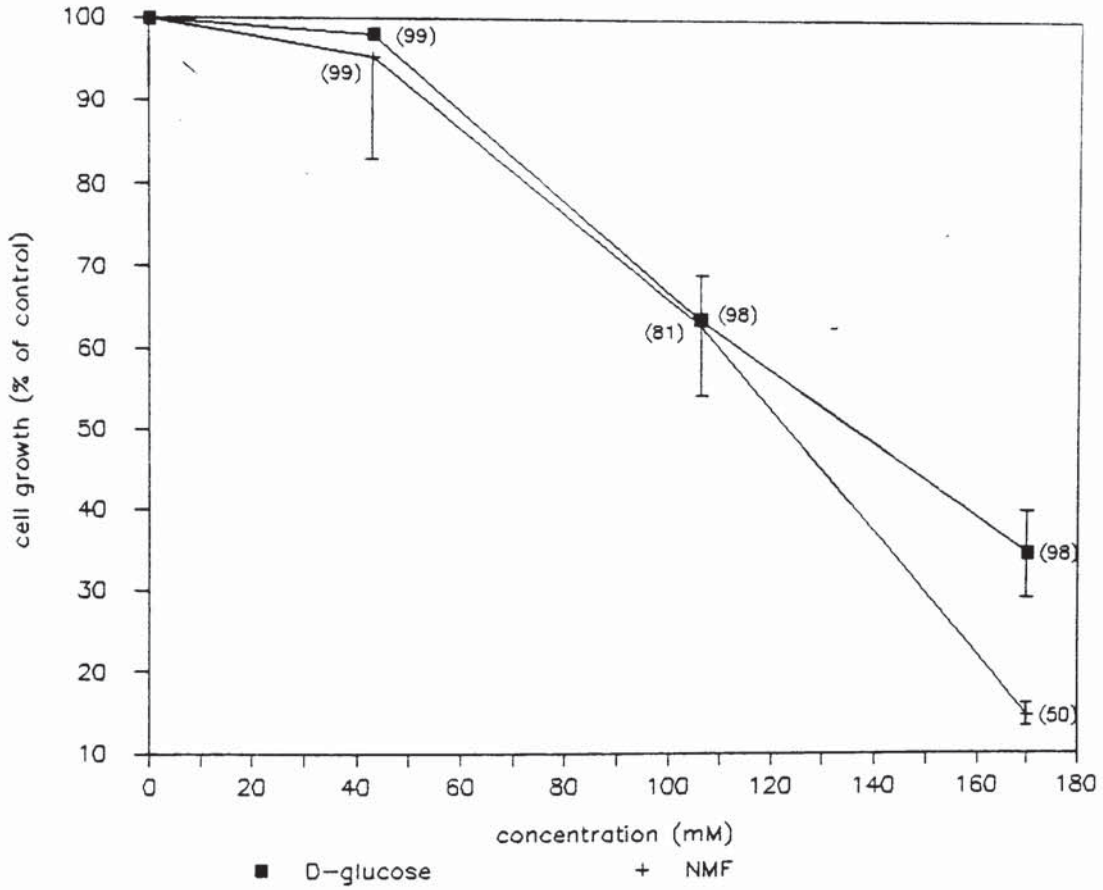


Figure 23 : Comparison of the effects of NMF and D-glucose on cell growth after incubation for 48h. Figures in parenthesis indicate % viability (n=6, ±SD).

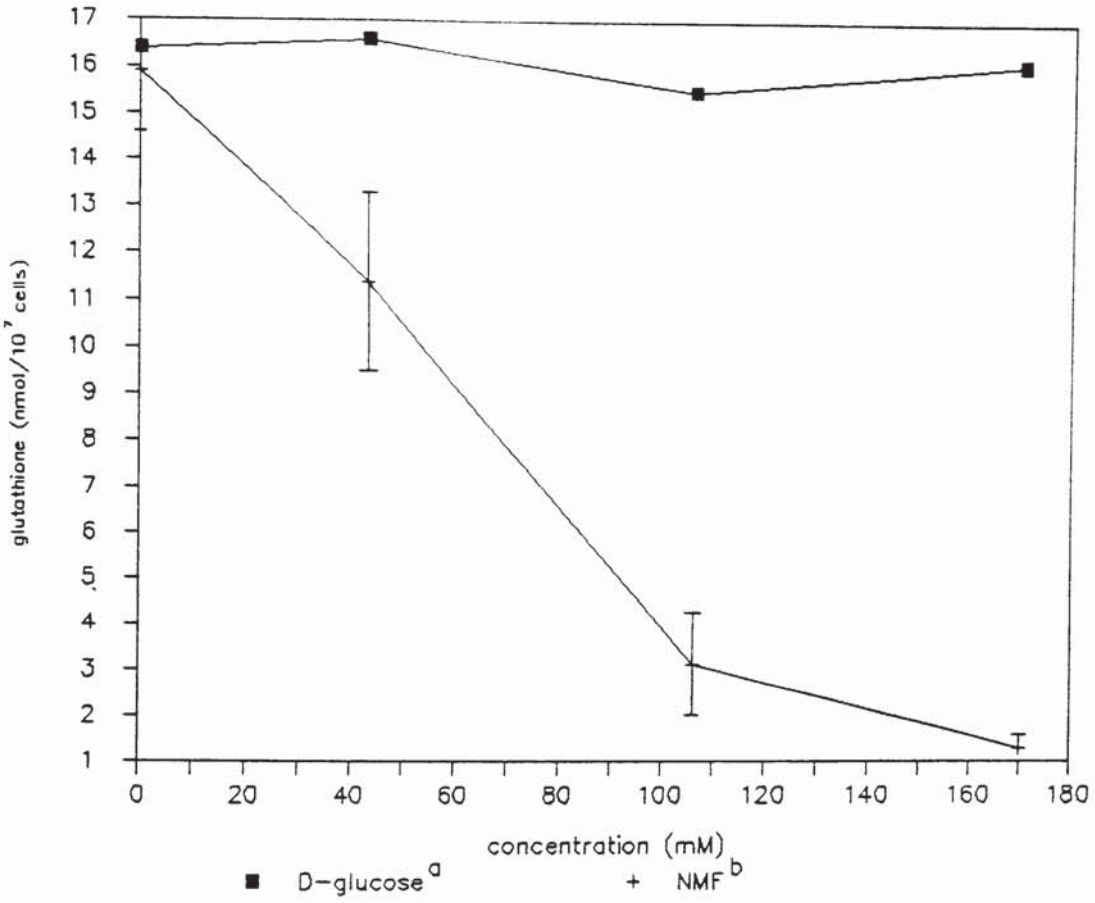


Figure 24 : Concentration dependent effects of NMF and D-glucose on the total intracellular glutathione levels after incubation for 48h . [a] n=2; [b] n=4, ±SD.

concentration (mM)	cell growth (% of controls)		
	NMF	DMF	NEF
43	93 ± 15 (98)	77 ± 14 (86)	46 ± 5 (80)
106	54 ± 5 (82)	15 ± 2 (57)	7 ± 1 (34)
170	16 ± 1 (48)	6 ± 1 (21)	6 ± 1 (30)

Table 7 : Comparison of the effects of NMF, DMF and NEF on cell growth after 48h incubation (n=4, ±SD). Figures in parenthesis are % viability.

4.VI The role of NMF metabolism in the in vitro cytotoxicity of NMF to TLX5 cells

4.VI.i Introduction

The concentration of NMF used in these studies (43 to 170mM) are considerably higher than the plasma concentration of NMF (7mM) found in mice after administration of an effective antitumour dose (400 mg/Kg) (Brindley et al, 1982). It is not known whether the activity of NMF in vitro is due to the parent drug or to a metabolite(s). Certainly in vivo the antitumour activity of NMF is thought to require metabolism (see section 1.V). NMF is metabolized in vivo by oxidation and conjugation with GSH and subsequent conversion to N-acetyl-S-(N-methylcarbamoyl)cysteine (Kestell et al, 1986b). Cleavage of the formyl C-H bond of NMF was shown to be the rate limiting step in this bioactivation route (Threadgill et al, 1986). In the studies of Threadgill et al, (1986) the formyl deuterated isotopomer of NMF (d-NMF) was used. The C-D bond of d-NMF was found to be metabolically more stable than the equivalent C-H bond of the NMF molecule. Comparison of the effects of NMF with that of d-NMF on GSH levels in TLX5 cells could enable an assessment of the importance of the formyl C-H bond fission for GSH depletion caused by NMF in this cell line.

This study also incorporated the use of [^{14}C] methyl labelled NMF ($^{14}CH_3NMF$) in order to establish whether there was metabolism of NMF.

4.VI.ii Results

TLX5 cells were incubated with NMF or d-NMF (106mM) over a 72h exposure period and the total intracellular glutathione content was compared with that of the control cells. NMF and d-NMF produced very similar profiles of glutathione depletion with time (figure 25).

To investigate if NMF was metabolized by TLX5 cells *in vitro*, cells were incubated with 106mM ($^{14}CH_3NMF$) (5uCi) for 48h. In another flask 106mM ($^{14}CH_3NMF$) (5uCi) was added to medium alone. At the end of the exposure period a saponin solution (40ug/ml) was added to the cultures to lyse the cells. Extracts of the medium were chromatographed, followed by autoradiographic analysis of the TLC plate. For both the TLX5 cell sample and the sample which contained medium alone, to which the radioactive drug had been added, there was one major component and three components in minute quantities, in both cases the Rf values were identical (figure 26).

4.VI.iii Discussion

The similarity of the GSH depletion caused by both NMF and d-NMF (figure 25), indicates that fission of the formyl C-H bond of NMF is not the rate limiting step for the fall of intracellular GSH in these cells. NMF does not react chemically with GSH and *in vivo* the prelude to conjugation with GSH is oxidation of the formyl carbon of NMF (Kestell *et al*, 1986b). The results suggest that NMF

conjugation with GSH is unlikely to occur *in vitro* in TLX5 cells.

The question remains unresolved, what mechanism is responsible for the depletion of intracellular TLX5 cell GSH by NMF. NMF does not cause an increase in GSSG (see section 4.III) and does not appear to undergo conjugation with GSH. The most likely explanation for the fall in GSH is leakage from the cells into the medium (see section 4.I).

No discernible metabolites could be identified on incubation of TLX5 cells with NMF (figure 26). It therefore appears that the *in vitro* effects of NMF are due to the parent drug itself and not a metabolite. If NMF is the active species *in vitro* and a metabolite is the active moiety *in vivo*, this could explain the difference in the concentration of NMF required for optimal growth inhibition *in vitro* and antitumour activity *in vivo*.

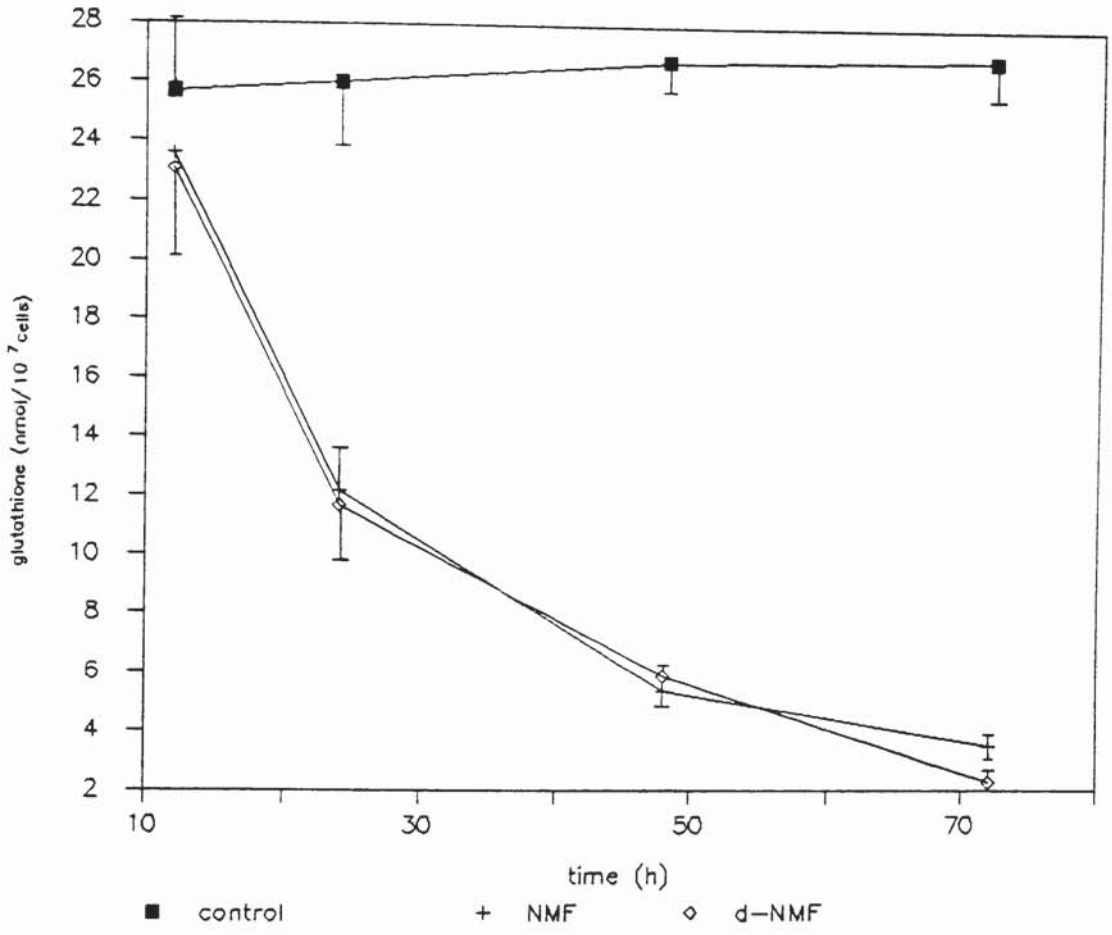


Figure 25 : Time course of the fall in the total intracellular glutathione levels induced by NMF or d-NMF (106mM) (n=4, ±SD).

Figure 26 : Autoradiograph of a TLC plate after incubation of cell culture or medium alone with 106mM $^{14}CH_3NMF$ for 48h.

PART TWO Studies of the effects of NMF on the TLX5 lymphoma *in vivo*.

SECTION 5: RESULTS AND DISCUSSION

SECTION 5: RESULTS AND DISCUSSION

5.I Studies on the antitumour activity and ultrastructure of the TLX5 lymphoma after administration of NMF

5.I.i Introduction

NMF has good antitumour activity against a number of murine tumours, including the TLX5 lymphoma. NMF also has antitumour activity against some human tumour xenografts (see section 1.IV). In this investigation the effects of NMF on the tumour volume of the TLX5 lymphoma and animal survival times were studied after the administration of one or two doses of NMF ip. Also the effects of NMF on the TLX5 lymphoma ultrastucture were investigated by transmission electron microscopy, as a comparison to the observed effects *in vitro*.

5.I.ii Results

The effect on the TLX5 tumour volume of a single ip injection of various doses of NMF is shown in figure 27. NMF caused a dose dependent regression of the TLX5 lymphoma; the minimum tumour size was measured 48h after administration of 200 to 800 mg/Kg NMF. At this time there was a significant difference in the tumour volume of NMF treated mice compared with control animals for 200 mg/Kg ($p < 0.01$)and for 400 and 800 mg/Kg NMF ($p < 0.001$). There was no significant difference in the tumour volume

regression caused by 400 or 800 mg/Kg NMF during the first 48h after injection of drug, but the recovery of tumour growth was slower at the higher dose. For each dose level of NMF the tumour volume increased after reaching the minimum size 48h after drug administration and eventually led to the death of the animals.

