FREE RADICALS AS POTENTIAL ANTITUMOUR AGENTS

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SUMMARY

The aim of this work was to use extremely low concentrations of free radical generating compounds as a "catalyst" to trigger endogenous free radical chain reactions in the host and to selectively eliminate neoplastic cells in the host.

To test the hypothesis, a number of free radical generating compounds were screened on several malignant cell lines in vitro to select model compounds that were used against tumour models in vivo. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and its derivatives were selected as the model compounds for in vivo experiments in view of their high cytotoxic potency against several malignant cell lines in vitro. The water soluble derivative, 2,2-diphenyl-1-(2', 4'-dinitro-6'-sulphophenyl) hydrazyl (DDSH) given by subcutaneous injections demonstrated significant antitumour activities against the MAC16 murine colon adenocarcinoma implanted subcutaneously in male NMRI mice at nanomolar concentration range.

40-60% of long term survival of over 60 days was achieved (compared with control survival of 20 days) with total tumour elimination. This compound was also active against both P388 leukaemia in male BDF1 mice and TLX5 lymphoid tumour in male CBA/CA mice at a similar concentration range. However, some of these animals died suddenly after treatment with no evidence of disease present at post mortem. The cause of death was unknown but thought to be related to the treatment. There was significant increase in serum level of malondialdehyde (MDA) following treatment, but did not correlate to the antitumour activities of these compounds. Induction of superoxide dismutase (SOD), and glutathione peroxidase (GPx) occurred around day 8 after the administration of DDSH.

Histological sections of MAC16 tumours showed areas of extensive massive haemorrhagic necrosis and vascular collapse associated with perivasular cell death following the administration of nanomolar concentration of DDSH which was probably compatible with the effects of free radicals.

It was concluded that the antitumour activities of these compounds may be related to free radical and cytokine production.

KEY WORDS; 2,2-DIPHENYL-1-PICRYLHYDRAZYL-DERIVATIVES, FREE RADICALS, ANTITUMOUR.

TO MY FAMILY AND PARENTS, WITH LOVE

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"For the Lord giveth wisdom: out of his mouth cometh knowledge and understanding"

Proverbs; Ch. 2 verse 6.

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ABBREVIATIONS

AA	Arachidonic acid
ADM	Adriamycin
AIDS	Acquired immunodeficiency syndrome
ALA	Alpha linolenic acid
ara-CTP	Arabinoside cytosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
t-BOOH	tert-Butyl hydroperoxide
B WT	Body weight
BSO	Buthionine sulfoximine
CAT	Catalase
CHL	Chlorambucil
CPH	N-Carbazylpicryl nitrogen free radical
CPH_2	N-Carbazylpicrylamine
DETAPAC	Diethylenetriaminepentaacetic acid
DGLA	Dihomogammalinolenic acid
DHA	Docosahexanoeic acid
DHFR	Dihydrofolate reductase
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl free radical
$DPPH_2$	2,2-Diphenyl-1-picrylhydrazine
DDSH	2,2-Diphenyl-1-(2',4'-dinitro-6'-sulphodiphenyl)hydrazyl free radical

$DDSH_2$	2,2-Diphenyl-1-(2',4'-dinitro-6'-sulphodiphenyl)hydrazine
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentanoeic acid
ESR	Electron spin resonance
5-Fu	5-Fluorouracil
FCS	Foetal calf serum
GLA	Gammalinolenic acid
GPx	Glutathione peroxidase
GSH	Glutathione reduced form
IL	Interleukin
INDO	Indomethacin
LA	Linoleic acid
LPO	Lipid peroxidation
LT	Leukotriene
MDR	Multiple drug resistance
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
NCI	National Cancer Institute
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NBT	Nitro blue tetrazolium
PUFA	Polyunsaturated acid
PG	Prostaglandin
PBS	Phosphate buffered saline
POA	Palmitoleic acid
RNA	Ribonucleic acid

RPMI	Rosewell Park Memorial Institute (medium)
SOD	Superoxide dismutase
SEM	Standard error of the mean
TNF	Tumour necrosis factor
TX	Thromboxane
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
UG	Ultroser-G

CHAPTER 1: GENERAL INTRODUCTION.

1.1. ONCOLOGY: PERSPECTIVE.

Oncology, the study of neoplastic disease, is generally referred to as the "cancer problem" (1,2,3). It is the study of a large variety of tumours of malignant nature with lethal potential.

An adequate understanding of cancer must begin with appreciation of the fact that cancer is not one disease but over 100 different diseases, each with its own characteristics and natural history.

The study of cancer may occur simultaneously at several levels. Basic researchers in chemistry, virology, and immunology attempt to discover and describe the insulting factors and cofactors; the earliest changes in DNA, RNA, and enzyme systems; and the changes in the host that permit transformation of cells and growth of malignant tumours. At another level epidemiologists, may seek to determine age-specific and sex-specific incidences of different types of cancer and relate them to ethnic, geographic, and enviromental factors. It is important to realise that death arising from cancer in the industrial countries is only second to death caused by cardiovascular diseases. In the Third World, it is the third commonest cause of death only after infectious and cardiovascular diseases (4). The incidence and pattern of cancer varies from country to country due to different local factors. Even in the same country the pattern varies from one area to another.

The size and scope of the cancer problem are best appreciated with numbers. About 29 in 100 males and 20 in 100 females will develop cancer before the age of 75 in the United Kingdom of which 10 in 100 males and about 6 in 100 females will develop lung and breast cancers respectively (5). Almost 56 million Americans now living will be diagnosed as cancer patients, one in every four, according to the present rate (2).

Although cancer is still the second most common cause of death in the Western World, it is probably no longer the most feared. That dubious distinction is now held by Acquired Immunodeficiency Syndrome (AIDS) which appears to be the subject of more lay and professional publications and discussions all over the world. Nonetheless, today and for the remainder of this century, more patients will probably die of cancer than from AIDS in the Western World.

Despite all these statistics, the primary goal in cancer research is centred on treatment. The questions usually asked by most

patients will be as follows. Has it spread? Can it be cured? Will I lose a breast or a leg or a lung? Will I be able to live normally after treatment? What are my chances? Where could it show up again?

Although the world is waiting anxiously for the "magic bullet", research in the treatment of cancer is complex and improvements in the results appear slowly and are expensive. The National Cancer Institute devoted 31% of its 1982 budget to preclinical and clinical research in the treatment of cancer (6). Most of it was spent on the development of new cytotoxic drugs and clinical investigation of chemotherapeutic agents that have shown preliminary evidence of effectiveness. Modest support is provided in research in radiotherapy, mostly with high linear energy transfer radiation such as neutrons. There is little support for surgical research, except with chemotherapy or immunotherapy as adjuvants. It has been said that surgery and perhaps radiotherapy have reached effectiveness, yet failure to control the primary tumour and its regional spread is not less a problem today than when it was first quantified by Suit (7) two decades ago.

Finally, it is important to realise that cancer will continue to challenge researchers from every discipline. It will need both their concerted efforts and money to finance the projects. Some of today's avenues of cancer research were easily recognizable when first found, whereas others required or still require a more distant perspective to appreciate their relevance. Some avenues lead nowhere; others dynamically trace the frontiers of progress. Holland (8) stated that 'the methodology of cancer research and the principle of cancer medicine are kinetic, but changes are incremental:"break through" a grievously overused word.' Since the future is defined by the past there is a great need for cancer researchers to define their goals more clearly in order to meet the difficult challenges posed by this dreadful disease. At the same time, we must realise that unusual or radical ideas that differ from the existing orthodox scientific knowledge are not easily accepted by the scientific world and hence are either rejected or get no support. Yet these are some of the areas in which radical changes in our knowledge and real scientific breakthrough may come about. This will continue to invite a lot of effort and determination from those scientists whose radical ideas may not readily be accepted. It must be remembered that where there is a will there is a way.

1.2. ADVANCES IN CANCER CHEMOTHERAPY.

The chemotherapy of malignant diseases refers to the use of cytotoxic drugs. The use of hormones and antihormones is included in this broad definition.

Following the use of both nitrogen and sulphur mustard gases in world war 1, scientists observed that in addition to their vesicant action and systemic effects, they inhibited cellular proliferation in the bone-marrow, lymphoid tissues, and the epithelium of gastrointestinal tract (9). In 1931, Adair and Bagg published the results of treatment with an alcoholic solution of mustard gas (dichlorodiethylsulphide) which they applied topically in 12 cases of tumours involving skin and in one case, a recurrent neurogenic sarcoma, they injected into the tumour (10). The few months of remissions which were attained in some patients brought a new hope for the future use of mustard gas in localised disease. The subsequent studies that were conducted on the chemistry and the biologic, physiologic and clinical effects of the agents were not published because of the secretive nature related to war agents. However, Philips who participated in the early studies, wrote a comprehensive review of the work done before 1950 (11). Thereafter, many reviews were published on the advances of chemotherapy. Among the notable ones were Hirschberg (12) who reviewed cancer therapy from 1945 to 1958, Oliverio and Zubrod (13); Gelhorn (14); Stock (16); and Zubrod (17). And the most recent ones are by Young (18); Bailar 111 and Smith (19).

Currently, the majority of chemotherapeutic agents fall in one of the following catagories: (a) alkylating agents; (b) antimetabolites; (c) alkaloids and antibiotics;(d) hormones and antihormones (e) miscellaneous drugs that includes enzymes like L-asparaginase and other drugs such as procarbazine, razoxane, mitoguazone, and levamisole (20).

Cytotoxic drugs are general cellular poisons which have a deleterious effect, to a greater or lesser degree, on normal cells and a variety of tumours. Because these drugs are potentially lethal, cancer chemotherapy is largely a compromise between toxic and therapeutic effects and great care must be exercised in its use. This care may include the following initial steps (40): (a) determination of safe dose range; (b) choice of appropriate route of administration; (c) awareness of the incidence and course of potentially life threatening toxicity; (d) awareness of routes of drug elimination and adjustment of the dose to accommodate organ dysfunction and (e) knowledge of drug interactions as influenced by dose and schedule to maximise favourable

interactions and minimise toxicity (20). At the same time the oncologist must be aware of the unique challenges presented by individual patients.

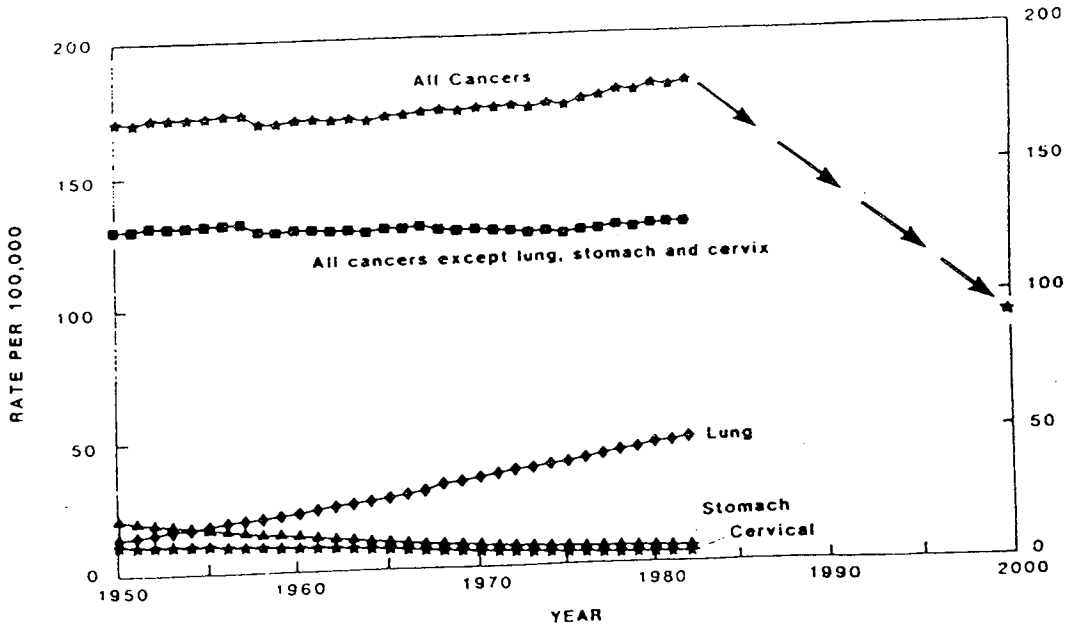
Although many effective anticancer agents are available, their curative potentials are still limited. Table 1.1 summarizes the effects of chemotherapy on various tumours to-date. In summary, chemotherapy is appropriate for certain tumours in which complete responses and prolongation of survival are high and cures are possible(group 1 in Table 1.1). On the other hand, survival may be prolonged but cure rarely occurs (group 2 Table 1.1). In a third group of tumours (group 3 Table 1.1) responses are high but short lived. Finally there is a group of tumours (group 4 Table 1.1) in which chemotherapy has had no impact and responses are few.

Table 1.1 *Effect of chemotherapy on various tumours (21)*

Group 1	*High complete response rates; cures common*
	Acute lymphoblastic leukaemia in children
	Hodgkin's disease
	Chorioncarcinoma
	Germ cell tumours
	Wilm's tumour
	Ewing's sarcoma (in conjuction with primary therapy)
Group 2	*High response rates: chemotherapy may prolong survival*
	Small cell lung cancer
	Ovarian cancer
	Breast cancer
	Acute myeloid leukaemia
	High grade non-hodgkin's lymphoma
	Osteosarcoma
Group 3	*Overall response rates at least 50%: chemotherapy has no definite effect on survival*
	Head and neck tumours
	Cancer of uterine cervix
	Bladder cancer
	Myeloma
Group 4	*Overall response rates < 40%: no demonstrable effect on survival*
	Soft tissue sarcoma
	Gastric carcinoma
	Non-small cell lung cancer
	Renal cancer
	Pancreatic cancer
	Hepatoma
	Melanoma
	Colorectal cancer

In view of the above data, many scientists and the general public want to know whether progress has been made against cancer since the early 1950s. The best single measure of progress against cancer is the change in age-adjusted mortality rate associated with all cancers combined in the total population (19) (figure1.1). Using this United State's statistic, it seems we are losing the war against cancer, despite the improvements in palliation and the extension of productive years of life. The improvements that may be noted in cases like cancer of the stomach have been achieved mainly by changes in life-style such as dietary changes. On the other hand, improvements in cervical cancer mortality is related to early diagnosis made possible by the screening tests. While continuing in the search for the "magic bullet", research emphasis may have to be shifted towards early diagnosis and prevention. However, newer forms of treatments may give us a hope in improving these statistics. The National Cancer Institute (NCI) has set up a goal of achieving 50% reduction in cancer-related mortality on an age adjusted basis by the year 2000 (19) (figure 1.1). With only 9 years to go from now and with no evidence of a downward trend, it is unlikely that the NCI will attain its stated goal. Despite this dismal outlook, there has certainly been better results in childhood neoplasms (21).

Fig. 1.1 Mortality from Cancer of All Sites and Selected Sites, 1950 through 1982, in the U.S.A Population. Age was adjusted to the U.S.A population of 1980. Extension to the year 2000 is shown to reflect the stated goal of the National Cancer Institute (19).



The curative potentials of these chemotherapeutic agents have been compromised by a variety of problems related to tumour sensitivity, access, and pharmacokinetics. Central to the causes of these failures, is drug resistance. Drug resistance may be intrinsic, acquired or induced and it may develop to one drug or it may occur simultaneously to multiple agents (multiple drug resistance-MDR). Table 1.2 summarises the clinical patterns of drug resistance and table 1.3 shows the tumours that are affected by drug resistance. Substantial progress has been made in our understanding of the mechanisms of drug resistance and the techniques to overcome the resistance.

Table 1.2 Clinical Pattern of Drug Resistance (65)

Intrinsic drug resistance	*Broad based*
	Universal resistance
	Rare tumour responses
	Specific
	In resistant tumours
	In sensitive tumours
Acquired or induced drug resistance	*Specific*
	Multidrug:
	Pleiotropic
	others
	Mixtures
	Specific-multidrug
	Pleiotropic-other multidrug

Table 1.3 Tumours Affected by Drug Resistance (66)

	New Cases	*Mortality*
Tumours intrinsically drug-resistant		
Digestive organs	227,500	122,350
Respiratory	168,300	144,250
Urinary organs	68,900	20,000
Brain and Cental nervous system	14,700	10,900
Total	**479,400**	**297,500**
Tumours with acquired or induced drug resistance		
Head and Neck tumours	30,200	9,050
Bone	2,100	1,300
Connective tissue	5,500	2,900
Skin	27,300	7,800
Breast	135,900	42,300
Genital	176,500	51,700
Endocrine	12,100	1,850
Leukaemia	26,900	18,100
Lymphoma	50.700	26,200
Total	**467,200**	**161,200**
Overall total	**946,600**	**458,700**

Intrinsic drug resistance is seen when tumours are first exposed to chemotherapeutic agents. Acquired or induced drug resistance is seen in initially responsive tumours, which over time no longer respond to drugs to which they were initially sensitive. This later type is commonly what investigators mean by the term drug resistance (25). Acquired resistance may be specific as with the development of methotrexate resistance related to gene amplification resulting in excess production of dihydrofolate reductase within the tumour (26), or it may be more broadly based as seen with the appearance of the MDR seen with the MDR-1 (P-170 glycoprotein) gene (41,42). And there is increasing evidence that mixtures of drug resistance patterns may occur in human tumours.

Intrinsically resistant tumours include those of digestive organs, such as hepatocellular carcinoma and cancer of the biliary tree. Tumours of the respiratory systems such as non-small cell lung cancers, and those of the urinary and central nervous systems namely, renal cell carcinoma and glioblastoma multiforme respectively, are included in this group. Tumours with intrinsic or primary resistance usually arise from duct cells or cells lining excretory organs (27). These cells may retain their malignant phenotype of detoxification, transportation and excretion of toxic compounds including chemotherapeutic agents (27). It has also been noted that even within tumour types that are generally thought to be broadly drug-resistant, there are occasional tumours that may show dramatic responses to chemotherapeutic agents. These may provide a clue of converting a heretofore uniformly resistant tumour into one commonly responsive (27).

Although a large number of specific mechanisms of drug resistance has been described in eukaryotic cell lines or in animal models, very few have been established in human tumours and their clinical significance documented. Those that have been identified include defective transport, altered drug activation, altered hormone receptor concentration or affinity, altered DNA repair, gene amplification, defective drug metabolism, altered target proteins, and altered intracellular nucleotide pools.

Defective transport or altered drug efflux has been documented as a mechanism for methotrexate resistance in human acute lymphoblastic leukaemia cells (28) and in adriamycin-resistant human ovarian carcinoma cells (29).

Altered drug activation has been described in human myeloblastic leukaemia in which there is a low level of the activating enzyme deoxycytidine kinase, which converts cytosine arabinoside to the

active triphosphate metabolite (ara-CTP). At the same time there is increased levels of degradative enzyme cytidine deaminase (30,31).

Altered binding affinity has been described as a mechanism for corticosteriod resistance in acute lymphoblastic leukaemia (32) and absence of oestrogen receptor has been found in hormone resistant breast cancer (33).

Resistance to alkylating agents and cisplatin seems to be due to altered DNA repair. Human ovarian cell lines resistant to melphalan demonstrate increased ability to repair melphalan damage (34). Futhermore, the potent inhibitor of alpha and beta DNA polymerase, aphidicolin has been found to inhibit repair of melphalan induced damage in these tumours by more than 50% (34).

Gene amplification as a mechanism for drug resistance in human tumours has been documented in methotrexate-resistant tumour cells with amplified dihydrofolate reductase(DHFR) gene copies. This has been found in cells from a patient with methotrexate resistant ovarian cancer (35), and in cells from a patient with small cell lung cancer in relapse after high dose methotrexate (36).

Similarly, altered target proteins have been described as mechanisms of resistance for both methotrexate and 5-fluorouracil in human cells (37,38), and defective drug metabolism has been found in methotrexate resistance secondary to defects in polyglutamation in human breast (39) and small cell lung cancers(35). Polyglutamation of methotrexate appears to allow methotrexate to accumulate preferentially in the absence of extracellular drug (35).

In addition to specific drug resistance, several mechanisms of MDR have been documented. Among these is the pleiotropic drug resistance associated with the MDR-1 gene and its protein product, the P-170 glycoprotein (41,42). This resistance occurs in malignant cells after exposure to a single agent derived from natural products such as anthracyclines, vinca alkaloids, actinomycin-D, and epipodophyllotoxins. Generally, a common finding in MDR cells is a decreased net intracellular accumulation of drug that is probably due to an increased, energy-dependent efflux mechanism (43). A net decrease in drug accumulation could result from several factors, including changes in influx or efflux, or could result indirectly from changes in binding to cellular constituents or metabolism (43). The majority of studies have

demonstrated that in MDR cells there is an increased rate of energy-dependent drug efflux mediated by the P-170 glycoprotein, to which, a variety of drugs, including calcium channel blocking agents bind (44,45,46,47,48).

There is yet another form of MDR frequently seen clinically in advanced cancers including ovarian cancer. This type of resistance is not associated with the classical MDR-1 resistance associated with the P-170 glycoprotein. In the ovarian cancers with this type of resistance the following has been documented both *in vitro* and *in vivo*: (a) the majority of specimens of ovarian cancer do not contain the MDR-1 gene; (b) the majority of cell lines established from MDR patients do not demonstrate adriamycin accumulation and MDR-1 amplification; and (c) the pattern of MDR seen in ovarian cancer is primarily not to natural products but rather commonly to alkylating agents, cisplatin and radiation (49).These human ovarian cell lines resistant to alkylating agents, cisplatin and irradiation have been demonstrated to contain elevated levels of glutathione(GSH) (49). These MDR cell lines, restored their sensitivity to alkylating agents either by nutritional depletion of glutathione precursors or by utilising a synthetic amino acid buthionine sulfoximine(BSO) which inhibits gamma-glutamyl-cysteine-synthetase resulting in GSH depletion (49). The exact mechanism by which GSH modulates the cytotoxicity of alkylating agents cisplatin and irradiation is unknown, probably, it increases drug metabolism via GSH-linked transferases(49). GSH may also facilitate repair of DNA cross-links (49).

There is a chance that as research progresses on uncovering the mechanisms of MDR in patients, a myriad of both specific and MDR mechanisms will be unravelled. It is also probable that mixtures of these mechanisms exist to produce the broad spectrum of resistance seen clinically.

There are several approaches currently being developed to overcome drug resistance. New drug analogues can now be screened in both sensitive and drug-resistant human tumour cell lines (50).

It has also been found that the common clinical resistance is more likely to be twofold to sixfold than 200-to 1000-fold seen in *in vitro* system. Therefore, drug regimens such as methotrexate-leucovorin (52) or high-dose cisplatin (53) have rationale even if the magnitude of drug dose increase is modest. However, techniques such as high-dose ablative therapy followed by bone-marrow transplantation could never be expected to be successful

unless clinically relevant drug resistance was relatively limited (54).

The use of calcium blockers such as verapamil or calmodulin inhibitors has had partial success in overcoming MDR-1 related drug resistance. It has been documented in *in vitro* studies in human ovarian cancer cell lines that adriamycin activity can be enhanced and its efflux reduced following incubation with verapamil (29). However, this result could not be reflected in the clinical trials because of unacceptable cardiac toxicity from verapamil (56). Subsesequent trials with other agents such as nifedipine and quinidine are underway (56).

There are attempts to reduce GSH in drug-resistant cells in order to alter resistance to alkylating agents and irradiation using BSO (49). There is an on going clinical trial in Europe to use aphidicolin succinate in order to overcome resistance to alkylating agents and cisplatin associated with an enhanced capacity to repair DNA damage (34).

Transfection of genes controlling protein products that would sensitise the resistant cells or provide potentially new lethal targets for chemotherapy are now being explored (59).

Other approaches to circumvent MDR are the use of biologic therapies. There is no proven cross-resistance of biological therapies to cell lines that when tested were resistant to chemotherapeutic agents. These include monoclonal antibodies linked to radioisotopes, natural toxins such as RicinA or pseudomonas exotoxin, or alternative therapies such as IL-2 alone, LAK cells and IL-2, combined (60).

Dose intensification in which the dose intensity is based upon the amount of drug administered over time (most commonly used is mg/m^2/week) has scored some success in overcoming drug resistance and better results. This includes the treatment of advanced breast cancer (61), advanced ovarian cancer (62), adjuvant therapies for both breast and colon cancer (63) and lymphomas (64). The major set back with dose intensity is that the dose-response relationship is likely to be a sigmoid curve with a threshold, a lag phase, and a plateau, and therefore, additional dose increase in certain responsive diseases (*i.e* Hodgkin's disease, and testicular carcinoma), would not be likely to produce additional improvement (65). On the other hand, in certain diseases, achievable dose intensities may not produce significant improvement if the dose intensity curve is relatively flat (*i.e* non-

small cell lung cancer). Finally, it is not yet clear what relationship exists between dose intensity and total dose of drug (65).

Regional use of chemotherapy has been tried in circumstances in which primary tumours or their metastases are confined to localised organs or particular regions of the body, or in situations in which a unique pharmacokinetic circumstance exists that favours rapid and localised regional clearance. This includes intra-arterial drug administration in cervical cancer, localised rectal carcinoma recurrences, intracarotid therapy for head and neck cancer, intracarotid administration of nitrosoureas for brain tumours (65) and intra-articular drug infusion in extremity soft tissue sarcoma or bone tumour to allow limb-sparing surgery (67,68). Other trials have included the use of implantable pump systems and catheters in perfusion therapy involving intra-arterial drug administration for primary and metastatic hepatic tumours (69). The conclusions from these trials were: (a) there is a higher response rate to intra-hepatic infusion therapy than to conventional therapy; (b) there is yet no evidence of a significant survival benefit (c) patients who have failed systemic therapy can respond to infusion therapy; (d) relapses usually occur systemically while liver disease remains controlled; (e) intra-hepatic infusion therapy can provide palliation for patients with metastatic disease confined to the liver and (f) local toxicity is substantial and includes bilary sclerosis, ulcer disease, and toxic hepatitis.

Intra-cavitary chemotherapy has been utilised for cancers confined to body cavities and for control of effusions in the peritoneum, pleura or pericardium. The rationale for such an approach is based upon the fact that for many chemotherapeutic agents the clearance from the body cavity is slower than the systemic clearance that results in a substantial differential concentration between the cavity and the systemic circulation. This approach has been used in the treatment of ovarian cancer in which disease remains confined in the intraperitoneal space. The drugs studied include 5-fluorouracil, adriamycin, cisplatin, melphalan and others. These have shown clear pharmacological advantage in minimal residual disease because of the limited penetration(1 to 3mm) of most drugs (70,71).

There has been a dramatic increase in the usefulness of hormones and antihormones in the management of some malignant diseases in the last few years. Prednisone and dexamethasone has been used for many malignant conditions such as in leukaemias, lymphomas, multiple myeloma and breast cancer, while stilbestrol has found its major application in prostatic carcinoma (72). The

antioestrogen tamoxifen and the antiadrenal aminoglutethimide are currently being used in the management of breast cancer and the progestational agent megestrol has come into widespread use in endometrial cancer (62). Recently, a new class of agents, luteinizing hormone-releasing hormone (LHRH) analogues are being used for prostatic cancer and are also being investigated for breast cancer therapy (62).

In conclusion, it is important to point out that this survey is not exhaustive and that there are many other areas of research on cancer treatment that has not been reviewed. Such areas include the use of differentiating agents, gene therapy, biological response modifiers and the role of free-radicals in cancer therapy which is the main theme of this thesis. It is clear that a new dawn is breaking in which our failures will act as a springboard to lay a firmer foundation for future success. We have acquired much knowledge and understanding in the last two decades on the mechanisms of carcinogenisis and the actions of most cytotoxic drugs. This will not only give a hope to the present generation but a promise to our future offsprings. We shall achieve this by reviewing our past and present achievements and to set up new avenues and goals for the future.

1.3 FREE RADICAL THERAPY OF CANCER: A LOW DOSE CONCEPT.

1.3.1 Introduction

In view of the present problems of cancer therapy, it is becoming clearer to scientists to search for new methods of treating neoplastic disorders. It is with this view that the concept of administering extremely low concentrations (10^{-3} -10^{-15} M) of free radical producing compounds to initiate a series of chain reactions in the host and for the products of this process to selectively destroy neoplastic cells with minimal side effects to the host was developed. There is no evidence from our literature survey that this type of work had been done before. It is important to acknowledge from the beginning that the birth of this idea is the result of hard work done by other scientists in increasing our understanding on this topic. Relevant facts on the subject are briefly reviewed followed by the setting of the hypothesis.

1.3.2 Background

Chemistry

A free radical is an atom or group of atoms with one or more unpaired electrons (73). Free radical reactions are generally chain reactions and most radicals are extremely reactive. Therefore, they ordinarily exist in very low concentrations of the order of 10^{-5} to 10^{-9}M or lower (74). Only radical reactions in which the dilute chain-carrying radicals are recycled will have useful rates. In these chain processes the radicals are generated in a step or steps called (a) *initiation* (b) *propagation* and (c) *termination* in which radicals are destroyed (75).

Initiation

$$LH \longrightarrow L^{\cdot} \qquad \text{(equation 1.1)}$$

Propagation

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \qquad \text{(equation 1.2)}$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \qquad \text{(equation 1.3)}$$

Termination

$$LOO^{\cdot} + L^{\cdot} \longrightarrow LOOL \qquad \text{(equation 1.4)}$$

$$LOO^{\cdot} + LOO^{\cdot} \longrightarrow LOOL + O_2 \qquad \text{(equation 1.5)}$$

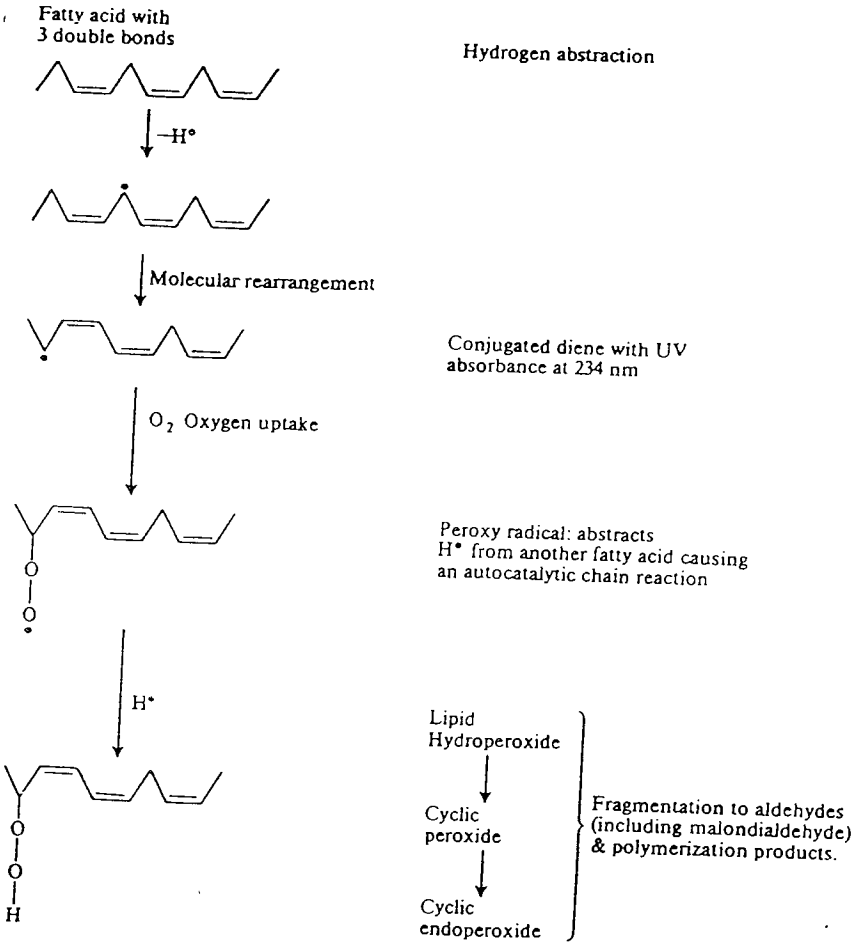
etc.

Excellent reviews of the mechanisms of initiation, propagation and termination of free radical reactions are available (74-76) and briefly summarised below.

The initiation reaction usually begins with the abstraction of a hydrogen atom from a polyunsaturated fatty acid (PUFA) resulting in the formation of a lipid radical ($L^{\cdot}$) (equation 1.1). The rearrangement of the double bonds result in the formation of conjugated dienes. The lipid radical reacts with oxygen (O_2) to form the lipid peroxyl radical ($LOO^{\cdot}$) (equation 1.2). The lipid peroxyl radical can abstract a methylene hydrogen from another PUFA to form a lipid hydroperoxide ($LOOH$) and a second lipid radical ($L^{\cdot}$) (equation 1.3). The lipid peroxyl radical can also cyclize to form a five membered lipid endoperoxide radical. The

lipid radical and lipid endoperoxide can react with O_2 and another PUFA to continue the radical chain reaction. The breakdown of lipid hydroperoxide and endoperoxide leads to the formation of numerous products including malondialdehyde (MDA) (fig. 1.2).

Fig. 1.2. *Initiation and propagation reactions of lipid peroxidation. The peroxidation of a fatty acid with three conjugated double bonds is shown (77).*



This process is called lipid peroxidation (LPO). The initiation process seems to be the most important step in the free radical mediated lipid peroxidation chain reactions. There are enzymatic as well as nonenzymatic processes in which lipid radicals are produced. Lipid peroxidation is usually investigated by measurements of the major peroxidation products, lipid hydroperoxide and conjugated dienes and of minor products, MDA, hexanal, fluorescent carbonylamine products and volatile hydrocarbons (fig.1.2).

Besides autoxidation of unsaturated fatty acids and radiation induced lipid peroxidation, a number of enzymes of diverse origin which includes cytochrome P-450 reductase (78), aldehyde reductase (48) and ketone reductase (79) are presumably involved in endogenous lipid peroxidation reactions. Various endogenous and exogenous organic compounds are reduced by the intracellular enzymes in one-electron transfer reactions, before they in turn reduce O_2 to $O_2^{\cdot -}$ (superoxide radical) (80). Thus, a cycle is formed of O_2 uptake at the expense of cellular reducing equivalents, notably NADPH, generating further active oxygen species. This process is called "redox recycle". Structures capable of "redox cycling" include catechols (81-83) and quinone anticancer drugs (such as doxorubicin and mitomycin C) which probably react with DNA by the formation of reactive oxygen species (84-87). O-naphthoquinone antibiotic beta-lapachone, is active by means of redox cycling of the quinone molecule, generating $O_2^{\cdot -}$, $HO^{\cdot}$ and H_2O_2 which lead to cytotoxic effects, particularly in cells low in antioxidant defense capacity as in the case of *Trypanosoma cruzi* (88).

In summary, numerous compounds which cannot all be mentioned here are able to maintain a redox cycle in the presence of redox catalysing enzymes (89). Because such enzymes are present in all biological systems (89,90) and are relatively nonspecific (91), the reactivities towards O_2 of the intermediates formed and the availability of oxygen species will itself decide whether reactive oxygen species will occur. In cancer chemotherapy, for example, the drug design is mainly based on the fact that tumour cells are less well supplied with oxygen and are in such hypoxic states, able to reduce quinoid and other reducible drugs in one-electron steps. They then form metastable intermediates which can reduce oxygen to form $O_2^{\cdot -}$ (92,93). The mechanisms of redox recycling of various drugs used in the experiments will be discussed in the relevant sections.

There is increasing evidence that the products formed during lipid peroxidation reactions are cytotoxic. The most important cell targets are nucleic acids, proteins (including oxygen scavenging enzymes) and membrane lipids (94,95).

The powerful antioxidant system of the cell maintains low steady state concentrations of oxygen metabolites. The enzymatic systems, include superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, glutathione S-transferases and catalase. The nonenzymatic are beta-carotene, alpha-tocopherol, vitamins A, ascorbic acid and glutathione just to mention a few. Further consideration on this system is discussed elsewhere in this thesis.

1.4 EVOLUTION OF THE HYPOTHESIS AND RELEVANT PHYSIOLOGICAL MECHANISMS OF FREE RADICAL REACTIONS

1.4.1 Introduction

It is evident from the above description that free radical and lipid peroxidation is essentially a degradative and pathological phenomenon; and there is no doubt that if lipid peroxidation proceeds to a substantial extent overwhelming the normally efficient protective mechanisms then gross cell damage can occur. However, there is increasing evidence that a low rate of lipid peroxidation occurs normally. Although present in low concentrations, hydrogen peroxide and superoxide are normal metabolites in the aerobic cells. The level of superoxide, which is more reactive, is maintained at 10^{-12} to 10^{-11} M by SOD (96), whereas the level of hyrogen peroxide which is less reactive, is regulated at concentrations up to 3 orders of magnitude greater, 10^{-9} to 10^{-7} M, depending on the hydrogen peroxide production (97). These are being generated by various biological processes in the cells.

From the above description it is clear that although uncontrolled free radical reactions have damaging effects on cells, free radicals play a very important part in the normal cell physiology. Oxidative influences on enzymatic reactions, plasma membrane potential, development and differentiation, and on tumour cells

are briefly discussed because of their interrelationship. Finally, the hypothesis is put forward and discussed.

1.4.2 Free radical and enzyme reactions

Free radical mechanisms have been proposed for various redox enzyme reactions (98-99). The reactions may be grouped into three types:

(a) enzymes that catalyse the formation of substrate free radicals;

(b) enzymes that promote the decay of free radicals and

(c) free radicals exist in the catalytic processes of enzymes.

The involvement of a free radical mechanism in enzyme reactions cannot be easily concluded even when free radicals are detected by ESR methods (99). Quantitative analysis is needed to answer the question whether the formation of the free radical is a main or side path in the enzyme reaction. However, it might be difficult to detect free radicals when free radical reactions occur as a transient activation process at a localised site of an enzyme-substrate complex. But their major function is probably to activate substrates through one-electron transfer reactions and in rare cases, by nucleophilic or electrophilic attack (98). The role of free radicals formed in enzyme intermediates is to facilitate electron transfer with a second substrate or produce a free radical of a second substrate.

1.4.3 Free radical and membrane potential

Plasma membrane potential is an important physiologic parameter dependent both upon cellular metabolism and upon the integrity of the cell membrane. Oxygen radicals have been shown to produce significant and early changes in the membrane potential. It was originally suggested in 1971 that oxygen radicals played a role in the generation of the membrane potential (103). This theory has not been widely accepted.

Indirect evidence suggests the possibility that changes in membrane potential might mediate the reported influences of oxygen radicals on cellular development. Parallel changes have been reported in rates of cyanide-resistant respiration, GSH concentration, superoxide dismutase activity (104) and membrane potential (105) during differentiation. Furthermore, both membrane potential(106) and "fluidity" (107) have been implicated in the regulation of normal cellular division. Since oxygen radicals are capable of modulating both membrane fluidity

(108) and membrane potential and since membrane potential influences lipid ordering in membranes (109), it is possible that these interrelationships between oxygen radicals and membrane potential could provide key links in both normal and abnormal cellular development.

1.4.4 Free radical, development and differentiation

The mechanisms by which selective genes become repressed or derepressed at critical times in development is presently unknown. It has been postulated that free radical generation by various metabolic pathways governs some developmental changes (104). There is also considerable evidence to suggest that ionic gradients and charge gradients (as discussed above) exert potent control over a variety of developmental events and that these gradients may themselves be influenced by oxidative metabolism (110). Therefore, development may be characterised as having metabolic, redox, membrane polarity, and ionic gradients.

Total SOD activity increases during the development of a variety of organisms (100,111,112). Other antioxidant enzymes such as catalase (47,101,102,111), GSH peroxidase (51,111) and the S-transferases also increase as development proceeds (55,57,58,111).

Cell division and cell proliferation are associated with increases in sulphydryl concentration (51). Differentiation to a non-mitotic, non-regenerative cell type occurs with a concomitant loss of GSH (114,115); differentiation to a cell type that retains a high regenerative potential (such as liver tissue) occurs without a significant loss of GSH and may be associated with an increase in GSH. Sulphydryl concentration increases in reproductive tissues during differentiation (116,117).

Ionic gradients play an important role in the cell cycle and development. Mitosis is dependent on calcium release and resequestration (118-122). Redox state affects the ability of cells to sequester calcium as well as other ions. Membrane polarity affects the entry of calcium into cells and its activity subsequent to entry. Ions can interact with chromatin to cause conformational changes and to affect the expression of pre-mRNAs. The karyoskeleton as well as free radicals have also been implicated as possible controlling elements in the processing and release of pre-mRNA from the nucleus (123).

The cytoskeleton of cells transmits information from the plasma membrane to the nuclear membrane and can affect changes in

gene expression. The integrity of the cytoskeleton and probably the nuclear karyoskeleton is influenced by membrane polarity, membrane fluidity, redox status, and ionic gradient (124,125). Chernavskii et al. (126) implicated free radical oxidation of membranes as a controlling element of the cell cycle. In their model, changes in membrane fluidity, resulting from membrane peroxidation and changes in lipid composition, are transmitted via the cytoskeleton to the nuclear envelope. Again, alteration in nuclear configuration was postulated to alter gene expression and, in this case, to regulate cell division. It seems therefore, that there is simultaneous interactions of all the components discussed, which may influence gene expression that affects the normal cellular development.

1.4.5 Free radical, lipid peroxidation, and cancer: a hypothesis

Several studies point to the existence of disturbance in the metabolism of the reactive species of oxygen in cancer cells. The following facts are established:

(a) in normal cells lipid peroxidation by-products interfere with and inhibit cell division. It has been shown that PUFAs inhibit the proliferation of smooth muscle cells, fibroblasts and foetal brain cells in culture and that the effect is overcome by antioxidants such as alpha-tocopherol and alpha-tocopherolquinone (127-132). Growth of rapidly dividing normal cells is also known to be inhibited by 4-hydroxyalkenals, which are aldehydic products of peroxidised membranes (133-139). Such substances inhibit DNA synthesis and consequently cell division, by blocking selectively the sulphydryl groups of DNA polymerase (140-145). Experiments similar to these reported above have been performed by using tumour cells in culture and transplanted tumours. It has been shown that arachidonic acid significantly reduces cell division in a human glioma cell clone in culture and that 4-hydroxyalkenals inhibit the growth of a large variety of tumour cells in culture as well as that of transplanted tumours (132,133,145,146).

(b) tumour subcellular organelles show a markedly decreased susceptibility to peroxidation, and it has been shown to correlate to the growth rate of the tumour (147).

(c) associated with such a high resistance to the action of oxy-radicals are the changed lipid composition of cellular membranes, whose content of PUFAs is particularly decreased. In addition, there are changes in the static and dynamic properties of the bilayer, that can lead to hiding, exposing and shedding of enzymatic proteins and /or receptors of the cell surface (148).

(d) cellular oxy-radical scavenging enzymes are markedly reduced (149).

Based on this evidence, the hypothesis that the abnormal oxy-radical metabolism of tumours plays a role in their growth can be reasonably sustained. Peroxidation by-products, that are greatly diminished in tumours, become unable to exert the normal controlling functions on cell division.

Prostaglandins(PG), thromboxanes(TX) and leukotrienes(LT) are products of lipid peroxidation through the so-called "arachidonate oxygenation pathways" (150-151). Both mast cells and macrophages avidly metabolise arachidonic acid to PG, TX and LT. Certain PGs are thought to modulate cell multiplication and differentiation as well as the immune response (152). TX, a potent vasoconstrictor, will decrease blood flow and therefore, may hinder metastasis of tumours. The LTs may affect tumour growth and spread. LTB4 especially seems to modulate immune function at very low concentrations (151). LTB4 is not only a potent chemotactic factor (153), but has also been shown to augment the killer function of lymphocytes (154,155); enhance the production of interleukins 1 and 2, and interferon as well as to induce suppressor lymphocytes (154,156). The interleukins, interferons and lymphocytes all have an established role in host defence, including the elimination of tumour cells (160).

Although tumour cells have low levels of peroxidation most of them have lost the capacity to induce MnSOD in response to high oxygen or superoxide concentration unlike the normal cells (157,158). In addition, they have relatively low levels of other antioxidant enzymes as compared to their normal counter-part as pointed out earlier. This is of special advantage to selectively target tumour cells to free radical attack.

Dormandy (159) argued that if transformation of normal into malignant tissue makes cells less peroxidizable; this failure of peroxidation, an essential component of the normal self-destruct mechanism, allows cancer cells to survive and multiply. And that, perhaps radiotherapy and many forms of cancer chemotherapy, powerful promoters of lipid peroxidation, are effective within limits because they prop up a cytotoxic process which is or should be, a built-in programme in every cell.

There is increasing evidence, therefore, that a low rate of lipid peroxidation, whilst not overwhelming normal cell defences and not causing significant disturbance to local membrane structure, may be of considerable physiological importance. Defense against

cancer is not fully understood up to-date but probably related to a surveillance system. The cellular and humoral immune defense mechanisms may not be competent to control problems that may arise within a cell. Instead, lipid peroxidation and its products may have a major role in this type of "internal surveillance "(i.e within the cell) including some control on gene functions. In view of this concept, the following proposals made below are the main theme of this thesis.

If the process of lipid peroxidation which physiologically occurs in normal cells but is low in malignant cells, is responsible for normal cell functions including the anticancer surveillance, then propping it up may help the body eliminate cancer and related disorders. This method would probably be more selective in destroying malignant cells because they lack the ability to induce some of the antioxidant enzymes such as MnSOD when challenged with oxidative species. In addition, malignant cells have relatively lower levels of most of the antioxidant enzymes compared to their normal counterparts. Since free radicals initiate chain reactions, only a few molecules of the free radical producing compound may be needed to start the chain reactions. As the chain reaction builds up, probably over several days, the normal body tissues would be able to induce protective antioxidant enzymes. The malignant cells which usually have low levels of these enzymes and are not capable of inducing them, would probably be selectively destroyed. This concept would apply to any similar situation where an organism is incapable of protecting itself from oxidative attack like the case of the *Trypanosoma cruzi* (88). It was pointed out earlier in this review that since most radicals are extremely reactive, they can only exist in very low concentrations, therefore, only radical reactions in which the dilute chain-carrying radicals are recycled will have useful rates. It is proposed that very low concentrations ranging from 10^{-3} to 10^{-15} M of the initiators be used. Long distance effects away from the original locus of peroxidation due to diffusion of secondary and tertiary products such as lipid alkoxy radicals and non-radical carbonyl compounds would enhance the effects. In addition, superoxide radical is relatively stable and can diffuse to regions of low pH such as in a tumour where it picks up a proton and is activated to $HO_2\cdot$. This would cause more reinitiation, building up the chain reactions in most parts of the body and damaging the malignant cells. In this way, the malignant growth may be brought under control.

1.5 AIM OF STUDY

The overall aim of the project was to evaluate the use of low concentrations of free radical producing compounds as anticancer agents both *in vitro* and *in vivo*. The preliminary data obtained from various compounds *in vitro* was used to form the basis for a more wide-ranging attempt to stimulate the endogenous production of free radicals in order to assess the effectiveness of such treatment in relation to cancer therapy. Data on the mode of action were also obtained. In general, the plan of investigation was as set out below:

(a) Effects of treatment of tumour cell suspensions with free radical producing compounds on cell growth *in vitro*.

(b) Studies on the activity of these compounds against tumour growth *in vivo*.

(c) Investigations concerned with improving their anticancer effectiveness.

(d) Evaluation of mode of action.

(e) Assessment of the levels of antioxidant enzymes.

CHAPTER 2: MATERIALS

2.1 Animals

Pure strain inbred NMRI mice were bred in the animal house of Aston University, Birmingham. Animals were fed *ad libitum* a rat and mouse breeding diet (Pilsbury's, Birmingham, West Midlands) and were given free access to water.

BDF_1 and CBA/CA mice (24-28g) were purchased from Banting and Kingman, Hull, U.K.

2.1.1 Tumour systems

The MAC tumours are a series of transplantable adenocarcinomas of the large bowel of mice from primary tumours induced by prolonged administration of 1,2-dimethylhydrazine (279). Tumours were passaged in pure strain NMRI mice (age 6-8 weeks). MAC16 tumour (doubling time; 10 days) was excised from donor NMRI mice, placed in sterile 0.9% NaCl and cut into fragments 1 x 2mm in size. Fragments were then implanted subcutaneously into the flank of mice using a trocar (Mike Wynter and Wayne Fleary Aston University). Sometimes 10^6-10^7 cells from respective cell cultures were injected subcutaneously or intramuscularly instead of tumour fragments. MAC16 is a poorly differentiated tumour.

