Chapter-I

Introduction and Review of Literature

Mitomycin
N-mustards
Bleomycin
Ellipticine
Proflavine
Netropsin
Bis-Acridines
Steroidal diamines

Nalidixic acid
Novobiocin
Streptolydigin
Rifampicin
1.1. Introduction:

Research into drug action at the molecular level must be concerned primarily with the physical interaction of a drug with its receptor and then with the events which connect this interaction to the pharmacological response. In so far as DNA can be regarded as a target for anticancer drugs, the study of its interaction with small molecules can be useful for our understanding of the mode of drug action (Denny 1989; Waring 1981; Wilson and Jones 1981; Hurley 1989). Thus, drug-nucleic acid interaction remains of vital interest, especially when considered within a process in which recognition of DNA by enzymes such as DNA topoisomerase II occurs (Chen and Liu 1994; Stacie et al., 1995; Krzysztof et al., 2004; Smitha et al., 2005).

We are concerned with two fundamental aspects of drug-DNA interactions: the structural basis leading to a preference for either intercalation or outside binding and the relationship between these binding modes and the induction or inhibition or DNA-topoisomerase II cleavable complex. The compounds examined in our studies are pyrimidothienoquinolines which are structurally related to ellipticine which has been isolated from Apocynaceae species (Paoletti et al., 1979) and has motivated important investigations to determine its antitumour properties (Festy et al., 1971; Didier et al., 1980; Auclair 1987 and 1988; Veronique et al., 2000; Frei et al., 2002).

We recently reported the promising anti proliferative activity associated with a new class of DNA intercalators based on a tetracyclic condensed quinoline system (Gopal et al., 2003 and 2004). Results from our laboratory are demonstrating that, dialkylamino and butylamino substitution at position 4 lead to the assumption that the length of the side chain could be responsible for metabolic activation and / or
covalent binding to DNA and moreover, the anti proliferative activity is strongly dependent on chromophore substitution (Gopal et al., 2003 and 2004). Recently Gopal and Veeranna studied DNA affinity properties of 4-benzylaminopyrimido [4,5:4,5]selenolo(2,3-b)quinoline, suggested that 4-benzylaminopyrimido[4,5:4,5] selenolo(2,3-b)quinoline / 4 - butylaminopyrimido[4,5:4,5]selenolo(2,3-b)quinoline (BA P SQ / BPSQ) binds with its long axis perpendicular to the long axis of the DNA helix and the same drug was able to bind to external sites, second mode once the intercalation sites were saturated at high concentration (Gopal and Veeranna 2004 and 2005). The mode of action of pyrimido[4,5:4,5]thieno/selenolo(2,3-b)quinolines remain unclear. Most of the drugs are intercalators, but other binding modes exist as well (Steven et al., 1989; Monnot et al., 1991; Guo-Dong Liu et al., 2002).

Structure-activity studies indicate that the dialkylamino, butylamino and Morpholino substitution at 4th position of pyrimidothienoquinolines contributes to stabilize the intercalation complex and thereby improves antitumour activity (Gopal et al., 2002, 2003 and 2004). Also, the 4-Aminopyrimido[4,5:4,5]thieno(2,3-b)quinolines / 8-Methly-4(3-diethylaminopropylamino)pyrimido[4,5:4,5]thieno(2,3-b)quinolines(APTQ/MDPTQ) may interfere with topoisomerase II activity by stabilizing the enzyme-DNA cleavable complex (Shenoy et al., 2006).

These considerations prompted us to explore a new series of pyrimidothieno quinoline type chromophores with potential DNA intercalating properties. We thought that drug-DNA interaction could be used as a guide to develop more active compounds in this series.
Five compounds viz, Pyrimido[4',5':4,5]thieno(2,3-b)quinoline (PTQ), Oxo pyrimido[4',5':4,5]thieno(2,3-b)quinolin-4(3H)-one (Oxo-PTQ), 8-Methoxypyrimido [4',5':4,5]thieno(2,3-b)quinolin-4(3H)-one (Methoxy-PTQ), 8-Chloropyrimido[4',5' :4,5]thieno(2,3-b)quinolin-4(3H)-one (Chloro-PTQ) and 4-Morpholinopyrimido [4',5' :4,5]thieno(2,3-b)quinoline (Morpholino-PTQ) were taken up for the present study (Figure 1).

The present studies were undertaken with the following main objectives:

- To examine the nature of interaction mechanism of PTQ, Oxo-PTQ, Methoxy-PTQ, Chloro-PTQ and Morpholino-PTQ with DNAs by measurement of the absorption spectrophotometry, spectrofluorimetric titration, melting behavior, viscosity and circular dichroism.

- To determine the relative activity of PTQ, Oxo-PTQ, Methoxy-PTQ, Chloro-PTQ and Morpholino-PTQ to inhibit cell proliferation, by evaluation using the MTT assay for cytotoxicity against the human cancer cell lines.

- To evaluate anticancer efficacy against B16 melanoma and to provide evidence of major antitumour activity for PTQ, Oxo-PTQ, Methoxy-PTQ, Chloro-PTQ and Morpholino-PTQ.

- To demonstrate the orientation of the intercalators in its binding site.

- To investigate topoisomerase II inhibitory activity of pyrimidothienoquino lines.

