Alkylating derivatives of oestrogens as potentially target-selecting cytotoxic agents

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ALKYLATING DERIVATIVES OF OESTROGENS

AS POTENTIALLY TARGET-SELECTING CYTOTOXIC AGENTS
ALKYLATING DERIVATIVES OF OESTROGENS

AS POTENTIALLY TARGET-SELECTING CYTOTOXIC AGENTS

by

PETER MORRISON LOCKEY

A DOCTORAL THESIS

Submitted in partial fulfilment of the requirements for
the award of Doctor of Philosophy of the Loughborough University
of Technology.

July 1986

Supervisor: Dr J.R. Traynor, B.Sc., Ph.D., M.P.S.

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The present work is a study of the structure-activity relationships and mode of action of oestrogens substituted with alkylating functions. These compounds are cytotoxic agents potentially selective for oestrogen-responsive tumours, since the oestrogen part of the molecule should lead to a concentration in tumour cells thereby imparting selectivity to the alkylating group.

An assay system was developed to test a series of compounds for the above properties. This system consisted of a cytotoxicity screen, using HeLa S3 cells, and a receptor binding assay using rat uterine cytosols to evaluate the compounds' selectivity. Selectivity was further assessed by use of the oestrogen-receptor containing cell lines MCF-7 and GH3.

The devised system was used to evaluate a series of oestrogen D-ring α-methylene-δ-lactones previously prepared in the Organic Chemistry Laboratory (Dr. L.S. Chagonda). Although strongly cytotoxic ($IC_{50} < 1\mu M$ against HeLa S3) these compounds show little affinity for the oestrogen receptor, and no apparent selectivity against MCF-7 and GH3 cells. This suggests that the 17-oxygen function is important for receptor binding, and modifications in this region of the oestrogen molecule result in loss of affinity for the receptor.

Consequently oestrogens retaining the 17-oxygen, but substituted at the 16-position with an α-methylene-γ-lactone ring were proposed. Three compounds of this type were prepared (Dr. G. Edge). These compounds are cytotoxic to HeLa S3 cells, though less so than the
D-ring α-methylene-δ-lactone oestrogens (IC$_{50}$ < 5μM). Two of these compounds exhibit a degree of affinity for the oestrogen receptor, and one of these has greater cytotoxic activity against MCF-7 cells than would be predicted for non-oestrogen compounds. However, it has not been possible to confirm the work on this active compound as it has four possible isomers, which may vary in selectivity. Work is presently underway to separate the isomers.

Two epoxide isomers derived from ethinyl oestradiol (prepared by Miss J.C. Gill) were also assessed. Both of these compounds are cytotoxic to HeLa S3 cells (at ~10μM levels), one being threefold more active. The more cytotoxic isomer also displays high affinity for the oestrogen receptor with an ED$_{50}$ of approximately 70 nM, i.e. greater than that of oestrone (ED$_{50}$ ~ 100 nM). This series of compounds is potentially very interesting and well worth further study, particularly if more active cytotoxic groups could be incorporated.

All of the alkylating oestrogen derivatives tested appear to form covalent links with the oestrogen receptor. The degree of covalent binding is apparently a function of both binding affinity and reactivity.

A study was also made of the possible mechanisms by which the compounds might block cell growth. Particular attention was paid to the role of thiol groups. All of the active compounds reduce cellular thiols and react with glutathione to some extent.

Thymidine uptake and incorporation into DNA, respiration, lysosomal enzymes and lysosomal membranes were examined. All of
these parameters were affected to varying degrees. However, the α-methylene-lactone compounds were found to be most active against Na⁺/K⁺ ATPase as determined by $^{86}\text{Rb}^+$ uptake into HeLa S3 cells and by effects on isolated preparations of the enzyme.
Abbreviations

Chemicals and Reagents:

ADP  adenosine diphosphate
ATP  adenosine triphosphate
DES  diethyl stilboestrol
DMSO dimethyl sulphoxide
DTNB 5,5' dithio-bis-2-nitro-benzoic acid
LDH  lactate dehydrogenase
NADH nicotinamide adenine dinucleotide, reduced form
PEP  phosphoenol pyruvate
PK  pyruvate kinase
TCA  trichloroacetic acid
TED  tris (hydroxymethyl) aminomethane
TE  tris, ethylenediaminetetraacetate buffer
TED  tris, ethylenediaminetetraacetate, dithiothreitol buffer

Parameters:

ED$_{50}$  concentration causing 50% displacement in competitive binding assays
IC$_{50}$  concentration causing 50% reduction in cell growth
SEM or sem standard error of mean

Cell lines:

GH$_3$  clone of rat pituitary tumour cell line
HeLa S3  clone of human cervical tumour cell line
MCF-7 human mammary carcinoma cell line
1. INTRODUCTION
1.1 Definitions of Cancer

Cancer is described as an abnormal growth of tissue without the constraints that characterise normal tissue growth. [1] The rate of growth in malignant lesions is not necessarily higher than the tissues from which they originate. [1] However the feedback control mechanisms which govern tissue size do not operate on malignant neoplasms, they become at least partly autonomous. [2] In general, malignant lesions can be distinguished from the benign form by several properties. [1,3]

(a) Benign neoplasms tend to show some degree of differentiation and resemble the tissues from which they originate in organisation. Malignant neoplasms are relatively undifferentiated.

(b) Benign lesions commonly form a surrounding capsule and are distinct from the surrounding tissues while malignant lesions invade and destroy surrounding tissue.

(c) Most importantly few benign lesions metastasise, while a common property of malignant neoplasms is an ability to form secondary lesions at sites distant from the primary.

In addition to localised destruction of healthy tissues, malignant lesions have a general deleterious effect on metabolism, this is termed cachexia. [3,4] One of the earliest observations on cancer cells was their increased rate of glycolytic metabolism. The resultant accumulation of lactate and its release into the bloodstream by malignant lesions is partly responsible for the
'wasting' effect seen in cancer. Production of inappropriate gene products such as ectopic hormones, or toxic metabolites add to this metabolic disruption.

From a clinical point of view, cancer is difficult to define in any useful sense. There are no absolute distinctions between malignant and benign neoplasms and only qualified generalisations can be made about their properties. [5] Cancer is better defined therefore in terms of its causes, and of the changes which occur at a cellular level which transform a normal cell to its neoplastic counterpart.
1.2 Causes of Cancer

Cancer has a genetic basis. All known carcinogens; chemicals, radiation and viruses cause mutations which lead to the transformation of normal cells. [2] Recently it has been demonstrated that the target for these carcinogens are specific genes, termed oncogenes, found in normal cells. [6,7] Oncogenes code for normal cell proteins, usually enzymes, such as protein kinases, or growth factors. When forced into an abnormal rate of expression oncogenes overproduce these proteins and this may cause the cell to become neoplastic. [8,9]

Chemical and radiation carcinogenesis occurs by mutation of a normal gene. [10] Point mutation of a single nucleotide base may be sufficient to convert a normal gene to one which causes cancer. [7] Such a point mutation has been implicated in a form of bladder cancer caused by several carcinogens, [7]. A large number of organic and inorganic compounds have been shown to be carcinogenic or are suspected of being so, some examples of these are shown in Figure 1.1.

Most of the organic chemical carcinogens undergo metabolic activation to an ultimate carcinogenic form which reacts with DNA. [7,10,11] This 'lethal synthesis' depends on the type of tissue and its metabolism, which may account for the specific carcinogenic activity of compounds on certain tissues. [10] For example vinyl chloride on the liver (angiosarcoma), and 2-naphthylamine and benzidine on
Figure 1.1. Compounds which are carcinogenic in humans.
the bladder. [10,11] The carcinogenic activity of a compound on a tissue will depend partly on the relative levels of the enzymes which activate the compound and those which detoxify it, as well as the reactivity of the compound itself. [12] Conversion to a reactive epoxide species is a common step in the metabolism of potential carcinogens. [10]

Polycyclic aromatic carcinogens have been most extensively studied, and are converted to electrophilic epoxide species in vivo. Vicinal diol-epoxides of the type shown in Figure 1.2a are believed to be the ultimate carcinogenic form of these compounds. Bay region vicinal diol-epoxides are the most reactive form of epoxide possible in this class of compound. [7,10]

Aflatoxins, which may cause some forms of liver cancer, are also converted to epoxides which subsequently react with cellular nucleophiles. [10] Aflatoxin B₁, a common contaminant of stored grain and nuts in Third World countries may be responsible for the high incidence of hepatic cancer and other liver disorders in these countries (Figure 1.2b).

More recently, steroid hormones have been implicated in the aetiology of certain cancers. [10] Synthetic oestrogens such as ethinyl oestradiol and diethylstilboestrol (DES) have been shown to form covalent links with proteins and nucleic acids. [13] Epoxide derivatives of these two compounds may be their ultimate carcinogenic species. (Figure 1.2 c,d) [10,13] Progestins, androgens and bile salts
Figure 1.2. Carcinogenic compounds and their possible metabolic derivatives.
may also have carcinogenic activity after conversion to their reactive epoxide analogs. [13,14]

A variety of viruses may cause cancer in humans. The acutely transforming retroviruses are capable of incorporating oncogenes from normal cells into their genomes. When the virus infects a cell it inserts its genome into the host cell as DNA. The viral promoter gene then initiates the synthesis of an oncogene product at the same time as its own components. The resulting overproduction of the oncogene protein causes conversion of the normal cell to a neoplastic cell. [15] The chronic transforming retroviruses act simply by inserting their genome next to a cellular oncogene in the host cell's DNA. Viral promoter genes then initiate over-expression of the oncogene. [15] Other viruses such as the hepatitis B virus, which causes liver cancer in humans, are known to insert themselves into the DNA of host cells but the mechanism of carcinogenesis is not understood. [16]

Rearrangement of chromosomes, or translocations in normal cells can also cause cancer, notably Burkitt's lymphoma the cells of which have a characteristically abnormal set of chromosomes. [17] This process is believed to initiate carcinogenesis in normal cells if the translocation causes insertion of a cellular oncogene next to a cellular promoter gene. [18]

A common characteristic of carcinogenesis is a latent period between initial contact with the carcinogen and development of a
A two stage process is believed to occur in carcinogenesis. (a) Initiation, which occurs on contact with the carcinogen, and (b) Promotion which may occur years after the initiation phase and results from environmental factors, usually contact with 'cocarcinogenic' compounds. [19]

**Differences between normal and neoplastic cells**

Biochemical differences between normal and neoplastic cells are of interest for three reasons.

