SYNTHESIS OF THIOL-DEPLETING AGENTS WITH SELECTIVE TOXICITY TO MALIGNANT CELLS

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by
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TO
MY PARENTS
AND
WIFE NEELAM

ABSTRACT

The present investigation was based on the following two observations. First, derivatives of styryl ketones and their corresponding Mannich bases have been found to possess antineoplastic activity with little or no affinity for the amino or hydroxyl groups found in nucleic acids. In particular the Mannich bases have a pronounced predilection for thiols and react approximately 240 times faster than the corresponding ketones. Hence these compounds may be free from undesired mutagenic and carcinogenic properties associated with present day alkylating agents. Second, L-histidinol which is a structural analog of the essential amino acid histidine has been found to protect normal cells from insult caused by various antineoplastic agents e.g. BCNU(N,N-*bis*-(2-chloroethyl)-N-nitrosourea), cyclophosphamide, *cis*-platinum etc..

(55a)

The aim of the present investigation was twofold and can be oulined as follows. It starts with the synthesis of a

compound(55a) which contains a Mannich base(a cytotoxic species) and L-histidinol. The Mannich base is a γ-piperidone with two arylidene groups attached to the piperidine ring adjacent to the carbonyl function. Second, the design, synthesis and antineoplastic evaluation of some potential alkylating species(A) was contemplated with a view to developing structure-activity relationships and of obtaining a compound with optimum activity.

(A)

To date, no success has been achieved in obtaining compound 55a. In the present report, three Series(Structure A) I(X=NH.HCl or NH), II(X=NCH_3.HCl or NCH_3) and III(X=$N^+(CH_3)_2$ X^-) were synthesized and evaluated against various tumour cell lines in vitro viz. P388 leukemic cells, L1210 leukemic cells and various human tumour cells. In a few cases, the compounds were evaluated against P388 lymphocytic leukemia in vivo. The results obtained did not produce any structure-activity relationships among different series or within a series. Variable results were obtained in various screens. Among the compounds tested against P388 leukemic cells in vitro, the activity ranged from 100 to 9700 times the activity of a reference compound BCNU.

The evaluation of various compounds in the *in vitro* L1210 screen produced the following results. In Series I, the compound with a 3,4-dichloro substituent(X=NH) was the most active compound(IC_{50}=0.3μM) and in fact was the most active derivative among the three series of compounds. It was more active than melphalan(taken as the reference drug). The bioactivity in Series III was found to be correlated with the MR(molecular refractivity) and π(hydrophobic) physicochemical parameters. A general increase in activity was observed with substituents having negative Hammett sigma values.

X-ray crystallographic studies were performed in an attempt to correlate the biological activity with the structural features of these compounds. Thermal denaturation studies revealed intercalation of the compounds of Series III into the minor groove of DNA, but this process was not found to be the sole mechanism of activity. No such binding was observed in the case of compounds of Series I or II. No correlation between cytotoxicity and DNA binding was evident.

Unsubstituted *mono*-benzylidene derivatives were synthesized and two of the compounds evaluated against P388 cells *in vitro* resulted in high albeit diminished cytotoxicity.

Various amides and carbamates of 3,5-*bis*-arylidene-4-piperidone were synthesized as prodrugs of the alkylating species i.e. the 3,5-*bis*-arylidene-4-piperidones. Among the

amides only one derivative(IC_{50}=0.3μM) was found to be more active than the parent drug(IC_{50}=2.5μM) and melphalan (IC_{50}=0.4μM) against L1210 leukemic cells *in vitro*. In the same system, all carbamates were found to be inactive with IC_{50} values greater than 43.7μM, except for one derivative which had an IC_{50} value of 0.15μM. The same compound was also found to be highly active in the *in vitro* NCI screen using human tumour cell lines and it has been taken up for stage II evaluation in an *in vivo* system.

Different *multi*-alkylating agents were prepared in which the additional alkylating arm was present as an *N*-acyl function in the drug molecule. No conclusive results could be obtained in relating cytotoxicity to the number of alkylating arms in these molecules.

β-Aminoalkylamides of 3,5-*bis*-arylidene-4-piperidone were prepared in order to produce water soluble analogs for two of the *multi*-alkylating agents which contained three alkylating arms. Different amines(constituting the β-amino functionality) having varying pKa values were used in order to govern their lability in releasing the active cytotoxic species by β-elimination(as in Mannich bases). The stability studies (pD=7.4, 37°C) using ^1H NMR spectroscopy of three of these representative compounds indicated an expected order of release of the cytotoxic species. Greater bioactivity was found with the compounds having slower rates of

decomposition. These compounds showed toxicity comparable to melphalan in several cases in both test systems i.e. the L1210 *in vitro* assay and NCI's human tumour cells *in vitro*. Once again at least one of the compounds with 3,4-dichloro substituents was found to be the most active compound, as was observed in the case of Series I.

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1.0.0.0 INTRODUCTION

1.1.0.0 Cancer and its Implications.

The word cancer was originally coined by Hippocrates, the ancient Greek physician, and is derived from the Greek word "karkinos" meaning "crab". It was so named because of its resemblance to a crab in that it tends to move erratically. In the present century, cancer strikes fear into the hearts of many and today it is a common cause of death, second only to diseases of the cardiovascular system(Pitot, 1986a). It has been defined as "a heritably altered, relatively autonomous growth of tissue"(Pitot, 1986b). Cancer basically is a disease characterized by disorder of cellular behaviour such as reduction or loss of effectiveness of the normal controlling influences that maintain cellular organization in the tissues. The defect lies within the cell itself and this fact has been proved by the observation that cancer cells when transplanted from an animal with a cancer to another of the same species, it continues to divide in the recipient and gives rise to a cancer indistinguishable from that of the donor animal(Scott, 1979). Tumours are tissue specific(Pierce et al., 1978); for example a tumour of the intestinal mucosa is identified as abnormally growing intestinal tissue and not abnormal lung epithelia even if metastasis from the intestine to the lung has occurred.

In general a malignant tumour is characterized by the following properties.

(1) Uncontrolled cellular proliferation and many but not all tumours exhibit very high rates of growth.
(2) A lack of cellular differentiation i.e. they may have lost the properties that characterize the cells of their tissue of origin.
(3) The ability to invade or the ability to infiltrate surrounding normal tissues through lymphatic and blood vessels.
(4) They often metastasize in the body through venous and lymphatic channels and establish and produce secondary tumour nodules at sites remote from the primary growth.

Benign tumours are distinguished from malignant tumours as follows. The former lack the ability to spread by infiltration and metastasis. Invasiveness and metastasis are the two major characteristics which have been suggested to make malignant tumour life threatening and leads to damage in the host at a variety of levels including creation of the following problems.

(1) Locally by pressure effects of tumour growth in the case of solid tumours.
(2) By destruction of tissues involved, both physically and in terms of their normal function.

(3) By systemic effects secondary to the localized growth.

Cancer research has long been mainly concentrated on the apparent uncontrolled growth of tumour tissues in relation to the normal counterpart. Tumours in general originate from the tissues that normally(or conditionally) proliferate by cell renewal mechanisms which maintain the steady-state size of tissues(Mackillop et al., 1983). Fig.1.1 represents the important functions of an idealized renewal system. The cell renewal system is critical in the tissue system in which cell production is balanced by cell loss and renewal proliferation occurs in the majority of human tissues. Stem cells as indicated in the Fig.1.1 represent the cell of origin i.e. a clone whose main functions are cell renewal and cell differentiation(Buick et al., 1977). By cell division stem cells can lead to population expansion, a population designated to perform the function of a particular tissue.

Apart from cell renewal populations, there are two other different types of cell populations which are present in animal tissues(Buick, 1984a).

(1) Cells in static populations do not undergo cell division in the postnatal life but instead undergo an increase in volume as the individual grows. The differentiated neurons of the central nervous system fall into this category.

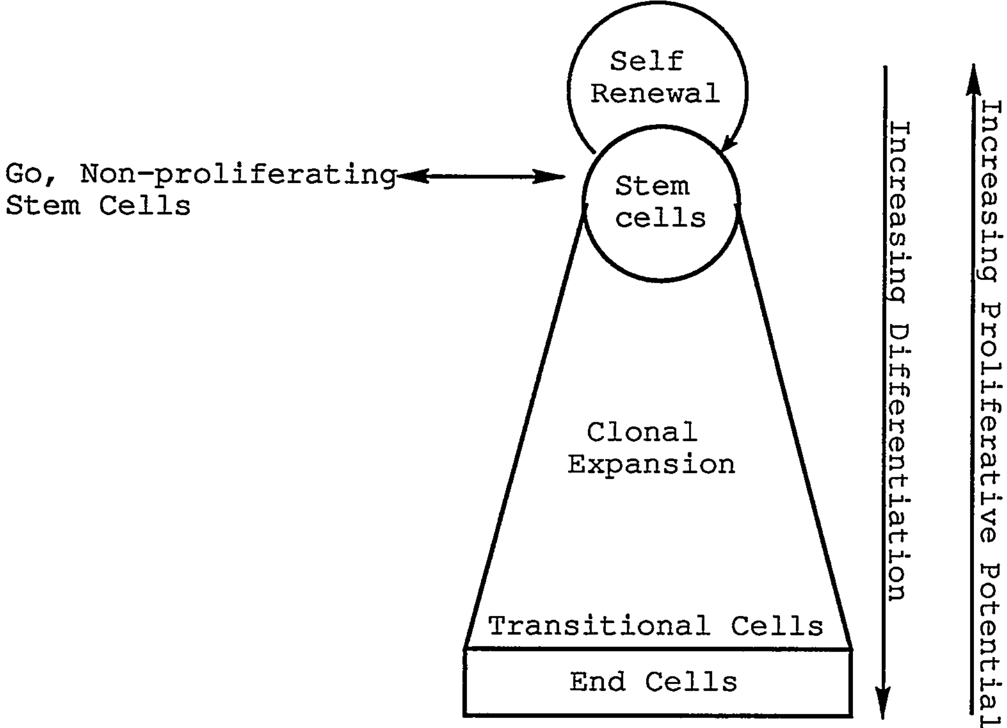


Figure 1.1 A schematic representation of the cell renewal system. Taken from Buick(1984b) and reproduced with permission of the copyright owner.

(2) Expanding populations have an increase in cell number that is complemented by an increase in the size of the total population. The rate of cellular proliferation decreases as the individual grows; for example the parenchymal cells of the liver and kidney as well as the exocrine and endocrine glands.

Cancer cells can be differentiated from normal cells on the basis of certain phenotypic derangements which are expressed as alterations in the morphological features and the amount and nature of the products of various cytological processes. These are often very useful in the diagnosis of the disease and are referred to as markers(Ruddon, 1981; Salmon, 1978). Some of these differences are listed below.

(1) Morphologically, cancer cells are more variable in size and shape than normal cells. The nucleus is often larger with more apparent chromatin and abnormal variation in the chromosome number and physical appearance can be seen microscopically.
(2) Both quantitative and qualitative changes in the cellular membrane surface composition are common. For example cells transformed by polyoma virus contained less N-acetylneuramic acid and N-acetylgalactosamine than their normal counterparts(Meezen *et al.*, 1969).
(3) Variation in the levels of certain enzymes e.g., enzymes involved in nucleic acid synthesis and metabolism. Other

enzymes for example γ-glutamyl transferase(GGT) have been observed in certain human epithelial tumours e.g., carcinoma of the larynx, oesophagus and urinary bladder (Fiala et al., 1980). An increase in levels of GGT was observed in tumours of prostrate, mammary glands and colon in quantities much greater than in the analogous normal tissues. GGT is responsible for the metabolism of amino acids i.e. their passage across the cell membrane(Orlowski and Meister, 1970) and the secretion of proteins(Binkley et al., 1975). This increase in GGT activity in turn can cause greater supplies of amino acids to cells and may become an important determinant in providing cancer cells with an advantage over normal cells enabling rapid growth, increased proliferation of the cells and/or accelerated glutathione hydrolysis(Fiala et al., 1980).

(4) The appearance of inappropriate gene products e.g., placental hormones and fetal antigens which are not normally seen in adult cells.

(5) Cancer cells undergo mitotic cell division more often than normal cells. Exceptions to this difference include the normal cells of bone marrow and gastrointestinal mucosa which show higher growth rates.

The exact cause of cancer is still unknown but it has been attributed to various possible agents e.g., viruses, chemical carcinogens and radiation. These agents in turn could induce

neoplastic transformation by a variety of processes(Pratt and Ruddon, 1979a).

(1) Genetic damage or mutation induced by chemicals or irradiation or both.
(2) Expression of abnormal genetic information induced by viral "oncogenes".
(3) The gain or loss of chromosomal material by neoplastic cells.
(4) Depression of "oncofetal" genes that are present but normally silent in adult cells.
(5) Alteration in the post-transcriptional "processing" of critical cellular macromolecules.

1.2.0.0 The Sulfhydryl Group and its Relationship to Cancer

Several studies have shown the importance of SH(thiol) groups in the complex process of cell division(Neufeld and Mazia, 1957; Sakai and Dan, 1959; Sandritter and Krygiver, 1959; Harris and Patt, 1969; Ord and Stocken, 1970; Schauenstein _et al_., 1977; Kosower and Kosower, 1978; Carow _et al_., 1982; Onfelt, 1983) and the "SH-SS" redox equilibrium was considered of the utmost importance i.e. a shift of equilibrium towards thiol formation could lead to cell division and when the equilibrium is towards the disulfide retardation of growth may occur. Hammett(1932) proposed that

the thiol group "is the naturally occurring, essential and specific chemical stimulus to cell division". However it is now widely accepted that it is not a single, causative, mitotic substance that is responsible for cell division, although high intracellular concentrations of thiols are required in plants, animals and possibly in micro-organisms for mitosis and division(Mazia, 1954; Mazia, 1959; Mazia, 1961a; Mazia, 1961b; Stern, 1956; Stern, 1959; Stern, 1960; Stern, 1962; Swann, 1957; Swann, 1958). Several careful studies have shown that thiols can, in certain cases, stimulate cell division under conditions where ordinary nutritional factors are absent (Harrington, 1967).

Accordingly, the participation and behaviour of mercapto groups in carcinogenesis have been studied and discussed in various reports(Needham, 1942; Brachet, 1950; Stern and Willheim, 1943; Calcutt, 1960; Calcutt, 1961; Calcutt and Coates, 1961; Calcutt and Doxey, 1962a; Calcutt and Doxey, 1962b; Calcutt et al., 1961). In general no consistent pattern of changes in thiol or thiol-disulfide levels in established tumours has emerged. Quite frequently high levels of thiols have been found in malignant cells(Lindler et al., 1963; Schauenstein et al., 1971; Nowak et al., 1972; Zakharova, 1979; Knock, 1981). The nonuniformity of thiol concentrations has been attributed to secondary effects associated with tumours in various stages of development and growth. In one of the studies(Fiala et al., 1976) hepatomas

induced by different chemicals led to variation in glutathione levels. The variations in glutathione levels were attributed to the differentiation state of hepatomas i.e. the greater the differentiation of the cells, the higher the levels of glutathione observed. Glutathione(GSH) is a tripeptide(γ-glutamyl-L-cysteinyl-glycine) which accounts for 90% of the intracellular nonprotein bound thiols(NPSH) (Wheeler et al., 1986).

A few reports have been published which indicate reduced thiol levels in certain types of malignant tissues(Nowak et al, 1974; Blagoeva and Balanski, 1982). In at least two studies(Dijkstra, 1964; Dijkstra and Pepler, 1964) a distinct increase in levels of soluble thiols accompanied the development and appearance of tumours in animals fed with carcinogenic dyes; this was not observed in animals fed with noncarcinogenic dyes. In other studies where resistance in the malignant cells occurred e.g., ovarian cancer and murine L1210 leukemia to alkylating agents such as melphalan, cisplatin(Ozols et al., 1987) and L-phenylalanine mustard (Suzukake et al., 1983) respectively, it was attributed to the elevation of intracellular glutathione levels compared to the sensitive cell lines from which they were derived.

Many carcinogens such as hydrocarbons, hormones, alkylating agents, nitrosamines, 4-nitro-quinoline-N-oxide, lactones, quinones, etc., react chemically with thiol groups both in

vivo and *in vitro*. Such interactions are suspected of being a part of carcinogenesis.

The relationship between concentrations of thiols and carcinogenesis has been critically examined by Harrington (1967), who proposed the following hypotheses. According to Harrington, at least one form of the carcinogenesis process commences with the initial inhibition of cell division by carcinogens reacting directly with thiol groups which are critical to cell division. Following this, high levels of free(acid soluble) thiols are produced as an overcompensatory response to the initial inhibition. These elevated levels of thiols are subsequently maintained and act as initiators of mitotic cell division (Swann, 1957; Swann, 1958).

Harrington(1967) further explained the specific mechanism involved in this form of carcinogenesis as the loss of feedback control of glutathione synthetase(Fig.1.2), the enzyme which was originally inhibited by the carcinogens. This leads to a depressed state in the biosynthesis of glutathione, which in turn causes an overproduction of glutathione. In other words, the carcinogen stimulates the formation of glutathione synthetase by limiting the synthesis of glutathione. It was suggested(Harrington, 1967) that this overproduction of a synthesizing system and its product, both involved in co-ordination of cell division, would result in

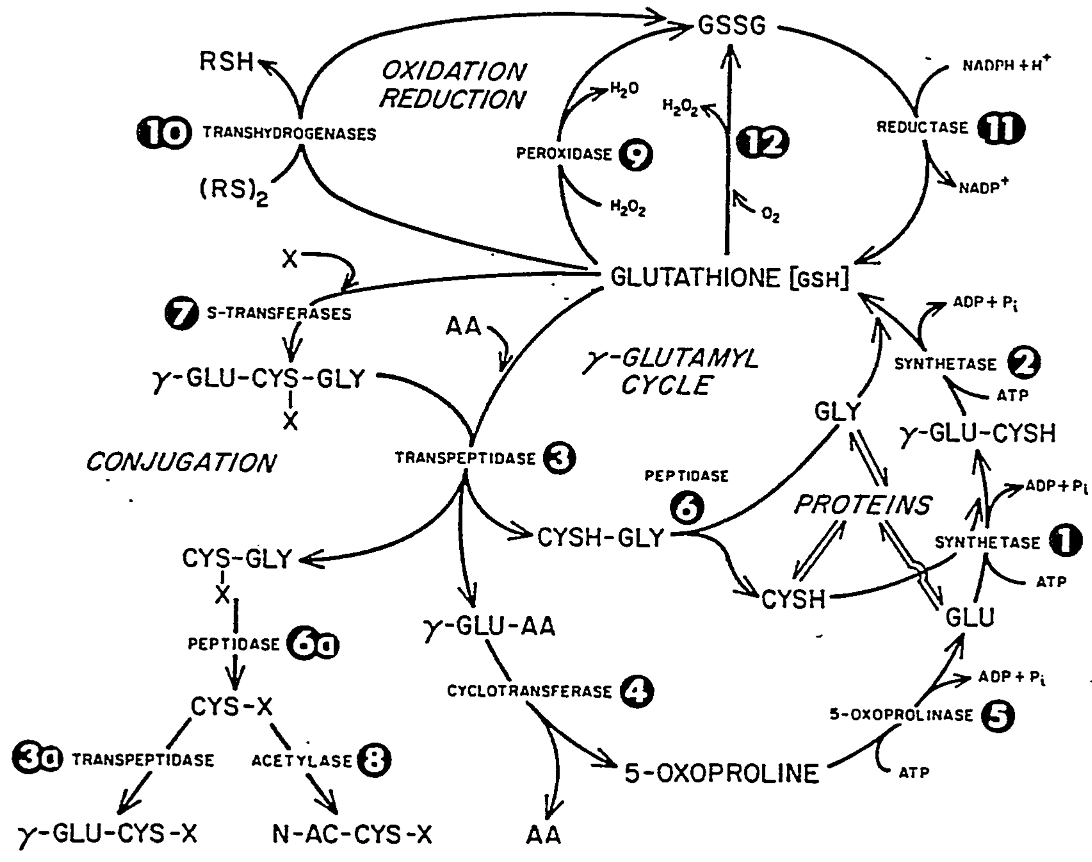


Figure 1.2 A schematic representation of metabolism and function of glutathione. Reaction : **1**. γ-glutamyl cysteine synthetase, **2**. GSH synthetase, **3** and **3a**. glutamyl transpeptidase, **4**. γ-glutamyl cyclotransferase, **5**. 5-oxoprolinase, **6** and **6a**. dipeptidase, **7**. GSH S-transferases, **8**. N-acetylase, **9**. GSH peroxidase, **10**. transhydrogenases, **11**. GSSG reductase, **12**. oxidation of GSH by oxygen(O_2); conversion of GSH to GSSG is also mediated by free radicals. Taken from Meister and Anderson(1983) and reproduced with permission of the copyright owner.

the loss of control of these processes with the development of cancer as an end result.