- To determine the clastogenic effects of PTQ, Oxo-PTQ, Methoxy-PTQ, Chloro-PTQ and Morpholino-PTQ in terms of chromosomal aberration and micronuclei induction.
Our findings may contribute to a new intercalation functional group to DNA targeted drug design, in addition to the understanding of the mechanisms of differentiation inducing effects of PTQ, Oxo-PTQ, Methoxy-PTQ, Chloro-PTQ and Morpholino-PTQ in the development of strategies to control growth of human cancer cells.
Figure 1: a. Pyrimido[4',5':4,5]thieno(2,3-b)quinoline (PTQ),

b. Oxopyrimido[4',5':4,5]thieno(2,3-b)quinolin-4(3H)-one (Oxo-PTQ),

c. 8-Methoxypyrimido[4',5':4,5]thieno(2,3-b)quinolin-4(3H)-one (Methoxy-PTQ),

d. 8-Chloropyrimido[4',5':4,5]thieno(2,3-b)quinolin-4(3H)-one (Chloro-PTQ),

e. 4-Morpholinopyrimido[4',5':4,5]thieno(2,3-b)quinoline (Morpholino-PTQ).
1.2. General Overview:

The biological activity of many low molecular weight antitumour agents appears to be related to their mode and specificity of interaction with particular DNA sequences. Such small molecules are of considerable interest in chemistry, biology and medicine. Most of the anticancer drugs employed clinically exert their antitumour effect by inhibiting nucleic acid (DNA or RNA) or protein synthesis. Inhibition can occur, for example, through cross-linking of bases in DNA or binding to and inactivation of DNA-related enzymes.

The majority of drugs used today in the treatment of cancer binds reversibly or irreversibly to DNA or induce DNA damage, either directly or via topoisomerase inhibition. DNA and associated proteins remain valid targets for cancer chemotherapy (Thurston 1999; Hurley 2002). Novel DNA intercalating (acridines, indolocarbazoles, naphthalimides) and alkylating agents (banzoacronycines, pyrrolo-benzodiazipines, distamycin conjugates) are still being developed (Catoen et al., 2004). DNA whether with two, three or four strands, still represents one of the most challenging bioreceptors for small molecules and a target of choice for the control of gene expression (Dervan 2001). The design of cytotoxic agents interfering with DNA metabolism is actively pursued.

Although it is well established that DNA binding is not sufficient to confer cytotoxic activities, interaction with DNA is often considered as a necessary criterion to maintain a cytotoxic effect, at least for some series of planar intercalating chromophores such as ellipticines, and acridines (Table 1). On the basis of this assumption, Tilakraj and Ambeker have recently reported the design of DNA-targeted
pyrimido[4',5':4,5]thieno/selenolo(2,3-b)quinolines (Tilakraj and Ambeker 1988; Nandeshiah and Ambekar 1998).

The interactions and DNA damage suspected to be involved in the cytotoxic process leading to antitumour activity are the following:

A. Reversible interactions:
   1. Intercalation between base pairs
   2. Outside binding

B. Covalent binding:
   1. Alkylation of bases
   2. Covalent binding of bulky compounds
      • At the intercalating sites
      • At the external sites

C. Generation of strand breaks:
   1. Through the generation of apurinic sites
      • From oxidative damage
      • From alkylation and occurrence of bulky adducts
   2. Through interaction with topoisomerase II

The difficulty in demonstrating the involvement of a given process in the expression of antitumour activity comes from the fact that most antitumour drugs exhibit several possibilities for damaging DNA for eg, ellipticine, covalently binds to DNA after being enzymatically activated. The cytochrome P-450 (CYP) isozymes known to be expressed at higher levels in tumours sensitive to ellipticine (i.e., breast cancer, renal cancer) than in peritumoural tissue, namely CYP3A4, CYP1A1 AND
CYP1B1 (Murray et al., 1993 and 1995; Patterson et al., 1999) were the most efficient in activating ellipticine to form covalent DNA adducts *in vitro* (Stiborova et al., 2001). Hence ellipticine might be considered a prodrug, whose pharmacological efficiency is dependent on its enzymatic activation in target tissues.

The major challenge in molecular pharmacology is, therefore, to determine the degree to which each individual event contributes to the cytotoxic activity of drugs. The most commonly used experimental approach consists of structure-activity relationship studies that should determine the critical parameters involved in a given biological effect. This approach requires (i) the synthesis of many derivatives in a homologues series in which, ideally, only one parameter varying and (ii) the use of appropriate biological models.

Ellipticine derivatives [Figure 2, Table 1] represent useful materials for such a purpose because more than 70 synthetic compounds of this group have been prepared and their structure-activity relationships have been screened (Le Pecq et al., 1974; Paoletti et al., 1979). In the view of their possible interaction with DNA, active ellipticines exhibit a striking analogy to anthracyclines and can be considered as representative of antitumour compounds that have potential multimodal mechanisms of cytotoxicity.

Recently, Tilakraj and Ambeker (Tilakraj and Ambeker 1988) have reported the synthesis of pyrimido[4',5':4,5]thieno(2,3-b)quinolines which have a structure that is very close to that of ellipticine. These compounds belong to a new class of linearly fused tetracyclic condensed quinoline system. Therefore, the present work of DNA intercalation and antitumour activity was undertaken.
The ellipticine nucleus is composed of a carbazole moiety linked to a pyridine ring, giving the appearance of a highly aromatized hydrophobic compound. The relatively simple structure of ellipticine has prompted chemists to design various structural modifications in order to obtain either more active derivatives or information on the structural moieties required for pharmacological activities. The main modifications have been:
• Hydroxylation at position 9 or 7.
• Quaternarization at the N-2 position by the addition of a methyl group or an aliphatic hydrophobic chain.
• Addition of methyl group or aliphatic chain at the position N-6 or C-1.
• Displacements of the methyl group form the C-11 position to the C-1 position, leading to the series of olivacines.
• Cyclization at C-10 and hydroxy-9 position in the presence of primary amines, leading to the series of oxazolopyridocarbazoles.