(a) They give clues to the causes of cancer
(b) They aid the early detection of cancer
(c) They may be exploitable therapeutically

The main differences so far uncovered in neoplastic cells relative to normal cells are summarised in Table 1.1.
Table 1.1. Biochemical changes in cancer cells relative to normal cells. [3,11,20]

Lower pH.
Greater free radical character.
Ectopic hormones.
Altered enzyme profiles.
Altered antigenicity.
Lower Ca\(^{+}\) and higher K\(^{+}\) levels
Elevated levels of methylated nucleosides.
Higher levels of plasma mucoproteins
   and mucopolysaccharides.
Altered mitochondrial structure.
Elevated levels of polyamines.
Greater need for exogenous Zn.
Higher biowater content.
Requirement for exogenous L-asparagine
   in some types (especially Leukaemias).
Altered levels of cyclic nucleotides.
1.3 Cancer Chemotherapy

Cancer chemotherapy involves the use of cytotoxic agents to kill neoplastic cells, or of hormones to slow their proliferation. The aim of chemotherapy is to eradicate neoplasms or at least to reduce the patient's neoplastic cell burden and improve their quality of life. [21]

Classes of anticancer drug, together with commonly used examples are shown in Table 1.2. The alkylating drugs, of major interest to the present work, are discussed in greater detail in the following section. The antimetabolites are active via inhibition of the metabolism of essential cellular intermediates, due to their structural similarity. Thus methotrexate,

\[
\text{Methotrexate}
\]

an analog of folic acid competitively inhibits dihydrofolate reductase. [22] An important consequence of this is loss of thymidine.
Table 1.2. Classes of anticancer agents

<table>
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<tr>
<th>Drug class</th>
<th>Examples</th>
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<tr>
<td>Alkylating agents</td>
<td>Cyclophosphamide, Myleran. (also see page 18)</td>
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<tr>
<td>Antimetabolites</td>
<td>Methotrexate, 5-Fluorouracil, 6-Mercaptopurine.</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Dactinomycin, Mitomycin C, Adriamycin.</td>
</tr>
<tr>
<td>Hormones</td>
<td>Glucocorticoids, Androgens, Oestrogens, Progestogens.</td>
</tr>
<tr>
<td>Vinca Alkaloids</td>
<td>Vincristine, Vinblastine.</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Cis-platinum, L-asparaginase, Hydroxyurea, Nafoxidine</td>
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5-Fluorouracil (5-FU) and 6-Mercaptopurine (6-MP) act as pyrimidine and purine analogs respectively. They are not themselves toxic, but become so on metabolism to their nucleotides. \[22,23\] They are believed to act via inhibition of the enzymes involved in nucleotide metabolism and hence reduce the pool of nucleotide triphosphates available for nucleic acid synthesis. 6-MP and 5-FU may be incorporated into DNA and RNA respectively, the contribution this makes to their cytotoxic activity is not clear. \[22,25,24\]

The antibiotic anti-cancer drugs (Figure 1.3) all act either directly or indirectly on DNA functions. Adriamycin and Dactinomycin are intercalating agents and reduce both DNA and RNA syntheses in treated cells. \[23,24\] Other drugs in this class, such as mitomycin C and bleomycin do not intercalate DNA but cause either cross-linking of DNA, in the case of mitomycin C, or strand breaks as with bleomycin. \[24\] The antibiotic class of drugs has been used increasingly in recent years because of the broad spectrum of malignant
Figure 1.3. Antibiotic anti-cancer drugs.
neoplasms they are active against.

The steroid hormones, Figure 1.4, are used in a variety of disease states, on their own and in combination with other chemotherapeutic agents. Androgens and oestrogens are extensively used in treatment of breast and prostate cancers respectively. [25] Glucocorticoids are used in treatment of certain leukaemias as well as myelomas, lymphomas and breast cancer. Progestins are used predominantly in treatment of endometrial cancer, but may also be used in breast and renal cancer. The steroid hormones are not curative. They produce remissions and may relieve symptoms. [25] They may also be used to reduce the side-effects of chemotherapeutic agents. [25]

The Vinca alkaloids (Figure 1.5) act as 'spindle poisons'. These drugs bind to specific sites on tubulin in microtubules resulting in their dissolution. The compounds are specific for the mitotic phase of the cell cycle, and are used against a variety of cancers. [23]

Several drugs used in anti-cancer treatment do not fit into any of the categories previously described and are grouped together in a miscellaneous class. Included in this group are enzymes, such as l-asparaginase which reduces levels of circulating asparagine. Neoplastic cells which are unable to synthesise this amino acid are effectively starved. [23] As would be expected this enzyme is only active against a rather narrow spectrum of malignancies and only affords remissions.
Figure 1.4. Natural and synthetic steroids used in cancer chemotherapy.
Vincristine: $R^1 = \text{CO}_2\text{CH}_3$

$R^2 = \text{CHO}$

$R^3 = \text{OCH}_3$

$R^4 = \text{COCH}_3$

Vinblastine: $R = \text{CO}_2\text{CH}_3$

$R = \text{CH}_3$

$R = \text{OCH}_3$

$R = \text{COCH}_3$

Figure 1.5. Vinca alkaloids used in cancer chemotherapy
Hydroxyurea inhibits the enzyme ribonucleotide reductase

\[ \text{H}_2\text{N} - \text{C} - \text{NH} - \text{OH} \]

Hydroxyurea

and kills cells that are synthesizing DNA. Thus cells in the S phase of the cell cycle are particularly susceptible. [26] Cis-platinums bind to DNA and inhibit its synthesis, they are believed to form intra-strand links with DNA. The cis-platinums are active in all stages of the cell cycle. [26]

\[ \text{H}_2\text{N}\text{.Pt}\text{.NH}_2 \]

Cis-platin

The choice of chemotherapeutic agent used depends on the type and state of the neoplasm. Use of combinations of chemotherapeutic drugs, and improved scheduling of administration has markedly increased the effectiveness of chemotherapy. [27,28]

Alkylation agents as cytotoxic compounds

Five major classes of alkylation agent are currently employed in cancer chemotherapy [29]; nitrogen mustards, nitrosoureas
triazenes, methane sulphonic acid esters and ethyleneimines. (Figure 1.6). All of these compounds are believed to react with nucleophilic hydroxy-, amino-, mercapto- or imidazole groups of proteins and nucleic acids. [11] The mechanism of action of the nitrogen mustards, the first of these drugs to be used clinically, has been more completely studied than any of the other classes.

Nitrogen mustards are believed to exert their major cytotoxic effect by inter-strand linking of DNA, [29,30] and are therefore most cytotoxic to rapidly proliferating cells. [23] Of the other classes the ethyleneimines, such as Triethylenethiophosphoramid (Thio-TEPA) have a mechanism of action similar to the nitrogen mustards. Nitrosoureas such as carmustine (BCNU) alkylate nucleic acids and also carbamoylates lysine residues of proteins, this dual action gives the nitrosoureas a broad spectrum of action against tumours which are resistant to the other alkylating agents. [11] Methane sulphonate esters such as Busulfan are considerably less reactive than nitrogen mustards; the alkyl oxygen bonds split and the main mechanism of action is by alkylation of the N-7 position of guanine. [29] Triazenes such as dacarbazine also alkylate the N-7 of guanine. Dacarbazine is demethylated, producing 5-amino-imidazole-4-carboxamide and a methyl radical. This radical is the active component which methylates the guanine of RNA and also attacks protein.

Although all of the compounds described are presently in clinical use, and are potent anti-tumour agents, they suffer from the disadvantage of indiscriminate reaction with biological nucleophiles.
Figure 1.6. Alkylating agents used in cancer chemotherapy
and thus have limited therapeutic indices. Selectivity of action of these compounds depends mainly upon the relative rates of tumour and normal cell proliferation. Since several types of normal body tissue cells replicate at the same rate, or faster than tumour cells, toxic side effects are often pronounced. To overcome this problem, research with the alkylating drugs has centred on producing compounds with target-selective activity. [11] To this end considerable work has been carried out, both on improving the selectivity of drugs already in clinical use, and on developing alternative alkylating drugs which might be more selective in action. Among the types of compound which have been considered in recent years are α-methylene lactones and to a lesser extent epoxides.

**α-Methylene lactones**

The alkylating activity of the α-methylene lactone function has been known for many years. Simple synthetic α-methylene-butyrolactone derivatives have been prepared for more than 30 years. [32,33,34] However, the biological properties of this class of compound were only investigated following their discovery as secondary metabolites in a variety of plants. [35] Cytotoxic sesquiterpene lactones, isolated during the course of a continuing search for anti-tumour agents from plants, were the first of the α-methylene lactone class of alkylating agents found to have anti-tumour activity. [35] More than 500 of these compounds have been
isolated from the Compositae, [36] many of them exhibiting cytostatic and cytotoxic activity against animal tumours \textit{in vivo} and against tumour cells in culture \textit{in vitro}, some of the more well known of these compounds are depicted in Figure 1.7. [35,37,38]

Structure-activity studies, both on the sesquiterpene lactones and on simpler model compounds have demonstrated that cytotoxicity \textit{in vitro} is dependent on the presence of at least one \(\alpha,\beta\)-unsaturated lactone group, with the \(\alpha,\beta\)-ethylenic linkage being exocyclic. [37,38] Endocyclic \(\alpha,\beta\)-unsaturated lactones are inactive. [39] The active compounds have been shown to undergo Michael type additions with biological nucleophiles. [39,40,41,42] Unlike the nitrogen mustards and other alkylating agents however, the \(\alpha\)-methylene lactones apparently do not react with the purine bases of DNA. Testing of the compounds \textit{in vitro} demonstrated a lack of reactivity with isolated DNA and also with purine nucleotides. [30] However, the possibility of metabolic activation of the compounds prior to interaction with DNA has not been excluded. [30,43,44] On the other hand the active sesquiterpene lactones react rapidly and apparently irreversibly with thiol bearing molecules, such as cysteine, to form stable adducts. [39] (Figure 1.8) Those compounds with endocyclic \(\alpha,\beta\)-unsaturated lactone groups react only slowly [39].
Figure 1.7. Cytotoxic sesquiterpene α-methylene lactone compounds.
Figure 1.8. Cysteine adducts of cytotoxic sesquiterpene α-methylene lactones.
Cytotoxic activity towards tumour cells in vitro is conferred simply by the presence of the reactive α-methylene group. However, additional functional groups are required for activity in vivo. [37] Thus, with few exceptions, simple model compounds for example Figure 1.9, have low activities against experimental tumours in vivo. [45,46]

Figure 1.9. Simple α-methylene lactone model compounds

The more complex terpenoid compounds, such as elephantopin and vernolepin, have poor therapeutic indices, and are too toxic for clinical use.

Elephantopin

Vernolepin
Attempts to produce compounds combining the low toxicity of the simple $\alpha$-methylenebutyrolactones with the potent tumour-inhibiting properties of the terpenoids have so far proved unsuccessful. [45,46,47]

The mechanism of cytotoxicity of the $\alpha$-methylene lactones is still unclear. Simple scavenging of cysteine and glutathione has been suggested, but is probably not of major significance. [45,47] Alkylation of essential intracellular thiols, and possibly of membrane proteins is likely to be of greater importance. [48,49]

Effects of sesquiterpene $\alpha$-methylene lactones on the activities of a number of sulphydryl containing enzymes have been reported. Reaction with essential thiols of these enzymes has been determined as the mechanism of inhibition. Enzymes involved in glycolysis, [50,51] nucleotide metabolism and DNA synthesis, [50,52] have all been shown to be inhibited.

Membrane functions, such as transport of amino-acids, sugars and nucleotides, [47,53] in addition to membrane-bound enzymes [54,44] are perturbed by reaction with $\alpha$-methylene lactones, and other thiol reagents. [54]

The ability to inhibit cellular respiration is another property of the sesquiterpene lactones. [58] Both basal and ATP stimulated respiration is significantly inhibited in isolated cells and in liver homogenates [58,59]. The mechanism of this activity is unclear, but may be caused either by alkylation of respiratory enzymes or of essential thiols on the mitochondrial membranes.
Lysosomal membranes are stabilised, and lysosomal enzymes inhibited by the sesquiterpene α-methylene lactones. This has prompted investigation of their properties as potential anti-inflammatory drugs. [60] The dose levels required for anti-inflammatory activity are considerably lower than those needed for anti-tumour activity in animals and hence toxic side effects are less evident. [60]

Sesquiterpene α-methylene lactones are also cytotoxic against microorganisms, both to bacteria and simple eukaryotes, [61] although concentrations higher than those required for anti-tumour activity are needed.