1.3.0.0 The Treatment of Cancer

The treatment of any disease depends mainly upon the methods available and on the goal to be achieved. In cancer treatment, the methods available include surgery, radiotherapy, chemotherapy, hormone therapy and immunotherapy. Each of these treatments is effective and have been used either alone or in combination. Yet at present there is no completely satisfactory method to treat all types of cancer. Surgery has been used successfully in eradicating primary or localized tumours. It is still the treatment of choice when the disease is localized. Radiotherapy can achieve the same effects in certain radiosensitive tumours. Chemotherapy is indicated where the cancer has metastasized to other areas of the body since it is the only systemic therapy which could attack microstases. In combination with surgery or radiotherapy or both(adjuvant therapy), chemotherapy has increased the survival rates for a number of solid tumours that were formerly treatable by only one therapeutic modality whereas immunotherapy has not been very successful and is still in the experimental stages. The type of treatment varies with each tumour. Ideally, cancer chemotherapy starts with an aim of curing or regressing the tumour resulting in the patient being returned to an active

life. On the other hand, if the tumour is disseminated and is nonresponsive to treatment, palliative therapy becomes the major objective.

The major drawback to the use of chemotherapeutic agents is their relative inability to select between neoplastic and non-neoplastic cells. The reason is largely the lack of qualitative differences between malignant and normal cells and hence most of the chemotherapeutic agents have been developed as antiproliferatives. The normal cells which are often affected are the cell renewal systems with high rates of cell division such as bone marrow, intestinal mucosa and hair follicles which bear the brunt of the toxic effects.

The other factors which often limit the clinical usefulness of a chemotherapeutic agent is the emergence of resistance. A number of cellular mechanisms have been proposed to be responsible for this drawback which include altered metabolism in the malignant cells, impermeability to the active species of the drug, increased activity of inhibited enzymes, increased cellular repair activity and others(Cline, 1971). The approach to overcome this problem has been to use larger doses of the same drug, but at the same time the use of higher doses is associated with higher incidences of toxic effects. One approach to delaying the emergence of resistant malignant cells is the rational use of several drugs with different modes of action in combination.

Drugs commonly used in cancer chemotherapy can be traditionally classified as follows.

(1) Alkylating agents e.g., nitrogen mustards, nitrosoureas, alkyl sulphonates

(2) Antimetabolites e.g., 5-fluorouracil, cytarabine, methotrexate

(3) Antibiotics e.g., actinomycin-D, mitomycin-C, bleomycin

(4) Vinca alkaloids e.g., vincristine(VCR), vinblastine(VLB)

(5) Enzymes e.g., L-asparaginase

(6) Hormones e.g., glucocorticoids, estrogens, androgens

(7) Radioactive isotopes e.g., phosphorus and iodine

(8) Miscellaneous agents e.g., cisplatin, hydroxyurea, procarbazine.

Fig.1.3 represents the mechanism and sites of action of various antineoplastic agents. Most of these agents interfere with synthetic processes and metabolic pathways in the cancer cells which are essential for cell division and for continued growth i.e. by inhibiting the synthesis of DNA, RNA and proteins. The toxic effects associated with these agents arise from the fact that any chemical and biological event that is mandatory for cancer cell functions is usually essential for normal cells as well, thereby making normal cells the potential targets for a cytotoxic drug. However, L-asparaginase is cytotoxic only to malignant cells. Normal

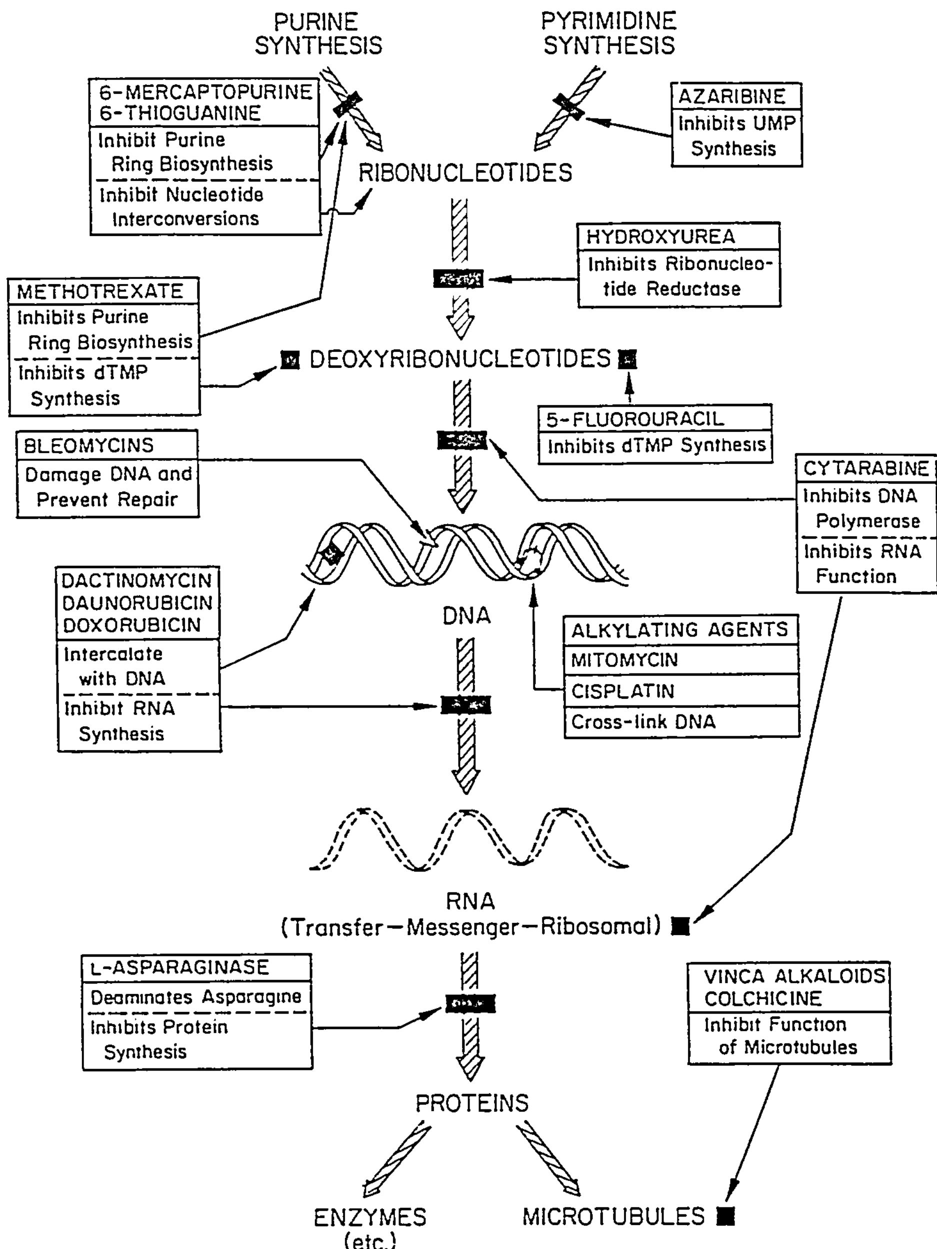


Figure 1.3 Sites and mechanism of action of various chemotherapeutic agents used against cancer. Taken from Calabresi and Parks (1980a) and reproduced with permission of the copyright owner.

cells contain asparaginase synthetase and are therefore able to produce their own asparagine. But some tumour cells lack this enzyme(a qualitative difference) and depend upon an exogenous supply of asparagine. The administration of L-asparaginase removes this exogenous source and the asparagine-sensitive deficient tumour cells therefore die. This enzyme has been used with moderate success in the treatment of human leukemia i.e. acute lymphocytic leukemia. Unfortunately, only a few tumours lack L-asparaginase (Patterson, 1975; Oettgen, 1975).

The antitumour agents affect the tumours by interfering with events occurring at different phases of the cell cycle. Accordingly, cytotoxic agents can be classified into two broad categories namely, cell cycle phase specific(Fig.1.4) and cell cycle phase nonspecific agents. The cell cycle phase specific agents are cytotoxic against cells in a single phase of the cell cycle. These agents are highly effective when the cells are actively dividing. And theoretically, multiple repeated fractions of a given drug(scheduling) should allow more access to relevant target cells than one large dose.

Cell cycle phase nonspecific agents have cytotoxic effects against various phases of the cell cycle. These agents produce their lethal effect by interfering with DNA-replication or DNA-repair which occur on subsequent exposure

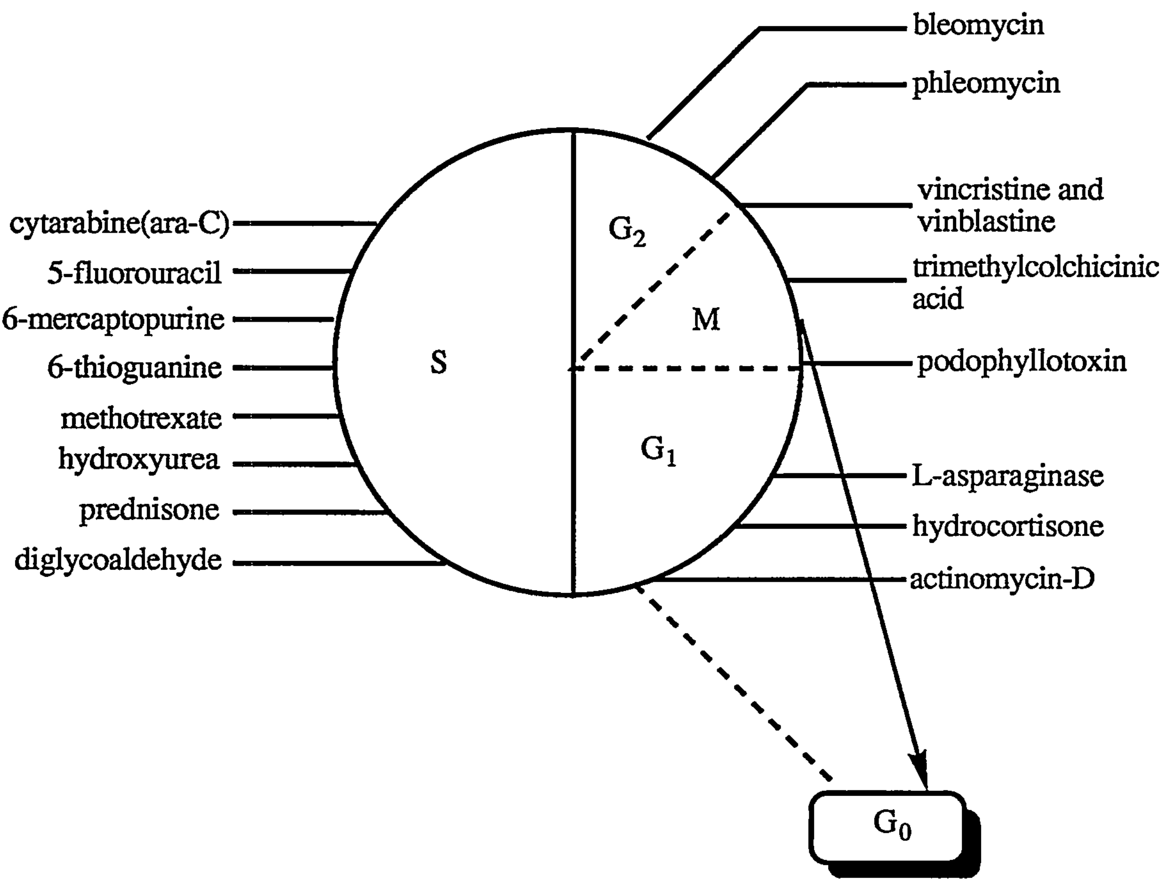


Figure 1.4 The main sites of action of the antineoplastic drugs in relation to the cell cycle.Where $\mathbf{G_1}$:presynthetic phase, **S**:synthesis phase(DNA synthesis), $\mathbf{G_2}$:postsynthetic phase, **M**:mitosis phase and $\mathbf{G_0}$:nonproliferative phase

to the drug. Theoretically, a single large dose of these agents should kill the same number of cells as multiple repeated fractions that total the same amount as one large dose. Fig.1.4 depicts the different stages of the cell cycle and the agents which are effective against them. Examples of cell phase nonspecific agents include the following groups of drugs.

(a) Antitumour antibiotics e.g., dactinomycin, doxorubicin, daunorubicin.
(b) Nitrosoureas e.g., semustine, carmustine, lomustine.
(c) Alkylating agents e.g., busulfan, chlorambucil, melphalan, mechlorethamine.
(d) Miscellaneous agents e.g., dacarbazine, cisplatin.

1.4.0.0 The Alkylating Agents and their Mechanism of Action

The alkylating agents are extremely reactive compounds that form covalent bonds with biomolecules which contain nucleophilic centres such as amino, sulfhydryl or imidazolyl moieties. These groups are found in proteins and nucleic acids and hence they are the potential sites of alkylation. But the evidence suggests that alkylation of nucleic acids, primarily DNA, is critical to the cytotoxic effects of these compounds(Wheeler, 1962; Crathorn and Robert, 1966). A variety of functionalities such as phosphate groups, the N^7-

atom of guanine and the oxygen atoms at positions 4 and 6 of thymine and guanine respectively in DNA interact with alkylating agents. In approximately 90% of the cases it has been observed that it is the N^7-atom of guanine which is alkylated(Creasy, 1981).

The various classes of alkylating agents that are employed in cancer chemotherapy include the following groups of compounds.

(1) Nitrogen mustards e.g., mechlorethamine(1), melphalan (2)
(2) Nitrosoureas e.g., carmustine(3), lomustine(4)
(3) Alkyl sulfonates e.g., busulfan(5)
(4) Aziridines e.g., triethylenemelamine(7), triethylenethiophosphoramide(6)
(5) Triazines e.g., 5-(3,3-dimethyltriazeno)-imidazole-4-carboxamide(8)
(6) Epoxides e.g., dianhydrogalactilol(9)

In general, alkylating agents can be divided into two groups namely, monofunctional and polyfunctional compounds. The monofunctional compounds have only one alkylating arm whereas polyfunctional compounds have more than one alkylating arm. Polyfunctional agents are more useful clinically since cytotoxicity has been found to be closely related to the extent of cross linkage(Roberts _et al_., 1968).

(1) (2)

(3) (4)

(5) (6) (7)

(8) (9)

There are different ways an alkylating agent can react with DNA as depicted in Fig.1.5. The reaction commences with an initial conversion to an active intermediate which frequently is a carbonium ion. This process may be illustrated by reference to mechlorethamine. At body pH(7.4), one of the 2-chloroethyl side chains loses a chloride ion by a first-order S_N1 intramolecular cyclization reaction. This reaction leads to the formation of a highly reactive ethylenimonium ion intermediate which then opens to release a carbonium ion which in turn reacts with various nucleophiles(Price, 1975). As mentioned earlier, the highly favoured position of attack is the N^7 atom of guanine. Guanine in DNA exists predominantly in the keto tautomer form and pairs with cytosine through hydrogen bonding. However, when the N^7 atom of guanine is alkylated(a quaternary ammonium nitrogen is now formed), the guanine residue becomes more acidic and the enol tautomer is favoured. Guanine in this form can pair with thymine. These chemical reactions result in faulty base pairing which may lead to possible miscoding. Alkylation of the N^7 atom of guanine can also labilize the imidazole ring thereby making it prone to opening or depurination by excision of guanine residues. In the case of bifunctional agents, the second arm can undergo a similar chain of reactions and thus alkylate a second guanine residue or another nucleophilic moiety such as an amino or a sulfhydryl group of a protein, leading to cross linking. Cross linking can occur between two nucleic acids of either the same strand

Figure 1.5 Mechanism of action of alkylating agents. Taken from Calabresi and Parks (1980b) and reproduced with permission of the copyright owner.

or two strands of a double helix. It can also occur between a nucleic acid and a protein. Alternatively, the second chain may react with water, a weak nucleophile, leading to overall monoalkylation of the DNA(Pratt and Ruddon, 1979b).

In general all these processes interfere with DNA-replication, RNA-transcription and eventually prevent cell division. The effects of alkylating agents are not restricted to any phase of the cell cycle and their activity does not depend on interference with DNA synthesis.

1.5.0.0 The Prodrug Concept and its Application to Alkylating Agents

The therapeutic potential of a new drug can be limited by various unfavorable processes commencing with the site of administration until it reaches its site of action as discussed in Section 1.7.0.0. There are a number of techniques that are employed in optimizing therapeutic potentials which have been discussed in detail in Sections 1.8.3.0 and 2.0.0.0. Prodrug design, earlier known as drug latentiation, is also a commonly applied technique that often results in enhancement of the therapeutic utility of a drug(Stella, 1975; Stella et al., 1985). In general, the prodrug approach involves derivatization of the parent drug and it has been used for the following reasons(Bundgaard and Hansen, 1981) namely to

(1) enhance bioactivity and passage through various biological barriers
(2) increase the duration of pharmacological effects
(3) decrease toxicity and adverse reactions
(4) improve organoleptic properties and
(5) improve the stability and solubility properties.