1.2.1. Anticancer Properties of Ellipticines:

All ellipticine derivatives, including the natural compounds ellipticine and 9-methoxyellipticine, are highly cytotoxic to malignant cultured cells (Stiborova et al., 2001 and 2004). Most of the 9-hydroxy derivatives are active on standard experimental tumours such as L1210 and P388 murine leukemia, melanoma B16, Sarcoma 180 and colon 38 (Le Pecq et al., 1976; Paoletti et al., 1978). The quaternarized drug 2N-methyl-9-hydroxyellitpicinium (NMHE) exhibits promising results for the treatment of osteolytic breast cancer metastasis, kidney sarcoma, and hepatoma (Juret et al., 1982; Paoletti et al., 1980; Caille et al., 1985). The main reasons for interest in ellipticines for clinical purposes are their limited toxic side effects and their complete lack of hematological toxicity (Diop et al., 1990).

1.2.2. DNA as the Target for Ellipticines:

The concept of intercalation was first introduced by Lerman (Lerman 1961) to explain the reversible, non-covalent binding of compounds with planar aromatic (most often heteroaromatic) extended chromophores to double helical DNA. The
essence of intercalation is the insertion of such a chromophore between adjacent base pairs of the DNA, thereby extending and stabilizing the double helix: Base-pair separation at an intercalation site thus increases from 3.4Å to 6.8Å (Berman and Young 1981, Wilson and Jones 1981). Processes of DNA transcription, translation, and replication can all be affected by intercalation; these effects have been rationalized by the decreased ability of the double helix to unwind consequent to drug binding. A number of cancers, especially leukemia’s, which are characterized by rapid cell proliferation, can have their growth preferentially inhibited by a variety of intercalating compounds as a consequence of their inhibition of DNA function. The effects produced by, in particular, the ellipticines, are such that they have a clinically exploitable window of therapeutic index, thereby discriminating between normal and some tumour cells. The intercalating compounds doxorubicin, mitoxantrone, amsacrine and actinomycin are all in current clinical use, with the anthracene–9, 10-dione, and doxorubicin having the widest spectrum of activity of any current anticancer drug. Thus the major interest in DNA-intercalating compounds has focused on their anticancer activity (Waring 1981; Zeeman et al., 1998; Hurley 1989; Traganos et al., 1991).

In general, intercalating drugs have a polarizable electron-deficient chromophore that is typically composed of three to four fused six-membered rings. A formal positive charge is common, located either on the central chromophore or attached to the substituents groups. The presence of substituents moiety appears to be a necessary precondition for antitumour action, although not for intercalative binding.
There is little evidence that these intercalating drugs have a DNA sequence selectivity that extends beyond the 2 or 3 base pair level. Thus, the sensitivity of some tumour cells to these compounds cannot readily be explained in terms of selective inhibition of tumour-specific DNA sequences (oncogenes). Rather, evidence has accumulated that the antitumour effects of such agents are related to a drug-induced stabilization of a cleavable DNA complex involving the enzyme DNA topoisomerase II, which results in lethal double-strand breaks (Holden 2001). Thus intercalation is currently considered to be an essential (although by itself insufficient) component of the anticancer action of these drugs, and is probably the primary recognition event prior to the formation of a ternary drug-DNA-enzyme complex.

The assumption that DNA may act as the primary target for ellipticines was based initially on the following physicochemical and biological evidence: The size and shape of the ellipticine chromophores closely resemble those of a purine-pyrimidine complementary base pair, providing favorable conditions for its intercalation in double stranded DNA. Furthermore, the polycyclic aromatic character of the molecule may result in tight interactions with appropriately conformed hydrophobic regions in DNA. Accordingly, ellipticine and most of its derivatives have been found to interact in vitro with DNA through an intercalation process with association constants $K_{\text{app}}$ on the order of $10^6 \text{M}^{-1}$ (Kohn et al., 1975; Le Pecq et al., 1974). Chromosomal abnormalities including achromatic gaps, chromatid breaks, sister chromatid exchanges and chromosome fragmentation appeared in cultured cells and in bone marrow cells from mice treated with ellipticines (Renault et al., 1987; Sakamoto-Hojo et al., 1988, Sakamoto-Hojo and Takahashi 1991).
A direct action on nucleic acids in cells also appeared to be likely because ellipticines have been found to interfere with the activity of DNA polymerase, RNA polymerase (Kohn 1983; Li and Cowi 1974), RNA methylase and more recently, topoisomerase II (Zwelling et al., 1982; Monnot et al., 1991; Le mee et al., 1998). These effects could be attributed either to the binding of the drugs to double-helical nucleic acids or to their direct effect on the enzymes.

1.2.3. Reversible Interaction with DNA:

On the assumption that DNA is the major target of the ellipticine derivatives, the rational approach to obtain drugs of increased activity in this series has been structural modifications to increase the apparent affinity ($K_{\text{app}}$) of the new derivatives to DNA. On the basis of crystallographic data, the hydroxylation of the ellipticine nucleus at position 9 was predicted to increase the $K_{\text{app}}$ value through the appearance of a hydrogen bond between the phenolic proton and the negatively charged phosphates (Courseille et al., 1974). This assumption has been experimentally verified for both basic and quaternarized ellipticines. Compared to their unhydroxylated counterparts, the 9-hydroxy derivatives exhibit a higher cytotoxicity on L1210 cultured cells. Significant antitumour activity was also found for the quaternarized drugs.

The active compounds exhibit an association constant value higher than $10^5$ M$^{-1}$ indicating that a high affinity for DNA is a necessary condition for antitumour activity. However, many drugs in these series, including 9-aminoellipticine and 6N-alkylellipticinium derivatives, which have $K_{\text{app}}$ values equal or higher than $2 \times 10^6$ M$^{-1}$, displayed low cytotoxic activity and no antitumour effect. Accordingly, nonlinear regression analysis of a large series of compounds has indicated that no significant
correlation exists between $K_{app}$ value for DNA and the cytotoxicity of the drugs on cultured cells.