400 mg/Kg NMF was an effective antitumour dose and this was used for later studies. The effect on the TLX5 lymphoma of a second dose of 400 mg/Kg NMF administered 24h after the first, was compared with that for a single dose (figure 28). The two dose regimen caused a greater tumour regression than the single dose with a maximum response 48h after the second dose at which time there was a significant difference ($p < 0.005$) from the tumour volumes of the mice which had received a single dose of NMF. The survival times of these animals is shown in figure 29. The values of T/C x100% for one and two doses of 400 mg/Kg NMF were 124 and 140 respectively.

Changes in the ultrastructure of the TLX5 lymphoma was studied by electron microscopy 24, 48 and 72h after mice had been injected with a single dose of 400 mg/Kg NMF (figure 30). The tumour had evidence of necrosis and apoptosis in the control samples, but increased cell death was observed in the NMF treated samples at each time interval after drug administration. There was, however, no difference in the extent of cell death at each time point examined after the administration of NMF. Typical necrotic cells in the early stages of cell death showed

swelling of cellular components, with chromatin flocculation and in the later stages progressive structural disintegration with chromatin changes of pyknosis, karyorrhexis and karyolysis. The apoptotic cells were more rounded than the viable tumour cells and the organelles remained intact, chromatin margination was also observed, the nucleolus fragmented with nuclear invaginations. Apoptotic bodies were also evident. A number of apoptotic cells were in the process of being phagocytosed by neighbouring tumour cells.

5.I.iii Discussion

A single ip dose of NMF caused a dose dependent regression of the TLX5 lymphoma (figure 27). The antitumour response was enhanced by a second dose of NMF injected 24h after the first (figure 28) which led to an increase in survival times of the mice (figure 29). The T/C x100% values (124 and 140) for one and two doses of 400 mg/Kg NMF respectively compare with the T/C x100% value of 178 for the 5 dose regimen of 400 mg/Kg NMF employed by Gate et al, (1986). Tumour regression was also reported for the murine M5076 reticulum cell sarcoma after administration of 200 mg/Kg NMF for ten days (Langdon et al, 1985c).

Tumour regression of the TLX5 lymphoma induced by NMF was due at least in part to cytotoxicity and cell death was evident on examination of the TLX5 lymphoma ultrastructure by electron microscopy, after

administration of 400 mg/Kg NMF (figure 30). Extensive necrosis and apoptosis were both present in the tumour samples from mice treated with 400 mg/Kg NMF, cell death was also observed for the control tumours, but to a lesser extent than the NMF treated samples. Evidence of apoptotic cells in vivo was different to the mode of cell death observed when TLX5 cells were incubated with NMF in vitro. Cell death of tumour cells from control mice was not surprising since solid tumours contain a proportion of dead cells, apoptosis being the major mode of cell death in solid tumours in vivo (Searle et al, 1982). Apoptosis is a significant factor in normal tissue regulation but it is increased by a number of cancer chemotherapeutic agents (Wyllie et al, 1980) which cause cytotoxicity. An earlier study of the antitumour activity of NMF against the sarcoma 180 showed evidence of cell swelling and pyknosis, typical of necrosis, which became progressively more pronounced with time, when 500 mg/Kg NMF was administered daily (Clarke et al, 1953).

The TLX5 lymphoma is sensitive to a number of cancer chemotherapeutic agents, other than NMF. In particular the TLX5 lymphoma has previously been shown to be sensitive to nitrosoureas (Gibson and Hickman, 1982), imidazotetrazines (Langdon et al, 1985b) and triazenes (Gescher et al, 1981).

The results indicate that NMF is an effective antitumour agent in the protocol used. A single ip dose of NMF (200 to 800 mg/Kg) caused tumour regression of the

TLX5 lymphoma and cell death occurred by necrosis and apoptosis.

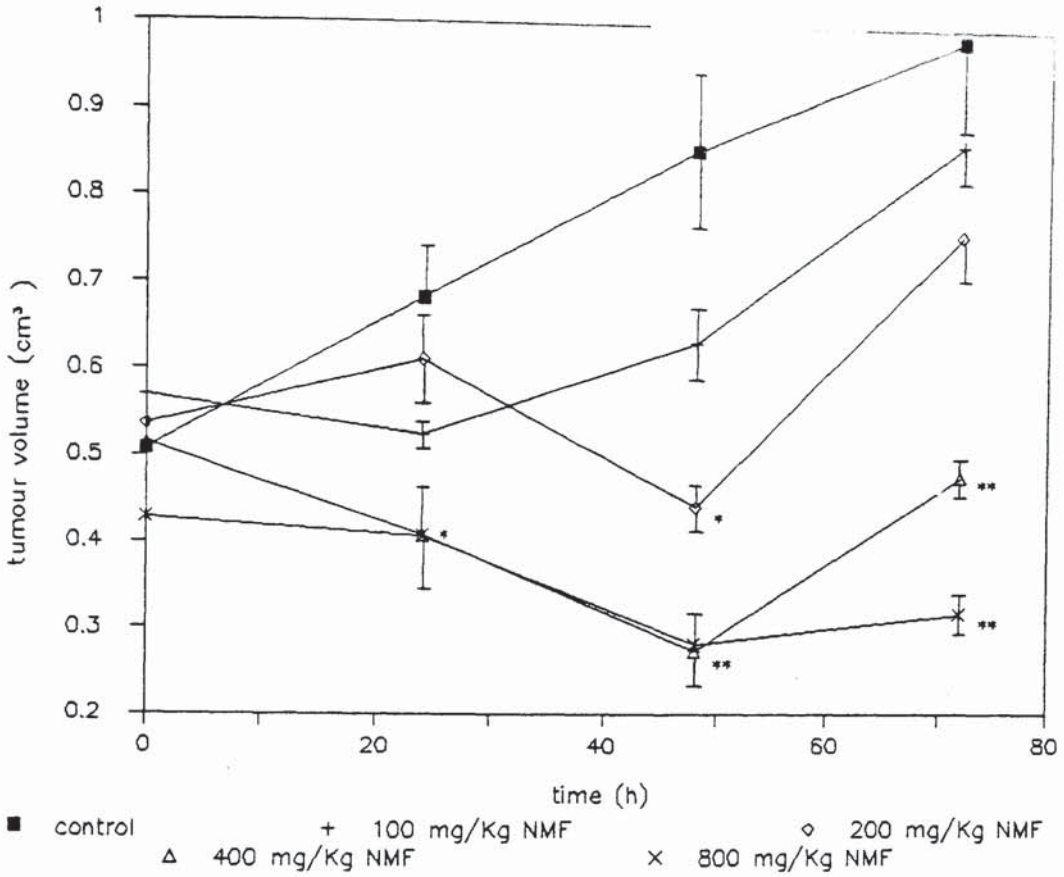


Figure 27 : Dose dependent effects of a single ip injection of NMF on TLX5 tumour volume (n=5, ±SE) * indicates that values are significantly different from control tumour volume $p < 0.01$ or ** $p < 0.001$ (student's t-test).

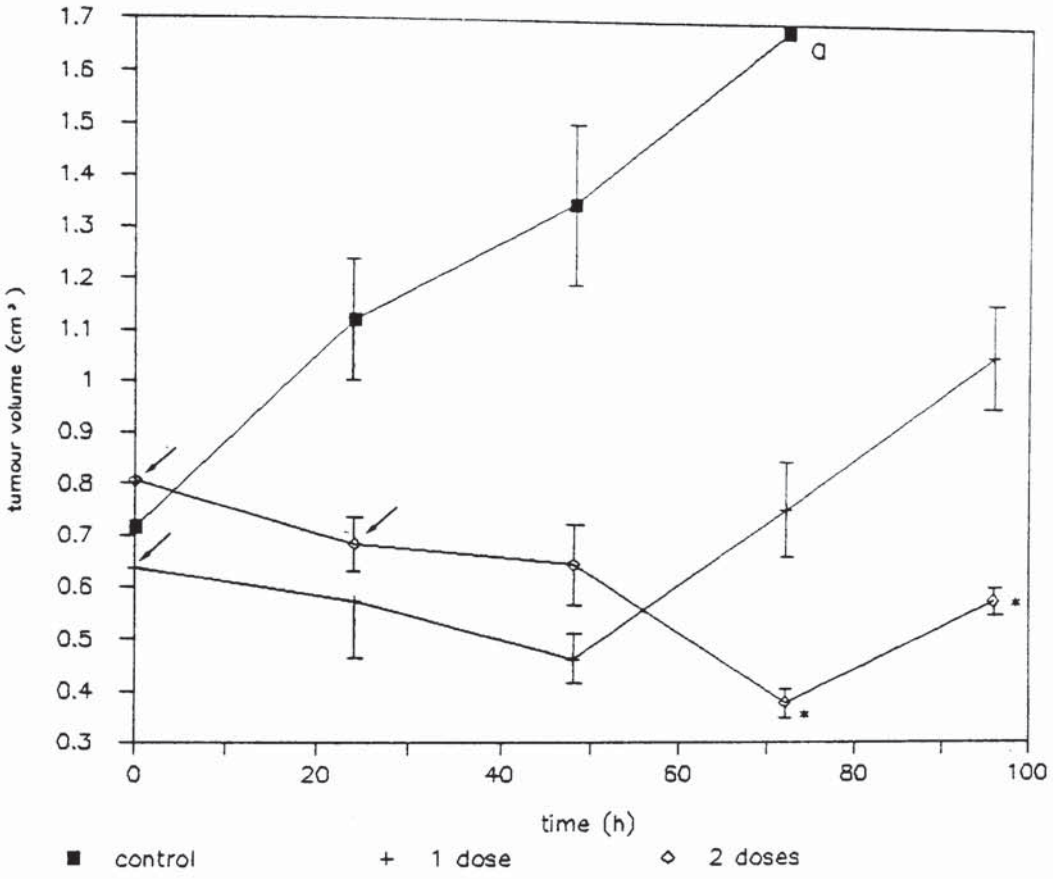


Figure 28 : Time dependent effects of one or two doses of 400 mg/Kg NMF ip on the TLX5 tumour volume. n=5, ±SE except, [a] n=1; * indicates values are significantly different from one dose $p < 0.005$ (student's t-test) / = injection of 400 mg/Kg NMF ip.

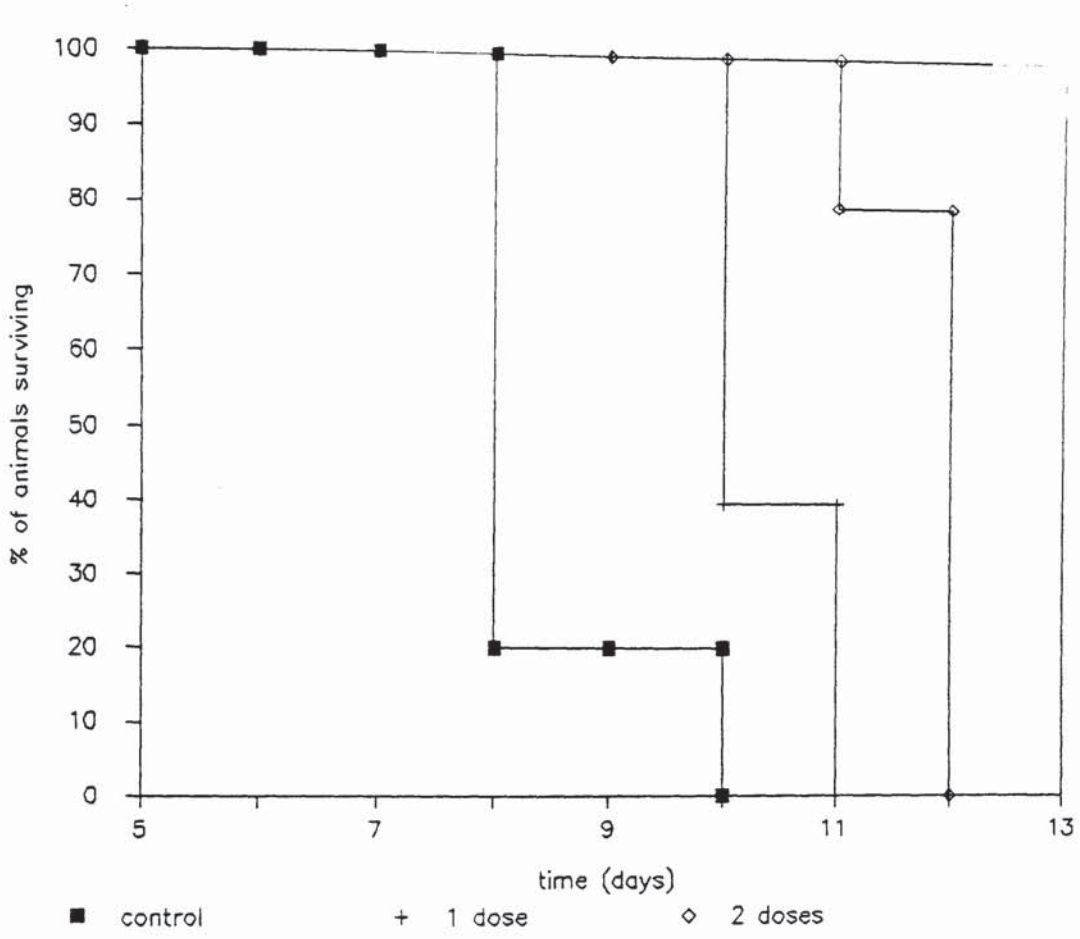
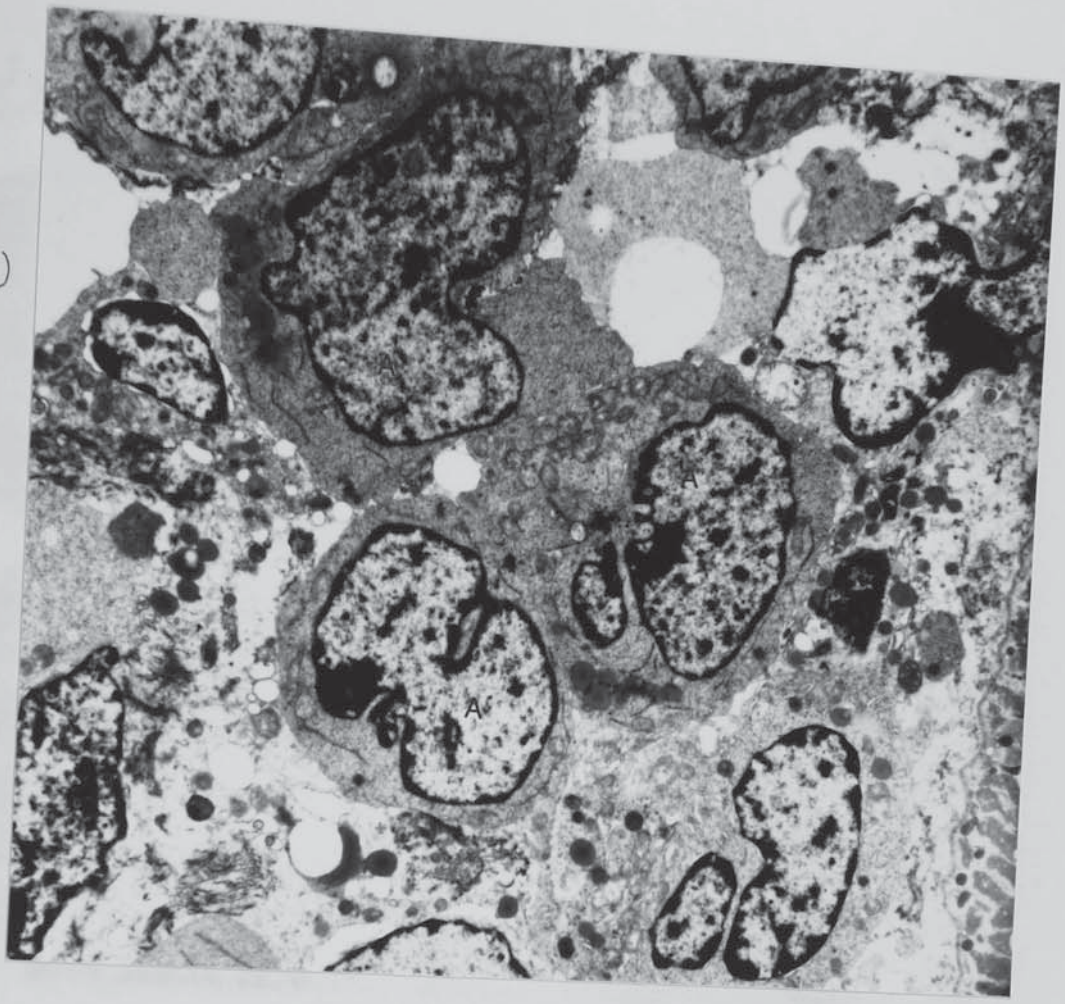