The wide structural diversity of the sesquiterpene α-methylene lactones raised the hope that they might show some selectivity of action through their special stereochemical and molecular structures. [47] This initial optimism has not been realised. As with the classes of alkylating drugs already in clinical usage, these plant products have proved largely indiscriminate in activity. [46] However, the α-methylene lactone alkylating group has potent anti-tumour activity, and might still be of clinical use if linked to a molecule which conferred selectivity.
Epoxides

The biological alkylating activity of the epoxides towards thiols and nucleophilic groups of DNA has been known for many years, mainly through research on their properties as carcinogens, as previously discussed. However, the alkylating activity of the epoxides has been employed in a number of anti cancer drugs (Figure 1.10) [62]

\[
\begin{align*}
\text{Ethoglucid} & \quad \begin{array}{c}
\text{Mitobronitol}
\end{array} \\
\end{align*}
\]

Figure 1.10. Epoxides used in cancer chemotherapy

Unfortunately these compounds have proved to be of only limited use in chemotherapy and several drugs have been withdrawn from clinical use because of their toxicity. Attempts to produce epoxides with a more selective action have proved unsuccessful.
1.4 Breast Cancer

Adenocarcinoma of the breast is one of the commonest forms of cancer in the developed world. It accounts for nearly 14% of all forms of cancer in the West. The disease has proved one of the most resistant forms of cancer to treatment. Five year survival rates in breast cancer patients have not significantly improved in the last 20 years. New forms of therapy are therefore constantly under review.

Hormonal therapy and Chemotherapy

Treatment of breast cancer following surgical removal of the primary tumour employs hormonal manipulations and/or chemotherapy, possibly in conjunction with radiotherapy. [25] The course of therapy depends on the oestrogen receptor state of the primary lesion. [65,64,65] Neoplasms with high oestrogen receptor levels (> 10 f moles mg\(^{-1}\) protein) designated ER+ are normally responsive to hormonal manipulations initially. Neoplasms with low receptor levels (< 3 f mol mg\(^{-1}\) protein, ER-) tend to respond more favourably to chemotherapy. [64,65] Approximately 60% of primary and 50% of metastatic lesions have measurable levels of oestrogen receptor. [66,67]

Prediction of which breast cancers are hormone responsive is obviously important for their subsequent treatment. The simple presence of oestrogen receptors does not positively identify hormone responsive lesions. [68] The predictive value of oestrogen receptor
assays on the primary lesion is improved by determining if the entire receptor mechanism is functional. [68] Several approaches are used to determine this, a) assay of oestrogen receptor-complexes associated with chromatin. [64,68] b) assay of levels of the various oestrogen receptor forms (45,55) [64] and c) assays of progesterone receptor levels.[69]

Hormonal therapy produces minimal side-effects compared to chemo therapy. [25] It is not curative but long disease-free remissions can be produced. [65] The type of endocrine therapy employed depends on the age and condition of the patient. Treatment of premenopausal women is ablative; ovariectomy to remove endogenous oestrogens. [70] This has the added benefit of reducing oestrogen-stimulated prolactin synthesis by the pituitary. [25] Prolactin stimulates growth in some types of breast cancer.

In postmenopausal women oestrogens may be given at supraphysiological levels. [25] These high levels of oestrogen have a direct inhibitory effect on the neoplasm by antagonising the effects of prolactin. [25] Androgens may also be used, depending on the age of the patient. [70] Anti-oestrogens such as tamoxifen and nafoxidine (Figure 1.11) are being used increasingly in

![Chemical structures of Nafoxidine and Tamoxifen](image)

Figure 1.11. Anti-oestrogens used in breast cancer therapy
postmenopausal women. [70] The mechanism of action of the anti-oestrogens is unclear. They may block the binding of oestrogens, or the tamoxifen-receptor complex may have a direct lethal effect. [25] Both tamoxifen and nafoxidine are lethal to some human breast carcinoma cell lines, such as MCF-7. [25] In addition the presence of an anti-oestrogen receptor has been detected in cells from a number of sources. [71] The function of this receptor is unknown, but lines of MCF-7 cells resistant to anti-oestrogens lack the receptor. [71]

Anti-cancer drugs of all the major classes are used in breast cancer chemotherapy. In ER- patients chemotherapy is the treatment of choice in all stages of the disease, as an adjuvant to surgery and in metastatic disease. However, in ER+ patients hormonal therapy is normally used as an adjuvant treatment and is replaced by chemotherapy when resistance to the endocrine manipulations arises during the metastatic stage of the disease. Combined use of hormones and chemotherapy with ER+ patients is also used and is apparently better than either treatment alone. However, it is as yet unclear if this treatment improves on the sequential use of hormones and chemotherapy. [25]

Combination chemotherapy, produces better results than any single drug. [65] However, no one combination regime appears markedly better than others despite trials with a large variety of combination treatments. [65] The CMF regime (cyclophosphamide, methotrexate, 5-fluorouracil) is one of the most widely used treatments for disseminated breast cancer. [65] CMF treatment is employed in
combination with other drug protocols as well as on its own. [65]

Progress in chemotherapy of breast cancer has been due less to improvement in drug design than to better use of existing agents. [72] Tailoring of chemotherapy to the individual patient, as well as better scheduling and dosage control increase the efficiency of treatment. [72] However, progress in these areas has served mainly to increase the length of remissions and improve the quality of life for the patient. Despite refinements in the application of endocrine and chemotherapy, management of breast cancer is achieved in less than 5% of patients 5 years after disease onset. [65]

Steroid Receptors: Structure and Function

The accepted mechanism of steroid hormone action on target cells has remained effectively unchanged for the past two decades. Steroid hormones are generally believed to bind to specific receptors in the cytoplasm which translocate to the nucleus by a temperature dependent 'activation' process, [73], (Figure 1.12). This mechanism may have to be revised in the light of recent findings. It is possible that steroid receptors, for oestrogens at least, exist either exclusively in the nucleus or in an equilibrium between cytoplasm and nucleus. [74,75,76] These findings may explain the seemingly anomalous reports of the presence of 'unfilled' oestrogen receptors in the nuclei of both normal and neoplastic cells. [63,73,77] The temperature-dependent activation of oestrogen receptors is also apparently a nuclear event since both activated and non-activated
Figure 1.12. Models depicting the possible cellular distribution of steroid hormone receptors.
forms are found in the nucleus. [78] (Fig. 1.12)

The mechanism by which the activated oestrogen-receptor complex (ER) alters gene expression in the chromatin of target cells is unclear. Binding of ER to chromatin leading to specific changes in m-RNA production has been demonstrated. [79] It is not known if the ER interacts directly with DNA, or via histone and non-histone proteins in the chromatin. [13,79,80] Some effects of steroid hormone action may be post-transcriptional; binding of ER to ribonucleoprotein in the nuclei and cytoplasm of target cells has been detected. [80] The ER may be involved in stabilization of the messenger RNA of hormone induced proteins and so facilitate their production. [80]

Oestrogen receptors exist as 4S monomers which commonly dimerise to 8S complexes at high salt concentrations.[13,81] On binding oestrogens the oestrogen - 4S complex is 'processed' to form a 5S molecule which binds to chromatin. [73] The mechanisms involved in the interconversions of the molecular forms of receptor are currently under study. [82] Cyclic nucleotide levels, and protein kinases which phosphorylate the receptor proteins, are involved in receptor binding. [83]
1.5 Steroid-Linked Cytotoxic Agents

The use of steroids as carriers of cytotoxic functions has been investigated for many years. It is an attractive route to increased selectivity since it exploits the known ability of target cells to concentrate steroids, via the hormone receptor mechanism. Cytotoxic-linked steroids should therefore be expected to concentrate in receptor positive neoplasms.

Initial work on steroid-linked alkylating drugs was undertaken primarily with a view to exploit the lipophilicity of the steroid nucleus, to improve drug transport, as well as directing the drug to specific target tissues. [84] The first steroid-linked alkylating compounds tested were relatively inactive, [84] with the exception of the cholesterol derivative phenestrin, (Figure 1.13) which was active against a number of experimental tumours in animals. [84] The success of phenestrin prompted the synthesis and evaluation of several nitrogen mustard and ethyleneimine steroidal compounds, (Figure 1.13) some of which reached the clinical trial stage. [84,85,86]. α-Methylene lactones linked to steroids were synthesised, following the work on the nitrogen mustard and ethyleneimine compounds, and found to be active against neoplastic cells in culture, in addition to animal systems. [86,87,88] (Figure 1.13).

Steroid-linked alkylating drugs up to this point had been produced without regard to their ability to bind to specific hormone receptors in the neoplasms they were designed to treat, such as malignancies of the breast and prostate. Improved design of these
Figure 1.13. Early nitrogen mustard and ethyleneimine and α-methylene lactone steroidal compounds.
drugs was possible with development of structure-activity profiles of steroid receptor binding sites using competitive binding assays. [89, 90] Research on the molecular biology of steroid hormone action has also contributed to development of this class of drug. [91, 92]

A systematic screening programme has been developed by several groups to assess oestrogen-linked alkylating drugs as possible agents in treatment of breast cancer. [93,94,95] The programme involves three steps.

(a) **In vitro** screening of compounds for receptor binding.

(b) Cytotoxicity assays on two human breast cancer cell lines; MCF-7 which has oestrogen receptors (ER+), and Evsa-T which does not (ER-).

(c) **In vivo** effects, on dimethylbenzanthracene (DMBA) induced mammary neoplasms in rats and the hormone dependent MXT-mouse mammary neoplasm.

However, not all workers agree with this mainly **in vitro** screening procedure. [96]

Several of a series of nitrogen mustard-linked oestrogens displayed activity in the first two phases of this programme. [93,97](Figure 1.14a). The drugs exhibited some degree of selectivity towards the ER+ cell line. The receptor binding of these compounds was poor compared to the parent oestrogens however, and further work is underway to improve their affinity for the oestrogen receptor and hence their selectivity. [98] More recently a series of thiourea-linked oestrogens were found to exhibit no selectivity against ER+ cells, this was attributed to their lack of receptor binding activity. [99] (Figure 1.14b).
Figure 1.14. Nitrogen mustard and thiourea derivatives of oestrogens.
Cytotoxic stilbene oxide anti-oestrogen compounds have also been tested for oestrogen receptor binding and for in vivo antitumour activities. [100] (Figure 1.15) The 3,3'-dihydroxy-α,β-dialkylstilbenes are anti-oestrogenic, and have strong mammary tumour inhibiting properties in animal systems. Unfortunately the corresponding oxides did not show increased activity, although their receptor binding was equivalent to the parent stilbenes. The lack of increased antitumour activity is attributed to the poor reactivity of the epoxides. [100]

A diverse series of hexestrol derivatives (Figure 1.16) initially synthesized as affinity labelling reagents for the oestrogen receptor,
are also currently undergoing tests to determine if they are selectively cytotoxic to ER+ cell lines. [101] A number of these compounds have been shown to alkylate and inactivate the oestrogen receptor.