1.2.4. Intercalation Versus Outside Binding:

In situations where DNA is the primary target for antitumour agents, the nature of the interaction between the drugs and DNA is likely to be a parameter, which may control their cytotoxic efficacy. DNA binding by drugs such as ellipticines may occur by external binding by electrostatic interactions, outside binding to either the minor or the major groove and intercalation between base pairs in these cases by hydrophobic interactions, hydrogen bonding and Vander wall’s forces. The hypothesis is that intercalation of ellipticines between DNA base pairs could be responsible for antitumour activity and has been supported by the fact that in this series, all drugs which are not able to intercalate exhibited a low cytotoxicity on cultured cells and no significant antitumour activity. Non intercalative ellipticines have been provided by structural modifications either by the hydrogenation of the pyridine ring which distorts the planar ellipticine nucleus, or the substitution of the hydrogen at position 9 by bulkier functional groups such as Br, F or NO$_2$. The inability of these drugs to intercalate was obvious from the low values of the slopes corresponding to the length increase of sonicated DNA following DNA binding (Saucier et al., 1971).

A summary of the results obtained with a large number of drugs clearly indicates that intercalation appears to be a necessary but not sufficient condition for antitumour activity. Very recent work moreover, has shown that the presence of a methyl group at the C-1 position of the pyridine ring of unhydroxylated ellipticumium resulted in the complete suppression of its ability to intercalate between DNA base pairs, probably
through the distortion of the planar shape of the chromophore (Moinet-Hedin et al., 2000). The two unhydroxylated isomers 1-methyl and 6-methylellipticinium, the ability to intercalate has no influence at all on the cytotoxic activity of the drugs. Additional information of the consequence of the intercalation process in cytotoxic and antitumour activity was obtained using oxazolopyridocarbazole derivatives as probes. These drugs, derived from NMHE through the covalent addition of primary amines at the C-10 position of 9-hydroxyellipticinium, are markedly stable and can be considered as representative of simple reversible intercalators.

![Diagram](a) (b) (c)

**Figure 3.** A three mode binding model of ellipticine to DNA. (a) Intercalative model, (b) External binding model, (c) Self stacking model.

### 1.2.5. Covalent Binding to DNA:

Stiborova and co-workers used two direct independent methods, namely $^{32}$p-post labeling and tritium labeled ellipticine to show that ellipticine binds covalently to DNA *in vitro* after CYP-catalyzed activation (Stiborova et al., 2001). The results clearly demonstrated that V79 cells expressing human CYP are appropriate model systems for
the bioactivation of the anticancer drug ellipticine to DNA-binding species. The active derivatives binding to DNA generated in the cells seem to be the same as those formed in vitro, because the HPLC profiles of the isolated DNA adducts from cells and from in vitro incubations are the same. In contrast to the in vitro incubations 1-2 additional minor adducts formed in cells expressing human CYP 3A4, may be by further oxidation of the intermediates generated by CYP or by other oxidative enzymes constitutively expressed in V79 cells.

Stiborova et al., have first time reported that one DNA adduct is formed independently of enzyme activation (Stiborova et al., 2001), while the major adduct is formed upon catalysis by CYP. The chromatographic conditions used to resolve the adducts are suitable for hydrophobic, bulky adducts (Stiborova et al., 1994; 1990; Nortier et al., 2000).

In the mid 1980's the group of B. Meunier studied DNA binding of the antitumour drug N2-methyl-9-hydroxyellipticinium, which was radioactively labeled, to DNA or RNA upon oxidation by e.g. horseradish peroxidase (Dugue et al., 1986; Auclair et al., 1986). The intermediate was found to be the 9-oxoderivative. In the case of CYP catalyzed activation of ellipticine another mechanism seems to occur, because these authors found the unhydroxylated ellipticinium derivative not to be oxidized and to bind only very weakly to DNA.

In addition to the two known mechanisms of DNA damage (intercalation of ellipticine into DNA and generation of DNA strand breaks by inhibiting mammalian topoisomerase II), recently Frei et al., have found a third one, covalent binding of enzymatically-activated ellipticine (Frei et al., 2002). Acute toxicity could be caused by
the inhibition of topoisomerase II activity, uncoupling mitochondrial oxidative phosphorylation and thereby disrupting the cells energy balance (Schwaller et al., 1995), or by CYP – independent metabolites.

1.2.6. Oxidative Damage:

The involvement of oxidative injury in the killing of cells was initially suspected from the so-called oxygen effect in the extent of toxicity induced by ionizing radiations and is supported by the following considerations: Hypoxic cells are usually resistant to both ionizing radiation and cytotoxic drugs.

Oxygen deficiency in solid tumours appears to be responsible for the ineffectiveness of chemotherapy. Most anticancer drugs including anthracyclines, ellipticines, bleomycin, mitomycin C and naphthoquinones have the ability to generate oxy radicals (Bachur et al., 1978; Trush et al., 1982; Hecht et al., 1978; Chin and Goldberg 1986). As such, they can undergo a variety of reactions including adding across unsaturated bonds, abstracting hydrogen from other molecules or combining among themselves to form other dimers. Another possibility to be considered is that the radical intermediate may be in an electronically excited state which could then transfer its electronic energy to acceptor molecules or utilize this energy to oxidize target molecules. In addition, free radical intermediates, such as the semiquinone anion, the azo anion, the nitro aromatic anion and the bipyridinium cation, can activate molecular oxygen by univalent reduction to the superoxide anion (O\(^{-2}\)), which in turn can readily dismutate to produce hydrogen peroxide (H\(_2\)O\(_2\)) (Trush et al., 1982).