Prodrug design has been defined as the chemical modification of a biologically active agent to form a new derivative which liberates the parent drug *in vivo*(Kupchan *et al*, 1965). The drugs which were in use, before this concept was recognized were called "accidental" or "empirical" prodrugs and examples include chloral hydrate(10) which is converted *in vivo* to its pharmacologically active central nervous system depressant, trichloroethanol. Similarly, methenamine(11) an urinary tract

$Cl_3C—CH(OH)_2$

(10)

(11)

antiseptic which releases formaldehyde(the active agent) in the normally acidic bioenvironment of the kidney. The prodrug concept of drug design is illustrated in Fig.1.6. The prodrug modification may involve formation of a simple ester derivative of an acidic parent drug or the inclusion of

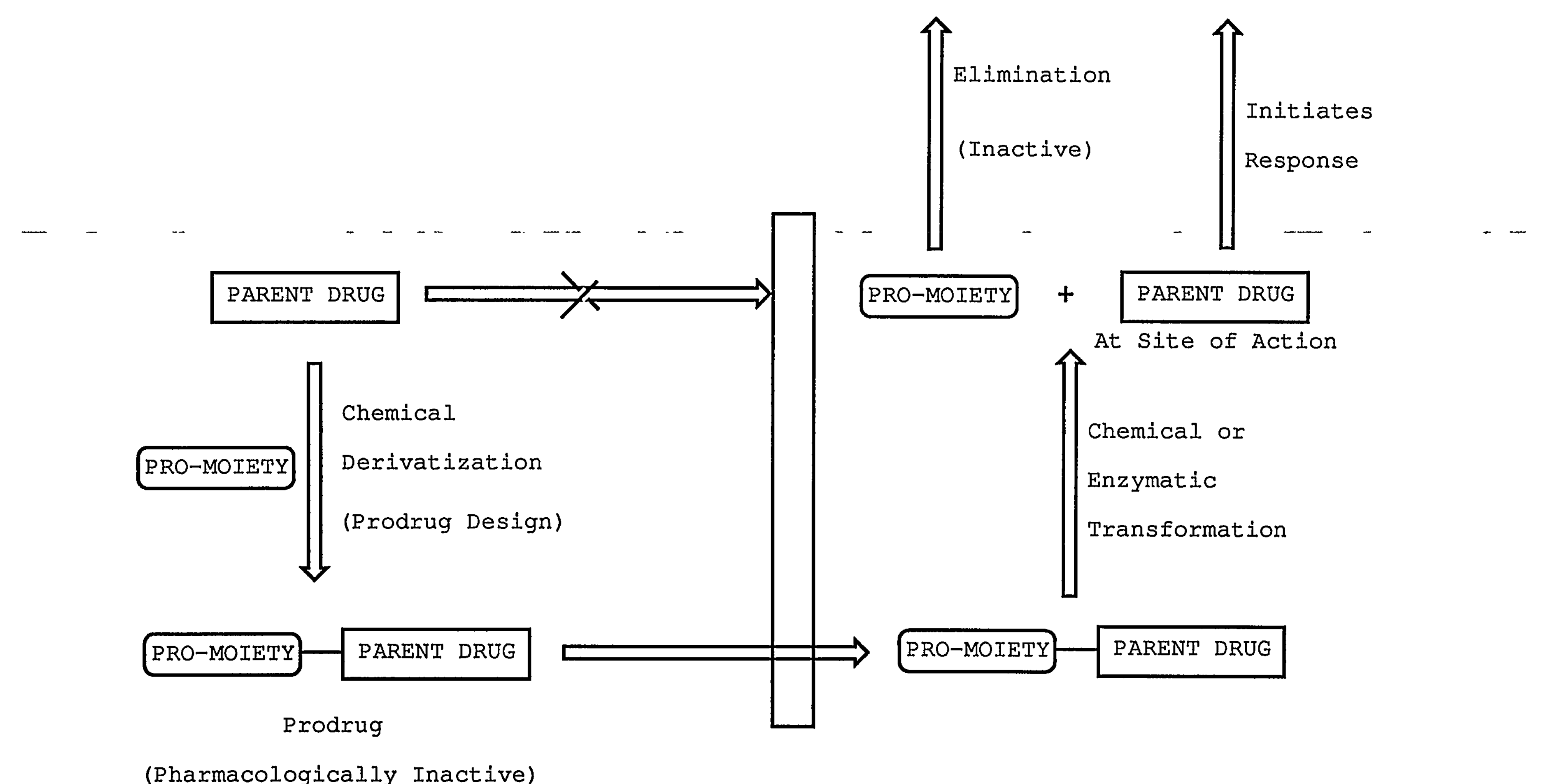


Figure 1.6 The Prodrug Concept

structural features of the promoiety requiring sophisticated chemical or biological mechanisms to generate the parent drug species. The primary requirements of a therapeutically useful prodrug are as follows(Riley, 1988).

(1) The prodrug itself should not have any significant pharmacological activity.
(2) Upon biotransformation, it should quantitatively yield the active parent drug and a pharmacologically inactive promoiety.
(3) The covalent bond between promoiety and the parent drug should be of balanced stability to permit pharmaceutical formulation of the prodrug.
(4) The covalent linkage must be labile enough to undergo either chemical or enzymatic cleavage *in vivo* at the desired site and/or time in the biological milieu.

The structures of most of the prodrugs are bipartite in nature consisting of a pharmacologically inactive promoiety covalently linked to the parent drug structure. Other prodrug types include tripartite prodrugs in which a promoiety and the parent drug are linked together by a special type of connector group(promoiety-link-parent drug). The tripartite prodrug concept has been used in cases where the problem of excessive stability of the covalent link in bipartite prodrugs reduces their ability to liberate the active drug *in vivo*(Carl *et al*., 1981)

As was mentioned earlier, there are problems associated with the use of alkylating agents e.g., the nitrogen mustards. These drugs play an important role in modern cancer chemotherapy but are nonselective owing to their high chemical reactivity and general toxicity. These agents have been referred to as general cell poisons and hence their biological effects are accordingly nonspecific. In the preparation of prodrugs of alkylating agents, various biochemical properties and enzymatic pattern differences between normal and cancerous cells have been explored to increase the targeting selectivity in the hope that with selective activation of the compounds in the cancer tissues, the cancerotoxic efficacy would also increase.

The application of the prodrug approach to alkylating agents led to the second generation of clinically useful antineoplastic drugs(Niculescu-Duvaz and Balaban, 1990a) such as cyclophosphamide(12), isophosphamide(13) and estracyst (14). Cyclophosphamide is the prototype of these compounds, although its mechanism of action does not correspond to the initial hypothesis. Cyclophosphamide is chemically inactive($t_{1/2}$>7 days) and is activated by microsomal enzymes. Isophosphamide, a triazene nitrogen mustard mitozolamide(15) and estracyst exhibit similar behaviour. According to the mechanisms of conversion to an active drug, prodrugs of

H
N
N$(CH_2CH_2Cl)_2$
P
O
O

(12)

CH_2CH_2Cl
N
NCH_2CH_2Cl
P
O
O

(13)

OPO_3H_2
O
$(ClH_2CH_2C)_2N$—C—O

(14)

N
N
N
N
N—CH_2CH_2Cl
H
O

(15)

H_2NOC
N
O_2N
NO_2

(16)

alkylating agents have been classified as follows(Niculescu-Duvaz and Balaban, 1990b).

(1) Compounds activated by oxidation. In this case activation of prodrugs is caused by enzymatic systems such as cytochrome P-450 dependant monooxygenases. The agents include cyclophosphamide, isophosphamide and mitozolamide.

(2) Compounds activated by reduction by enzyme systems such as nitro-reductases e.g., CB 1954(16) or other NADPH dependant reductases. Benzo- and napthoquinone derivatives(17,18,19 and 20) are activated by these enzymatic systems. These agents were designed to achieve a selectivity against hypoxic(cancer) cells because of their higher reducing potential as opposed to normal cells.

(3) Compounds activated by enzymatic hydrolysis. Urethane nitrogen mustards e.g., estracyst belong to this class of agents.

(17) R = CH_3, OCH_3

R^1= H, $H_3C(CH_2)_{14}$

(18) R = H, CH_3, $CH_3(CH_2)_{14}$-

X = Cl, CH_3COO-

(19) R = Cl, Br, CH_3COO-

(20) R = Cl, CH_3, CH_3COO-

1.6.0.0 Intercalating and Nonintercalating DNA Binding Agents

A great majority of clinically used antineoplastic agents exert their cytotoxic actions by interfering with the functions of DNA. For example, antimetabolites and certain other compounds inhibit DNA replication either directly or indirectly. Alkylating agents, on the other hand, react chemically with DNA by forming covalent bonds thereby interfering with DNA replication and RNA transcription by causing breakage of both the DNA molecule and also the cross linking of the DNA double helix. These interactions eventually prevent cell division.

Recently another class of agents with direct action on DNA include agents which mediate their cytotoxic action by binding noncovalently(unlike alkylating agents) and reversibly to DNA. These agents ultimately lead to either inhibition of nucleic acid synthesis or initiate DNA breakage and prevent repair phenomena. These compounds can be further

subdivided into two broad categories namely, the ones which bind to DNA by intercalation of one or more planar, aromatic portions of these molecules between the DNA base pairs and the agents which bind to DNA but do not show any evidence of intercalation i.e. nonintercalators(Baguley, 1982).

1.6.1.0 Intercalators

The antitumour antibiotics, for example actinomycin-D, adriamycin and bleomycin, form a typical class of agents which owe at least part of their cytotoxicity to DNA intercalation. Intercalation is usually associated with molecules which have relatively flat polycyclic areas. In this process the intercalating agent slips into the space between two base pairs by unwinding the deoxyribose-phosphate backbone of the helix(Fig.1.8). From X-ray diffraction studies it has been found that the distance between the centres of the atoms in adjacent base pairs on a strand is 3.36Å(Fig.1.7)(Albert, 1985c). The additional space needed for an intercalator to fit in comes from unwinding of the double helix. In the case of ethidium bromide(21), an aminoacridine, the unwinding angle of the double helix is 12°(Fuller and Waring, 1964), which in turn creates an additional space of 3.36Å for ethidium bromide to occupy. The intercalated portion of the drug lies perpendicular to the helical axis and binds to the base pairs above and below by van der Waals and other noncovalent forces. Additional

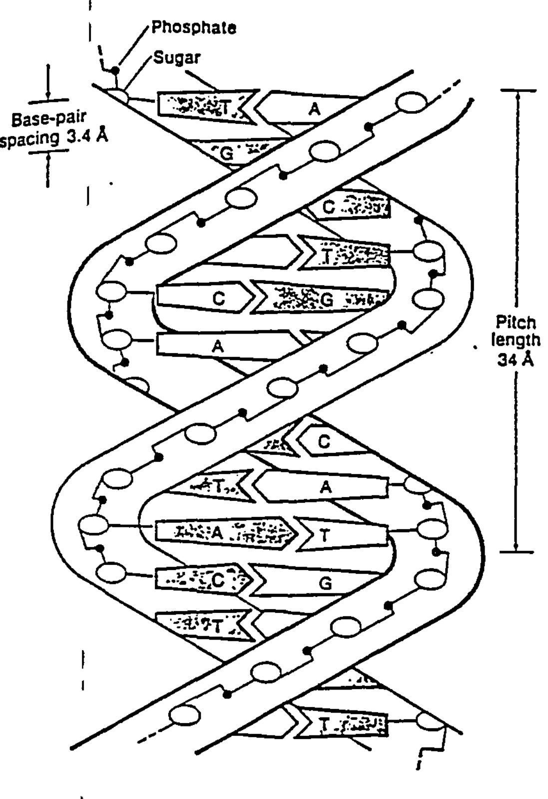


Figure 1.7 DNA double helix indicating arrangement of base-pairs. Taken from Zubay and Marmur(1988b) and reproduced with permission of the copyright owner.

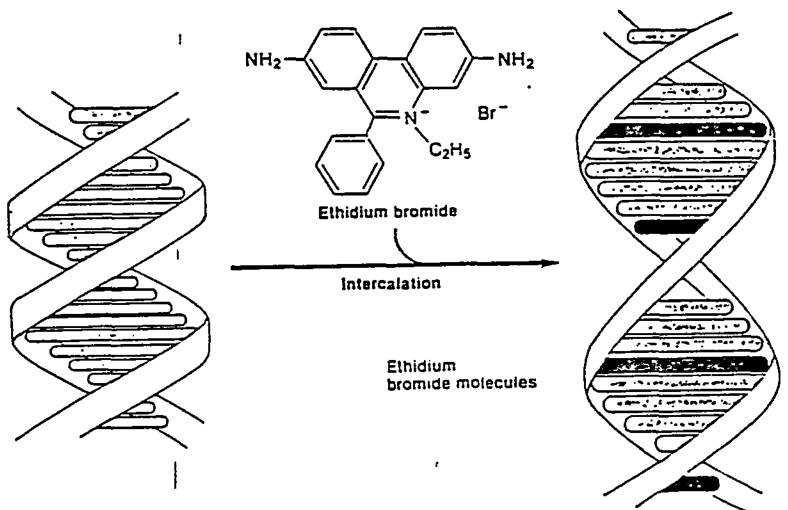


Figure 1.8 DNA intercalated with ethidium bromide. Taken from Zubay and Marmur(1988c) and reproduced with permission of the copyright owner.

(21)

binding can occur between the nonintercalated portion of the drug and the DNA outer surface. For example daunorubicin(22)

(22)

which is an intercalator was suggested to have a specific hydrogen bond between the 4'-hydroxy group of its sugar moiety and a phosphate group on the DNA. In addition an electrostatic attraction between the 3'-amino group and DNA phosphate anion is thought to occur(Pigram et al., 1971). The evidence for intercalation comes from the changes in the

properties of DNA such as an increase in viscosity, decrease in buoyant density and increase in thermal denaturation temperature which in turn reflects unwinding, lengthening and stiffening of the double helical structure. These changes are used as an indication of the occurrence of intercalation. Other indicators include chemical shifts in the ^{1}H-NMR spectrum of base pairs and the drug molecule(Remers, 1984). The lethal action of intercalating agents is usually caused by impairment of DNA as a template rather than direct action on enzymes or competition with cofactors. For example, ethidium bromide and daunorubicin inhibit DNA-dependent DNA polymerase I and DNA-dependent RNA polymerase from E.coli (Waring, 1975; Di Marco et al., 1965), whereas actinomycin-D selectively inhibits DNA-dependent RNA polymerase(Reich et al., 1961; Behr and Hartmann, 1965). And hence cell division (mitosis) fails to occur.

1.6.2.0 Nonintercalators

Nonintercalators in general bind to DNA without inserting any part of the bound molecules between the base pairs. They may interact in the helical grooves or along the surface of the phosphate backbone of the double helix. In general the major groove(Fig.1.9 and 1.10) is utilized by the control proteins (repressor and promoters)(Takeda et al., 1983; Ohlendorf et al., 1982) whereas minor groove(Fig.1.9 and 1.10) specificity is shown by xenobiotics(Zimmer and Wahnert, 1986).

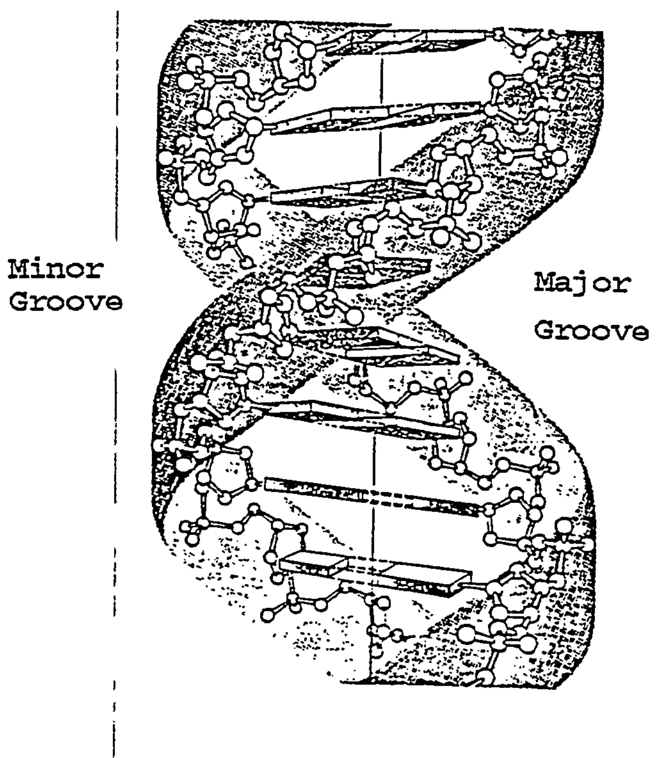


Figure 1.9 Major and minor grooves as seen in the DNA double helix. Taken from Zubay and Marmur(1988a) and reproduced with permission of the copyright owner.

MAJOR GROOVE
12Å WIDE

MINOR GROOVE
6Å WIDE

GUANINE : CYTOSINE

MAJOR GROOVE
12Å WIDE

MINOR GROOVE
6Å WIDE

ADENINE : THYMINE

Figure 1.10 The Major and minor grooves present in the DNA helix. Taken from Stryer(1988) and reproduced with permission of the copyright owner.

Although alkylating agents can act by nonintercalation mechanisms, the emphasis here pertains to agents which bind noncovalently or physically to DNA without intercalation. Compounds in this class include the antitumour antibiotics such as distamycin(23), netropsin(24) and synthetic compounds

(23)

(24)

e.g. diarylamidines (25, 26), phthalanilides(27), bisguanylhydrazones(28) and bisquaternary ammonium heterocycles(29). Many of these nonintercalators bind selectively to poly (dA.dT) and a few bind selectively to poly(dG.dC) synthetic DNAs'. Among the most widely studied and best defined drugs are netropsin and distamycin. Netropsin and distamycin bind specifically to dA.dT base pairs in the minor groove. It has been suggested that dA.dT specificity in the case of

(25) X : NHN=N Berenil

(26) X : CH=CH Stilbamidine

(27)

(28)

(29)

netropsin is a result of potential hydrogen bonding by the hydrogen donating group of the carboxamide system i.e. >CONH with O(2) atoms of thymine and N(3) atoms of the adenine bases along a sequence of four to five dA.dT pairs within the minor groove(Zimmer and Wahnert, 1986).

The same tests which indicate the process of intercalation are used to differentiate between nonintercalation and intercalation. The most promising evidence for nonintercalation is the absence of unwinding of closed circular DNA combined with positive evidence of DNA binding(Zimmer, 1975; Braithwaite and Baguley, 1980). The structural requirements of these compounds to act as nonintercalators has been studied in detail by Baguley(1982). The two basic features are coplanarity of certain groups and the presence of two quaternary centres. Substitution of one strongly charged group by an amine led to reduction in *in vitro* toxicity and abolished antileukemic activity in mice.

Apart from antitumour activity, many of the nonintercalative DNA binding drugs exhibit a broad spectrum of antiviral, antibacterial and antiprotozoal activity(Zimmer and Wahnert, 1986). In most cases these agents have very limited clinical use at present because of their toxicity. Various *in vitro* studies have shown that the cytotoxic effect of these agents could be attributed to the inhibitory effect on the DNA-

dependant nucleic acid synthesis caused by direct binding of the nonintercalators to the DNA template.

1.7.0.0 Major Processes involved in Drug Action

For a drug to produce its action it normally has to travel through a very complex route. Many competing events take place between the introduction of a drug and its final interaction with an intended target i.e. a specific receptor in which the desired response is required. The frequent failures to find a simple relationship between chemical structure, physical properties and biological action may be accounted for in terms of this complex nature of biological systems. The complicated process of drug action can be divided into three discrete phases namely the pharmaceutical, pharmacokinetic and pharmacodynamic phases(Ariens and Simonis, 1974). Fig.1.11 illustrates these phases of drug action and are discussed below in further detail.