Oestrogens with linked alkylating groups may have a dual action on receptor positive tumours. They may alkylate and inactivate the receptor, effectively acting as an anti-oestrogen, or they may bind non-covalently to the receptor and be released in the target cell nucleus, to alkylate cellular nucleophiles. There is evidence that both mechanisms may be involved in the action of these drugs. Irreversible binding, indicating possible covalent attachment, to the oestrogen receptor has been noted in several test compounds. [95] Thiol groups on the oestrogen receptor play an important role in ligand binding, and may be located in the binding site itself. [102,103] Alkylating oestrogens could react with receptor thiols during the binding process. In this way the alkylating compounds act as anti-oestrogens by blockade of the receptor. This process is sufficient to inhibit growth in some hormone responsive tumours in vivo, and cell lines in vitro. [104,105]

Dissociation of cytotoxic oestrogens from receptors and subsequent reaction with cell components is preferable in tumours which although containing ER are not oestrogen dependent. To this end the cytotoxic oestrogen should not bind covalently to oestrogen receptors. [106]

The development of steroid-linked cytotoxic drugs as selective anti-cancer agents, has only become quantitative relatively recently.
The initial results seem encouraging, but there is room for considerable improvement in the selectivity of this class of drug. This should prove possible using newer methods for screening potential drugs of this class. Also the continued research on the mechanisms of steroid hormone receptor action in cells should prove helpful in new drug design.
1.6 **Scope of Work**

The aim of this project was to produce compounds selectively toxic towards oestrogen receptor containing neoplasms, as agents for the treatment of breast cancer. It was proposed to do this following the scheme described below.

(1) To design an *in vitro* assay system to evaluate the cytotoxicity and potential selectivity of alkylating derivatives of oestrogens.

(2) To use this system to examine a series of D-ring α-methylene-δ-lactone derivatives of oestrogens produced by the Organic Chemistry Section at Loughborough.

(3) To use the structure-activity data so gained in the design of compounds with improved selectivity.

(4) To evaluate the mode of action of these and related compounds in an attempt to identify potential biochemical targets.
2. MATERIALS AND METHODS
2.1 Materials

2.1.1 Cell Culture

Cell Lines: HeLa S3 and GH3 cells were supplied by Flow Laboratories (Irvine, Scotland). MCF-7 cells were kindly provided by the Cell and Tissue Culture Laboratory ICI Pharmaceuticals Division, Alderley Park.

Media and Plastics: Culture media, serum and balanced salt solutions were obtained from Flow Laboratories. Plastics (75 cm² flasks and 25 cm² dishes) were obtained from Corning (Stone, England).

Instrumentation: Cells were maintained in a Grant incubator, Aseptic procedures were performed in an Howarth Laminar Flow cabinet. A Coulter Counter Model ZB was used for cell counting, with Isoton II balanced electrolyte solution, Coulter Electronics, Luton, England.

2.1.2 Steroid Binding Assays

Animals: Female Wistar rats (<50 g) obtained from the Animal Unit, Sutton Bonington, University of Nottingham, were used for all uterine receptor binding studies.

T.E.D. Buffer: (Tris(hydroxymethyl)aminomethane) 10 mM
Ethylenediaminetetraacetate (E.D.T.A.) 1.5 mM
Dithiothreitol 0.5 mM
Adjusted to pH 7.4 with dilute HCl

Radiochemicals: [6,7-³H] Oestradiol (49 Ci mmol⁻¹) was supplied by Amersham International (Amersham, England).
Chemicals and Biochemicals: All Chemicals and Biochemicals used in binding assays were purchased from Sigma or Aldrich, (Gillingham, England). PCS scintillation fluid was supplied by Amersham International.

Instrumentation: Cytosols were prepared using an MSE Superspeed MK II centrifuge. Radioactivity was measured using a Phillips PW 4700 liquid scintillation counter.

Pyruvate kinase activity: Pyruvate kinase/lactate dehydrogenase enzyme preparation was supplied by Sigma.

Imidazole Buffer: Imidazole 50 mM

KCl 120 mM

MgSO₄ 30 mM

pH adjusted to 7.6 using dilute HCl

Chemicals and Biochemicals for the enzyme assay were obtained from Sigma.

2.1.3 Biochemical Studies

Animals: Female Sprague - Dawley rats supplied by the Animal Unit, Nottingham University were used as a source of liver tissue for oxidative phosphorylation and lysosomal membrane studies.

Microorganisms: Escherichia coli NC1B 9001, Candida albicans A39 (clinical isolate obtained from Boots, Nottingham) and Penicillium chrysogenum CMI 26211 were used in assays to assess toxicity on prokaryotes and simple eukaryotes.
Media:

**Fungal and Yeast Media.**

- Maltose 38.0 g l\(^{-1}\)
- Yeast Extract 2.5 g l\(^{-1}\)
- Mycological Peptone 8.0 g l\(^{-1}\)
- Malt Extract 20.0 g l\(^{-1}\)
- pH adjusted to 5.0

**Nutrient Broth:**
- Beef Extract 10.0 g l\(^{-1}\)
- Neutralized Bacteriological Peptone 10.0 g l\(^{-1}\)
- NaCl 5.0 g l\(^{-1}\)
- pH adjusted to 7.5

All media ingredients for culture of micro-organisms were obtained from Oxoid Ltd., (Basingstoke, England).

Buffers

**Oxidative Phosphorylation**

**S.T.E. Buffer:**
- Sucrose 250 mM
- Tris 1 mM
- E.D.T.A. 1 mM

**Assay Buffer:**
- KCl 75.0 mM
- Tris 50 mM
- KH\(_2\)PO\(_4\) 12.5 mM
- MgCl\(_2\) 5 mM
- E.D.T.A. 1 mM
- α-ketoglutarate 10 mM
- pH adjusted to 7.4

**Na\(^+\)/K\(^+\) ATPase assay:**
- Tris 92 mM
- NaCl 60 mM
- E.D.T.A. 0.1 mM
- KCl 5 mM
- MgSO\(_4\) 5 mM
- pH adjusted to 7.5
2.1.4 Electron Microscopy

Fixative solution: Gluteraldehyde 25 ml
Formaldehyde 15 ml
H₂O 10 ml
0.32M KCl/0.04M Mg Acetate 50 ml

Fixative stock solution was mixed with an equal volume of Earle's balanced salt solution before use.

Washing solution: Sodium cacodylate 0.05 M
KCl 0.08 M
Mg Acetate 0.01 M

Veronal Acetate Buffer: Na Acetate 0.24 M
Na Barbitone 0.14 M
NaCl 0.6 M

5 ml of the basic solution was mixed with 7.0 ml 0.1M HCl, 13.0 ml H₂O and 0.25 ml 1M CaCl₂ to give a pH of 6.1.
2.2 Methods

2.2.1 Maintenance of Cell Lines

**HeLa S3 cells**

HeLa S3 (cervical carcinoma) cells were grown in Eagle's Minimum Essential Media supplemented with 10% foetal bovine serum, 2mM L-glutamine, 1% non-essential amino acids and the antibiotics penicillin (50 IU ml\(^{-1}\)) and streptomycin (50 \(\mu\)g ml\(^{-1}\)).

Cells were incubated at 37°C with a 5% CO\(_2/\)air humidified atmosphere. Under these conditions the generation time was approximately 24 hours (Results, Figure 3.1). Stock cells were grown in 75 cm\(^2\) flasks and subcultured at 3-5 day intervals. Cells were removed from monolayers using a 0.25% trypsin solution, and harvested with Earle's Balanced Salt solution. For biochemical assays cells were removed by scraping with a 'policeman' to detach monolayers.

**GH\(_3\) Cells**

GH\(_3\) cells (rat pituitary adenoma) cells were grown in Ham's F-10 Media supplemented with 2.5% foetal bovine serum, 15% donor horse serum, 2mM L-glutamine, penicillin (50 IU ml\(^{-1}\)) and streptomycin (50 \(\mu\)g ml\(^{-1}\)). The cells were incubated as for HeLa S3 cells. Generation time was approximately 33 hours, (Results, Figure 3.1).
Storage of Cells

Cell lines were prepared for storage in liquid nitrogen by a modification of the method described by Paul. [107] Cells were harvested and resuspended in media containing 15% serum and 10% dimethyl sulphoxide to a density of $5 \times 10^6$ cells/ml. 6 ml Aliquots in storage tubes were cooled in an ice/water bath for 30 minutes, then transferred to a freezer at -20°C for 4 hours and finally to storage in liquid nitrogen vapour.

Cell Counting

Cells were counted using a Coulter Counter model ZB with a 140μM diameter orifice tube. Cells were first sized, Figure 2.1, and appropriate instrument settings used thereafter to count HeLa S3 or GH3 cells.

2.2.2 Cytotoxicity assays using cell cultures

HeLa S3 cells

Compounds were assayed following the Cancer Chemotherapy National Service Centre (CCNSC) protocol for KB cells [108] Cell numbers were determined as described by Coulter Counter. Growth inhibition curves were plotted from mean cell counts after 48 hours. $2.5 \times 10^5$ Cells in 5 ml media were seeded in 25 cm² dishes. After incubation for 24 hours the media was changed. Control dishes were counted to give baseline cell numbers and test compounds added in 25 μl DMSO (0.5% final concentration).
Figure 2.1. Size distribution of GH3 (○) and HeLa S3 (●) cells.
Compounds were screened initially at concentrations between 10 and 0.5 µg ml\(^{-1}\). Those which significantly inhibited growth below 10 µg ml\(^{-1}\) were re-assayed to produce dose vs inhibition of growth curves. Compounds with little or no activity at 10 µg ml\(^{-1}\) were re-assayed at 20 µg ml\(^{-1}\) in triplicate.

**GH\(_3\) cells**

Activity of compounds against GH\(_3\) cells was determined by a modification of the procedure described for the HeLa S3 line. To take account of the longer generation time of the GH\(_3\) cells, a drug contact time of 66 hours was used, after the initial 24 hour incubation.

**MCF-7 cells**

Assays using MCF-7 cells (performed at I.C.I.) were carried out in 1 ml multiwell tissue culture dishes. 1.5 x 10\(^5\) Cells were seeded in 1 ml of medium (containing dextran/charcoal stripped serum) and incubated for 24 hours. Following incubation the media was replaced with 1 ml of fresh media containing test compounds added in 10 µl DMSO (0.5%). Control wells were counted in triplicate to determine baseline numbers. A model 2M Coulter Counter was used with open channels; counts were corrected for background.

A drug contact time of 72 hours after the initial 24 hour incubation was used.
2.2.3 Steroid Binding Methodology

Receptor assays

Uterii from oestradiol benzoate-treated immature (2 day old) rats (0.16 µg/s.c. in arachis oil daily x 3) were homogenised in TED buffer at 4°C. The homogenate was centrifuged at 100,000 g for 1 hour (4°C) and the cytosol used immediately. Cytosol (150 µl) was incubated with varying concentrations of competing ligands added in TED buffer (50 µl) and [³H] oestradiol (35nM, 50 µl) in TED buffer at 30°C for 30 minutes. Parallel incubation of cytosol (150 µl) and 50 µl of a solution of diethylstibosreol (5 µM) in TED was used to determine the specific binding of [³H] oestradiol. All tubes were then cooled in ice/water for 15 minutes after which 200 µl of a suspension of dextran coated charcoal (250 mg% Norit-A, 2.5 mg% dextran) in TED buffer was added and the tubes allowed to stand for 20 minutes in ice/water with occasional vortex mixing. Tubes were centrifuged (2000 g x 5 minutes) and 200 µl aliquots of supernatent were added to 5 ml PCS and counted. Counting efficiency was 30-35%.

Cytosol containing oestrogen binding sites was obtained from GH3 cells by a modification of the method according to Roos et al. [109] Attached cells were washed 3x with TED buffer 4°C, scraped from the plates and suspended at a final concentration of 10⁷ cells ml⁻¹. The suspension was sonicated, (2 x 5 second bursts) at 4°C, and the resulting
suspension was centrifuged at 100,000g for 1 hour. The supernatants were used immediately in binding assays, as described for rat uterine cytosols.