The generation of free radicals, including secondary molecular oxygen-derived radicals presents a danger to cells because radicals are capable of interacting
with, and subsequently damaging the entire array of biomolecules, which constitute cells. Moreover, radical initiated processes are particularly deleterious because they are conservative and propagative; that is, radical interaction with cell constituents may produce secondary and tertiary free radicals derived from lipids, amino acids, glutathione, ascorbic acid and components of nucleic acids. The cumulative effects of such a cascade of radical initiated reactions may be the immediate death of cells, possibly resulting in tissue necrosis and fibrosis or may be more subtle and delayed as evidenced by the development of neoplasmas.
Free Radical Generation

Molecular oxygen derived radicals

Drug activation

Dietary alterations and/or prior drug exposure

Drugs and other xenobiotics

Cellular metabolic activating systems

H₂O

Ionizing radiation

UV Light

Drug activation

Molecular oxygen derived radicals

Drug activation

Enzymatic and Non-Enzymatic Cellular Defenses

Superoxide dismutase

Ascorbate

Glutathione

Uric acid

Catalase

GSH Peroxidase

GSSG Reductase

α-Tocopherol

Enzymatic and non-enzymatic cellular defenses

INTERACTION WITH TARGET MOLECULES

Repair mechanisms

Altered cellular metabolism

Organelle damage

CELLULAR DAMAGE

POSSIBLE MANIFESTATIONS OF ENHANCED FREE RADICAL GENERATION WITHIN CELLS AND TISSUES

- Carcinogenesis and Mutagenesis
- Chemotherapeutic effect (Antineoplastic, Bactericidal, Parasiticidal)
- Photosensitivity and Photoallergy
- Radiation sensitization
- Red cell hemolysis, Tissue necrosis and Fibrosis

General reaction scheme illustrating how enhanced free radical generation within cells may result in altered cellular function.
In the ellipticine series, the generation of oxyradicals has been found to occur in vitro at alkaline and near neutral pH values during the auto-oxidation of the 9-hydroxy derivatives as follows.

The reaction occurs according to an oxidasic reaction, i.e., using molecular oxygen as the electron transfer process, generating intermediates, the superoxide anion (O$_2^-$) and the phenoxy radical of the drug, which further dismutates to produce hydrogen peroxide and the quinone-9-oxoellipticine, respectively. The process is accelerated markedly by superoxide dismutase and manganese ions.

In agreement with this hypothesis, nuclear DNA damage suspected to result from oxy radical production, such as alkali-labile sites and chromosome aberrations was detected in cultured L1210 cells and in bone marrow cells from mice treated with 9-hydroxy ellipticine (Paoletti et al., 1979). The inability of hydroxyellipticines to induce oxidative damage to DNA was suggested as well by the in vitro determination of strand breakage of super coiled DNA upon addition of drugs (Malvy et al., 1986). In contrast to bleomycin and adriamycin, 9-hydroxyellipticines are not able to produce any detectable strand breaks despite their ability to produce oxy radicals in the presence of DNA, as detected by the spin trapping technique. This can be explained either by the low flux of
oxy radicals produced through auto-oxidation compared to redox cycling or more likely, by the DNA site at which oxy radicals were produced. At the present time, it can be reasonably assumed that cellular DNA damage resulting from the effect of hydroxyellipticine did not result from the action of oxy radicals. It should be noticed as well that in contrast to drugs such as anthracycline none of the ellipticine derivatives subjected to efficient redox cycling, produce any cardiotoxic effect in experimental animals or in humans.

1.2.7. Interaction with Topoisomerase II:

Anticancer drugs targeting topo II are some of the most widely used chemotherapeutic agents. These drugs include doxorubicin, etoposide and ellipticine (Fortune and Osheroff 2000). In a broad sense, the drugs targeting topo II can be classified as either topoisomerase poisons or topoisomerase catalytic inhibitors. Transient double strand DNA break is characteristic of topo II reaction. The topo II poisons increase the steady state levels of this transient intermediate. They do this either by increasing the rate of DNA cleavage (ellipticine) or by inhibiting the rate of DNA religation (etoposide, amsacrine). In either case, these drugs convert the enzyme in to a DNA damaging agent. This is a stoichiometric relationship because there is the potential for one DNA double strand break for every drug-stabilized topo II enzyme. Thus sensitivity to the topo II poisons is dependent on high levels of enzyme. The more enzyme, the more DNA damage on other types of anticancer drugs and inhibit the activity of the enzyme. Drugs interfering with the catalytic activity of the enzyme deprive the cell of the enzyme’s ability to decatenate chromosomal DNA strands prior to mitosis. Cell growth is therefore altered. For these catalytic inhibitors, the reverse sensitivity
pattern holds. Cells with low levels of topo II are the most sensitive to the topo II catalytic inhibitors and cells with high topo II levels are most resistant. The topo II poisons resemble the action of the quinolones on *E. coli* gyrase and topo IV, and the catalytic inhibitors resemble the action of the coumarins.

The primary mechanism of action is to increase the concentration of the normally short lived cleaved DNA-intermediate that occurs during the topoisomerase II reaction. Theoretically, they could do this either by increasing the forward rate of the cleavage reaction or inhibiting the reverse (religation) reaction rate. In fact, accumulating data indicate that the topoisomerase II poisons can be divided into two classes based on which part of the cleavage reaction they affect. These are drugs that increase the amount of cleaved intermediate by stimulating the forward reaction and these are those that increase the amount of cleaved intermediate by inhibiting relication. The drugs that have clearly been shown to inhibit relication are etoposide, teniposide and amsacrine (Fortune and Osheroff 2000). Azotoxin, ellipticine appear to stimulate the forward reaction rate (Cline *et al.*, 1997; Froelich-Ammon *et al.*, 1995). Ellipticine which inhibits reigation, recent steady state and pre-steady enzymatic assays have allowed for a precise determination where in the reaction cycle the drug acts (Holden 2001). It appears that the drug acts to either inhibit the release of the first ADP from the enzyme or the hydrolysis of the second ATP. The transport of DNA through the G segment occurs prior to the inhibitory effects. Therefore the enzyme in the presence of drug is able to pass one DNA strand through the break in the G segment.