1.7.1.0 The Pharmaceutical Phase

This phase deals with the chemical and physical environment of the drug prior to its absorption into the living system namely the dosage form of the drug. This phase can be referred to as the phase of development of a drug into a delivery system. The delivery system can be one of the traditional forms such as tablets, capsules, injections,

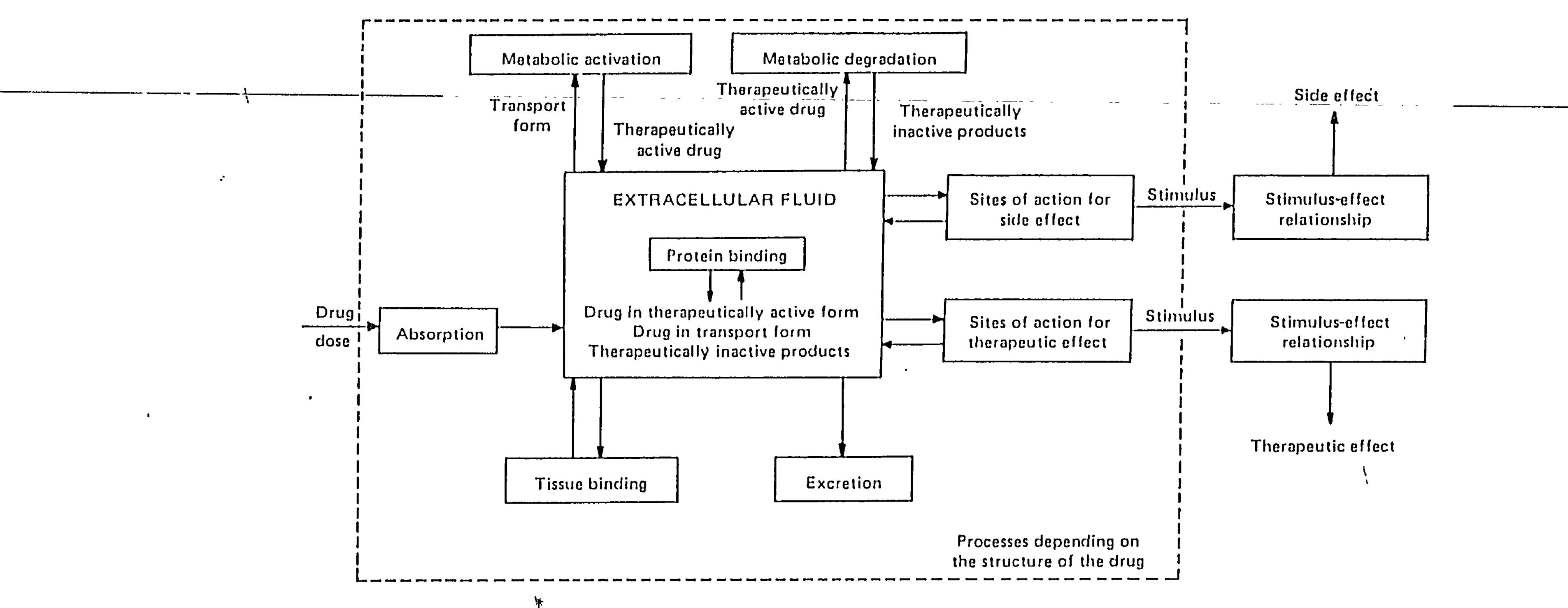


Figure 1.11 Major processes involved in drug action. Taken from Ariens(1966) and reproduced with permission of the copyright owner.

creams/ointments etc., or one of the new drug delivery modes such as transdermal delivery patches or implanted devices. The two main barriers encountered in this phase while developing a dosage forms include

(1) the aesthetic properties of a new molecule may limit its usefulness e.g., odour, taste, pain at the site of injection, gastrointestinal irritability, etc..
(2) formulation problems e.g., the instability of the drug or because of its physicochemical properties, the drug cannot be incorporated into a particular type of dosage form.

The important concern of this phase is the availability of the active drug for absorption.

1.7.2.0 The Pharmacokinetic Phase

After the drug has been released from its formulation it enters the pharmacokinetic phase which is the period during which the drug is transported to its target organ. This phase of drug action includes absorption, distribution, biotransformation and elimination of the drug. Immediately upon release, the drug gets absorbed into the blood and is distributed to various tissues in the body by making its passage through numerous membranes. If the site of action is in the central nervous system, it will have to pass through

very restricted barriers made of membranes e.g., the blood-brain barrier. The different characteristics of the drug molecule which could influence its passage through membranes include its molecular size and shape, solubility at the site of absorption, degree of ionization and relative solubility of its ionized and unionized forms.

While in the blood, a drug can bind to the blood proteins and become exposed to metabolic attack especially on its passage through the liver. An agent that is totally and strongly bound to the plasma proteins particularly albumin has no access to cellular sites of action nor can it be metabolized and eliminated. Since the drug is distributed throughout the body, only a small fraction of it will be available at the appropriate target and some of the drug could elicit responses from other tissues as well.

Metabolism exists primarily to modify the structure of any invading compound in the body so that it can be more readily eliminated. This process involves a number of enzymes which are capable of derivatizing a range of foreign structures or xenobiotics. The drug metabolites produced are usually more polar and less lipid soluble than the parent compound and thus excretion is enhanced. Biotransformation often results in inactivation of the compound but sometimes it results in the formation of metabolites with pharmacological activity, which could be either similar to or different from those of

the parent drug molecule. In addition, these metabolites may be responsible for important toxic effects that follow drug administration.

Advantage sometimes has been taken of drug metabolizing enzymes by administration of an agent in an inactive form e.g., cyclophosphamide is a prodrug which is used in cancer chemotherapy. If the drug metabolites are active, termination of action takes place by further biotransformation or by excretion of the active metabolites in the urine.

The major importance of this phase lies in the bioavailability of the active drug molecule to the target organ.

1.7.3.0 The Pharmacodynamic Phase

This phase deals with the drug-receptor interaction and may be defined as 'the study of the biochemical and physiological effects of drugs and their mechanism of action'(Gilman et al., 1980). It is known that the affinity of a drug for a specific macromolecular component of the cell(receptor) and its intrinsic activity are intimately related to its chemical structure. The minor modifications in the drug molecule (structurally specific) may not alter all action and effects of a drug equally whereas stereoisomers in particular can result in major changes in pharmacological properties. This

is the phase of interest to the medicinal chemist, where one may explore structure-activity relationships and try to develop a congener with a more favorable ratio of therapeutic to toxic effects or to improve on more acceptable secondary characteristics of the parent drug. The major objective of this phase is to optimize the biological effect.

After the drug has initiated a response, normally it is eliminated from the body. The time taken for it to be eliminated varies enormously between individual drugs. The more water soluble the original drug is, the more rapidly it is eliminated directly whereas very lipophilic drugs are taken up by the fatty tissues of the body and are retained there for considerable periods of time. Indirectly, the lipid soluble drugs are converted to more water soluble derivatives by metabolic processes and are then eliminated.

The major routes of elimination are through urine and faeces. The excretory organs eliminate polar compounds more efficiently than substances with high lipid solubility. Substances excreted in the faeces are mainly compounds which are unabsorbed after oral ingestion or drugs and metabolites which are excreted in the bile and are not reabsorbed from the intestinal tract. Anaesthetic gases are usually excreted through the lungs in expired air. Various other routes of elimination include sweat, tears and milk.

1.8.0.0 Approaches to the Development of New Anticancer Drugs

The fundamental goal of the drug designer of anticancer drugs is to exploit the molecular features of cancer cells which are distinct from those of normal cells. In practice, it has proved difficult to identify these unique features of cancer cells. This situation is unlike antibacterial agents such as penicillins and cephalosporins(β-lactam antibiotics) which inhibit a transpeptidase reaction involved in the biosynthesis of a peptidoglycan(an important component of bacterial cell wall) since this process is not carried out by mammalian cells. This biochemical difference results in the destruction of microorganisms while the host cells are spared. It has therefore been difficult to design antineoplastic agents rationally(Montgomery, 1979) with the exception of some antimetabolites(Hellman, 1983). Despite the dearth of many well defined processes to be exploited for the design of novel drugs, useful agents continue to be produced.

Most of the current cytotoxic agents used clinically have been procured as a result of random screening for tumour-inhibitory activity. It has been the major source of new leads giving rise to different chemical structures from natural products and synthetic compounds(Doyle, 1980). Natural products derived from plant or bacterial sources represent around one quarter of the anticancer agents in

use(Farmer, 1985). Today while seeking for a new lead or new structure in the process of drug design, different approaches can be used and are as follows.

1.8.1.0 Lead Discovery by Design

This technique has been mainly employed in forming enzyme inhibitors. In this case it is important to have a proper understanding of the reaction mechanism and the structure of the enzyme. The structural information can be obtained by X-ray crystallography. It is also required that the enzyme be therapeutically relevant, involved in the rate limiting reactions and there should be a convenient source of material with a straightforward assay.

The overall strategy is based on the consideration of a transition state(ES) formed by reversible binding of the substrate(S) to the enzyme(E) and that "an enzyme will bind the transition state of the reaction which it catalyses many order of magnitudes more tightly than it will bind the substrate"(Transition State Analogue Theory)(Lindquist, 1975). So, a rationally designed "inhibitor resembling the transition state species would be much more tightly bound to the enzyme than the substrate is to the enzyme"(Bernhard and Orgel, 1959). The aim can be to achieve enzyme inhibition through reversible or irreversible mechanisms. Among the reversible classes of inhibitors, the transition state

analogue approach has been discussed by Wolfenden(1978) and Lienhard(1973). Advantage has been taken of the binding interactions available to the transition state for the enzymatic process and it is expected to bind more tightly than the substrate although it is reversible and competitive. For example, inhibition of adenosine deaminase has been achieved by 30, which is a compound that mimics the proposed transition state 31(Wolfenden, 1969; Cha et al., 1975). Methionine sulfoximine 32 inhibits glutamine synthetase after phosphorylation generates the transition state analogue 33 of the proposed intermediate 34 (Baldwin, 1987a). Although there are numerous examples of the successful design of transition state analogues, most of them have failed to be developed into useful drugs due to problems with transport, metabolic stability and biological half life(Baldwin, 1987a). It has been suggested by Goodford(1984) that along with other requirements as mentioned above, the appropriate physicochemical properties be incorporated into drug design using the principles of Hammett and Hansch.

Another approach to enzyme inhibition is called suicide inhibition. In this process the enzyme first acts on the inhibitor resulting in the generation of a species which is capable of blocking the enzyme either reversibly or irreversibly. It is irreversible blocking which is more common. This process is devoid of the nonspecific alkylation

(30)

(31)

(32)

(33)

(34)

associated with the haloacyl type of active site-directed irreversible agents(Burger, 1983) e.g., inhibition of GABA(γ-aminobenzoic acid) aminotransferase by 4-amino-5-fluoro-pentanoic acid(Silverman and Levy, 1981) and the inhibition of aromatic amino acid decarboxylase by α-vinyl DOPA(Metcalf and Jund, 1977).

1.8.2.0 Lead Discovery by Random and Directed Screening

Screening here refers to the testing of compounds and mixtures from various sources in a relevant assay. The test compounds can be procured from various sources including synthesis, proprietary sample collections, samples from other laboratories, microbiological sources, toxins and plant and marine extracts. The structurally novel, innovative leads are most often found from naturally acting compounds.

An approach alternative to or as a supplement to totally random approach is the hypothesis-based screen. This strategy uses a mechanistic hypothesis or structural information about a natural ligand, substrate or inhibitor as the starting point for compound selection. As shown in Fig.1.12, substructure searching is carried out to find compounds for testing that contain the particular arrangement of the atoms thought to be important for the biological response.

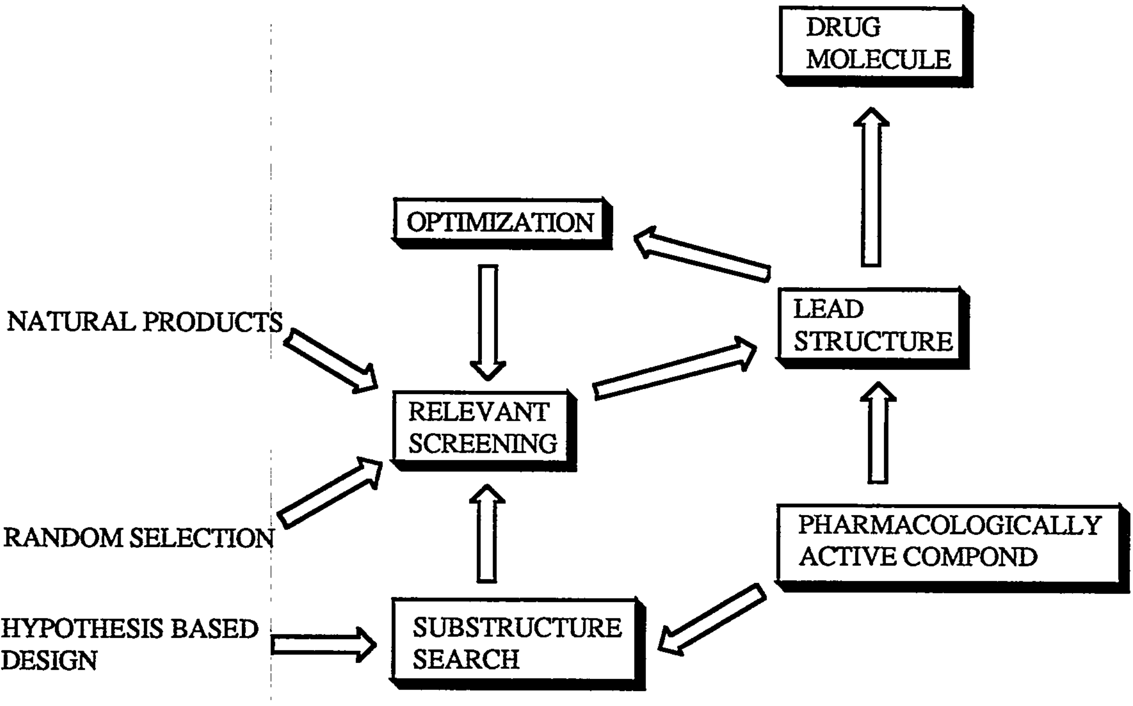


Figure 1.12 Pathway representing the central role of lead discovery and optimization process in the drug discovery process. Taken from Baldwin(1987b) and reproduced with permission of the copyright owner.

Another approach called directed screening, is based on the properties of known compounds and includes molecular modifications with a view to improving the parent drug in terms of potency or side effects and to exploit an unexpected biological activity. Maxwell(1984) divided such molecular modifications of known agents into two classes.

(1) The "enlightened" approach. This hypothesis is based and directed towards overcoming deficiencies of the parent drug.
(2) The "unenlightened" approach. The main goal of this procedure is to discover a biologically active analogue that may provide by chance an improvement over the parent compound.

1.8.3.0 Lead Development of New Anticancer Drugs

In general, when the lead structure has been obtained it is subjected to systematic molecular modifications in an endeavor to improve its therapeutic potential as a cancer chemotherapeutic agent and to elucidate biochemical and pharmacological mechanisms of action.

Molecular modifications can be designed to achieve some specific goals such as to(Ariens, 1971):

(1) develop more potent analogues with respect to anticancer activity
(2) eliminate or minimize the side effects responsible for host toxicity
(3) separate the components of the spectrum of action, such as host toxicity and antineoplastic activity into separate molecular entities
(4) develop analogues with differences in tissue specificity and
(5) modulate the pharmacokinetic properties of the compound in order to alter either dose-effect or time-concentration relationships.

These objectives can be achieved in one of the three ways namely a systematic empirical approach, a hypothesis based approach or a combination of both techniques(Baldwin, 1987a).

1.8.3.1 Systematic Empirical Approach

In this approach physicochemical parametric variations such as partition coefficient and charge distribution are carried out on the lead compound. It is done by preparing a series of compounds keeping in view their synthetic feasibility. The compounds prepared are then evaluated for their desired activity. The interpretation of results then leads to a more refined series and this process continues until optimum biological activity has been obtained. There are different

stepwise-optimization procedures available which include the Topliss decision tree, Hansch selection grid method, sequential simplex method, Fibonacci series and Hansch cluster analysis. Almost all of these techniques involve substitution on the aromatic rings and side chains. Some of these techniques make use of computer technology, so the choice of technique is dependent upon accessibility to computers though each technique is associated with intrinsic limitations. No single technique is completely successful. Some of these techniques will be discussed in detail in the following sections.

1.8.3.2 Hypothesis Based Lead Development

One of the hypothesis based strategies involves bioisosteric replacement and the new groups when introduced into a bioactive molecule would be expected to produce a compound with similar chemical, physical and biological properties. With no two substituents alike, this type of substitution could result in changes of size, shape, electronic distribution, partition coefficient, solubility, pKa, chemical reactivity, susceptibility to metabolism and hydrogen bonding capabilities. But bioisosterism in general results in improved potency, selectivity, duration of action, bioavailability and/or a reduction in toxicity. A variety of bioisosteric replacements have been used in optimization of bioactivity and it can be exemplified by cimetidine(Ganellin,

1982). The isosteric replacement of the thiourea moiety of metiamide(35) by a cyanoguanidine group resulted in a H_2-receptor antagonist cimetidine(36). Cyanoguanidine was chosen because of its similarity in physicochemical properties with the thiourea moiety and this replacement resulted in the low incidence of granulocytopenia associated with metiamide.

(35) X = S

(36) X = NCN

The symbiotic approach involves the molecular combination(or hybridization) of two mutually complementary bioactivities into a single entity. One of the advantages of a hybrid molecule over two different independent entities when given in combination, lies in their pharmacokinetic properties. With two different drugs, each pharmacological activity depends on individual absorption, metabolism and excretion whereas it is not the case with a hybrid molecule. This approach has been particularly useful in the cardiovascular area where a variety of hybrids have been produced and studied, for example labetolol(37), an α,β-adrenoceptor antagonist.

$$H_2N-C(=O)-C_6H_3(OH)-CH(OH)-CH_2-NH-CH(CH_3)-CH_2-CH_2-C_6H_5$$

(37)

The prodrug approach is another procedure involving hypothesis based lead development. This technique takes advantage of metabolic processes whereby an inactive prodrug molecule by action of different enzymes is converted into an active drug molecule by releasing it from its carrier group. This approach has primarily been explored(see section 1.5.0.0) with an aim to improve bioavailability and tissue selectivity, as in the case of cancer chemotherapy. Cyclophosphamide(12), an antitumour drug used clinically, was first synthesized as a potential prodrug that would be acted upon by phosphamidases present in high concentration in some tumour cells. This characteristic of some tumour cells would result in the release of the active drug molecule i.e. a nitrogen mustard, specifically in cancer cells.

Soft drugs is another approach which takes advantage of metabolic processes. Soft drugs may be defined as "biologically active, therapeutically useful chemical compounds with a characteristic of undergoing predictable and controlled <u>in vivo</u> destruction to nontoxic moieties"(Bodor, 1984). Soft drugs are based on the hypothesis that

predictable and controlled metabolism can be achieved by avoiding oxidative metabolism which can lead to the formation of reactive and toxic metabolites and the soft drug approach takes advantage of hydrolysis as a process for the deactivation of drugs. This approach can be exemplified by a β-adrenergic blocking agent esmolol(38). This compound has a β-receptor inhibitory property on smooth muscles as an ester but hydrolysis to the acid results in a dramatic decrease in β-adrenoceptor affinity(Erhardt _et al._, 1982). Esmolol can also be given by infusion and as soon as the infusion is stopped, blockade of the β-receptors rapidly disappears.