P388, lymphocytic leukaemia, was passaged in BDF1 mice every seven days by injecting intraperitoneally ascitic tumour from the donor mouse. This tumour was originally induced in 1955 in a DBA/2 mouse by painting with 3-methylcholanthrene (280).

TLX5, is a fast growing lymphoma which was originally induced in the thymus of CBA mice by X-irradiation (24). It was passaged as an ascites tumour in CBA/CA mice intraperitoneally every seven days.

2.2 Cell lines

The L929, sensitive to TNF, and the L929R, with induced resistance to TNF, cell lines were kindly donated by Dr. N. Mathews, Dept. of Microbiology, UWIST, Cardiff, Wales. They have a doubling time of 18 hours and grow as a monolayer.

The HL60, a human myelomonocytic leukaemia (originally classified as promyelocytic) donated by Patterson Laboratories, Manchester was grown as a cell suspension culture with a doubling time of 24 hours.

K562, human chronic myelocytic leukaemia, donated by Charing Cross Oncology Dept. was grown as a cell suspension culture with a doubling time of 24 hours.

GM892, human lymphoblastoma, supplied by the cell bank Aston University was grown as a cell suspension culture with a doubling time of 22-26 hours.

Raji, a Burkitt lymphoma, supplied by the cell bank Aston University, was grown as a cell suspension culture with a doubling time of 22-26 hours.

MAC16, a mouse colon adenocarcinoma, was donated by Drs. J A Double and M Bibby of University of Bradford, and was grown as a monolayer/suspension culture with a doubling time of 36 hours.

All the cell lines were screened for mycoplasma and maintained in RPMI-1640 medium supplemented with either 10% foetal calf serum (FCS) or 1% ultroser-G (a chemically defined medium) in 95% air, and 5% CO_2 at 37°C.

2.3 Gases

The following gases were purchased from BOC Ltd, London:

nitrogen

oxygen

argon

air: carbon dioxide (95:5)

2.4 Purchased materials, chemicals and reagents

Aldrich Chemical Company Ltd Gillingham Dorset U.K.

ammonium hydroxide

ammonium chloride

azobenzene

p-benzoquinone

t-butylhydroperoxide

n-butanol

chloroform

carboxy-PROYL free radical

carbazole

carbon-tetrachloride

crystal violet

p-chloronitrobenzene

5,8-dihydroxynaphthoquinone

diphenylhydrazine

dioxane

diphenylpicrylhydrazyl free radical

diphenylpicryl hydrazine

dichlorofluorescein

duodocane thiol

doxorubicin

diazomethane

ethanol

ether

galvinoxyl free radical

hexane

5-fluorouracil

methyl ethyl ketone peroxide

nitro blue tetrazolium

ligroin

lead dioxide

phenylhydrazine

phosphotungstic acid

quinzarin

sulfonazo 111

zinc dust

Sigma Chemical Company Ltd, Poole, Dorset U.K.

albumin (bovine)

boron trifluoride solution in methanol (14%wt/v)

butylated hydroxytoluene

catalase

chlorambucil

diethylenediaminepenta-acetic acid

dimethylforamide

ethylenediaminetetra-acetic acid (EDTA), disodium salt

all the fatty acids /fatty acid standards used in the experiments

glutathione-reduced

glutathione reductase

glutathione peroxidase

indomethacin

lipoxygenase (soya-bean)

nicotinamide adenine dinucleotide phosphate reduced (NADPH)

superoxide dismutase

sodium nitrite

sodium azide

trypan blue

vitamin E

xanthine

xanthine oxidase

BDH Chemical Ltd, Poole, Dorset U.K.

acetic acid (glacial)

benzophenone

copper sulphate

calcium carbonate

calcium chloride

dimethylsulfoxide

D.P.X., neutral mounting medium

2,4-dinitrophenylhydrazine

dipotassium hydrogen orthophosphate, trihydrate ($K_2HPO_4.3H_2O$)

hydrochloric acid

ferrous chloride

ferric chloride

methanol

oleum

potassium dihydrogen orthophosphate (KH_2PO_4)

potassium nitrate

picryl chloride

sodium hydroxide (pellets)

sodium chloride

sulphuric acid

Gibco, Paisely, Scotland

foetal calf serum

RPMI 1640 (with 25mM HEPES and L-glutamine)

ultroser-G

Flow laboratories, Herts, U.K.

ITS Pre-mix

ITS Pre-mix plus

Oxoid Ltd, Basingstoke, Hants, U.K.

phosphate buffered saline

Coulter Electronics Ltd, Luton, Beds, U.K.

Isoton 11 balanced electrolyte solution (azide free)

Merck, Darmstadt, U.K.

Partisil-10

Supelco,Surrey, U.K.Ltd

GP3% SP-2310/2%SP-2300 on Chromosorb WAW

2.5 Gifts

Mitozolomide was synthesised and donated by May and Baker, Dagenham U.K and was stored at 25°C.

Recombinant human Tumour Necrosis Factor-alpha (TNF), 6 x10^7 U/mg, was donated by Boehringer Ingelheim Ltd, Bracknell, Berkshire and was stored at 4°C.

2.6 Solutions

Phosphate buffered saline

Oxoid PBS	5 tablets
Distilled water	to 500ml

This gave a solution equivalent to 0.8g sodium chloride, 0.2g potassium chloride, 1.15g disodium hydrogen orthophosphate dihydrate, 0.2g potassium dihydrogen orthophosphate per litre, pH 7.4.

0.05 M phosphate buffer (pH 7.8)

A) 0.1 M KH_2PO4: 13.609g

Distilled water to 500ml

B) 0.1M K_2HPO4 8.7g

Distilled water to 500ml

Take 21ml of (A) and 230ml of (B), check the pH, adjust if necessary, then make up to 500ml with distilled water.

Preparation of solutions for Superoxide Dismutase assay

1) Sodium carbonate: (pH 10.2) 1.590g

Distilled water to 100ml

2) EDTA: 0.117g

Distilled water to 100ml

3) NBT: 1.17mg

Distilled water 3ml

4) Xanthine: dissolve 45.63mg

in a little 1M NaOH

Distilled water to 100ml

Preparation of solutions for assay of Glutathione Peroxidase

1) 0.9mM NADPH: 7.5mg

Distilled water to 1ml

2) 15 mM GSH: 110.2mg

Distilled water to 2ml

3) H_2O_2: dilute 30%W/V H_2O_2: H_2O 1:10

4) NaN_3 10mg

Distilled water to 10ml

5) Phosphate buffer/EDTA (pH 7.0) 0.9306g EDTA

to 500ml 0.05M PO_4 buffer.

Preparation of solution for assay of Catalase

1) H_2O_2-phosphate buffer: 0.160ml H_2O_2 (30%W/V)

to 100ml 0.05M PO_4 buffer

Reagents for Protein determination

Reagent A: 2% Na_2CO_3 in 0.1M NaOH solution;

Reagent B 0.5% $CuSO_4.5H_2O$ in 1%Na.K. tartrate solution

Folin's reagent: dilute 1:1 with distilled water

Bovine serum albumin standard solution 1mg/ml

2.7 Compounds synthesised at Aston University

2.7.1 The synthesis of linoleic hydroperoxide

LOOH was synthesised by the method of Hamberg and Samuelsson (15).

Linoleic acid was incubated with soya-bean lipoxygenase enzyme in 0.04M NH_4OH-NH_4Cl buffer (pH 9.0) in an oxygen atmosphere (500mg substrate, 75mg enzyme, activity 8000U/mg, total volume 500ml).

After incubation for 1 hr the hydroperoxide was extracted with ether (after acidification to pH3.0), dried, reduced in volume and methylated with diazomethane. The methylated ester of the hydroperoxide was then purified by chromatography on Kieselgel H using 10% ether in hexane as the eluting solvent. Fractions containing the hydroperoxide were pooled concentrated and analysed by thin layer chromatography on silicic acid using hexane (30:70 V/V) as the developing solvent. This showed that the methyl ester of the hydroperoxide was homogeneous when visualised using ultra-violet light, ferrous thiocyanate spray, and chromic acid spray followed by charring.

High performance liquid chromatography was carried out, at ambient temperatures, on the purified material as follows. A stainless steel column (4.6 mm (internal diameter) x 500 mm) packed with Partisil-10 (Reeve Angel Scientific Ltd, London). The column was activated using anhydrous ethanol and equilibrated with the eluting solvent (0.5% anhydrous ethanol in dry hexane)

before chromatography of the samples. Solvent was delivered at 4 ml/min via an injection head using a Waters 6000 constant flow pump. The effluent was monitored at 235 nm using an ultra-violet spectrophotometer.

The pure hydroperoxide was stored under argon at -20°C.

2.7.2 Synthesis of potassium salt of 2,2-diphenyl-1-(2',4'-dinitro-6'sulphophenyl) hydrazyl (DDSH) and hydrazine homologue (DDSH2)

This compound was first prepared by Ikrina and Matevosyan (22) and was obtained as follows.

p-Chloronitrobenzene 16 g was heated with 36 ml of 25% oleum and 36 ml of concentrated H_2SO_4 for 20 hours at 125°C. The mixture was cooled, and 20.1g of KNO_3 were added slowly, keeping the temperature between 40 and 60°C. It was then heated for 4 hours at 110°C, and poured on to ice, when yellow needles of 2,4-dinitro-6-sulphochlorobenzene potassium salt separated out.

2,4-dinitro-6-sulphochlorobenzene potassium salt 6 g was dissolved in 20ml water containing 4g of $CaCO_3$, added to 5g of 1,1- diphenylhydrazine in 20ml dioxan, and the mixture was heated on a water-bath for 24 hours. The $CaCO_3$ was filtered off and dark red crystals of 2,2-diphenyl-1-(2',4'-dinitro-6'-sulphophenyl)hydrazine potassium salt separated out; and was recrystallized from 95% aqueous dioxan. The yield was about 44%. The melting point was 187°C consistent with the literature value of 187-188°C with decomposition (22).

Elemental analysis:

$C_{18}H_{13}KN_4O_7S \cdot H_2O$

Calculated % C-44.44; H-3.09; N-11.52; S-6.59

Found % C-44.65; H-3.24; N-11.46; S-6.64

The free radical was generated by oxidizing a solution, containing 2g in 10ml dioxan, with excess lead dioxide (20 times by weight) for 4 hours. The solution was filtered and concentrated. Addition of ether precipitated blackish-purple crystals of the free radical. The precipitate was dried in vacuum. The yield was about 52%. On heating the product it began to change at 115°C and decomposed

vigorously at 118°C, consistent with the literature values 110-120°C with decomposition (22). Thin-layer chromatography was performed on both products.

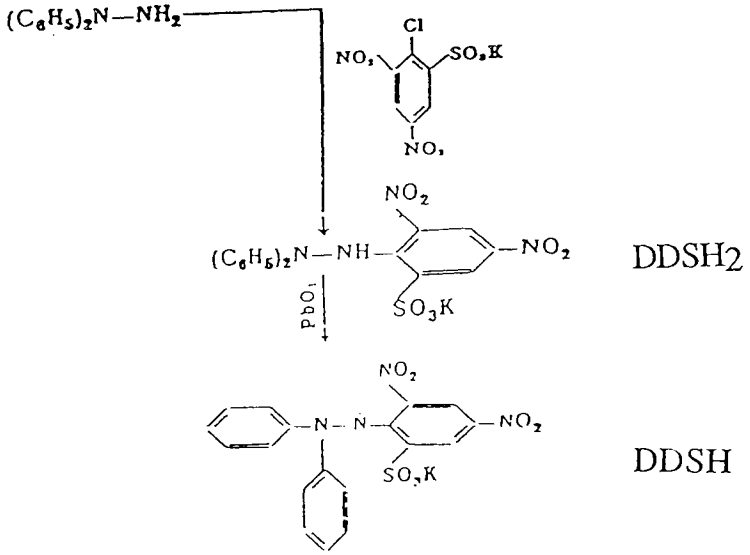
Elemental analysis:

$C_{18}H_{12}KN_4O_7S \cdot (C_2H_5)_2O$.

Calculated % C-48.80; H-4.07; N-10.35; S-5.91

Found % C-48.71; H-3.87; N-10.56; S-5.87

Synthetic pathway:



2.7.3 Synthesis of N-carbazylpicryl nitrogen (CPH) and carbazyl picrylamine (CPH_2).

The compound was first synthesised by Matevosyan *et. al.* (23) and was obtained as follows.

0.03 mole of sodium nitrite was added to 0.02 mole of carbazole in glacial acetic acid and was stirred continously for 2 hours at room temperature. At the end of this period the mixture was heated to 40-45°C and another 0.005 mole of sodium nitrite was added. The mixture was cooled to 8-10°C and the nitrosocarbazole

precipitated out. This was filtered and recrystallized from ligroin (b.p. 50-60°C). Light green crystals were isolated, and dried in a vacuum desiccator.

To a solution of 0.12 mole of nitrosocarbazole in ether was added three times the amount by weight of glacial acetic acid. 1.2 mole of zinc dust was added to the solution of nitrosocarbazole while stirring vigorously. The temperature was kept between 8-10°C. The mixture was stirred for 2 hours; solid separated and the ether solution, after washing with a saturated of sodium bicarbonate, was dried with calcium chloride. The aminocarbazole was separated from the ether solution as a hydrochloride salt by passing in a stream of dry hydrogen chloride. The resulting hydrochloride was transformed into the free base by treatment with a hot alcoholic solution of 25% ammonia and the amine precipitated from the solution. After recrystallizing from ethanol colourless crystals of N-aminocarbazole (carbazylamine) were obtained.

0.05 mole of picryl chloride in chloroform was added to a solution of 0.1 mole of carbazylamine in chloroform. The mixture was boiled for 20-30 minutes and reduced to a third the original volume. To this twice solution the amount of ethanol was added and the mixture was boiled for another 20-30 minutes. The brick red precipitate of N- carbazylpicrylamine, which formed was filtered off and recrystallised from a mixture of chloroform and alcohol (1:3) and dried in vacuum. The yield was about 68%. The melting point was 238°C (with decomposition), consistent with literature values (239-241°C) (23).

Elemental analysis:

$C_{18}H_{11}O_6N_5$

Calculated % C-54.96; H-2.80; N-17.81

Found % C55.14; H-2.85; N-17.84

To a solution of 0.05 mole of carbazylpicrylamine in dry chloroform was added 20 times the amount (by weight) of lead dioxide and 0.5 mole of ignited sodium sulphate. The mixture was shaken for 2 hours. The dark violet solution of N-carbazylpicryl nitrogen free radical was separated from the solid, and the chloroform was rotary evaporated from the mother liquor to form a crystalline paste which was dried at room temperature in a

vacuum. The melting point was 218°C (with decomposition), consistent with literature values 215-217°C (23).

Elemental analysis:

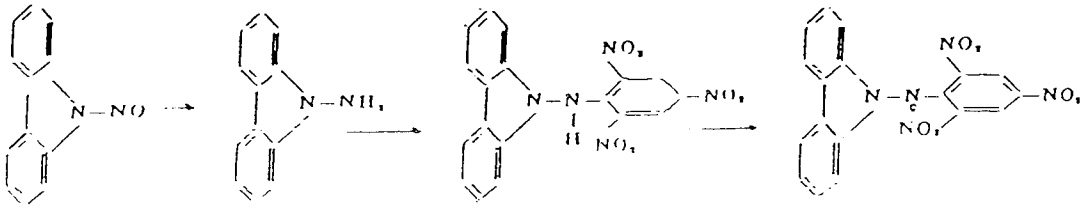
$C_{18}H_{10}O_6N_5$

Calculated % C-55.10; H-2.55; N-17.86

Found % C-55.23; H-2.51; N-17.94

Thin layer chromatography was performed on both compounds.

Synthetic pathway:



CPH_2 CPH

CHAPTER 3: METHODS

3.1 Introduction

There has been much interest in lipid peroxidation in relationship to its importance as a primary mechanism of tissue injury (161-163). Lipid peroxidation is a free radical-mediated process that results in the degradation of unsaturated fatty acids to complex varieties of products (167). A lot of studies have centred around the peroxidation reactions of arachidonic acid, which results not only in marked changes in membrane structure and function, but also in the production of mediators such as the prostaglandins (PGs), leukotrienes (LTs), epoxy- and hydroxy- derivatives, and carbonyl degradation products (164-166). These carbonyl degradation products include alkanals, alkenals, and 4-hydroxyalkenals (167). The latter groups of compounds react with thiol groups and can inhibit DNA synthesis and cell division (174).

There are a variety of procedures that can be used to stimulate lipid peroxidation in tissues, isolated cells, and suspensions of intracellular organelles. They include exposure to ionising radiation, the addition of transitional metal salts or complexes, incubation with peroxides like hydrogen peroxide or cumene hydroperoxide, administration of or incubation with carbon tetrachloride and several other substances (176). This would lead to production of reactive intermediates such as singlet oxygen and superoxide radicals. The only general method of detecting free radicals in biological systems is by electron spin resonance (esr), a technique that permits the determination of the structure and concentration of the free radical (177). Other methods used are discussed elsewhere in the thesis.

Several anticancer drugs are known to bring about their tumoricidal actions by a free radical dependent mechanism. A majority of the studies has reported that adriamycin (doxorubicin), mitomycin C, bleomycin etc., augument free radical generation and lipid peroxidation (175,178). Other studies have reported the cytocidal actions of PUFA to be mediated by a free radical mechanism (168,169,179).

In this section, the mechanisms of free radical formation of the compounds used in the investigations will be reviewed and this will provide the rationale for choosing them in the study. These compounds fall broadly into these groups (see appendix 11 for their structural formulae):

(a) unsaturated fatty acids,

(b) anticancer quinones,

(c) azo anion and hydrazyl free radicals,

(d) nitroaromatic anion free radicals,

(e) thiyl free radical,

(f) other stable free radicals,

(g) miscellenous- mitozolomide, chlorambucil, 5-fluorouracil(5-FU).

3.1.1 Unsaturated Fatty Acids

The role of polyunsaturated fatty acids (PUFAs) and peroxides has already been discussed in relation to lipid peroxidation. (see the Introduction, Section 1.3.2).

3.1.2 Hydroperoxides and Peroxides

It has been established that most of the transitional metals catalyse the decomposition of peroxides and this has been reviewed (185-187). For example iron catalyses the decomposition of hydroperoxides as shown below:

$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO^{\cdot} + HO^{-} \quad \text{(equation3.1)}$$

$$Fe^{3+} + ROOH \longrightarrow Fe^{2+} + ROO^{\cdot} + H^{+} \quad \text{(equation3.2)}$$

sum: $2ROOH \qquad RO^{\cdot} + ROO^{\cdot} + H_2O$ (equation3.3).

Lipid peroxidation is known to be catalysed by haemoglobin, iron protophyrin, and other naturally occuring iron salts and complexes (188) and it appears certain that this type of catalyst is very important in any oxidations that occur in vivo. It is also known that ferric ions can be reduced by other natural materials, such as ascorbic acid, hence maintaining the redox reactions.

In the presence of unsaturated lipid, these free radicals ($ROO^{\cdot}$ and $RO^{\cdot}$) can participate in the propagation of lipid peroxidation (185).

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There are two general free-radical mechanisms for the anticancer properties of many quinone compounds that have been proposed (283). It has been postulated that the semiquinone of these antitumour compounds are sufficiently stable to enter the nucleus, where they may intercalate and/or react with DNA and/or with other macromolecules (171).

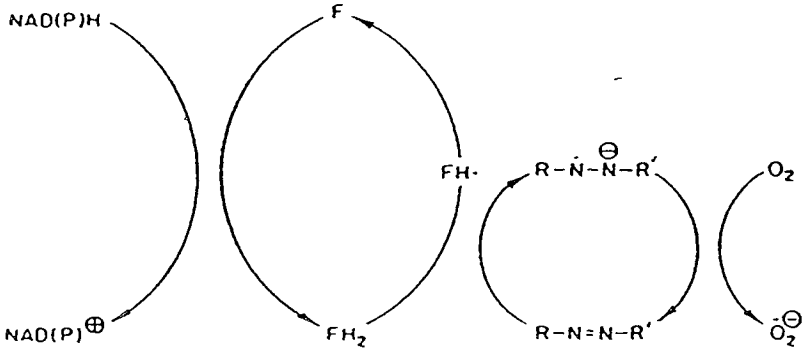
The second mechanism of antitumour activity may involve the quinone catalysed superoxide-derived species, such as the hydroxyl radical causing DNA scission (172).

3.1.4 Azo and Hydrazyl Free Radicals

Azo compounds have mainly been used as dyes (199). Among the compounds in this group is the diazonaphthol, sulfonazo 111, which is structurally related to monoazo food dye Red No. 2. Sulfanazo 111 is used in the titrimetric determination of sulphates and organic sulphur (199). Its esr spectrum is characteristically similar to that of an azo radical (200). Using a similar analysis the *g* value of the sulfanazo111 metabolite is indistinguishable from the *g* value of the azobenzene radical or of chemically related diphenyl picryl hydrazyl (DPPH) (199). DPPH is a stable free radical used as a *g* value standard in esr spectra (199). There is no work reported up to-date in literature of its cytotoxic effects to malignant cells.

It has been proposed by Hernandez et al. that cytochrome P-450 reductase is the primary azo-reductase in rat hepatic microsomes (202). Therefore, the azo-compound probably accepts one electron from this flavoprotein to form the azo free radical 202). And in the presence of oxygen, the azo-compound is probably reduced to a free radical which would react with oxygen to form superoxide radical with the regeneration of the parent compound (202). In such a scheme there would be no net reduction of the azo-compound since the parent compound would be reformed. The stimulation of oxygen uptake was partly reversed by superoxide dismutase and catalase, implying the presence of both superoxide and hydrogen peroxide respectively (fig.3.2). The oxidation of the hydrazine intermediate could be responsible for the generation of superoxide.

Fig. 3.2 Proposed mechanisms of azo compound-mediated production of superoxide radical and oxygen consumption. It assumes that the azo free radical precedes the formation of the hydrazine intermediate, and that the free radical is the species that reacts with oxygen (199).



Phenylhydrazine and other drugs which induces red blood cell haemolysis in individuals with a glucose-6-phosphate dehyrogenase deficiency probably act via peroxidation initiated by the production of superoxide radicals produced during their metabolism (201,203). These individuals are unable to regenerate reduced glutathione, the cofactor for GPx leaving them especially susceptible to drug-induced haemolytic anaemia. Superoxide dismutase is reported to inhibit phenylhydrazine-induced red blood cell haemolysis and peroxidation (201,203).

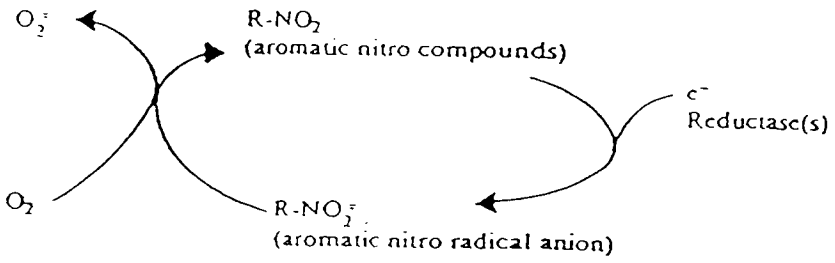
3.1.5 Nitroaromatic Free Radical

There are a number of nitroaromatic compounds in clinical use. They include the antibacterial 5-nitrofurans and the trichomonicidal 5-nitrothiazole (204).

Their activity has been proposed to depend on reductive activation of nitroreductases (191,192,205). Nitroreduction is also necessary for the nitro-compound-induced bacterial mutations and the associated damage to DNA (193). Carcinogenic nitrocompounds require the nitro group for their carcinogenicity

(206). Flavoproteins have been suggested to play a role in their metabolism. Several studies have been conducted on the mechanisms of metabolism of these compounds. The results are all consistent with (a) the formation of nitroaromatic radicals under aerobic conditions, (b) the rapid air oxidation of these radicals resulting in the catalytic generation of superoxide and superoxide-derived species, and (c) the oxygen inhibition of nitroreductases (204,207) (fig.3.3).]

Fig.3.3 *Proposed scheme of redox cycling of nitro-aromatic compounds (207).*



3.1.6 Thiyl Free Radicals

There is no esr evidence that thiyl free radical ($RS^{\cdot}$) is enzymatically formed, although the oxidation of thiol compounds by peroxidase in the presence of Mn^{2+} and certain phenols may form thiyl radicals (210). Thiyl free radicals can be formed by the reaction of sulphydryl compounds with transition metals (211) (equation 3.4).

$$\mathbf{RSH + Me^{n} \longrightarrow RS^{\cdot} + Me^{n-1}} \quad \text{(equation 3.4)}$$

This has been observed during the reaction of Cu^{2+} with penicillamine, copper is reduced to Cu^{1+} implying the formation of the thiyl free radical (212,213). It has been argued that the thiyl radical has a life time longer than several minutes (204). Thiyl

radicals play important roles in the mechanisms of radiation damage and radioprotective process (215).

3.1.7 Miscellenous Compounds

For comparative purposes, the actions of the above free radical producing compounds have been compared with non-free radical producing compounds such as chlorambucil, an alkylating agent and 5-FU a antimetabolite. In addition, both galvinoxyl and carboxy-PROXYL stable free radicals were used for comparative purposes with DPPH.

3.1.8 Antioxidants

An antioxidant is a compound which decreases the rate of autoxidation reaction. They can be classified as (a) preventive- that is, an antioxidant which prevents the initiation of the autoxidative process. (b) Chain-breaking or primary antioxidant is one which scavenge the chain-propagating free radicals (216) (**table 3.1** below).

Table 3.1 Major antioxidants (modified from reference216).

Preventive antioxidants:

Some metal chelators

Superoxide dismutase

Catalase

Glutathione peroxidase

Glutathione reductase

Glucose-6-phosphate dehydrogenase

Chain-breaking antioxidants:

Glutathione

Ascorbate

Ubiquinone

Beta-carotene

Alpha-tocopherol

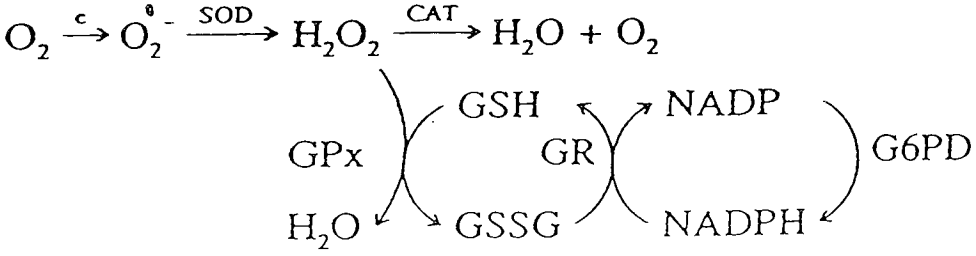
Synthetic-butylated hydroxytoluene and butylated -hyroxyanisole.

3.1.9 Preventive antioxidants:

The antioxidant enzyme system consists of copper- and zinc-containing superoxide dismutase (Cu, ZnSOD), manganese-containing superoxide dismutase (MnSOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glucose-6-phosphate dehyrogenase (G6PD) (217).

These enzymes function to protect against toxic oxygen radicals produced during normal metabolism and after oxidative insult as shown in figure 3.4 below.

***Figure 3.4** Antioxidant enzymes in the cellular defense systems (217).*



3.1.9.1 Superoxide Dismutase: (EC1.15.1.1)

This was first discovered by McCord and Fridovich in 1969 (218). The enzyme dismutases two molecules of superoxide to form hydrogen peroxide and oxygen. The SOD family consists of four metallo-forms; two containing copper and zinc, one manganese and one iron. Cu,ZnSOD is found in the cytosol of most eukaryotic cells (219), a different form of Cu,ZnSOD is found in extracellular fluids, where it is called EC-SOD for extracellular SOD (220,221). MnSOD is located in the mitochondrial matrix as well as in bacteria(222). While FeSOD and MnSOD share considerable amino acid homology, they are very different from both Cu,ZnSOD (224). Cu,ZnSOD is sensitive to cyanide, but resistant to chloroform-ethanol treatment. In contrast, MnSOD is resistant to cyanide, but destroyed by treatment with chloroform plus ethanol(222). The human genes which encode Cu,ZnSOD and MnSOD are found on chromosome 21q22.1 and 6q21, respectively (223).

There are a number of methods used for the assay of SOD, but the recommended one is the method described by Fridovich and Beauchamp (194). In the assay, a flux of superoxide is generated

by the action of xanthine oxidase on xanthine, and nitro-blue-tetrazolium (NBT) is used to detect the radical. The reduction of formazan formation is used as the basis of the assay for SOD. The method has a high sensitivity to measure SOD in crude homogenates of tumour cells with low SOD activity. The NBT assay also has the ability to distinguish between Cu,Zn-SOD activity and Mn-SOD activity. This is important because tumour cells have been found to be generally (but not always) low in Cu,Zn-SOD activity, but always low in Mn-SOD activity when compared to a differentiated normal cell counterpart (157,158). Thus, measurement of total SOD activity is not enough; Mn-SOD should be measured for comparison. In the cytochrome *c* assay of SOD, Mn-SOD can not be assayed because the amount of cyanide that is used to inhibit Cu,Zn-SOD will inhibit the cytochrome *c* reduction (but not NBT reduction). Lastly, it has been found that NBT assay has a comparative sensitvity to immunoassay of SOD (195).

3.1.9.2 Catalase: CAT (EC1.11.1.6)

It is one of the oldest known enzymes. It was named by Loew in 1901 (225). The enzyme catalyzes the reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2. \qquad \text{(equation 3.5)}$$

Most aerobic cells contain this enzyme. In animals CAT is present in most body organs, being especially concentrated in liver and erythrocytes. At the subcellular level, CAT is found mostly in peroxisomes (80%) and cytosol (20%). The usual form of CAT has a MW of about 240,000 and consists of four protein subunits, each containing a haeme [Fe(111)-protophyrin] group bound to its active site. Dissociation of the molecule into its subunits causes loss of activity(226). The inhibitors of CAT include azide, cyanide, 3-amino-1,2,4-triazole, reduced glutathione (GSH) and dithiothreitol (227,228). The gene encoding human CAT is found in chromosome 11p13 (223).

The catalase activity is assayed by a photometric method (208). In this assay the decomposition of H_2O_2 is followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of the catalase activity.

To avoid inactivation of the enzyme during the assay (usually 30 sec) or formation of bubbles in the cuvette due to the liberation of O_2, it is necessary to use around 10mM H_2O_2 concentration. The H_2O_2 concentration is critical in as much as there is direct proportionality between the substrate concentration and the rate of decomposition. Measurements can be carried out between 0^o

and 37°C; however, 20°C is recommended. The optimum pH varies from 6.8 to 7.5; 7.0 is recommended.

3.1.9.3 Glutathione peroxidase: GPx (EC1.11.1.9).

It was first described in 1957 by Mills (229). The enzyme catalyzes the oxidation of GSH to GSSG at the expense of hydrogen peroxide:

$$H_2O_2 + 2GSH \longrightarrow GSSG + H_2O \quad \text{(equation 3.6)}$$

By its selenium (Se) dependency, GPx can be divided into two forms: Se-dependent GPx and Se-independent GPx. Se-dependent GPx is a tetramer of MW84,000 with very high activity towards both hyrogen peroxide and organic hydroperoxides. It contains one residue of selenocysteine per mole at each of the active sites and is found both in the cytosol (70%) and mitochondria (30%). Inhibitors include iodoacetate, cyanide and superoxide radicals (230, 231).

The Se-independent GPx are the GSH S-transferases (GST, EC2.5,1.18). They were originally observed in the catalysis of the first step in the formation of the mercapuric acids (232). The enzymes are dimers of MW approximately 50,000 with at least 7 different forms of subunits and 8 isoenzymes towards organic hydroperoxides but none at all towards hydrogen peroxide. They have multiple functions, but are mainly involved in the biotransformation of xenobiotics (230,233) and detoxification of carcinogens (234). The intracellular distribution was found to be cytosolic and mitochondrial. The gene encoding Se-dependent GPx is located in chromosome 3p13-q12 while the gene encoding Se-dependent GPx is to be found on 6p12.2 and 11q13-qter (223).

GPx assay is determined by a modified procedure of Pinto and Bartley (209). Oxidized glutathione is converted to the reduced form with glutathione reductase and NADPH. The decrease in absorbance at 340 nm is as a measure of enzyme activity. As the reaction rate depends on the steady-state level of GSH, any factor influencing GSH regeneration, e.g.,by significantly decreasing glutathione reductase activity, will affect the determination.

3.1.10 Chain-breaking antioxidants

These scavenge the chain propagating free radicals of autoxidation reactions: carbon-centred radicals $R^{\cdot}$, and peroxy $ROO^{\cdot}$(242). This may be achieved by reductive, electron acceptor or additive reactions.

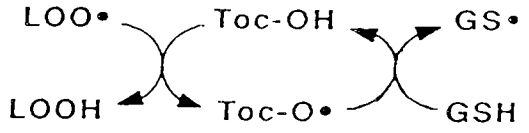
Chain-breaking reductive antioxidant scavenge the peroxy radical R O O ·, an oxidising radical. Alpha-tocopherol, butylated hydroxytoluene (BHT) and -hydroxyanisole (BHA), fall into this category (242).

$$ROO^{\cdot} + AOH \longrightarrow ROOH + AO^{\cdot} \quad \text{(equation 3.7)}$$

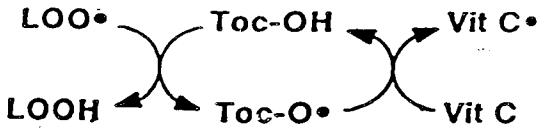
The autoxidation has been retarded because ROOH has been formed from ROO· without reaction with RH and continuing the free radical chain reaction. The antioxidant free radical is of low reactivity and is reduced back to its active form by ascorbic acid and /or reduced glutathione-dependent mechanism (244) as illustrated below in figure 3.5 a and b.

Figure 3.5 a and b; *antioxidant reactions.* ***a),*** *Regeneration of vitamin E Toc-OH) via glutathione-dependent mechanism.* ***b),*** *Ascorbic acid (vit C) regeneration of vitamin E. LOO·, lipid peroxyl radical; LOOH, lipid hydroperoxide (244).*

a



b



Chain-breaking electron acceptor antioxidants scavenge chain propagating radicals which are good reductants. For example, in autoxidation where superoxide is a chain propagation radical, oxidised quinones (Q) may scavenge superoxide. Ubiquinone and related compounds may function as an antioxidant of this class (242).

$$Q + O_2^{-} + H^{+} \longrightarrow SQ^{\cdot} + O_2 \quad \text{(equation 3.8)}$$

$$AO + O_2^{-} \longrightarrow AO^{-\cdot} + O_2 \quad \text{(equation 3.9)}$$

Beta-carotene is now thought to act as an antioxidant in low oxygen concentration, scavenging alkyl radicals by an addition mechanism (242).

$$AO^{\cdot} + R^{\cdot} \longrightarrow AOR \qquad \text{(equation 3.10)}$$

The fate of the adduct is not yet understood.

It has been observed that despite the low levels of antioxidants in tumour cells, there is a substantial increase in chain-breaking antioxidant activity especially in Novikoff and Yoshida tumours which have high levels of alpha-tocopherol (242). These liver cell tumours accumulate a much higher intracellular concentration of alpha-tocopherol than found in normal hepatocytes and this greatly reduce the rate of lipid peroxidation in these cells.

Metal ions particularly ferric and cupric ions may initiate autoxidation process. Chelators of these metal ions saturate the coordination sphere of the ion and make it kinetically inert to the initiation process. The iron storage protein ferritin, the metal ion chelators diethylenetriaminepentaacetic acid (DETAPAC) and desferrioxamine are in this class of antioxidant (247,248).

N-Acetyl-cysteine an antioxidant of protein, is used in the treatment of drug toxicity such as paracetamol poisoning(249).

Propyl gallate is a synthetic antioxygen antioxidant that can be used to suppress autoxidation (251).

3.1.11 Methods used in the assay of lipid peroxidation

Many different methods have been used for the estimation of products of lipid peroxidation. In table 3.2 the principal methods are listed. The main method used in this thesis is the MDA (or TBA-thiobarbituric acid) which is described in details elsewhere in this thesis. However, there are some relevant points that needs discussion.

The TBA reaction is the most frequently used to determine the extent of lipid membrane peroxidation, but it has many limitations

The method is not specific for free MDA, and many other substances (including tissue aldehydes, sugars, etc.,) that occur in biological specimens give postive reaction with TBA (214). These other substances include decomposition products that arise during the heating stage of the reaction of the tissue sample with TBA.

Accordingly the TBA method is described as measuring MDA or "MDA-like substances".

In view of the low specificity of the TBA assay, Yagi (235), devised a method of isolating lipid peroxides in the biological

sample from other TBA reacting substances. The principle of this method is to isolate the lipids by precipitating them with protein using phosphotungstic acid-sulphuric acid system and to determine their amount by TBA reaction in acetic acid. By this procedure, TBA-reacting substances other than lipid peroxides are easily eliminated and the reaction product assayed by the absorption at 532 nm. This method has become widely used for the determination of peroxide products in blood and tissues. On the other hand the TBA method is quite simple, very easy to use and is quite sensitive. It may be calibrated against malonaldehyde bisdimethyl acetal.

Table 3.2. *Methods for the Assay of Lipid Peroxidation (250 and references there in).*

Reaction Product	Assay Method
1.Conjugated dienes	a) Increase in A_{233}. b) Assay of ^{14}C labelled Dielo-Alder adduct c) Oxidation index (A_{233}/A_{215})
2. Hydroperoxides	Iodometric method
3. Conjugated trienes, aldehyde, etc.	Increase in A_{270}
4. Malondialdehyde (MDA)	a) Reaction with thiobarbituric acid (TBA) by colorimetry (A_{532}) b) High performance liquid chromatography (HPLC) c) Fluorescence of TBA.MDA adduct; Ex-532 nm; Em-533 nm d) UV-spectrophotometry with A_{max} at 245 nm (e_{245}= 30000 at pH4.65) e) Spectrofluorometry of Schiff bases (Ex: 360-390nm; Em: 440-470 nm)
5. Loss of lipid subsrate	Gas chromatography
6. Oxygen uptake during lipid peroxidation	Oxygen uptake
7. Oxidative reactions associated to lipid peroxidation (singlet oxygen decay)	Detection of chemiluminescence by using sensitive photon detectors.
8. Hydroxyl aldehydes (carbonyl containing substances including hydroxyalkenals)	Derivatization with 2,4-dinitrophenylhydrazine, separation & estimation by TLC and HPLC
9. Alkanes (ethane, pentane)	Gas chromatography

The TBA method has been directly compared with other methods of measuring lipid peroxidation, such as diene conjugation, chemiluminescence, oxygen uptake, lipid hyroperoxide content, etc., and the comparison has been satisfactory enough to warrant continued usage of this method. It is good to keep in mind that only unsaturated fatty acids with three or more methylene interrupted double bonds can ultimately form MDA, and the variation in MDA production may be a reflection of the lipid composition rather than the susceptibility to lipid peroxidation (214).

The direct measurement of lipid hydroperoxides has an advantage over the TBA method in that it permits a more accurate comparison of lipid peroxide levels in dissimilar lipid membranes. However, its use is limited by the fact that lipid hydroperoxides in biological membranes are transient species that are exposed to factors that catalyze their breakdown. In vitro, transition metals, particularly in their reduced state, and haemoprotein, facilitate the decomposition of hydroperoxides (185-187). Addition of metal chelators affords protection against metal catalyzed hydroperoxide decomposition and should be present during isolation and storage of membranes to be assayed for hydroperoxide levels using the iodometric assay. The measurement of lipid hydroperoxide content as an indication of lipid peroxidation has not been so widely used as other methods such as diene conjugation or MDA content. However, recent development of sensitive HPLC methods for lipid hydroperoxides may change the picture very soon (214).

The detection of conjugated dienes in unsaturated lipid is a sensitive assay that can be used to study lipid peroxidation. However, detection of small amounts of conjugated dienes poses problems, since the diene absorption (233 nm) appears as a small shoulder on a high background absorption. The use of double derivative spectroscopy can improve the situation (236). Also some workers have used the ratio of absorption at 233 nm and 215 nm for correction of background (237).

Finally, more than 95% of diene conjugation in fresh human material was shown to be due to a single fatty acid residue. Contradicting the assumption that diene conjugation means lipid peroxidation, this fatty acid is not a peroxide, or a peroxidation product: it is a simple isomer of linoleic acid with the double bond shifted from 9 and 12 to the 9 and 11 position (238). The steric configuration of the double bond is *cis-cis* in linoleic acid and *cis-trans* in the isomer.

3.2 METHODS IN VITRO

3.2.1.Cell cultures

All cells were maintained as suspension cultures in RPMI-1640 medium supplemented with 10% FCS (mycoplasma free) in a humidified chamber gassed with 95%air/5%CO_2. Cells were employed between 10 and 30 passages depending on the cell-line. For testing the cytotoxic/cytostatic potentials of various agents cells were suspended in RPMI-1640 medium supplemented with either appropriate concentrations of FCS or a chemically defined medium (ultroser-G or ITS-Premix) and passaged twice before exposing them to various agents.

All cell-lines were initially suspended at a cell density of 1 x 10^5 cells/ml except the MAC16 cell-line which was suspended at 5 x 10^5 cells/ml before adding the various agents by serial dilutions (see appendix 1 for the normal growth curves of various cell lines used in the study). The cells were incubated at 37° C in 95%air/5%CO_2 for four days. Appropriate solvent/or additive controls were included in each experiment. The experiments were repeated at least three times.

3..2.2 Cell Count

Cell counts were determined by a Coulter counter (model ZBI). Briefly, cells were uniformly mixed by shaking the tissue culture flask gently and breaking up clumps of cells by repeated pipetting with a 1ml Gilson pipette several times when necessary. 200 ul of the cell suspension was diluted with 980 ul (i.e, 50x dilution) in balanced salt solution (isoton) for cell count. The cells were counted in two aliquots of 0.5 ml each. The cell density per ml was calculated after making an appropriate dilution factor.

3.2.3 Assay of cell viability by the exclusion of trypan blue.

Cells were centrifuged in a 1.5 ml microcentrifuge tube for 20 seconds at 11,600 g using a MSE centrifuge, and most of the supernatant removed. To the pellet was then added 5 ul of 0.1% trypan blue dye in phosphate-buffered saline (pH 7.4), and this was mixed using a Fisons whirlmixer, before pipetting a drop onto a microscope slide and viewing with a 10x eyepiece and 40x objective on an Olympus microscope. Non-viable cells had taken up the dye and appeared blue in colour whereas viable cells were able to exclude the dye and remained colourless. At least 300 cells were counted per sample, and the percentage of viable cells calculated.

3.2.4 Data analysis

The results were reported as the mean ID_{50} plus or minus the standard error of the mean. The *p* values were computed by student *t*-test.

3.2.5 Fatty acids

The effects of various fatty acids were studied on the HL-60 myelomonocytic leukaemic cell line growing in 1% ultroser-G. The fatty acids included palmitoleic acid (cis-9-hexadecanoic acid) (POA) (C16:1 n-9), linoleic acid (cic-9-cis-12-octadecadienoic acid) (LA) (C18:2 n-6), gammalinolenic acid (6,9,12–octadecatrienoic acid) (GLA) (C18:3 n-6), dihomo-gammalinolenic acid (8,11,14-eicosatrienoic acid) (DGLA) (C20:3 n-6), arachidonic acid (5,8,11,14-eicosatetraenoic acid) (AA) (C20:4 n-6), alpha-linolenic acid (ALA) (9,12,15-octadecatrienoic acid) (C18:3 n-3), 5,8,11,14,17-eicosapentanoeic acid (EPA) (C20:5 n-3), and 4,7,10,13,16,19-docosahexanoeic acid (DHA) (C22:6 n-3).

Stock solutions of the fatty acids were made in absolute ethanol at a concentration of 100 mM and stored in darkness at -20° C after gassing with high grade argon. For the experiments, fatty acids were prepared from the stock solutions and by appropriate serial dilutions added to cell suspensions in various tissue culture flasks. The rest of the experiment was carried out as previously described (section 3.2.1). A cell count was performed on day four.

3.2.6 Fatty acids, antioxidants and cell density

The above experiment (section 3.2.5) was repeated using BHT as an antioxidant. A stock solution of both compounds was made in absolute ethanol and an appropriate dilution made in order to achieve a final concentration of 5 uM of BHT in the cell cultures before adding the fatty acids. The fatty acids tested were POA, LA, AA, and EPA. The experiments were carried out in 1% ultroser-G at cell densities of 1×10^5 and 4×10^5 cells/ml.

3.2.7 Serum and serum substitutes

The effects of POA and LA acids were studied using the following supplements: (a) 10% FCS, (b) 2% ultroser-G (c) and ITS- Premix. All experimental procedures was as described above (sections 3.2.1).

3.2.8 Lipid peroxidation-TBA test

5uM and 1 nM concentration of POA, LA, GLA, EPA and DHA were added to the HL60 cells (5 x 10^5 cells/ml) in 1%ultroser-G. Unsupplemented control received a <2% final concentration of ethanol. All samples were incubated in parallel. At indicated times, medium and cells were separated and assayed for MDA according to the CRC protocol (286).

In this method, 10^6 cells were suspended in PBS (pH7.0) and were mixed with TBA reagent consisting of 0.375% TBA and 15% TCA (trichloroacetic acid) in 0.25 M hydrochloric acid. The reaction mixture of sample and TBA reagent was placed in a boiling water-bath for 15 min, cooled, and centrifuged at 12,000 *g* for 10 min. The absorbance of the supernatant was measured at 532 nm using PBS as a reference. Absorbance was converted to picomoles of MDA-Eq from a standard curve generated with 1,3,3,tetramethoxypropane. The experiment was performed in triplicate. Medium (2ml) from which the cells had been removed was processed in a similar manner.

3.2.9 Hydroperoxides and Peroxides

The following experiments were carried out in a similar manner as descibed above (section 3.2.1). The following compounds were used as typical representatives: (a) linoleic hydroperoxide; (b) *t*-butylhydroperoxide; (c) and methyl ethyl ketone peroxide.

Stock solutions of these compounds were prepared fresh in absolute ethanol just before use to reduce the risk of decomposition.

3.2.10 Fatty acid analysis: the effect of supplements on fatty acid composition

The fatty acid composition of the MAC16, HL60, and K562 cells was analysed by gas-liquid chromatography (GLC) when cells were grown in either 2% ultroser or 10% FCS and compared with the fatty acid profiles of the supplements themselves.

The cells were washed three times with PBS (pH7.4) and 10^6 cells were placed in a clean hyrolysis tube to which 2.5 ml of 5% NaOH/50% methanol was added. The lid was screwed tightly and the the tubes placed in a beaker of boiling water for 30 min. The tube was removed, and cooled to room temperature. The sample was acidified by adding a few drops of concentrated hydrochloric acid (checked with litmus or pH paper). The fatty acids were

transmethylated by treating the sample with 2.5 ml of boron trifluoride solution in methanol (14%wt/vol.) at 80° C for about 5 min with the lid put on loosely. The fatty acid methyl esters were extracted with 5 ml of chloroform:hexane mixture (1:4). The upper layer was removed and rotary evaporated to dryness. The methyl esters were redissolved in 200 ul of hexane and 1 ul was injected onto the column. Gas chromatography of the fatty acid methyl esters was performed on a Pye Unicam Series 204 chromatograph equipped with a flame ionisation detector, with a 2 m long column of 6 mm internal diameter packed with GP3% SP-2310/2%SP-2300 on 100/120 Chromosorb WAW (Supelco, UK LtD). The column was operated under a temperature programme, at 150°C initially, but later increasing to 220° C, at a rate of 2° C/min with a gas flow of 30 ml/min of helium through the column. For quantitative analysis, the samples were injected with a known quantity of 17:0 methyl ester standard. The peaks were identified by comparison of the retention times with those of authentic standards. Changes in the composition of the fatty acids were expressed as a percent by weight of the content relative to C18:0.