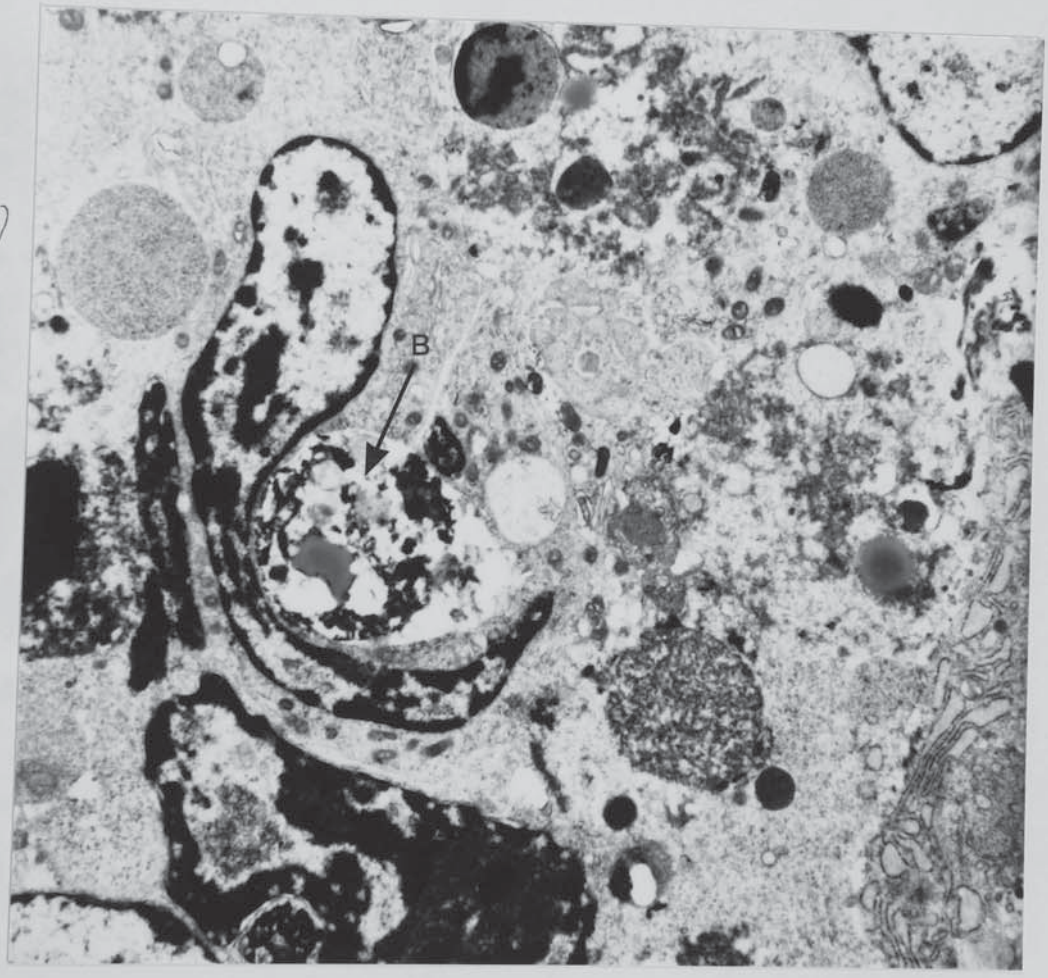
Figure 29 : Survival times of mice after the implantation of 1 x 10^7 TLX5 cells sc in the inguinal region, treated with one or two doses of 400 mg/Kg NMF ip on day 5 or days 5 and 6 respectively (n=5).

Figure 30 : Transmission electron micrographs of sections from the TLX5 lymphoma (a) control (magnification x4,600), (b) control (magnification x6,900), (c) 24, 48 or 72h after administration of 400 mg/Kg NMF ip (magnification x6,900), (d) 24, 48, or 72h after administration of 400 mg/Kg NMF ip (magnification x4,600). A=intact cells; B=apoptotic cell being phagocytosed by a neighbouring cell; C=early stage of apoptosis; D=karyorrhexis; E=pyknosis; F=widespread cell death.

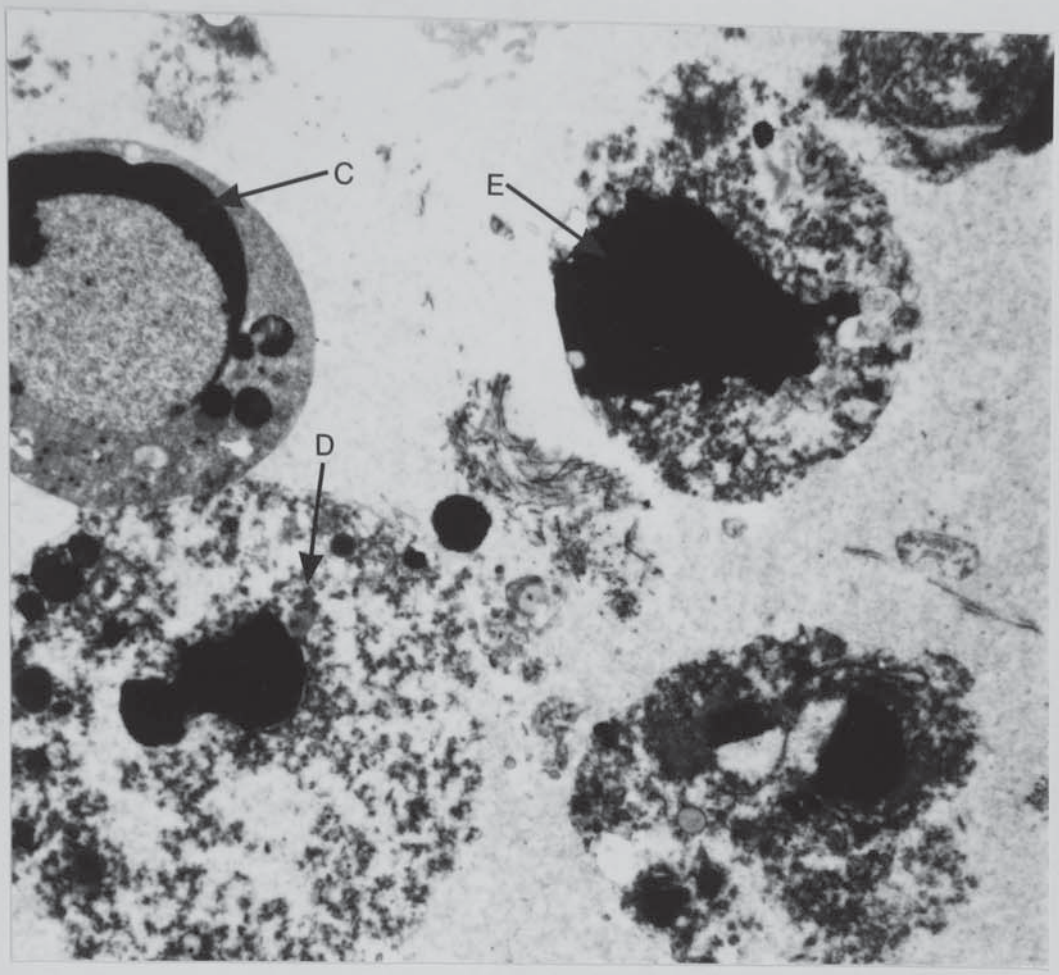
(a)



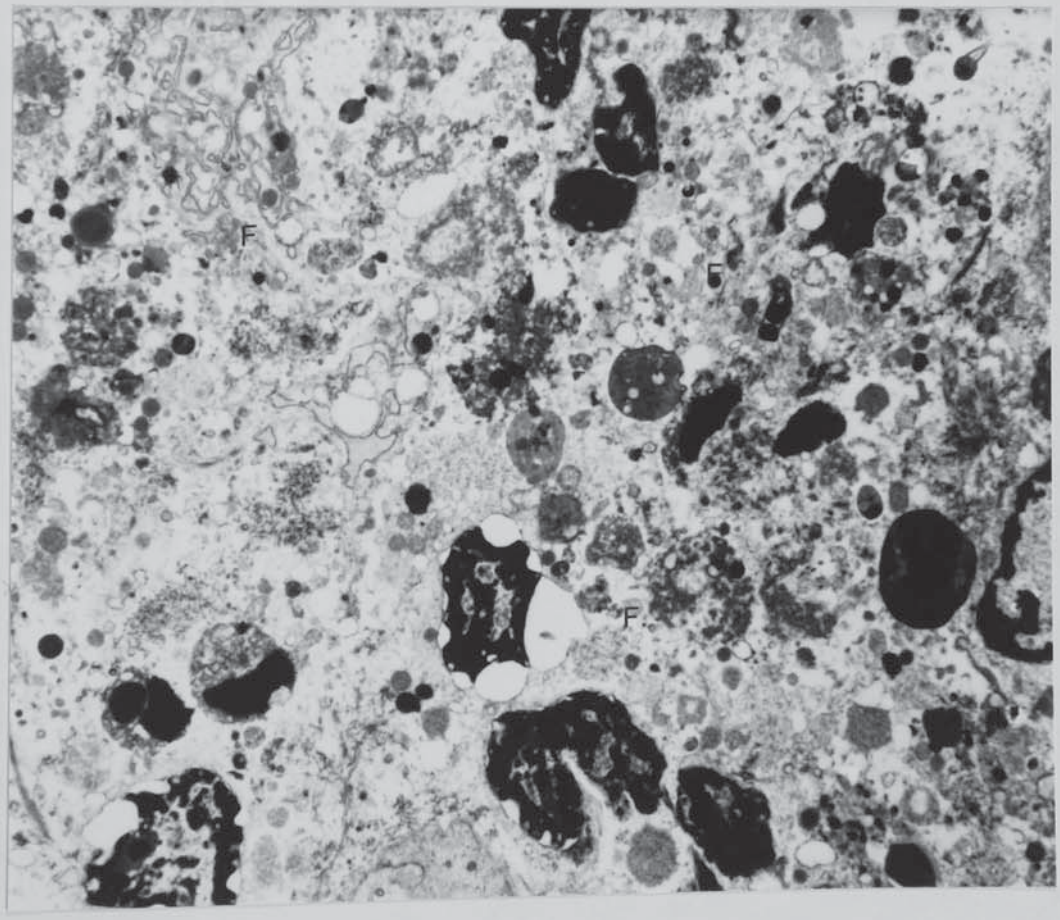
(b)



(c)



(d)



5.II Studies on the effects of NMF on the cell cycle of the TLX5 lymphoma

5.II.i Introduction

TLX5 cells which were incubated with NMF *in vitro* were arrested in the G_1 phase of the cell cycle, which was at variance with the observations for the DLD-1 clone A cells, for which all phases of the cell cycle were affected equally by NMF (see section 4.II). There is however a paucity of information on the effects of NMF on the cell cycle of tumours *in vivo*. Therefore the effects of NMF on the cell cycle kinetics of the TLX5 lymphoma were investigated.

5.II.ii Results

An effective antitumour dose, when administered as a single ip injection, was 400 mg/Kg NMF (see section 5.I). This dose was administered to female CBA/CA mice which had been implanted with 1×10^7 TLX5 cells 5 days earlier and the time dependence of the effect of NMF on the cell cycle kinetics of the TLX5 cells was analysed (figure 31). There was an increase in the proportion of cells in the S phase, with a concomitant decrease in the G_1 phase population, there was no change in the number of cells in G_2M. The accumulation of cells in early S phase reached a maximum 24h after administration of NMF, there was a 19% increase in the S phase population compared to control cells at this time. 48h after drug administration the

cell cycle distribution was similar to controls. Representative DNA histograms of control tumour cells and cells 24h after injection of 400 mg/Kg NMF are shown in the inset of figure 31.

The administration of a single ip dose of NMF elicited a dose-dependent decrease in the volume of the TLX5 lymphoma (see section 5.I). In order to investigate whether there was a dose-dependent accumulation of TLX5 cells in S phase after treatment with NMF, mice were injected with a single dose of drug and cells were analysed 24h later (figure 32). There was no correlation between the dose of NMF and accumulation of cells in S phase. The number of cells in S phase reached a peak at a dose of 200 mg/Kg NMF and there was a 5% decrease in the mean proportion of cells in S phase at the more effective antitumour dose of 800 mg/Kg.

To investigate whether there was a cumulative increase in the S phase population of cells, a repeat dose of NMF was administered. Mice were injected with 400 mg/Kg NMF and 24h later a second dose was administered, the time dependent effect on the TLX5 cell cycle was analysed after the second dose (figure 33). Cells were observed traversing the S phase 2h and 8h after the second dose; by 24h the overall S phase population had decreased to levels that were similar to control cells, there was however, a minor accumulation of cells in early S phase.

A common side effect of cancer chemotherapeutic agents is their toxicity to the bone marrow; NMF is

unusual in that it is not myelosuppressive at optimal antitumour doses in mice (Langdon et al, 1985a; Langdon et al, 1985c). In order to investigate whether the absence of myelosuppression correlated with a lack of effect on the cell cycle, the action of 400mg/Kg NMF on bone marrow cell cycle was investigated 24h after injection (table 8). There was no significant cell cycle perturbation of the bone marrow cells.

The cell cycle data shown in figures 31 to 33 and table 8 were obtained by FCM. The rise in the number of TLX5 cells in early S phase after treatment with NMF could be the consequence of either inhibition or an increase in DNA synthesis. To resolve which of these two alternatives was taking place, the incorporation of thymidine into cells was investigated. Mice were injected with 400 mg/Kg NMF; 24h later the TLX5 tumours were removed and a single cell suspension was prepared. Cells were then incubated for 30 min with [^{3}H] thymidine (7.5uCi). The amount of radioactivity incorporated into the TLX5 cells was measured by a scintillation counter. Cells from the same sample were fixed onto slides and the thymidine labelling index (TLI) was measured by autoradiography. The extent of DNA synthesis as measured by these two techniques in the NMF treated cells was 2.2 or 2.7 times greater than that seen in control cells for TLI and amount of radioactivity incorporated respectively (table 9). The results indicate that the S phase accumulation of the TLX5 cells after administration of NMF was due to an increase

in DNA synthesis, rather than an inhibition.