O
H_3CO
O
$NHCH(CH_3)_2$
OH

(38)

On the other hand, hard drugs are prepared to completely avoid metabolism since low toxicity has been found to be associated with metabolically stable compounds. Chemically reactive species such as epoxides and _N_-hydroxy arylamines formed as a result of oxidative metabolic pathways alkylate nucleophiles in proteins and nucleic acids. The formation of these active species has been avoided by the replacement of hydrogens by fluorine in olefins sensitive to epoxide formation or by the introduction of steric constraints to

prevent enzymatic attack(Ariens, 1980; Ariens and Simonis, 1982).

Site-specific delivery is one of the recent approaches being used to achieve selectivity in the case of antitumour chemotherapy. In this case the bioactive molecule is covalently attached to a target specific carrier molecule depending upon its affinity for cancerous cells. For example, various anticancer drugs have been linked to estrogens, antibodies, dextrans, synthetic polymers and DNA. A daunomycin-DNA complex bound covalently had reduced toxicity compared to daunomycin while retaining its antitumour properties.

1.9.0.0 Quantitative Structure-Activity Relationships (QSAR)

It has been known for quite some time that within a series of related compounds the potency of a biological response can be related to the physicochemical properties(hydrophobic, electronic and steric effects) of the molecules in a quantifiable manner(Albert, 1985a). In this approach it is assumed that the compounds are exerting their effects by an identical mechanism. Using this approach it is possible to delineate the optimum molecular features required to produce a particular type of response. Based on these findings, several correlations and approaches have been proposed with

respect to investigations on quantitative activity relationships in drug design.

1.9.1.0 The Hammett Correlation

The Hammett correlation defines the quantitative relationship between chemical reactivity of the side chain and the electronic nature of the substituents(i.e. electron releasing or electron withdrawing) in an aromatic ring system(Scheme 1.1).

Scheme-1.1

In Scheme 1.1, R is a reaction site on the side chain attached to an aryl ring and X is a _meta_ or _para_ substituent. Hammett excluded _ortho_ substituents on the grounds that there would be specific steric interactions between the reaction site and substituents which would not be amenable to a regular quantitative treatment. The relationship known as the Hammett Equation is as follows(Equation 1.1)(Hammett, 1937) which is an example of a linear free energy relationship (LFER).

$$\log \mathbf{k}/\mathbf{k_o} = \rho\sigma \text{ ....(1.1)}$$

The letter **k** in Equation 1.1 represents either the equilibrium constant $\underline{K}$ or the rate coefficient $\underline{k}$ of the ionization and $\mathbf{k_o}$ and **k** are the corresponding values for the unsubstituted(i.e. for X=H) and substituted compounds respectively. The symbol ρ(rho) is a constant for a particular reaction under study and is independent of the nature of X. The letter σ(sigma) is a substituent constant which quantifies the effect of a substituent group(X) on the chemical reactivity of the side chain. It depends only on the nature of X and whether the substituent is in the meta or para positions. It is independent of the nature of R. The sigma(σ) value represents a combination of both inductive and resonance effects.

The sign and magnitude of the two constants sigma(σ) and rho(ρ) provide insight into the reaction under investigation. A positive sigma(σ) value represents an electron-withdrawing substituent and a negative value indicates an electron-donating substituent. The magnitude of sigma(σ) is a measure of the strength of the electron withdrawing or electron donating abilities of the substituents. Whereas a positive rho(ρ) value indicates that the reaction is accelerated or the equilibrium is favoured by electron withdrawal, a negative value shows just the opposite. The magnitude of rho(ρ) shows the sensitivity of the reaction towards substituent changes. The Hammett constants for a variety of

substituents have been reported(Hansch et al., 1973; Lewis, 1986). The Hammett plot between k/k_0 against sigma(σ), usually gives a straight line with a slope which is defined by rho(ρ).

1.9.2.0 The Hansch Approach

The Hammett approach, however, is empirical and fails to distinguish readily between the relative importance of electronic, steric and physical interactions of the individual substituents(Stenlake, 1979). In order to overcome this difficulty, Hansch and Fujita(1964) adapted the Hammett Equation(Equation 1.1). Hansch's approach is primarily based on two assumptions.

(1) The movement of a bioactive compound from its site of application to its site of action is dependant on the partition coefficient of the drug.

(2) The effects of the substituent on the molecular interaction are related to the biological response and are equal to an additive effects of the substituents on the model system.

As most generally applied, the Hansch model expresses the biological response as a function of hydrophobic, electronic and steric factors and it may be written in the general form of Equation 1.2.

$$\log(1/C) = k_1\pi^2 + k_2\pi + k_3\sigma + k_4E_S + k_5 \quad .....(1.2)$$

In Equation 1.2, C is the concentration of drug required to produce a standard measurable biological effect. The standard is an arbitrary value and can be taken for example as the concentration of drug that causes (a) 50% inhibition of activity of a target enzyme or (b) a selected percentage increase in life span of tumour bearing animals compared to untreated animals. Sigma(σ) is the sum of the Hammett substituent constants for the compound and represents the electronic contribution to bioactivity. E_S is the sum of the Taft's steric constants for each substituent on the aromatic ring of the drug molecule. The constants k_1-k_5 indicate the relative importance of each parameter in the system under study and are determined by regression analysis after several compounds have been synthesized and tested for their biological activity. The hydrophobicity constant(π, a substituent constant) was devised by Hansch and it can be defined by a Hammett-like relationship as in Equation 1.3.

$$\pi_X = \log P_X/P_H = \log P_X - \log P_H \quad ....(1.3)$$

In this case P_X and P_H are the octanol-water partition coefficients for the substituted and unsubstituted compounds respectively. The sign of the pi(π) value indicates the position of the compound on the continuum of solubility. A positive pi(π) value denotes that the substituent group

prefers hydrophobic solvents and a negative value means that the group enhances aqueous solubility. Groups making an equivalent contribution are referred to as isolipophilic groups. So if lipophilicity is an important criterion in determining the biological activity, then the substitution of one isolipophilic group for another may allow retention of activity. The importance of partition coefficients in the development of cancer chemotherapeutic agents has been reviewed(Cain, 1975) and great stress has been laid on the lipophilicity and pi(π) constants in the development of analogues of cancer chemotherapeutic agents.

In the design of drugs it has been suggested that the partition coefficient determines the rate of drug migration to the site of action(Penniston et al., 1969) with drug movement being considered a partitioning process between lipid-rich and lipid-poor biophases. In general a compound with an optimum partition coefficient reaches the desired site most rapidly and in higher concentration than compounds with suboptimal or supraoptimal partition values(Kozarich et al., 1979). This behavior explains the parabolic shape(i.e. the π^2 factor in the Hansch Equation) in plots of logπ vs the biological response.

In a standard Hansch approach, an initial series of 6 to 12 compounds is prepared with substituents that cover the widest possible variations in π, σ and E_s values which are evaluated

for the desired biological activity. A regression analysis is then performed in order to approximate the dependency of biological responses on each parameter(Craig, 1971). The results of this initial analysis then determine the selection of a second series of analogues designed to attain the maximally active derivative of the series. The major limitations to this approach are firstly, the synthesis and screening of a large number of compounds to obtain a meaningful correlation and in further deciding the next step to achieve the optimization of activity and secondly, the need for access to computer facilities.

1.9.3.0 The Topliss 'Decision-Tree' Approach

This approach is based on the Hansch principles and is one of the non-mathematical and relatively simple approaches used in drug design in order to optimize the activity of the lead compound(Topliss, 1972). The approach can be applied to molecular modification of both aromatic rings and side chains based on σ, π and E_S values. It involves the stepwise synthesis and evaluation of a series of compounds starting with the parent unsubstituted aryl derivative of known bioactivity. Then a 4-chloro analogue with higher pi(π) and sigma(σ) values than the parent compound is prepared. A decision tree is followed depending upon the potency of its biological activity. If the 4-chloro derivative is more active than the parent compound, then the 3,4-dichloro

derivative is suggested for synthesis since it will further increase the magnitude of the pi(π)and sigma(σ)values. Alternatively, if the 4-chloro analogue is less active then the parent unsubstituted compound, a 4-methoxy derivative is prepared in order to decrease the pi(π)and sigma(σ)values. The 4-methyl analogue is prepared if the 4-chloro analogue is equipotent to the parent compound. In this case it is suggested that a favorable positive pi(π)effect is being counterbalanced by an unfavorable negative σ effect. This procedure is continued in a stepwise fashion by systematically varying the parameters following the tree as shown in Fig.1.13, until optimum activity is achieved.

A similar decision tree was devised for the side-chain alkyl substitution as shown in Fig.1.14, and it is followed until optimization of the activity of the lead compound is obtained.

Topliss(1977) proposed another non-computerized method for unfused and fused aromatic rings. This technique is of greater value where the compounds under investigation are relatively easy to synthesize and the biological results cannot be obtained so quickly. This technique has been referred to as the evaluation of data using a potency order table. The procedure requires the initial synthesis and screening of five compounds namely the unsubstituted parent derivative and the 4-chloro, 3,4-dichloro, 4-methoxy and 4-

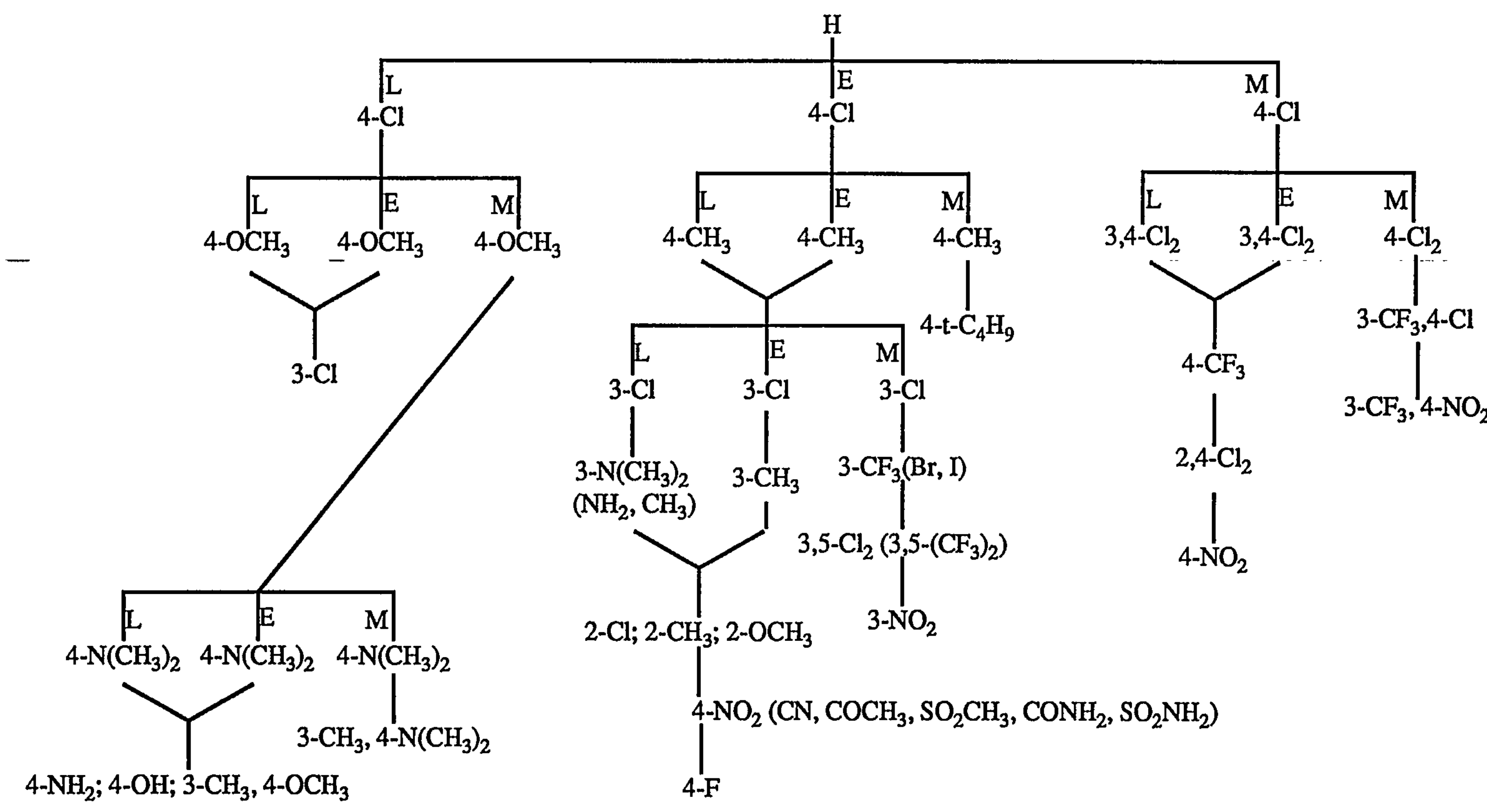


Figure 1.13 Topliss scheme for substituent selection on an aromatic ring. **M**:more active, **E**:equipotent, **L**:less active. The descending lines indicate the sequence of compounds to be considered for synthesis. Brackets indicate alternatives. Taken from Topliss(1972) and reproduced with permission of the copyright owner.

CH_3

L: i-C_3H_7 — H; CH_2OCH_3; $CH_2SO_2CH_3$

E: i-C_3H_7
- L: C_2H_5; E: C_2H_5 — $CHCl_2$; CF_3; CH_2CF_3; CH_2SCH_3
- M: C_2H_5

M: i-C_3H_7
- L: cyclo-C_5H_9; E: cyclo-C_5H_9 — cyclo-C_4H_7(CH_2-cyclo-C_3H_5) — tert-C_4H_9
- M: cyclo-C_5H_9 — cyclo-C_6H_{11} — $CH_2C_6H_5$ — $(CH_2)_2C_6H_5$

Figure 1.14 Topliss scheme for substituent selection on an aliphatic chain. **M**:more active, **E**:equipotent, **L**:less active. The descending lines indicate the sequence of compounds to be considered for synthesis. Brackets indicate alternatives. Taken from Topliss(1972) and reproduced with permission of the copyright owner.

methyl analogues. Depending upon their biological activity, they are ranked in order of decreasing potency. This order is then compared with the data in a potency order table(Table 1.1), which in turn would indicate whether a parameter or a combination of parameters is playing the major role responsible for the desired bioactivity. The information so obtained is used in conjunction with Table 1.2, in order to determine which substituent should next be placed in the aryl ring. The information obtained from the screening of this second set of analogues may give support of the parameter dependence and can be utilized to generate the optimally active compound.

Table 1.1 Potency order for various parameter dependencies[a].

Substituents	Parameters									
	π	$2\pi-\pi^2$	σ	$-\sigma$	$\pi+\sigma$	$2\pi-\sigma$	$\pi-\sigma$	$\pi-2\sigma$	$\pi-3\sigma$	E_4[b]
3,4-Cl_2	1	1-2	1	5	1	1	1-2	3-4	5	2-5
4-Cl	2	1-2	2	4	2	2-3	3	3-4	3-4	2-5
4-CH_3	3	3	4	2	3	2-3	1-2	1	1	2-5
4-OCH_3	4-5	4-5	5	1	4	4	4	2	2	2-5
H	4-5	4-5	3	3	5	5	5	5	3-4	1

[a]Taken from Topliss(1977) and reproduced with permission of the copyright owner.
[b]Unfavourable steric effect from 4-substitution.

Table 1.2 New substituent selection[a].

Probable operative parameters	New substituent selection
π, $\pi+\sigma$, σ	3-CF_3, 4-Cl; 3-CF_3, 4-NO_2; 4-CF_3; 2,4-Cl_2; 4-cyclo-C_5H_9; 4-cyclo-C_6H_{11}
π, $2\pi-\sigma$, $\pi-\sigma$	4-$CH(CH_3)_2$; 4-$C(CH_3)_3$; 3,4-$(CH_3)_2$; 4-$O(CH_2)_3CH_3$; 4-$OCH_2C_6H_5$; 4-$N(C_2H_5)_2$
$\pi-2\sigma$, $\pi-3\sigma$, $-\sigma$	4-$N(C_2H_5)_2$; 4-$N(CH_3)_2$; 4-NH_2; 4-NHC_4H_9; 4-OH; 4-$OCH(CH_3)_2$; 3-CH_3, 4-OCH_3
$2\pi-\pi^2$	4-Br; 3-CF_3; 3,4-$(CH_3)_2$; 4-C_2H_5; 4-$O(CH_2)_2CH_3$; 3-CH_3, 4-Cl; 3-Cl; 3-CH_3; 3-OCH_3; 3-$N(CH_3)_2$; 3-CF_3; 3,5-Cl_2
ortho effect	2-Cl; 2-CH_3; 2-OCH_3; 2-F
other	4-F; 4-$NHCOCH_3$; 4-$NHSO_2CH_3$; 4-NO_2; 4-$COCH_3$; 4-SO_2CH_3; 4-$CONH_2$; 4-SO_2NH_2

[a]Taken from Topliss(1977) and reproduced with permission of the copyright owner.

1.10.0.0 Potential of α,β-Unsaturated Ketones as Antineoplastic Agents

A number of compounds containing the α,β-unsaturated carbonyl group exhibit antibacterial, antiviral, antifungal and antitumour activity by virtue of their ability to react with cellular nucleophiles(Dimmock and Wong, 1976; Osato and Hanno, 1953). In general, α,β-unsaturated ketones have been classified as biological alkylating agents which alkylate strong nucleophiles including thiols and amines but preferentially thiols (Kupchan et al., 1970; Friedman et al., 1965). As will be discussed later, this property of α,β-unsaturated ketones has been explored to avoid the possible mutagenic and carcinogenic activities associated with other alkylating agents.

A large variety of naturally occurring compounds of plant origin such as quinones(Lin et al., 1972; Lin et al., 1973; Lin et al., 1974) and sesquiterpenoid lactones(Hartwell and Abott, 1969; Kupchan, 1970a; Jewers et al, 1973) have been shown to possess antitumour and cytotoxic activity. The activity of these compounds has been attributed, in part at least, to the alkylating ability of the unsaturated keto function and/or to be associated with the interference of redox potential mechanisms in the living cell(Dimmock and Wong, 1976). Most of these biologically active compounds contain in their molecule the α-methylene-γ-lactone moiety

along with other functional groups such as the epoxide, chlorohydrin, unsaturated ester, unsaturated lactone and unsaturated ketone groups(Kupchan et al, 1970). Initially Kupchan(1970b) suggested that cytotoxicity of these sesquiterpinoid lactones was due to the conjugated α-methylene-γ-lactone moiety. Later Lee and co-workers(1971) demonstrated that it was the α,β-unsaturated keto system which was responsible for the observed activity, whether it is present in the form of a ketone or lactone. In addition, they showed that among the nucleophiles investigated, thiols were the most reactive toward the olefinic bonds of several sesquiterpinoid lactones. On selective reduction of the olefinic bond of the α,β-unsaturated ketone moiety of a sesquiterpinoid lactone helenalin(39) 2,3-dihydrohelenalin (40) was formed which has a 46 fold decrease in the cytotoxicity against growth of cells from the human epidermoid carcinoma of the larynx compared to helenalin(Lee et al., 1972). Further, the mono-cysteine adducts 43 and 44 derived from elephantopin(41) and eupatundin(42) respectively showed appreciable cytotoxicity but the bis-cysteine adducts were essentially devoid of growth-inhibitory activities (Kupchan et al., 1970).