3.2.11 Diphenyl-picryl-hydrazyl (DPPH) and diphenyl-picryl-hydrazine ($DPPH_2$)

The effects of these compounds were studied on the MAC16, K562, Raji, GM892, and the HL60 cell-lines in 1% ultroser-G. All cell culture conditions and procedures were the same as described above (section 3.2.1). Stock solutions were made in high grade acetone before adding them by serial dilution to the cells. A solvent control was also performed.

3.2.12 Hyrazyl derivatives

These compounds were tested under similar experimental conditions and procedures described above (section 3.2.1). The MAC 16 cell-line in 1% ultroser-G was used. Stock solutions were made in high grade acetone before adding them to the cells by serial dilutions. These compounds were: (a) carbazyl-picrylnitrogen (CPPH) and carbazyl-picrylamine ($CPPH_2$) (b) water-soluble derivatives, 2,2-diphenyl-1-(2',4'-dinitrophenyl-6'-sulphophenyl)hydrazyl (DDSH) and 2,2-diphenyl-1-(2',4'-dinitrophenyl-6'-sulphophenyl)hydrazine ($DDSH_2$) (see appendix 11 for structural formulae).

3.2.13 $DPPH_2$ and synthetic antioxidants

The MAC16 cell-line in 1% ultroser-G was used. 5uM BHT and indomethacin (INDO) were used either individually or in combination. Stock solutions of these compounds were made in absolute ethanol. BHT and INDO controls were also performed. The rest of the experimental conditions and procedures were as described above (section 3.2.1).

3.2.14 $DPPH_2$ and antioxidant enzymes

The MAC16 cell-line in 1% ultroser-G was used. The enzymes were diluted in PBS (pH7.4) and filter-sterilised. 130 IU/ml SOD, and 60 IU/ml CAT were used individually or in combinations. $DPPH_2$ was added immediately by serial dilutions. Enzyme controls were also performed. The rest of the experimental conditions and procedures were as described above (section 3.2.1).

3.2.15 DPPH, $DPPH_2$ and lipid peroxidation

TBA-test was carried out exactly as desribed above (section 3.2.8) using 1 uM and 1 nM of both DPPH and $DPPH_2$. The MAC16 cell line in 1% ultroser was used.

3.2.16 Other compounds

The following compounds were also tested on the MAC16 cell line in 1% ultroser-G. Stock solutions were prepared in appropriate solvents (in parenthesis) and the experimental conditions and procedures were as described above (section 3.2.1).

The compounds were grouped as follows:

(a) hydrazines: phenylhydrazine and diphenylhydrazine

(both in ethanol);

(b) quinones: *p*-benzoquinone (water), quinzarin (ethanol),

5,8-dihydroxynaphthoquinone (ethanol) and

adriamycin (doxorubicin) (water);

(c) azo-componds: azobenzene (ethanol) and sulfonazo111

(water);

(d) nitro-aromatic: picryl chloride (ethanol);

(e) other stable free radicals: galvinoxyl and carboxy-PROXYL free radicals (both in acetone)

(f) thiyl radical: 1-dodecanethiol (ethanol);

(g) others: 5FU (ethanol), chlorambucil (ethanol) and

mitozolomide (DMSO).

3.3 METHODS INVIVO

3.3.1.Chemotherapy

Male NMRI mice were transplanted with the MAC16 tumour as described in section 2.1.1 (page 23). Tumour bearing animals were allocated by restricted randomisation into groups of 5. Animals with tumours measuring 40 mm^3 or more were used for therapy. Tumour volumes and the weights of the animals were assessed daily. The tumour volume was measured with 2-dimensional calipers and calculated from the formula $a^2 \times b/2$ where a is the smaller diameter and b is the larger one (286). Any animal which lost more than 30% of the original body weight for more than 48 hr or with the tumour exceeding 1000 mm^3 was sacrificed. Special permission was obtained from the Home Office to allow the monitoring of animals whose tumours had ruptured as a result of the chemotherapy. The tumours would usually form a dry scab within 24 hr. However, if the animals condition deteriorated or continued to bleed from the site of rupture it was sacrificed. Solvent treated controls were used. The results were expressed in terms of average survival time. Relevant photographs of the tumours were taken.

The compounds were injected subcutaneously at the opposite flank far from the tumour as a single or a three-day dose schedule The concentration ranged from 2.5×10^{-5} to 2.5×10^{-17} mol/kg body weight. Stock solutions were made in appropriate solvents. For the administration, the compounds were serially diluted in very clean sterile glass containers containing appropriate volumes of sterile double-distilled deionised water under a laminar flow hood. The injection was administered using sterile disposable needles and syringes.

The following compounds were tested:

(a) hydrazyl derivatives: DPPH, $DPPH_2$, and DDSH;

(b) fatty acids: POA, LA, GLA, AA, ALA, and EPA;

(c) hydroperoxides and peroxides: MEKP and *t*-BOOH;

(d) quinones: PBQ and ADM;

(e) others: chlorambucil.

3.3.2 P388 Leukaemia

The cells were grown intraperitoneally in male BDF_1 mice as described above (section 2.1.1). DDSH was administered subcutaneously as a single dose schedule one day after the tumour transplant. The concentrations ranged from 2.5×10^{-5} to 2.5×10^{-17} mol/kg body weight. The preparation of the DDSH for the treatment was similar to the procedures described above (section 3.3.1). The animals were weighed daily. The response was assessed by the survival time of the treated groups versus the controls. However, if any animal died suddenly without any physical evidence of disease such as gain in weight due to malignant ascites, a post mortem was carried out. Animals which appeared sick or distressed were sacrificed according to the Home Office regulations.

3.3.3 TLX5 Lymphoid tumour

The tumour cells were transplanted intraperitoneally into male CBA/CA mice and treated with DDSH administered subcutaneously on the third day after the transplant. The concentrations ranged from 2.5×10^{-5} to 2.5×10^{-17} mol/kg body weight. The experimental conditions and procedures were similar to that described above (section 3.3.1).

3.3.4 TBA assay

Serum MDA was determined by method described by Yagi (235). For each concentration there were 9 animals. Three animals from each group were bled every third day from the tail vein. The blood was allowed to stand to clot. After centrifugation at 3,000 rpm for 10 min, 20 ul of the serum was mixed gently with 4.0 ml of N/12 H_2SO_4. To this solution, 0.5 ml of 10% phosphotungstic acid was added and mixed. After allowing to stand at room temperature for 5 min, the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was discarded, and the sediment was mixed with 2.0 ml of N/12 H_2SO_4 and 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged at 3,000 rpm for 10 min. The sediment was suspended in 4.0 ml of distilled water, and 1.0 ml of TBA reagent added. TBA regent was a mixture of equal volumes of 0.67% TBA aqueous solution and glacial acetic acid. The reaction mixture was heated for 60 min at 95° C in a water bath. After cooling with tap water, 5.0 ml of *n* - butanol was added and the mixture was vigorously mixed with a whirl mixture.

After centrifugation at 3,000 rpm for 15 min, the *n*-butanol layer was taken for fluorometric measurement at 553 nm with 515 nm excitation.

The fluorescence intensity of the standard solution, was obtained by reacting 0.5 nmol of tetramethyoxypropane with TBA reagents. The rest of the procedures were similar to that of the samples.

Calculation:

Taking *F* as the fluorescence intensity of the standard, and *f* that of the sample, the lipid peroxide level (Lp) was expressed in terms of MDA:

Serum Lp = 0.5 x f/F x 1.0/0.02 = f/F x 25 (nmol/ml of serum).

The MDA was assayed for 9 days.

3.3.5 TNF Assay

TNF was determined by an *in vitro* cell cytotoxicity assay similar to that described by Ruff and Gifford (241). L929S cells were seeded at a concentration of 3 x 10^4 per well into 96-well flat-bottom microtitre trays in 100 ul RPMI 1640 medium containing 10% FCS, and incubated at 37°C overnight under an atmosphere of 5%CO_2 in air. The medium was then removed and was replaced with varying dilutions (from 1 to 11) of mouse serum and actinomycin D (1ug/ml), to a final volume of 100ul. The sera were obtained on day 6 from BDF1 mice transplanted with P388 leukaemia treated with 2.5 x 10^{-5} or 2.5 x 10^{-11} mol/kg body weight of DDSH by a single subcutaneous injection. There were at least 5 animals per group. Controls contained only RPMI 1640 medium and actinomycin D. Internal standards contained RPMI 1640 medium with 1U of recombinant human TNF and actinomycin D. The plates were reincubated for 16 to 18 hr and were rinsed with phosphate-buffer saline. The cells were fixed with 200 ul of methanol for 10 min and were then stained with distilled water and left to dry in a stream of warm air. 50 ul of 33% acetic acid was added to each well to elute the dye and the plates were enumerated spectrophotometrically at 570 nm on a Titerek Multiskan. The percent cell cytotoxicity was calculated using the following formula, as described by Flick and Gifford (243):

$$\% \text{ cell cytotoxicity}_{dil} = (A_{con} - A_{dil})/A_{con} \times 100$$

Where % cell cytotoxicity$_{dil}$ is the amount of cell destruction at any particular dilution of the TNF-containing serum, A_{con} is the absorbance of control wells, and Adil is the absorbance of wells at a particular dilution of TNF. Dose-response curves were generated from assays performed in triplicate.

All serum samples were assayed on the TNF-resistant cell line, L929R, in order to ensure that any cell cytotoxicity was solely due to TNF.

3.3.6 Antioxidant Enzymes

3.3.6.1 Tissue preparation

Animals were killed by cervical dislocation. The tissues were excised, weighed and rapidly homogenized in 2 volumes of ice-cold 50 mM phosphate buffer, (pH 7.8). The homogenate was then sonicated at 4°C for 10-20 sec at 125 W using an MSE sonic oscillator. The supernatant collected after centrifugation at 18,000 *g* for 20 min was immediately frozen and later used for the determination of enzyme activity. A Beckman spectrophotometer was used for the assay.

Enzyme activity is expressed as a function of total cellular protein which was determined by the method of Lowry using bovine serum albumin as a standard (section 3.3.7).

The tissues studied were the (a) MAC 13, MAC 16 and MAC 26 tumours, (b) livers from NMRI and BDF_1 mice before and after treatment.

3.3.6.2 SOD assay

Reaction mixtures containing 0.05 M potassium phosphate buffer (pH 7.8), 1.0 mM EDTA, 0.1 mM xanthine, 0.056 mM NBT was used to zero the recorder. After adding 20 ul of xanthine oxidase to the above mixture, the absorbance at 560 nm was recorded for about 50-60 sec against a blank containing all ingredients in the above mixture except xanthine oxidase. Between 20-50 ul of sample which had been diluted 1 in 20 was added to the reaction mixture to give a 40-60% inhibition of NBT reduction by xanthine oxidase. After adding the sample, the cuvette was mixed thoroughly and the change in absorbance recorded for about 3-5 min. Each sample was measured in duplicate.

The % inhibition by SOD of NBT reduction is equal to the change in absorbance produced after addition of the sample divided by the

change in absorbance after addition of xanthine oxidase. A calibration curve was constructed using commercially-prepared SOD.

One unit of SOD is defined as that amount of enzyme that will inhibit the reduction of NBT by xanthine oxidase by 50% under the assay conditions.

3.3.6.3 Catalase assay

A sample containing 3.0 ml of H_2O_2-phosphate buffer (15 mM H_2O_2-50 mM phosphate buffer) and 10-40 ul of substrate was read against a blank containing 3.0 ml phosphate buffer and the same volume of substrate at 240 nm. The time required to decrease the optical density of the sample from 0.450 to 0.4 was measured.

Calculations:

The activity was calculated by using the method of Bonnichsen (289). With very short reaction times and relatively high enzyme concentrations the decrease of the H_2O_2 concentration with time due to the action of catalase corresponds to a first order reaction:

The rate constant (k) for the overall reaction is given by:

$$k = (1/dt)(\ln S_1/S_2) = (2.3/dt)(\log S_1/S_2) \quad \text{(equation 3.11)}$$

where $dt = t_2 - t_1$ = measured time interval and S_1 and S_2 = H_2O_2 concentrations at times t_1 and t_2.

From equation 3.11 it follows that a decrease in optical density from 0.450 to 0.400:

$$K = 2.3/t \log F_{initial}/F_{final} = 2.3/t \log 0.450/0.400 = 2.3/t\ 0.05115 = 0.1175/t\ sec^{-1} \quad \text{(equation 3.12)}$$

where F= optical density at 240 and 250nm

2.3 = factor to convert from Ln to log.

The value K can be converted to any catalase units.

A unit is the amount of enzyme which liberates half the peroxide oxygen from a hyrogen peroxide solution of any concentration in 100 sec at 25°C. The unit of enzyme activity is therefore related to the half-life time T of a first order reaction.

For T the following formula is usually valid:

$$T = Ln2/K = 0.693/K \qquad \text{(equation 3.13)}$$

The relationship between the observed half-life time T observed and the enzyme activity is:

1 unit $= 100/\ observed = K\ observed/\ 6.93\ x10^{-3}$

or referring to equation 3.12:

$0.1175/t\ x\ 6.93\ x10^{-3} = 17/t$ = Units/ assay mixture

3.3.6.4 GPx assay

The reaction mixture contained the following solutions: 1.931 ml 0.05 M phosphate buffer, 100 ul enzyme sample, 28 ul glutathione reductase and exactly 100 ul 15 mM GSH, 741 ul 15 mM NaN_3. 100 ul NADPH solution was added and the hydroperoxide-independent consumption of NADPH was monitored for about 3 min. The overall reaction was started by adding 12 ul of H_2O_2 solution and the decrease in absorption at 340 nm was monitored for 2-4 min. The nonenzymatic reaction rate was correspondingly assessed by replacing the enzyme sample by buffer. The reaction rate of the latter system was subtracted from that of the former to determine the true enzymatic activity.

One unit of GPx is defined as the number of micromoles of NADPH oxidized per min calculated on the basis of the molar absorptivity for NADPH at 340 nm of 6.22 $x10^3$ mol^{-1} cm^{-1}. The activity is calculated by measuring change in absorbance with time; then using the extinction coefficient for NADPH which is given above to calculate the number of micromoles of NADPH oxidized to NADP per min.

3.3.7 Protein estimation (Lowry)(290)

A calibration curve was prepared by using standard bovine serum albumin (concentration ranged from 10 ug to 500 ug). 5 ml of alkaline CuSO4 was added to all calibration tubes plus any samples, mixed and left for 10 min at room temperature. 0.5 ml of Folin's reagent was added to each tube, mixed and left at room temperature for at least 30 min. The absorbance was read at 750 nm against a blank containing 1 ml of water, 5 ml alkaline CuSO4 and 0.5 ml of Folin's reagent.

Concentration of standard bovine serum albumin was plotted against absorbance to give calibration curve and the unknown concentration of protein in the samples were read off.

3.3.8 Histology

Tissues were cut to appropriate sizes and preserved in 10% formal saline for at least 24-48 hr depending on the size. After being processed through the normal histological procedures, paraffin-wax sections were cut, fixed, stained with haematoxylin and eosin and mounted in DPX before viewing under a light microscope. Relevant photographs were taken. The films were processed in the department of photograpy at Aston University.

CHAPTER: 4 RESULTS AND DISCUSSIONS

RESULTS

4.1 The effects of unsaturated fatty acids on the growth of HL60 cell line in vitro.

Unsaturated fatty acids play a major role in the initiation of free radical chain reactions via lipid peroxidation (168,169,179). Their effects on the proliferation of HL60 cells under various experimental conditions were studied in vitro. Cell counts were performed during the exponential growth phase on day 4 and ID_{50} determined. The growth inhibition was a function of many variables such as media supplements, cell density, the presence of oxidant and antioxidant.

It was found that the majority of the fatty acids tested inhibited the growth of HL60 cells with γ–linolenic acid (GLA) being the most potent inhibitor except linoleic acid (LA) which had no effect on the growth of HL60 cells in the presence of 10% FCS except after the addition of ferrous chloride and ascorbic acid. This inhibition was greater following the addition of ferrous chloride plus ascorbic acid than with ferrous chloride alone ($p<0.001$) There was also more growth inhibition when cells were grown in the presence of 2 % ultroser-G (UG) (a chemically defined medium) than in 10 % FCS (tables 4.1 and 4.2). The growth inhibition caused by both palmitoleic acid (POA) and LA in the presence of 2 % UG was reversed by butylated hydroxytoluene (BHT) and that of POA in the presence of 10% FCS was similarily reversed by BHT ($p<0.05$). The initial cell density used in the experiments was $1x10^5$ cells/ml.

Our previous studies had revealed that POA had a biphasic cytocidal effect on the growth of HL60 cells in the presence of 2 % UG (figs. 4.1 c, d). The first cytotoxicity occurred at a concentration of 10^{-4} M while the second one was at a concentration of 10^{-9} M. LA had no cytotoxic effect on the growth of HL60 cells at a concentration of 10^{-4} M in the presence of 10 % FCS, but it was cytotoxic at a concentration of 10^{-10} M (figs. 4.1 a, b). The cytotoxicities that occurred at these low concentrations were abolished by the presence of BHT. This unique cytotoxic effect at these low concentrations was observed on 3 occassions with each of the fatty acids out of over 20 experiments and therefore, it was not reliably reproducible. However, it provided a great encouragement to investigate this phenomenon further using other free radical generating compounds.

Lipid peroxides are stable products of lipid peroxidation. They participate in the initiation and propagation of free radical chain reactions. The inhibitory effects of linoleic hydroperoxide (LOOH), methyl ethyl ketone peroxide (MEKP) and t-butyl hydroperoxide (t-BOOH) on the growth of HL60 cells were assessed in the presence of 2 % UG and 10% FCS. The initial cell density was $1x10^5$ cells/ml. It was found that these compounds were as potent as GLA in inhibiting the growth of HL60 cells in either media (table 4.2). This growth inhibition was not reversed by BHT.

Previous study had revealed that the cytotoxicity of PUFAs on various cell lines varied with the initial cell density (180). The influence of cell density and media on the inhibitory effects of POA and t-BOOH on the growth of HL60 cells is summarised in table 4.3. The initial cell density was $4x10^5$ cells/ml. The media were supplemented with either FCS or serum substitutes which included UG, ITS Premix (chemically defined medium containing 5ug insulin, 5ug transferrin, 5ng selenous acid per ml of supplemented medium), ITS+ Premix (chemically defined medium containing 6.25ug insulin, 6.25ug transferrin, 6.25ng selenous acid, 1.25mg bovine serum albumin, and 5.35ug linoleic acid per ml of supplemented medium). The inhibitory effects of POA and t-BOOH on the growth of HL60 cells were reduced compared with the effect at an initial cell density of $1x10^5$ cells/ml in the presence of 10% FCS or 2% UG (table 4.3). The growth inhibition caused by both POA and t-BOOH at the initial cell density of $4x10^5$ cells/ml in the presence of either 10% FCS or 2% UG was completely abolished by the addition of BHT. However, in the presence of ITS Premix (no albumin) BHT had no effects in reversing the growth inhibition of POA on HL60 cells while abolishing that caused by t-BOOH. Both POA and t-BOOH had no inhibitory effects on the growth of HL60 cells in the presence of ITS+ Premix.

PUFAs have been shown to inhibit tumour cell growth in vitro probably via free radical generations (168,169,179). MDA determination is one of the methods used to assay free radical reactions in biological systems. The effects of palmitoleic acid (POA), linoleic acid (LA), and arachidonic acid (AA) on the production of MDA by HL60 cells grown in 2% UG were evaluated (fig. 4.2). There was significant production of MDA at a concentration range of 10^{-6} to 10^{-4} M of AA. At a concentration of 10^{-4} M there was continued production of significant but decreasing quantities of MDA up to 24 hr. The production of MDA at a concentration of 10^{-12} and 10^{-9} M was not significant. MDA

production by both POA and LA was statistically significant when compared with the controls at concentrations of 10^{-9} and 10^{-10} M respectively. This only occurred when there was a concurrent cytotoxicity exhibited by these fatty acids at these low concentrations. The values were 300 ± 13.2 ($p < 0.05$), and 120 ± 18.4 ($p < 0.05$) nmol/ml of medium for POA and LA respectively. These values were determined on day 4 of incubation. The cytotoxicity at these low concentrations were very unpredictable and not reproducible (figs. 4.1a,b,c and d).

Table 4.1. Effects of PUFAs on the growth of HL60 cells in vitro. The ID_{50} values were determined on day 4 of incubation. The initial cell density was $1x10^5$ cells/ml. n=3-6.

	10 % FCS		2 % Ultroser-G		
Fatty acids	Conc. x 10^{-4} M	± SEM	Conc. x10^{-4} M	±SEM	[a] p <
GLA	0.5	0.3	0.2	0.1	0.05
DGLA	3.0	0.3	ND		
AA	2.0	0.1	0.9	0.1	0.01
ALA	6.0	0.3	ND		
EPA	2.0	0.2	0.7	0.3	0.01
DHA	4.0	0.2	ND		

ND= not determined, a= indicates when ID_{50} in 2%UG was statistically different from ID_{50} in 10%FCS.

Table 4.2. Influence of BHT and ferrous chloride on the ID_{50} values of fatty acids and peroxides towards HL60 cells in vitro. These values were determined on day 4 of incubation. The initial cell density was $1x10^5$ cells/ml. n=3-6.

	10 % FCS		2 % Ultroser-G		
Compounds	Conc. x 10^{-4} M	± SEM	Conc. x 10^{-4} M	± SEM	^{a} p <
POA	5.0^b	0.3	2.0^b	0.1	0.005
POA+BHT	7.0^b	0.3	4.0^b	0.1	
LA	NE		5.0^b	0.2	0.001
LA+BHT	NE		7.0^b	0.3	
LA+$FeCl_2$	5.0^c	0.2	ND		
LA+$FeCl_2$ +Ascorbate	2.0^c	0.1	ND		
LOOH	0.5^d	0.1	ND		
LOOH+BHT	0.6^d	0.1	ND		
MEKP	0.4^e	0.1	0.3^e	0.2	NS
MEKP+BHT	0.6^e	0.2	0.5^e	0.1	
t-BOOH	0.6^f	0.3	0.5^f	0.2	NS
t-BOOH +BHT	0.6^f	0.2	0.7^f	0.3	

ND=not determined, NE=no effect, NS=not significant, a= indicates when ID_{50} in 2% UG was statistically different from ID_{50} in 10% FCS. $^b p<0.05$, $^c p<0.001$, d,e,f=not significant (compares ID_{50} values with and without BHT)

Table 4.3. Influence of initial cell density and different media on the ID50 values of POA and t-BOOH on the growth of HL60 cells in vitro. These values were determined on day 4 of incubation. The initial cell density was 4 x10^5 cells/ml. n= 3-6.

	10 % FCS		2 % Ultroser-G		ITS Premix		ITS+ Premix
Fatty acids	Conc. x10^{-4}M	± SEM	Conc. x10^{-4}M	± SEM	Conc. x10^{-4}M	± SEM	Conc. x10^{-4}M
POA	3.0^a	0.1	4.0^c	0.1	5.0	0.1	NE
POA+BHT	NE		NE		5.0	0.1	NE
t-BOOH	2.0^b	0.1	4.0^d	0.2	1.0	0.1	NE
t-BOOH +BHT	NE		NE		NE		NE

NE= no effect, $^a p<0.005$, $^{b,c,d} p<0.001$ (indicates when ID50 at 4x10^5 cells/ml was statistically different from ID50 at cell density of 1x10^5 cells/ml).

Fig. 4.1 a and b. a, Dose response curve of linoleic acid (LA) on the growth of HL60 cells in vitro. The cells were grown in 10% FCS and the cell counts were performed on day 4 of incubation. The cell count for the control was 16.3 x 10^5 ±1.9 cells/ml. b, % viability. The control had a viability of 95% ± 1.2. The results are expressed as the mean ± s.e.m. In 3 experiments out of over 20 experiments cytotoxicity occured at 10^{-10} M LA, otherwise, the viability at this concentration was similar to that of the control..

Fig. 4.1a.

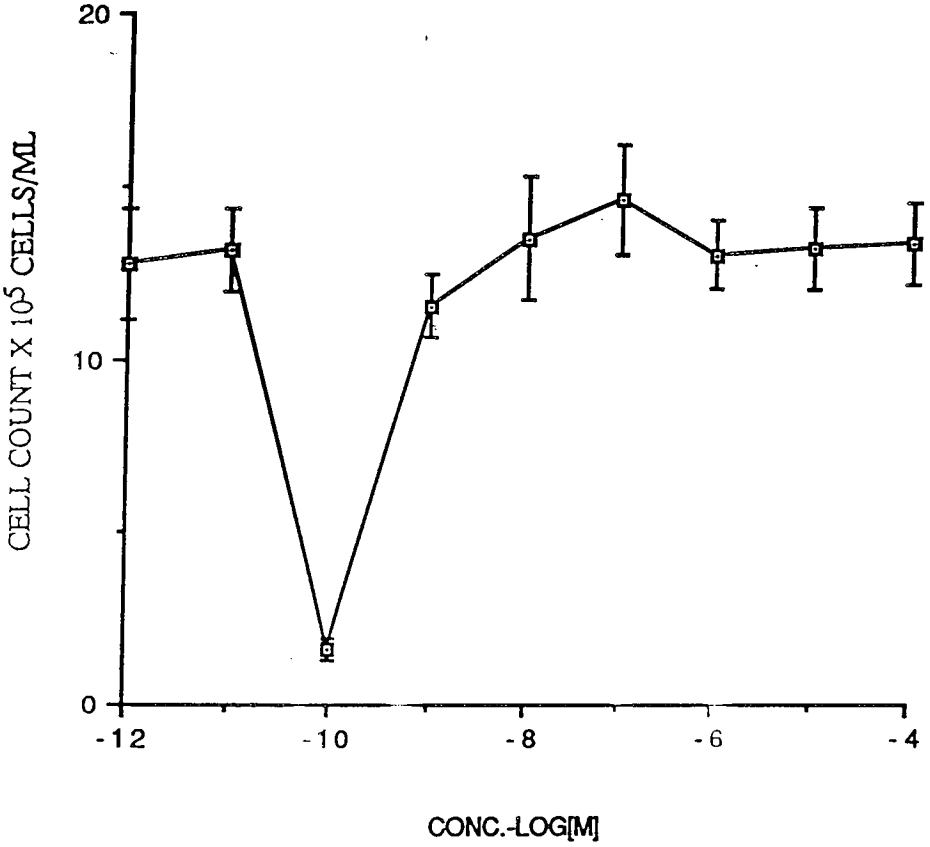


Fig. 4.1b.

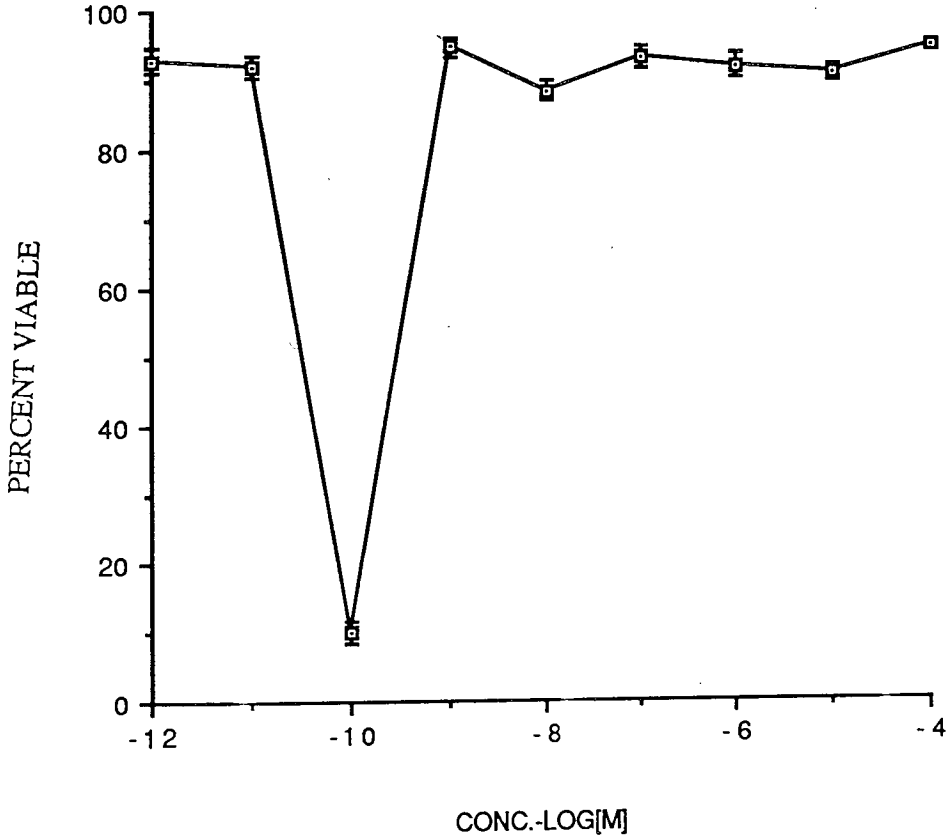


Fig. 4.1 c and d. c, Dose response curve of palmitoleic acid (POA) on the growth of HL60 cells with and without BHT in vitro. The cells were grown in 2% ultroser G and the cell count was performed on day 4 0f incubation. The counts for the controls with and without BHT were 6.3 x 10^5 ± 1.2, and 6.2 x 10^5 ± 1.5 cells/ml respectively. d, % viability with and without BHT. The viability of the controls with and without BHT were 91% ± 1.8, and 92% ± 1.5 respectively. The results are expressed as the mean ± s.e.m. In 3 experiments out of over 20 experiments cytotoxicity occured at 10^{-9} M POA, otherwise, the viability at this concentration was similar to that of the control.

Fig. 4.1 c.

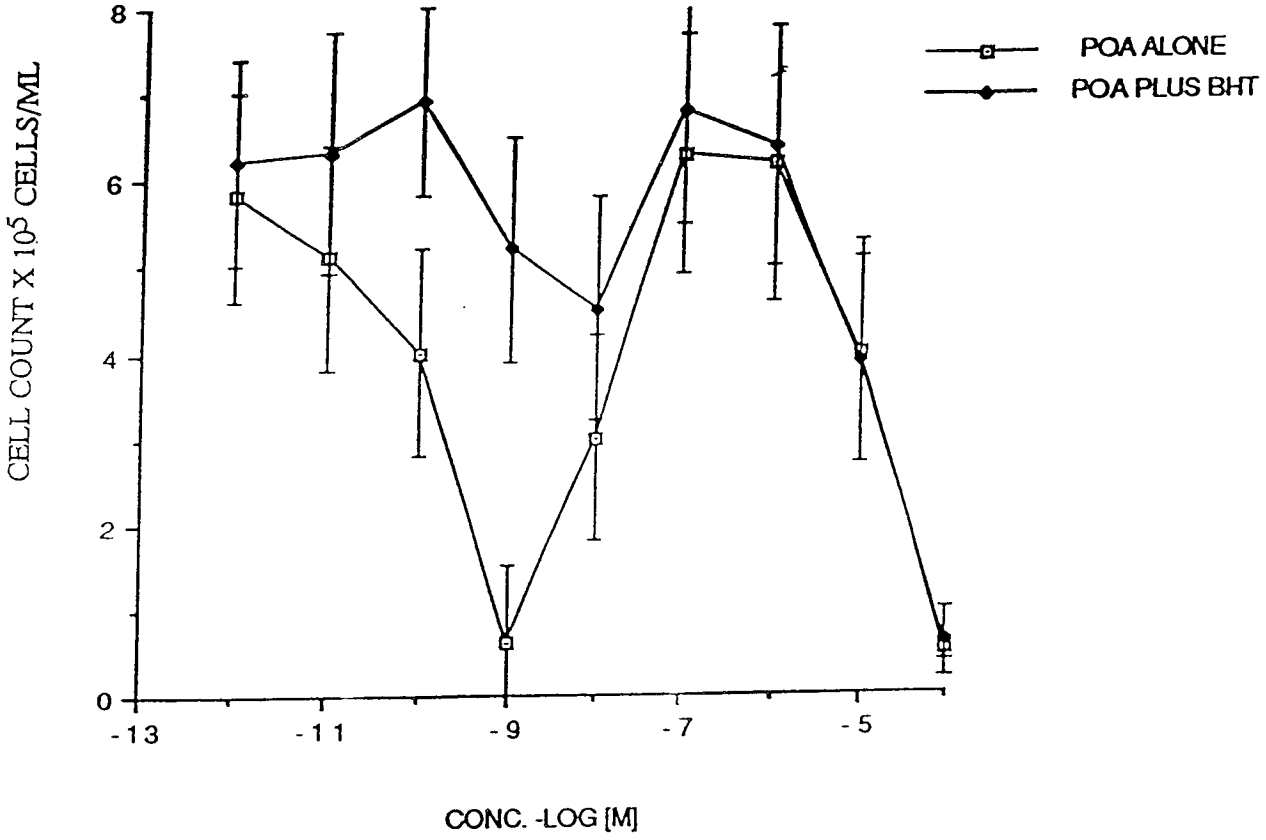


Fig. 4.1 d.

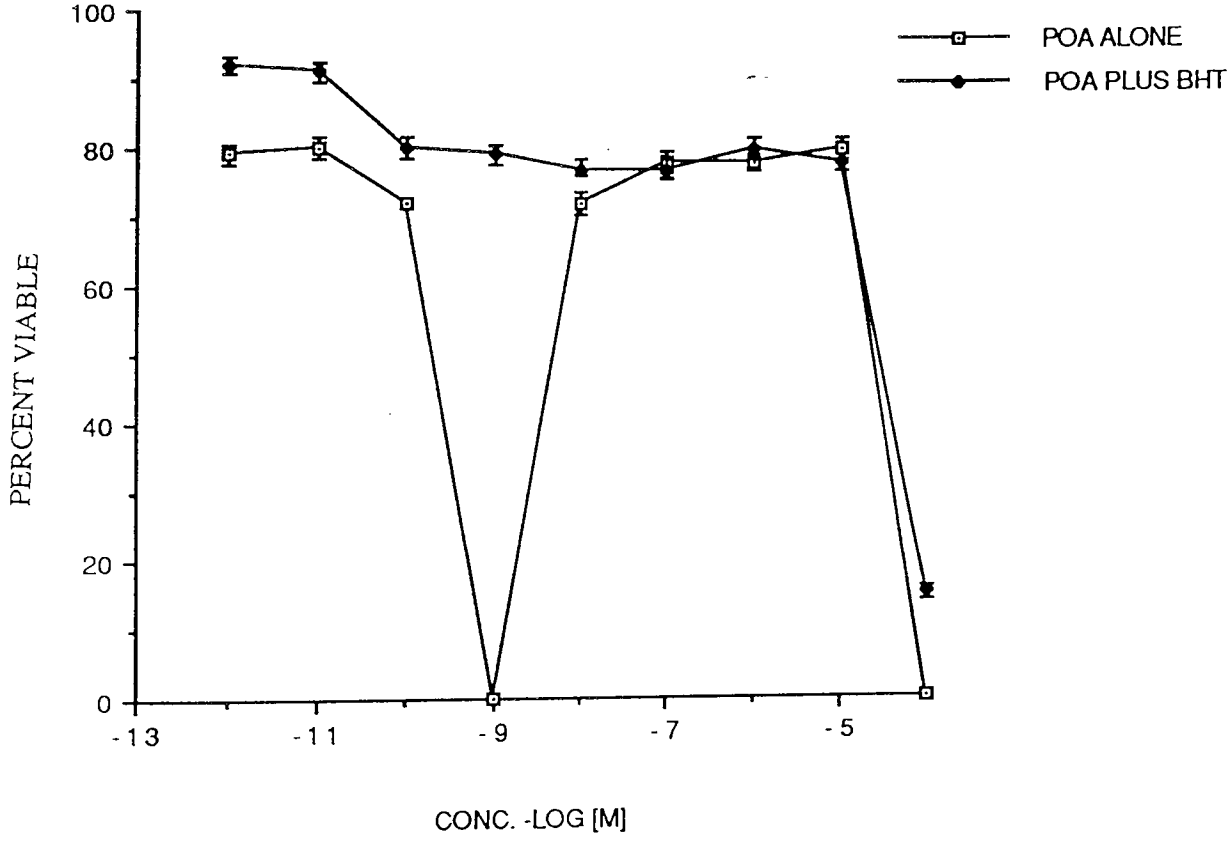
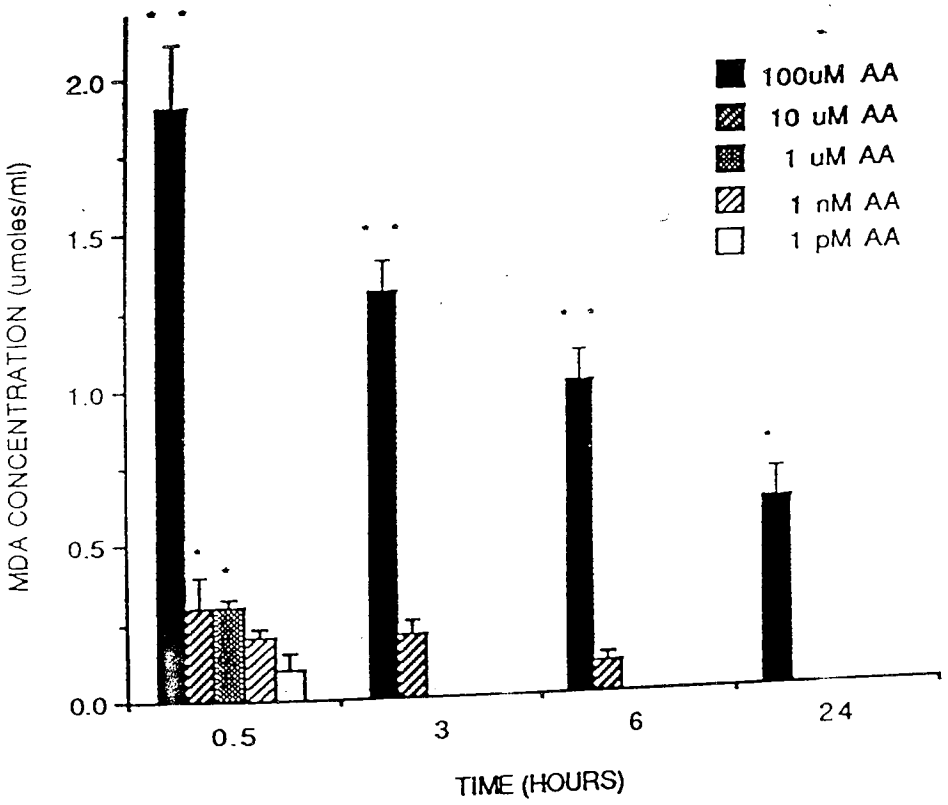

100
80
60
40
20
0
PERCENT VIABLE
-13
-11
-9
-7
-5
CONC. -LOG [M]
POA ALONE
POA PLUS BHT

*Fig. 4.2. Effect of arachidonic acid on the production of MDA by HL60 cells in vitro. The MDA concentration for the control was 0.031±0.001 umol/ml. The results are expressed as the mean ± s.e.m. n=3. Significance versus control: * p <0.05, ** p< 0.005.*



DISCUSSION

4.2 Cytotoxic effects of fatty acids and organic peroxides on HL60 cell line

The cytotoxic effects of the unsaturated fatty acids tested depended on the following factors:

a) the type of fatty acid;

b) additives;

c) cell density.

The most potent fatty acid was GLA (C18:3 n-6) followed by AA (C20:4 n-6), EPA (C20:5 n-3), DGLA (C18:4 n-6), DHA (C22:6 n-3), and POA (C16:1 n-9). LA (C20:2 n-6) was the least active.

All the fatty acids tested are both essential fatty acid (EFA) and PUFAs except POA. PUFAs are fatty acids which have two or more double bonds linking carbon atoms in the molecules (181). EFAs are PUFAs that cannot be synthesised by the body and therefore must be taken in the diet (182). There are two series of EFAs, the n-6 derived from cis-linoleic acid and the n-3 derived from alpha-linolenic acid (see the Introduction). The numbers indicate the position of the first double bond from the omega (methyl) end of the molecule.

According to the results those fatty acids with 3, 4 and 5 double bonds were the most potent in inhibiting the growth of HL60 cells. Although POA is neither a PUFA or an EFA it was quite active against HL60 cells. It was demonstrated using AA that the inhibitory effects of some of the fatty acids may be related to the MDA production and hence the degree of lipid peroxidation that they initiate (fig.4.2). AA which has 4 double bonds produced significant amounts of MDA even at low concentrations in contrast to POA and LA which have 1 and 2 double bonds respectively. Although it was found that both POA and LA exhibited a unique cytotoxic action at concentrations of 10^{-9} and 10^{-10} M, it was not readily reproducible (figs. 4.1 a,b,c,d). However, the mechanism of this unique cytotoxicity seemed to be related to a free radical mechanism in view of its reversibility by BHT and the significant MDA production. There is great doubt about the significance of this observation, unless, it can reliabily be reproduced in future.

It was quite evident that the effectiveness of LA in 10% FCS was enhanced by the addition of $FeCl_2$ and ascorbate. These two

compounds are known to have catalytic activity in enhancing the process of lipid peroxidation (239,240,245). It was also noted that the effectiveness of these fatty acids depended on the nature of medium supplements. Fatty acids are known to bind to albumin. The amount of albumin in ultroser-G (UG) is much less than the amount in FCS (35-50g/litre) (246). The total protein concentration in UG is 1.5 mg/ml (manufacturer's information) of which albumin is a major component. Increasing the initial cell density by 4 times reduced the growth inhibitory potency of POA both in the presence of UG and 10% FCS (table 4.3). This inhibition was reversed by the addition of BHT. This may suggest that lipid peroxidation and therefore, a free radical mechanism may be involved in the cytotoxicity of POA. However, even at this high cell density, when ITS Premix which has no albumin was used as medium supplement, POA was active independent of the presence of BHT. In contrast, when ITS+ Premix which has albumin (1.25 g bovine serum albumin /litre of medium-manufacturer's information) was used as a medium supplement, POA lost its cytotoxic activity. This seems to suggest that in the absence of albumin there may be other factors involved in the cytotoxicity of POA and probably of other fatty acids. These other factors may involve the detergent effect of the fatty acids rupturing cell membranes. Cellular debris were formed within 30 minutes of adding POA in the presence of ITS Premix and no such debris were formed in the presence of any supplement which contained albumin. BHT did not abolish the inhibition caused by POA at a lower initial cell density (1 x 10^5 cells/ml) in the presence of either 2% UG or 10% FCS, probably, because the concentration of fatty acid per cell was greater and therefore, overcame the antioxidant influence of BHT.

Many reports have been published on the PUFA-induced cytotoxicity against tumour cells and its relation to lipid peroxidation (168,169,179,180). In one report selective cytotoxicity of PUFAs to cancer cells was noted (180). Among the PUFAs tested, gamma-linolenic acid (GLA), arachidonic acid (AA), and eicosapentanoeic acid (EPA), were the most effective at the concentration tested (60uM) and docosahexanoeic acid (DHA) was least effective, despite the presence of 6 double bonds. Linoleic acid (LA) and alpha-linolenic acid (ALA) had intermediate effects. The broad conclusion that was drawn from these studies was that the cytotoxic potential of these PUFAs depended on, but was not directly proportional, to the number of double bonds in the carbon chain and that GLA was the most cytotoxic PUFA with greatest selectivity. There was also evidence that the effectiveness of a given PUFA in killing cancer cells correlated with the intracellular

thiobarbituric acid reactive materials (TBARM) content which is a measure of free radical generation. GLA and AA with 3 and 4 double bonds respectively generated the most TBARM and this correlated with their cytotxicity. DHA with 6 double bonds was the least effective, either in generating TBARM or in killing the malignant cells. Iron and copper accelerated the rate of cell death, whereas, antioxidants such as vitamin E and butylated hydroxyanisole (BHA) inhibited the effect of GLA dose dependently. They concluded that the effectiveness of a given fatty acid in killing cancer cells correlated with the extent of lipid peroxidation of the added fatty acid in the cells. These findings are in agreement with our results. However, the fatty acids were dissolved in ethanol and added directly to the cell cultures in these studies. Contrary to these results in which PUFAs were dissolved in ethanol and added directly to cells in tissue culture, complexes of LA with bovine serum albumin (BSA) stimulated the growth of human breast cancer cell line *in vitro* (254). The LA-BSA complex is more similar to the physiological conditions than does the addition of fatty acid dissolved in ethanol. This is consistent with reports that LA enhance tumour growth (255). This may account for the lack of activity of POA against HL60 cells in the presence of ITS+ Premix (discussed below).

LOOH, MEKP and t-BOOH were very potent inhibitors of the growth of HL60 cells independent of the medium supplements at a seeding cell density of 1 x 10^5 cells/ml (table 4.2). Using t-BOOH as a model compound (i.e hydroperoxides) the growth inhibitory effect of t-BOOH was reduced when the cell density was increased by 4 times. This inhibition was abolished by BHT independent of the medium supplements at this cell density. This observation may suggest that t-BOOH mediates its action mainly through the production of free radicals and BHT was able to abolish its action even in the presence of ITS Premix, which contains no albumin. This is contrary to the action of POA under similar conditions in the presence of ITS Premix, comfirming our suggestion that the action of POA and possibly other unsaturated fatty acids is dual in nature. This could be via a free radical mechanism and a detergent effect. Selenium is a component of GPx which is responsible for the elimination of hydroperoxides from cells. Selenium is present in ITS Premix and it may contribute significantly to the antioxidant action of BHT to the action of t-BOOH to HL60 cells. This antioxidant effect was magnified in the presence of ITS+ Premix, which abolished the activity of both POA and t-BOOH. ITS+ Premix has both selenium and albumin (manufacturer's information) (see the above discussion on the

influence of albumin on the effect of fatty acids on malignant cells in culture).

Despite the presence of 6 double bonds, DHA is not as potent as other fatty acids with fewer double bonds. The presence of 6 double bonds would mean that DHA would be more readily oxidisable. It has been suggested that the greater the number of double bonds the greater the repulsive forces between adjacent molecules (256), and since the process of initiation of a free radical reaction involves the abstraction of a methylene hydrogen of two adjacent molecules it would therefore, require the close proximity between two molecules to provide the best chance of a free radical reaction. This would depend on the optimum number of double bonds giving minimal repulsive forces. GLA seems to provide the best configuration for free radical chain reactions while DHA has the most disadvantageous configuration.

A vital question with respect to this result is whether or not lipid peroxidation represents a coincidental outcome of radical-induced damage, or if lipid peroxidation products are directly deleterious to the cells. At this stage, it is not possible to be more specific as to the precise nature of the material that is actually toxic to the cells. Tumour cell death may result from the production of specific peroxide species and /or derived radicals or degradation product(s) that is (are) found in different levels in the tumour cells depending on the fatty acid substrate.

The results suggest that HL60 cell sensitivity to cytotoxic unsaturated fatty acids and peroxides varies with their ability to stimulate lipid peroxidation. In addition, mechanical rupture of cell membranes due to the detergent effect of the fatty acids especially in the absence of albumin may augment cell death. These findings are in agreement with the results reported by others (reveiwed in the Introduction section).

There is some evidence of an insignificant level of lipid peroxidation occurring at very low concentrations of AA (10^{-9} and 10^{-12} M) (fig.4.2). Although, this may not have an important role in the in vitro situation, it may play a crucial role in initiating free radical chain reactions in vivo, and this is the fundamental goal of this study.

RESULTS

4.3 The effects of DPPH and derivatives on the growth of various cell lines in vitro.

The inhibitory potency of the stable free radical DPPH and its derivatives was assessed against the growth of various malignant cell lines under various experimental conditions *in vitro*. Cell counts were performed during the exponential growth phase on day 4 and the ID50 values were determined. Unless stated, otherwise, the cells were all grown in 1% ultroser-G (chemically defined medium).