5.II.iii Discussion

A single dose of 400 mg/Kg NMF ip caused an increase in the number of TLX5 lymphoma cells in the S phase, the maximum effect was observed 24h after injection of NMF (figure 31). The increase in the S phase population of cells was not related to the dose of NMF administered (figure 32) and therefore the cell cycle effect was not directly proportional to the antitumour response (see section 5.I).

The increase in the population of TLX5 cells in the S phase of the cell cycle after administration of NMF *in vivo* is in contrast to the early G_1 phase arrest of TLX5 cells observed when cells were incubated with NMF *in vitro*. The difference between the activity of NMF on the cell cycle *in vitro* and *in vivo* suggests that different mechanisms of action may be occurring in each case.

The accumulation of cells in S phase occurs in response to a number of anticancer agents that inhibit DNA synthesis: for example nucleotide analogues such as 5-aza-2'-deoxycytidine (Chabot and Momparler, 1986), cytosine arabinoside (Waldman *et al*, 1985) and 2-bromo-2'-deoxyadenosine (Huang *et al*, 1986) or methotrexate which inhibits the de novo synthesis of nucleotides (Bokkerink *et al*, 1986).

The increase in the proportion of TLX5 lymphoma cells in S phase caused by NMF was due to an increase in DNA

synthesis rather than due to inhibition of this process (table 9). The procedure used to measure the incorporation of thymidine required that cells were first disaggregated to a single cell suspension prior to incubation with [^{3}H] thymidine. The *in vitro* labelling method meant that cells were in culture for 1.25h in the absence of NMF or metabolites before analysis of radioactivity. It could be argued that the TLX5 cells were in fact blocked in early S phase and that during the time *in vitro* in which NMF or its metabolites were absent from the medium, that cells were able to reverse the block and commence DNA synthesis. This was postulated by Painter, (1977) to explain why non-DNA damaging inhibitors of DNA synthesis such as hydroxyurea and nickel chloride cause an increase in DNA synthesis after exposure to the agent followed by removal of the drug from the medium. However, recent reports suggest that cells which are blocked in early S phase or at the G_1/S interface require a number of hours after the drug is removed before cells can precede to DNA synthesis. Swiss 3T3 cells blocked at the G_1/S border by hydroxyurea commenced DNA synthesis 5h after the drug was removed from the medium (Das, 1981). A multienzyme complex is thought to be required for DNA synthesis which is produced at the start of S phase (Pardee *et al*, 1986), this complex is labile and breaks down when cells are blocked in S phase and several hours are required for cells to re-enter this "committed state" after the drug causing the S phase arrest is removed (Das,

1981).

Antitumour drugs such as methotrexate and 5-fluorouracil which modulate nucleotide pools, can increase the incorporation of [^{3}H] thymidine into tissue 24h after administration of the drug (Lambert and Eriksson, 1979) as observed here with NMF. Agents which damage DNA such as cyclophosphamide, CCNU and chlorambucil cause a decrease in the rate of DNA synthesis (Lambert and Eriksson, 1979; Painter, 1977) and cells usually arrest in the G_2 phase (Charcosset, 1986).

The increase in DNA synthesis of TLX5 lymphoma cells after administration of NMF occurred at a time when there was a decrease in the tumour volume and evidence of widespread cell death (see section 5.I). A similar increase in DNA synthesis occurs in the liver in response to the hepatonecrotic agent, carbon tetrachloride, which is associated with liver regeneration (Higgins et al, 1984). Indeed, an increase in DNA synthesis in the mouse liver following administration of carbon tetrachloride was related to the extent of hepatotoxicity (Doolittle et al, 1987). For TLX5 cells the rate of TLX5 cell death caused by NMF exceeded the increase in proliferation as measured by [^{3}H] thymidine incorporation. [^{3}H] Thymidine incorporation into cells is not, however, necessarily a good indicator of cell proliferation. Beta lactam antibiotics enhanced [^{3}H] thymidine incorporation into K562 erythroleukaemia cells which corresponded to DNA synthesis. However, this increased DNA synthesis occurred

when there was an inhibition of cell proliferation (Cottegnoud and Neftel, 1986).

It has been suggested that incorporation of [^{3}H] thymidine into lymphoid cells can occur when the cells are not in S phase (Neckers et al, 1985). However, in the case of the TLX5 lymphoma cells treated with NMF, [^{3}H] thymidine incorporation corresponded to the S phase as measured by FCM.

At present it is unknown whether the increase in DNA synthesis caused by NMF in vivo is a common effect of the drug on tumours other than the TLX5 lymphoma. The effect of NMF on the cell cycle of a number of tumours is worthy of investigation since actively transcribing DNA is more sensitive to the damaging effects of x-irradiation than inactive DNA (Chiu and Oleinick, 1982) and this may be a contributory factor in the increased radiosensitivity of some tumours after prior administration of NMF (Dexter et al, 1984; Iwakawa et al, 1987).

A second dose of 400 mg/Kg NMF administered 24h after the first did not cause a cumulative increase in the S phase population of TLX5 cells (figure 33). Therefore the biochemical modulation produced by NMF or metabolite(s) may be self-limiting. Alternatively, the TLX5 cells which were recruited into DNA synthesis after the administration of the first dose of 400 mg/Kg NMF may subsequently die and the remaining cells may be resistant to stimulation of DNA synthesis by a second dose of NMF.

The mechanism by which NMF caused an increase in the

DNA synthesis of the TLX5 lymphoma cell is unknown. Earlier studies on the mechanism of antitumour activity of NMF suggested that nucleic acid metabolism was affected by the drug, although it was not clear from these reports how this occurred or what bearing it had on the antitumour response (see section 1.IV).

Many of the agents that cause gene amplification interfere with DNA metabolism (Hamlin et al, 1984). Under cytotoxic conditions accelerated gene amplification can take place : such a cellular response could ostensibly increase the chance of cell survival since it may enhance the probability of producing a cell with drug resistance (Varshavsky, 1983a). Varshavsky, (1983b) suggested that diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) acts as an "alarmone" and is produced in response to premature arrest of a replication fork, caused by lesions in the DNA or by inhibition of enzymes involved in DNA synthesis. The alarmone in turn increases the probability of re-initiation of DNA replication. However, the role of Ap_4A as an alarmone has been questioned recently (Segal and Le Pecq, 1986).

The incorporation of [^{3}H] thymidine into cells can be enhanced by mutagens and carcinogens which produce DNA damage that is often repaired by excision of the affected sites, followed by regeneration of the previously damaged regions by unscheduled DNA synthesis (UDS). If UDS occurred in the TLX5 lymphoma cells when mice were dosed with NMF then non-S phase DNA replication would occur

(Bose and Allison, 1987). Non-S phase DNA synthesis seems unlikely since there was a good correlation between the proportion of S phase cells assessed by FCM and [^{3}H] thymidine incorporation measured as disintegrations per minute or TLI (figure 31 and table 9).

The cell cycle of bone marrow cells was not significantly affected by NMF *in vivo* (table 8). This lack of bone marrow cell cycle perturbation by NMF would be expected since the drug does not cause myelosuppression in mice (see section 5.II.ii).

Overall it can be concluded that NMF treatment causes an initial S phase accumulation of TLX5 lymphoma cells which is due to an increase in DNA synthesis, but this cell cycle perturbation does not correlate with the cytotoxic activity of the drug against this tumour.

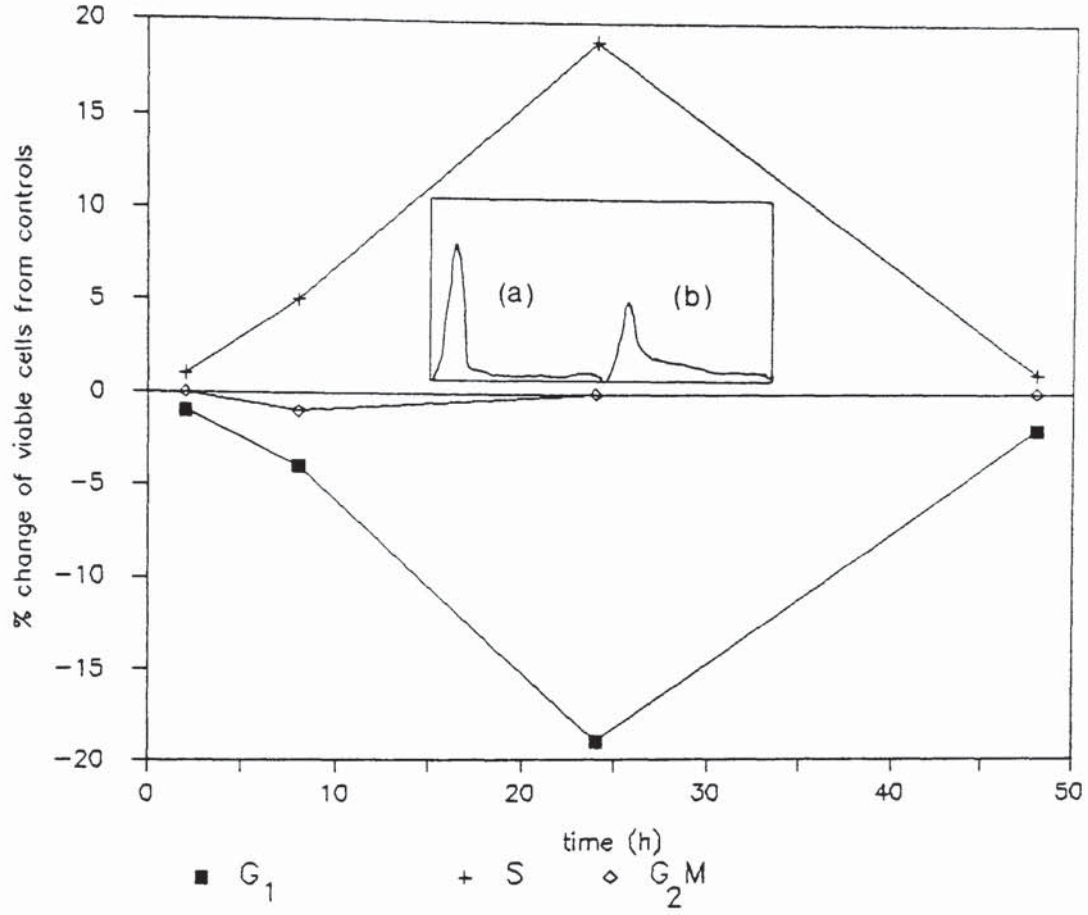


Figure 31 : Time dependent effects of 400 mg/Kg NMF ip on the cell cycle phases of TLX5 lymphoma cells (means of two independent experiments). Representative DNA histograms of (a) control and (b) 24h are shown inset.

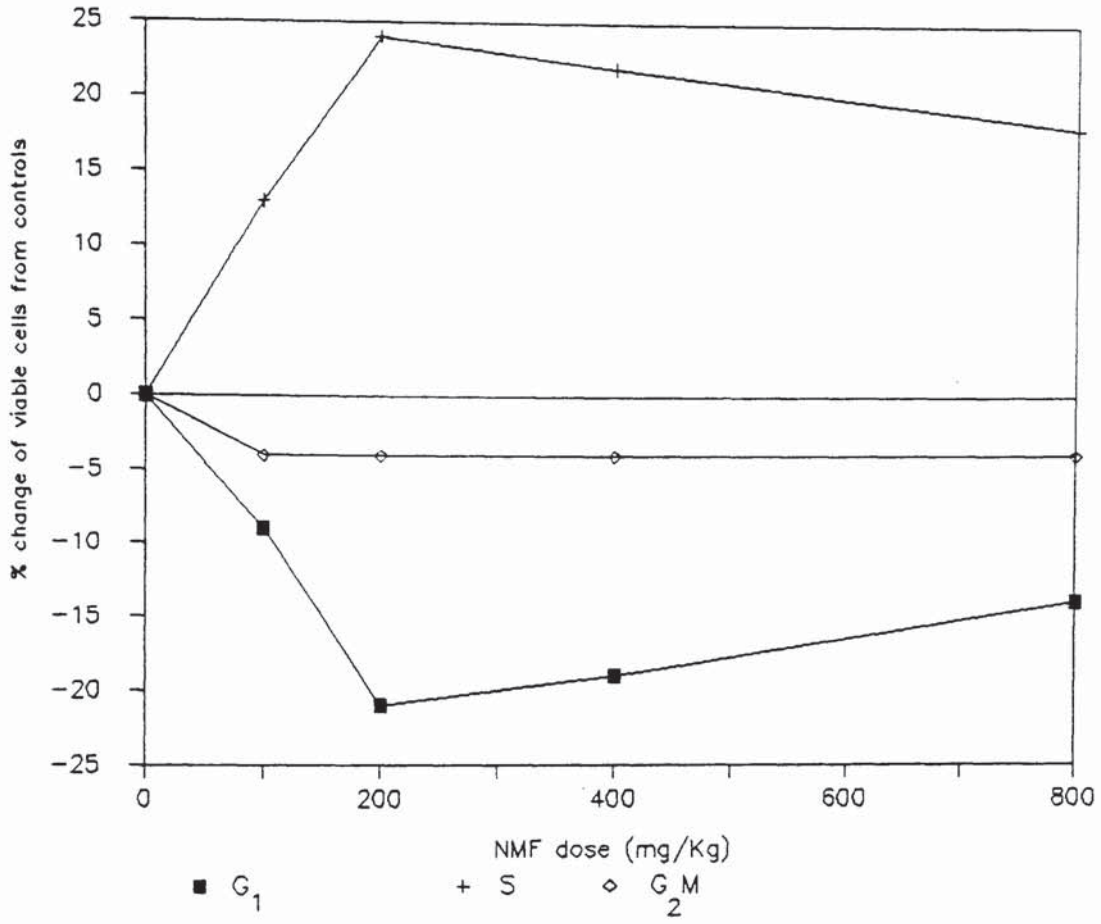


Figure 32 : Dose dependent effects on the cell cycle phases of TLX5 lymphoma cells, 24h after ip administration of NMF (mean of three independent experiments).