Similarly, synthetic derivatives containing the α-methylene-butyrolactone group were found to possess antitumour activity and representative compounds in the series formed adducts

H
O
O
O
OH

(39)

H
O
O
O
OH

(40)

O
O
O
O
O
O
O

(41)

HO
H
O
O
O
HO
O
O

(42)

O
O
O
O
O
O
O
S
$H_3N^{\oplus}$
$COO^{\ominus}$

(43)

HO
H
O
O
O
HO
O
S
O
$COO^{\ominus}$
$H_3N^{\oplus}$

(44)

with sulfhydryl compounds such as mercaptoacetic acid and cysteine(Rosowsky et al., 1974).

On the other hand, the antitumour activity of prostaglandins of series "A"(PGA's) has been found to be associated with the α,β-unsaturated keto moiety present in the cyclopentane rings of their molecules. It was observed that on reaction of PGA_2(45) with increasing concentrations of glutathione, progressive reduction of the ability of PGA_2 to inhibit DNA synthesis by B16a melanoma cells occurred(Honn and Marnett, 1985).

(45)

(46)

Furfural acetone(46) a non-cyclic derivative had been shown to increase the life span of animals by approximately 17% when tested against Ehrlich ascites tumour in mice(Furst et al., 1954). Dimmock and Taylor (1975) observed a low level of toxicity in the case of compounds (47) towards an epidermoid carcinoma of nasopharynx in vitro, whereas compounds 48 and 49(Dimmock et al., 1975) were found to be devoid of any antitumour activity in vivo. This lack of activity was attributed to the poor aqueous solubility of these compounds.

To increase aqueous solubility, the corresponding Mannich bases were prepared. The subject of Mannich bases as applied to α,β-unsaturated ketones will be discussed in later sections.

$$R_1,R_2\text{-}C_6H_3\text{—}(CH{=}CH)_n\text{—}C({=}O)\text{—}(CH_2)_5\text{—}CH_3$$

(47) $R_1 = R_2 = Cl, N(CH_3)_2, N^+(CH_3)_3\ I^-$; n = 1

(48) $R_1 = R_2 = H$; n = 1

(49) $R_1 = R_2 = H$; n = 2

Recently, α,β-unsaturated ketones have been explored for their possible antimicrotubular activity(Edwards et al., 1990). The area of exploration is based on the studies that a sulfhydryl residue is present at a colchicine(50) binding site and that sulfhydryl reagents interfere with the microtubular assembly(Aikeda and Steiner, 1978; Lee et al, 1981). In this latter study it was found that an intact α,β-unsaturated ketone moiety was important for inhibiting the tubulin polymerization process since the reduction of a carbonyl group(52) or a double bond(53) of a representative compound 51 led to diminished activity.

H_3CO, H_3CO, H_3CO, $NHCOCH_3$, O, OCH_3

(50)

H_3CO, H_3CO, H_3CO — C(=O) — CH = CH — $N(CH_3)_2$

(51)

H_3CO, H_3CO, H_3CO — CH(OH) — CH = CH — $N(CH_3)_2$

(52)

H_3CO, H_3CO, H_3CO — C(=O) — CH_2 — CH_2 — $N(CH_3)_2$

(53)

2.0.0.0 RATIONALE OF THE PRESENT INVESTIGATION

2.1.0.0 Introduction

The majority of the present generation antineoplastic agents have no particular specificity whatsoever toward tumour cells and their detrimental effect is due to their action on proliferating cells in the cell cycle. In general anticancer drugs are most useful against malignant tumours with a high proportion of dividing cells. That is the reason why most of the drugs are primarily effective against leukemias and lymphomas which have the highest fraction of dividing cells among tumours. The most common malignant tumours, however, are "solid" tumours including those of the colon, rectum, lung and breast. These tumours usually have a low proportion of dividing cells and therefore are less susceptible to treatment by drugs alone.

Because of the involvement of these agents with DNA many of the antineoplastic agents have the potential of being mutagenic and carcinogenic(Harrap et al., 1981). This in turn prevents the administration of a sufficient quantity of the drug to eradicate the tumour completely. The most common finding of chemotherapy is that tumours respond poorly or not at all to the present day drugs. If they do show a significant regression they often regrow once the drug has had to be discontinued because of excessive toxicity.

Nevertheless, there have been improvements in the survival rates of some of the rare tumours e.g. choriocarcinoma, teratoma and acute leukemia of childhood which are now being cured by chemotherapy but only rarely are common tumours e.g. breast, lung and gastrointestinal cancers eradicated. Thus there is a need for new selective anticancer agents. The synthesis of analogues of drugs with known anticancer activity is one of the obvious way of designing new drugs. This particular "molecular tinkering" has been especially useful for understanding and determining the characteristics responsible for drug-receptor affinity, clinically undesirable side effects, antitumour activity and how various molecular modifications could alter the pharmacological response of a drug molecule(lead compound). The principal goal in cancer drug development is to develop new selective anticancer agents and this may be accomplished by forming agents which could be directed towards other intracellular targets such as mitochondria, the cell membrane or any other key enzyme function significant for the tumour cells. In reality only few such differences are known and the majority of them are quantitative rather than qualitative. Selective concentration in tumour cells can also be achieved by improving the selectivity of the present day drugs either by forming prodrugs(see Section 1.5.0.0) provided activating enzymes are only present in the tumour cells or if some means could be found by which the sensitive tissues of the host be

protected from the adverse effects of the drug at the same time as the effect of the drug on the tumour was not altered.

Recently L-histidinol, emerged as an agent with such characteristics. It has been found to act as a protective agent for normal cells when used in combination with conventional anticancer drugs and at the same time it has been found to enhance the vulnerability of tumourigenic cells(see Section 2.2.3.0).

Various other approaches have been explored and are part of the ongoing research for site-directed chemotherapeutic attack towards tumour cells and principally involves targeting of drugs e.g. by forming drug-carrier conjugates. Advantage has been taken of the excessive demand for exogenous substrates by tumour cells than their normal cell counterparts and increased endocytosis of extracellular material(Pratt and Ruddon, 1979c). It was suggested that selective toxicity might be achieved by complexing cytotoxic drugs with appropriate carriers, whose entry into the cell would depend on endocytosis(DeDuve et al., 1974). It led to the development of DNA-drug complexes(Trouet et al., 1972) e.g. DNA-daunorubicin and DNA-adriamycin complexes, which have been shown to cause increases in therapeutic indices over the respective free drug against L1210 leukemia in mice(Henry, 1974). Cell-like phopholipid vesicles(liposomes) (Gregoriadis, 1977) containing nitrogen mustards(Rutman et

al., 1976) and ara-C(Mayhew et al., 1976), have been shown to be more effective than the free drug in experimental animal tumour systems. Attempts have been made to direct drugs to tumours by attachment of the drugs to monoclonal antibodies raised against a specific tumour receptor based on the increasing evidence that tumour tissues synthesize substances that are either absent from or produced in extremely limited amounts by the original normal parent tissue. These compounds are called TAAs' i.e. tumour-associated antigens or tumour markers e.g. chlorambucil-antitumour globulin noncovalent complex has been found to be more effective than chlorambucil alone against mouse Ehrlich ascites carcinoma and EL4 lymphomas(Ghose et al., 1983). Several other targeting procedures are being investigated to achieve the ultimate goal of selectivity and include for example, the use of macromolecules(serum albumin) as carriers (Zaharko et al., 1979) as well as targeting of antineoplastic agents using magnetic albumin microspheres(Widder et al., 1983).

The quest for agents which could be directed to tumours so as to avoid interaction with cytoplasmic and nuclear amino and hydroxy groups which form a major portion of in vivo biological nucleophiles(e.g. DNA) led to the development of α,β-unsaturated ketones as a new class of antineoplastic agents. α,β-Unsaturated ketones emerged as a class of alkylating agents which had marked predilection for thiols in contrast to amino and hydroxy containing compounds(Waddell et

al., 1983; Baluja et al.,1964). The ability of α,β-unsaturated ketones to react with thiols to give β-ketothioethers has been invoked in several studies to explain the antineoplastic activities of a number of carbonylene containing compounds (Lee et al., 1972; Dimmock and Wong, 1976; Honn and Marnett, 1985). It is conceivable that tumours with larger fraction of proliferating cells would be more susceptible to this class of compounds, since thiol levels may be more abundant just prior to and during mitosis(Emmelot, 1964). This unique selectivity of the conjugated enones toward thiols indicates that these compounds may have one or more advantages over the clinically used alkylating agents where carcinogenic(Farmer, 1982) and mutagenic(Cairns, 1980) properties are of major concern. This is because interaction with functional groups of nucleic acids may be absent(Ashby, 1978).

Earlier work from these laboratories had shown that α,β-unsaturated ketones possessing general formula 54 were found to have little antineoplastic activity in vivo against P388 murine leukemia(Dimmock and Smith, 1980). This test system has been claimed to be a good predictor of clinical anticancer activity(Johnson and Goldin, 1975). However, conversion of a number of the ketones with structure 54a to the corresponding Mannich bases 54b revealed that some of these derivatives had significant activity as well as murine

R_1, R_2 — C₆H₃ — CH=CH—C(=O)—CH(R_3)—$(CH_2)_4CH_3$

(54)

54a. $R_1 = R_2 = H, Cl, N(CH_3)_2$ etc.; $R_3 = H$

54b. $R_1 = R_2 = H, Cl$; $R_3 = CH_2N(CH_3)_2$ .HCl

toxicity in the P388 screen(Dimmock and Taylor, 1975). One of the chlorinated derivatives(R_1=2-Cl; R_2=4-Cl) met the criterion for activity in the P388 prescreen with an increase in the life span of mice by 30%(25% was considered significant) at a dose of 18mg/Kg(Dimmock and Taylor, 1975; Dimmock _et al._, 1979). As well the Mannich bases displayed _in vitro_ cytotoxicity to human epidermoid carcinoma of nasopharynx(Dimmock _et al._, 1979). This potentiation in the effect by forming the Mannich bases of the corresponding α,β-unsaturated ketones was attributed to the greater avidity for thiols by 54b than 54a. This view was supported by an observation that a Mannich base which was active in the P388 screen reacted with the thiols cysteine and glutathione but it did not react with lysine and guanine(Dimmock _et al._, 1983). In addition, the Mannich bases 54b reacted approximately 240 times more rapidly with ethanethiol than

the corresponding enones 54a when similar substituents were present in the aryl ring(Dimmock *et al.*, 1980b). In addition Dimmock and co-workers(1980a) found that some Mannich bases derived from conjugated styryl ketones did not show any mutagenicity when examined in the Ames test while various chalcones were reported to inhibit the mutagenic activity of benzo(a)pyrene(Torigoe *et al.*, 1983). Apart from antineoplastic activity, Mannich bases also demonstrated greater inhibition of mitochondrial respiration in Morris hepatomas as compared to those from normal hepatocytes(Dimmock *et al.*, 1980a).

In the present studies, the aim was firstly to prepare some Mannich bases of various *bis*-styryl ketones and secondly to prepare a derivative incorporating the features of *L*-histidinol. As has been described previously, this compound may enable selectivity towards malignant cells to be achieved. The compound was to be designed in such a way that *L*-histidinol is released *in vivo* before the alkylating agent could produce its effect. The concept of this cascade effect is discussed below in detail.

2.2.0.0 L-Histidinol and a Cascade Effect

(55a)

The aim of the present investigation was to prepare 55a and related compounds, which will cascade *in vivo* to release

(1) L-histidinol, which as mentioned earlier is a compound which protects some normal cells but not the corresponding malignant cells against lethal effects of various anticancer drugs(Warrington, 1986),

(2) a thiol alkylator, which will reduce the level of NPSH(non-protein bound thiols) which may make malignant cells more vulnerable to the alkylating agents and

(3) a cytotoxic alkylating agent i.e. a Mannich base which will exert its biocidal effect by reacting with critical thiols necessary for cell division(Harrington, 1967), preferably in malignant cells.

2.2.1.0 Alkylating Agents

The alkylating agents chosen were 4-piperidones which may be viewed as internal Mannich bases(β-aminoketones) which have one or generally two arylidene groups attached to the piperidine ring adjacent to the carbonyl function. The nitrogen atoms in the target compounds will be acylated which on hydrolysis will liberate the alkylating agents which will be protonated at physiological pH.

Earlier studies from this laboratory have demonstrated that Mannich bases had pronounced toxicities to mice and certain of the compounds displayed significant potencies against murine P388 lymphocytic leukemia(Dimmock and Taylor, 1975). This bioactivity was attributed to the avidity of Mannich bases for thiols. These compounds accordingly would react with thiols by a 1,4-addition reaction(Michael type addition) followed by rearrangement of the resultant enol to give the corresponding saturated ketones i.e. the β-ketothioethers (e.g. Ij)(Dimmock et al., 1990). This being the case, then electronic and steric factors should affect the extent of interaction with thiols. To test this hypothesis a Series I of bis-alkylators i.e. analogues of Ia were proposed by putting different substituents i.e. electron-donating and electron-withdrawing atoms or groups on the aryl rings of the arylidene moieties in order to seek a correlation between structure and activity. The compounds with electron-

(I)

Compound	R_1	R_2	X
Ia	H	H	HCl
Ib	SCH_3	H	HCl
Ic	Cl	H	HCl
Id	CH_3	H	HCl
Ie	OCH_3	H	HCl
If	$N(CH_3)_2$	H	–
Ig	Cl	Cl	–
Ih	$COO^{(-)}K^{(+)}$	H	–

(Ii)

(Ij)

withdrawing substituents in the aryl rings would be more susceptible to thiolation by withdrawing electrons from the olefinic group and making it more electrophilic and hence more reactive than compounds with electron-donating substituents. A related analogue(compound Ii) was also proposed.

As mentioned earlier, comparison of the second order rate constants for the reaction of a model thiol ethanethiol with a series of α,β-unsaturated ketones of general structure 54a and a series of its corresponding Mannich bases of general structure 54b, showed that compounds of Series 54b reacted approximately 240 times more rapidly than the corresponding enones 54a(Dimmock *et al.*, 1980b) when similar substituents were present on the aryl ring. It was suggested that this difference in chemical reactivity could be attributed to the following two factors.

(1) The quadrivalent nitrogen of Series 54b exerts a positive inductive effect which stabilizes the reaction intermediate($54b^1$).

(54b[1])

(2) The stabilization of the reaction intermediate by an N^{+}-----O interaction or by hydrogen bonding O-----H (54b[2]).

(54b[2])

These effects would increase the electrophilicity of the β-carbon atom in the Mannich base, which is absent in the corresponding enones 54a.

Therefore in the present investigation, a Series of compounds II and two related analogues IIi and IIj were prepared where the secondary amino group of Ia was replaced by a weaker base i.e. a tertiary amino group. The relativebasicities of Ia and

(II)

Compound	R	X
IIa	H	HCl
IIb	SCH_3	HCl
IIc	$COO^{(-)}K^{(+)}$	H_2O
IId	$N(C_2H_5)_2$	-
IIe	OCH_3	-
IIf	F	-
IIg	$N(CH_3)_2$	-
IIh	OH	-

(IIi)

(IIj)

IIa could be deduced from a consideration of the pKa values of piperidine and N-methyl piperidine namely 11.22 and 10.08 respectively(Albert and Serjeant, 1962). One could say that IIa should have fewer molecules in the ionized form at physiological pH and thus be less reactive and predictably less toxic than Ia.

A Series of 3,5-bis-arylidene-1-methyl-4-piperidone methohalides III was envisaged. Since these quaternary compounds would be completely ionized at physiological pH, they should be more reactive than compounds of Series I and Series II. This means that they could have higher bioactivity than either of Series I and II. In addition two related analogues were considered, first by replacing the aryl rings of IIIa by pyridyl functions(compound IIIj) and second by the insertion of an ethylene spacer arm group between the benzylidene function and the piperidine ring(compound IIIi). Compound IIIi was considered in order to study the receptor interaction requirements.

It was proposed to examine some of the compounds in Series III for their DNA binding properties. Since it is generally considered that drug molecules become associated at receptor surfaces by the alignment of active sites of the receptor at complementary functions of the molecule, a fixed distance between active sites requires a corresponding specific disposition of the complementary groups on the molecule if

(III)

Compound	R	X
IIIa	H	Br
IIIb	$N(CH_3)_2$	I
IIIc	$N(C_2H_5)_2$	I
IIId	OCH_3	I
IIIe	OH	I
IIIf	SCH_3	I
IIIg	F	I
IIIh	$COO^{(-)}K^{(+)}$	I

(IIIi)

(IIIj)

effective drug-receptor site interaction is to occur. Thus examination of structure-activity relationships should yield information relevant to understanding of the binding site of the receptor. So a further related Series namely IV was

(IV)

Compound	R	n
IVa	$N(CH_3)_2$	3
IVb	$N(CH_3)_2$	5
IVc	H	3
IVd	H	5

suggested in order to study the structural requirements for drug-receptor interaction.

Ideally, compound 55a after releasing L-histidinol will give rise to VIIIe the olefinic bond of which should be more reactive towards nucleophilic attack by thiols at position 1

(VIIIe)

than position 2 since the N-atom is acylated. To test this hypothesis, compound 56 was proposed in order to compare its reactivity with that of VIIIe toward thiols. On hydrolysis of the amidic bond of compound VIIIe, it would liberate compound Ia and in this derivative the olefinic double bonds will be activated by the protonation of the N-atom at physiological pH.

(56) (56a)

Compound 58 is a dienone but it lacks any group capable of forming a tetravalent nitrogen atom. It would be expected to be either inactive or less active toward nucleophilic attack than any of the compounds under consideration. At the same time compound 57 was envisaged, in which the carbonyl group

of Ia has been reduced, to observe if the keto group is required for bioactivity. Compound 56a, an intermediate in the synthesis of compound 56, was also considered as a candidate for screening.

(57) **(58)**

When tested against P388 leukemia cells in vitro vide infra, compound Ia was found to be 5800 times more active than the established anticancer agent BCNU[N,N'-bis-(2-chloroethyl)-N-nitrosourea] but it was found to be inactive in an in vivo P388 screen. This inactivity in part could be attributed to the poor aqueous solubility of compound Ia and related analogues of the Series I and II. So in order to produce in vivo activity, compounds 59 and 60 were suggested to be prepared and screened.

$(H_3C)_3\overset{\oplus}{N}$ … $\overset{\oplus}{N}(CH_3)_3$ R X . $2I^{\ominus}$

(59) R = CH_3; X = -

(60) R = H ; X = HCl

(61)

The potential mono-alkylators Vb,c with only one arylidene group were also proposed in order to obtain some perception of the importance of one or two alkylating arms. Theoretically, one would expect mono-alkylators to be less reactive than bis- or multi-alkylators. Compound (61), a mono-alkylator with no nitrogen atom in the cyclic ring and Va, an intermediate in the synthesis of compound Vb, were also proposed for screening.

(V)

Compound	R	X
Va	$COCH_3$	–
Vb	H	HCl
Vc	CH_3	HCl

Finally, if thiol alkylation were responsible, at least in part, for cytotoxicity then the addition of a further site of alkylation should enhance cytotoxicity and the following

compounds 62, 63 and VIIId,g,h(p100) were proposed to be more cytotoxic than either mono- or bis-alkylators.

(62)

(63)

At the same time Series VI was suggested which has the potential of being multiple alkylators and are quaternary ammonium salts(with a permanent pH-independent positive charge on the nitrogen atom). Variation of the length of the alkylene chain may reveal optimal DNA binding properties.