**Role of thiols**

To determine if reaction with thiol groups in cytosols reduces availability of drugs for binding during competitive binding assays, bromoacetone treated cytosols were used as described by Katzenellenbogen. [101]

The effect of bromoacetone on specific binding in cytosols was first determined. Uterii from three oestradiol-benzoate treated rats were excised and the horns divided. The horns were then randomly split; three were homogenised in TED buffer, three in buffer without dithiothreitol (TE buffer). The homogenates were centrifuged (100,000g x 1 hour) and the cytosols used immediately.

Cytosol in TE buffer was assayed for sulphhydryl groups; 200 µl cytosol added to DTNB solution (1 µ mole in 2.8 ml phosphate buffer) and the absorbance measured at 412 nm. The cytosol was then divided in two. Half was treated with a four-fold excess of bromoacetone, which reduced thiols to below measurable levels and then specific binding in both treated and untreated aliquots was determined as described previously. Cytosol in TED buffer was assayed for specific binding as normal.

Receptor binding of drugs in bromoacetone-treated cytosols was determined by preparing cytosol in TE buffer and treating with four
fold excess of bromoacetone. Drugs were used in the binding assay at 2.5 μM. Dose/response curves for diethylstilboestrol were also measured to ensure that the characteristics of the oestrogen receptor were not altered by the treatment.

**Reversibility of drug/receptor binding**

Reversibility of drug/receptor binding was determined according to the method described by Katzenellenbogen. [101] This method makes allowance for any loss of receptor binding that may occur due to denaturation of the receptor protein.

Cytosol was prepared as described from immature rat uterii. 150 μl Aliquots were incubated with saturating concentrations of ligands, control tubes contained 30 nM oestradiol, in a final volume of 250 μl. The tubes were incubated for 16 hours at 30°C. A parallel incubation was first 'protected' with 30 nM oestradiol (1 hour, 4°C), to fill the receptor binding sites, before addition of the alkylating compounds.

After 16 hours incubation all the tubes were cooled to 4°C and treated with dextran/charcoal suspension (200 μl) as described previously, to remove unbound ligands.

Two 150 μl aliquots of supernatant were taken from each tube. One aliquot was incubated with 7 nM [3H]-oestradiol and the other with 7 nM [3H]-oestradiol plus 1 μM 'cold' oestradiol in a final volume of 250 μl. The tubes were incubated for a further 16 hours.
at 30°C. After this final incubation the tubes were cooled to 4°C, and treated with dextran/charcoal suspension. 200 µl Aliquots of the supernatants were added to 5 ml PCS and counted.

**Effects of drugs on allosteric enzyme activity** (Pyruvate kinase)

Pyruvate kinase (PK) activity was measured in a coupled assay system, with lactate dehydrogenase (LDH) at 25°C in 50 mM imidazole buffer pH 7.6 by spectrophotometric determination of NADH disappearance at 340 nm. [110]

Assay mixtures consisted of 2 mM adenosine diphosphate (ADP), 3 mM phosphoenolpyruvate (PEP) and 1 mM nicotinamide adenine dinucleotide reduced form (NADH) in a total volume of 3.0 ml. Reactions were started by addition of 0.1 ml PK/LDH solution, (10 µg enzyme/ml in buffer). The reaction was followed using a SP 500 spectrophotometer linked to a Vitatron chart recorder, constant temperature was maintained in the spectrophotometer by a water jacketed cuvette rack. Compounds were added in DMSO up to a final concentration which did not exceed 10%. DMSO up to a concentration of 10% did not adversely affect activity.

2.2.4 **Biochemical Studies**

**Electrophilic activity of compounds**

Each compound under test (1 µmole) in 0.9 ml DMSO was added to 2.1 ml phosphate buffer pH 7.0 at 25°C containing 1 µmole of glutathione. 200 µl Samples were taken at intervals and unreacted
glutathione determined by Ellman's method \textsuperscript{[111]} as follows; samples were added to 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) solution (1 μmole in 2.8 ml phosphate buffer pH 7.0) and the change in absorbance measured at 412 nm.

**Effect of drugs on cellular thiol levels**

2 x 10\textsuperscript{6} Cells in 5 ml media were placed in 25 cm\textsuperscript{2} dishes and incubated overnight. Drugs were added in 25 μl DMSO (0.5%), and the cell incubated for various times. Attached cells were washed twice with Isoton (4°C), harvested and suspended in 5 ml Isoton. The suspension was centrifuged (600 g x 5 minutes), and resuspended in 500 μl Isoton.

Cell surface thiols were determined by reacting 100 μl of cell suspension with 1.4 ml DTNB solution (1 mM in phosphate buffer pH 7.0) for 5 minutes at room temperature. The reaction was stopped by centrifugation (600 g x 5 minutes) and absorbance of the supernatant measured at 412 nm.

Total cell thiols were determined by sonicating the remaining cell suspension. The sonicate (100 μl) was added to 4.1 ml DTNB as above. After centrifugation (11,000 g x 5 minutes) the absorbance was measured at 412 nm.

Viability of cells after treatment with drugs was measured after staining with nigrosin.
Lysosomal membrane stability and lysosomal enzyme activity

Labilisation or stabilisation of lysosomal membranes was assessed by determining the release of the marker enzyme aryl sulphatase. [112]

Livers from female Sprague/Dawley non-fasted rats (approx. 250 g) were removed, weighed, and homogenised in 0.25 M sucrose -0.02 M Tris pH 7.4, (4°) to give a 10% homogenate using a TR 1-R K41 glass-teflon homogeniser. The homogenate was centrifuged (600 g x 10 minutes, 4°C), and the supernatants diluted with an equal volume of the same buffer and centrifuged (1500 g x 15 min, 4°). The supernatants were used immediately as the source of lysosomes.

A suspension of the lysosome-containing liver fraction was warmed to 25°C for 5 minutes, then 0.2 ml samples were added to 2 ml buffer with or without drug (added in DMSO, 0.5% final concentration) and incubated at 37° for 1 hour. 0.5 ml Samples of the incubation mixture were then used in the enzyme assay as follows:-

Aryl sulphatase activity was determined by adding 0.5 ml of incubation supernatant to 1 ml of 0.2 M acetate buffer pH 5.8. Incubations (37°C, 20 mins) were initiated by addition of 50 μl substrate (p-nitrocatechol sulphate, 25 mg/ml in distilled H2O), and terminated by addition of 100 μl of 4N sodium hydroxide. Absorbances were measured at 510 nm, and compared with total activity obtained as follows:-

An aliquot of the lysosomal liver fraction was incubated with
0.2% triton x - 100 for 1 hour at 37° to totally disrupt lysosomal membranes. The resulting preparation was then assayed for aryl sulphatase activity, as described above, but in the presence of drugs, (added in DMSO, 0.5%).

**Oxidative Phosphorylation**

Respiration in 10% liver homogenates of rats was measured using a Clark Oxygen Electrode, with a 2 ml reaction vessel volume, connected to a Vitatron recorder:-

Livers from female Sprague/Dawley rats were excised, weighed and homogenised in STE buffer pH 7.4 at 4°C to give a 10% homogenate (w/v). The homogenate was centrifuged (600 g x 10 mins, 4°) and the pellets discarded. The supernatants were centrifuged (8,500 g x 10 min, 4°) the pellets resuspended in buffer and centrifuged again under the same conditions. The resulting mitochondrial pellet was resuspended in STE buffer, approximately 1 ml per gram of original tissue to give a suspension of 20-30 mg of protein per ml (Lowry estimation). [113]

The O₂ electrode was calibrated by addition of saturated dithionite solution (100 μl) to fresh assay media in the vessel, to give a full scale deflection. Reactions were initiated by addition of 100 μl of mitochondrial preparations to 2 ml of assay media and the rate of state 4 respiration measured for several minutes, 100 μl of a 5.6 mM ATP solution in assay media was then added to obtain the
state 3 rate of respiration. Effects of compounds on the rates of respiration were measured both by addition of the drugs to the reaction vessel (in 10 μl DMSO, 0.5%), and by preincubation of the mitochondrial preparation with drug before use.

\[ ^{3}\text{H} \] Thymidine uptake and incorporation in HeLa S3 cells.

Uptake and incorporation of \[^{3}\text{H} \] thymidine was determined according to the techniques described by Woynarowski and Konopa [43]

(a) Uptake of \[^{3}\text{H} \] thymidine:

HeLa S3 cells in log phase of growth were harvested, resuspended in Minimum Essential Media, (MEM) for suspension cultures (pH adjusted to 7.4), to give a stock cell suspension of \(1.05 \times 10^6 \) cells ml\(^{-1}\). Stock solutions of \[^{3}\text{H} \] thymidine in MEM were made up with labelled and 'cold' thymidine to give a solution of 0.45 n moles thymidine (0.75 μCi) ml\(^{-1}\).

Incubations were initiated by addition of stock cell suspension to thymidine solutions in the ratio 1:2 to give an inoculate containing 3.5 \( \times \) 10\(^5\) cells, 0.3 μmoles, 0.5 μCi ml\(^{-1}\). Incubations were at 37°C in a shaking water bath, 70-80 oscillations per minute, for varying times and with varying concentrations of test drug, (added in DMSO, 0.5%).

After incubation 0.5 ml aliquots of cell suspension were collected by filtration using a vacuum manifold on Whatman GF/C glass fibre filters. The filters were washed with isoton 4° (3 x 5 ml), placed in scintillation vial inserts and counted in 5 ml, PCS.
(b) **Incorporation of \[^{3}\text{H}\] Thymidine:**

Cells were harvested and suspended in ice cold MEM for suspension cultures to a density of $3.5 \times 10^5$ cells/ml. \[^{3}\text{H}\] Thymidine was added to give a concentration of $2\ \mu\text{Ci}\ \text{ml}^{-1}$ and the cells incubated for 1 hour at $6^\circ\text{C}$. The suspension was then centrifuged ($600\ \text{g} \times 5\ \text{mins, } 4^\circ\text{C}$), and the cells washed twice with ice cold media and resuspended in fresh media. Drugs were added (DMSO) and the cells were incubated, under the same conditions as described above, for 30 minutes. Incubation was terminated by removal of 0.5 ml aliquots of suspension and the cells collected by centrifugation ($600\ \text{g} \times 5\ \text{mins}$). The supernatant was then aspirated and the cells resuspended in 200 $\mu\text{l}$ ice cold $10\%\ (\text{w/v})$ TCA and left overnight ($4^\circ\text{C}$). Precipitated macromolecules were centrifuged ($11,500\ \text{g} \times 5\ \text{min}$) and resuspended in 250 $\mu\text{l}$ 5% TCA and hydrolysed by heating ($70^\circ\text{C},\ 1\ \text{hour}$). 200 $\mu\text{l}$ samples were then counted in 5 ml PCS.

**Na\(^+\)/K\(^+\) Adenosine triphosphatase (Na\(^+\)/K\(^+\) ATPase) activity**

**a) Isolated enzyme assays**

ATPase from HeLa S3 cells in addition to commercial enzyme preparations was used to determine the effects of drugs. ATPase from HeLa cells was prepared from cells harvested in early log phase. The cells were resuspended in distilled water and sonicated (3 x 5 sec bursts), and the protein concentration adjusted to approximately 0.5 mg ml\(^{-1}\). (Lowry estimation). This suspension was then used in the enzyme assay. Commercial enzymes were adjusted to give an enzyme activity of 0.05 units ml\(^{-1}\).
ATPase activity was determined by measurement of the release of inorganic phosphate according to the method of Martin and Doty. [114] Enzyme suspension (0.1 ml) was added to 0.8 ml of Tris buffer pH 7.5 with or without drug (added in DMSO). Reaction was started, after 10 minutes equilibration at 37°C, by addition of 0.1 ml ATP solution (40 mM in distilled water) and terminated by the addition of 1 ml of cold 10% TCA.