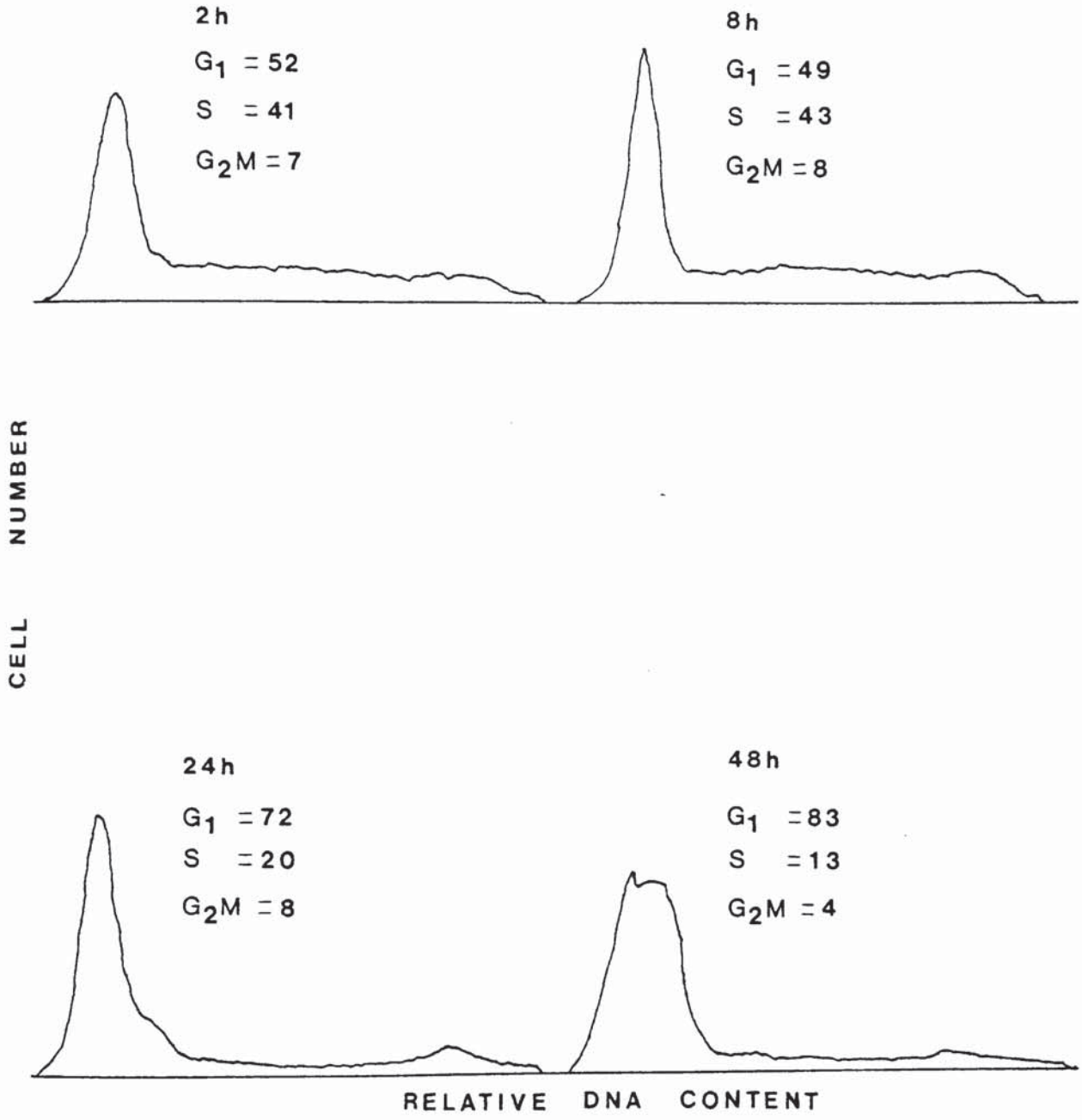


Figure 33 : Representative DNA histograms of TLX5 cells at time intervals after the administration of a second dose of 400 mg/Kg NMF ip injected 24h after the first dose. Values are expressed as the % of viable cells.

sample	G_1	S	G_2M
control	74 ± 2	19 ± 2	7
NMF	77 ± 1	15 ± 1	8 ± 1

Table 8 : Percentage of bone marrow cells in different phases of the cell cycle, 24h after administration of 400 mg/Kg NMF ip (n=3, ±SD).

sample	TLI (%)	counts (DPM)
control	20 ± 2	28090 ± 6049
NMF	43 ± 5	75501 ± 34097

Table 9 : [^{3}H] thymidine incorporation measured by TLI or radioactivity associated with the acid insoluble fraction of 3 x 10^5 cells, 24h after the administration of 400 mg/Kg NMF ip (n=4, ±SD).

5.III Studies on the effects of NMF on the glutathione status of the TLX5 lymphoma

5.III.i Introduction

Glutathione has an important role in a number of cellular functions (see section 1.V). In rodents and man, NMF is oxidized at the formyl carbon causing the formation of a reactive species, possibly methyl isocyanate, which reacts with GSH and is excreted in the urine as N-acetyl-S-(N-methylcarbamoyl)cysteine (see section 1.V).

The hepatotoxicity of NMF in mice corresponded to a decrease in liver GSH levels. The depletion of GSH and the extent of hepatic damage was exacerbated by BSO, while GSH repleting agents abrogated the toxicity (see section 1.V). When NMF metabolism was impaired, the hepatotoxicity and also the antitumour activity of the drug was reduced (Masuda et al, 1986).

When 303 mg/Kg/day NMF was administered by constant infusion, GSH levels were depleted to 22% of control HCT-15 human colon carcinoma tumours after 48h of treatment with NMF which led to a 78% inhibition of tumour growth 7 days after therapy commenced. Coinfusion of 200 mg/Kg/day N-acetylcysteine prevented the NMF mediated loss of GSH, but an effect on tumour growth was not observed (Chatterjee et al, 1987).

This study investigated the effects of NMF on the glutathione status of the TLX5 lymphoma, in order to establish whether there was a relationship between the

ability of this compound to modulate tumour glutathione levels and its antitumour activity.

5.III.ii Results

The time dependent effect on the total glutathione content of the TLX5 lymphoma after a single ip dose of 400 mg/Kg NMF was investigated (figure 34). The nadir of glutathione content was reached 24h after injection of NMF (64.4 ± 4.1% of control glutathione levels), this level of glutathione was maintained for a further 24h. To investigate whether a repeat dose of NMF had a further effect on TLX5 lymphoma glutathione levels a second dose of 400 mg/Kg NMF was administered 24h after the first, no further depletion in the total glutathione content of the TLX5 lymphoma was observed 24h after the repeat dose.

The relationship between the dose of NMF and TLX5 glutathione content measured 24h after drug administration is shown in figure 35. There was no significant difference in the total glutathione levels for the dose range of 100 to 800 mg/Kg NMF.

The effect of 400 mg/Kg NMF on the GSSG content of the TLX5 lymphoma is shown in table 10, there was no significant difference between the NMF treated and control samples. All GSSG values were <3% of the total glutathione levels.

Mice were injected with the GSH synthesis inhibitor BSO (1,600 mg/Kg ip) and the time course of tumour glutathione depletion was measured (figure 36). Depletion

of glutathione reached a minimum 6h after treatment (46.5 ± 7.0% of control glutathione levels) and glutathione depletion was maintained at this value for the length of the study (48h). In order to establish whether prior depletion of GSH enhanced the antineoplastic activity of NMF, 1,600 mg/Kg BSO was administered to mice and 6h later 400 mg/Kg NMF was injected, the tumour volume was then assessed daily (figure 37). BSO treatment alone had no antitumour activity. BSO in combination with NMF produced a small enhancement in tumour regression ($p < 0.05$). However, 400 mg/Kg NMF may not be a suitable dose to compare tumour volumes with or without addition of BSO, since 800 mg/Kg NMF did not exacerbate TLX5 tumour regression in comparison with the lower dose of 400 mg/Kg (see section 5.I). A lower dose of NMF (100 mg/Kg) was therefore administered to mice after an injection of 1,600 mg/Kg BSO 6h earlier. In this case no significant change in tumour volume occurred for control mice, NMF alone or NMF in combination with BSO (figure 38).

5.III.iii Discussion

The total glutathione level of the TLX5 lymphoma was reduced to 64.4 ± 4.1% of control values 24h after a single ip dose of 400 mg/Kg NMF. Depletion of the glutathione to this extent was maintained over the next 24h and was not enhanced by a repeat dose of NMF (figure 34). The profile of glutathione changes in the TLX5 lymphoma is different to those of the liver. The same

dose of NMF (400 mg/Kg) caused a depletion of the hepatic glutathione levels by 59.8%, 1h after administration to Balb/c mice (Gescher et al, 1982) or a lower dose of NMF (200 mg/Kg) depleted hepatic glutathione levels to 53% of control livers 2h after drug administration to CBA/CA mice (Pearson et al, 1987a), the strain of mouse used in these studies. After hepatic glutathione levels had reached a minimum at 2h, in the study of Pearson et al, (1987a) there was a rapid recovery of glutathione to control levels.

The sustained depletion of the TLX5 lymphoma glutathione content caused by NMF could be significant in the antitumour response. Chatterjee et al, (1987) maintained the glutathione levels of the HCT-15 human colon carcinoma tumours at 47% of controls by constant infusion of 151 mg/Kg NMF which caused a 50% tumour growth inhibition after 7 days. However, there was no correlation between the dose of NMF administered and the glutathione content of the TLX5 lymphoma (figure 35), although there is a dose dependent relationship for antitumour activity (see section 5.I). The results for the TLX5 lymphoma suggest that glutathione levels are not an important factor in determining the antitumour activity of NMF against this tumour.

NMF administration did not affect the GSSG levels of the TLX5 lymphoma (table 10), which is in accordance with previous observations for the liver (Pearson et al, 1987a). Although GSSG can be released from tissues (Sies

and Akerboom, 1984), this seems unlikely to account for the results observed.

The glutathione content of the TLX5 lymphoma was depleted to <50% of control tumour levels 6h after administration of 1,600 mg/kg BSO and GSH was maintained at this level for at least 48h (figure 36). A repeat dose of 1,600 mg/Kg BSO is lethal to CBA/CA mice (Chubb D, unpublished). The glutathione content of NFSa tumours decreased to a minimum of 45% and then gradually recovered to 75% of control tumour levels, 12 and 24h after the injection of 5 mmole/Kg (1,112 mg/Kg) BSO ip (Ono et al, 1986). A dose of 2.5 mmole/Kg BSO decreased the GSH levels of 3 murine tumours; minimum levels (55 to 65% of control tumours) were reached 10 to 12h after drug administration. GSH levels recovered to control values by 48h (Lee et al, 1987). Liver and kidney glutathione levels were depleted to 65% of control values at 2 to 4h after administration of 1,600 mg/Kg BSO (Drew and Miners, 1984). The results from these studies show that the changes in GSH content of tumours brought about by BSO administration appear to depend on the tumour cell type, as it does for normal tissues (Ono and Shrieve, 1986). The GSH depletion in the TLX5 lymphoma caused by BSO or NMF may reflect a slow metabolic turnover of the non-protein thiol in this tumour.

The inhibition of glutathione biosynthesis by BSO did not effect the growth of the TLX5 lymphoma; it also had no significant effect on the activity of 100 mg/Kg NMF but

did marginally augment the antitumour response of 400 mg/Kg NMF (figures 37 and 38). The results contrast with the significant delay in the growth of EMT6 mammary tumours after a single dose of 4mmole/Kg BSO (Miller and Henderson, 1986) and the significant cell kill of this tumour by the cytotoxic agents cyclophosphamide and mitomycin c after administration of 5mmole/Kg BSO (Ono and Shrieve, 1986). However no direct correlation was found between tumour glutathione content and the effects on EMT6 tumour cell growth (Miller and Henderson, 1986).

In conclusion, the results indicate that NMF causes extended depletion of GSH in the TLX5 lymphoma; this manifestation alone, however, does not correlate with the antitumour response to the drug. The results are different to those reported for the liver, for which glutathione depletion caused by NMF was proportional to the degree of hepatotoxicity (Pearson et al, 1987a). The results are in accordance with the suggestion that the biochemical mechanisms which convert N-alkylformamides into heptotoxins are different from those mechanisms which are responsible for the antitumour activity, although metabolism is required for both hepatotoxicity and the antineoplastic activity (Kestell et al, 1987; Masuda et al, 1986).

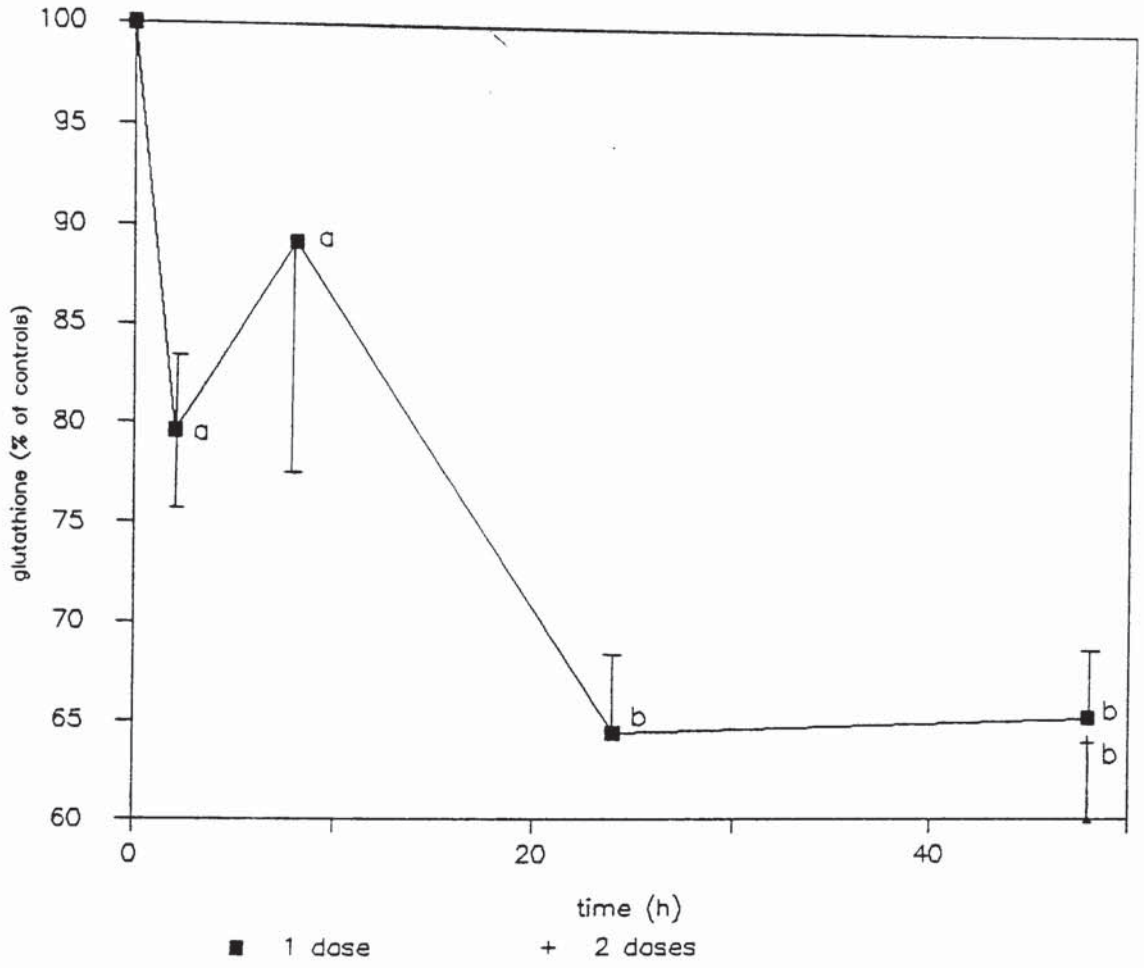


Figure 34 : Time dependent effects of one or two doses of 400 mg/Kg NMF ip on the total glutathione content of the TLX5 lymphoma. [a] n=3, ±SE; [b] n=5, ±SE.