The topo II poisons convert the enzyme into a DNA damaging agent. The interesting possibility that this type of topoisomerase mediated DNA damage in the
presence of an anticancer drug may actually represent a normal cellular pathway involved in cell death has recently been suggested (Sabourin and Osheroff 2000). Several types of DNA damage such as base mismatches, deaminated cytosines and the loss of pyrimidine residues, induce topo II DNA cleavage in a manner analogue to the topoII poisons. Perhaps a cell that has suffered from, irreparable DNA damage uses topo II function to cleave DNA at the damaged areas, which then cycles the cell to an apoptotic death. The topo II targeted anticancer drugs may simply take advantage of this pathway.

1.2.8. Anticancer Drug-Induced Apoptosis:

The phenomenon of apoptosis, which is morphologically distinct from that of necrotic cell death, was first described by Kerr (Kerr et al., 1972). In the cell undergoing apoptosis extra cellular stimuli induce nuclear condensation and cellular shrinkage preserving the cell membrane, whereas necrosis is characterized by destruction of the cell membrane via an increase in osmotic pressure from outside the cell (Green and Reed 1998; Gross et al., 1999). Apoptosis is therefore known as programmed cell death and follows the activation of signal transduction (Evan and Littlewood 1998; Thornberry and Lazebnik 1998). In the cancer cells the growth balance which is determined as the ratio between the rate of cell proliferation and incidence of apoptosis is uncontrolled, and the increase in abnormal proliferation of cancer cells causes tumour invasion and metastatic potential. Cell proliferation and apoptosis in tumour growth is regulated by several oncogenes and tumour suppressor genes associated with the cell cycle, as well as by apoptotic related genes such as those of p53 and Bcl-2 families (Ferreira et al., 1999; Reed 1998; Cao and Korsmeyer 1998).
Apoptosis is an important phenomenon in cancer chemotherapy, because anticancer drugs exert their antitumour effect against cancer cells by inducing apoptosis. The apoptotic body which is characterized by nuclear condensation and cell shrinkage is subsequently dealt with phagocytosis by tumour associated macrophages.

Although the rate of apoptosis, which is assessed in terms of the apoptotic index, is not necessarily high in tumour tissues, the induction of apoptosis is correlated with tumour response and clinical outcome in cancer patients (Cassinelli et al., 2001; Kim et al., 2000). Resistance to apoptosis causes a decrease in the sensitivity of cancer cells to drugs, resulting in the failure of chemotherapy. Since the induction of apoptosis following chemotherapy is associated with the activation of pro-apoptotic genes and the suppression of anti-apoptotic genes, attenuation of pro-apoptotic genes and increase in anti-apoptotic genes causes resistance to apoptosis. Thus to increase the therapeutic effect of cancer chemotherapy, the assessment of molecular mechanisms and targeting apoptotic related genes may lead to new strategies for the enhancement of the antitumour effect against target organs.

The recent reviews on molecular mechanisms of anticancer drug-induced apoptosis revealed two pathways (1) drug induced apoptosis mediated by death receptor-dependent and (2) independent pathways which are related to the release of cytochrome C through voltage-dependent anion channels in the mitochondrial inner membrane. The release of cytochrome C is the central gate in turning on / off apoptosis and is regulated by the interaction of pro-apoptotic proteins, including Bid, Bax and Bak and anti-apoptotic proteins including Bcl-2 and Bcl-XL (Krajewski et al., 1995; Ogura et al.,)

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and a specific class of inhibitors of apoptosis proteins including Akt, survivin and heat shock proteins.

The caspase cascade is activated by the release of cytochrome C which is initiated by the formation of apoptosomes, consisting of procaspase-9, Apaf-1 and cytochrome C in the presence of dATP and results in the activation of caspase-9 and caspase-3 (Li et al., 1997; Hu et al., 1998), thereby leading to apoptosis. The introducing proapoptotic genes and the inhibition of anti-apoptotic proteins can enhance drug sensitivity. The later process is mediated by antisense oligonucleotides and is associated with apoptosis; the signal transduction pathways that are triggered by their central gate in mitochondria play a critical role in anticancer drug-induced apoptosis. The modulation of signal transduction pathways targeting the proteins involved in these signal transduction pathways using antisense inhibitor of apoptosis proteins (IAPS) and growth factor antibodies may be a good strategy for enhancing therapeutic efficacy of anticancer drugs in cancer chemotherapy.
Common pathways of apoptosis.
1.2.9. Genotoxicity of Antitumour Drugs:

Evaluation of new chemical entities for genotoxicity is an important part of the safety analysis of new drug substances. Clastogenicity and mutagenecity are fairly well understood processes and methods for the routine evaluation of these endpoints for the mandated ICH testing battery. It has been recognized, however that positive gene-tox test results could be the result of so-called “epigenetic” effects wherein DNA damage arises secondarily to other events such as lysozomal breakdown, perturbations in cell cycling or nucleotide metabolism or inhibition of topoisomerase II. These secondary effects are usually very difficult to distinguish from true drug/DNA interactions and, because of this, the interpretation of positive gene-tox findings is often unclear. Uncertainties of this type often affect decisions regarding further development of new pharmaceuticals.