The results show that the radical DPPH and its hydrazine homologue, as well as carbazylpicrylamine (CPH_2), behave almost identically as very potent growth inhibitors of the MAC16 cell line in vitro (table 4.4). DPPH and $DPPH_2$ exhibited a similar effect on the growth of other cell lines in vitro, with the K562 leukaemia and the Raji lymphoma cell lines being the most sensitive (tables 4.5 a, b, c and d). These compounds are water insoluble. However, when the growth inhibitory effect of the water soluble derivatives, DDSH and its hydrazine homologue, were tested against the MAC16 cells, they were found to be about 300 to 400 times less potent than both DPPH and $DPPH_2$ ($p<0.001$) (table 4.4).

Serum affects the activities of many drugs especially those which are bound to albumin. When the influence of various serum concentrations on the growth inhibitory effect of $DPPH_2$ on the MAC16 cell line was assessed, it was found that there were no significant differences in the inhibitory potency of $DPPH_2$ at the various serum concentrations tested (table 4.6).

The dose-response curves for the effects of DPPH and $DPPH_2$ on the MAC16 cell line grown in 1% UG have flat segments corresponding to the concentrations of DPPH and $DPPH_2$ of 10^{-9} to 10^{-4} M, while that of K562 was between 10^{-10} to 10^{-4} M (figs. 4.3 and 4.4). These segments represented the concentrations of DPPH and $DPPH_2$ where cell viability was less than 10% using trypan blue exclusion method (data not shown). For cells grown in FCS the segment corresponded to the concentrations of $DPPH_2$ of 10^{-7} to 10^{-4} M (fig. 4.5).

The effects of antioxidants involve neutralisation of damaging radical species that may eventually cause cell death. The influence

of both enzymatic and nonenzymatic antioxidants on the growth inhibitory effect of $DPPH_2$ on the MAC16 cells in vitro was assessed. The antioxidants used included SOD, catalase (CAT), BHT and indomethacin. They all enhanced the inhibitory effect of $DPPH_2$ on the MAC16 cell line except SOD which significantly reversed the inhibition ($p<0.001$) (tables 4.7 a,b and figs. 4.6 and 4.7). The enhancement of the growth inhibition being most pronounced with a combination of both BHT and indomethacin.

Table 4.4. Effects of DPPH and derivatives on the growth of MAC16 cells in vitro. The ID50 values were determined on day 4. The cells were grown in 1% ultroser-G. The initial cell density was $5x10^4$ cells/ml. n=3-9

Compound	Conc. x 10^{-9} M	± SEM
DPPH	0.3	0.9
$DPPH_2$	0.8^{NS}	1.0
CPH_2	0.5^{NS}	0.3
DDSH	43.0*	1.5
$DDSH_2$	30.0*	1.3

*Significance versus ID50 of DPPH: *p<0.001, NS= not significant.*

Table 4.5 a,b,c,d. Effects of DPPH and its hydrazine homologue on the growth of various cell-lines in vitro. The ID50 values were determined on day 4. The cells were grown in 1% ultroser-G. The initial cell count was 1 $x10^5$ cells/ml. n=3-6

Table 4.5 a. HL60 cells.

Compound	Conc. x 10^{-10} M	± SEM
DPPH	0.2	0.9
$DPPH_2$	0.9^{NS}	0.3

NS=not significant (versus ID50 of DPPH).

Table 4.5 b. GM 892 cells

Compound	Conc. x 10^{-10} M	± SEM
DPPH	1.0	0.9
$DPPH_2$	0.5^{NS}	0.6

NS=not significant (versus ID50 of DPPH).

Table 4.5 c. Raji cells

Compound	Conc. x 10^{-11} M	± SEM
DPPH	4.7	0.5
$DPPH_2$	3.5^{NS}	0.7

NS= not significant (versus ID50 of DPPH).

Table 4.5 d. K562 cells

Compound	Conc. x 10^{-12} M	± SEM
DPPH	1.3	0.3
$DPPH_2$	0.9^{NS}	0.5

NS= not significant. (versus ID50 of DPPH)

Table 4.6. Effects of $DPPH_2$ on the growth of MAC 16 cells in the presence of different concentrations of FCS. The ID_{50} values were determined on day 4 and the initial cell density was 5 x 10^4 cells/ml. n=3

%FCS	Conc. x 10^{-9} M	± SEM
2.5	1.2^{NS}	0.3
5.0	0.8^{NS}	0.3
10	1.0	2.5
20	0.01^{NS}	1.6

NS= not significant (versus ID_{50} in 10% FCS).

Table 4.7 a and b. Influence of antioxidants on the ID50 values of $DPPH_2$ towards MAC16 cells in vitro on day 4. The cells were grown in 1% ultroser-G. The initial cell density was 5 x 10^4 cells/ml. The concentrations of the antioxidants were: BHT 5uM; indomethacin 5uM; SOD 100 U/ml; and CAT 56 U/ml;. n=3.

Table 4.7 a.

Compound	Conc. x 10^{-11} M	± SEM
$DPPH_2$	20.0	0.6
$DPPH_2$+BHT	1.0*	0.3
$DPPH_2$+INDO.	2.0*	0.3
$DPPH_2$+INDO+BHT	0.4*	0.3

*Significance versus ID_{50} of $DPPH_2$: *p<0.001.*

Table 4.7 b

Compound	Conc. x 10^{-11} M	± SEM
$DPPH_2$	16.0	0.2
$DPPH_2$ + SOD	80.0*	0.3
$DPPH_2$ + CAT	1.0*	0.1
$DPPH_2$ + SOD + CAT	4.0*	0.2

*Significance versus ID_{50} of $DPPH_2$: *P<0.001.*

Fig 4.3. Dose response curve of DPPH, $DPPH_2$ and CPH_2 on MAC 16 cells grown in 1% ultroser-G in vitro. The cell counts were performed on day 4 of incubation. The cell count for the controls for DPPH and $DPPH_2$ were 3.8 x 10^5 ±0.3 cells/ml and 2.6 x 10^5 ±0.1 cells/ml for CPH_2. The results are expressed as the mean ± s.e.m. n=3-6.

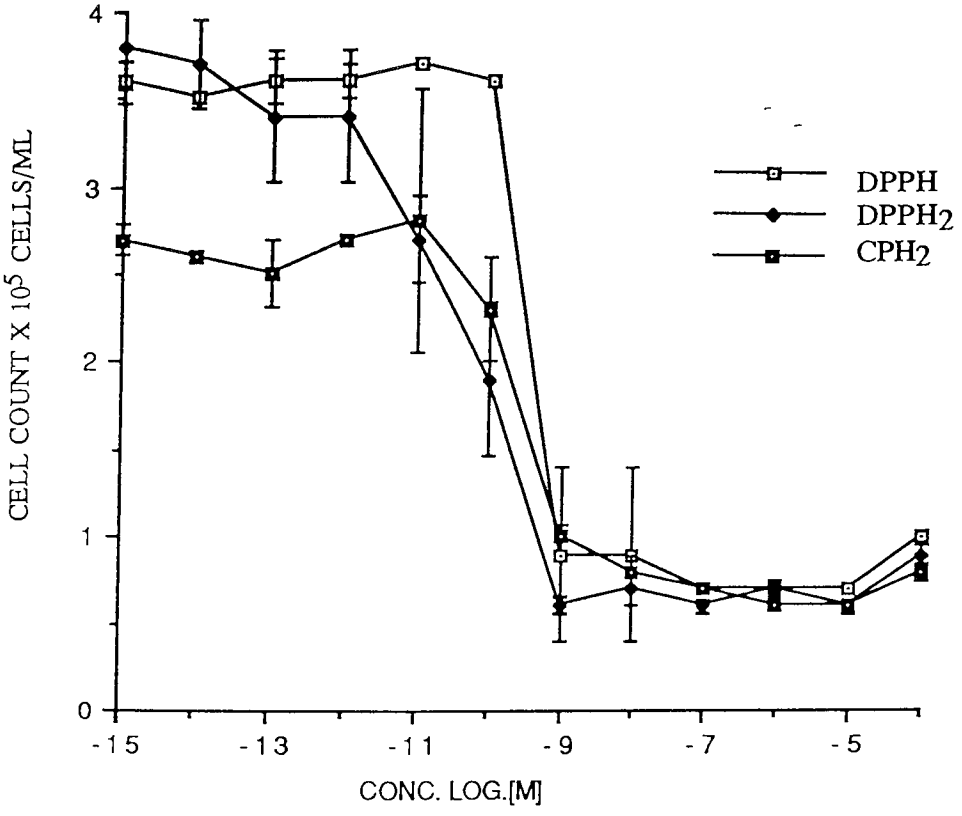


Fig. 4.4. Dose response curve of DPPH and $DPPH_2$ on the K562 cell line in vitro. The cells were grown in 1% ultroser-G. The cell counts were performed on day 4 of incubation. The cell count for the control was 4.4 x 10^5 ± 0.1 cells/ml. The results are expressed as the mean ± s.e.m. n=3.

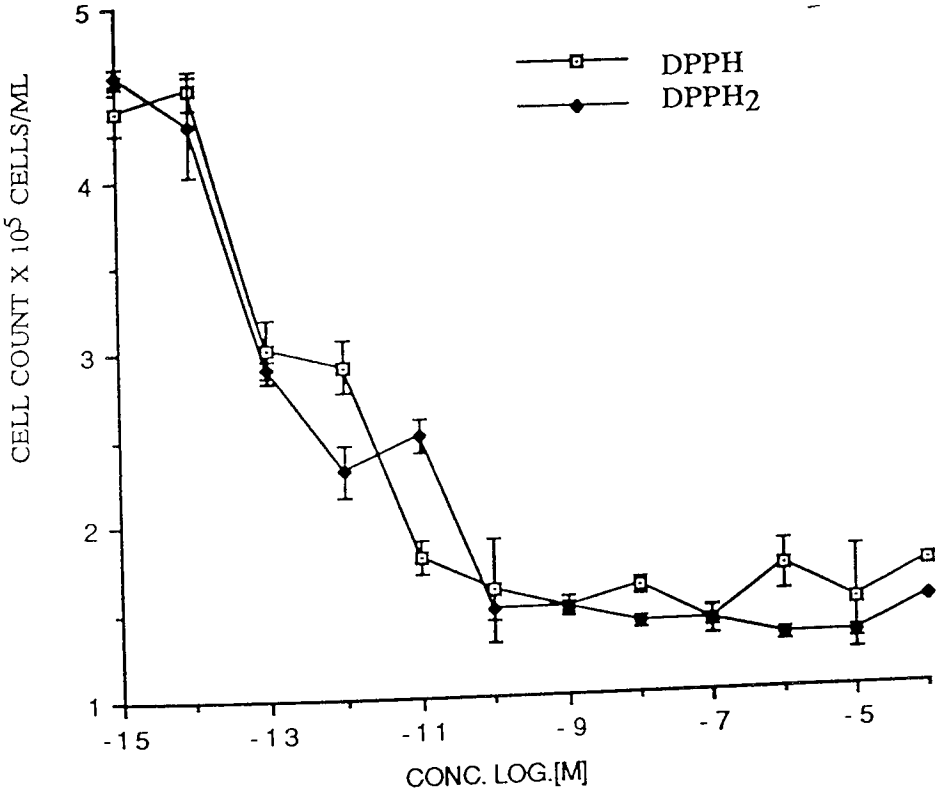


Fig. 4.5. Dose response curve of $DPPH_2$ on the MAC16 cell line grown in various concentrations of FCS in vitro. The cell counts were performed on day 4 of incubation. The cell count for the control for both 2.5% and 5% FCS was 7.4 $x10^5$ ±0.1 cells/ml; that of 10% and 20% FCS was 5.9 x 10^5 ±0.1 cells/ml. The results are expressed as the mean ± s.e.m. n=3.

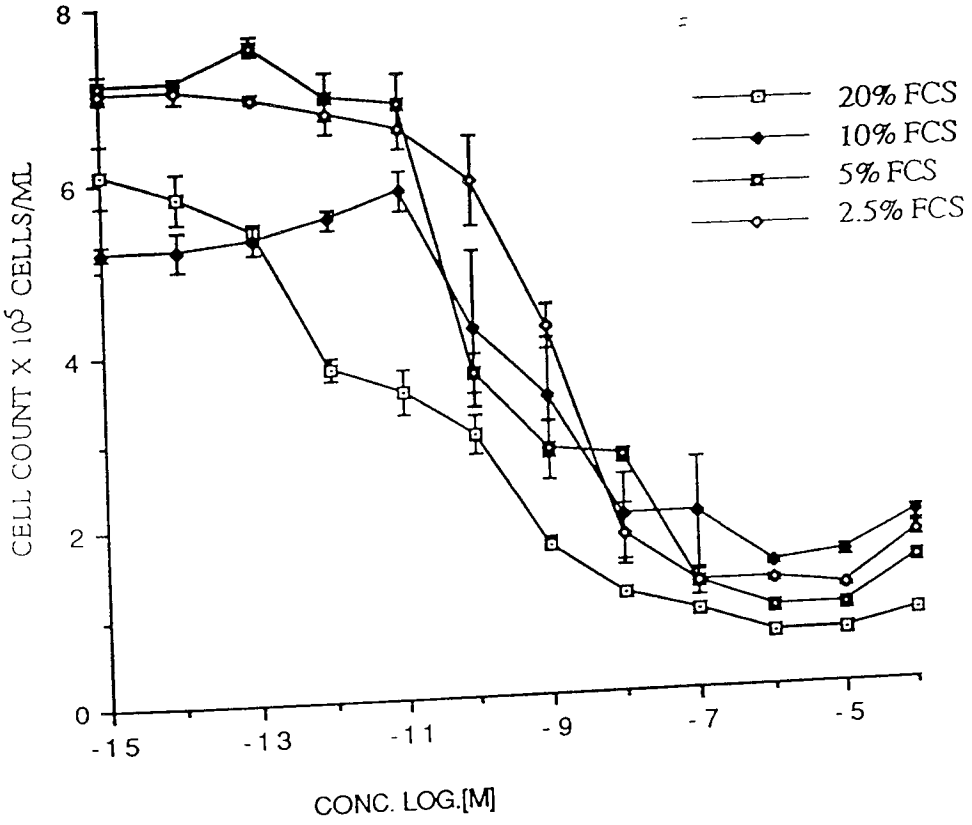


Fig. 4.6. The influence of BHT and indomethacin on the cytotoxicity of $DPPH_2$ on MAC16 cell line grown in 1% ultroser-G in vitro. The cell counts were performed on day 4 of incubation. The cell count for the control was 4.7 x 10^5 ±0.3 cells/ml. The results are expressed as the mean ± s.e.m. n=3.

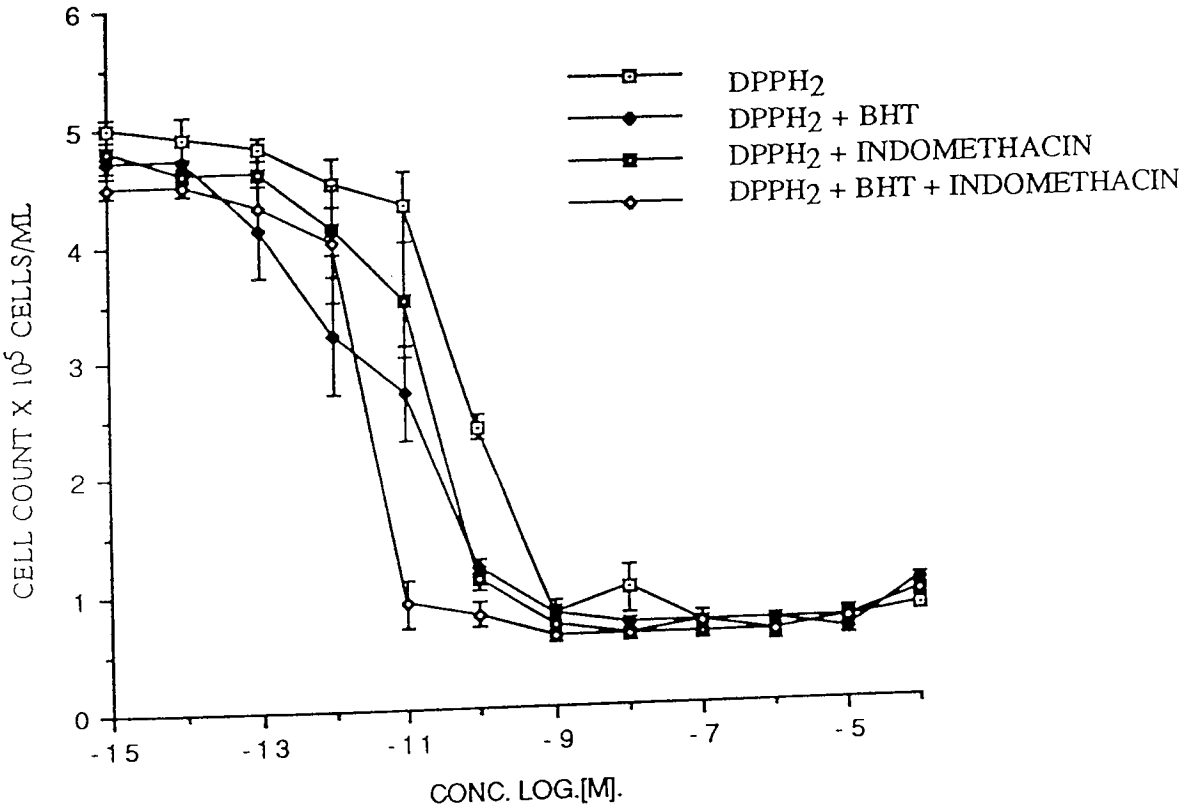
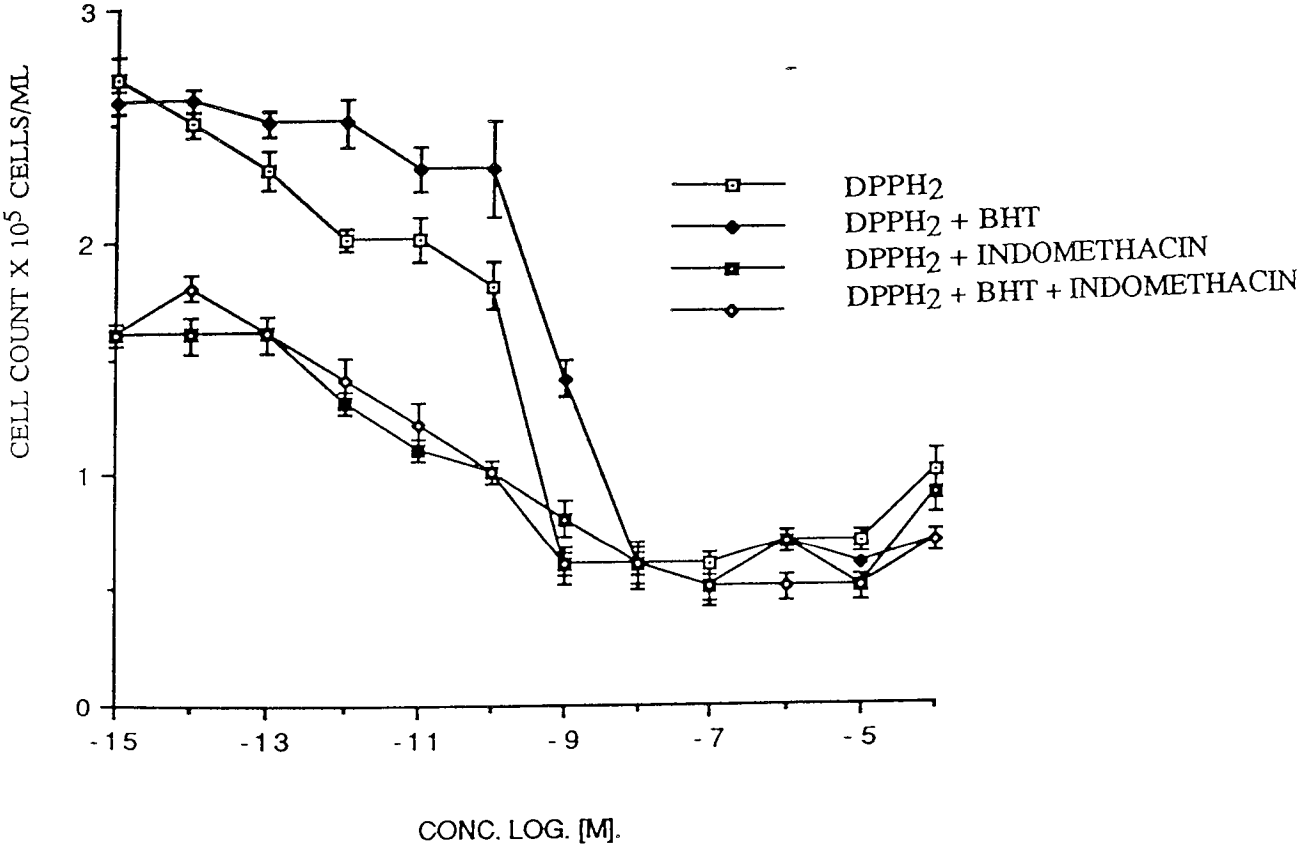


Fig. 4.7. Dose response curve of the influence of SOD and catalase on the effects of $DPPH_2$ on the growth of MAC16 cell line in 1% ultroser-G in vitro. The cell counts were performed on day 4 of incubation. The cell count for the control was 2.7 x 10^5 ±0.3 cells/ml. The results are expressed as the mean ± s.e.m. n=3.



4.4 The effects of DPPH and its derivatives on various cell lines in vitro

DPPH, $DPPH_2$ and CPH_2 behaved almost identically as very potent inhibitors of the growth of the MAC16 and other cell lines *in vitro* in the presence of 1% UG (table 4.4 and 4.5 a,b,c,d). To date, there is no work reported in the literature regarding the antineoplastic activity of this group of compounds. However, since DPPH is a stable free radical, it is likely that its mechanism of action may be related to the generation of free radicals in the cells. Dearden et al. investigated the kinetics of hydrogen abstraction from amino acids using DDSH (252). They concluded that hydrogen abstraction appeared to occur exclusively from the alpha-carbon atom to an amino or imino group. Moreover, the rate of abstraction was found to be an inverse function of the interaction between the amino acid and water, so that hydrophobic regions of a protein should yield hydrogen more readily than would a polar moiety. Russel et al. have shown that DPPH abstracts hydrogen from the -OH group of phenols and the >NH group of secondary aromatic amines to yield the corresponding hydrazine (257), while Proll and Sutchiffe found that it abstracted hydrogen from the carbon atoms of some simple aliphatic compounds (258). Hydrogen abstraction has also been shown to occur from the alpha-carbon atoms of amino acids and peptides (259). However, all these studies were done in systems that did not contain cells and DPPH which is a stable free radical could carry out this hydrogen abstraction directly without further metabolic activation, but this would not account for the similar potency observed with the hydrazine derivatives ($DPPH_2$ and CPH_2). It seems more likely that in a biological system with cells these compounds are activated by enzymatic systems, probably NADPH-cytochrome c (P-450) reductase (reviewed in the Introduction), by which superoxide radicals are generated. The superoxide radicals in turn could be involved in the generation of other free radicals which would damage the cells. Different cell lines probably, have different abilities to metabolise these compounds to generate free radicals accounting for the variation in sensitivity of different cell lines to these compounds. On the other hand, these cell lines may have differing capacity to produce antioxidant enzymes to destroy the free radicals being generated and hence susceptibility to free radical damage. The hydrophobic nature of these compounds probably provides them with better penetration to the critical sites where they can generate free radicals and cause damage. This would probably account for the reduced activity of the water soluble derivatives,

DDSH and $DDSH_2$ *in vitro*. Although a free radical mechanism was the most favourable explanation to account for the cytotoxicity of these compounds it was difficult to demonstrate. There was no statistically significant MDA production using TBARM assay in cell culture using the MAC16 or the HL60 cells (data not shown). BHT, indomethacin and catalase were not able to reverse the cytotoxicity caused by $DPPH_2$ on the MAC16 cell line. In fact these compounds enhanced the cytotoxicity of $DPPH_2$ (table 4.7, fig.4.6 and 4.7). The only evidence of free radical involvement was the reversal of the cytotoxicity caused by low concentrations of $DPPH_2$ by SOD but this was ineffective at higher concentrations of $DPPH_2$.

BHT is both a chain-breaking antioxidant and an inhibitor of the lipoxygenase pathway, while indomethacin inhibits the cyclooxygenase pathway (260). This would imply that the eicosanoids which are products of these pathways are not involved in the cytotoxicity of these compounds. In addition, the failure of catalase to abolish the cytotoxicity probably implies that hydrogen peroxide did not play a role in the cytotoxicity. Catalase is known to contain iron and therefore, the enhanced cytotoxicity by catalase could probably be due to the catalytic action of iron to propagate free radical reactions.

It has been reported in the literature that DPPH and $DPPH_2$ are very potent uncouplers of oxidative phosphorylation in whole mitochondria (261). They exhibit many of the same effects on respiratory kinetics and ATPase as does 2,4 -dinitrophenol, but at low concentration near 10^{-7} M, and appear to act at a mechanistic locus common to other " true " uncouplers of oxidative phosphorylation.

A recent proposal by Wang and co-workers on the mechanism of oxidative phosphorylation has centred around the possibility of free radical participation (262). They based their hypothesis on model experiments, implicating imidazolyl and possibly flavinyl species, endogenous to the mitochondrial composition, as active participants in the coupling scheme. With such a hypothetical mechanism in mind, future studies need to examine, in more detail, whether the mechanism of uncoupling is related to the antineoplastic activity of DPPH and its derivatives.

Although the ID_{50} of $DPPH_2$ on the MAC16 cells is similar both in the presence of UG or FCS, $DPPH_2$ had a more cytostatic than a cytocidal action in the presence of FCS (table 4.6 and fig.4.5). There may be several reasons for this effect. Since FCS is a natural product from animals, it may have more antioxidants (both

enzymatic and non-enzymatic) than UG and therefore, inhibit the cytotoxic effect of $DPPH_2$ on cells if the mechanism of cytotoxicity is via free radical production. Although there is more albumin in FCS than in UG, there are no reports in the literature to suggest that albumin would affect the activity of this group of compounds and hence it would be very difficult to draw any conclusion on the effects of albumin on their activity.

There was a reduction of the concentration of PUFAs after 18 hr following the treatment of the MAC16 cells in culture with DPPH and $DPPH_2$ (table 4.9 d). However, this reduction in the concentration of PUFAs might have occurred as a result of cellular injuries inflicted by the drugs causing lipid peroxidation.

Although the evidence to suggest that the cytotoxicity of DPPH and its derivatives involved free radical mechanism(s) were circumstantial, this group of compounds provided the best tool to test the antitumour effects of low concentrations of free radical generating compounds *in vivo* in view of their potency at extremely low concentrations.

RESULTS

4.5 The effects of various compounds on the growth of the MAC16 cells in vitro.

Reactive oxygen species are generated during the metabolism of many organic compounds. This study was to compare the growth inhibitory effects of a variety of organic compounds on the MAC16 cell line in vitro. Most of the compounds used are known to generate free radicals during their metabolism (see the Introduction for the review). The cells were grown in 1% ultroser-G (chemically defined medium) and the ID50 values were determined during the exponential growth phase on day 4 of incubation. The results are summarised on table 4.8 (see appendix 11 for the structural formulae).

Adriamycin (doxorubicin), 5,8-dihydroxynaphthoquinone, 5-fluorouracil (5-FU), and picryl chloride were the most active compounds in the group against the MAC16 cell line. In contrast, the least active compounds included the stable free radicals, carboxy-PROXYL and galvinoxyl [2,6-di-tert-butyl-α-(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-tolyloxy], mitozolomide, and azobenzene. Sulfonazo 111 tetrasodium salt [3,6-bis(2-sulphophenylazo)-4,5-dihydroxy-2,7-naphthalenedisulfonic acid tetrasodium salt] had no effects on the growth of the MAC16 cell line *in vitro* at the highest concentration tested of 10^{-4} M. In comparison to adriamycin or 5FU, chlorambucil, duodocane thiol, 1,4-dihydroxyanthraquinone, p-benzoquinone, diphenylhydrazine and phenylhydrazine had intermediate effects.

Table 4.8. ID50 values of the effects of various compounds on the growth of MAC16 cell line in vitro. The cells were grown in 1% ultroser-G. The cell counts were performed on day 4 of incubation. The initial cell density was 5 x 10^4 cells/ml. n=3

Compound	Conc. x 10^{-7} M	± SEM
adriamycin (doxorubicin)	0.6	0.4
5,8,dihydroxy-naphthoquinone	0.8	0.6
5-fluorouracil	0.8	0.4
picryl chloride	7.8	0.6
chlorambucil	33.0	0.5
duodocane thiol	35.0	0.8
1,4,dihydroxy-anthraquinone (quinizarin)	50.0	0.1
p-benzoquinone	47.0	0.6
phenylhydrazine	80.0	0.5
diphenylhydrazine	90.0	0.3
carboxy-PROXYL-stable free radical	225.0	0.9
galvinoxyl-stable free radical	400.0	0.3
mitozolomide	300.0	0.5
azobenzene	500.0	0.1
sulfonazo111	*NE*	

NE = no effects.

4.6 The effects of various compounds on the MAC16 cells in vitro

The majority of compounds used here are known to generate free radicals from the reports in the literature and their proposed mechanisms of action have already been discussed briefly in the Introduction in chapter 3. These compounds fall into the following categories:

a) those known to generate semiquinone radicals (80,83). This includes doxorubicin, 5,8-dihydroxynaphthoquinone, 1,4-dihydroxyanthraquinone and parabenzoquinone.

It is clear, however, that some other feature in addition to free radical formation must contribute to the antitumour activity of the quinone antitumour drugs, or else why do not all quinones exhibit a similar antitumour activity? This other feature might be the selective binding of the compounds to DNA or other critical sites in the cell. Site-specific covalent binding of the quinone to DNA followed by direct enzymatic reduction, or reduction by diffusible cellular reductant, might allow the formation of damaging semiquinone or oxygen radicals in close proximity to the DNA (196). In this model the location of the DNA damage is determined by the specificity of original quinone binding. Metal ions, particularly iron, have been known to increase radical formation by several antitumour quinones, and DNA could be involved at the critical site of damage (196). Generation of oxygen radicals not preceded by site specific binding of quinone could be responsible for nonspecific cellular toxicity. This may be the mechanism of anthracycline toxicity to the heart. The high rate of oxidative metabolism in the heart makes it a prime target for oxygen radical damage, perhaps, mediated by lipid peroxidation (196).

The attention given to DNA in the nucleus as the site of action of antitumour compounds should not be allowed to obscure the possibility that there could be other sites in the cell where antitumour quinones produce their cytotoxic effects. The cell surface membrane is a potential site for the action of a number of anticancer drugs. Several quinone metabolising flavoenzymes are located at the surface membrane (197) and they give rise to quinone mediated cell surface damage and toxicity. As an example, the inhibition of the cell surface receptor for epidermal growth factor has been reported to occur during quinone metabolism (198).

Despite the large number of reports of radical formation by antitumour quinones we are still not in a position to answer the question of whether radical formation is responsible for the antitumour activity of this class of compounds. The evidence for such an association is strong but other mechanisms make equal claims for attention. It is possible that, ultimately, a combination of mechanisms, including radical formation, will prove to be responsible for quinone antitumour activity.

b) compounds known to generate azo anion and hydrazyl free radicals (199,200). This included phenylhydrazine, diphenylhydrazine, azobenzene and sulfonazo 111.

c) compounds known to generate nitro-aromatic anion free radicals such as picryl chloride (207)

d) and duodocane thiol is a compound known to generate thiyl radicals (210)

It is documented that the azo anion, hydrazyl and nitroaromatic free radicals in the presence of oxygen produce superoxide radicals in biological systems (204). The superoxide may participate in free radical reactions and cause damage to biological molecules including vital structures such as membranes and DNA. The azo ion, hydrazyl, nitroaromatic and thiyl radicals of these compounds if sufficently stable to enter the nucleus may react with DNA or with other macromolecules to cause cell damage. The activities of these compounds may vary depending on their transportation and metabolism at critical sites where these radicals are eventually generated to induce biologcal damage.

Carboxy-PROXYL and galvinoxyl are stable free radicals but were 10^5 times less active than DPPH which is also a stable free radical. Therefore, this may suggest that other factors may be involved in the antitumour activity of DPPH other than a free radical mechanism only.

5-FU, chlorambucil and mitozolomide were used for comparative purpose.

Following the screening of these free radical generating compounds, it was decided that DPPH and its derivatives would provide the best model compound for *in vivo* experiments.

RESULT

4.7 Fatty acid analysis using gas chromatography

Unsaturated fatty acids are susceptible to lipid peroxidation and they form a major part of cell membranes.

This study was designed to investigate the relationship between fatty acid composition of the media and that of cells grown in them and how the fatty acid composition would affect the susceptibility of these cells to free radical generating compounds. The K562 cell line was about 100 times more sensitive to DPPH and $DPPH_2$ than the MAC16 cell line *in vitro* (tables 4.4 and 4.5 d). It was important to find whether there was a relationship between the increased sensitivity of the K562 cell line to DPPH and $DPPH_2$ and its fatty acid profile. An increase in the proportion of unsaturated fatty acids in comparison to the fatty acid profile of the MAC16 cell line would render the K562 cell line more susceptible to free radical attack and therefore, the increased sensitivity to both the DPPH and $DPPH_2$. This was on the assumption that both the DPPH and $DPPH_2$ exerted their antitumour effect by a free radical mechanism.

There was a significant difference between the fatty acid composition of FCS and ultroser-G (table 4.9a). FCS had a higher proportion of PUFAs, and C18:1 n-9 than ultroser-G, which had a higher proportion of C18:2 n-6. The fatty acid composition of the MAC16 cells in culture reflected the fatty acid composition of the media in which they were grown (i.e. 10% FCS and 1% ultroser-G) (table 4.9b). In addition, there was no significant difference between the fatty acid composition of the MAC16 and the K562 cell lines grown in 1% ultroser-G except for the MAC16 cells which had a higher proportion of C16:1 n-9 and C18:1 n-9 (table 4.9c).

Following the treatment of the MAC16 cell line with 10^{-6} M DPPH and $DPPH_2$ respectively, there was a significant decrease in the proportion of C18:1 n-9, C20:4 n-6, and C22:6 n-3 with a relative rise of C18:0 and C16:0 in the DPPH and $DPPH_2$ treated groups respectively (table 4.9d).

Table 4.9a. Fatty acid composition of 10% FCS and 1% ultroser-G using gas chromatographic analysis. The results are expressed as % weight of total fatty acid content ± s.e.m. n =3-6.

	10% FCS		1% Ultroser-G		
Fatty acid[a]	Weight %	± SEM	Weight %	± SEM	[b] P<
16:0	20.9	1.6	21.2	0.3	NS
16:1 n-9	4.4	0.7	8.3	1.0	0.01
18:0	13.7	1.7	21.5	0.6	NS
18:1 n-9	29.6	0.7	20.6	0.4	0.001
18:2 n-6	3.4	0.5	20.9	0.6	0.001
20:4 n-6	5.0	0.5	2.9	0.2	0.05
20:5 n-3	1.4	0.2	2.3	0.4	NS
22:4 n-6	3.7	0.2	1.0	0.1	0.001
22:5 n-6	3.7	0.2	0.6	0.2	0.001
22:5 n-3	1.6	0.3	0.3	0.1	0.001
22:6 n-3	3.4	0.7	1.2	0.1	NS

[a] *Fatty acids are abbreviated in the usual manner: the first number indicates the number of carbon atoms and is followed by the number of double bonds; the number after n- indicates the number of carbon atoms between the methyl end of the molecule and the first double bond on this side; NS= not significant; b= significance versus values in 10% FCS.*

Table 4.9b. Fatty acid composition of the MAC16 cell line grown in 10% FCS and 1% ultroser-G (UG) using gas chromatographic analysis. The results are expressed as % weight of the total fatty acid content ± s.e.m. n=3-6.

	MAC16 CELLS IN 10% FCS		MAC16 CELLS IN 1% UG		
Fatty acid[a]	Weight %	± SEM	Weight %	± SEM	[b]P<
16:0	17.0	1.0	20.3	0.4	NS
16:1 n-9	3.0	0.3	9.6	1.3	0.01
18:0	19.2	0.5	5.7	0.6	0.001
18:1 n-9	31.2	1.0	22.1	1.8	0.01
18:2 n-6	2.6	0.3	13.4	0.9	0.001
20:4 n-6	6.3	0.6	5.0	0.3	NS
20:5 n-3	1.2	0.1	1.7	0.2	NS
22:4 n-6	2.9	0.2	0.6	0.1	0.01
22:5 n-6	3.2	0.1	0.8	0.2	0.01
22:5 n-3	2.1	0.1	1.3	0.1	NS
22:6 n-3	6.6	0.3	5.2	0.3	NS

[a] *Fatty acids are abbreviated in the usual manner: the first number indicates the number of carbon atoms and is followed by the number of double bonds; the number after n- indicates the number of carbon atoms between the methyl end of the molecule and the first double bond on this side; NS= not significant; b= significance versus values in 10% FCS.*

Table 4.9c. Fatty acid composition of the MAC16 and the K562 cell lines grown in 1% ultroser-G using Chromatographic analysis.The results are expressed as % weight of total fatty acid content ±s.e.m. n=3-6.

	MAC 16 CELLS		K562 CELLS		
Fatty acid[a]	Weight %	± SEM	Weight %	± SEM	$b_{P<}$
16:0	20.3	0.4	18.5	1.2	NS
16:1 n-9	9.6	1.3	6.9	0.3	0.05
18:0	5.7	0.6	8.7	1.6	NS
18:1 n-9	22.1	1.8	12.1	0.1	0.05
18:2 n-6	13.4	0.9	11.7	0.2	NS
20:4 n-6	5.0	0.3	4.3	0.1	NS
20:5 n-3	1.7	0.2	2.3	0.3	NS
22:4 n-6	0.6	0.1	1.4	0.7	NS
22:5 n-6	0.8	0.2	0.9	0.2	NS
22:5 n-3	1.3	0.1	1.8	0.3	NS
22:6 n-3	5.2	0.3	7.0	0.5	NS

[a]Fatty acids are abbreviated in the usual manner: the first number indicates the number of carbon atoms and is followed by the number of double bonds: the number after n- indicates the number of carbon atoms between the methyl end of the molecule and the first double bond on this side; NS= not significant; b= significance indicates when the K562 value was statistically different from the MAC16 value.

Table 4.9d. Fatty acid composition of the MAC16 cell line grown in 1% ultroser-G, 18 hr after treating with 10^{-6} M DPPH and $DPPH_2$, using gas chromatographic analysis. The results are expressed as % weight of total fatty acid content ± s.e.m; n=3.

	Control		DPPH Treated			$DPPH_2$ Treated		
Fatty acid[a]	Weight %	± SEM	Weight %	± SEM	$b_{P<}$	Weight %	± SEM	$c_{P<}$
16:0	20.3	0.4	25.6	1.2	NS	29.3	1.7	0.05
16:1n-9	9.6	1.3	6.5	0.6	NS	9.7	0.8	NS
18:0	5.7	0.6	10.0	0.5	0.01	2.3	1.1	0.01
18:1n-9	22.1	1.8	12.6	0.6	0.05	8.3	0.3	0.01
18:2n-6	13.4	0.9	11.7	0.8	NS	11.4	0.2	NS
20:4n-6	5.0	0.3	1.6	0.2	0.01	1.8	0.6	0.01
20:5n-3	1.7	0.2	1.8	0.3	NS	0.9	0.7	NS
22:4n-6	0.6	0.1	1.0	o.2	NS	1.3	0.3	NS
22:5n-6	0.8	0.2	0.8	0.1	NS	1.1	0.2	NS
22:5n-3	1.3	0.1	0.2	0.1	0.05	0.9	0.1	NS
22:6n-3	5.2	0.3	2.4	0.2	0.01	3.3	0.1	0.01

[a]Fatty acids are abbreviated in the usual manner: the first number indicates the numger of carbon atoms and is followed by the number of double bonds: the number after n- indicates the number of carbon atoms between the methyl end of the molecule and the first double bond on this side; NS= not significant; b and c = significance versus control.

DISCUSSION

4.8 Fatty acid analysis

The gross lipid composition of the MAC16 and the K562 cell lines, reflected the fatty acid profile of the media in which they were grown (tables 4.9 a b and c). The results also showed that the fatty acid composition of the MAC16 tumour implanted in the NMRI mice reflected the fatty acid composition of the serum which in turn, reflected the fatty acid profile of the diet which animals had been fed (data not shown).

Reports in the literature confirm our results that the fatty acid composition of cells in culture depended upon the composition on the fatty acid composition of the medium (264). It has been shown however, that cells in culture could exhibit very different fatty acid patterns according to their embryological origin and physiological status, especially, proliferative tumour cells which usually contain much less PUFAs when compared with cells in primary culture (265).

Although the K562 and the MAC16 cell line grown in 1% ultroser-G had nearly identical fatty acid profiles, the K562 cell line was about 100 times more sensitive to DPPH and $DPPH_2$ than the MAC16 cell line *in vitro* (tables 4.4, 4.5 d and 4.9 c). This may suggest that other factors may be involved in the antitumour activity of DPPH and $DPPH_2$. This may include the transportation and the metabolism of these compounds in the cell.

The decrease in the proportions of the unsaturated fatty acids, namely, C18:1 n-9, C20:4 n-6, and C22:6 n-3 following the addition of DPPH and DPPH2 to the culture media may suggest that the process of lipid peroxidation might have taken place in the culture, although the results of the MDA assay done concurrently were statistically insignificant (table 4.9 d and data for MDA assay not shown). However, the changes in the fatty acid profile of the MAC16 could have resulted from either the cell injuries inflicted by the drugs causing the oxidation of fatty acids or from the free radicals being generated initially and causing the eventual damage to the cells.

RESULTS

4.9.The effects of chemotherapy on the survival of tumour bearing mice.

Although the stable free radical, DPPH, and its hydrazine homologue, $DPPH_2$, as well as carbazyl picrylamine (CPH_2) were very potent inhibitors of the growth of a wide range of malignant cell lines *in vitro*, they were inactive *in vivo* against the MAC16 tumour transplanted in male NMRI mice (data not shown). In contrast, DDSH, a water soluble derivative, which was far less active *in vitro*, exhibited antitumour activity *in vivo* against the MAC16 tumour transplanted in male NMRI mice at very low concentrations when administered either as a single or a 3 day dose schedule by subcutaneous injections (figs.4.9 a and 4.10.a). The concentrations ranged from 2.5×10^{-5} to 2.5×10^{-17} mol/kg body weight. There was an overall improvement in survival of the treated groups compared with the controls with both dose schedules. Using a single dose schedule there was significant improvement in survival at concentrations of DDSH of 2.5×10^{-5}, and 2.5×10^{-10} to 2.5×10^{-16} mol/kg body weight, while this was achieved at concentrations of 2.5×10^{-5} to 2.5×10^{-10} and 2.5×10^{-14} mol/kg body weight in the groups that received a 3 day dose schedule. In both groups the longest survival of 60 days was achieved at a concentration of DDSH of 2.5×10^{-10} mol/kg body weight, with a survival of 60% and 40% in the single and a 3 day dose schedules respectively (figs. 4.9 b and 4.10 b). One early death from fighting was recorded in the group that had received a single dose schedule.

In contrast, a high dose schedule did not improve the survival of tumour bearing male NMRI mice transplanted with the MAC16 tumour (fig.4.10 e). In this case the tumours grew despite the treatment and none ruptured.

The average tumour volumes on initiation of therapy were about 50 mm^3 and 200 mm^3 in the single and the 3 day dose schedules respectively. The administration of DDSH retarded the growth of the tumours and caused their eventual rupture (figs. 4.9 c and 4.10 c). However, in both control groups the tumours continued to grow until they either ulcerated or reached the end point volume of 1000 mm^3 at which the animals were sacrificed. It took an average of 12 and 19 days after treatment for the tumours to rupture following the administration of 2.5×10^{-10} mol/kg body weight of DDSH as a 3 day or single dose schedule respectively. The tumours in both groups did not cause cachexia and the

animals had an average weight of 30 g at the time of treatment. Following treatment, the animals in both groups lost weight rapidly, while the control groups had relatively stable weights despite the growth of their tumours (figs. 4. 9 d and 4.10 d). The weight lost reached a maximum at about day 19 following the administration of a single dose of DDSH and about day 14 in the group that recieved a 3 day dose schedule. These periods coincided with the average time intervals taken for the tumours to rupture following treatment. Thereafter, it took about one to two weeks for the animals whose tumours had ruptured to reach pretreatment body weights. This weight lost was probably drug induced because the animals in the control groups maintained relatively stable body weights despite the growth of their tumours.

The sequence of events following the administration of low concentrations of DDSH given subcutaneously are illustrated in **figs. 4.11 a, b** and **c. a**, Before treatment ; **b**, within 24 hr after a complete tumour rupture. There was a dry scab formed at the site of rupture. There was no evidence of active bleeding which usually occurs after tumour ulceration. **c**, Approximately 72 hr after the rupture. The healing process was nearly complete and there was no evidence of residual tumour. Similar changes were observed in instances in which the tumour ruptured following the administration of other free radical generating compounds.

Histological sections of the MAC16 tumours growing in NMRI mice are illustrated in **figs. 4.12 a, b** and **c**. The sections were stained with Haematoxylin and Eosin. **a**, Normal appearance of the MAC16 tumour from control (magnification x100). There were viable cells surrounding a blood vessel next to the area of tumour necrosis. **b**, Appearance of the MAC16 tumour from NMRI mouse treated with 2.5×10^{-5} mol/kg body weight of DDSH (magnification x 100). A similar appearance to the control except that the tumour necrosis was slightly more extensive. **c**, Appearance of the MAC16 tumour from a NMRI mouse treated with 2.5×10^{-10} mol/kg body weight of DDSH (magnification x 25) and **d**, same section at x 100 magnification. In addition to the more extensive haemorrhagic tumour necrosis there were dead tumour cells in the perivascular areas. Most of the blood vessels have collapsed and have deposits of haemosiderin.

It was proposed that the antitumour effect of DDSH against the MAC16 tumour *in vivo* was by a free radical mechanism and if this proposal was valid, other compounds known to generate free radicals could have a similar antitumour effect against the MAC16

tumour. A study was designed to test the proposal in which several compounds that are known to generate free radicals were used to treat the MAC16 tumour implanted in male NMRI mice. The compounds included several unsaturated fatty acids, MEKP, t-BOOH, and p-benzoquinone. Chlorambucil, an alkylating agent, was included as a negative control. Most of these compounds had exhibited antitumour effects against the MAC16 cell lines *in vitro* (table 4.8).

The results are summarised in fig. 4.13 a. There was a statistically significant improvement in the survival of animals treated with 2.5×10^{-5} mol/kg body weight LA, EPA and 2.5×10^{-11} mol/kg body weight t-BOOH. There was one long term survivor in the group that received 2.5×10^{-11} mol/kg body weight GLA but the average survival of the group was not different from controls. Most of the animals had partial tumour rupture although there were a few complete tumour ruptures in the groups that had long term survivors. Most of the tumours ruptured 5 to 7 days after treatment (data not shown). The tumours that partially ruptured left large residual masses and the animals had to be sacrificed. These animals lost weight initially in the first 6 days after treatment including the solvent treated controls. However, most of their weights stabilised after the tumour rupture (fig 4.13 b).

The influence of DDSH on intraperitoneal ascites tumours was assessed using the P388 leukaemia and TLX5 lymphoma transplanted in male BDF1 and male CBA/CA mice respectively. The study was designed to investigate whether extremely low concentrations of DDSH administered by subcutaneous injection would inhibit the growth of intraperitoneal tumours. This would further point to an indirect antitumour mechanism(s) of action of low concentrations of DDSH. The animals were divided into two groups and treated with a single dose schedule of 2.5×10^{-5} and 2.5×10^{-11} mol/kg body weight DDSH administered subcutaneously. The dose selection was based on the results of pilot studies. There was no improvement of survival in any of the groups treated (figs. 4.14 a, b, c and 4.15 a, b, c). Two out of five BDF1 mice which received 2.5×10^{-11} mol/kg body weight DDSH died suddenly on day 8 after treatment. The two animals that died had no ascites at post mortem (figs. 4.14 a,b,c). On the other hand, three out of five CBA/CA mice that received 2.5×10^{-11} mol/kg body weight of DDSH also died suddenly two on day four and one on day five after treatment. All the three animals that died had no ascites (figs. 4.15 a,b,c). In addition, one CBA/CA mouse that received 2.5×10^{-5} mol/kg body weight of DDSH was

found dead on day 6 after treatment and no ascites was present at post mortem. We confirmed the occurrence of the sudden death on several occassions at concentrations ranging from 2.5×10^{-5} to 2.5×10^{-16} mol/kg body weight of DDSH using BDF1 mice transplanted with P388 leukaemia (data not shown). In view of the progressive disease, the BDF1 and CBA/CA mice were all sacrificed 9 and 6 days after the treatment respectively.