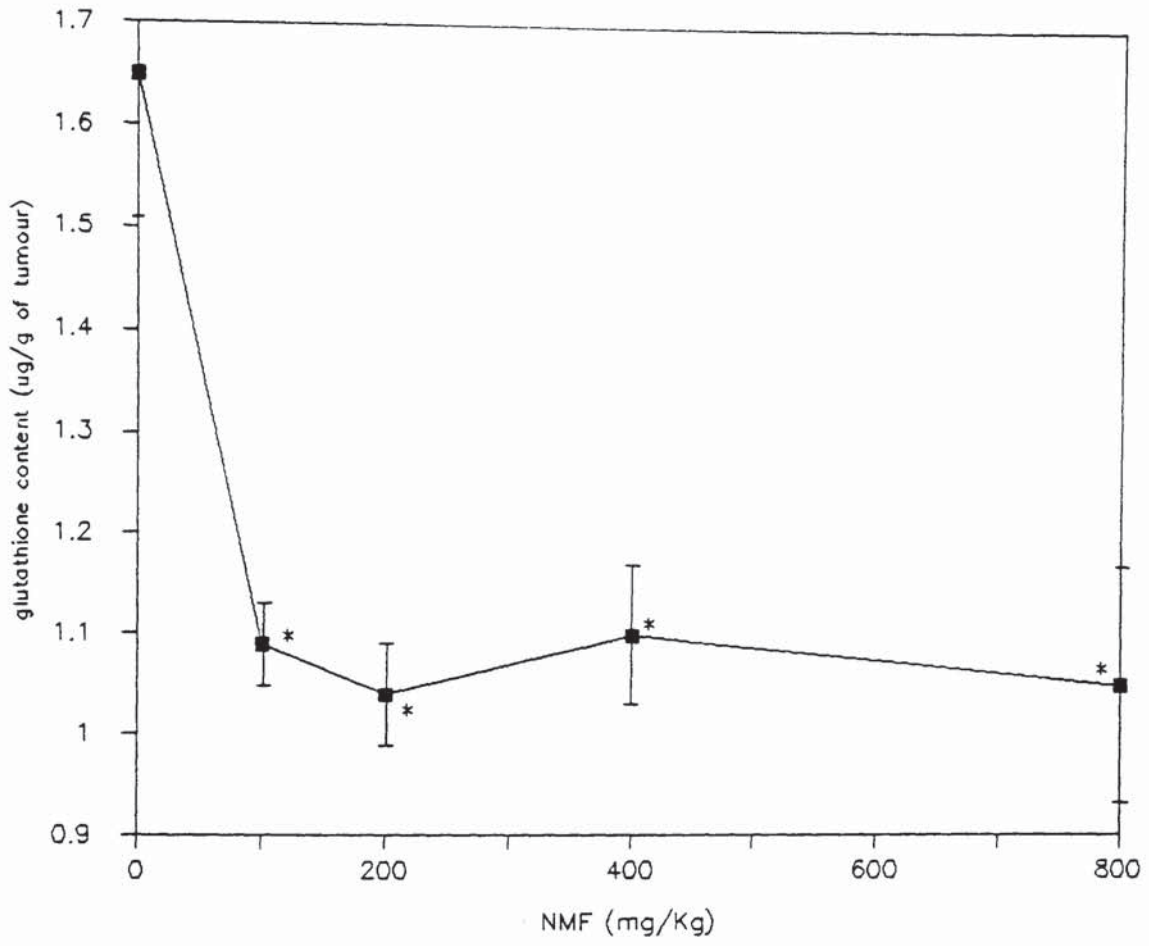


Figure 35 : Dose dependent effects of NMF on the total glutathione content of the TLX5 lymphoma after ip administration (n=3, ±SE); * indicates that values are significantly different from control glutathione levels $p < 0.05$ (student's t-test).

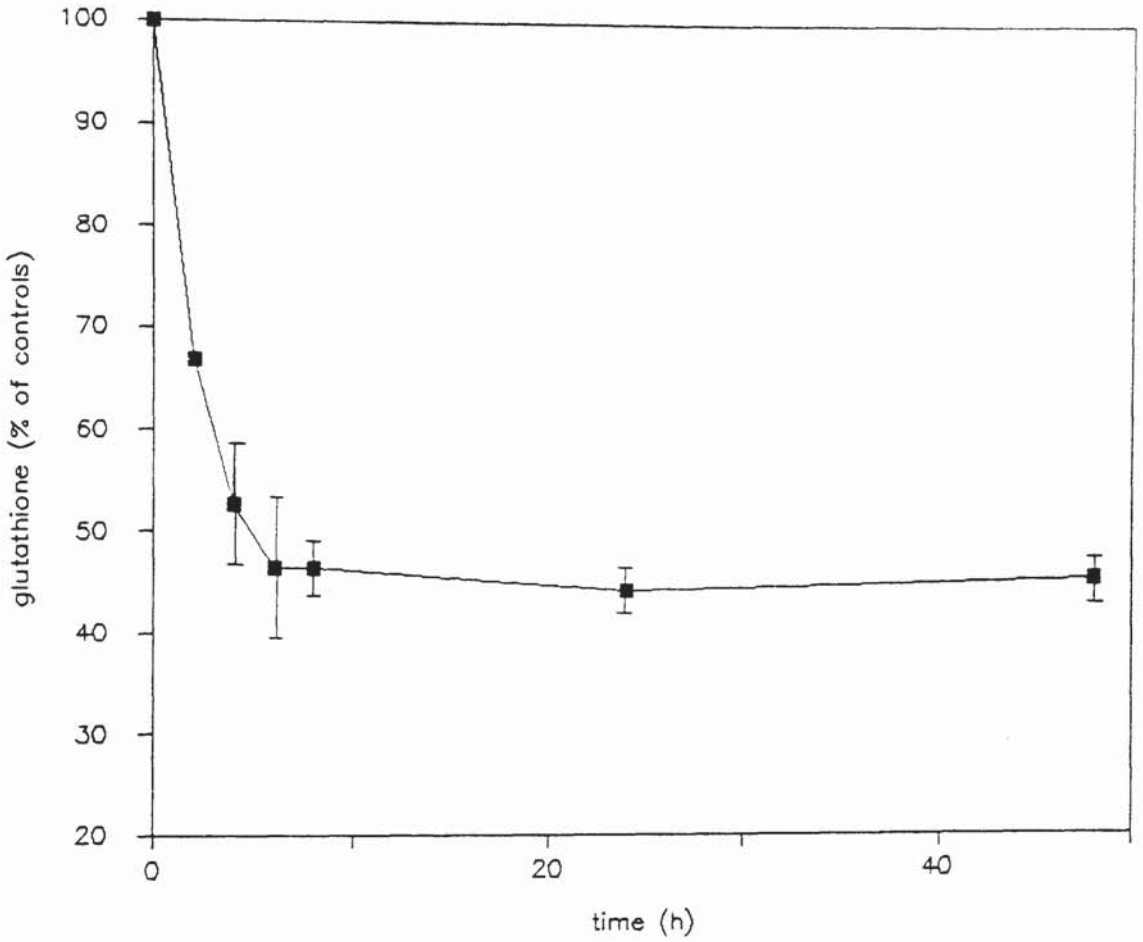


Figure 36 : Time dependent effects of 1600 mg/Kg BSO ip on the total glutathione content of the TLX5 lymphoma (n=3, ±SE).

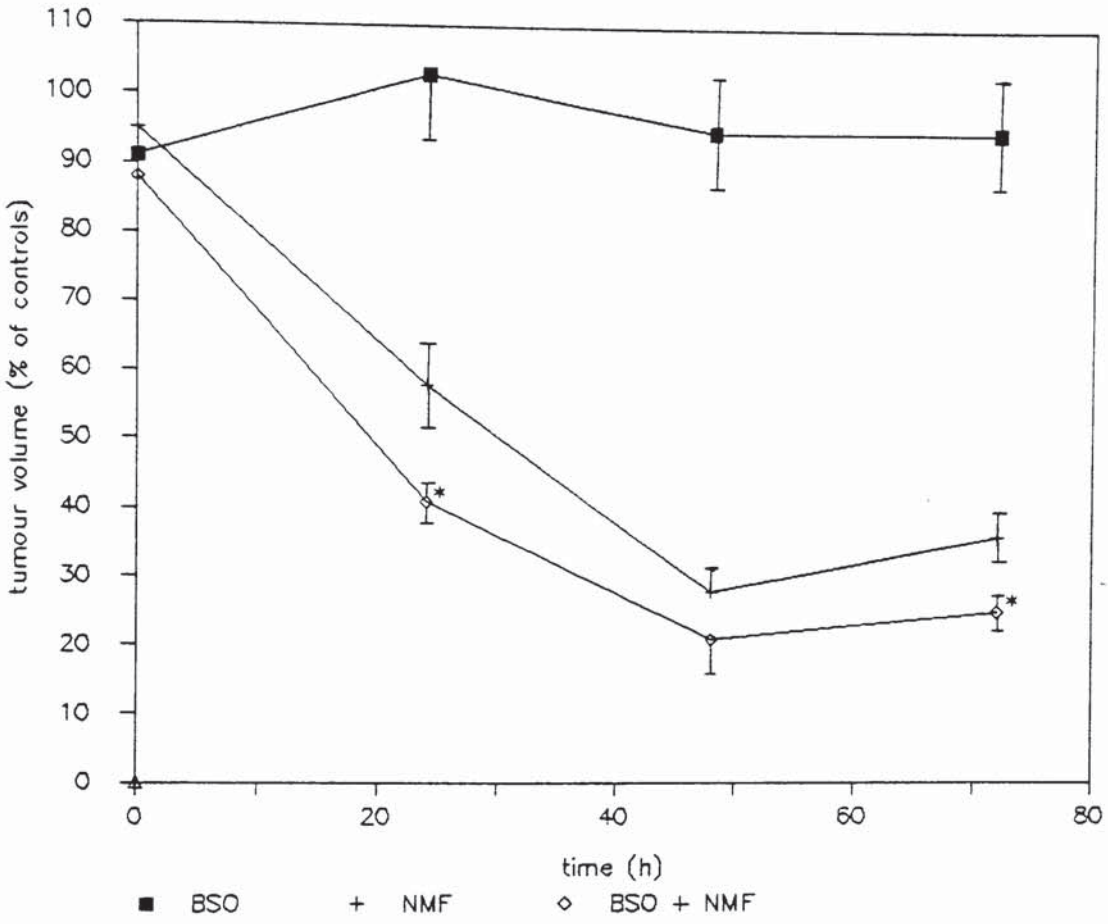


Figure 37 : The effects of 1600 mg/Kg BSO ip and 400 mg/Kg NMF ip alone or in combination on the tumour volume of the TLX5 lymphoma (n=5, ±SE); * indicates that values are significantly different from NMF alone $p < 0.05$ (student's t-test).

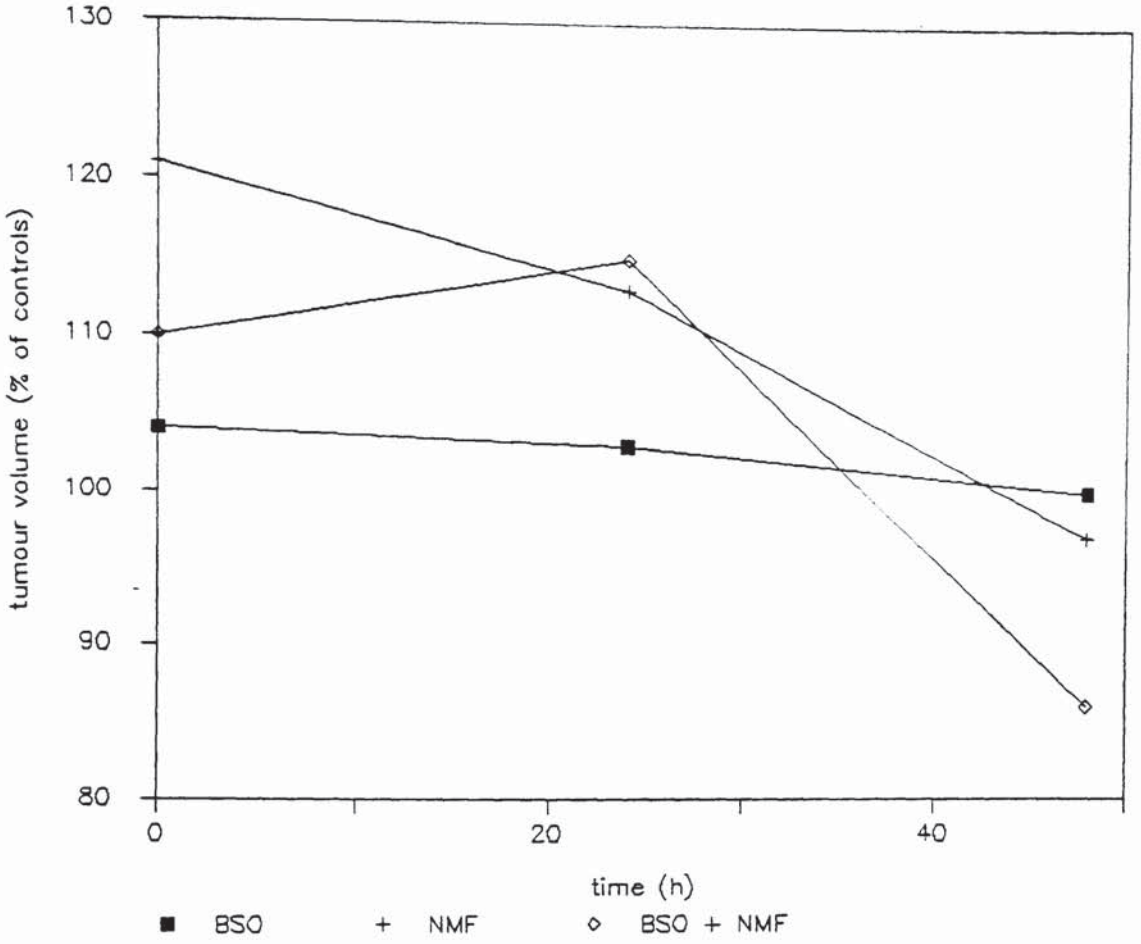


Figure 38 : The effects of 1600 mg/Kg BSO ip and 100 mg/Kg NMF ip alone or in combination on the tumour volume of the TLX5 lymphoma (n=5).

sample	time after NMF administration (h)	GSSG content (nmol/g)
control	2	13.1 ± 3.5[a]
NMF	2	10.8 ± 2.3[a]
control	24	21.0[b]
NMF	24	18.0[b]

Table 10 : GSSG content of the TLX5 lymphoma after administration of 400 mg/Kg NMF ip. [a] n=3, ±SE; [b] n=2.

5.IV A comparative study of the effects of NMF, DMF and NEF on the TLX5 lymphoma tumour growth and cell cycle kinetics

5.IV.i Introduction

NMF, DMF and NEF are good inducers of differentiation for certain cell lines in vitro; these formamides also caused growth inhibition and G_1 phase arrest of TLX5 cells (see section 4.V). In one of the first studies of the antitumour activity of formamides against the Ehrlich ascites tumour in vivo, NMF exhibited the greatest activity (Furst et al, 1955). NMF also has good antineoplastic activity against the TLX5 lymphoma, whereas NEF has only marginal activity and DMF is inactive when given in a five day dose schedule (Gate et al, 1986). The antitumour activity of the three formamide analogues was investigated after a single ip dose of drug and the cell cycle of TLX5 lymphoma cells was analysed in order to compare their activity in this particular cell type.