(VI)

Compound	n
VIa	2
VIb	3
VIc	4

2.2.1.1 Substituted Amides and Carbamates of 3,5-*bis*-arylidene-4-piperidones

The blood brain barrier(BBB), like many other body membranes, exhibits the characteristics of a lipid-like boundary. The permeability of this barrier to a wide array of drugs and other xenobiotics is proportional to the latter's octanol/water partition coefficient and also inversely proportional to the molecular weight right up to a cut-off point at about 400(Albert, 1985b). Brodie and co-workers (1960) investigated a number of compounds of diverse

structures and physiological properties to evaluate the factors of lipid solubility and the degree of ionization for the passage of drugs into cerebrospinal fluid(CSF) and they drew the following conclusions.

(1) Lipid solubility is the rate limiting factor with drugs that are mainly unionized in biological fluids e.g. plasma. These compounds penetrate the blood-CSF boundary at rates related to the lipid to water partition coefficients of the unionized molecules.
(2) The degree of ionization is the rate limiting factor with compounds that are highly ionized in plasma. These drugs enter the CSF at rates roughly parallel to the proportion of drug unionized at pH 7.4.
(3) Although both lipid solubility and the degree of ionization are important in governing passage of drugs into the CSF, lipid solubility is notably the dominant characteristic, since the relevance of the degree of ionization is probably a consequence of the poor lipid solubility of organic ions.

Ions like the quaternary ammonium group(cation) and the sulfonic acid function(anion) penetrate the brain and CSF much more slowly than do lipid soluble non-ionized drug molecules(Brodie _et al_., 1960; Hansson and Schmiterlow, 1961a; Hansson and Schmiterlow, 1961b; Levine, 1959; Mayer and Bain, 1956; Rall and Zubrod, 1960). In our present

studies, compound Ia emerged as a useful lead compound when screened in the _in vitro_ P388 screen. It had approximately 5800 times the cytotoxicity of the established anticancer agent BCNU(_vide infra_). Being an amine, the passage of compound Ia through the BBB would be disfavored by its protonation at physiological pH. One approach to overcome this problem in order to improve its therapeutic utility, has involved the development of prodrugs which are potential precursors of the cytotoxic drugs. So, it was suggested to prepare and screen different amides(VIIIa-c,i) and carbamates(Series VII) as bipartite prodrugs with enhanced delivery properties in order to observe if these compounds produced any activity against CNS tumours. Since there appears to be no carbamate specific enzymes present _in vivo_ in contrast to amides which can be acted upon by nonspecific amidases, these carbamates would be poorly hydrolyzed if at all. Thus aryl carbamates with different **p**-substituents on the aryl ring were suggested with a view to facilitating their hydrolysis _in vivo_ especially the compounds with electron-withdrawing substituents on the aryl ring of the carbamate carrier group. Compounds VIIIi and VIIi, which are intermediates in the synthesis of compound 59, can be regarded as prodrugs of compound If and thus they were also suggested to be screened for bioactivity.

(VII)

Compound	R_1	R_2
VIIa	H	C_2H_5
VIIb	H	CH_2CCl_3
VIIc	H	C_6H_5
VIId	H	$C_6H_4(4OCH_3)$
VIIe	H	$C_6H_4(4CH_3)$
VIIf	H	$C_6H_4(4Cl)$
VIIg	H	$C_6H_4(4NO_2)$
VIIh	H	$CH_2\text{-}C_6H_5$
* VIIi	$N(CH_3)_2$	C_2H_5

(VIII)

Compound	R_1	R_2	R_3
VIIIa	H	H	CH_3
VIIIb	H	H	CCl_3
VIIIc	H	H	CF_3
VIIId	H	H	$CH{=}CH_2$
VIIIe	H	H	$CH{=}CH{-}COOH$
VIIIf	H	H	CH_2CH_2COOH
VIIIg	H	H	$CH{=}CH{-}C_6H_5$
VIIIh	Cl	Cl	$CH{=}CH_2$
VIIIi	$N(CH_3)_2$	H	CH_3
VIIIj	H	H	$CH_2CH_2COOCH_3$

2.2.1.2 Mannich Bases

Several drugs show poor and variable oral absorption characteristics as a result of insufficient aqueous solubility and absorption becomes dissolution rate limited(Mattock *et al.*, 1977; Poole, 1979). The procedures commonly used for improving the rate of dissolution of such drugs involve modification of the physical or physico-chemical characteristics of the drug e.g. reduction of the particle size and use of various formulation techniques. In recent years, the alternative prodrug approach has been explored to enhance the aqueous solubility by producing more hydrophilic and hence more soluble derivatives. An ideal prodrug should be readily soluble(>5%) in water at physiological pH, be sufficiently stable in aqueous solution to allow long term storage and yet it should be converted quantitatively and rapidly *in vivo* to the active parent drug molecule(Jensen *et al.*, 1990).

Ideally it is important that prodrug consideration should be invoked in the drug design process at an early stage. It is much easier and less expensive to undertake a logical, structural-physical-chemical based prodrug screening at an early discovery phase than to go back after lengthy toxicity and formulation studies(Bodor, 1981).

To overcome the associated problem of poor aqueous solubility, Mannich bases(β-amino ketones) have been proposed as potentially useful prodrug candidates for α,β-unsaturated ketones(Dimmock and Wong, 1976). Mannich bases in general have been shown to yield water soluble and rapidly dissolving derivatives and according to the prodrug approach they have been shown to liberate the corresponding α,β-unsaturated ketones(parent compound) by deamination(Blicke, 1942; Riviere, 1960). Alternatively, Mannich bases have been shown to undergo the reverse Mannich reaction(Riviere, 1960). The two mechanisms are summarised in Scheme 2.1 below. Mannich

$$R{-}C({=}O){-}CH_2CH_2{-}N(CH_3)_2 \longrightarrow R{-}C({=}O){-}CH{=}CH_2 + HN(CH_3)_2$$

$$R{-}C({=}O){-}CH_2CH_2{-}N(CH_3)_2 \longrightarrow R{-}C({=}O){-}CH_3 + HN(CH_3)_2 + HCHO$$

Scheme 2.1

bases are generally formed by reacting a compound possessing an acidic proton with an aldehyde and a primary or secondary aliphatic or aromatic amine. The process can be referred to as aminomethylation. By selection of an appropriate amine component, Mannich base prodrugs with varying degrees of *in vivo* lability can be obtained(Bundgaard, 1985) e.g. *N*-Mannich bases prodrugs of *NH*-acidic compounds derived from secondary

amines have been shown to have very high aqueous solubilities in the salt form whereas the ones derived from primary amines did not show increased solubility even as salts. This disparity in behavior has been attributed to the occurrence of intramolecular hydrogen bonding in the latter derivatives(Stella et al., 1980).

In the present studies, a series of Mannich bases namely Series IX and a compound 64 were proposed primarily to improve on the aqueous solubility of the parent drug molecules. In Series IX, as mentioned earlier, different amines form a part of the carrier molecule in order to optimize the aqueous solubility and kinetics of release of the parent drug molecule. In the case of compound 64 on the other hand, an amine(compound Ia) is the parent drug molecule. A related analogue compound IXk which is the quaternary ammonium salt of compound IXa was also suggested to be prepared. This derivative would be expected to undergo a Hofmann elimination under physiological

(64)

(IX)

Compound	R_1	R_2	R_3	R_4
IXa	H	H	H	$-N(CH_3)_2$
IXb	H	H	H	$-N(C_2H_5)_2$
IXc	H	H	H	—N (pyrrolidinyl)
IXd	H	H	H	—N (piperidinyl)
IXe	H	H	H	—N (morpholinyl) O 1/2 H_2O
IXf	H	H	H	$-NH(CH_3).HCl$
IXg	H	H	H	$-NH_2.HCl$
IXh	Cl	Cl	H	$-N(CH_3)_2.HCl$
IXi	Cl	Cl	H	—N (morpholinyl) O .HCl
IXj	H	H	C_6H_5	$-N(CH_3)_2.HCl$
IXk	H	H	H	$-N^+(CH_3)_3Br^-$

conditions namely pH 7.4 and a temperature of 37°C to liberate the parent olefin(compound VIIId).

2.2.2.0 Spacer Arm

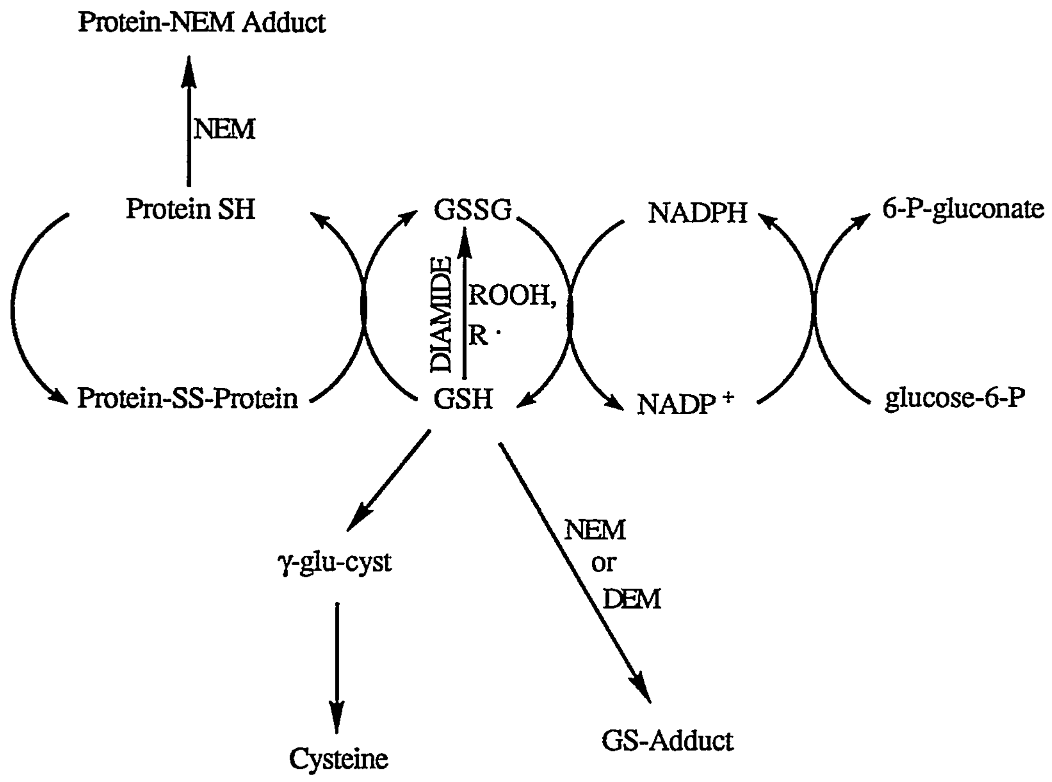
The objective of the spacer arm, as mentioned earlier is to reduce the levels of NPSH(non protein bound thiols) in cells. The high levels of NPSH, 90% of which is reduced glutathione(GSH)(Revesz, 1985), has been reported to be a limiting factor in reducing the cytotoxicity of a wide variety of antineoplastic agents(Wheeler *et al.*, 1986; Nutr. Rev., 1989). A wide variety of chemotherapy drugs with many mechanisms of action are used in cancer treatment. Redox active drugs such as bleomycin, adriamycin and *neo*-carzinostatin are capable of producing hydroxy radicals, superoxide and hydrogen peroxide(Sies *et al.*, 1983) and at least part of their cytotoxicity is mediated through these oxygen related species(Russo *et al.*, 1986). Such species are detoxified by GSH/GSH peroxide, catalase and superoxide dismutase. The nitrosoureas in addition to their alkylating activity inhibit glutathione reductase(Babson and Reed, 1978) a crucial enzyme that maintains cellular GSH in the reduced state. Alkylating agents such as melphalan and cisplatin are detoxified in several ways by GSH. GSH reacts directly with such reagents in a non catalyzed nucleophilic reactions, yielding GSH/drug adducts. Reaction of the drug with GSH results in a net lowering of the available drug concentration

capable of inflicting cytotoxicity. Drug/GSH adducts are rapidly metabolized and expelled from the cell and thus the active drug is detoxified before it exerts any cytotoxicity. It is of interest to note that increased levels of glutathione have been shown to be potentially useful as an anticarcinogen(Chen et al., 1988).

In one case of ovarian cancer, the development of in vitro resistance to melphalan and cisplatin has been attributed to the 2 to 3 fold elevation in intracellular glutathione levels compared to the sensitive cell lines from which they were derived(Ozols et al., 1987). In a similar study of the association between drug resistance and GSH and GSH-related enzymes, it was shown that adriamycin-resistant human breast cancer cells had very high activities of GSH transferase(≈ 80 fold compared with parallel cell lines)(Batist et al., 1986).

Selective sensitization of tumour cells to cytotoxic agents by glutathione depletion has been invoked in various studies(Hales, 1981; Olson et al., 1980; Hamilton et al., 1985; Ishikawa et al., 1990). In all cases so far, GSH depletion prior to drug exposure has afforded increased sensitization. Glutathione depletion itself does not result in loss of cell viability; rather it renders cells more sensitive to the cytotoxic effects of a variety of electrophilic and free radical reagents. As glutathione is depleted a greater proportion of these agents can interact

with cellular proteins via cysteinyl residues(Streeter et al., 1984). Different strategies have been employed for varying the concentration and redox state of glutathione in cells. These strategies are summarized in Scheme 2.2.



Scheme 2.2

One strategy has been to add reagents which form covalent adducts with thiols e.g. N-ethylmaleimide(NEM). NEM reacts rapidly with cellular thiols including protein thiols to form covalent adducts. However, the high reactivity of NEM has been found to afford little specificity(Smyth et al., 1960) and resulted in considerable toxicity. Diethyl maleate(DEM)

and phorone are much less reactive than NEM and have been used to deplete glutathione preferentially(Plummer *et al.*, 1981), since they are substrates for glutathione S-transferase(Chasseaud, 1976; Van Doorn *et al.*, 1978). Glutathione S-transferase an enzyme responsible for the glutathione conjugation of xenobiotics is one of the major detoxification processes in phase II metabolism. Ethacrynic acid on the other hand is converted to a substrate for glutathione S-transferase in hepatocytes and has been used to deplete glutathione in these cells(Meredith and Reed, 1982).

Another approach has been to limit the synthesis of glutathione and rely on normal catabolic pathways for depletion. This has been accomplished by restriction of cysteine availability(Suzukake *et al.*, 1982) and by use of inhibitors of glutathione synthesis such as buthionine sulfoximine(BSO)(Griffith, 1981; Griffith and Meister, 1979).

A third approach involves oxidation of GSH to GSSG, a process which is normally reversed within a few seconds unless the reductive capacity of the cell is suppressed. Diamide has been the most popular reagent for this purpose(Kosower *et al.*, 1969), although diamide has been shown to exhibit nonspecific reactivity(Harris *et al.*, 1971).

Finally, a fourth approach has been to limit the reductive capacity of the cell by inhibiting glutathione reductase with

BCNU metabolites(Meredith and Reed, 1982) or by decreasing the capacity for NADPH generation by inhibiting the glucose-6-phosphate dehydrogenase with dihydroepiandrosterone(Lopez-S and Rene, 1973). While each of these approaches is useful in depleting glutathione levels at different stages of glutathione metabolism, the present work has been focussed on the following two observations.

(1) GSH depletion prior to drug exposure results in sensitization of the cells to chemical insult.
(2) Use of DEM as a reagent for thiol perturbation.

In case of compound 55a, after the L-histidinol moiety has been released by ester hydrolysis, the spacer arm according to the cascade theory(as hypothesized) would act as a thiol depletor. This would lead to sensitization of malignant cells before the activated cytotoxic species(compound Ia) is released. The spacer arm in the case of compound 55a may be regarded as a modified acyclic analogue of the established thiol depletor DEM. Alternatively, the haloacyl function (compound 55c) was suggested to constitute a thiol alkylator (spacer arm).

Because of the numerous unsuccessful attempts at preparing L-histidinol ester(compound 55a), a dihydro analogue(compound 55b) was suggested to be prepared and screened.

(55)

Compound	R_1	R_2
55a	H	$-CH=CHCOOCH(NH_2)CH_2-$ (imidazol-4-yl; N, NH)
55b	H	$-CH_2CH_2COOCH(NH_2)CH_2-$ (imidazol-4-yl; N, NH)
55c	$-COOCH(NH_2)CH_2-$ (imidazol-4-yl; N, NH)	$-CH_2Br$

2.2.3.0 L-Histidinol : Esterification and different possible Attachment Sites to the Alkylating Moiety

As discussed earlier, one of the major limitations to cancer chemotherapy is the failure of most antineoplastic agents to act specifically against tumour cells. Many of the present day anticancer agents act directly against rapidly proliferating cells regardless of their origin. A consequence of this mechanism of action is that normal host tissues e.g. bone marrow and intestinal mucosa which also proliferate rapidly, are also sensitive to the cytotoxic effects of these agents which usually prevents the administration of a sufficient quantity of the drug to eradicate the tumour completely(Hoagland,1982; Valeriote and van Putten, 1975). The most common finding of chemotherapy is that tumours respond poorly or not at all to present day drugs. If they do show a significant regression they often regrow once the drug has had to be discontinued because of excessive toxicity to non-malignant cells of the myelopoietic, gastrointestinal and epidermal tissues.

While drugs which are currently available do possess the ability to kill tumour cells, they could be made considerably more effective if their selectivity was improved. In other words, it is important to find some means by which the sensitive non-malignant tissues of the host could be

protected from the action of antineoplastic drugs, but at the same time the effect of drug on the tumour can be retained thereby improving significantly the therapeutic index of anticancer agents. This would not only allow more aggressive treatment regimens but also reduce the deleterious side effects of many cancer chemotherapeutic agents.

Recently the emergence of L-histidinol, a structural analog of the essential amino acid L-histidine has been found to possess such required characteristics. It has been found to arrest reversibly the growth of non-malignant cells with normal phenotype in a G_0-like noncycling state. At the same time it does not cause any similar cessation of growth of tumourogenic cell lines and, as a consequence, it makes them more susceptible to **S**-phase drugs(Warrington, 1978; Warrington and Fang, 1982; Warrington and Hechtman, 1977; Warrington and Muzyka, 1983; Warrington *et al*., 1977). Furthermore these exceptional properties of L-histidinol have been found to be retained in tumour bearing animals. When used in combination with antineoplastic agents, L-histidinol has been reported to increase both specificity and efficacy of alkylating agents like BCNU[N,N'-*bis*-(2-chloroethyl)-N-nitrosourea],cyclophosphamide, *cis*-platinum and antitumour antibiotics such as daunorubicin. Further, it has been found to diminish the associated *in vivo* myelotoxicity of agents like cytosine arabinoside and 5-fluorouracil. In combination with L-histidinol, these drugs have been shown to provide

curative treatment for tumour-bearing animals under conditions where these drugs on their own have little or no impact on survival. It has been suggested that L-histidinol/anticancer drug combinations approach to chemotherapy could be effective with a variety of clinically relevant antineoplastic agents(Warrington, 1986).