In view of the sudden deaths, a study was conducted to find whether the death of these animals were drug related. Non-tumour bearing BDF1 mice consisting of 5 animals per group were treated with either sterile distilled deionised water or 2.5×10^{-11}, 2.5×10^{-5} mol/kg body weight of DDSH. There was no death recorded in either group although the animals that received 2.5×10^{-5} mol/kg body weight lost weight in the first 5 days of treatment and regaining thereafter (fig. 4.14 e). The study was terminated on day 20 after treatment. Therefore, the results suggested that the cause of death was related to both the therapy and presence of tumour.

*Fig. 4.9a. Average survival time of the male NMRI mice transplanted with the MAC16 tumour and treated with DDSH administered as a single subcutaneous injection. 5 animals per group. The results are expressed as the mean ±s.e.m. Significance versus solvent treated controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.*

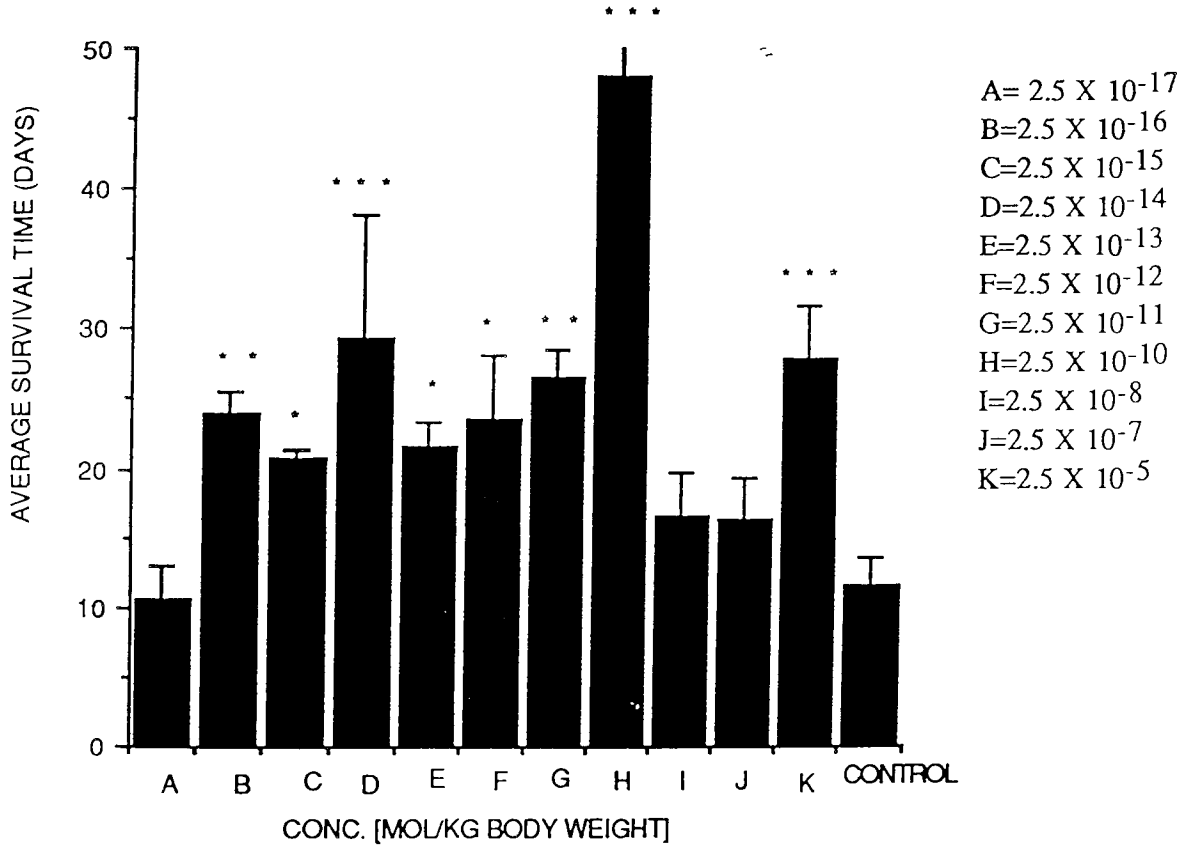
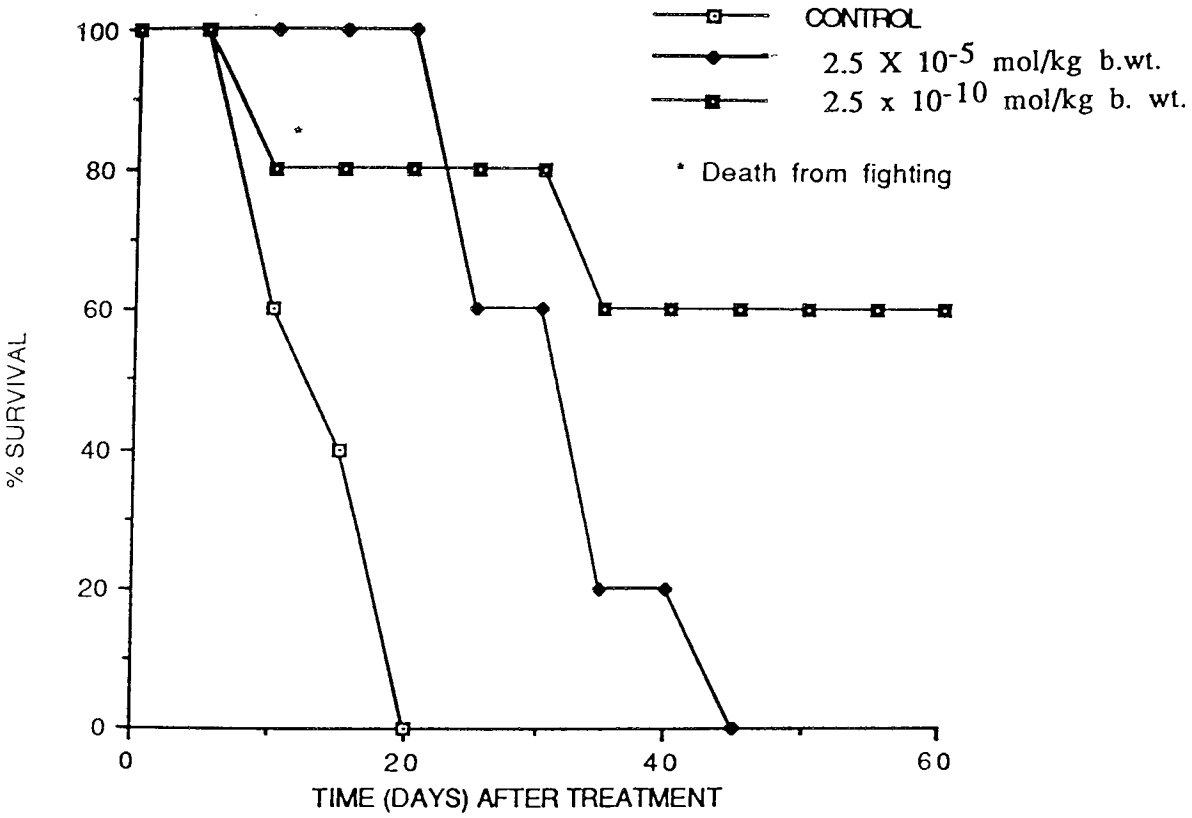


Fig. 4.9b. Survival curve of male NMRI mice implanted with the MAC16 tumour and treated with DDSH administered as a single subcutaneous injection. 5 animals in the group.



Figs. 4.9 c. The influence of 2.5 x 10-10 mol/kg body weight of DDSH on the growth of the MAC16 tumours. The results are expressed as the mean ± s.e.m. 5 animals per group.

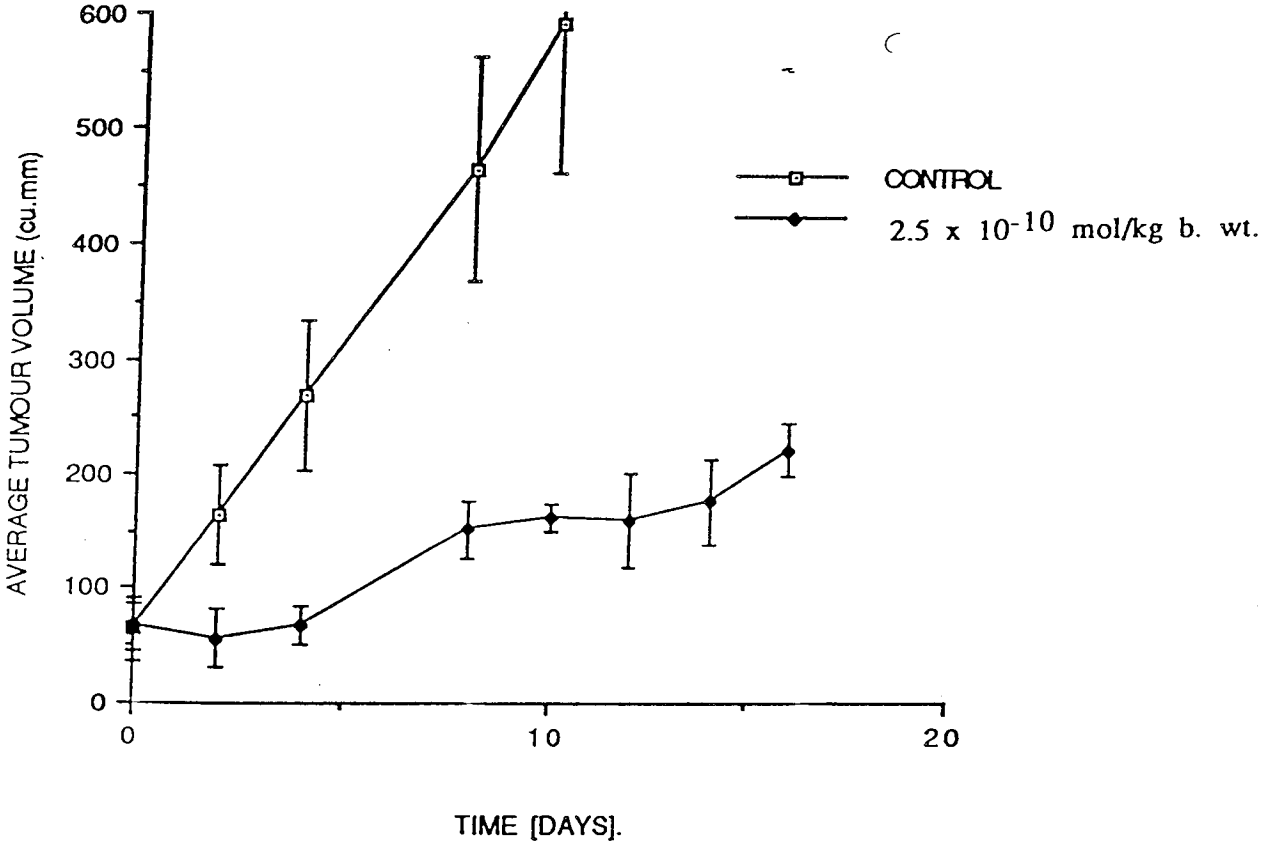


Fig. 4.9 d. Average weight of male NMRI mice carrying the MAC16 tumour after treatment with 2.5 x 10^{-10} mol/kg body weight of DDSH. 5 animals per group.

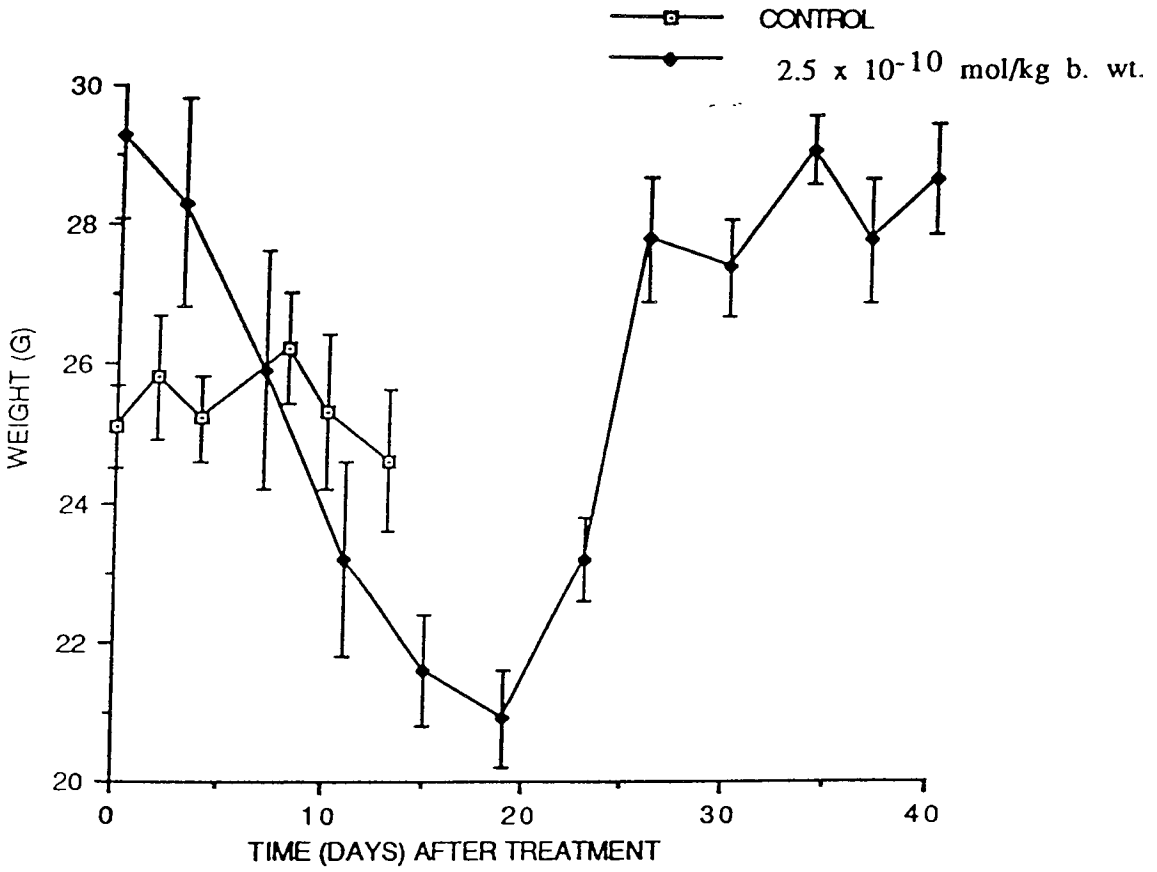


*Fig. 4.10 a. Average survival time of male NMRI mice implanted with the MAC16 tumour and treated with DDSH administered as a three day course by subcutaneous injection. The results are expressed as the mean ± s.e.m. 5 animals per group. significance versus solvent treated controls: * $p < 0.05$.*

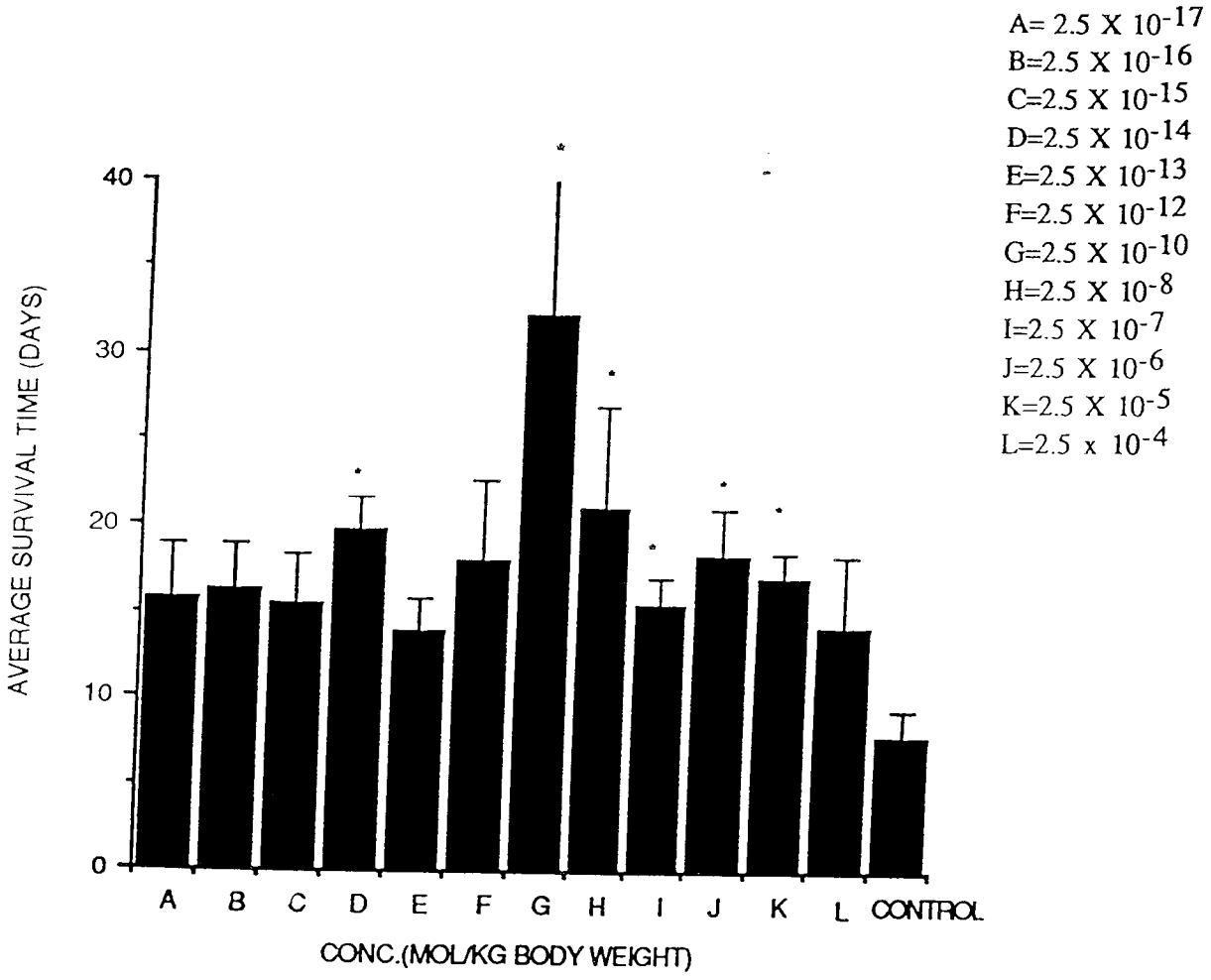


Fig. 4.10 b. Survival curve of male NMRI mice implanted with the MAC16 tumour and treated with DDSH administered as a 3 day course by subcutaneous injection. 5 animals in the group.

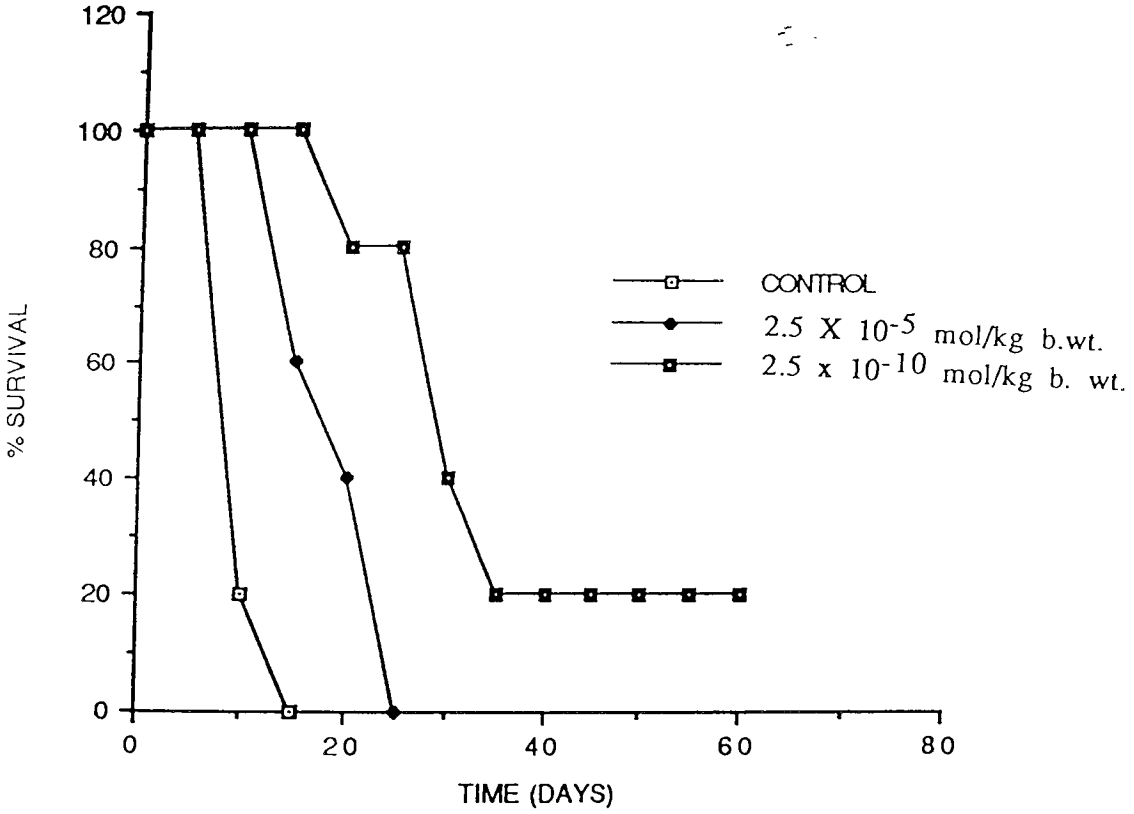


Fig 4.10 c. The influence of a 3 day course of 2.5 x 10^{-10} mol/kg body weight of DDSH on the growth of the MAC16 tumour. The results expressed as the mean ± s.e.m.

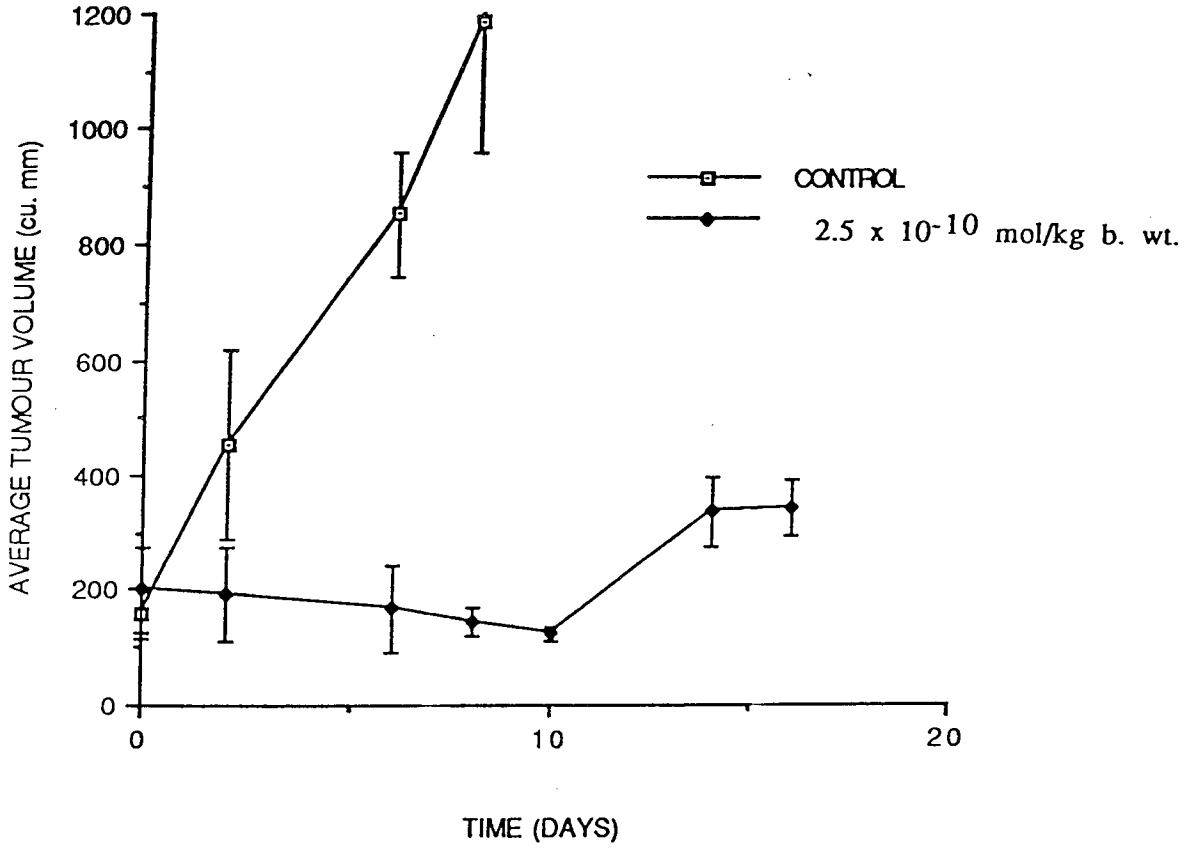


Fig. 4.10 d. Average weight of male NMRI mice carrying the MAC16 tumour after treatment with a 3 day course of 2.5 x 10^{-10} mol/kg body weight of DDSH. The results are expressed as the mean ± s.e.m. 5 animals per group initially.

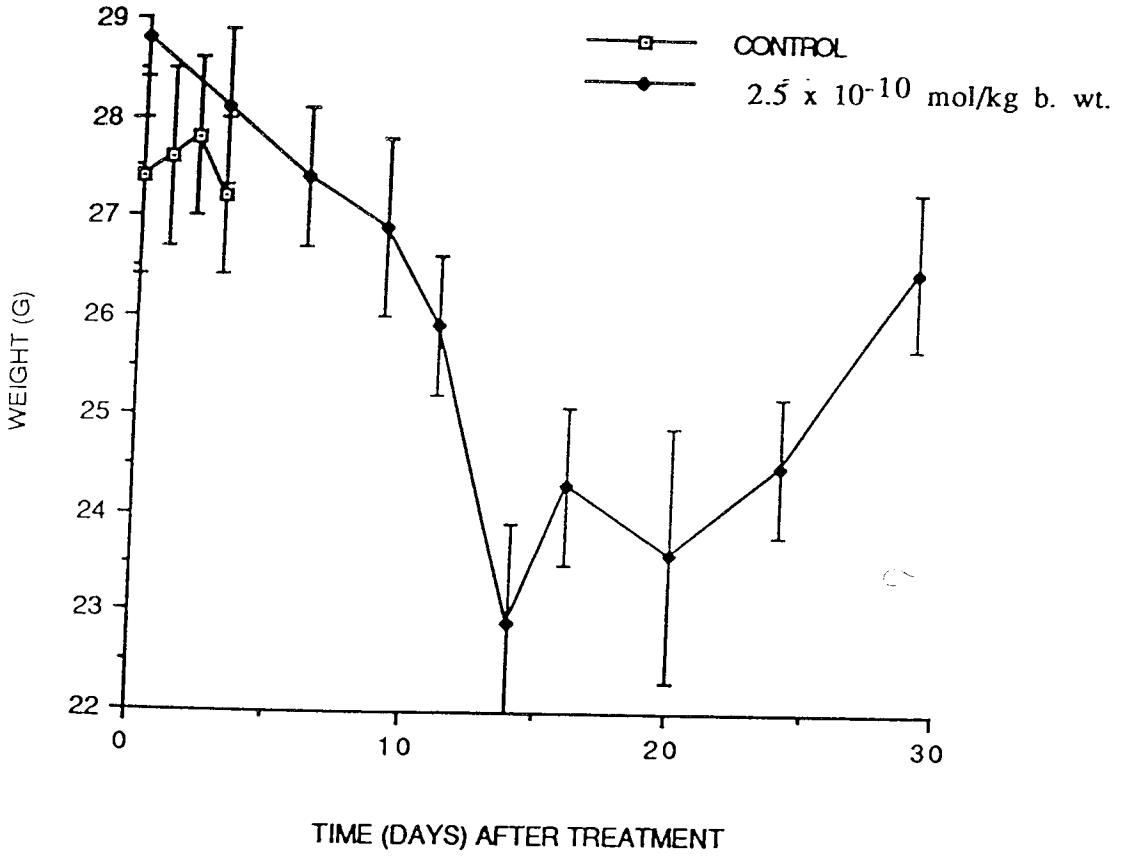
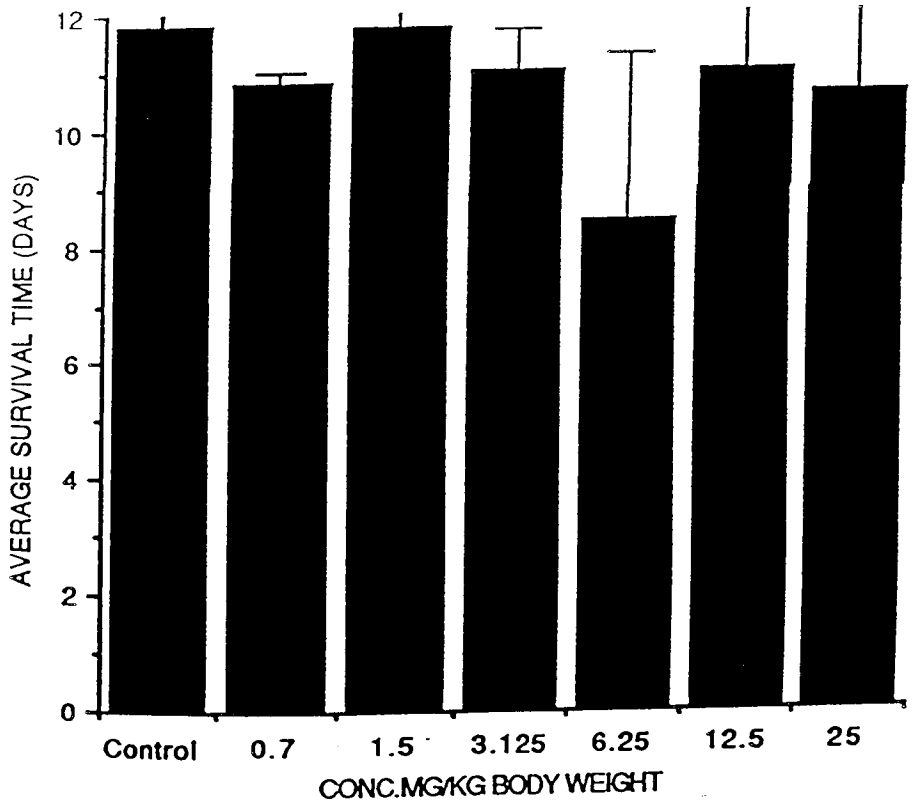


Fig. 4.10 e. Average survival time of male NMRI mice transplanted with the MAC16 tumour after treatment with high dose of DDSH administered by a single subcutaneous injection. The results are expressed as the mean ± s.e.m. 5 animals per group.



The effects of chemotherapy on the MAC 16 tumour.

Fig. 4.11 a. A control NMRI mouse with the MAC 16 tumour on the right flank.

Fig. 4.11 b. NMRI mouse 24 hr after a complete tumour rupture.

Fig. 4.12 b. The MAC 16 tumour section from NMRI mouse that was treated with 2.5 x 10^{-5} mol/kg body weight of DDSH (x 100 magnification, Haematoxylin and Eosin).

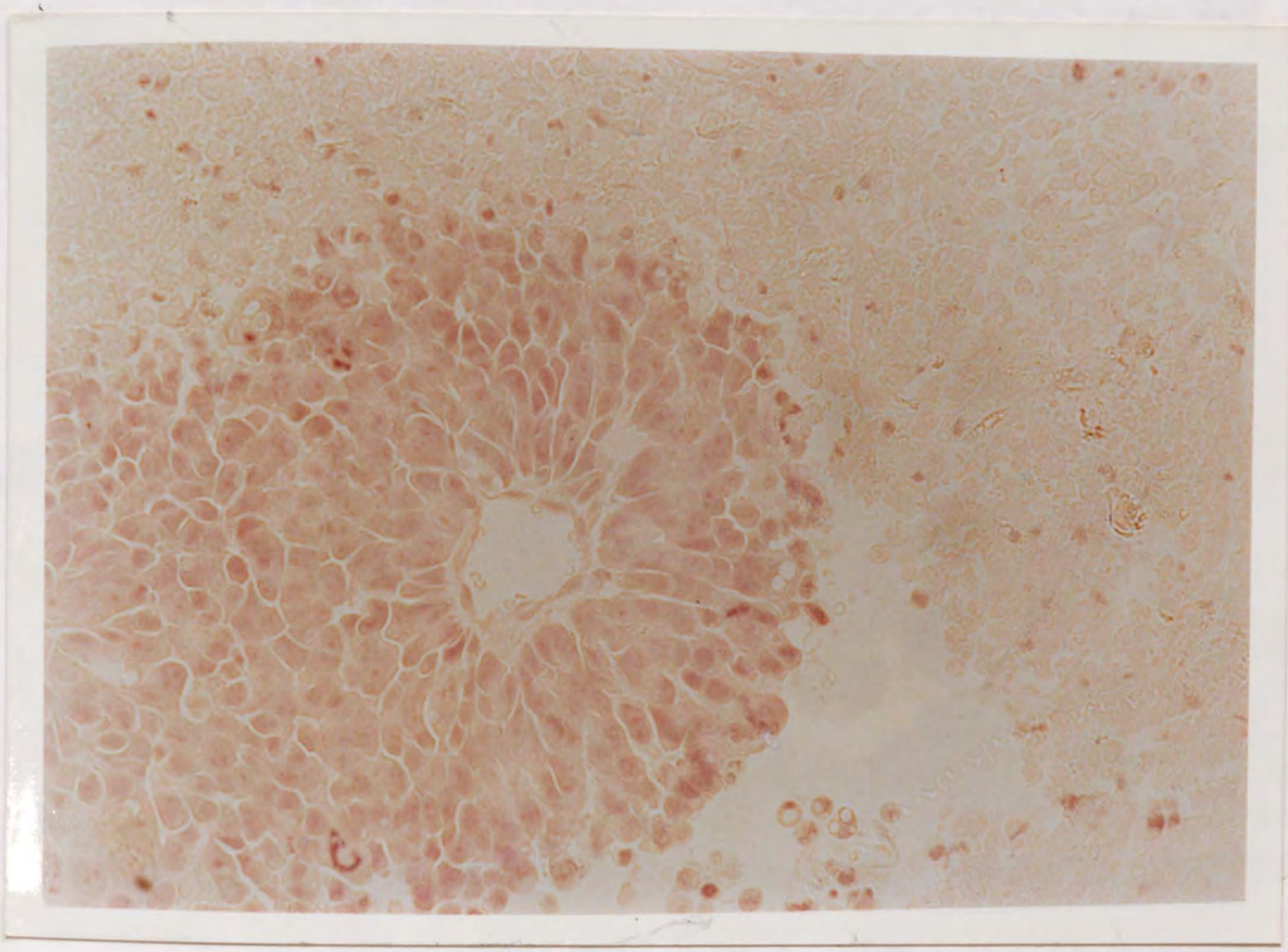


Fig. 4.12 c. The MAC 16 tumour section from NMRI mouse that was treated with 2.5 x 10^{-10} mol/kg body weight of DDSH (x 25 magnification, Haematoxylin and Eosin)..

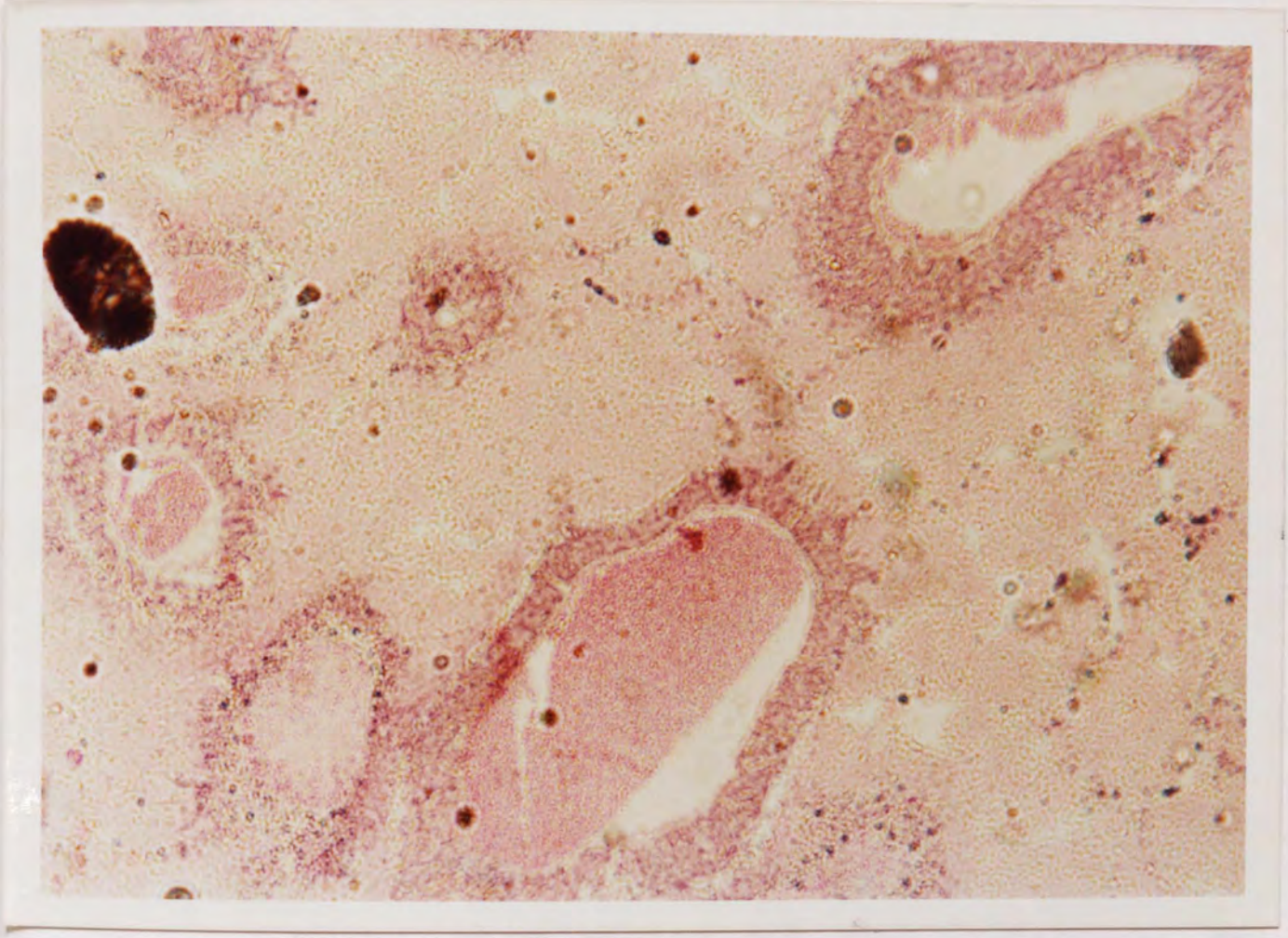


Fig. 4.12 d. The MAC 16 tumour section from NMRI mouse that was treated with 2.5 x 10^{-10} mol/kg body weight of DDSH (x 100 magnification, Haematoxylin and Eosin).

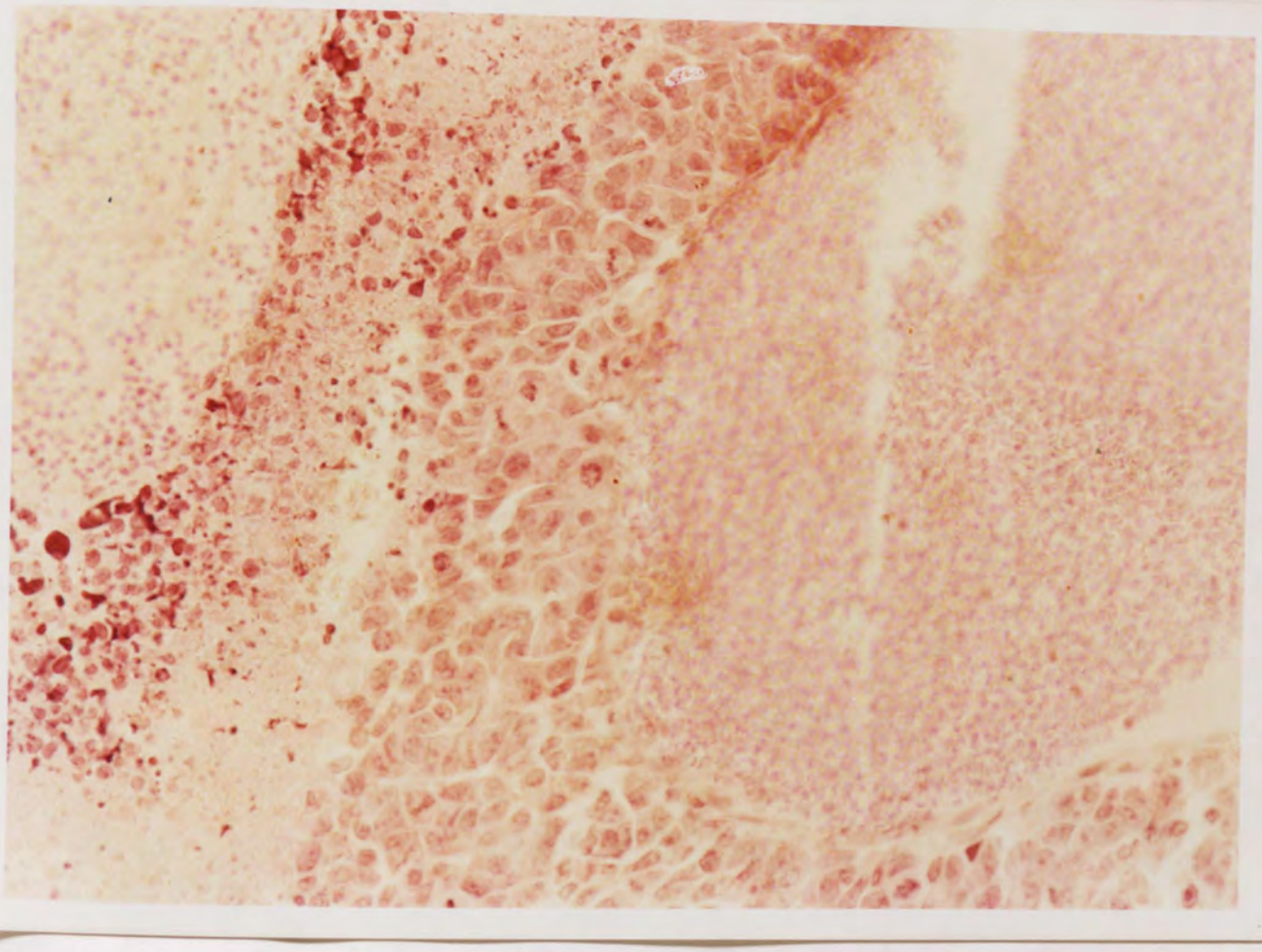
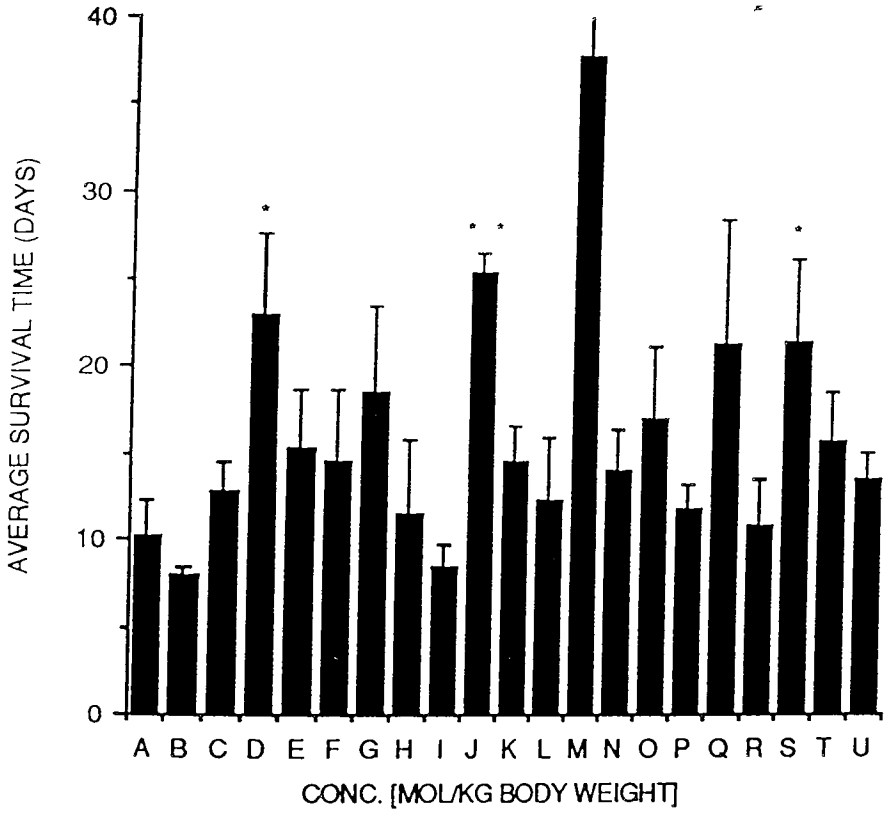
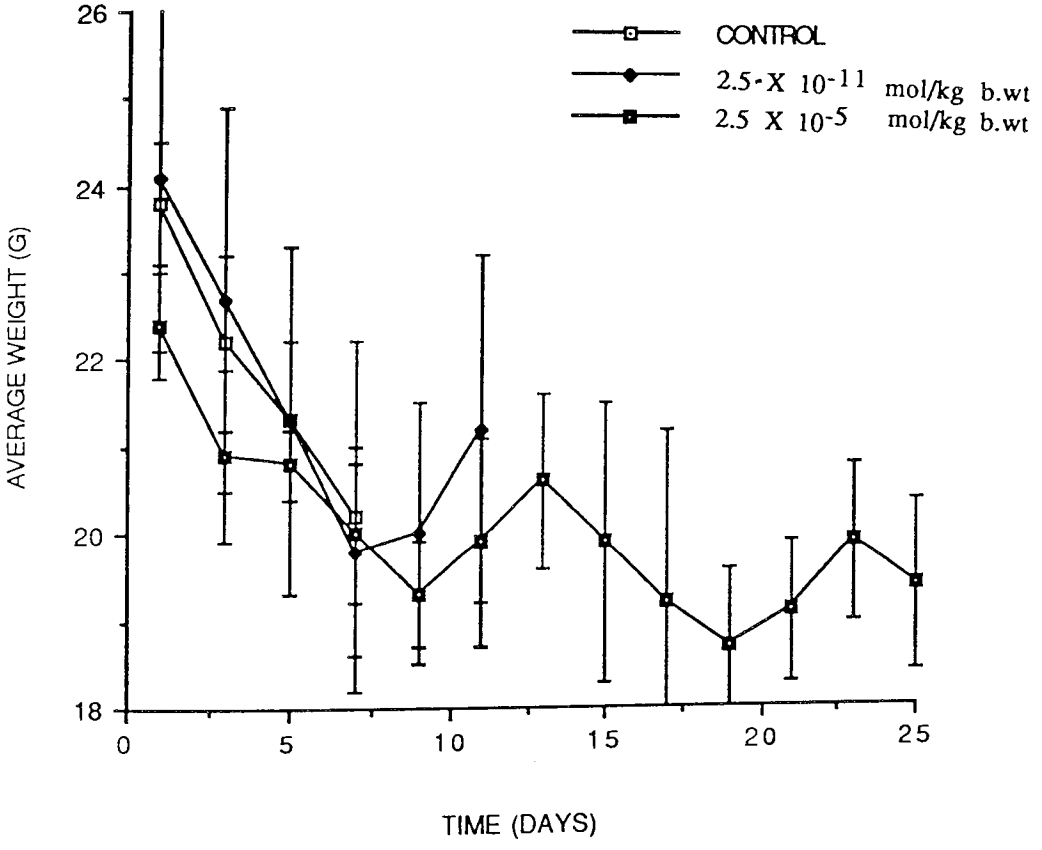


*Fig 4.13 a. Average survival time of male NMRI mice transplanted with the MAC16 tumour after treatment with various agents. The results are expressed as the mean ± s.e.m. 5 animals per group. Significance versus solvent treated controls : * p <0.05, ** p < 0.001.*



A=	CONTROL
B=POA	2.5 X 10^{-5}
C=POA	2.5 X 10^{-11}
D=LA	2.5 X 10^{-5}
E=LA	2.5 X 10^{-11}
F=AA	2.5 X 10^{-5}
G=AA	2.5 X 10^{-11}
H=ALA	2.5 X 10^{-5}
I=ALA	2.5 X 10^{-11}
J=EPA	2.5 X 10^{-5}
K=EPA	2.5 X 10^{-11}
L=GLA	2.5 X 10^{-5}
M=GLA	2.5 X 10^{-11}
N=MEKP	2.5 X 10^{-5}
O=MEKP	2.5 X 10^{-11}
P=PBQ	2.5 X 10^{-5}
Q=PBQ	2.5 X 10^{-11}
R=t-BOOH	2.5 X 10^{-5}
S=t-BOOH	2.5 X 10^{-11}
T=CHLORAMB	2.5 X 10^{-5}
U=CHLORAMB	2.5 X 10^{-11}

Fig 4.13 b. Representative curve of the average weight of male NMRI mice carrying the MAC16 tumour after treatment with a single dose of EPA. The results are expressed as the mean ± s.e m. 5 animals per group.