5.IV.ii Results

Table 11 shows the effects of NMF and DMF on the tumour volume of the TLX5 lymphoma after administration of a single dose of 400 mg/Kg ip. NMF caused regression of the TLX5 lymphoma. DMF had no effect on the TLX5 tumour volume compared with control tumours. NEF (400 mg/Kg ip) was lethal to the mice, animals died 24h to 48h after administration of the drug (data not shown).

The cell cycle of TLX5 tumour samples were analysed 24h after injection of the formamide analogue. NMF caused an increase in the number of cells in S phase, DMF produced a 5% increase in the proportion of cells in S phase, but NEF had no significant effect on the cell cycle distribution of TLX5 lymphoma cells (figure 39).

5.IV.iii Discussion

The only formamide analogue tested which caused tumour regression of the TLX5 lymphoma was NMF. DMF showed no significant activity (table 11). The results for NMF and DMF agree with those previously reported for the Ehrlich ascites tumour and TLX5 lymphoma. NMF and to a lesser extent, DMF caused an increase in the population of S phase cells (figure 39). The result is surprising in view of the speculative mode of action, involving metabolism (see section 1.V). The metabolic pathway of NMF and NEF, which involves oxidation of the formyl moiety and leads to N-acetyl-S-(N-alkylcarbamoyl)cysteine formation, is not an important route for DMF in rodents (Mraz et al, submitted). Instead for DMF metabolic N-methyl hydroxylation is the quantitatively important pathway (Kestell et al, 1987).

In conclusion, of the formamide analogues investigated, NMF, NEF and DMF, only NMF caused regression and significant S phase accumulation of TLX5 lymphoma cells. NEF (400 mg/Kg ip) was lethal to CBA/CA mice.

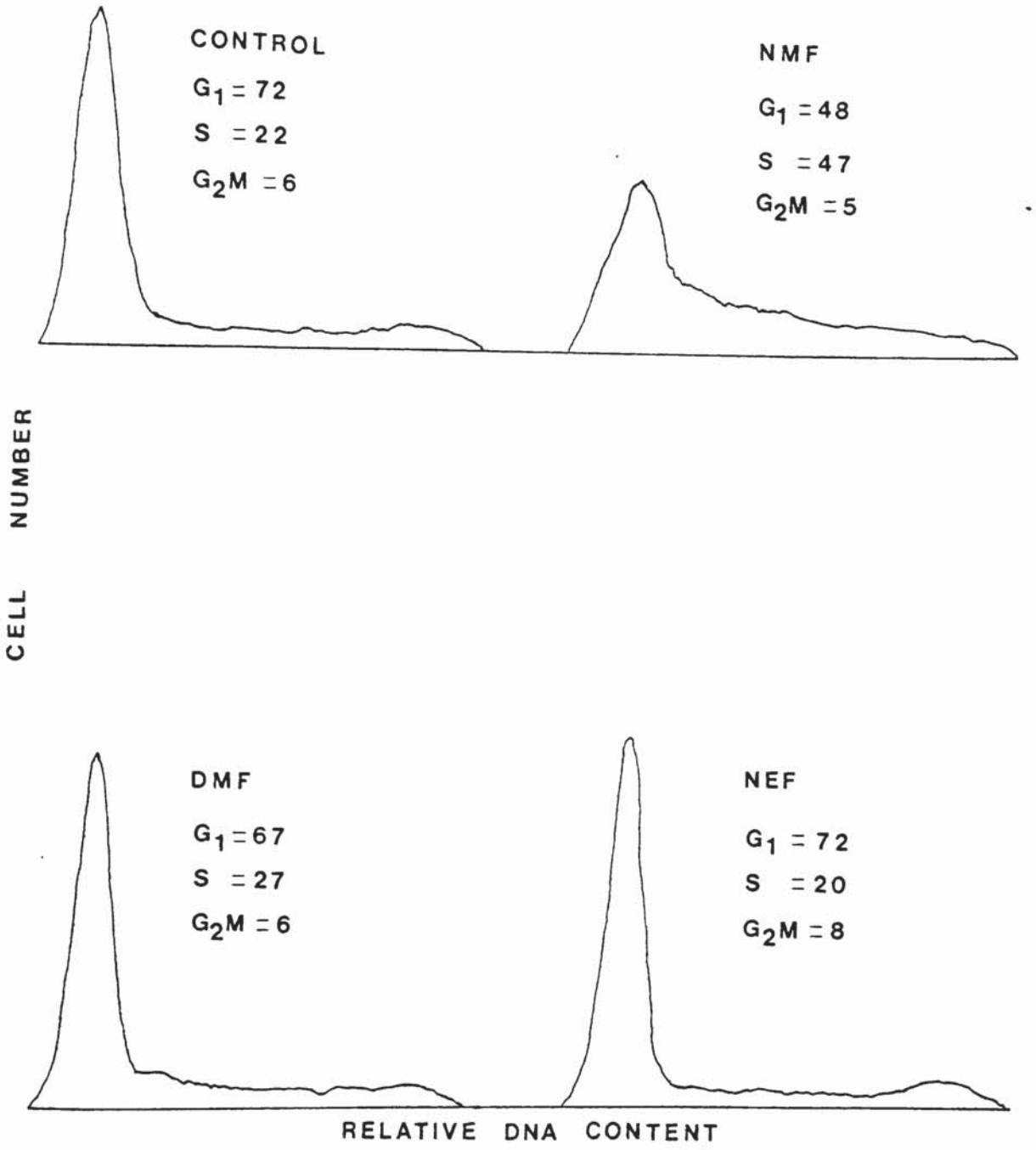


Figure 39 : Representative DNA histograms of TLX5 lymphoma cells, 24h after the administration of NMF, DMF or NEF (400 mg/Kg ip). Values are expressed as the % of viable cells.

time after drug administration (h)	tumour volume (mm^3)		
	control	NMF	DMF
0	412 ± 74	426 ± 34	352 ± 48
24	553 ± 85	400 ± 60	588 ± 40
48	862 ± 32	323 ± 47	864 ± 39
72	919 ± 86	500 ± 77	870 ± 66

Table 11 : Comparison of the effects of a single administration of NMF or DMF (400 mg/Kg ip) on the TLX5 tumour volume (n=5, ±SE).

5.V A distribution study of NMF or metabolites and the role of metabolism in the antitumour activity of NMF

5.V.i Introduction

The maximum plasma concentration of NMF in mice after a single ip injection of 400 mg/Kg is 7mM (Brindley et al, 1982). The aim of this study was to determine the tissue distribution of NMF or metabolites following the administration of a single dose of 400 mg/Kg ip $^{14}CH_3NMF$ (5uCi). Distribution of NMF and total radioactivity in the TLX5 lymphoma was compared with that for the liver and kidney, two tissues which have previously been studied (Matook et al, 1984) and the bone marrow a tissue to which NMF is not toxic at this dose (Langdon et al, 1985a).

It was previously argued that NMF required metabolic activation in order to exert its antitumour effect. If fission of the C-H formyl bond is important for this activity, then d-NMF should be less active as an antitumour agent than NMF; this hypothesis was tested.

5.V.ii Results

Female CBA/CA mice were implanted with 1 $x10^7$ TLX5 cells sc in the inguinal region, 5 days later mice were administered 400 mg/Kg ip $^{14}CH_3NMF$ (5uCi). Mice were killed 4h later and organs were removed for analysis of tissue levels of NMF and metabolites. At this time after NMF administration, the drug is distributed throughout the body (Matook et al, 1984). Sections (20um thick) from

various depths of the TLX5 tumour were cut and the amount of radioactivity was qualitatively assessed by autoradiography. Radioactivity was found throughout the tumour, including the poorly vascularized and dead regions. The total amount of radioactivity which corresponded to NMF or metabolites was quantified (table 12). The mean levels present in the liver, kidney, TLX5 lymphoma and bone marrow were 1.82, 1.36, 1.47 and 9.46 ug/mg protein respectively. The amount of NMF in the samples was measured by gas chromatography (table 13) and the mean levels obtained were; liver, kidney, TLX5 lymphoma and bone marrow 0.72, 0.91, 1.25 and 4.23 ug/mg protein respectively.

CBA/CA mice were injected with NMF or d-NMF (400 mg/Kg), 5 days after mice were implanted with 1 $x10^7$ TLX5 cells sc. Regression of the TLX5 lymphoma occurred for both drugs, the maximum effect on tumour volume occurred 2 days after drug administration (figure 40). However, tumour regression caused by NMF was significantly greater ($p < 0.001$) than that for d-NMF, which led to T/C x100% values of 102 and 113 for d-NMF and NMF respectively (data not shown).

5.V.iii Discussion

Radioactivity associated with NMF or metabolites was found throughout the TLX5 lymphoma (autoradiography of tumour sections) and in the liver and kidney, 4h after administration of 400 mg/Kg $^{14}CH_3NMF$ (table 12 and table

13). A similar study was performed by Barlow, (1982) in CBA/CA mice. In this report the results were expressed as the amount of NMF or equivalents per weight of tissue. If the results of Barlow, (1982) were converted into ug/mg protein using the mean values of protein per weight of tissue obtained from this study then the amounts of NMF derived radioactivity approximate to 1.6 in the case of the liver and 0.7 ug/mg protein for the kidney, 4h after administration of 400 mg/Kg $^{14}CH_3NMF$. This compares to the values in table 12 of 1.82 ± 0.37 and 1.36 ± 0.29 ug/mg protein for liver and kidney respectively. In the study of Barlow, (1982) the maximum tissue radioactivity for the TLX5 lymphoma was found 8h after drug administration (about 0.7 ug/mg protein), which is less than the value of 1.47 ± 0.39 ug/mg protein at 4h after injection (table 12). However, the study of Barlow, (1982) used a different protocol and therefore tumour size and blood supply to the tumour may have varied from this study. Also the tumours would have been obtained from different sources which may lead to heterogeneity.

The total radioactivity was not significantly different in the liver, kidney or TLX5 lymphoma. The liver and kidney have a good blood supply and it could have been anticipated that the more poorly vascularized TLX5 lymphoma would have lower drug levels, but figure 40 showed that radioactivity was distributed throughout the tumour.

The tissue levels of NMF have previously been

investigated by Matook et al, (1984). They administered 300 mg/Kg NMF to male heterozygous Swiss Webster mice and measured NMF levels after 4h; their values for the liver and kidney were 1.83 ± 0.57 and 4.82 ± 0.52 ug/mg protein compared with 0.72 ± 0.21 and 0.91 ± 0.35 ug/mg protein (table 13) in this study. The difference in results may be due to the strain of mice used. Indeed, mouse susceptibility to NMF induced hepatotoxicity is strain specific (Pearson et al, 1987a).

NMF does not cause myelosuppression in mice at effective antitumour doses (Langdon et al, 1985a). The levels of NMF or equivalents (9.46 ± 1.41) and NMF (4.23 ± 0.71) ug/mg protein found in the bone marrow, are considerably higher than the corresponding values for the liver, kidney or TLX5 lymphoma, these results do not correlate with the difference in toxicity. However, the time profile of total radioactivity in the bone marrow is not known and it may be that the toxic metabolite is not present in the bone marrow or is present in minute concentrations. Also, detoxification may be increased in the bone marrow, although the GSH levels in the bone marrow of CBA mice are similar to those of the TLX5 lymphoma (5.7 ± 2.2 (Adams et al, 1985) or 6.8 ± 2.3 nmol/10^7 cells (data not shown)) for bone marrow, compared with 8.8 ± 0.3 nmol/10^7 for TLX5 cells (data not shown)).

The ratio of total radioactivity to NMF was different for the various tissues studied (tables 12 and 13). The ratio for liver was 2.5:1, compared with 1.5:1 for kidney

and 1.2:1 for the TLX5 lymphoma. This may reflect on the increased rate of metabolism of NMF in the liver accompanied by the rapid decrease in hepatic glutathione (Pearson et al, 1987a) and by covalent binding of NMF metabolites to hepatic proteins (Pearson et al, 1987b). A study of the rate of NMF metabolism in the TLX5 lymphoma may show that there is a slow metabolism of the drug which might help to explain the change in GSH levels with time for the tumour after mice were administered NMF (see section 5.III). Also, metabolites of NMF formed in the liver may circulate to the TLX5 lymphoma. There is presumably no accumulation of NMF in the TLX5 tumour with time, since plasma concentration versus time profiles were identical for mice with or without a TLX5 lymphoma (Brindley et al, 1982).

There was a significant difference in tumour regression of the TLX5 lymphoma caused by 400 mg/Kg d-NMF compared with the same dose of NMF (figure 40). These results suggest that fission of the C-H formyl bond of the NMF molecule is an important regulatory step in the antitumour activity of the drug, although it is still unknown whether the active species is the product of oxidation of the formyl moiety of NMF or a metabolite formed after conjugation with GSH, or indeed a combination of both. d-NMF was less active than NMF on a 5 day dose regimen (see section 5.I) in the TLX5 tumour using survival times as the end point (Chubb D, unpublished) and for another tumour, the M5076 reticulum cell sarcoma, a

dose of 200 mg/Kg d-NMF was equivalent to 100 mg/Kg NMF in terms of tumour volume regression (Chubb D, unpublished). d-NMF was also less hepatotoxic than NMF as measured by enzyme leakage (Threadgill et al, 1987). These results are in accordance with the observations of Masuda et al, (1986) who suggested that NMF metabolism was required for the hepatotoxicity and antitumour activity of the drug.

In conclusion, radioactivity was found in all parts of the TLX5 lymphoma 4h after administration of 400 mg/Kg $^{14}CH_3NMF$ ip. At this time the distribution of NMF or metabolites was similar for the TLX5 lymphoma, liver and kidney, but levels were significantly higher in the bone marrow.

Fission of the formyl C-H bond of the NMF molecule appears to be a regulatory step in the formation of the active antitumour species.