Several types of assays are available for the detection and quantification of covalent drug/DNA interactions. However, neither a suitable cellular assay has been developed for the detection of non covalent interaction such as intercalation, nor has the genotoxic significance of intercalative binding has been fully characterized. Specific questions remaining to be addressed relate to (1) fully defining the structural characteristics conferring intercalative ability, (2) assessing the genotoxic and carcinogenic effects of intercalative binding and (3) determining the mechanism of those genotoxic effects. Because no cellular assay has been developed for demonstrating DNA intercalation, these three questions have been difficult to examine directly. Certainly the genotoxicity of classical aromatic tricyclic fused ring compounds has been well established and these agents are known to be frameshift mutagens as well as clastogens. The clastogenic activity of these agents is usually attributed to inhibition of DNA
topoisomerase (Denny 1989). It is clear, however, that there is wide structural variation among compounds that, at least in naked DNA systems, are capable of intercalative DNA binding (Wilson 1988; Strekowski et al., 1992). Structural motifs for intercalation among these groups are very diverse and not always immediately obvious. Therefore, the assessment of new polycyclic chemical entities as DNA intercalators may provide important information upon which predictions of genotoxicity might be based.

A method allowing the detection of drug intercalation in intact cells has gradually evolved. The glycopeptide antibiotic bleomycin exhibits strong antitumour activity through the induction of DNA double strand breaks. These breaks form when bleomycin complexed with ferrous ion, becomes activated by molecular oxygen, binds the minor groove of DNA, and makes DNA strand breaks by hydrogen abstraction (Strekowski et al., 1992; Kozarich and Stubbe 1985; Hechr 1986). It was shown as early as 1975 (Bearden and Haidle 1975) that intercalating agents altered the structure of DNA in such a way as to make it more susceptible to the DNA strand breaking activity of bleomycin invitro. This finding was later used by Strekowski (Strekowski et al., 1987, 1988 and 1991) and Wilson (Wilson et al., 1981) to define the structural features associated with intercalative binding studies designed to discover drugs that could amplify the antitumour effects of bleomycin clinically. It was subsequently demonstrated that intercalating agents increased the sensitivity of bacteria and mammalian cells to bleomycin induced cell killing and DNA damage (Grigg et al., 1984; Agostin et al., 1984; Pavelie et al., 1985). A further extension of the method was employed by Hoffmann (Hoffmann et al., 1993, 1994; Hoffmann and Littlefield 1995) to assess the
ability of various agents to protect against bleomycin-induced micronucleus formation of in cultured G₀ human lymphocytes.

Intercalation and groove binding should be viewed as two variable-depth potential wells on a continuous energy surface. The mode with the most favorable free energy for a particular ligand will depend on the DNA sequence and conformation as well as on the specific molecular features of the bound molecule. The usfused aromatic compounds may be fully or only partially sandwiched between DNA base pairs in the intercalation complexes. Either way, a relaxed DNA structure in the vicinity of the intercalation complex is produced.

It is believed that intercalative binding of such agents with DNA induces favorable conformational changes of the double helix for an increased affinity of the active bleomycin complex and favorable stereochemical fit for the bleomycin-mediated chemistry. As a result, a larger fraction of the active bleomycin complex interacts with DNA under such conditions and the scission of one strand of the double helix is enhanced. The observed preferential double strand scission of DNA has been explained in terms of a second strand degradation in the vicinity of the first damage because DNA is structurally relaxed in this region. Evidence has been presented that similar structural changes facilitate interaction of bloemycin with DNA (Strekowski et al., 1992).

1.2.10. Pharmacological Properties of Anticancer Drugs:

Prostaglandins (PGs) have been shown to mediate the inflammatory response locally and modulate a variety of physiological responses systematically (Davies et al., 1994). The conversion of arachidonic acid to prostaglandins is mediated by the two step action of prostaglandin H synthase (PGHS), commonly known as cyclooxygenase
(COX). The first committed step in this process is the oxidative cyclisation of arachidonic acid to PGG$_2$, which is followed by peroxide reduction to PGH$_2$ at the second distinct binding site. The moieties are then converted to PGD$_2$, PGE$_3$, PGF$_2$, and PGF$_{12}$ or thromboxanes (TBX). The specific prostaglandin produced is determined in part, by the particular cell type under consideration. In the past it was thought that COX was a single enzyme present constitutively in most cells. This led to the widely held notion that inhibition of cyclooxygenase would unavoidably lead to both beneficial and detrimental effects. However, recently it was observed that COX activity dramatically increased in inflammatory states and that cellular COX activity could be induced by inflammatory cytokines and endotoxins. This suggested that a second form COX existed, an inducible form (COX-2), which is expressed during inflammatory conditions. The constitutive form (COX-1) produces physiologically important PG's and is present in tissues such as the gastrointestinal tract and kidney. Despite recognition of cyclooxygenase's mechanism of action thirty years ago, the enzyme, COX-1 was first cloned only in 1988. The second isoform, COX-2 was reported three years later. Both the isozymes have about the same affinity ($K_m$) and capacity ($V_{max}$) to convert arachidonic acid to PGH$_2$, COX-2 is able to metabolize C18 and C20:3 fatty acids, additionally. Protective PGs which preserve the integrity of the stomach lining and maintain normal renal function in a compromised kidney are synthesized by COX-1. It is also constitutively expressed in cultured endothelial and vascular smooth-muscle cells. Although, COX-1 is constitutively expressed playing a house-keeping role, in many tissues under basal conditions, its expression may also be regulated. Conversely, COX-2 was thought to be an inducible enzyme, generally not present (or minimally so) in most tissues. Rather, its expression is
considered to be associated with an inflammatory response and other pathophysiological states. More recent investigations, however, reveal that COX-2 plays a key role in a wide range of physiological processes including organogenesis, brain and nerve function, reproduction, bone metabolism, salt and water handling, rennin release, angiogenesis and apoptosis. It is present constitutively in the brain and the spinal cord, where it may be involved in nerve transmission, particularly for that pain and fever. PGs made by COX-2 are also important in ovulation and in the birth process (Ebehart et al., 1994). Thus the dichotomy of a constitutive COX-1, with solely “house-keeping” functions under physiological conditions, and an inducible-COX-2, accounting for prostanoid production in disease states, is an over simplification of biological reality.