Figs 4.14 a b,and c. Survival time of male BDF1 mice inoculated intraperitoneally with P388 leukaemia and treated with DDSH administered as a single injection by a subcutaneous injection. a, controls, b, treated with 2.5 x 10^{-5} mol/kg body weight of DDSH, c, treated with 2.5 x 10^{-11} mol/kg body weight of DDSH. The results are of individual animals.

Fig. 4.14a. Control

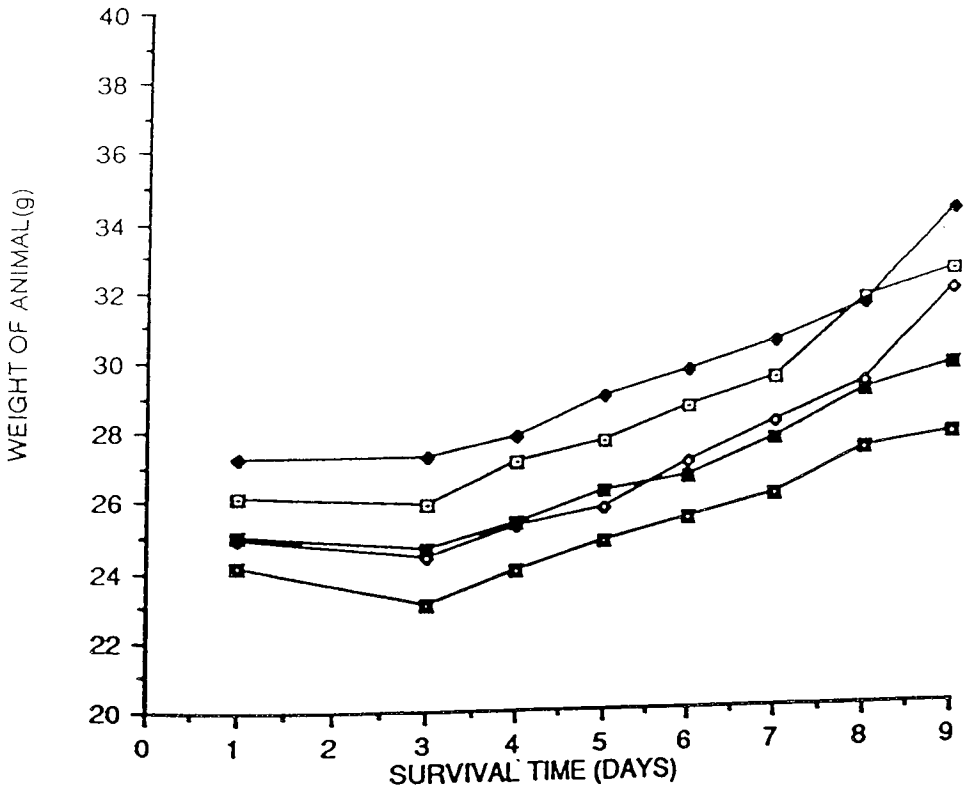
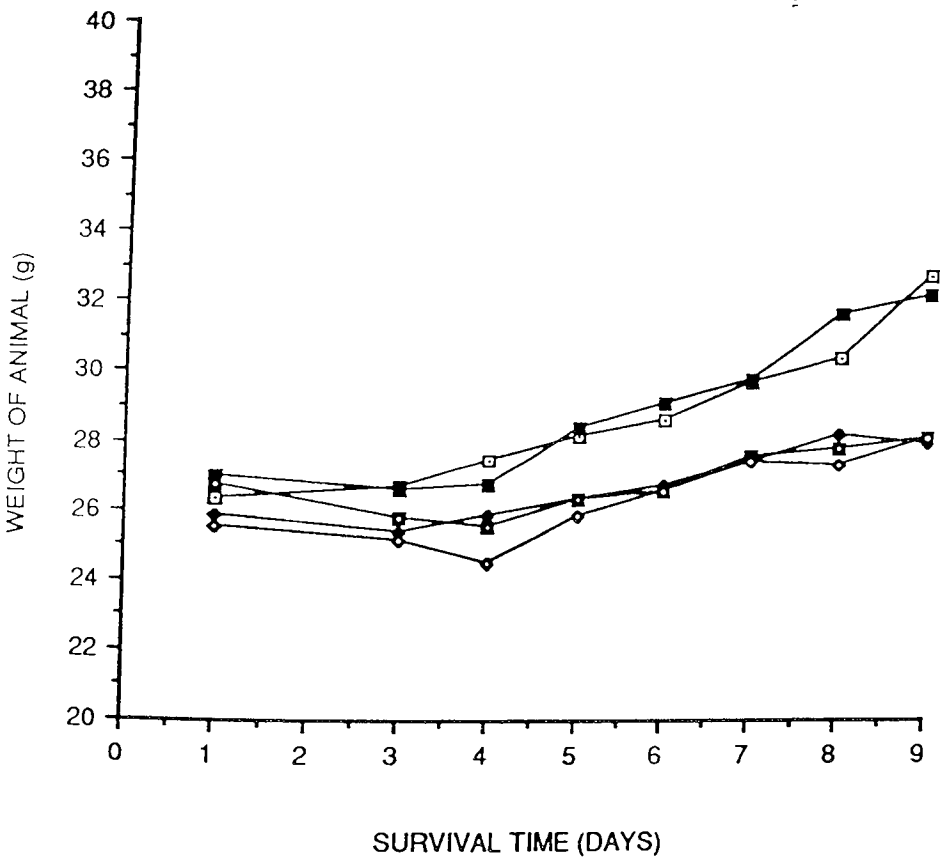


Fig. 4.14b.


40
38
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34
32
30
28
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24
22
20
0
1
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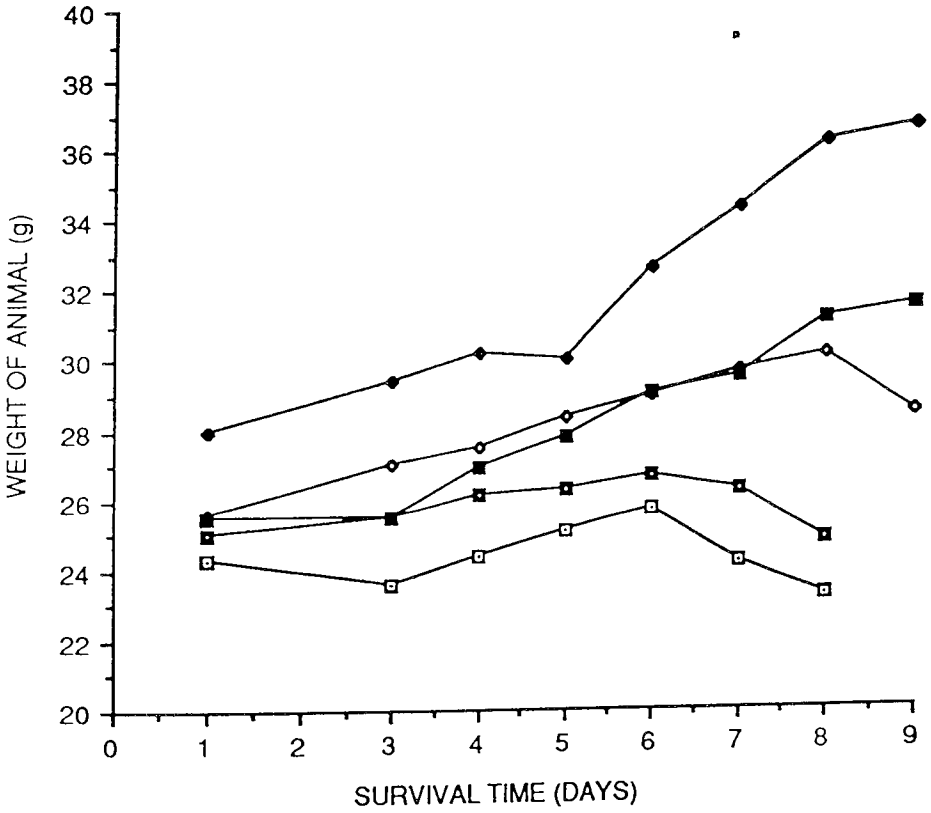
4
5
6
7
8
9
WEIGHT OF ANIMAL (g)
SURVIVAL TIME (DAYS)

Fig.4.14c.


WEIGHT OF ANIMAL (g)
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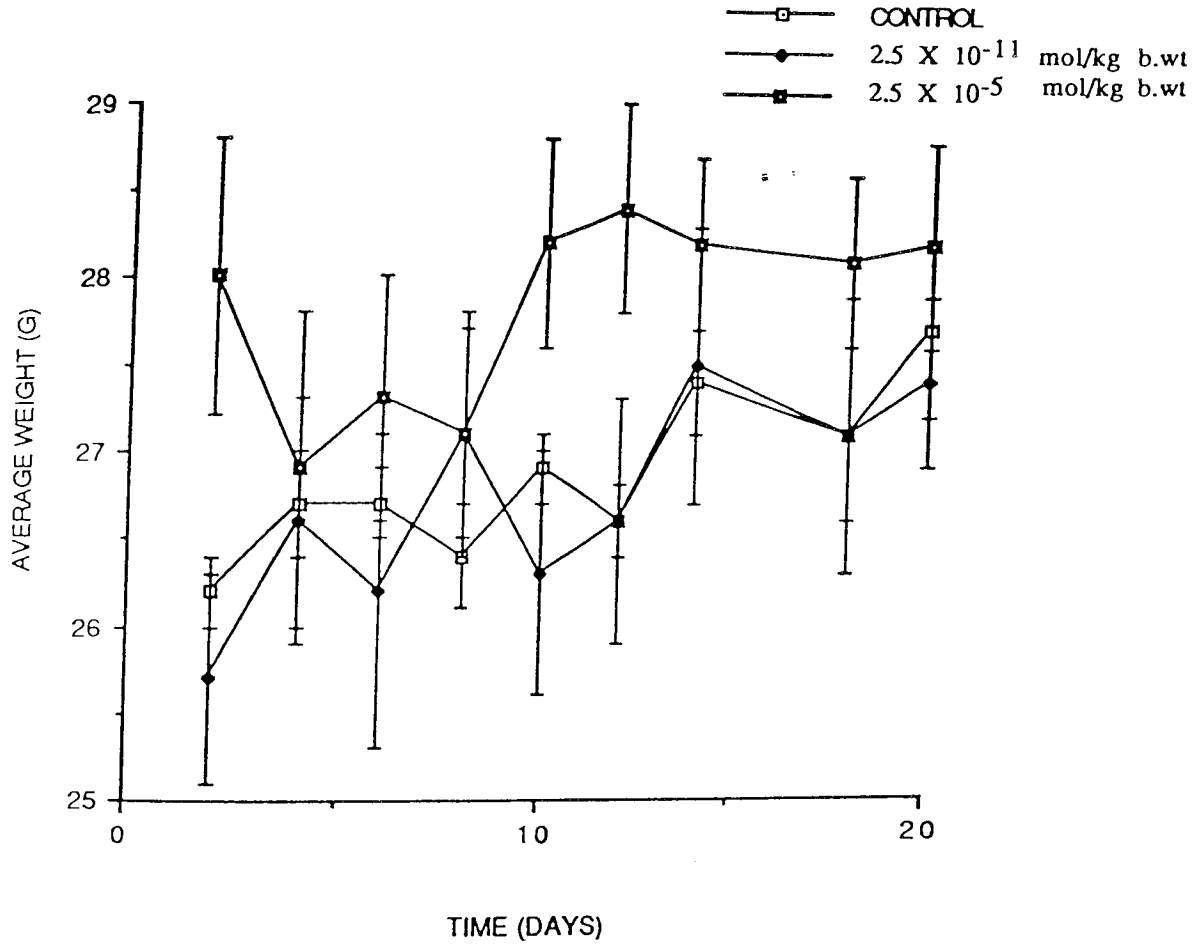
9
SURVIVAL TIME (DAYS)

Fig. 4.14 e. Average weight of non-tumour bearing male BDF1 mice treated with DDSH administered as a single subcutaneous injection. The results are expressed as the ± s.em. 5 animals per group.



Figs. 4.15 a, b and c. Survival time of male CBA/CA mice inoculated intraperitoneally with TLX5 lymphoma and treated with DDSH administered by a single subcutaneous injection: a, controls, b, treated with 2.5 x 10^{-5} mol/kg body weight of DDSH, c, treated with 2.5 x 10^{-11} mol/kg body weight of DDSH. The results are of individual animals.

Fig. 4.15a. Control.

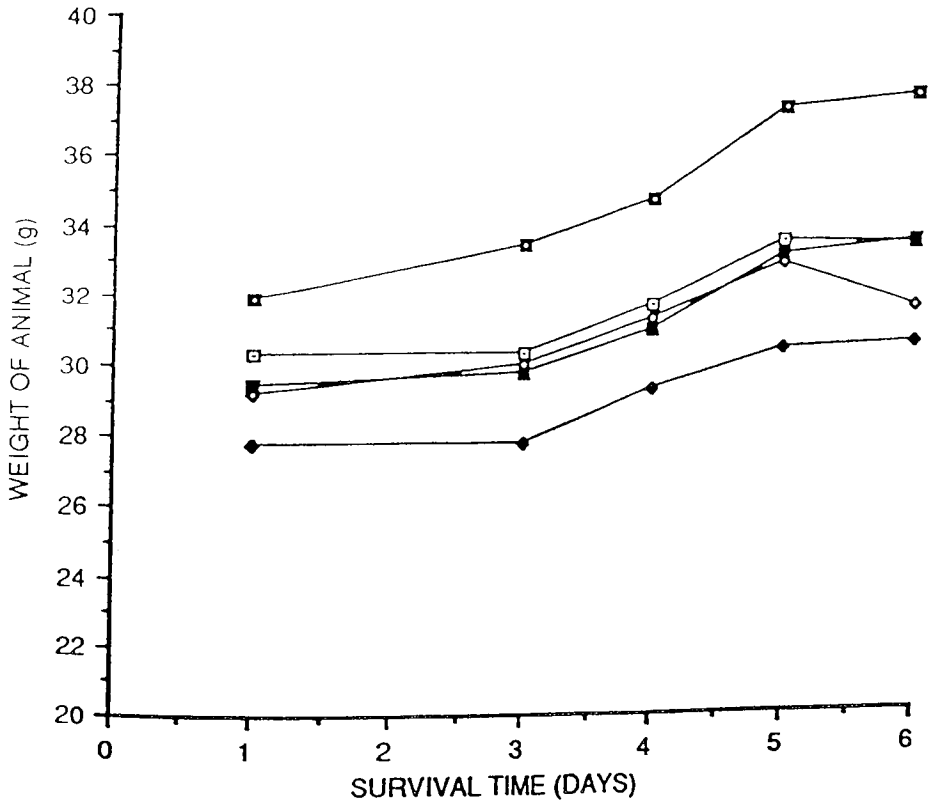
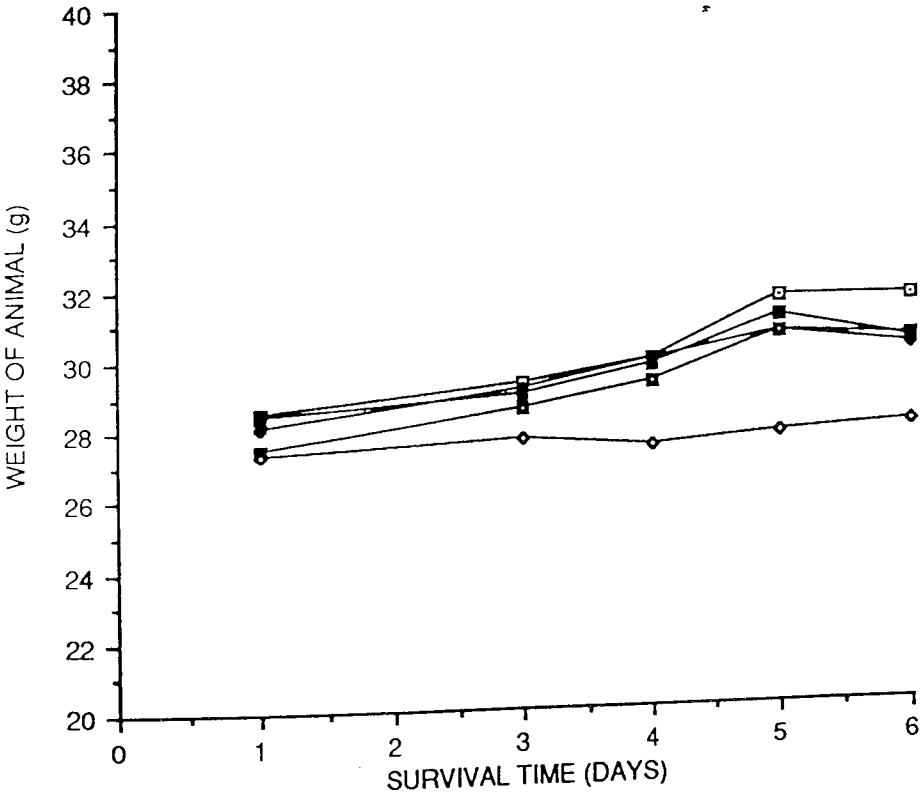


Fig. 4.15b.


WEIGHT OF ANIMAL (g)
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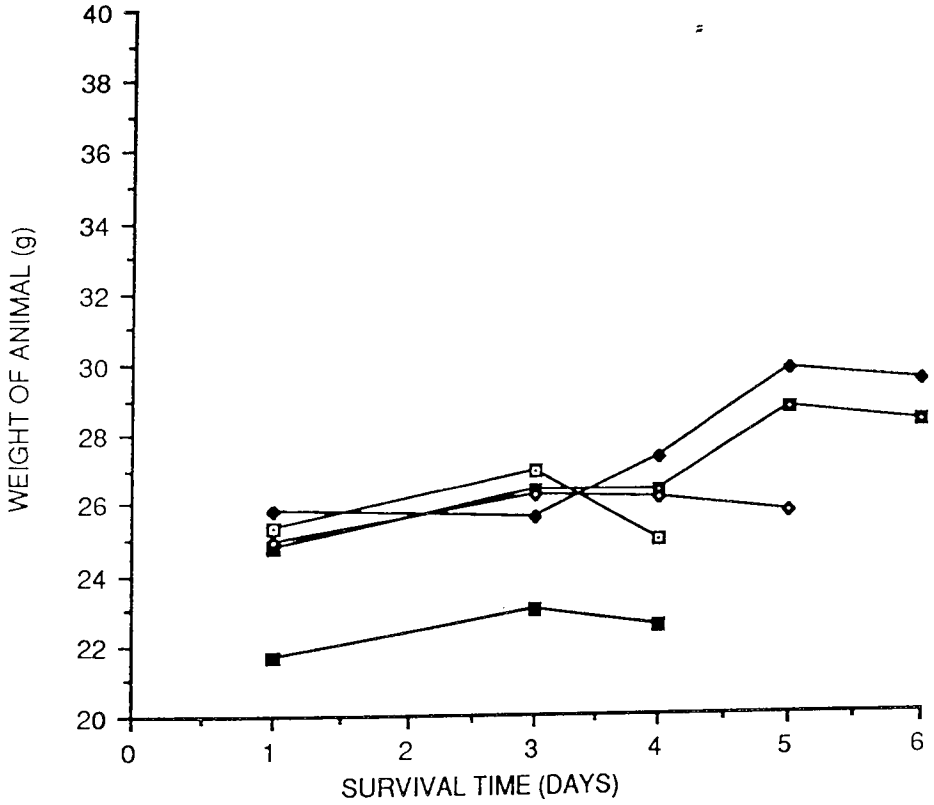
6
SURVIVAL TIME (DAYS)

Fig. 4.15c.


WEIGHT OF ANIMAL (g)
40
38
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4
5
6
SURVIVAL TIME (DAYS)

RESULTS

4.10 Serum TNF assay.

The antitumour activities of some cytokines such as TNF and interferons are believed to be mediated via free radical mechanisms(273,274,275). It was considered that DDSH may mediate its antitumour effect through a number of mechanisms, of which TNF might be involved in veiw of the extensive tumour necrosis that has been observed following the administration of DDSH. This necrosis was akin to that caused by the administration of FAA. In addition, BDF1 mice became inactive and were shivering following treatment with 2.5×10^{-11} mol/kg body weight of DDSH before their death. These animals did not have evidence of disease at post mortem. Patients that have been treated with TNF usually develop fever and rigors similar to the situations observed in these mice. We studied the effects of serum from these mice on TNF sensitive and resistant L929 cell line in vitro. The serum was collected 6 days after treatment with 2.5×10^{-11} mol/kg body weight of DDSH. There was a statistically insignificant cytotoxicity caused in various wells in both cell lines and since it occurred in both cell lines, it probably did not arise from TNF (figs. 4.16 a and b).

Fig. 4.16 a. Effect of serum from male BDF1 mice inoculated intraperitoneally with P388 leukaemia cell line on TNF sensitve L929 cell line in-vitro. Animals had been previously treated with DDSH and serum collected 6 days after treatment. 1 unit TNF was used as internal standard. 5 animals per group. The results are expressed as the mean ± s.e.m.

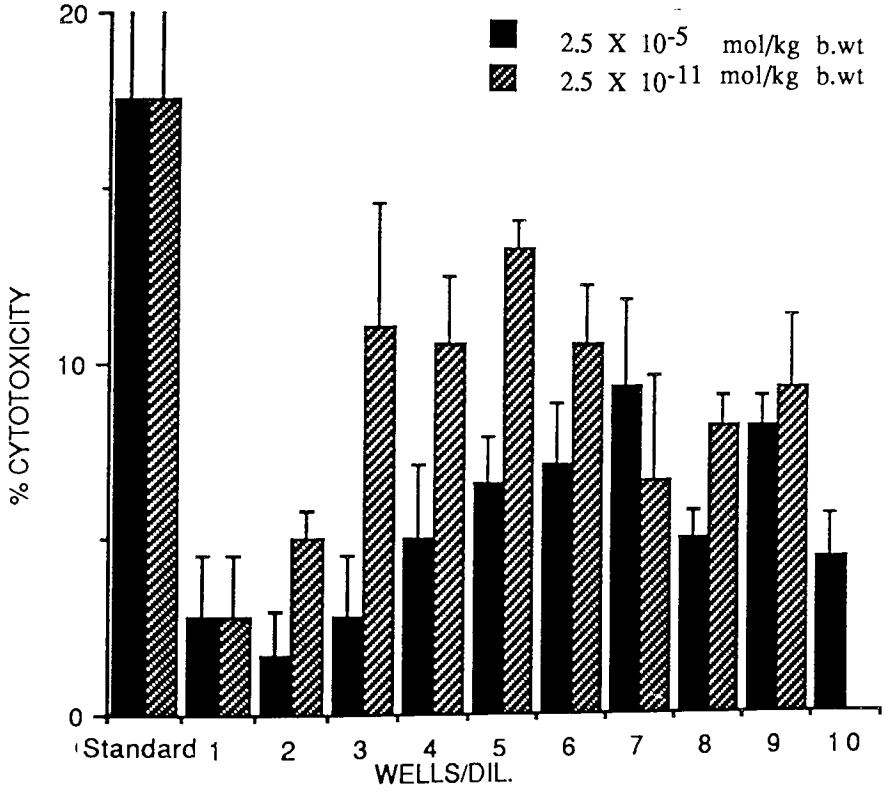
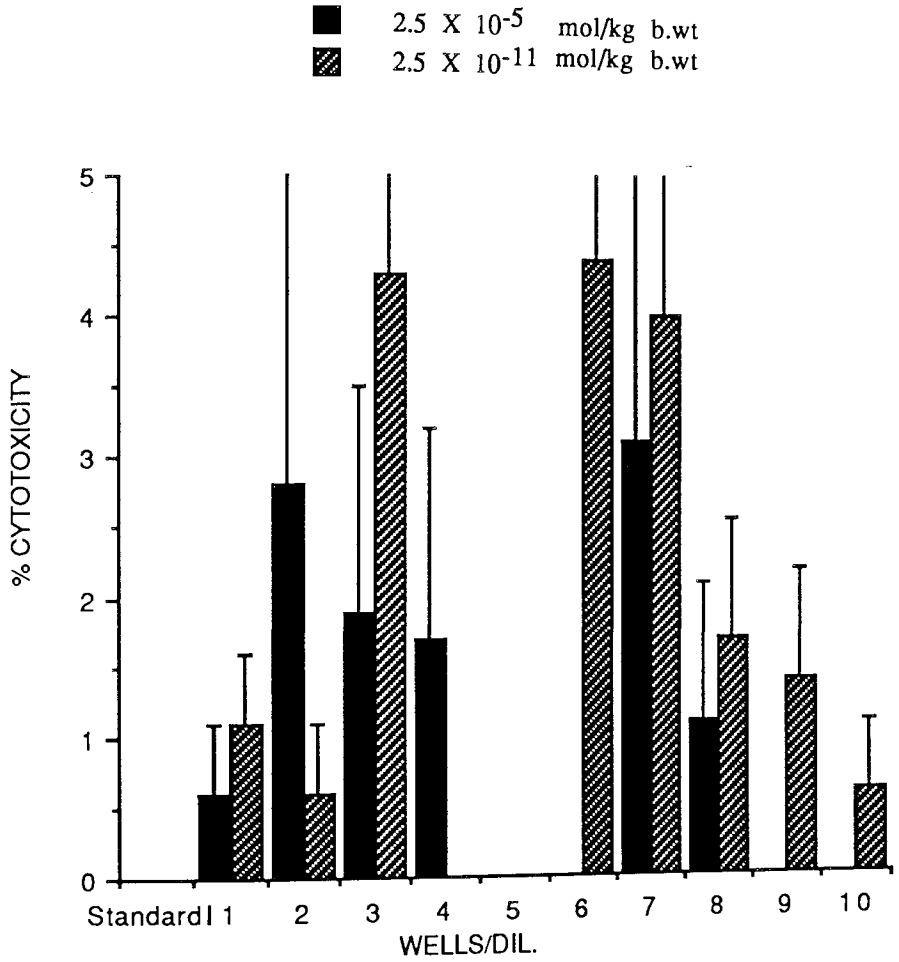


Fig. 4.16 b. Effect of serum from male BDF1 mice inoculated intraperitoneally with P388 leukaemia cell line on TNF resistant L929 cell line in-vitro. Animals had been previously treated with DDSH and serum collected 6 days after treatment. 1 unit of TNF was used as internal standard. 5 animals per group. The results are expressed as the mean ± s.e.m.



DISCUSSION

4.11 The effects of chemotherapy on the survival of tumour bearing mice

The MAC 16 tumour is resistant to most of the standard chemotherapeutic drugs except thioTEPA. 5FU, and cyclophosphamide exhibited a small antitumour effect and no response to methyl-CCNU (MeCCNU) or mitozolomide (266). Previous experimental chemotherapy studies have shown that flavone acetic acid (FAA) is the most active compound against the subcutaneous MAC tumours when administered by a two dose schedule split by seven days and is highly active against the MAC16 tumours by this treatment schedule (267). Control of tumour growth is accompanied by control of tumour associated cachexia.

DPPH and its hydrazine homologue did not have any antitumour effect against the MAC16 tumour *in vivo* probably due to their water insolubility. However, the more hydrophilic DDSH, which was far less active *in vitro*, had a significant effect in improving the survival of tumour bearing mice given as a single or 3 dose schedule by subcutaneous injections (figs. 4.9 a,b and 4.10 a,b). The optimal concentration was 2.5 x 10^{-10} mol/kg body weight of DDSH in both dose schedules. The sequence of events that followed such an injection was relative stabilisation of tumour volume (figs. 4.9 c and 4.10 c). Thereafter, some tumours softened and darkened followed by sudden tumour rupture. The tumour rupture was either partial or complete. Complete tumour rupture was when no residual macroscopic tumour was left at the tumour site, while in a partial tumour rupture there was evidence of residual disease. The tumour rupture occurred independent of the tumour volume. A dry scab formed within 24 hr of the tumour rupture and a complete healing within 72 hr of a complete tumour rupture (figs. 4.11 a,b,c). In a partial tumour rupture the disease would always recur. The animal remained comfortable with no evidence of bleeding from the tumour site in contrast to what happens in tumour ulceration. The animal gained weight following a tumour rupture (figs. 4.9 d and 4.10 d). In some cases a second rupture followed a first partial tumour rupture. Nearly all tumours that partially ruptured regrew and were resistant to repeat course of treatment.

It has been suggested that tumour vasculture may be important in the action of FAA. The haemorrhagic necrosis produced in mouse tumours by FAA has been likened to the effects seen with tumour necrosis factor (TNF) where massive haemorrhagic

necrosis occurs starting as early as 2-4 hr after administration (267,268). In FAA treatment significant haemorrhagic necrosis seems to be restricted to tumours growing at a subcutaneous site. Further studies with subcutaneous tumours have shown that these responses are associated with a reduction in tumour blood flow (270,271) and the resultant necrosis is likely to be at least in part due to ischaemic injury. The underlying mechanisms of blood flow reduction is a matter of speculation but a recent study by Mahadevan et al. has confirmed the involvement of TNF (272). It was suggested that the cytotoxicity of TNF was mediated through free radical generation (273) and therefore the effect of free radical generation on tumour vasculture may be similar to that of TNF independently of the elevation of endogenous TNF production. In the MAC16 tumours treated with DDSH, similar changes akin to the effect of FAA were observed (figs. 4.12 a,b,c,d). The tumours usually darkened and softened before rupturing suggesting the occurence of haemorrhagic necrosis within the tumour. It is proposed that this necrosis is associated with the production of free radicals. Free radical production would have multiple effects. There would be direct damage to the vascular endothelia of blood vessels supplying the tumour resulting in bleeding and tissue ischaemia. Free radicals and their products would also cause direct damage to the tumour cells. Finally, products of free radical reactions are known to modulate the immune system (273,274,275). This may involve the activation of natural killer (NK) cells and the production of cytokines such as interferon and TNF (273,274,275). These would all have deleterious effects on the survival of tumour cells. Although the TNF assay was unrevealing (figs. 4.16 a,b), the involvement of TNF in the antitumour activity of DDSH could not be excluded unless a more sensitive and specific method of assay was used to detect TNF. The use of monoclonal antibodies would provide such a tool.

In comparison to DDPH and its hydrazine homologue, DDSH was far less active against the MAC16 tumour *in vitro* but had a significant antitumour effect *in vivo*. It was proposed that the antitumour activity at low concentrations was mediated through a free radical mechanism. Most free radicals are extremely reactive; they exist at very low concentrations in the the order of 10^{-5} to 10^{-9} M or lower (74). At higher concentrations, the termination process of chain reactions would dominate because radical recombination requires nearly no energy. Therefore, only radical reactions in which the dilute chain-carrying radicals are recycled would have useful rates. In this chain process, the radicals were initially generated in a step or steps called 'initiation process' by the DDSH administered. A concentration of DDSH of 2.5×10^{-10}

mol/kg body weight probably provided the optimal concentration for the initiation process. Subcutaneous sites provided an ample 'depot' of fatty acids which were substrates for lipid peroxidation and they probably played an important role in the propagation of free radical chain reactions and cytotoxicity. From this subcutaneous 'depot' many other radicals and hydroperoxides were probably generated and travelled to remote sites to reinitiate and propagate further free radical reactions. This included sites in the tumours resulting in their destruction. It is evident from the histology that in addition to the haemorrhagic necrosis probably resulting from vascular damage, there was evidence that some tumour cells surrounding the blood vessels were dead (figs. 4.12 c,d). This cell death was probably caused by cytotoxic compounds generated from various sites by free radical reactions and passed to the blood stream which caused the direct destruction of tumour cells surrounding the blood vessels. Perivascular cell death usually occurs following the adminstration of compounds that exert their effects directly through the blood stream such as cyclophosphamide. The cytotoxic agents generated might be either free radicals or the products of free radical reactions which include carbonyl compounds such as aldehydes. In this case, DDSH would just be acting like a 'catalyst' to initiate the processes that would allow the host to produce endogenous compounds that eliminate the tumour. The usefulness of low concentrations of DDSH as an antitumour agent was further reinforced when high concentrations of DDSH was used, there was no improvement in the survival of tumour bearing animals (fig.4.10 e).

The underlying cause of the sudden death of some of the tumour-bearing male BDF1 and male CBA/CA mice following the administration of 2.5×10^{-11} mol/kg body weight of DDSH remained speculative (figs. 4.14 a.b,c and 4.15 a,b,c). We suggested that the cause of death in some of these animals was probably due to 'tumour lysis syndrome' (276). This syndrome occurs most commonly in diseases with large tumour burdens and high proliferative fractions that are exquisitely sensitive to cytotoxic treatment. These disorders include high-grade lymphomas, leukaemias with high leukocyte counts, and less commonly solid tumours. The tumour lysis syndrome occurs as a result of the rapid release of intracellular contents into the blood stream which then increase to life-threatening concentrations. The syndrome is characterised by hyperuricaemia, hyperkalaemia, hyperphosphataemia, and hypocalcaemia. The consequences of hyperuricaemia and hyperphosphataemia is acute renal failure. Lethal cardiac arrhythmias are the most serious consequences of

hyperkalaemia. Elevated serum phosphates may decrease renal function which leads to further reductions in urinary excretion of potassium and phosphate. Hypocalcaemia as a result hyperphosphataemia may cause muscle cramps, cardiac arrhythmias and tetany. This syndrome has been described in human beings but it could equally occur in animals with such tumours undergoing chemotherapy. There was no strong evidence to support the involvement of this syndrome in the death of some of these animals except to measure the serum electrolytes in similar future experiments. Non-tumour bearing BDF1 mice which received a similar therapy did not die implying that this death was related to both the treatment and tumour (figs. 4.14 e). This observation gave further evidence of the indirect actions of DDSH. Although the tumours were inoculated intraperitoneally and the drug was administered at extremely low concentrations subcutaneously, it had an effect in eliminating the intraperitoneal disease in the animals that died suddenly.

When other free radical generating compounds were administered subcutaneously as single injections to NMRI mice bearing the MAC16 tumour statistically significant improvements in survival were achieved in mice that recieved 2.5×10^{-5} mol/kg body weight of EPA, and 2.5×10^{-11} mol/kg body weight of t-BOOH. There was long term survival over 100 days in one animal that had recieved 2.5×10^{-11} mol/kg body weight of GLA, although there was no statistically significant improvement in survival of the group (fig.4.13 a). Although most of these compounds are known to generate free radicals in biological systems, their weak antitumour effect may suggest that the antitumour effect of DDSH might not solely be related to the generation of free radicals but may involve some other mechanisms such as the uncoupling of oxidative phosphorylation (see section 4.4). However, their weak antitumour effect could be related to other factors such as the transportation and the metabolism of these compounds within the tumour cells.

Presently, the major side effects of this therapy are the severe loss of weight observed in the tumour bearing NMRI mice (figs. 4.9 d and 4.10 d) and the unexplained sudden death that occurred following the treatment of the tumour bearing BDF1 and CBA/CA mice (figs. 4.14 a,b,c and 4.15 a,b,c). Other organ toxicity such as cardiotoxicity (277), pulmonary damage (287), hepatotoxicity (288) and oxygen toxicity (253) usually associated with the administration of free radical generating compounds can not be ruled out and these possibilities will be investigated in future.

These results will provide a foundation on which to base our future work to explore and improve this novel method of treating malignant diseases. We will also concentrate in defining more precisely the mechanisms of action, toxicities, and improving the efficacy of this group of compounds that has shown promising results.

RESULTS

4.12 Serum MDA production in mice after treatment with DDSH.

Free radicals and the products of free radical reactions are thought to play an important role in mediating the antitumour actions of free radical generating drugs (175,178). Malondialdehyde (MDA) is one of the products of free radical reactions in biological systems and its assay provides one of the methods used to quantitate free radical reactions in biological systems. We measured the serum MDA levels in mice that had been treated with either DDSH or other free radical generating compounds using the microassay method of Yagi (235), namely fluorometry using thiobarbituric acid (TBA). For this purpose, 0.05 ml of the serum specimen was usually used.

Following the administration of DDSH to male NMRI mice transplanted with the MAC16 tumour, there was a significant increase in the serum levels of MDA after 3 hr in both groups of animals that were treated (fig. 4.17 a). However, in the group that received 2.5 X 10^{-10} mol/kg body weight of DDSH, the serum MDA concentration continued to rise and it peaked 8 days after treatment, while in the group that received 2.5 x 10^{-5} mol/kg body weight of DDSH their serum MDA levels dropped from day 5 onwards. The solvent treated controls also showed a peak level of MDA 4 days after treatment, which dropped rapidly from day 5 onwards. A similar variation of serum MDA levels was observed when non-tumour bearing male NMRI mice were treated with DDSH (fig. 4.17 b). The adminstration of vitamin E with DDSH reduced the overall serum MDA levels in tumour bearing animals, although, there was a significantly increased production of serum MDA on day 3 and 5 when compared with vitamin E treated controls (fig 4.17 c).

The cause of sudden death in tumour bearing male BDF1 and male CBA/CA mice following treatment with DDSH was unknown. Experiments were designed to find any correlation between the serum MDA levels and the sudden death. Serum MDA levels were measured in both tumour and non-tumour bearing male BDF1 mice following the administration of DDSH. There was significant increase in serum MDA levels 6 days after treatment in tumour bearing animals only (fig. 4.18). These tumour bearing animals were sacrificed 7 days after treatment because they became very ill and the rest were sacrificed on day 9. At post mortem, 3 out of the 5 tumour bearing animals that were treated with 2.5 x 10^{-11} mol/kg body weight DDSH had no ascites and the remaining two

had ascites. All the controls and those that received 2.5 x 10^{-5} mol/kg body weight of DDSH had ascites. Therefore, the increased serum MDA levels did not correlate with the antitumour activity of DDSH.

The antitumour activities of other free radical generating compounds were studied (fig. 4.13 a) and attempts were made to find any correlation between the antitumour effects of these compounds in male NMRI mice transplanted with the MAC 16 tumour and the serum MDA levels after treatment with these agents. The serum MDA levels were measured 8 days after treatment with either 2.5 x 10^{-11} or 2.5 x 10^{-5} mol/kg body weight of each of these compounds administered by a single subcutaneous injection. The following compounds were found to cause significant increase in serum MDA levels on day 8: 2.5 x 10^{-11} and 2.5 x 10^{-5} mol/kg body weight of GLA, t-BOOH, 2.5 x 10^{-11} mol/kg body weight of MEKP and 2.5 x 10^{-5} mol/kg body weight of chlorambucil (used as a negative control) (fig.4.19). However, a statistically significant improvement in survival was achieved only in animals treated with 2.5 x 10^{-5} mol/kg body weight of LA, EPA and 2.5 x 10^{-11} mol/kg body weight of t-BOOH (fig. 4.13 a). The improved survival did not correlate with increased serum MDA levels except in the group that received 2.5 x 10^{-11} mol/kg body weight of t-BOOH. Therefore, serum MDA levels appear not to correlate with the antitumour activity of this group of compounds.

*Fig. 4.17 a. Serum MDA concentration in male NMRI mice transplanted with the MAC 16 tumour after treatment with DDSH administered by a single subcutaneous injection. Day 1 MDA values determined 3 hr after treatment. 5 animals per group.The results are expressed as the mean ± s.e.m. Significance versus solvent treated mice: * $p< 0.05$, ** $p< 0.01$.*

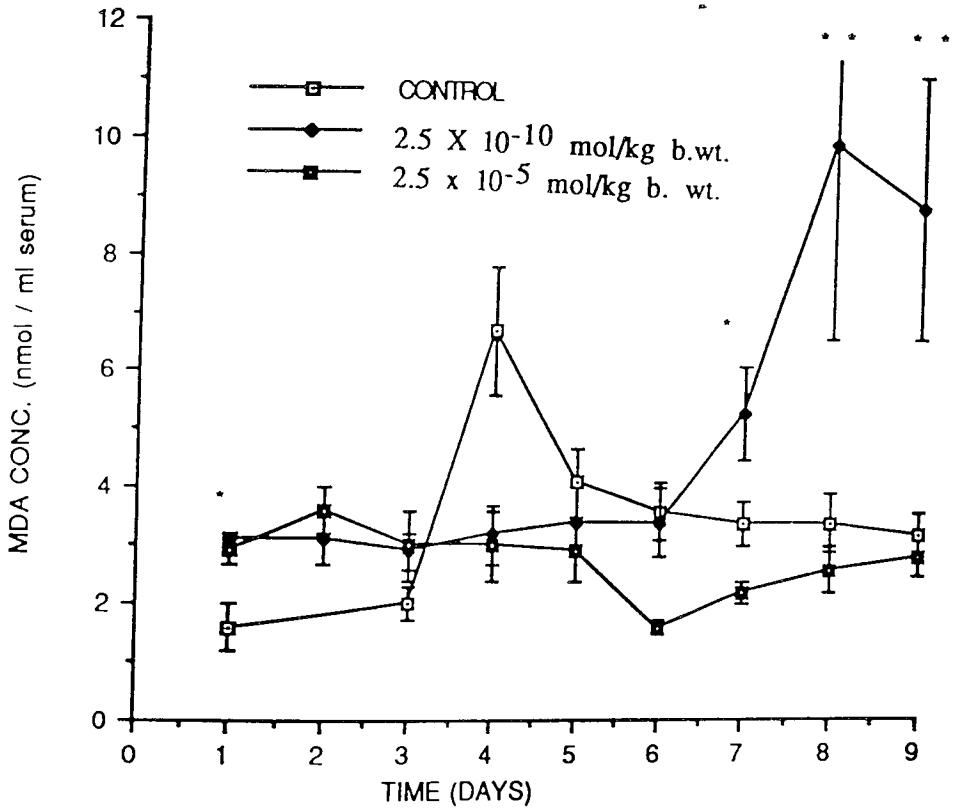


*Fig. 4.17 b. Serum MDA concentration in male NMRI mice without tumour after treatment with DDSH administered by a single subcutaneous injection. Day 1 MDA values determined 3 hr after treatment. 5 animals per group.The results are expressed as the mean ± s.e.m. Significance versus solvent treated control mice: * p< 0.05, ** p< 0.005, *** p< 0.001.*

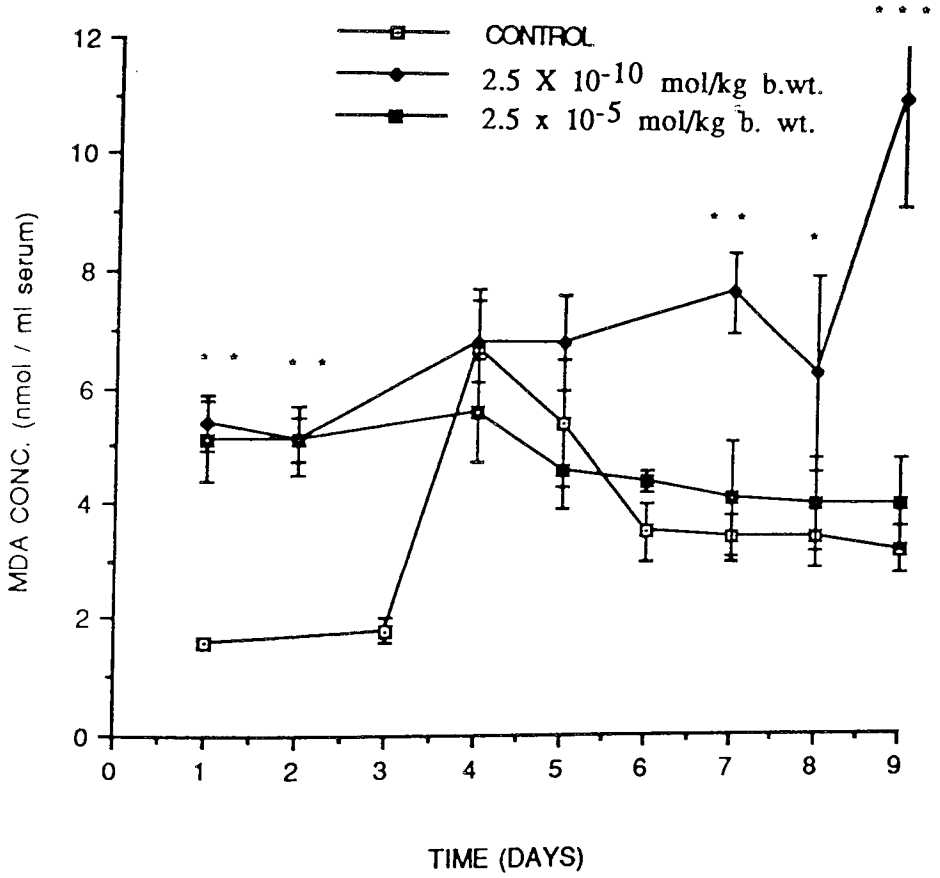


*Fig. 4.17 c. Serum MDA concentration in male NMRI mice transplanted with the MAC 16 tumour after treatment with DDSH and 1 mg /kg body weight of vitamin E administered by a single subcutaneous injection. The vitamin E was mixed with the DDSH before the administration. 5 animals per group. The results are expressed as the mean ± s.e.m. Significance versus viitamin E treated control mice: * $p < 0.05$.*