Reaction mixture (1 ml) was taken and added to tubes containing 2.5 µl of H₂O saturated isobutanol/benzene (1:1 mixture) and 1 ml of ammonium molybdate solution (1.25% in 0.5 M H₂SO₄). The tubes were vortex mixed for 15 seconds each then centrifuged, 2000 g x 3 mins, to separate the layers. 1 ml of the isobutanol/benzene layer was taken and added to tubes containing 2 ml of ethanol-H₂SO₄ (980 ml ethanol : 20 ml conc H₂SO₄) and mixed, 0.1 ml of a solution of stannous chloride (40 g SnCl₂ in 100 ml conc HCL diluted 200 x with 0.5 M H₂SO₄) were added and the colour allowed to develop. The absorbances of the solutions were measured at 730 nm.

(b) ⁸⁶Rb⁺ uptake by HeLa S3 cells

2.5 x 10⁵ Cells in 5 ml media were seeded in 25 cm tissue culture dishes and incubated for 24 hours. After 24 hours the media was replaced with fresh media containing 0.5 µCi ⁸⁶Rb⁺ ml⁻¹. Drugs were added in 25 µl DMSO (0.5%). After incubation for various times the media was aspirated and the cells washed with Tris buffer pH 7.4 (3 x 1 ml). ⁸⁶Rb⁺ was extracted in 0.5 ml 5% TCA solution. A 250 µl sample
of extract was then added to 5 ml PCS scintillation fluid and radioactivity counted.

**Toxicity of drugs against microorganisms**

The effects of the compounds on growth of bacteria, fungi and yeasts was determined.

(a) *Escherichia coli* (E. coli)

E. coli starter cultures were grown in nutrient broth overnight in a shaking water bath at 37°C. 1 ml Aliquots of starter culture were then innoculated into tubes containing 5 ml nutrient broth and allowed to reach log phase by incubating for 2 hours at °C. At this (zero) time the turbidity in control cultures was measured at 650 nm. Test compounds were added in 50 µl DMSO and the cultures incubated for a further 3 hours when the turbidity was again measured. Inhibition of growth was defined as a slowing of the turbidity increase compared with control tubes.

(b) *Candida albicans*, and *Penicillium chrysogenum*

Slope cultures of the organisms were transferred to 50 ml of fungal media and incubated at 30°C for 48 hours. 0.5 ml Aliquots were then transferred to tubes containing 4.5 ml nutrient media. Steroids and α-methylene lactones were added in 125 µl DMSO (2.5%), and the cultures incubated at 30°C for a further 48 hours when the turbidities were measured at 650 nm. Inhibition of growth was determined from reductions in turbidity over 48 hours in the case of C. albicans and by dry weight.
2.2.5 Electron Microscopy

(a) Scanning Electron Microscopy

1.0 x 10^6 Cells in 5 ml media were added to 25 cm² tissue culture dishes, containing 33 mm diameter coverslips, and incubated for 24 hours to allow the cells to adhere to the coverslips. After 24 hours the media was replaced and compounds added in 25 µl DMSO. The cells were incubated for a further 24 hours.

Treated and untreated cells were then washed with Earle's balanced salt solution (2x) and with washing solution (2x). The cells were then covered with fixative solution at 4°C and left overnight. After fixation the cells were rinsed with balanced salt solution (2x) and then with distilled water (3x). The fixed cells were dehydrated in a vacuum dessicator overnight.

The coverslips with attached cells were removed and gold coated (10 nm). The cells were then examined in an Alpha 9 scanning electron microscope [115], at a magnification of 1500x.

(b) Transmission Electron Microscopy

2 x 10^6 Cells in 10 ml media were added to 75 cm² tissue culture flasks and incubated for 24 hours, when the media was replaced and compounds added in 50 µl DMSO. The cells were incubated for a further 24 hours.

Treated and untreated cells were washed with Earle's balanced salt solution (2x) and washing solution (2x). Fixative solution (4°C) was then added and the cells were fixed overnight at 4°C. After fixing the cells were rinsed with washing solution (2x), collected by
scraping and centrifugation (600 g x 5 mins) and the cell pellets mounted by mixing (1:1) with 4% purified agar at 50°C. After cooling the agar was cut into 1 mm cubes which were left to rinse overnight in washing solution. The cells were then rinsed for 15 minutes in veronal acetate buffer, and stained, first with osmium tetroxide (1% in veronal acetate buffer) for 1 hour and then with uranyl acetate (2% in veronal acetate buffer) for 1 hour.

After staining, the cells were dehydrated by washing with ethanol solutions; 25% (1 x 20 minutes), 50% (1 x 20 minutes), 75% (2 x 15 minutes) and 100% (2 x 15 minutes). Following dehydration the cells were embedded by immersion first with ethanol/propylene oxide (1:1) for 15 minutes, then propylene oxide for 20 minutes, propylene oxide/resin (1:1) for 30 minutes at 60°C, and finally resin for 60 minutes at 60°C.

The agar blocks were then transferred to resin filled embedding capsules, and placed at the apex of the capsule cone. The capsules were left at 60°C overnight to allow the resin to harden. The mounted cells were sectioned, and sections were supported on copper grids, then stained with Reynold's lead citrate to increase contrast. The sections were examined with an EM6B transmission electron microscope, at a magnification of 3000x.
2.3 Compounds Under Investigation

Vernolepin and Elephantopin were a kind gift of Dr. Sneden, University of Virginia, U.S.A.

![Vernolepin](image)

![Elephantopin](image)

Estramustine was a gift of A.B. Leo, Helsingborg, Sweden.
ORG 4333 (11β-chloromethyloestradiol) was a gift of Organon, Morden, England.

Estrolactone (1) and Estrolic acid (2) were synthesised according to the method described by Westerfeld.
The following compounds 4-17 were synthesised by L.S. Chagonda in the Organic Chemistry Laboratory of this Department, according to the method of Yamada. [116] Brief analytical data is given for all new compounds, for further data see reference 137.

4. 3-Methoxy-16-methylene-17-oxo-17a-oxa-D-homo-estra-1,3,5(10)-triene.

\[ \text{mp 167-169°. } [\alpha]_D + 6° (c, 0.01). \text{ MS 312. 1725 (M, C}_{20}\text{H}_{24}\text{O}_3), } \]
\[ \text{calc M, 312, 1725.} \]
\[ \text{Anal C}_{20}\text{H}_{24}\text{O}_3: \text{ calc\%: C, 76.89; H, 7.74} \]
\[ \text{Found: 76.50; 7.90} \]

5. 3-Acetoxy-16-methylene-17-oxo-17a-oxa-D-homo-estra-1,3,5(10)-triene.

\[ \text{m.p. 167-170°. } [\alpha]_D = 0 (c, 0.02). \text{ (M, C}_{21}\text{H}_{24}\text{O}_4), \text{ calc M 340. 1675; 298. 1586 (M-C}_2\text{H}_2\text{O, C}_{19}\text{H}_{22}\text{O}_3) \text{ calc 298. 1569.} \]
6  3-Hydroxy-16-methylene-17-oxo-17a-oxa-D-homo-estra-1,3,5(10)-triene.

\[
\begin{align*}
\text{HO} & \\
\text{m.p. 226-230°. MS 298.1567 (M C_{19}H_{22}O_3) calc. M 298.1569 (BP)}
\end{align*}
\]

7  3-Methoxy-17-oxo-17a-oxa-D-homo-estra-1,3,5(10)-triene.

\[
\begin{align*}
\text{CH}_3\text{O} & \\
\text{m.p. 167-172°. MS 300.1724 (M C_{19}H_{24}O_3), calc. M 300.1726.}
\end{align*}
\]

8  3-Acetoxy-17-oxo-17a-oxa-D-homo-estra-1,3,5(10)-triene.

\[
\begin{align*}
\text{AcO} & \\
\text{m.p. 156-60° (literature 149-151).}
\end{align*}
\]
9  3-Hydroxy-17-oxo-17α-oxa-D-homo-estra-1,3,5(10)-triene.

\[ \text{HO} \]

m.p. > 265° (Lit 335-340°). MS (LR) 286 (M C_{18}H_{22}O_{3})

10  3-Methoxy-16-methyl-17-oxo-17α-oxa-D-homo-estra-1,3,5(10)-triene.

\[ \text{CH}_3\text{O} \]

m.p. 147-150°. MS 314.1882 (M C_{20}H_{26}O_{3}) calc. M 314.1881.

11  3-Methoxy-16-(hydroxymethylene)-17-oxo-17α-oxa-D-homo-estra-1,3,5(10)-triene.

\[ \text{CH}_3\text{O} \text{OH} \]

m.p. 242-245°. MS 328.1674 (M C_{20}H_{24}O_{3}), calc. M 328.1675.
12 3-Methoxy-16-propyldene-17-oxo-17a-oxa-D-homo-estra-1,3,5(10)-triene.

m.p. 176-179°, \([\alpha]_D^\circ + 30^\circ\) (C, 0.01). MS 340.2039 (M C\(_{22}\)H\(_{28}\)O\(_3\)), calc. M 340.2039.

13 3-Acetoxy-16-(acetoxyethylene)-17a-oxo-17a-oxa-estra-1,3,5(10)-triene.

m.p. 215-218°.

14 3-Hydroxy-16-(Hydroxymethylene)-17-oxo-17a-oxa-D-homo-estra-1,3,5(10)-triene.

m.p. 262-265°. MS 314.1519 (M C\(_{19}\)H\(_{22}\)O\(_4\)) calc. M 314.1518.
15 3-Methoxy-16-(phenylthiomethyl)-17-oxo-17α-oxa-D-homo-estra-
1,3,5(10)-triene.

Oil. MS 422.1929 (M C₂₆H₃₀S0₃), calc. M 422.1916.

16 3β-Acetoxy-16-benzyl-17-oxo-17α-oxa-D-homo-5α-androstane.

16a 3β-Hydroxy-16-benzylidene-17-oxo-17α-oxa-5α-androstane

\[
\text{HO}
\]

m.p. 120-122° [α]D 128° (C, 0.01). MS 394.2504 (M C_{26}H_{34}O_{3}), calc. M 394.2508

17 3β-Acetoxy-16-benzylidene-17-oxo-17α-oxa-5α-androstane.

\[
\text{AcO}
\]

m.p. 200-202°. [α]D -88° (C 0.01). MS 436.2622 (M C_{28}H_{36}O_{4}),
calc. M 436.2617.

Anal C_{28}H_{36}O_{4}; calc. %: C, 77.03; H, 8.31

Found : 76.50; 7.90
Compounds 18-20 were synthesised by G. Edge in the Organic Chemistry Laboratory of this department according to the method described by Öhler [86,117].

18. 4-(3-hydroxyoestra-1,3,5(10)-triene-17-on-16-yl)-2-methylene-4-butanolide.

\[
\text{m.p. 226-228°C, MS 366.1840 (M} \text{C}_{23}\text{H}_{26}\text{O}_4) \\
\text{Anal (C}_{23}\text{H}_{26}\text{O}_4): \text{calc: C}\,75.4\%, \text{H}\,7.3\%
\]
\[
\text{Found: C}\,75.1, \text{H}\,7.1
\]

19. 4-(3-methoxyoestra-1,3,4(10)-triene-17-on-16-yl)-2-methylene-4-butanolide.

\[
\text{m.p. 173-174, MS 380.1988 (M} \text{C}_{24}\text{H}_{28}\text{O}_4) \\
\text{Anal (C}_{24}\text{H}_{28}\text{O}_4): \text{calc: C}\,75.8\%, \text{H}\,7.4\%
\]
\[
\text{Found: C}\,75.9, \text{H}\,7.1
\]
20. 3,17β-dihydroxy-4'-methylene-3'-oxo-ostra-1,3,4(10)-triene-16-spiro-2'-oxolane.

m.p. 213-215°C, MS 354 (M\textsubscript{1}C\textsubscript{22}H\textsubscript{24}O\textsubscript{4})

Anal (C\textsubscript{22}H\textsubscript{24}O\textsubscript{4}): calc%: C, 74.6%, H, 7.3%

Found: C, 74.4, H, 7.5

Compounds 21a and 21b were synthesised by J.C. Gill in the Organic Chemistry Laboratory of this department.