In a recent study(Warrington and Fang, 1989) L-histidinol has been found to reverse multidrug resistance(MDR) in the colchicine resistant(CH^R) variant CH^RC5 chinese hamster ovary cell line. This cell line has been been found resistant to colchicine, daunorubicin and vinblastine and the multidrug resistance has been attributed to the overexpression of a **P**-glycoprotein(170,000 dalton) by regulating the transport of various agents in or out of the MDR cells(Gerlach et al., 1986)

Keeping in view the remarkable property of L-histidinol and the ubiquitousness of esterases in vivo, it was suggested to prepare compound 55a wherein the L-histidinol moiety is present in the form of an ester. Compound 55a, according to our cascade theory, would lead to sensitization of the tumour cells before the thiol alkylator and cytotoxic species are released to produce their required effects(Section 2.2.0.0). Alternatively, it was suggested that the L-histidinol moiety could be attached as an ester to the aryl ring of the alkylating agent 55c(a cytotoxic species) containing a

nuclear hydroxy function especially, in which case a haloacyl functionality is used as the thiol depletor.

Having known that compound Ia is 5800 times more reactive than the established anticancer agent BCNU[N,N'-*bis*-(2-chloroethyl)-N-nitrosourea] when screened against P388 leukemia cells *in vitro*(*vide infra*) and to see if *L*-histidinol does exacerbate the cytotoxicity of Ia, which would validate our cascade hypothesis, it was suggested that compound Ia be screened in combination with *L*-histidinol against P388 cells *in vitro*.

2.3.0.0 *mono*-Basic and *bis*-Basic Mannich Salts of Aryl Alkyl Ketones and Styryl Alkyl Ketones

Many of the present anticancer agents used in cancer chemotherapy discussed earlier, cause several adverse side effects on organ systems other than neoplasms especially the hematopoietic and gastrointestinal systems(Calabresi and Parks, 1980c). In addition, paradoxically they may induce neoplasia and other mutations via their intercalation with amines and hydroxy groups present in the DNA(Harrap *et al*., 1981). It is self evident from this fact alone that there is a need for new selective anticancer drugs which could be directed against other intracellular targets such as mitochondria, the cell membrane or other key cellular processes which are of particular significance to the tumour

cells. It has been known for a long time that Mannich bases(β-amino ketones) possess a wide variety of biological activities including antineoplastic activity and often they are more effective than their corresponding α,β-unsaturated ketones in producing similar bioactivities(Dimmock and Wong, 1976).

Mannich bases are a class of compounds, many of which are capable of undergoing deamination to give unsaturated ketones. In addition, Mannich bases of conjugated styryl ketones already possess the α,β-unsaturated keto group. Compounds either possessing the enone function or capable of generating it by deamination(Dimmock and Wong, 1976; Mollica et al., 1970) or by possibly undergoing elimination-addition reactions(Andrisano et al., 1967) have a marked affinity for thiols but not amino or hydroxy group which are present in nucleic acids. Hence these derivatives should be bereft of the carcinogenic and mutagenic properties associated with a number of clinically used alkylating agents.

Most of the earlier work from our laboratories involved the synthesis and evaluation of mono-basic and bis-basic Mannich salts of styryl alkyl ketones and aryl alkyl ketones. Many clinically useful anticancer alkylating drugs have two alkylating arms i.e. are bifunctional with two sites for chemical reactivity, and bifunctional alkylators are generally more effective than mono-alkylators(Nobles and

Burckhalter, 1958; Wilman and Connors, 1983). Therefore in an attempt to prepare molecules with the features that could improve antineoplastic activity and also exhibit reactivity toward thiols, two small groups of compounds namely Series X and Series XI were initially suggested to be prepared and screened.

One would expect the two series of compounds to release cytotoxic species in a cascade manner(Scheme 2.3), firstly one of the alkylating arms(mono-alkylator(i) in the case of Series X and a bis-alkylator(ii) in the Series XI) and subsequently the second similar cytotoxic species(as shown in Scheme 2.3) which could hinder repair of drug-induced damage caused by the first alkylating species. In order to predict the effectiveness of the alkylating moieties which will be produced, different aromatic substituents were suggested to be placed in the aryl ring in order to seek correlations between bioactivity and both the Hammett(σ) and the Hansch hydrophobic(π) values of the aryl substituents. So depending upon the results, appropriate selection of substituents could be made in order to prepare more active analogues using the "Topliss Tree Approach".

A number of Mannich bases as mentioned earlier can undergo β-elimination to generate the active cytotoxic species i.e. the

$$R_1\text{-}C_6H_3(R_2)\text{-}C(=O)\text{-}CH_2CH_2\text{-}N(CH_2CH_3)\text{-}CH_2CH_2\text{-}C(=O)\text{-}C_6H_3(R_2)\text{-}R_1 \cdot HBr$$

(X)

Compound	R_1	R_2
Xa	H	H
Xb	Cl	Cl
Xc	OCH_3	H

$$R_1\text{-}C_6H_3(R_2)\text{-}CH{=}CH\text{-}C(=O)\text{-}CH_2CH_2\text{-}N(CH_2CH_3)\text{-}CH_2CH_2\text{-}C(=O)\text{-}CH{=}CH\text{-}C_6H_3(R_2)\text{-}R_1 \cdot HCl$$

(XI)

Compound	R_1	R_2
XIa	H	H
XIb	Cl	Cl
XIc	OCH_3	H

R_1 R_2 —X—C(=O)—CH_2 CH_2—N(—CH_2 CH_3)—CH_2 CH_2—C(=O)—X— R_1 R_2

HCl

β - elimination

R_1 R_2 —X—C(=O)—CH_2 CH_2—NH—CH_2—CH_3 HCl + H_2C= CH—C(=O)—X— R_1 R_2

C_2H_5 NH_2 . HCl

R_1 R_2 —C(=O)—CH=CH_2

(i)

R_1 R_2 —CH=CH—C(=O)—CH=CH_2

(ii)

Scheme 2.3

corresponding α,β-unsaturated ketones(Dimmock and Wong, 1976; Mollica *et al.*, 1970) such as(Scheme 2.4) the dienone 66 from 65a(Dimmock *et al.*, 1980a) and 65b(Edwards *et al.*, 1983). Earlier Dimmock and co-workers(1979) had shown that the dienone 68 derived from 67 lacked both antineoplastic activity and murine toxicity.

In contrast, the similar dienones 66 generated from 65b have been proposed as the active species when 65b was screened against HVH(herpes virus hominis) infection in mice(Edwards *et al.*, 1983). At the same time it should be borne in mind that while attempting to correlate biological activity with the chemical structures, a plethora of variables could affect the biological results especially the physicochemical characteristics such as aqueous solubility for example which could indirectly affect the compounds' permeability through cells since Mannich bases in some cases have been prepared as prodrugs to improve aqueous solubility(Dimmock and Wong, 1976).

In an attempt to clarify whether or not the enone is the major active species responsible for bioactivity, the preparation of the following two unsubstituted compounds 69 and 70 were suggested whereby the presence of four geminal methyl groups would prevent β-elimination and thus no enone would be formed.

R_1, R_2-C_6H_3–CH=CH–C(=O)–CH_2 CH_2–NR_2 → R_1, R_2-C_6H_3–CH=CH–C(=O)–CH=CH_2

(65) **(66)**

65a. $NR_2 = N(CH_3)_2$ .HCl; $R_1 = R_2 = H$

65b. NR_2 = N(morpholino)O .HCl; $R_1 = R_2$ = Cl, CN, NO_2, CF_3

R_1, R_2-C_6H_3–CH=CH–C(=O)–CH(–CH_2–$N(CH_3)_2$. HCl)–$(CH_2)_4$–CH_3 → R_1, R_2-C_6H_3–CH=CH–C(=O)–C(=CH_2)–$(CH_2)_4$–CH_3

(67) **(68)**

R_1 = 4-Cl, 3-Cl; R_2 = H, 4-Cl

Scheme 2.4

(69)

(70)

If we consider that the enones derived from the corresponding Mannich base are the species responsible for bioactivity, then the other way of enhancing the effect of the Mannich base would be by forming quaternary salts of Mannich bases. This is a rational process for the following reasons. Firstly the strong positive inductive effect of the ammonium group would render the carbon atom of the adjacent methylene group more acidic and secondly it would be a good leaving group. Hence, the quaternary ammonium halides derived from Mannich bases would be expected to undergo elimination at a faster rate than the corresponding tertiary amine Mannich bases. It has been reported that while crystallizing quaternary salts of Mannich bases, they readily undergo β-elimination to produce the corresponding enones(Stenlake et al., 1989) and

when tested against HVH(herpes virus hominis) in infected mice, the quaternary salts were found to be more active than the corresponding Mannich bases which are tertiary amines. This disparity in activity has been attributed to the release of the corresponding enones <u>in vivo</u> from the quaternary salts(Edwards <u>et al</u>., 1983)

Therefore in the present investigation the two Series XII and XIII of quaternary halides of the corresponding tertiary Mannich bases Series X and XI respectively were suggested to be prepared and screened. In addition it was also suggested to prepare the following two quaternary salts 71 and 72 of the corresponding dimethyl analogues(Mannich bases) 69 and 70 respectively.

(71)

(72)

$$R_1,R_2\text{-}C_6H_3-\overset{O}{\overset{\|}{C}}-CH_2CH_2-\overset{\oplus}{N}(CH_3)(CH_2CH_3)-CH_2CH_2-\overset{O}{\overset{\|}{C}}-C_6H_3\text{-}R_1,R_2 \quad Br^{\ominus}$$

(XII)

Compound	R_1	R_2
XIIa	H	H
XIIb	Cl	Cl
XIIc	OCH_3	H

$$R_1,R_2\text{-}C_6H_3-CH{=}CH-\overset{O}{\overset{\|}{C}}-CH_2CH_2-\overset{\oplus}{N}(CH_3)(CH_2CH_3)-CH_2CH_2-\overset{O}{\overset{\|}{C}}-CH{=}CH-C_6H_3\text{-}R_1,R_2 \quad Br^{\ominus}$$

(XIII)

Compound	R_1	R_2
XIIIa	H	H
XIIIb	Cl	Cl
XIIIc	OCH_3	H

2.3.1.0 Conjugated Styryl Ketones : Styryl Alkyl Ketones

$$R_1(R_2)C_6H_3-CH{=}CH-C({=}O)-C(R_3)(R_3)-H$$

(XIV)

Compound	R_1	R_2	R_3
XIVa	OCH_3	H	H
XIVb	Cl	Cl	H
XIVc	H	H	CH_3

These compounds were prepared to serve as precursors for the preparation of their corresponding Mannich bases. The rationale for the synthesis of the Mannich bases is discussed in detail in the preceeding section.

3.0.0.0 DESCRIPTION OF THE EXPERIMENTAL WORK

3.1.0.0 Materials and Methods

Drying of reagents, solvents and compounds

Drying was carried out according to reported procedures (Vogel, 1978a). The organic extracts were dried over anhydrous magnesium sulfate or sodium sulfate. The pure compounds after preliminary drying by aspirator were eventually dried in an Aberhalden drying pistol(small samples) or using a vacuum oven manufactured by Lab Line Instruments(larger samples) over vanadium pentoxide and submitted for analysis.

Weighing and dissolution of compounds

Weighings were carried out on Mettler AE100(digital) and Mettler type H6T analytical balances. Dissolution of compounds was facilitated using a S/P® Vortex mixer(American Scientific Products).

Freeze-drying

Freeze drying was performed on a Labonco^{R} freeze dryer.

Chromatography

Column chromatography was carried out using Merck's silica gel 60, 70-230 mesh size(ASTM). Eastman chromatogram sheets, type 13181(silica gel with fluorescent indicator) and kieselgel 60 F_{254}(silica gel) sheets from Merck were used to perform thin layer chromatography(TLC). TLC's were run in four different solvent systems containing varying proportions of benzene and methanol(viz., 95:5, 85:15, 65:35 and 50:50) unless otherwise stated in the text. Reverse phase TLC was used for quaternary compounds and was performed on Whatman MKC18F reverse phase TLC plates of 200 μ thickness. The solvent systems used included benzene:acetic acid(80:20, 95:5, 50:50), chloroform:acetic acid(98:2, 95:5), chloroform: methanol(90:10) and chloroform:methanol:acetic acid(90:5:5, 80:10:10) as well as several other combinations for better separation of the reaction products.

Melting points

Melting points were observed visually on a Gallenkamp MF-370 instrument and are uncorrected.

Elemental analysis

The samples were analyzed by Mr.K.Thoms, Department of Chemistry, University of Saskatchewan using a Perkin-Elmer

CHN elemental analyzer. Some of the samples were also analyzed by Guelph Chemical Laboratories Ltd., Ontario.

pH Measurements

All pH determinations were carried out using a METROHM 632 pH meter(Brinkman) equipped with a microelectrode. Before each determination, the meter was calibrated with coloured buffer solutions of pH 4.00, 7.00 and 10.00 obtained from BDH Chemicals.

Infrared(IR) spectroscopy

IR samples were prepared as KBr discs and spectra were recorded on a Beckman Acculab$^{T.M.}$4 spectrophotometer.

Ultraviolet(UV)-visible(VIS) spectroscopy

UV spectra were recorded on a Gilford Response$^{T.M.}$ UV-VIS or a Schimadzu(UV-265) UV-VIS spectrophotometers.

Nuclear magnetic resonance(NMR) spectroscopy

A Varian T-60 instrument was used to procure routine NMR spectra. High resolution NMR spectra were obtained by Dr.M.Duffy on a Bruker AM-300 FT NMR spectrophotometer equipped with a variable unit(BVT-1000) and Aspect 3000

computer located in the Department of Chemistry, University of Saskatchewan. DSS(sodium 2,2-dimethyl-2-silapentane-5-sulfonate) was used as the internal standard when solutions were made in deuterium oxide and TMS(tetramethylsilane) was employed when other organic deuterated solvents were used. Chemical shifts(δ) are reported in ppm with reference to the internal standards. The nature of the absorptions are specified as **s** for singlet, **br.s.** for broad singlet, **d** for doublet, **t** for triplet, **q** for quartet and **m** for multiplet.

Biological evaluations

Measurement of non-protein bound thiol levels

The assay for the glutathione content of mouse livers was carried out in our laboratory by Mrs.S.Wonko.

Antineoplastic evaluations

The *in vitro* antineoplastic evaluation against P388 leukemic cells was carried out in the laboratory of Dr.R.C. Warrington, Department of Biochemistry, University of Saskatchewan. The *in vitro* antineoplastic evaluation against L1210 leukemic cells was carried out in the laboratory of Dr. T.M.Allen, Department of Pharmacology, University of Alberta. The prescreening antineoplastic evaluation against P388 lymphocytic leukemia in CD_2F_1 mice and various human tumour

cell lines in an *in vitro* system was carried out under the auspices of the Developmental Therapeutic Program, Division of Cancer Treatment, National Cancer Institute, Bethedsa(USA) using their protocols(Geran *et al.*, 1972; Boyd, 1989).

Thermal denaturation profiles

DNA binding studies using thermal denaturation profiles were carried out in the laboratory of Dr.J.S.Lee, Department of Biochemistry, University of Saskatchewan.

Stability studies

Stability studies were carried out by Dr.M.Duffy using a Bruker AM-300 FT NMR spectrophotometer. The work was carried out in the Department of Chemistry, University of Saskatchewan.

X-ray crystallographic studies

These studies were carried out by Mr.Z.Jia under the supervision of Dr.J.W.Quail of the Department of Chemistry, University of Saskatchewan in the laboratory of Dr.L.T.J.Delabare, Department of Biochemistry, University of Saskatchewan.

3.2.0.0 Synthesis of 3,5-*bis*-arylidene-4-piperidone hydrochlorides and free bases

3.2.1.0 General method of preparation of 3,5-*bis*-arylidene-4-piperidone hydrochlorides(Table 3.1, Ia-e)

These compounds were prepared according to a literature procedure(Kuettel and McElvain, 1931). To a stirred solution of 4-piperidone hydrochloride monohydrate(1.54 g, 0.01 mol) in glacial acetic acid(58 ml) was added the appropriate aryl aldehyde(0.021 mol). Anhydrous hydrogen chloride gas was passed through the solution until it was saturated; external cooling was applied to ensure that the temperature of the reaction mixture did not rise above 25°C. Stirring was discontinued and external cooling was removed allowing the mixture to rise to room temperature. After 24h , the crystals were removed by filtration, washed with glacial acetic acid and then anhydrous ether and dried. The crude product was purified by recrystallization and its purity checked by TLC using a solvent system of benzene : methanol(85 : 15). The structures of these compounds were confirmed by NMR and IR spectroscopy and elemental analysis. The spectroscopic data of one of the representative compounds Ia is as follows.

NMR(60 MHz) : δ(DMSO-d_6) 8.07-7.90(s, 2, C<u>H</u>, olefinic), 7.72-7.50(s, 10, $C_6\underline{H}_5$, aromatic), 4.63-4.38(br.s.,4, $C\underline{H}_2$, cyclic).

IR(KBr) : 1675(C=O), 1610, 1585 and 1450(conjugated -C=C-Ph).

3.2.2.0 General method of preparation of 3,5-*bis*-arylidene-4-piperidones(Table 3.1, If,g)

The hydrochloride salts of If and Ig could not be characterized satisfactorily by elemental analysis even after repeated crystallizations. Thus they were converted to the corresponding tertiary amine bases by dissolving the hydrochloride salts in boiling aqueous ethanol(50% v/v) and to this hot solution a sufficient amount of aqueous sodium bicarbonate solution(10% w/v) was added to liberate the free bases. On cooling, the resulting suspensions of crude products were filtered, washed with aqueous ethanol(95% v/v) and dried. The crude products thus obtained were purified by recrystallization using an appropriate solvent system. The NMR spectral data generated for a representative compound If is as follows.

NMR(60 MHz) : δ($CDCl_3$) 7.77-7.62(s, 2, C<u>H</u>, benzylidene), 7.42-7.12(s, 2, C<u>H</u>, **o**-aromatic, J=9 Hz), 6.75-6.50(d, 4, C<u>H</u>, **m**-aromatic, J=9 Hz), 4.15-4.02(br.s., 4, C$\underline{H}_2$, cyclic), 3.00-2.88(s, 12, N(C$\underline{H}_3$)$_2$).

3.2.3.0 Synthesis of dipotassium 3,5-*bis*-(4'-carboxybenzylidene)-4-piperidone(Table 3.1, Ih)

This compound was prepared by a literature procedure(McElvain and Rorig, 1948) with some modifications. A solution of potassium hydroxide(2.19 g, 0.039 mol) in water(15 ml) was added dropwise to a mechanically stirred solution of 4-piperidone hydrochloride monohydrate(1.54 g, 0.01 mol) and 4-carboxybenzaldehyde(3.15 g, 0.021 mol) in aqueous ethanol(95% v/v, 150 ml). The temperature of the reaction mixture was maintained below 15°C throughout this addition. The reaction mixture was stirred for an additional 6h and was allowed to attain ambient temperature. The precipitated crude product was filtered, washed with aqueous ethanol(95% v/v), dried and purified by recrystallization from ethanol-water. The title compound obtained was characterized by NMR spectroscopy and elemental analysis. The NMR spectral data of the title compound is as follows.

NMR(60 MHz) : $\delta(D_2O)$ 7.45-7.05(d, 4, C$\underline{H}$, **o**-aromatic, J=10 Hz), 7.73-7.45(t, actually doublet with third internal peak of C$\underline{H}$-olefinic protons, 6, **m**-aromatic, J=10 Hz), 3.93-3.42(br.s., 4, C$\underline{H}_2$, cyclic).