During the past few years, selective inhibitors of COX-2 enzyme have emerged as important pharmacological tools for the treatment of pain and inflammation. But the recent findings of the involvement of COX-2 enzyme in some key physiological functions, as described above, have complicated the scene regarding the use of selective /specific COX-2 inhibitors as anti-inflammatory agents. At the same time, these findings have opened new doors for the treatment of certain other ailments as well. Further, higher concentrations of COX-2 have been implicated in several other disease states other than the inflammation, such as colonic polyposis (Ebehart et al., 1994), various forms of cancer (Tsujii et al., 1997), alzheimer’s disease and vascular retinosis following angioplasty. Prevalence of higher concentrations of COX-2 enzyme than the normal in these diseases states has opened avenues for the possible therapeutic mitigation of these diseases. Involvement of COX-2 expression in these diseases and the role COX-2 inhibitors could play as potential therapeutic agents in future.
1.2.11 COX-2 in Cancer:

Several lines of evidence suggest that COX-2 enzyme plays an important role in carcinogenesis. Increased amounts of COX-2 are commonly found in both premalignant tissues and malignant cancers of the head and neck, oesophagus and lung (Wilson et al., 1998, Wolff et al., 1998). COX-2 expression is induced in a variety of cells leading to high levels of prostaglandin production. Such aberrant expression of COX-2 have been reported in murine and human breast cancer, human colon cancer, lung cancer, head and neck cancer and pancreatic cancer (Tsujii et al., 1997). There is extensive evidence, beyond the findings that COX-2 is commonly over expressed in tumours, to suggest that COX-2 is mechanistically linked to the development of cancer. COX-2 can affect multiple mechanisms that are important in carcinogenesis. It may aid in carcinogenesis, by altering the normal cellular processes like angiogenesis, cell proliferation, immunomodulation, carcinogenic metabolism and apoptosis.

Angiogenesis is essential for the proper nourishment and oxygen supply to highly dividing and proliferating cells. Normal blood vessel endothelium expresses COX-1 isozyme whereas angiogenic blood vessel endothelium expresses COX-2 isozyme. COX-2 and thromboxane A2 receptor dependent signaling pathways have been shown to activate cellular invasion and angiogenesis. Recently, levels of COX-2 were found to correlate with both VEGF expression and tumour vascularisation in head and neck cancers. This finding in human tissues is consistent with prior evidence that over expression of COX-2 in epithelial tissues leads enhanced production of vascular growth factors and the formation of capillary like networks. Evidence has been provided that COX-2 derived prostaglandins contribute to tumour growth (Masferrer et al., 2000) by
inducing newly formed blood vessels (neo angiogenesis) that sustain tumour cell viability and growth (Wolff et al., 1998) Prostaglandins appears to increase cell proliferation with the help of biological modifiers like polyamines. Increased polyamine levels are associated with DNA synthesis which results from ornithine decarboxylase activity. Over expression of COX-2 in colorectal tumour cell lines caca-2 results in increased metastatic potential (Tsujii et al., 1997). The growth of tumours typically is associated with immune suppression. Colony stimulating factors released by tumour cells activate monocytes and macrophages to synthesize PGE2 which inhibits the production of immune lymphokines, T and B cell proliferation and the cytotoxic activity of natural killer cells. PGE2 also inhibits the production of tumour-necrosis factor while inducing the production of IL-10, which has immunosuppressive effects. High concentrations of certain prostaglandins like PGE2 attenuate host’s immune response preventing the killing of malignant cells. PGE2 shows a potent immunosuppressive effect by acting as a negative feed back inhibitor, thereby inhibiting T cell activity, lymphocytic mitogenesis, macrophage activity, antibody production, production of cytokines by immune cells and natural killer cell activity. COX-2 inhibitors, when given immediately after tumour transplant in animal models decrease PGE2 levels and tumour growth. The peroxidase component of COX isozymes has broad specificity and can oxidize a variety of xenobiotics including certain procarcinogens and carcinogens. Classes of compounds like aflatoxins, halogenated pesticides, polycyclic hydrocarbons, heterocyclic amines etc. are acted upon by the peroxidase component of COX to form mutagenic carcinogens. The byproducts of the oxidation of arachidonic acid like malondialdehyde, are highly reactive and form adducts with DNA that may initiate cancer. COX inhibitors along with antioxidants may play a
role to protect the cells and DNA from the damage (Chinery et al., 1998). Overproduction of prostaglandins like PGE2 by COX-2 may also send improper signals in the cells thereby stimulating cell growth inappropriately or reducing apoptosis. Bcl-2 gene is an important gene in regulating apoptosis. Murine intestinal cell lines like RIE-S showed that over expression of COX-2 in cancerous cells was associated with the over expression of Bcl-2 gene, which prolongs cell's life by inhibiting apoptosis. The exact relationship between COX-2 overexpression, production of prostaglandins and expression of Bcl-2 gene in cancer is yet to be investigated. Inhibition of COX isozymes results in decreased production of PGs from their substrates like arachidonic acid, leading to the accumulation of the substrate. Arachidonic acid when present in increased concentration in the cells is supposed to stimulate the fragmentation of DNA and conversion of sphingomyelin to ceramide in the cells, which is a known inducer of apoptosis.

Selective COX-2 inhibitors have the desired property of interfering with tumourigenesis in experimental systems. Inhibition of COX-2 by celecoxib delays tumour growth and metastasis in xenograft tumour models as well as suppresses basic fibroblast growth factor 2 (FGF-2)-induced neovascularisation of the rodent cornea (Masfeerer et al., 2000).