21a. (20R) 3,17β-dihydroxy-20,21-epoxy-19-norpregna-1,3,5(10)-triene

m.p. 154-158°C, [α]\textsubscript{D} + 41.8°, (c, 1.0). MS 314.1880. (M\textsubscript{1}C\textsubscript{20}H\textsubscript{26}O\textsubscript{3})

Anal (C\textsubscript{20}H\textsubscript{26}O\textsubscript{3}): calc%: C, 76.4%, H, 8.3%

Found: C, 76.5, H, 8.4
21b (20S) 3,17β-dihydroxy-20,21-epoxy-19-norpregna-1,3,5(10)-triene

m.p. 184-200°C, [α]D + 46.6°, (C, O.9), MS 314.1876 (M1C20H26O3)

Anal (C20H26O4): calc%: C, 76.4%, H, 8.3%
Found: C, 76.5, H, 8.4
3. RESULTS
3.1 The Assay Systems

3.1.1 Cytotoxicity

(a) HeLa S3 cells

The HeLa S3 line was used to evaluate the general cytostatic activity and cytotoxicity of compounds. The population doubling time of this cell line is approximately 24 hours (Figure 3.1).

The sesquiterpene compounds elephantopin and vernolepin were found to have IC₅₀ values of the same order as those previously reported. [37,43] (Figure 3.2) These compounds were used as standard non-selective α-methylene lactones for comparison purposes.

(b) MCF-7 and GH₃

The population doubling time of MCF-7 cells is reported as approximately 36 hours in stripped serum. [118]. The GH₃ cell line has a population doubling time of approximately 33 hours (Figure 3.1). Unlike the HeLa S3 line, both cell lines have measurable levels of functioning oestrogen receptors. [109, 119] Only the MCF-7 line is growth responsive to oestrogens however. [118]

Vernolepin exhibits a similar degree of cytostatic activity toward GH₃ cells as against HeLa S3, (Figure 3.3). The IC₅₀ value obtained for elephantopin against MCF-7 cells, (Figure 3.4) is approximately tenfold that obtained against the HeLa S3 line. The modified assay procedure used with the MCF-7 cell line with greater numbers of
Figure 3.1. Growth of HeLa S3 (●) and GH3 (▲) cells. Means of 3 determinations.
Figure 3.2. Effect of Elephantopin (○) and Vernolepin (▲) on HeLa S3 growth. Means of 4 determinations ± SEM.
Figure 3.3. Effect of Vernolepin on GH3 growth. Means of duplicate determinations, which did not vary by more than 10%.
Figure 3.4. Effect of Elephantopin on MCF-7 growth. Means of 4 determinations ± SEM. Standard errors less than symbol size.
innoculum cells, may be responsible for the higher IC\textsubscript{50} value obtained.

3.1.2 Receptor-Binding Assays

Binding assays using rat uterine and GH\textsubscript{3} cytosols gave results for standard compounds which compared well with literature values. [120,121] Oestradiol and DES were found to have ED\textsubscript{50} values for [\textsuperscript{3}H]-oestradiol displacement close to values previously quoted for this assay system, (Figure 3.5).

The above assay methods were used to evaluate candidate compounds following the scheme:

\begin{align*}
\text{Cytotoxicity toward HeLa} \rightarrow \text{receptor binding} \rightarrow \\
\text{cytotoxicity towards GH}_{3}\text{ and/or MCF-7 cells}.
\end{align*}
Figure 3.5. Displacement of $[^{3}\text{H}]$-oestradiol (7 nM) from rat uterine cytosols by diethylstilboestrol (•) and oestradiol (○). Means of 3 determinations. Standard errors less than symbol size.
3.2 Cytotoxicity Studies

3.2.1 D-ring-a-methylene-6-lactone oestrogens

The compounds examined (Figure 3.6) were as described in section 2.3. The D-ring-a-methylene-δ-lactone compounds 4, 5 and 6 have IC50 values against HeLa S3 cells in the same range as elephantopin, but lower than the nitrogen mustard compound estramustine. (Figure 3.7, Table 3.1).

The importance of the a-methylene-δ-lactone function to cytotoxicity was investigated using a series of D-ring lactones and derivatives. Values obtained are given in Table 3.1. The corresponding lactones of 4, 5 and 6, that is compounds 7, 8 and 9 (estrolactone) have greatly reduced activity. Similarly substitution of the a-methylene group, as in compounds 10, 11, 12, 13 and 14, also reduces cytotoxicity. On the other hand, the thiophenol adduct of 4, compound 15, retains considerable activity, (Figure 3.8).

The saturated A-ring compounds such as 16, 16a and 17 are relatively non-toxic, even at higher concentrations, 20μg ml⁻¹ (> 60μM).

Studies with certain of the D-ring-a-methylene-δ-lactones were also performed using GH3 and MCF-7 cells. (Table 3.2) While compounds 5 and 6 were highly toxic to GH3 cells (Figure 3.9), in line with the HeLa S3 cells, 5 (6 was not examined) was tenfold less active against MCF-7 cells, (Figure 3.10) as with the findings of
Figure 3.6. D-ring α-methylene lactone steroids and related compounds.
11. R = CH₃, R = OH
12. R = CH₃, R = C₂H₅
13. R = Ac, R = oAc
14. R = H, R = OH

Figure 3.6. Continued......
Figure 3.7. Effect of 4 (○) 5 (△) 6 (▲) and Estramustine (●) on HeLa S3 growth. Means of 6 determinations ± SEM.
Table 3.1. Effect of D-ring α-methylene lactone steroids and related compounds on HeLa S3 growth

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)ᵃ</th>
<th>% Inhibitionᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elephantopin</td>
<td>0.64±0.01</td>
<td></td>
</tr>
<tr>
<td>Vernolepin</td>
<td>3.50±0.10</td>
<td></td>
</tr>
<tr>
<td>Estramustine</td>
<td>3.39±0.20</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.74±0.02</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.29±0.01</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.49±0.03</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>48±3.5</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>50±2.5</td>
</tr>
<tr>
<td>9. Estrolactone</td>
<td></td>
<td>30±1.0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>63±5.6</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>27±2.7</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>9±0</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>22±1.0</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>14±2.7</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>1.9±0.07</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>55±1</td>
</tr>
<tr>
<td>16a</td>
<td></td>
<td>42±3.9</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>42±1.1</td>
</tr>
</tbody>
</table>

ᵃ Values are means of 3 or more determinations.

ᵇ Values represent % inhibition of growth at 20μg ml⁻¹ (=60μM) the highest dose tested, means of 3 determinations ± SEM
Figure 3.8. Effect of 4 (○) and the thiophenol adduct 15 (●) on HeLa S3 growth. Means of 6 determinations ± SEM.
Figure 3.9. Effect of 5 (*) and 6 (△) on GH₃ growth. Means of duplicate determinations, which varied by 5% at most.
Figure 3.10. Effect of $S$ on MCF-7 growth. Means of 3 determinations ± SEM.
elephantopin on this cell line.

Consequently the influence of initial inoculum cell numbers on the observed activity of a cytotoxic compound was investigated using HeLa S3 cells, (Figure 3.11) Compounds 5 and 6 was used at a concentration of 0.5 µM. This concentration of 5 is sub-lethal under the standard assay conditions for HeLa S3 cells (c.f. Figure 3.7).

As expected, the saturated A ring compound 16 was toxic only at high levels against MCF-7 cells.

Table 3.2. Effect of D-ring-α-methylene-δ-lactones and related compounds on GH3 and MCF-7 cell growth.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (µM)</th>
<th>GH3</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.28</td>
<td>2.1±0.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>&gt;30</td>
<td></td>
</tr>
<tr>
<td>Elephantopin</td>
<td>-</td>
<td>5.1±0.0</td>
<td></td>
</tr>
<tr>
<td>Vernolepin</td>
<td>5.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ORG 4333</td>
<td>-</td>
<td>&gt;30</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2. 16-(α-methylene-γ-lactone)-substituted oestrogens.

As a development of the work on α-methylene-lactone linked compounds, oestrogens containing an α-methylene-γ-lactone function linked at the 16-position were investigated. The compounds examined are given below (Figure 3.12).
Figure 3.11. Effect of initial cell numbers on inhibition of HeLa S3 growth by compound 5 (0.5 μM). Means of 5 determinations ± SEM.

Controls open bars.
Compounds 18 and 20 are approximately one tenth as active against HeLa S3 cells as the D-ring α-methylene-δ-lactone series (Figure 3.13, Table 3.3). Compound 19 has much lower activity, and is of the same order as the inactive, substituted α-methylene-δ-lactones, i.e. >60μM. A similar order of activity is seen against GH3 cells (Figure 3.14, Table 3.3).

In the MCF-7 cell assay, 18 has similar activity as against HeLa S3 and GH3 (Figure 3.15). Taking into account the effect of initial cell numbers in the MCF-7 assay, this would suggest that 18 shows enhanced cytotoxicity toward the oestrogen-receptor containing MCF-7 line. (A sample of 20 was not available for testing on MCF-7 cells).
Figure 3.13. Effect of 18 (○) and 20 (▲) on HeLa S3 growth. Means of 6 determinations ± SEM for 18. Means of duplicate determinations for 20. A further repeat assay of 20 gave an IC₅₀ of 2.5μM.
Figure 5.14. Effect of 18 (*) and 20 (▲) on GH₃ growth. Means of 2 determinations, values varied by less than 5%.
Figure 3.15. Effect of \(18\) on MCF-7 growth. Means of 3 determinations \(\pm\) SEM.
Table 3.3. Effect of 16-(α-methylene-δ-lactone) substituted oestrogens on cell growth

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
<th>HeLa S3</th>
<th>GH₃</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>12.3±0.1</td>
<td>12.9</td>
<td>10.4±0.4</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>(24.6±9.4)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>6.0</td>
<td>7.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Elephantopin</td>
<td>0.64±0.1</td>
<td>-</td>
<td>5.1±0</td>
<td></td>
</tr>
</tbody>
</table>

*a Figure in parentheses represents % inhibition of growth at 20μg/ml (52.6μM), mean of 3 determinations ± SEM.

3.2.3. 17α-epoxyethano-oestrogens

As an alternative to the α-methylene lactone alkylating group, 17α-epoxyethano oestrogens were assessed for activity using the assay systems developed.

Against HeLa S3 cells compounds 21a and 21b (Figure 3.16) have activities of the same order as the 16-(α-methylene-γ-lactone) oestrogens (Figure 3.17). Isomer 21a is approximately three times as active as its diastereomer 21b against HeLa S3 cells.
The activities of the compounds against GH3 cells (Figure 3.18) are similar to those against HeLa S3. (The epoxides were not available for testing against MCF-7 cells).