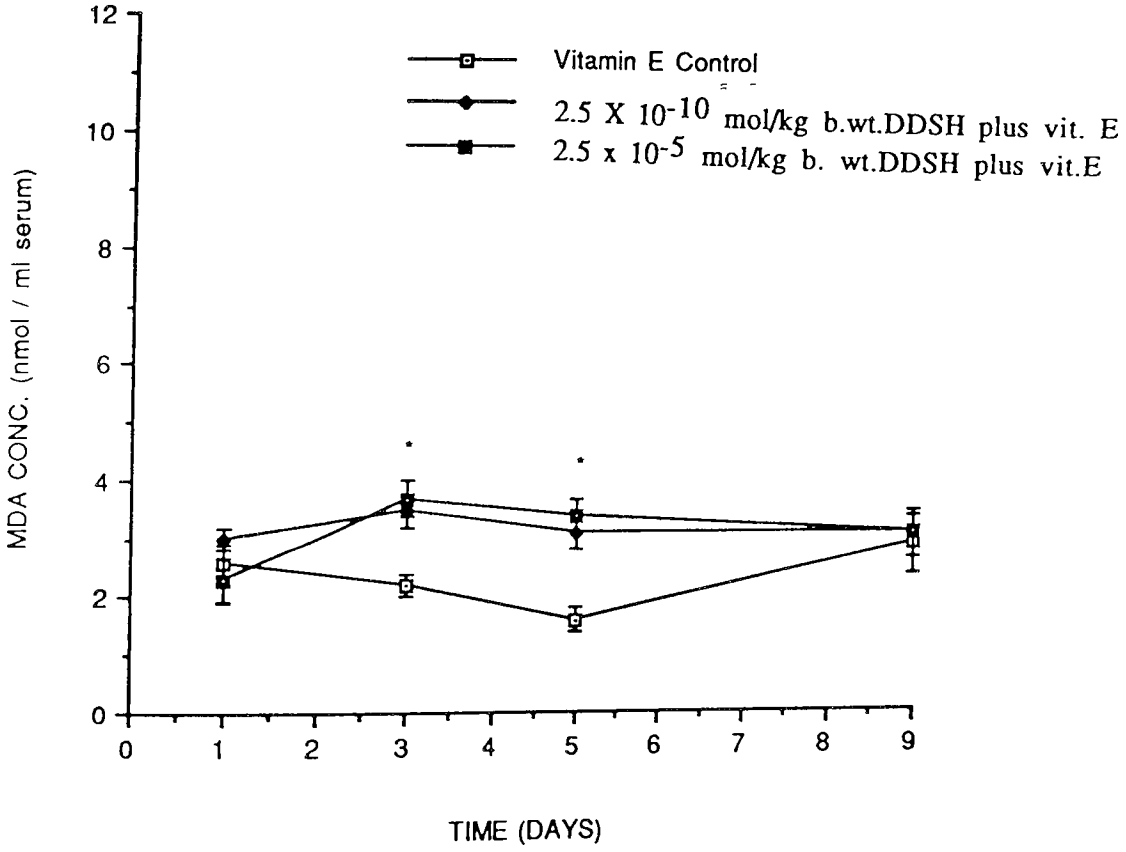


*Fig. 4.18. Serum MDA concentration in male BDF1 mice inoculated intraperitoneally with P388 leukaemia cell line and in those without tumour after treatment with DDSH administered by a single subcutaneous injection. 5 animals per group. The results are expressed as the mean ± s.e.m. Significance versus control male BDF1 mice with P388 leukaemia. * P < 0.001.*

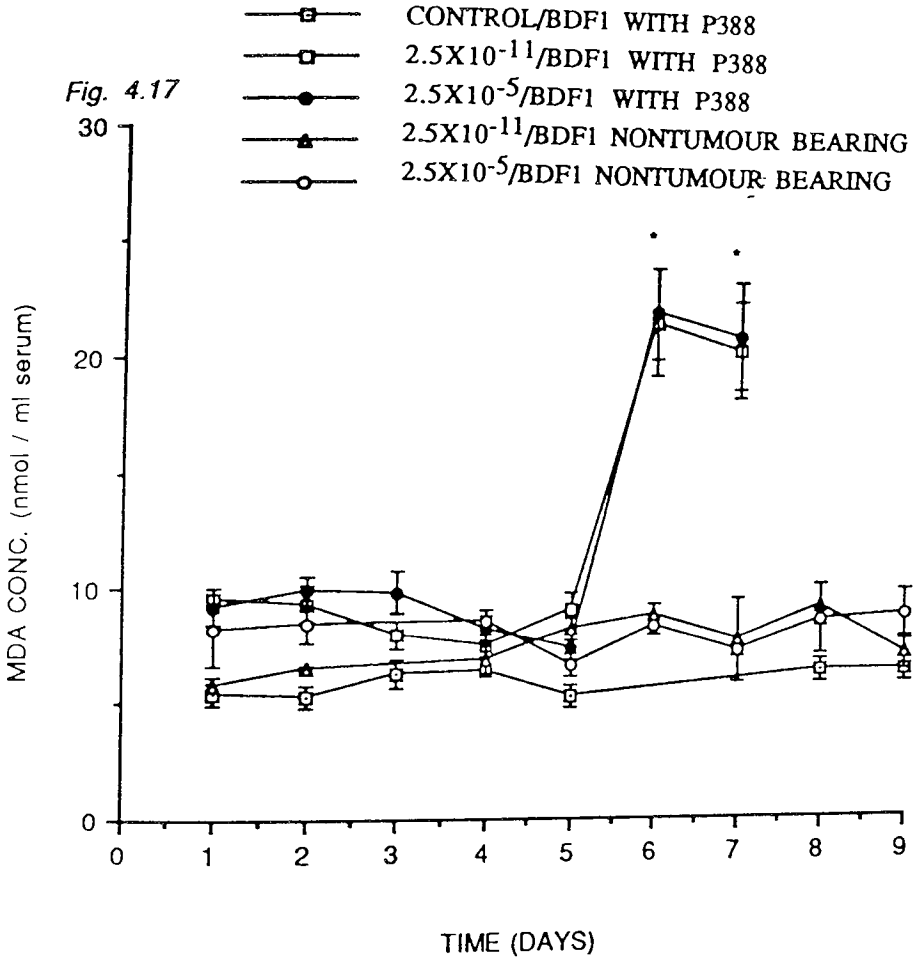
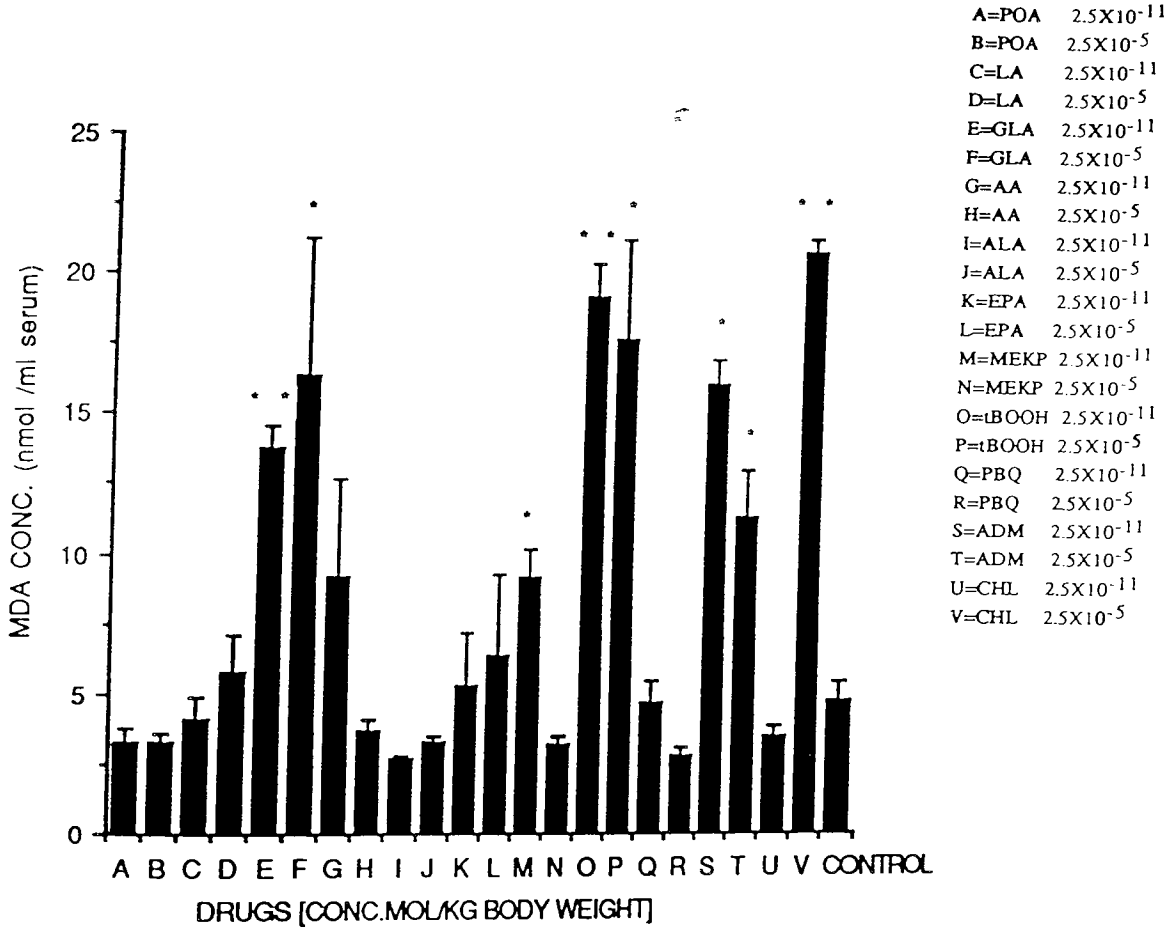


*Fig. 4.19. Serum MDA concentration on day 8 in male NMRI mice transplanted with the MAC 16 tumour after treatment with various agents administered by a single subcutaneous injection. 5 animals per group. The results are expressed as the mean ±s.e.m. Significance versus solvent treated control mice: * $p < 0.05$, ** $p < 0.005$.*



DISCUSSION

4.13 Malondialdehyde (MDA) assay

Several anticancer drugs are known to bring about their tumouricidal actions by a free radical dependent mechanisms (175,178) and there is an increasing appreciation to find reliable methods to quantify the process of lipid peroxidation in *vivo*. MDA determination has been used routinely and in particular, the "TBA test" to detect and quantify lipid peroxidation in a wide array of sample types (250). Our results reported here suggested that following chemotherapy with low concentration of DDSH and other free radical generating compounds, there was a significant increase in serum MDA levels from animals in certain experimental groups below:

(1) The tumour and non-tumour bearing male NMRI mice after 3 hr and peaking 8 days after treatment only in the groups that received 2.5×10^{-10} mol/kg body weight of DDSH (figs. 4.17 a and b).

(2) The tumour bearing male BDF1 mice peaking 6 days after treatment (fig. 4.18).

(3) The tumour bearing male NMRI mice treated with 2.5×10^{-11} and 2.5×10^{-5} mol/kg body weight of GLA, t-BOOH, 2.5×10^{-11} mol/kg body weight of MEKP and 2.5×10^{-5} mol/kg body weight of chlorambucil measured 8 days after treatment (fig. 4.19). Chlorambucil was used a negative control and therefore, the significant increase in the serum MDA level following its adminstration might have occurred as a result of direct tissue damage leading to lipid peroxidation. This may imply that any significant increase in serum MDA level following the adminstration of 2.5×10^{-5} mol/kg body weight of a cytotoxic drug in the NMRI mice may result in significant tissue damage that could cause a significant increase in the serum MDA levels. Therefore, such results must be interpreted with caution.

The best survival of 40-60%, 60 days after treatment was achieved after the adminstration of 2.5×10^{-10} mol/kg body weight of DDSH (figs. 4.9b and 4.10b) which correlated with the DDSH concentration that gave a significant increase in the serum MDA level in the male NMRI mice.

In contrast, a statistically significant increase in the serum MDA level occurred only in the tumour bearing male BDF1 mice that

received 2.5×10^{-11} and 2.5×10^{-5} mol/kg body weight of DDSH and not in the non-tumour bearing animals (fig. 4.18). Although these tumour bearing animals with the significant increase in the serum MDA levels had an identical MDA concentration curve, only 3 out of the 5 animals had no ascites and all the animals that received 2.5×10^{-5} mol/kg body weight of DDSH had ascites. In this case, there was no correlation between the antitumour response and the serum MDA levels.

Although the NMRI and the BDF1 mice were both treated with DDSH, only the tumour and the non-tumour bearing NMRI mice that received 2.5×10^{-10} mol/kg body weight of DDSH, and the tumour bearing BDF1 mice that received 2.5×10^{-11} and 2.5×10^{-5} mol/kg body weight of DDSH showed a significant increase in the serum MDA levels compared with solvent treated controls. There is no clear explanation presently, to account for this type of response, although with reference to the nature of free radicals reactions, the higher the dilution the longer the life span of these radicals in solutions. Since the recombination energy of a radical to radical reaction is nearly zero, the higher the dilution the greater the molecular dispersion and the less the chance of the recombination reactions and therefore, the greater the propagation chain reactions (74). Therefore, a concentration of 2.5×10^{-10} mol/kg body weight would be better than 2.5×10^{-5} mol/kg body weight of DDSH in sustaining a free radical chain reaction before the termination reactions. This was probably reflected in the higher serum MDA levels in both the tumour bearing and non-tumour bearing NMRI mice and the better survival in tumour bearing NMRI mice that received 2.5×10^{-10} than 2.5×10^{-5} mol/kg body weight of DDSH. In contrast, the significant increase in the serum levels of MDA occurred only in the tumour bearing BDF1 mice that received 2.5×10^{-11} and 2.5×10^{-5} mol/kg body weight of DDSH and not in the non-tumour bearing animals. The lysis of the P388 leukaemic cells as a result of the chemotherapy probably provided the major source of MDA production in the tumour bearing BDF1 mice. In addition, the breakdown products of these cells might have caused further damage to other vital organs including the kidneys causing a further rise in the serum MDA and probably the sudden death that occurred in some of these animals. The difference in the serum MDA levels of both the non-tumour bearing BDF1 and NMRI mice can not be explained at the moment, unless, either the metabolism of MDA or the sensitivity to free radicals in both strains of mice are different. However, presented below are some of the major problems faced in MDA determination and

interpretation which may account for some of the anomalies in our MDA results.

There is an extensive body of literature as to the ability of MDA and especially, TBA reactivity to serve as diagnostic indices of the occurrence and extent of lipid peroxidation (40,170,173,183,184). Various studies have demonstrated the variable nature of the correlation among MDA production, and other potential indices of lipid peroxidation. Some published results concluded that MDA determination and the TBA test are excellent means to evalute the extend of lipid peroxidation (263,268) while others maintain that MDA indices/or TBA reactivity cannot be used to quantify lipid peroxidation (278,281,282). The MDA analysis is limited in practice by several important considerations: (a) MDA yield as a result of lipid peroxidation varies with the nature of the PUFA peroxidised (especially its degree of saturation) and the peroxidation stimulus (281,283); (b) only certain lipid oxidation products decompose to yield MDA (170,284); (c) MDA is only one of several (aldehydic) end products of fatty peroxide formation and decomposition (285,291); (d) the peroxidation enviroment influences both the formation of lipid-derived MDA precursors and their decomposition to MDA (285,291); (e) MDA itself is a reactive substance which can be oxidatively and metabolically degraded (292-296); (f) oxidative injury to nonlipid biomolecules has the potential to generate MDA (293,294).

In addition, the yield of MDA from lipid oxidation products is invariably low, some 30% in the case of thromboxane synthase reaction (297,298) and even less (< 5%) from autoxidation products which are nonvolatile MDA precursors (170,278,281). It is particularly noteworthy in the light of frequent literature references to fatty hydroperoxide determination as MDA (299-302) that hydroperoxides per se decompose to MDA with particularly low efficiency under both physiological conditions and conditions which drive their breakdown (170,278,281,300).

Given these considerations, and even with the most favourable analytical techniques for MDA quantification at hand (table 3.2), MDA measurement can represent at best a narrow perspective on the complex process of fatty peroxide formation and decomposition. The perspective is also more than a bit selective in that MDA reflects only certain lipid peroxidation products (40,170,281) and is itself one of many (aldehydic) end products from a particularly late point in the degradative reactions of lipid peroxidation (291,303-305).

The many factors which influence TBA-test response to fatty peroxide-derived MDA, along with the nonspecificity of the TBA test toward MDA (291) and the wide variety of nonlipid-related, TBA-postive materials, prohibit general conclusion that the TBA test is a useful method for detecting and quantifying lipid peroxidation. In addition, no one version of TBA test is generally suitable for all applications. In some cases, TBA-test response may correlate well with the MDA content of a peroxidised lipid sample (282,306), but in other cases no quantitative or qualitative correlation exists (170,278,281,307). No clear explanation covering all such discrepant reports can be provided, largely due to the indirect relationship between lipid peroxidation and TBA-test signal (whether measured by UV or fluorescence spectrophotometry or as a specific TBA-derived pigment) (308). Neither the high sensitivity of the TBA test towards MDA nor the economy and experimental ease of the test procedure can justify regarding a TBA-test response as indicative of the presence or amount of lipid-derived MDA (let alone fatty hydroperoxide) without confirmatory chemical evidence under precise experimental conditions employed. Rather a positive TBA-test response is indicative only of the presence of thiobarbituric acid reactive substances (TBARS) in the sample and by itself gives no quantitative or chemical information whatsoever on the source or nature of the TBARS (40,281).

The complexity of the molecular transformations associated with lipid peroxidation (309,310) and the many factors influencing the nature and amounts of primary and secondary products formed make it particularly difficult to rely solely on secondary end product analyses such as MDA determination or TBA reactivity when attempting to quantify peroxidative lipid injury in biological samples and living systems. In such cases, many diverse factors which affect MDA formation and TBA-test responses from lipid peroxidation products may be operating simultaneously (if not synergistically), inviting analytical artifacts so extreme that TBA-test response bears no quantitative relationship whatsoever to the oxidative injury status of a tissue being examined (40,281). With biological materials, it appears prudent to consider a positive TBA-test response per se as nothing more than an empirical indicator of the potential occurrence of peroxidative lipid injury and not as a measure of lipid peroxidation (311). Likewise, although the direct isolation and quantification of MDA provides more substantial evidence of fatty peroxide formation and decomposition, the absolute amount of MDA need not reflect the fatty hydroperoxide content of the biological matrix. For example, interpretation of TBA numbers and MDA levels as indicators of

fatty acid hydroperoxide mass in biological systems has led to grossly misleading overestimates of the peroxide tone of human plasma (312-314). Although different methods of MDA determinations have limitations, measurements of different classes of peroxidation is desirable and manadatory when studying lipid peroxidation in biological samples and living systems. It is now evident that although the TBA-test is simple to perform, it is difficult to interpret.

RESULTS

4.14 Assay of antioxidant enzymes

The enzymatic defense systems against free radical attack includes three basic enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). The basic function of these enzymes is to convert superoxide radical produced during metabolism into water.

A number of studies have been performed on antioxidant enzymes in tumour cells and point out that tumour cells have abnormal activities of antioxidant enzymes when compared to appropriate control cells (157,158). Although SOD is an inducible enzyme, it has been found to remain low in tumour cells despite attempts to induce it with superoxide (157,158). Therefore, lack of inducibility would provide the basis of selectively killing malignant cells using free radical generating compounds while protecting normal cells which have the capacity to induce SOD. A number of experiments were designed to investigate the following parameters:

Firstly, to determine the levels of these enzymes in the various solid MAC tumours. It would help in future studies to correlate the tumour sensitivity to low concentrations of free radical generating compounds to the levels of these enzymes in the various MAC tumours. In this respect, it was found that the concentration of SOD, expressed per mg of total soluble cell protein was 5-10 times higher in the MAC16 tumours than the MAC13 or the MAC26 tumours (fig. 4.20 a). The difference in the activities of catalase in the MAC solid tumours were statistically insignificant, although the MAC16 showed a slightly higher value than the rest (fig. 4.22 a). On the other hand, the activities of GPx in the MAC13 tumours was about 5 times the activities in the MAC16 or the MAC26 (fig. 4.23 a).

Secondly, since it has been reported that even a high oxygen or superoxide concentration does not induce SOD in tumour cells, we wanted to find out whether the adminstration of low concentrations of DDSH would induce SOD in the MAC16 tumours and in the livers of NMRI mice. This would provide circumstantial evidence of free radical generation by DDSH and a basis of selectively killing the malignant cells while preserving normal tissues if the enzyme induction occurred only in the liver but not in the tumour. When male NMRI mice implanted with the MAC16 tumours were treated with DDSH, there was a significant increase in the tumour content of SOD in animals treated with 2.5×10^{-10}

mol/kg body weight of DDSH 8 days after treatment when compared with the solvent treated controls (fig. 4.20 b). This rise in tumour SOD was not suppressed by the administration of vitamin E although serum MDA was suppressed (figs 4.17 c and 4.20 c). To eliminate the influence of the MAC16 tumour on the liver SOD, non-tumour bearing NMRI mice were used and their liver SOD activities were assayed 8 days after the administration of DDSH. Again there was a rise in SOD activities in both treated groups but this was statistically significant only in the group which received 2.5×10^{-10} mol/kg body weight of DDSH (fig. 4.21 a). In contrast, the difference in the activities of CAT and GPx in the liver of non-tumour bearing male NMRI mice following the administration of DDSH was statistically insignificant compared with the solvent treated controls (figs. 4.22 b and 4.23 b). The activities of CAT and GPx in the MAC16 tumour were not determined since our investigations were directed more in determining the activities of SOD in veiw of the several reports in the literature about its inducibility in malignant and not in the normal tissues in response to high concentrations of superoxide radicals.

Thirdly, following the death of male BDF1 mice inoculated with P388 leukaemia after the administration of DDSH, a study was designed to assess the activities of the liver antioxidant enzymes and to compare the activities of these enzymes in the livers of male BDF1 mice to those of the male NMRI mice before and after the treatment. The male NMRI mice had shown remarkable tolerance to the treatment and this could have been due to the high activities of antioxidant enzymes in their tissues that might have protected them from the toxicity of free radicals and their products. To avoid the influence of tumour, the activities of these enzymes were assayed in the livers of non-tumour bearing BDF1 mice 8 days after the administration of DDSH. There was a significant induction of SOD in the livers of BDF1 mice that received 2.5×10^{-11} mol/kg body weight of DDSH when compared with the solvent treated controls (fig. 4.21 b). The activities of SOD in the livers of both non-tumour bearing male BDF1 and the male NMRI mice before and after treatment with DDSH were similar (figs. 4.21 a and 4.21 b). On the other hand, the activities of CAT in the livers of non-tumour bearing male BDF1 mice in the treated group was not significantly different from those of the solvent treated controls (fig. 4.22 c). These values were similar to those found in the livers of non-tumour bearing NMRI mice (fig.4.22 b). In contrast, there was a significant rise in the activities of liver GPx in non-tumour bearing BDF1 mice that were treated with DDSH compared with the solvent treated controls, which had no

increase in MDA and no death (fig. 4.23 c). The rise was much higher in the group of animals that received 2.5 x 10^{-11} mol/kg body weight of DDSH. The activities of GPx in the livers of non-tumour bearing male NMRI mice before and after treatment with DDSH were about 6 times the activities in the livers of the solvent treated control non-tumour bearing male BDF1 mice (fig. 4.23 b). The GPx activities in the livers of male BDF1 mice were able to rise to this high values only after the administration of 2.5 x 10^{-11} mol/kg body weight of DDSH to the BDF1 mice. The results are summarised below.

The MAC16 tumour had the highest activities of SOD in comparison to those of the MAC13 or MAC26, while GPx was highest in the MAC13 tumour. The activities of CAT were about the same in the solid MAC tumours. The SOD was induced in the MAC16 tumour following the administration of 2.5 x 10^{-10} mol/kg body weight of DDSH contrary to the findings in other studies that they remain low in tumours despite attempts to induce it with superoxide. In addition, this enzyme was induced in both the livers of male NMRI and BDF1 mice following the administration of 2.5 x 10^{-10} and 2.5 x 10^{-11} mol/kg body weight of DDSH respectively. There was no induction of CAT in both the livers of NMRI and BDF1 mice following the administration of the same treatment. The pretreatment GPx levels in livers of male NMRI mice were high and low in the livers of BDF1 mice. The levels in the livers of NMRI mice did not rise further following treatment while those of BDF1 mice rose.

Fig. 4.20a. Concentration of SOD in the MAC13, MAC16 and MAC26 tumours from male NMRI mice. 5 samples per assay. The values are expressed as the mean ± s.e.m.

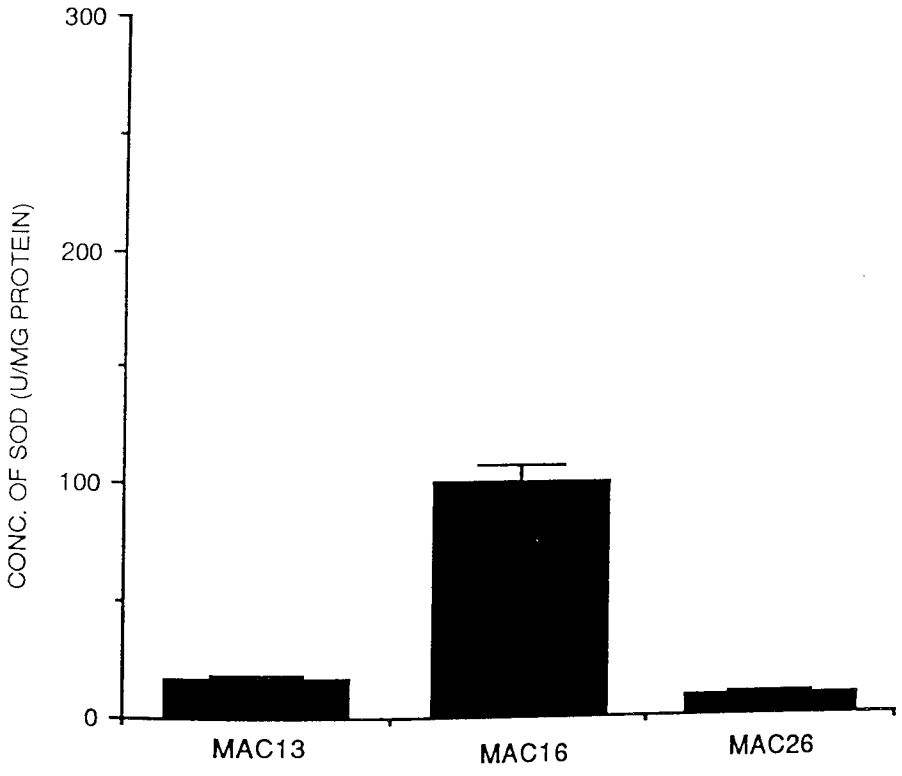


*Fig. 4.20b. Concentration of SOD in the MAC16 tumours from male NMRI mice treated with DDSH administered by a single subcutaneous injection. The assay was performed 8 days after treatment. 5 samples per assay. The values are expressed as the mean ± s.e.m. Significance versus solvent treated controls: * $p < 0.05$.*

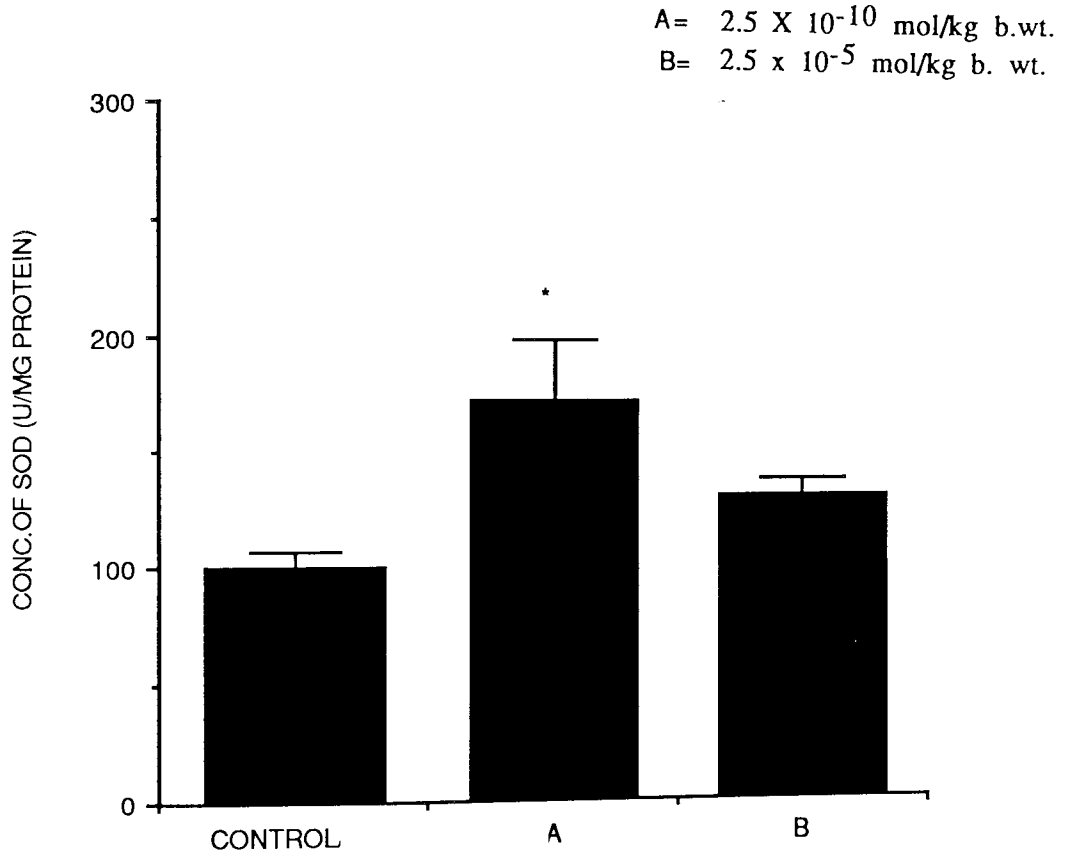


*Fig. 4.20c. Concentration of SOD in MAC16 tumours from male NMRI mice treated with DDSH and 1mg /kg body weight of vitamin E administered by a single subcutaneous injection. The DDSH was mixed with the vitamin E. The assay was performed 8 days after treatment. 5 samples per assay. The values are expressed as the mean ± s.e.m. Significance versus vitamin E treated control: * p <0.05.*

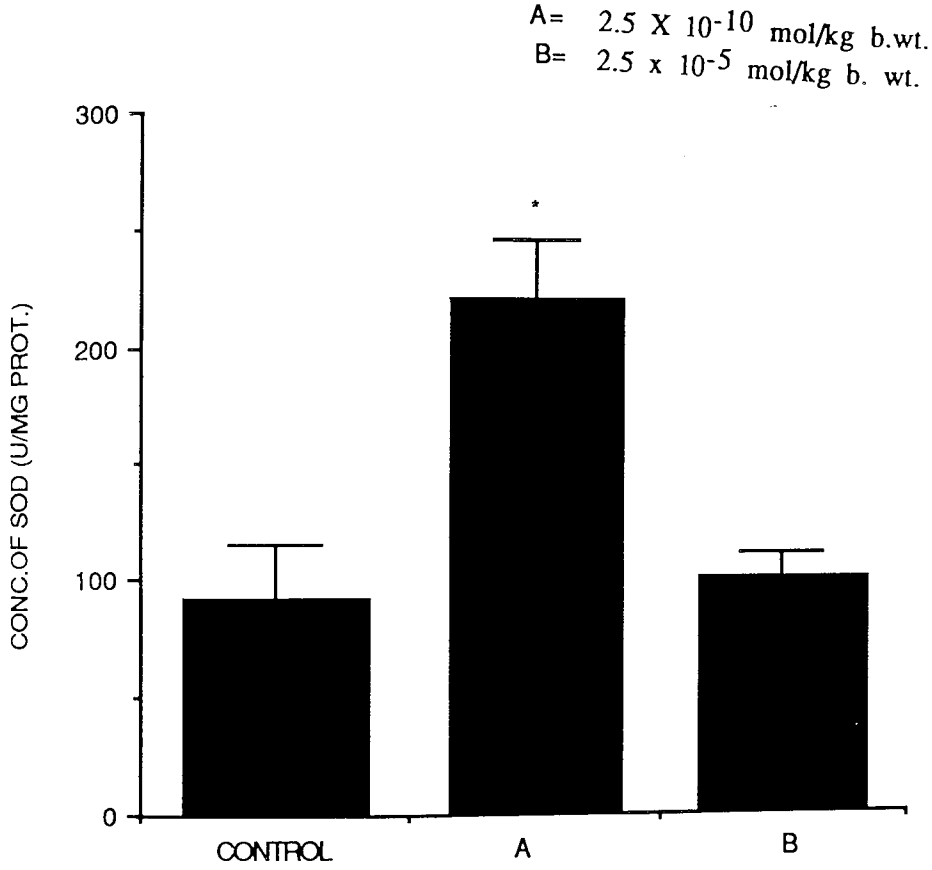


*Fig. 4.21a. Concentration of SOD in the liver of male NMRI mice treated with DDSH administered by a single subcutaneous injection. The assay was performed 8 days after treatment. 5 samples per assay. The values are expressed as the mean ± s.e.m. Significance versus solvent treated controls: * $p < 0.05$.*

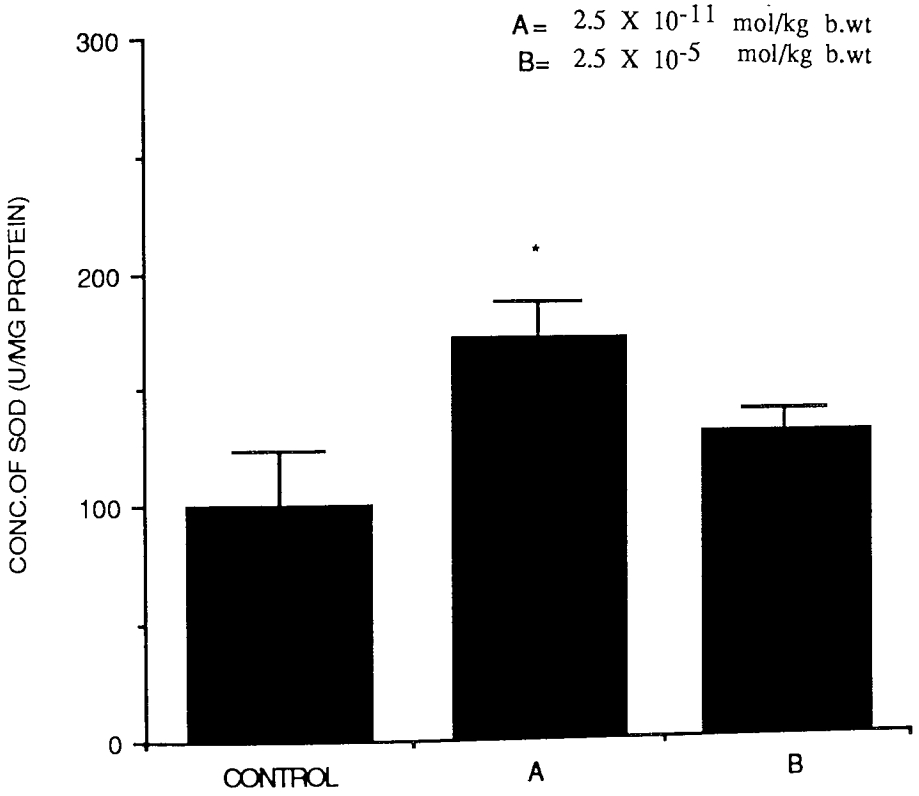


*Fig. 4.21b. Concentration of SOD in the liver of male BDF1 mice treated with DDSH administered by a single subcutaneous injection. The assay was performed 8 days after treatment. 5 samples per assay. The values are expressed as the mean ± s.e.m. Significance versus solvent treated controls: * $p < 0.05$.*

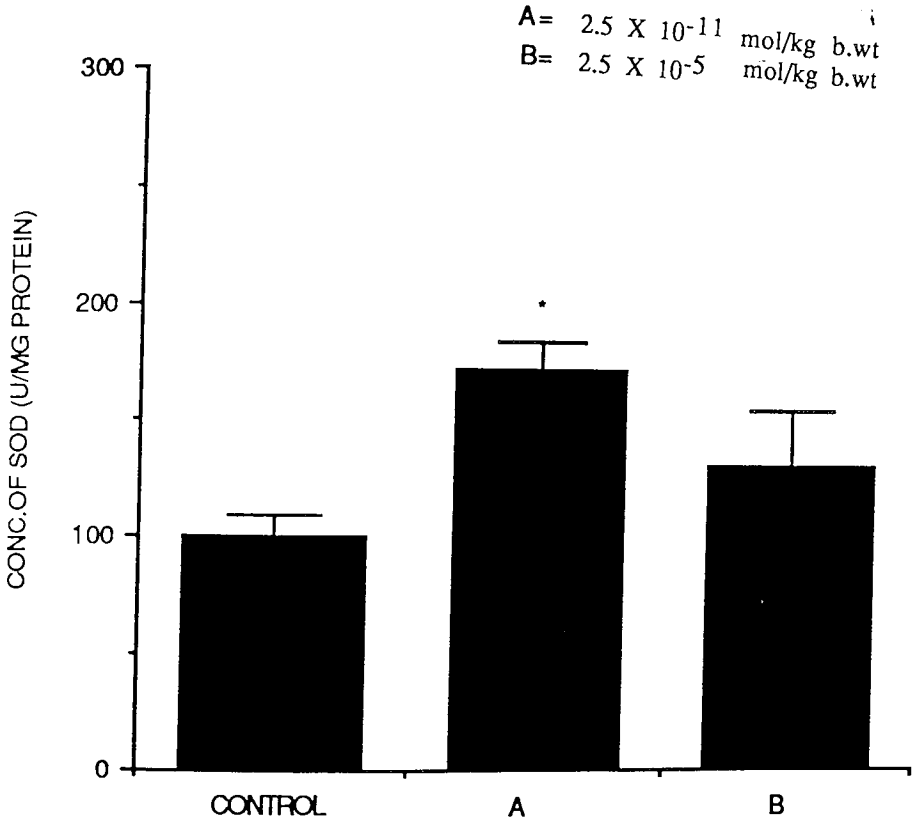


Fig. 4.22a. Concentration of catalase in the MAC 13, MAC16 and MAC26 tumours from male NMRI mice. 5 samples per assay. The values are expressed as the mean ± s.e.m.

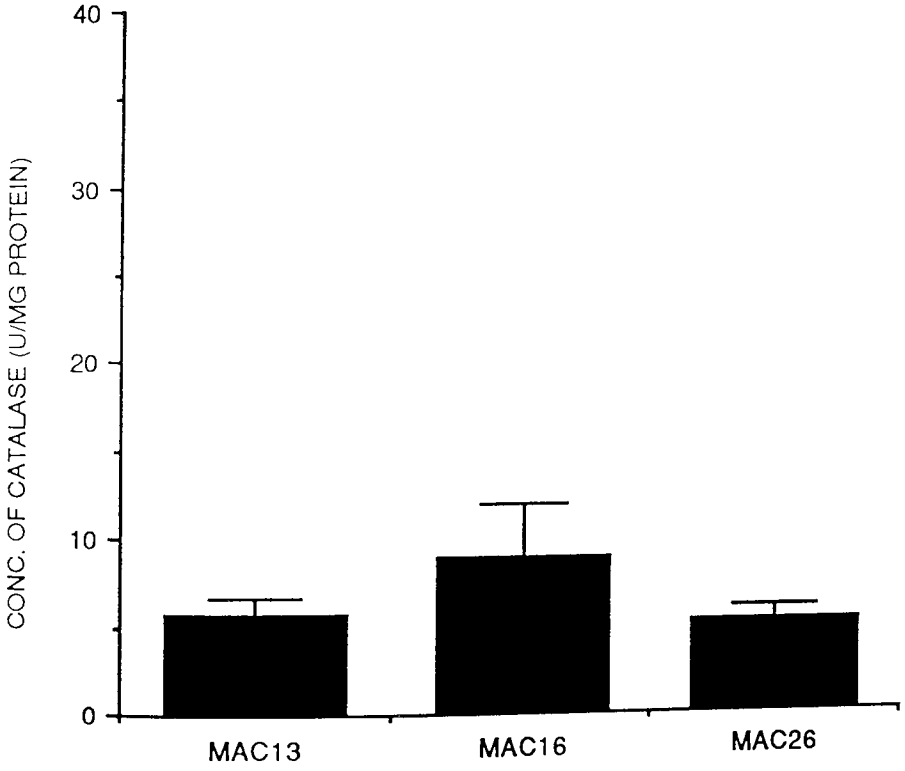


Fig. 4.22b. Concentration of catalase in the liver of male NMRI mice treated with DDSH administered by a single subcutaneous injection. The assay was performed 8 days after treatment. 5 samples per assay. The values are expressed as the mean ± s.e.m.

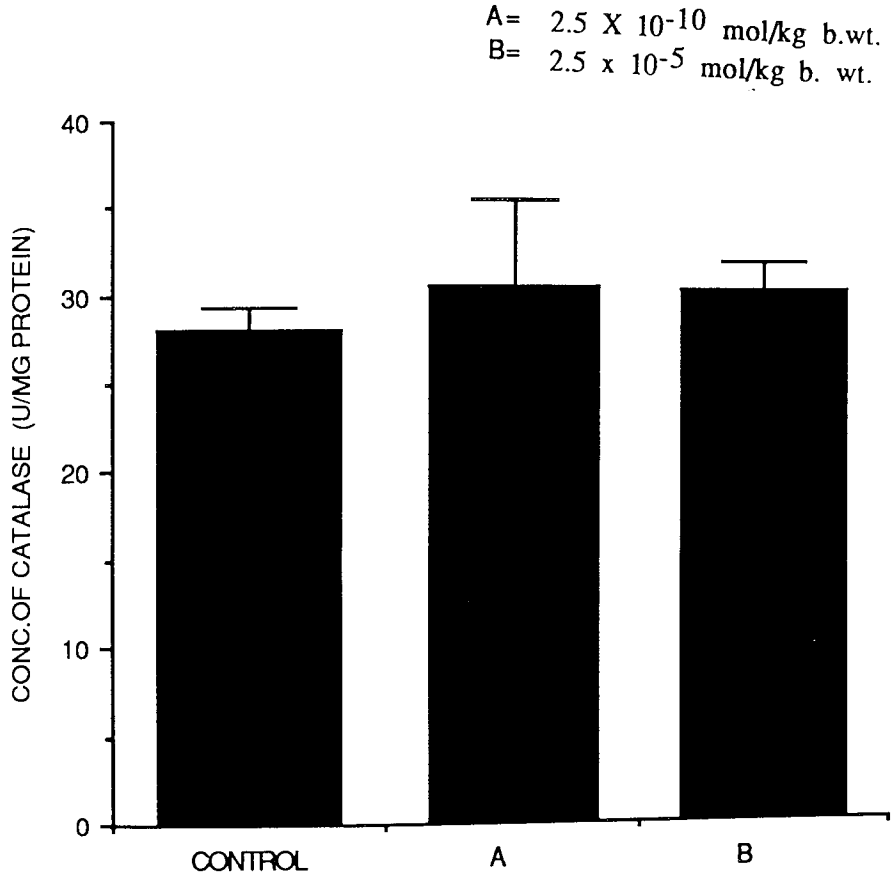


Fig. 4.22c. Concentration of catalase in the liver of male BDF1 mice treated with DDSH administered by a single subcutaneous injection. The assay was performed 8 days after treatment. 5 samples per assay. The values are expressed as the mean ± s.e.m.

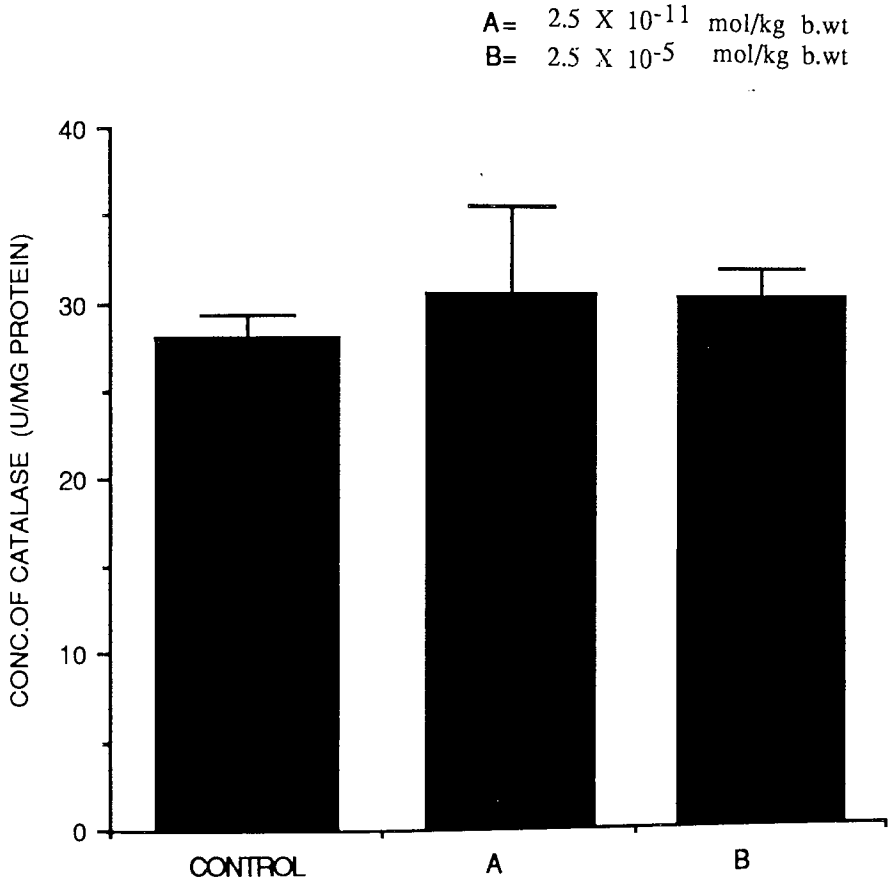


Fig. 4.23a. Concentration of GPx in the MAC13, MAC16 and MAC26 tumours from male NMRI mice. 5 samples per assay. The values are expressed as the mean ± s.e.m.

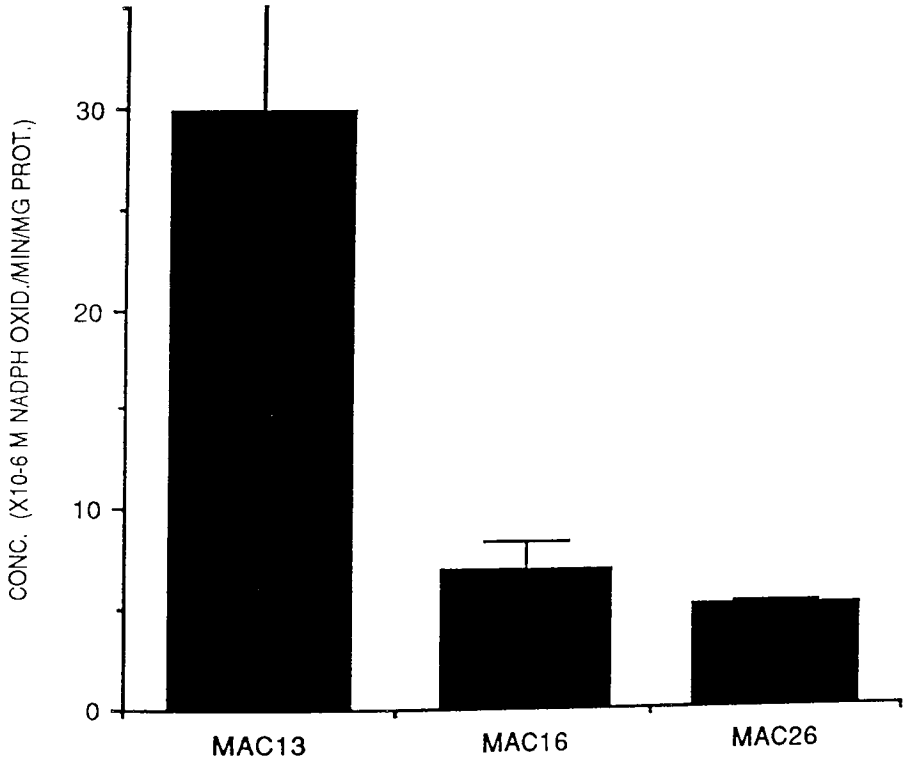


Fig 4.23b. Concentration of GPx in the liver of male NMRI mice treated with DDSH administered by a single subcutaneous injection. The assay was performed 8 days after treatment. 5 samples per assay. The values were expressed as the mean ± s.e.m.