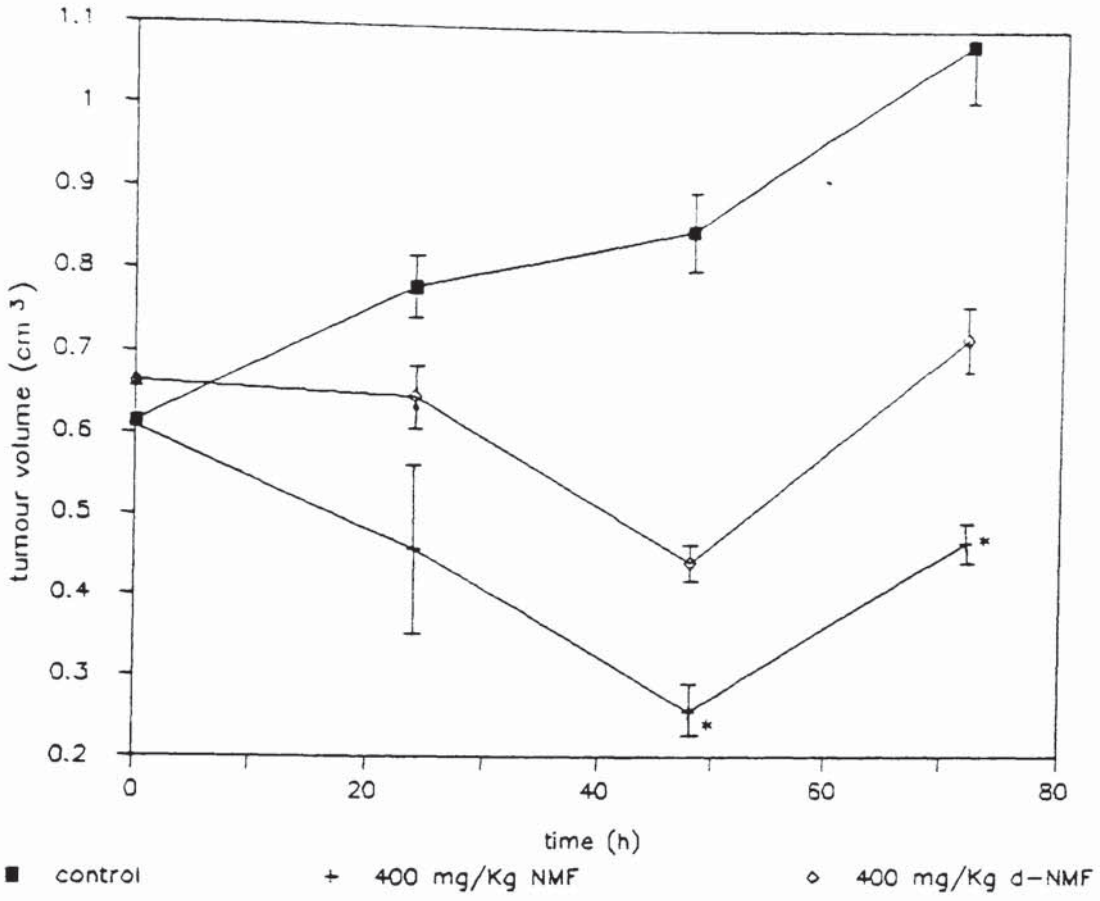


Figure 40 : Comparison of the effects of NMF or d-NMF (400 mg/Kg ip) on the TLX5 tumour volume (n=5, ±SE) * indicates that values are significantly different from d-NMF $p < 0.001$ (student's t-test).

mouse	liver	kidney	TLX5 tumour	bone marrow
1	1.84	0.93	1.02	11.30
2	2.34	1.43	2.07	10.61
3	1.76	1.31	1.57	8.82
4	1.29	1.37	1.34	8.18
5	1.86	1.75	1.35	8.36
mean (±SD)	1.82 ± 0.37	1.36 ± 0.29	1.47 ± 0.39	9.46 ± 1.41

Table 12 : Tissue levels of NMF or metabolites measured 4h after the administration of 400 mg/Kg ip $^{14}CH_3NMF$ (5uCi/mouse). Values are expressed as ug NMF or equivalents/mg protein.

mouse	liver	kidney	TLX5 tumour	bone marrow
1	0.58	0.36	0.57	4.26
2	1.07	1.07	2.05	5.41
3	0.63	0.86	1.53	4.10
4	0.55	0.98	1.04	3.58
5	0.77	1.30	1.05	3.82
mean (±SD)	0.72 ± 0.21	0.91 ± 0.35	1.25 ± 0.56	4.23 ± 0.71

Table 13 : Tissue levels of NMF measured 4h after the administration of 400 mg/Kg ip $^{14}CH_3NMF$ (5uCi/mouse). Values are expressed as ug/mg protein.

SECTION 6: GENERAL DISCUSSION

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In the following, the findings obtained in in vitro and in vivo experiments (sections 4 and 5) will be compared and placed into the context of previous studies of the mode of antineoplastic action of NMF.

Polar solvents such as NMF are good inducers of differentiation in vitro. A number of leukaemia cell lines undergo terminal differentiation, whereas some non-leukaemic cell types acquire more benign characteristics, when exposed to NMF in vitro (see section 1.II).

NMF caused growth inhibition and a decrease in viability of TLX5 cells, when incubated with drug in vitro (see section 4.I). The decrease in the growth rate of TLX5 cells induced by NMF was associated with arrest of cells in the early G_1 phase of the cell cycle (see section 4.II). A decrease in the growth rate and accumulation of cells in the G_1 phase of the cell cycle are events which are typical of terminal differentiation or the acquisition of a more benign phenotype (see section 1.II). When NMF was removed from the medium, the viable TLX5 cells proliferated, retained their tumourigenic potential and the cell cycle distribution of cells returned to control profiles (see section 4.IV). The results suggest that TLX5 cells that remained viable were not terminally differentiated, but resided in a quiescent state that was reversed when NMF was removed from the medium. The TLX5 cells that died after incubation with NMF may have

terminally differentiated; this possibility cannot be discounted, since markers of terminal differentiation were not assessed.

The demonstration that DMF and NEF can cause growth inhibition and G_1 phase arrest of TLX5 cells in culture, similar to that observed with NMF, suggests that they may share a common mechanism of action (see section 4.V). Since D-glucose caused growth inhibition of TLX5 cells in a concentration and time dependent manner, similar to NMF, it appears that the osmolarity of the medium is a regulatory factor in TLX5 cell growth in culture.

The depletion of intracellular GSH in TLX5 cells induced by NMF was similar to that observed for human colon carcinoma cell lines. However, this decrease in GSH pool size was not responsible for the growth inhibition of TLX5 cells, unlike in the case of human colon carcinoma cells in which both events appeared to be related (see section 4.III).

The activity of NMF against the TLX5 cells <u>in vitro</u> appeared to be due to the parent drug rather than an active metabolite (see section 4.VI).

The cell membrane is a possible locus of action of polar solvent induced growth inhibition (see section 1.II). NMF caused a dose and time dependent increase in the membrane permeability of TLX5 cells (see section 4.I). The increase in the membrane permeability appeared to be an early event in the pathway of NMF induced growth inhibition and cell death. Indeed, membrane leakage was

detected prior to uptake into the cell of the commonly used indicator of cell viability, trypan blue. An increase in membrane permeability would probably coincide with changes in the structure of the cell membrane. Alterations in the morphology of the cell membrane, could alter normal membrane biochemistry and may prevent the ability of growth factors to bind to the cell membrane receptors (see section 4.I). If mitogens were unable to bind to the cell membrane receptors, the cascade of events which are initiated at the plasma membrane and normally lead to cell proliferation, would be prevented. Growth arrest of cells in the G_1 phase of the cell cycle would then occur (Pardee et al, 1986).

The early G_1 phase arrest of the TLX5 cells induced by NMF suggests that the G_0 to G_1 phase transition is affected (Lord, 1986). Cells arrested in the G_0/early G_1 phase will be growth factor dependent for proliferation; therefore, if growth factor binding to the plasma membrane receptors is altered, this will prevent the transition of cells from early G_1 to late G_1 phase.

There was no direct evidence in these studies that NMF interacted with the genome. NMF caused a G_1 phase arrest of TLX5 cells, which corresponded to a decrease in the synthesis of DNA. A decrease in the rate of DNA synthesis may, however, be due to an indirect action of NMF. An effect for example at the cell membrane could subsequently lead to a decrease in the synthesis of DNA.

Two of the early studies into the mechanism of action

of NMF were performed on the prokaryote, E.coli. Skipper et al, (1955), reported that NMF (250 to 340mM) caused growth arrest and inhibition of purine biosynthesis in E.coli. Tomisek et al, (1969), found that growth inhibition of the E.coli caused by NMF (280mM) was associated with a marked inhibition of pyrimidine biosynthesis and to a lesser extent, purine biosynthesis.

The findings of Skipper et al, (1955) and Tomisek et al, (1969) suggest that NMF decreased the rate of DNA synthesis in E.coli cells, in a similar manner to that observed for the TLX5 cells on incubation with NMF. It is not known whether NMF effects the purine and/or pyrimidine levels in the TLX5 cells.

The effects of NMF on oncogene expression of the TLX5 cells was not investigated in this study, but considering the relevance of oncogenes in the growth and differentiation of cells and the previously reported changes in the levels of oncogene expression for DLD-1 clone A cells caused by NMF (see section 1.II), such an avenue of research is worthy of investigation in the TLX5 cells.

A single ip dose of NMF (200 to 800 mg/Kg) caused significant regression of the TLX5 lymphoma and an increase in animal survival times, when the tumour was grown sc in CBA/CA mice (see section 5.I). The tumour regression was paralleled by cell death, typical of apoptosis and necrosis. NMF was also significantly more effective than DMF or NEF as an antitumour agent against

the TLX5 lymphoma. There was no indication of myelosuppression caused by NMF, as measured by the absence of a significant effect on the cell cycle of bone marrow cells (see section 5.II), despite the high levels of radioactivity originating from NMF in the bone marrow (see section 5.V). These results for the TLX5 lymphoma are in accordance with the reported data for antitumour activity and lack of myelosuppression caused by NMF and other formamides (see section 1.IV).

NMF treatment caused an initial increase in DNA synthesis in the TLX5 lymphoma cells. The increase in the number of TLX5 cells in the S phase of the cell cycle, did not correlate, however, with the cytotoxic activity of NMF against the TLX5 lymphoma (see section 5.II).

Further experiments tested the hypothesis that a metabolite of NMF is responsible for its activity. d-NMF was less effective than NMF as an antitumour agent against the TLX5 lymphoma; it therefore appears that fission of the formyl C-H bond of the NMF molecule is a rate limiting step in the genesis of the active antitumour species of NMF (see section 5.V). The result confirms the idea that NMF requires metabolic activation _in vivo_ to exert its antitumour activity (see section 1.V).

It remains to be elucidated, what is the active antitumour species after the administration of NMF. The unknown metabolic intermediate, perhaps methyl isocyanate, arising from the oxidation of the formyl group of NMF (Kestell _et al_, 1986b) could conceivably be the active

moeity. Methyl isocyanate is a potent toxin which can probably be detoxified by conjugation with GSH. NMF caused a decrease in the GSH levels of the TLX5 lymphoma which did not correlate with tumour regression. The antitumour activity of NMF was, however, enhanced by the administration of the GSH depleting agent, BSO (see section 5.III). This enhanced antitumour activity of NMF after lowering the tumour GSH levels, may reflect a decreased ability to detoxify reactive xenobiotics, such as methyl isocyanate.

As discussed in the introduction (see section 1.V), the formation of N-hydroxymethylformamide (HMF) from NMF is thought to be a detoxification pathway and HMF is not active against tumours which are sensitive to NMF *in vivo* (Cooksey *et al*, 1983). The endogenous amine, methylamine which is also a metabolite of NMF, is similarly thought to be an unlikely antitumour agent.

It is possible that the active antitumour species of NMF *in vivo* may be one of the metabolites formed after the conjugation of the reactive oxidation product of NMF with GSH. Skinner *et al*, (1958) found that S-carbamoylcysteine was an effective antitumour agent against some murine leukaemias. Nemeth *et al*, (1978) reported that the amino acid derivative, S-ethyl-carbamoylcysteine, was an active antitumour agent against some animal tumours. Furthermore, a single ip dose of 200 mg/Kg S-ethyl-carbamoylcysteine caused severe cell damage to Ehrlich ascites cells, which resulted in

cell death, 24h after administration of drug. Restoration of Ehrlich ascites cell growth began 48h after drug administration. S-ethyl-carbamoylcysteine (200 mg/Kg) also caused an increase in the DNA content of Ehrlich ascites cells, 72h after drug administration. The cell death and increase in DNA content of the Ehrlich ascites cells are similar to the results obtained when the TLX5 lymphoma was treated with NMF (see sections 5.I and 5.II). The increase in the DNA content of the Ehrlich ascites cells observed with S-ethyl-carbamoylcysteine occurred at a time of tumour regeneration, rather than an overall decrease in tumour size, which was observed with the TLX5 tumour after administration of NMF.

S-carbamoylcysteine and S-ethyl-carbamoylcysteine are structurally similar to the probable NMF metabolite S-(N-methylcarbamoyl)cysteine (Threadgill et al, 1987). S-(N-methylcarbamoyl)cysteine could be present in the TLX5 tumour after administration of NMF and could therefore, possibly be an active antitumour species.

The in vivo studies conducted into the possible mechanism of antitumour activity of NMF (see section 1.IV) utilized a number of different regimens of dosing. Sartorelli and LePage, (1958) administered NMF (200 mg/Kg/day) on days 1, 2, 4, and 6 after implantation of 10^7 TA3 ascites carcinoma cells into mice. They reported a cross resistance of NMF with a tumour which was resistant to azaserine, an inhibitor of the de novo synthesis of purines.

The studies of Barclay et al, (1954), Barclay and Garfinkel, (1957) and Eidinoff et al, (1961) used a single dose of NMF, like the studies reported in this thesis. Eidinoff et al, (1961) administered 500 mg/Kg NMF to rats bearing HS#1 and HEp#2 human tumour transplants; there was a slight increase in the synthesis of pyrimidines in the tumour, as well as in the liver, intestine and spleen, 7h after administration of NMF. Since a time study of change in pyrimidine levels was not reported, it is unknown whether the synthesis of pyrimidines increased further, which may have a bearing on possible increases in DNA synthesis.

Barclay et al, (1954) administered a single dose of 2 g/Kg NMF to male Wistar rats; 24h later the rats were killed. There was a 10 fold increase in the incorporation of adenine (A) and guanine (G) into liver nucleic acids. This does not indicate that there was an increase in DNA synthesis, however, since there was no change in the thymine incorporation in the liver DNA. In a later study, Barclay and Garfinkel, (1957) administered 2 g/Kg NMF to Wistar rats and 24h later administered ^{14}C-formate, to measure the extent of purine biosynthesis. The levels of A and G were measured 24h later (48h after administration of NMF). In the Murphy-Sturm rat lymphosarcoma, which is insensitive to NMF, there was no change in the incorporation of A or G into nucleic acids, whereas a sensitive tumour, the Walker carcinosarcoma 256, showed decreased A and G incorporation into nucleic acids,

however, incorporation of these bases increased in the liver. The authors postulated that the effect on the liver was due to a toxic action which led to regeneration of liver cells. A similar toxic action and regeneration of the TLX5 lymphoma may occur in CBA/CA mice after administration of NMF.

From the reported studies of purine and pyrimidine synthesis after a single administration of NMF, it appears that an increase can occur in both of the DNA precursors. In relation to the observed increase in recruitment of TLX5 cells into DNA synthesis, 24h after the administration of 400 mg/Kg NMF, an increase in both pyrimidine and purine synthesis would be required to augment an increase in the deoxyribonucleotides required for DNA synthesis.

An increase in DNA synthesis renders cells more susceptible to the damaging effects of x-irradiation (Chiu and Oleinick, 1982). The activity of NMF as a radiosensitizer (see section 1.V) may be the result of an increase in actively transcribing DNA, as well as a decrease in detoxification due to a fall in GSH.

In conclusion, this study indicates that there are differences in the mechanism of antitumour activity of NMF in vitro and in vivo. NMF caused a reversible decrease in the growth rate and G_1 phase arrest of murine TLX5 lymphoma cells in vitro. The effects induced by NMF in vitro appeared to be due to the parent drug. No structural specificity was required to induce growth

inhibition. GSH depletion induced by NMF was not a prerequisite for growth inhibition of TLX5 cells, unlike the reports for the human colon carcinoma cell lines. NMF caused tumour regression and cell death of the TLX5 lymphoma *in vivo*. This antitumour activity of NMF required metabolic activation to produce the cytotoxic species. The antitumour activity of NMF was marginally increased by the GSH depleting agent, BSO. NMF also increased DNA synthesis in the TLX5 lymphoma but this effect did not correlate with the antitumour response.

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