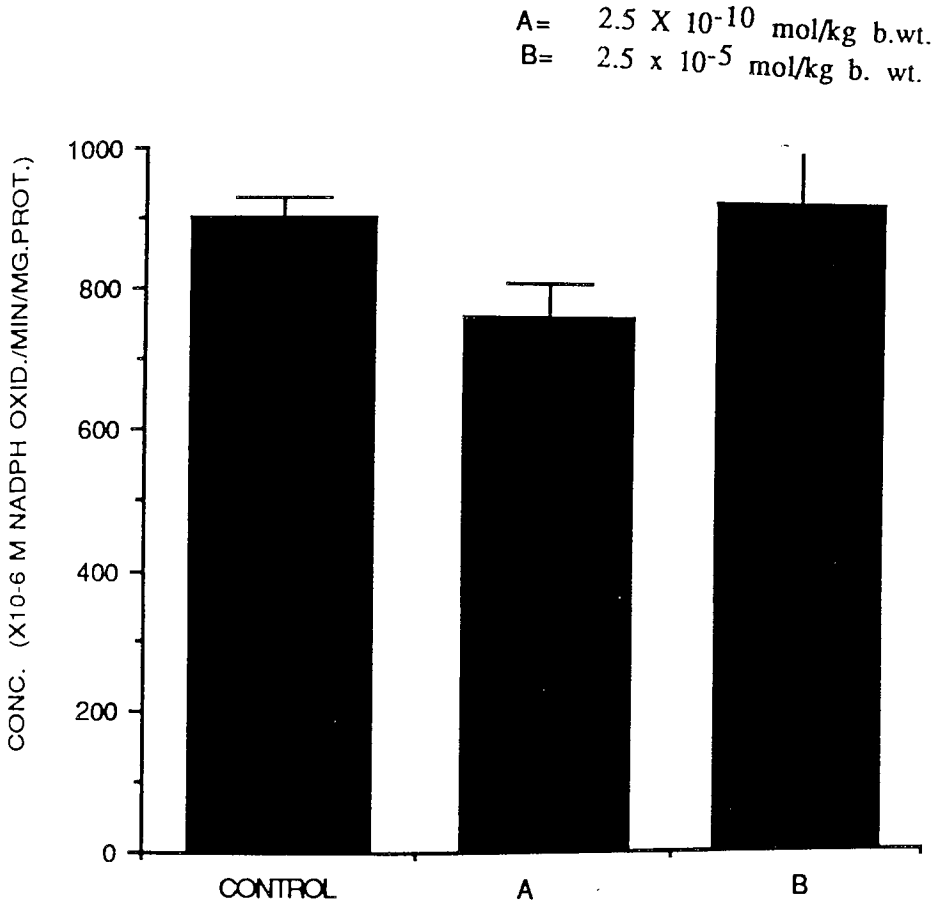
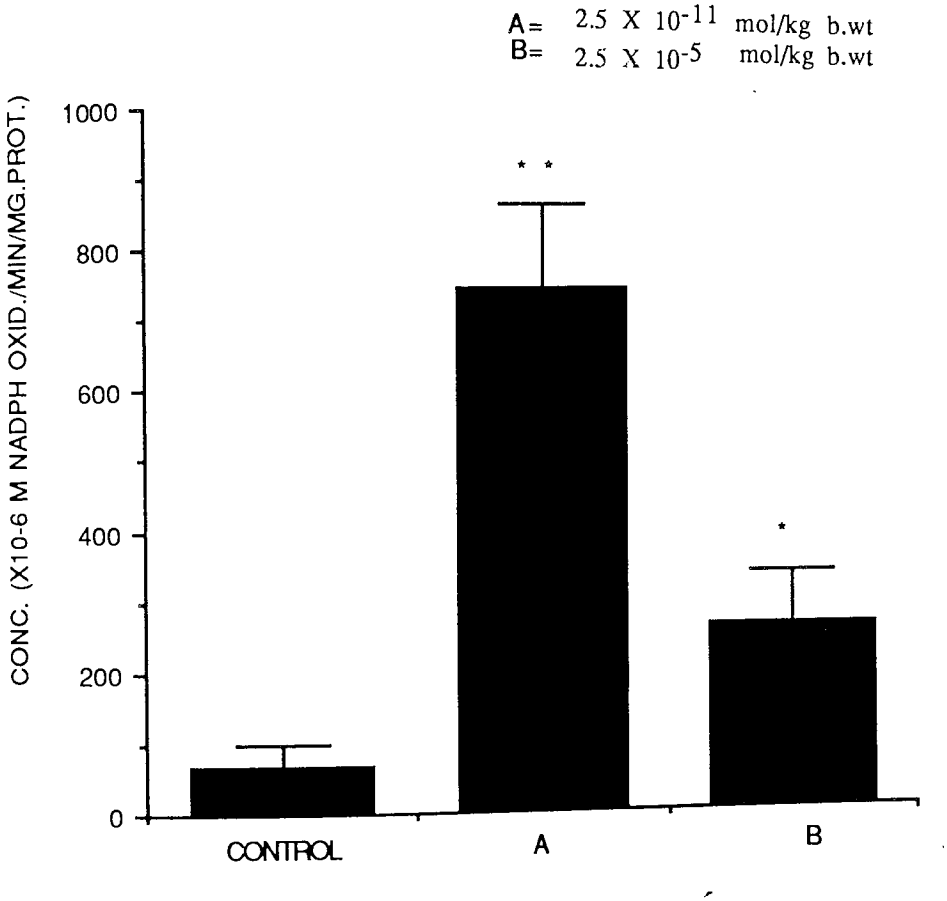


*Fig. 4.23c. Concentration of GPx in the liver of male BDF1 mice treated with DDSH administered by a single subcutaneous injection. The assay was performed 8 days after treatment. 5 samples per assay. The values are expressed as the mean ± s.e.m. Significance versus solvent treated controls: * $p < 0.05$, ** $p < 0.005$.*



DISCUSSION

4.15 Antioxidant enzymes assay

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are antioxidant enzymes which play fundamental roles in the protection of tissues against free radical damage. The activities of these enzymes have been compared in the various MAC tumours and their ability to be induced following the administration of DDSH to male NMRI and male BDF1 mice has been assessed.

The MAC16 and the MAC13 tumours had the highest activities of SOD and GPx respectively, while the activities of catalase was about the same in all the three MAC solid tumours (figs. 4.20 a, 4.22 a and 4.23 a). Although these values may reflect the relative activities of these enzymes in these tumours, it may not be the actual value because of tissue heterogeneity. The MAC16 tumour is very necrotic and therefore this assay may underestimate the actual amount of the enzyme present. The MAC26 is the least necrotic tumour among the MAC solid tumours and these concentrations may reflect the true values. Enzyme assays in tumour tissues are more difficult than in tissues like the liver because of the difficulty in preparing good tissue homogenates. The cell membranes are difficult to disrupt and the connective tissue is tough.

There are no significant differences in the response rates of the MAC tumours to standard anticancer drugs (nitrosoureas, cyclophosphamide, and 5FU) in the NMRI and nude mice host (268). These tumours are generally resistant to most anticancer drugs but no direct correlative study has been conducted to relate the resistance of free radical generating drugs to the activities of antioxidant enzymes. However, the high activities of SOD in the MAC16 may contribute to the resistance of this tumour to free radical generating drugs in cases which failed to respond to a second dose of DDSH after partial tumour rupture.

Since a number of agents which cause increased oxygen radical production in cells, such as adriamycin, paraquat, bleomycin and molecular oxygen have been shown to cause an increase in SOD activities (315), its activities in the MAC16 tumour grown on the male NMRI mice were evaluated following the administration of DDSH. It has been found that although SOD enzyme is an inducible enzyme, it always remained low in tumours (157,158). It was considered that this would have provided a basis of selectively killing malignant cells without causing any major damage to the

normal tissues. Unfortunately, there was a statistically significant increase in the tumour SOD activity in the animals that were treated with 2.5 x 10^{-10} mol/kg body weight of DDSH when compared with solvent treated controls (fig. 4.20 b). Vitamin E administration did not suppress the rise in the SOD activity probably because the free radical reactions that might have been initiated by the administration of DDSH overwhelmed the antioxidant capacity of the vitamin E (fig. 4.20 c). There was also a similar increase in the activities of SOD in the livers of NMRI mice that had received a similar therapy but the levels of CAT and GPx were not significantly different from those of solvent treated controls (figs. 4.21 a, 4.22 b and 4.23 b). The influence of the administration of DDSH on the activities of CAT and GPx in the MAC16 tumour was not evaluated. It is very difficult to draw strong conclusions about the capacity of the MAC16 to induce SOD in response to free radical attack because the tumour is an heterogenous tissue. There are other normal tissues within the tumour such as connective tissues that may have a better capacity to induce SOD in response to free radical challenge and therefore, the high activity of SOD enzyme. It is difficult to pinpoint the actual source of SOD in this heterogenous system. This can only be overcome by using pure tumour cells from cell culture for this type of assay. Nonetheless, if this observed increase in the activities of SOD is real, it may be related to the tumour resistance that has been documented following the adminstration of drugs that augument the generation of free radicals such as adriamycin and mitomycin C (315). Mechanistic studies have shown that anthracyclines drugs such as adriamycin, intercalate into DNA bases, form reactive metabolites which bind covalently to cellular macromolecules, interact with cell membranes causing lipid peroxidation, inactivate a variety of enzymes, induce topoisomerase 11-dependent DNA strand breaks, and form semiquinone free radicals and oxy-radicals (316-324). Mitomycin C, a quinone-containing antibiotic active in the treatment of solid tumours, contains an aziridine ring and acts as a bioalkylator inducing cross-links in DNA molecules. While in the absence of molecular oxygen the alkylation of biopolymers by mitomycin C is likely to be the major pathway for tumour cell cytotoxicity, in the presence of oxygen, oxy-radical dependent toxicity is also expected (325). The formation of oxy-radicals from mitomycin C, following activation by either liver microsomal enzymes or by chemical reducing agents, has been well documented, and several recent reports have indirectly linked the production of oxy-radicals by mitomycin C to tumour cell death (324,326). Although not universal in human tumours or in human tumour cell lines, increased activities of antioxidant enzymes in multidrug-resistant

tumour cells are associated with decreased sensitivity to anthracyclines and mitomycin C (315). Coupled with the observations that drug-sensitive cells generate more superoxide and hydroxyl free radicals in the presence of these drugs than drug-resistant variants, a reasonable argument can be constructed supporting the involvement of enzymatic activation to drug free radicals and oxygen radical generation in the antitumour activity of these agents. Although convincing evidence has been obtained using tumour cell lines in culture, there is a paucity of information about the possibility that this mechanism of tumour killing is operational in human cancer *in situ*. The same argument can be extended to explain the antitumour effect of DDSH in view of the SOD enzyme induction which might have occurred in response to free radical attack. It is also important to consider the emergence of tumour resistance to free radicals in the MAC16 tumour following a partial tumour rupture because these tumours failed to respond to a repeat course of DDSH.

The influence of DDSH administration on the liver antioxidant enzyme activities of the male NMRI mice were compared to the activities of these enzymes in the liver of male BDF1 mice in view of the sudden death that occurred in some of the tumour bearing BDF1 mice following therapy with DDSH. The activities of SOD in the livers of male BDF1 mice were similar to the levels in the liver of male NMRI before and after the administration of DDSH with a significant increase occurring in the animals that were treated with 2.5×10^{-10} mol/kg body weight (figs. 4.21 a,b). There was no significant increase in the activities of CAT and this was similar to the activities in the livers of male NMRI mice before and after treatment (figs. 4.22 b,c). In contrast, the activities of GPx in the livers of male BDF1 mice were about 20 times less than the activities of GPx in the livers of male NMRI before treatment with DDSH. The activities of GPx significantly increased in the livers of the male BDF1 mice following the administration of DDSH, with a greater increase occurring in the group that received 2.5×10^{-10} mol/kg body weight and reached the level of GPx activities in the liver of male NMRI mice (figs. 4.23 b,c). It has been reported that chronic exposure to nonlethal concentration of paraquat in vivo produced increases in gluthathione-dependent enzymes that exceeded the increases in SOD (327). Paraquat is a potent herbicide which undergoes a cyclic redox reaction with oxygen to produce superoxide radicals, singlet oxygen, hydroxyl radicals, hydrogen peroxide lipid peroxides, and disulphides (327-330). It was suggested in the report that glutathione-dependent enzymes alone can contribute to protection against paraquat and increases in activities can occur independent of alterations in SOD activities.

The mechanisms by which GPx and transferase protects against paraquat is not clear. GPx and transferase, besides protecting against hydrogen peroxide and lipid hydroperoxides also protects cells against a wide variety of damaged macromolecules, including DNA peroxides, hydroperoxyeicosatetraenoic acids, and prostaglandins (331-333). Our results are in agreement with these reports and it seems GPx and other glutathione-dependent enzymes play a greater role in protection against free radical toxicity than any other enzymes. It is most probable that because of the low pretreatment activities of GPx in the livers of BDF1 mice, vital organs were subjected to lethal injuries by free radical reactions and by the time these enzymes reached protective levels following induction, irreversible damage had already been inflicted and therefore, the sudden death. If the magnitude of the enzyme induction reflects the extent of free radical generation, then the BDF1 mice that received 2.5×10^{-11} mol/kg body weight of DDSH generated more free radicals than those that received 2.5×10^{-5} mol/kg body weight in view of the greater SOD and GPx induction. Therefore, the BDF1 mice that were treated with 2.5×10^{-11} mol/kg body weight of DDSH sustained greater injuries than those that were treated with 2.5×10^{-5} mol/kg body weight and therefore, the greater the numbers of sudden death that occurred at this concentration. The sudden death only occurred in tumour bearing animals and there was none recorded in non-tumour bearing animals that received a similar treatment. The lysis of P338 leukaemic cells by the free radicals in these animals might have provided a source of lipid peroxides and damaged macromolecules, including DNA peroxides, hydroperoxyeicosatetraenoic acids, and prostagladins. They might have all contributed to the sudden death of these animals. The tumour bearing NMRI mice escaped these injuries because their tumours popped out and in addition they had a higher baseline activities of all the antioxidant enzymes tested which were able to protect them better from free the radical attacks.

The finding that GPx is elevated, but CAT is not, does not rule out the involvement of hydrogen peroxide. It has been known for years that GPx, and not CAT, is the primary protective enzyme against hydrogen peroxide (336). This differential protection has two components. There is a difference in reactivity of the enzymes to hydrogen peroxide, and GPx has the lower Km value (337). Also, there is a difference with intracellular location. CAT is confined primarily to peroxisomes (338). GPx, in contrast, is found throughout the cell with the major activity in the cytosol (339,341). Observations by others confirm that GPx, and not CAT,

is important in detoxifying hydrogen peroxide in human leukocytes (340).

This was our preliminary contribution to define the protective response of tumour and host to free radical injuries. It is hoped that in conjunction with other studies, it will help to lay a foundation by which we shall enhance the generation of free radicals in tumours without causing irrepairable damage to the normal tissues.

4.16 GENERAL DISCUSSION AND CONCLUSIONS

The experiments described were designed to test the concept of using extremely low concentrations of free radical generating compounds as a "catalyst" to trigger free radical chain reactions in the host. The parameters used were as follows:

(a) to screen the cytotoxic effects of a number of free-radical generating compounds against malignant cell lines *in vitro*;

(b) to confirm whether the mechanisms responsible for some of their cytotoxic effects was related to free radical generation *in vitro*;

(c) to compare the cytotoxic effects to the activities of other compounds which are known not to generate free radicals;

(d) to select a few of these compounds and test the effects on tumours in animal models;

(e) to assess the mechanisms of action *in vivo*;

(f) and to assess the induction of antioxidant enzymes in both the tumours and host animals.

It was important to establish whether low concentrations of DDSH and other compounds that are known to generate free radicals actually generated free radicals *in vivo*. This was a very crucial question to answer because the original concept was based on this idea. It was an hypothesis that contradicts the law of mass action, a fundamental law in chemistry which forms the basis of modern chemotherapeutic principals. "The bigger the dose the better the response." To prove directly the existence of free radicals in a biological system is usually very difficult because they are very reactive and very short lived. Spin trapping is the standard method employed to detect the presence of free radicals in a biological system (342). However, circumstantial evidence is usually used to prove the involvement of free radicals in any reaction. The method employed was to inhibit the effects of free radicals by the use of enzymatic and non-enzymatic antioxidants (343). In addition, evidence of the induction of antioxidant enzymes pointed very stongly to free radical involvement. Although the existance of free radicals in a biological system may be proven, it would even be more difficult to find if its existence was an 'effect or a cause'. Many agents can cause direct tissue injury and this may result in lipid peroxidation, or free radical metabolites may be formed as possible toxic intermediates (144). The two events are very difficult to differentiate because they

usually co-exist. In this study, the following facts were in favour of a primary free radical involvement although they were circumstantial.

These compounds exhibited their antitumour effects at very low concentrations and the best response was obtained in the nanomolar concentration range. The high dose of DDSH did not display any antitumour effect against the MAC16 tumour. The antitumour activities was not only displayed against the MAC16 tumour which was implanted subcutaneously in the NMRI mice, but it was able to eliminate the P388 leukaemic cells, an ascitic tumour that was inoculated intraperitoneally in the BDF1 mice, at nanomolar concentrations despite the drug being administered by a subcutaneous injection. The antitumour effect against the MAC16 tumour implanted in the NMRI mice was also exhibited by very low concentrations of some other compounds that are known to augument free radical generation. It is very difficult to imagine how such an extremely low concentration of these compounds would have a direct antitumour effect. The involvement of an endogenous mediator(s) would be the only sensible and logical explanation for such an effect and we proposed that such a mediator(s) must involve directly or indirectly free radical(s). Slater argued that lipid peroxidation is often a very damaging and degradative process; if lipid peroxidation overwhelms the normally efficient cellular defence systems then it can cause cytotoxicity and cell death (144). However, the established biological effects of very low concentrations of products of lipid peroxidation allows the contention that small rates of lipid peroxidation in normal cells may have important physiological consequences. One such consequence could be the effect of 4-hydroxy-alkenals, such as 4-hydroxy-nonenal on DNA-synthesis and on thiol-dependent reactions (139). In this way endogenous production of low levels of 4-hydroxy-alkenals could act as a 'coarse control' on DNA synthesis; in contrast, inhibition of lipid peroxidation might then be expected to be associated with a stimulation of cell division (344). There is no doubt on the toxic effects of free radical reactions and we concluded that the sudden death that occurred in some of the tumour bearing BDF1 mice could have been due to some free radical mechanism(s).

Histological sections of these tumours showed areas of massive haemorrhagic necrosis and vascular collapse akin to the effect of flavone acetic acid (FAA) on the MAC16 tumour (267). In addition, there was perivascular cell death which is not seen following the adminstration of FAA. It was concluded that the mechanisms of action was both direct and indirect. The direct mechanism

probably involved free radicals /or by-products and this would have direct cytotoxic effects on the tumour cells, especially those in the perivascular areas. The indirect mechanism was probably due to free radical associated vascular damage causing ischaemic necrosis of the tumour.

Tumour necrosis factor (TNF) is thought to mediate the antitumour effects of FAA (268), and the antitumour effects of TNF is probably mediated through a free radical mechanism(s) (273). We could not rule out completely the involvement of TNF in our study. Other cytokines such as interferons (IFNs), in addition to their antiviral action, have been shown to inhibit cell proliferation, induce differentiation of some tumour cells, activate natural killer (NK) cells and macrophages, and modulate phagocytosis (273-275). The exact mechanism(s) by which IFN can bring about these pleiotropic actions is not known. Previous studies showed that IFN can augment free radical generation in the cells (273,275). Free radicals can stimulate lymphocyte mitogenically and activate macrophages and NK cells (274). It is also known that activated macrophages and polymorphs produce oxidative metabolites, such as hydrogen peroxide, which is responsible for a sterilising action against microorganisms and cytotoxic activity against tumour cells (245). Free radicals are also known to inhibit cell division (139). Since IFN can augment free radical generation, it is suggested that free radicals mediate some of the actions of IFN. These are some of the proposed mechanisms that were considered by which the low concentrations of DDSH and other agents known to augment free radical generation might have exhibited their antitumour effects.

Although neither MDA determination nor TBA-test response can generally be regarded as a diagnostic index of the occurrence/ extent of lipid peroxidation, MDA/TBA reactivity may act as an indicator of lipid peroxidation (311). Our results demonstrated a significant increase in MDA concentration following the administration of DDSH and some of the free radical generating compounds. The increase in the MDA levels was suggestive of free radical reactions although it did not completely correlate with the antitumour effects of these compounds.

Recently, it has been reported that flavone acetic acid (FAA) stimulated the production of nitric oxide from activated peritoneal macrophages (346). This is via an L-arginine-dependent pathway which is usually induced to produce nitric oxide when endotoxin (347) and tumour necrosis factor-α (348) are used as second signals to activate macrophages. Earlier studies showed that macrophage activation for expression of cytotoxicity against

tumour cells required several signals including a priming signal and a triggering signal (349-352). This would lead to the NADPH-mediated oxidation of a terminal guanidino nitrogen atom of L-arginine, which is induced by these differentiating signals, leads to the synthesis of the cytotoxic product nitric oxide, which subsequently reacts with oxygen and water to produce nitrite and nitrate (353,354). Nitric oxide produced by the activated macrophages in vitro is directly cytotoxic, possibily by forming iron-nitrosyl complexes which inhibit iron-containing enzymes in target cells (346). It was further suggested that low concentration of nitric oxide produces relaxation of vascular endothelia and hypotension similarly to the action of FAA. The hypotension causes tumour ischaemia and necrosis (346). The tumour necrosis and cytotoxicity caused by the administration of low concentration of DDSH was very similar to the effects of FAA (figs. 4.12 a,b,c,d). If nitric oxide production is stimulated by FAA, this would imply that some of the antitumour action of DDSH was mediated by the production of nitric oxide. In this case, the priming signal for the macrophage activation is the tumour, while the triggering signal is the low concentration of DDSH. In addition, it was noted that the MDA production was statistically significant in the tumour bearing BDF1 mice, while the nontumour bearing BDF1 mice did not produce significant quantities of MDA following the administration of DDSH (fig. 4.18). The priming signal in this case was the ascitic tumour which probably activated the peritoneal macrophages and the administration of low concentration of DDSH might have acted as the triggering signal. This proposal warrants further investigation.

In view of the induction of SOD and GPx, it could be argued that this induction could have occurred as a response to free radical generation following the administration of DDSH. The increase in the activities of SOD in the MAC16 tumour provides futher evidence that some of the free radicals were probably being generated within the tumour. Although the arguments to support the generation of free radicals *in vivo* as a result of the administrations of the DDSH and other free radical compounds may be strong, there is very little evidence to confirm that a free radical mechanism(s) was operational in bringing about the antitumour effects of these compounds. Even if a free radical mechanism(s) were to be involved in the antitumour effects of these compounds, there is no evidence of selectivity because the SOD was induced in both the MAC16 tumour and the livers of the these mice. This is in contrast to the reports in the literature which documented the lack of inducibililty of SOD in tumours (157,158).

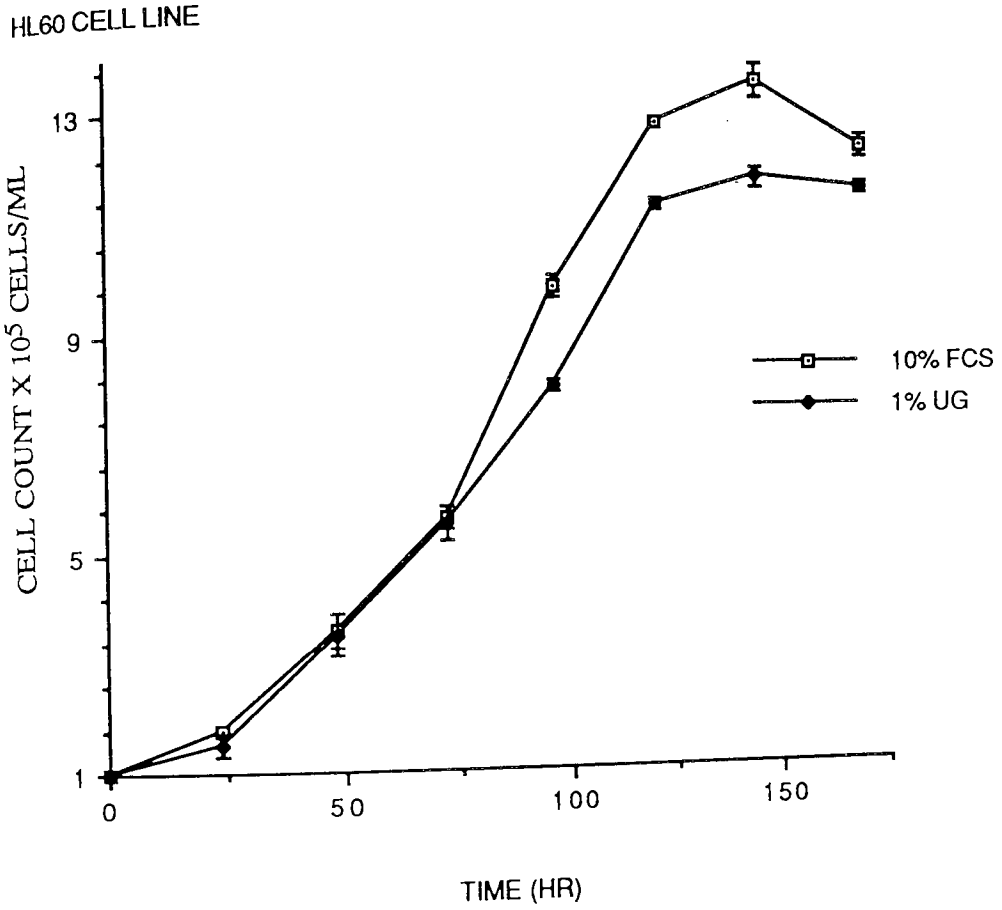
DPPH, and its hydrazine homologue, are known to be uncouplers of oxidative phosphorylation (261) and we considered the possibly that this may play a part in the antitumour effects of DDSH since the uncoupling of oxidative phosphorylation is thought to involve a free radical mechanism(s) (262). However, this would not account for the antitumour effects of low concentration of other compounds that were used in this studies. There is no documentation (to my knowledge) which may suggest that these other compounds may cause uncoupling of oxidative phosporylation. There would be no basis of selectivity, if uncoupling of oxidative phosphorylation were to be the main mechanism of antitumour effects.

In conclusion, although we have attempted to demonstrate that low concentrations of DDSH and other compounds when administered in vivo may cause the generation of free radicals, it was difficult to establish whether the free radicals that were being generated were responsible for the antitumour effects and the toxicity of these compounds. Even though the mechanism(s) of the antitumour effect of low concentration of DDSH is not clear, because of the similarities in the action of DDSH, FAA and TNF, we concluded that its mechanisms of action may involve: (a) the modulation of the immune system including the induction of cytokines, (b) effect on the tumour blood supply and (c) the direct cytotoxic action of free radicals and their products. We could not establish the basis for selectivity. However, the antitumour effects of low concentrations of these compounds warrants further evaluation because it will provide a new approach in the management of neoplastic diseases and other disorders.

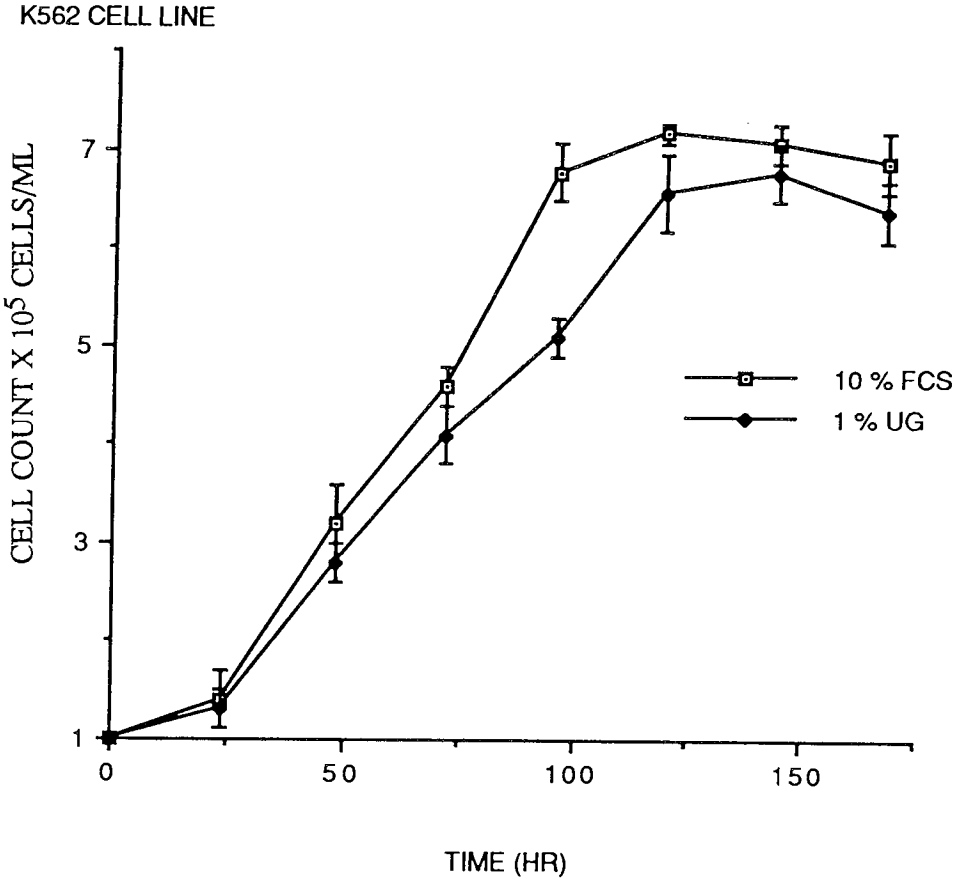
APPENDICES

APPENDIX

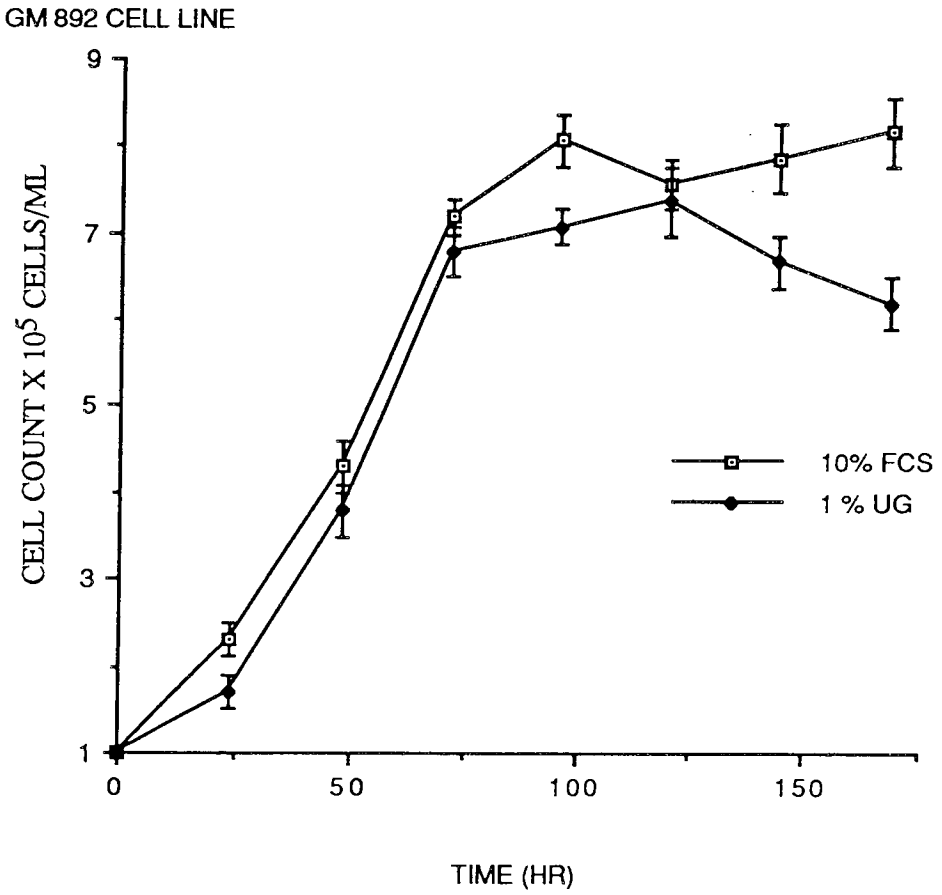
1A Growth curve of HL60 cell line in 10 % FCS and 1 % UG.



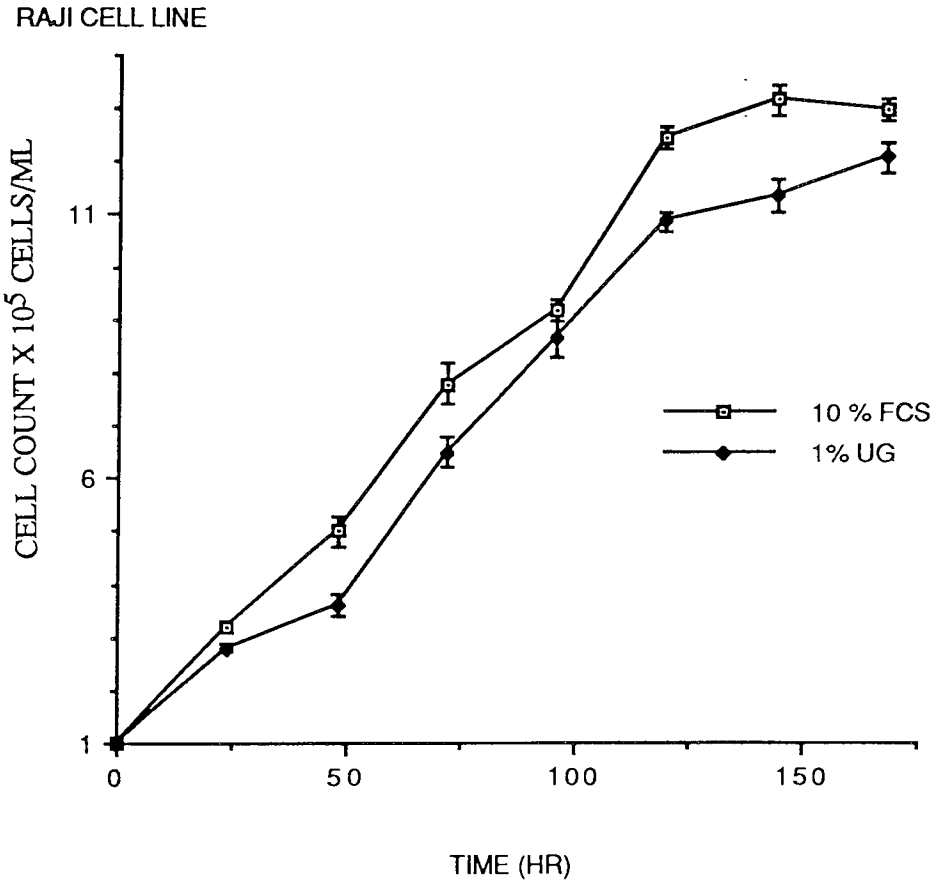
1B. Growth curve of K562 cell line in 10% FCS and 1% UG.



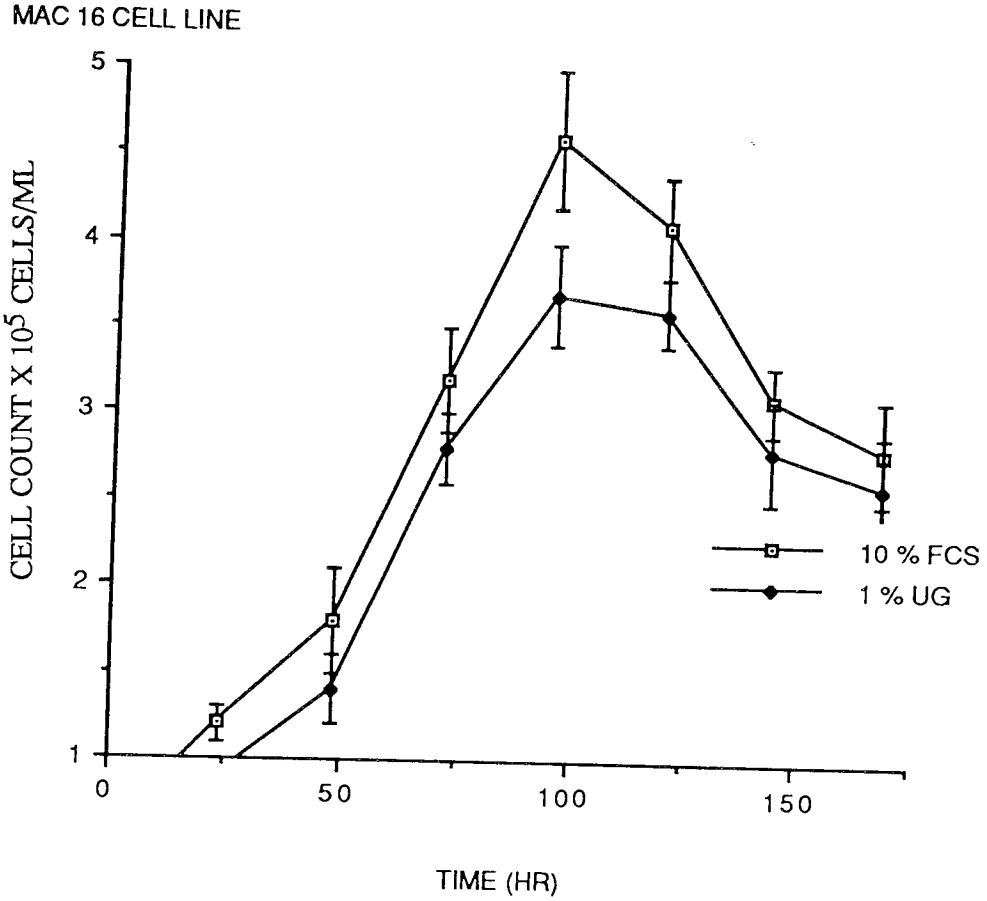
1C. Growth curve of GM892 cell line in 19% FCS and 1% UG.



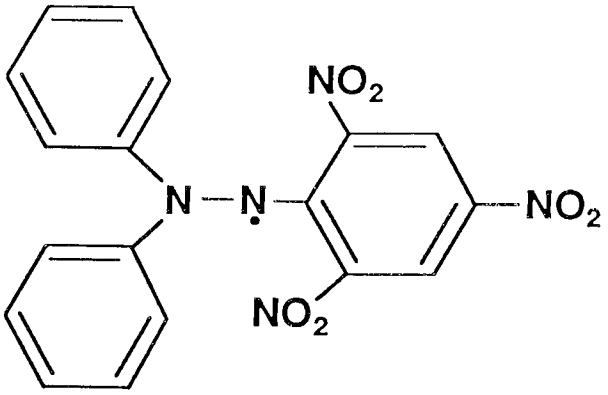
1D. Growth curve of Raji cell line in 10% FCS and 1% UG.



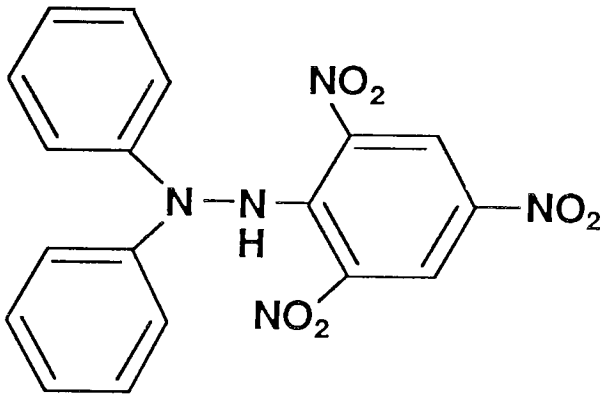
1E. Growth curve of MAC16 cell line in 10% FCS and 1% UG.



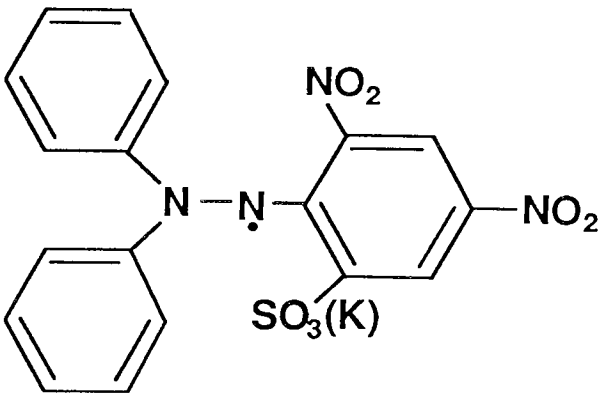
APPENDIX 11. STRUCTURAL FORMULAE OF SOME OF THE COMPOUNDS USED IN THE STUDY.



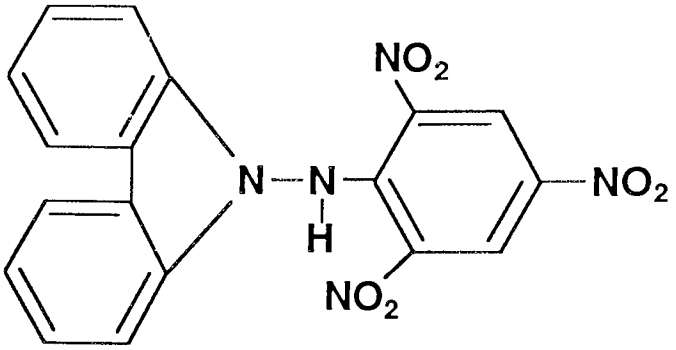
1,1–Diphenyl–2–picryl–hydrazyl (DPPH)



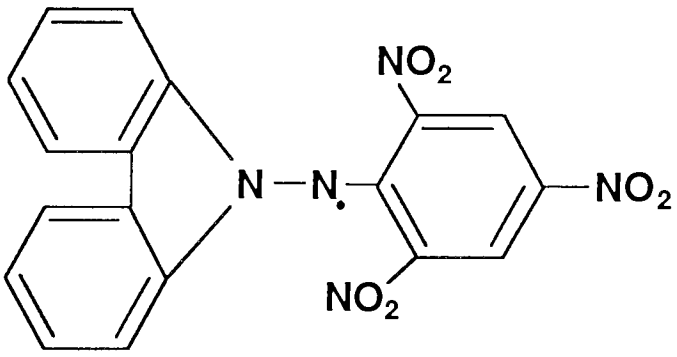
1,1–Diphenyl–2–picryl hydrazine ($DPPH_2$)



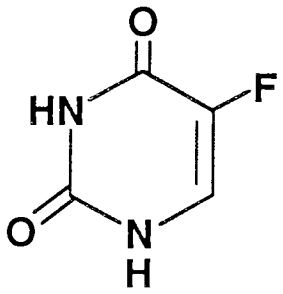
2,2–Diphenyl–1–(2',4'–di nitro–6' Sulphophenyl) hydrazyl Potassium salt (DDSH)



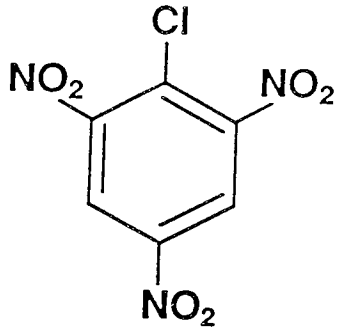
Carbazylpicrylamine (CPH_2)



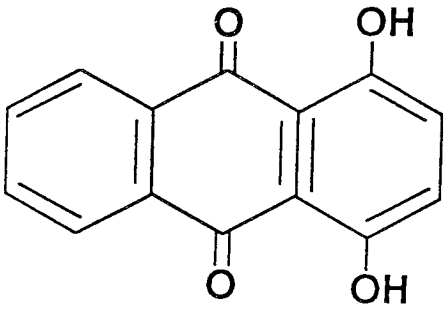
Carbazylpicryl Nitrogen (CPH)



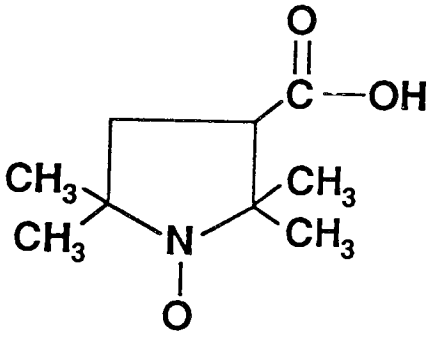
5–Fluorouracil



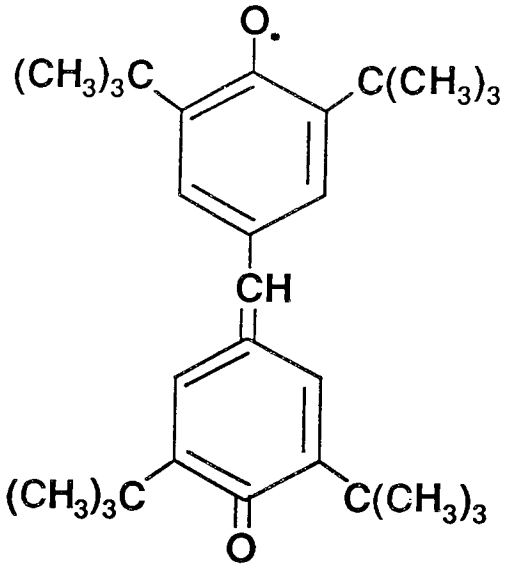
Picryl chloride



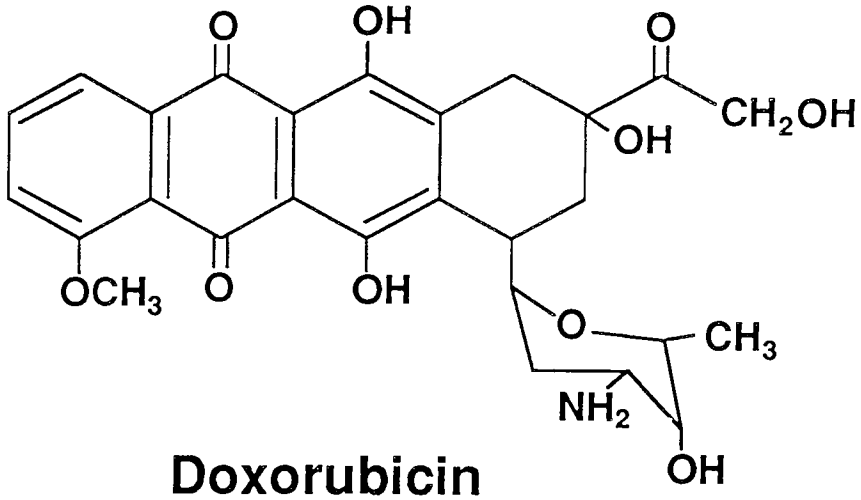
Quinizarin



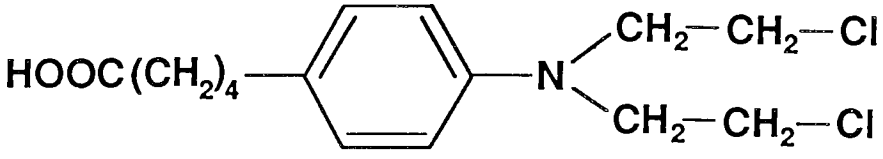
3–Carboxy–PROXYL



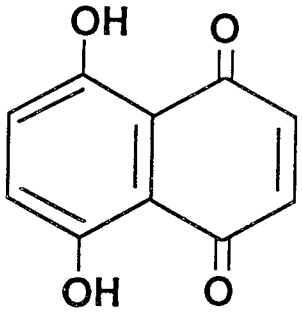
Galvinoxyl



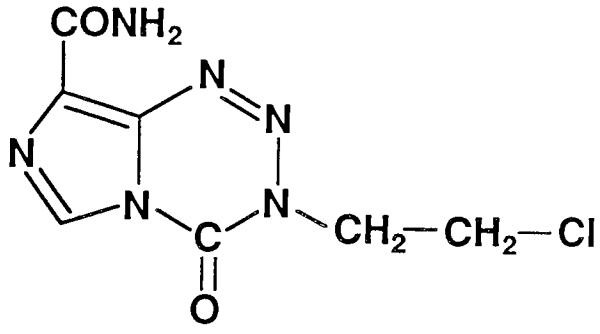
Doxorubicin



Chlorambucil



5,8–Dihydroxy–1,4–naphthoquinone



Mitozolomide

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