Table 3.4. Effects of 17α-epoxyethano compounds on cell growth

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (µM)</th>
<th>HeLa</th>
<th>GH3</th>
</tr>
</thead>
<tbody>
<tr>
<td>21a</td>
<td>6.5±1.9</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>21b</td>
<td>18.6±2.0</td>
<td>17.7</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.17. Effect of 21a (●) and 21b (▲) on HeLa S3 growth. Means of 2 or 4 determinations. Inset shows initial study of effect of 21a and 21b on HeLa S3 growth. Means of duplicate determinations.
Figure 3.18. Effect of 21a (△) and 21b (○) on GH<sub>3</sub> growth. Means of duplicate experiments.
3.3 Receptor Binding Studies

3.3.1. D-ring α-methylene-γ-lactone oestrogens

The series of D-ring compounds 4, 5 and 6 display negligible receptor binding to rat uterine cytosol at 2.5μM, (Figure 3.19). To exclude reaction with cytosolic thiols, receptor binding of the compounds was reassayed in cytosol treated with bromoacetone dimethylacetal to remove free thiols. Treatment with bromoacetone dimethylacetal reduces specific binding in cytosols by only 15% (Figure 3.20). Further, receptor binding of DES is substantially unaffected, (Figure 3.21). However receptor binding of 4, 5 and 6 does not increase in treated cytosol, (Figure 3.22).

Receptor binding of the parent lactones 7, 8, 9 (estrolactone) and estrolic acid 2 was also assayed, (Figure 3.23). All of these compounds have a low affinity for uterine oestrogen receptors.

Receptor binding of estrolactone, and estrolic acid was assayed in GH3 cytosol, (Figure 3.24). Both compounds exhibit similar lack of affinity binding in GH3 and rat uterine cytosol.

It was of interest to note that one of the two unsaturated A-ring compounds 16 exhibited affinity for the oestrogen receptor, (Table 3.5) despite lacking a phenolic A-ring.

The enzyme pyruvate kinase, with an allosteric binding site for oestrogens, was used as an alternative oestrogen-sensitive system. The activity of this enzyme is inhibited by oestrogens.
Figure 3.19. Displacement of $[^{3}\text{H}]-\text{oestradiol}$ (7nM) by 4, 5 and 6 (2.5µM) from normal uterine cytosols. Means of 4 determinations ± SEM
Figure 3.20. Specific binding of $[^3H]$-oestradiol in rat uterine cytosols. 1 = control, 2 = cytosol in buffer minus dithiothreitol, 3 = cytosol in buffer minus dithiothreitol plus bromoacetone. Means of 3 determinations ± SEM.
Figure 3.21. Displacement of $[{}^3\text{H}]$-oestradiol by diethylstilboestrol from normal (•) and bromoacetone dimethylacetal treated (▲) cytosols. Means of 6 determinations ± SEM. Standard errors less than symbol size.
Figure 3.22. Displacement of $[^3H]$-oestradiol by 4, 5 and 6 from normal (open bars) and bromoacetone dimethylacetal treated (hatched bars) uterine cytosols. Means of 6 determinations ± SEM. Drug concentration 2.5 μM.
Figure 3.23. Displacement of $[^{3}\text{H}]$-oestradiol by estrolic acid (2), estrolactone (9), 7 and 8 from uterine cytosols. Means of 3 determinations ± SEM. Drug concentration 2.5 μM.
Figure 3.24. Displacement of $[^3\text{H}]$-oestradiol by estrolic acid (2) and estrolactone (9) from uterine (open bars) and GH3 (hatched bars) cytosols. Means of 3 determinations ± SEM. Drug concentration 2.5 µM.
Table 3.5. Displacement of $[^3H]$-oestradiol from rat uterine cytosol

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (nM)$^a$</th>
<th>% Oestradiol bound$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol</td>
<td>10.6±0.6</td>
<td></td>
</tr>
<tr>
<td>DES</td>
<td>5.6±0.1</td>
<td></td>
</tr>
<tr>
<td>DES</td>
<td>4.1±0.2$^c$</td>
<td></td>
</tr>
<tr>
<td>Estramustine 1 (Estrolactone)</td>
<td>57±8</td>
<td></td>
</tr>
<tr>
<td>Estramustine 2 (Estrolic acid)</td>
<td>66±3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>88±7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>86±3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>99±3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>100±0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>95±4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>100±0</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>7.3±6</td>
<td></td>
</tr>
<tr>
<td>16a</td>
<td>100±0</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>100±0</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>265±27$^d$</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3400±100</td>
<td></td>
</tr>
<tr>
<td>21a</td>
<td>79±11</td>
<td></td>
</tr>
<tr>
<td>21b</td>
<td>724±89</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ ED$_{50}$ values are means (3 or more determinations) ± SEM.

$^b$ Values are % $[^3H]$-oestradiol bound at 2.5µM, 3 or more determinations ± SEM.

$^c$ In bromoacetone dimethylacetal treated cytosol.

$^d$ Subsequent assays showed lower affinity, (Figure 3.28).
The inhibition recorded for DES (Figure 3.25) compares well with the literature values. [122] Compounds 4, 5 and 6 have no effect on pyruvate kinase activity at concentrations up to 100μM, confirming their lack of oestrogenic activity.

3.3.2. 16-(α-methylene-γ-lactone) oestrogens

These compounds did displace [3H]oestradiol binding in rat uterine cytosols. Compound 18 was found to have considerably greater affinity for the uterine oestrogen receptors than 20 in initial studies, as shown in Figure 3.26.

Further samples of 18, produced by crystallisation from the same mother liquor, showed much reduced receptor binding activity, (Figure 3.27).

3.3.3. 17α-epoxyethano oestrogens

These compounds were relatively potent in inhibiting the binding of [3H]-oestradiol to cytosols, though considerable difference was seen between the isomers 21a and 21b. Thus 21a (ED50 = 79±11nM) is approximately ten times more active in the receptor binding assay than 21b (ED50 = 724±89nM), (Figure 3.28).

A comparison of the relative activity of all the compounds examined is given in Table 3.5.

3.3.4. Irreversible Binding

The extent to which the various derivatives irreversibly bind to the oestrogen receptor is given in Figure 3.29. The affinity
Figure 3.25. Effect of diethylstilboestrol on pyruvate kinase activity. Single determinations.
Figure 3.26. Displacement of $[^3H]$-oestradiol by 18 (●) and 20 (○) from uterine cytosols. Means of 4 determinations ± SEM.
Figure 3.27. Displacement of $[^3\text{H}]$-oestradiol by different samples of 13 from uterine cytosols, means of 3 determinations ± SEM. Drug concentration 2.5 $\mu$M.
Figure 3.28. Displacement of $[^3H]$-oestradiol by 21a (•) and 21b (○) from uterine cytosols. Means of 3 determinations ± SEM.
Figure 3.29. Irreversible binding of ORG 4333 (30μM), 18 (15μM), 20 (15μM), 21a (2.5μM), 21b (15μM). Means of 3 determinations ± SEM.
labelling reagent ORG 4333 has an activity in this system close to reported values. [123] The rank order of irreversible binding of compounds under investigation is $2_{1a} > 18 > 20 > 2_{1b}$. This is similar to the order of binding affinity, namely $2_{1a} > 18 > 2_{1b} > 20$. 
3.4. Mechanisms of Action of Alkylation Agents

3.4.1. Effect on thiol levels

(a) Electrophilic activity - reaction with glutathione

Vernolepin is the most reactive of the compounds tested, followed by elephantopin, (Figure 3.30). Similar results have been reported for reaction of these compounds with cysteine. [37] The order of reactivity of the D-ring α-methylene-δ-lactones, 4, 5 and 6, follows their cytotoxicity, 5, 4 > 6, (Figure 3.31).

Estramustine, the 16-(α-methylene-γ-lactone) oestrogens 18, 19 and 20 and the 17-epoxyethano-oestrogens 21a and 21b reacted slowly or not at all with glutathione under the assay conditions, (Figures 3.32 and 3.33)

(b) Addition of Glutathione to media

Glutathione at 1mM reduces the effect of compound 4 on cell growth, and prevents cell death at higher doses of 4 (Figure 3.34). These effects are seen only at relatively high concentrations of glutathione, i.e. at a thousand fold excess over 4.

(c) Effects of compounds on cellular thiol levels

Over 24 hours vernolepin rapidly depletes HeLa S3 thiols to an equilibrium level of approximately 70% of control for surface and 55% for intracellular thiols, (Figure 3.35). However, observed over a shorter time scale this effect is apparently more complex. An initial sharp drop in thiol levels is followed by a recovery
Figure 3.30. Time course for reaction of vernolepin (•) and elephantopin (△) with glutathione, control (○). Means of 3 determinations 1.0 μ moles drug in 3 ml. Standard errors less than symbol size.
Figure 3.31. Time course for reaction of D-ring steroidal α-methylene lactones 4 (●), 5 (▲) and 6 (▲) with glutathione, control (○).
Means of 3 determinations. 1.0 μ moles drug in 3 ml.
Standard errors less than symbol size.
Figure 3.32. Time course for reaction of estramustine (A) and 16-(α-methylene-γ-lactone)-substituted oestrogen 15 (●) with glutathione, control (○) 19 and 20 produced virtually identical results to 18. Means of 2 determinations 1.0 μ moles drug in 3 ml.
Figure 3.33. Time course for reaction of 17a-epoxyetheno-oestrogens 21a (●) and 21b (▲) with glutathione, control (○). Means of 2 determinations. 1.0 µ moles drug in 3 ml.
Figure 3.34. Effect of glutathione (1 mM, hatched bars) on inhibition of HeLa S3 growth by 4 at 0.8μM and 1.0μM. Means of 3 determinations ± SEM.
Figure 3.35. Effect of vernolepin (20μM) on cell surface (●) and intracellular (●) thiol levels. Means of 3 determinations ± SEM.
Figure 3.36. Effect of vernolepin (20μM) on cell surface (▲) and intracellular (●) thiol levels. Means of 3 determinations ± SEM.
period to reach the equilibrium level, (Figure 3.36). A similar effect has been observed with vernolepin and elephantopin on P815 leukaemia cells. [124]

An incubation period of 4 hours to reach equilibrium was used with compounds at 10, 20 and 50μM, (Table 3.6). Ability to deplete thiol levels followed the same order as cytotoxicity and electrophilic activity with glutathione, and was dose-dependent.

Inhibition of HeLa S3 growth by α-methylene lactone compounds occurs over an equally short period. Thus 4 reduces growth below control levels after 3 hours exposure, (Figure 3.37). Growth rate is retarded even when cells have been exposed to 4 for short periods, followed by its removal, (Figure 3.38).

3.4.2. Effects on Na⁺/K⁺ ATPase

The dependence of ⁸⁶Rb uptake on Na⁺/K⁺ ATPase activity is demonstrated by ouabain inhibition, (Figure 3.39). Accumulation of ⁸⁶Rb⁺ reaches equilibrium after approximately 3 hours, (Figure 3.39).

An incubation period of 3 hours was taken, using varying concentrations of drugs, to produce dose/response curves for inhibition of ⁸⁶Rb⁺ uptake. The sesquiterpene α-methylene lactone vernolepin was active in preventing ⁸⁶Rb⁺ accumulation, reaching a maximum effect after approximately 30 minutes, (Figure 3.40). Indeed, vernolepin and the steroidal α-methylene-δ-lactone 4 were most active in this system, 4 being approximately three times as active as vernolepin, (Figure 3.41). The parent lactone 7 is relatively inactive affording only 25% reduction in uptake at a concentration of 100μM, (Figure 3.42).
Table 3.6. Reduction of surface (S) and intracellular (I) thiol levels in HeLa S3 cells after 4 hrs exposure to \(\alpha\)-methylene lactones.

% Control Values

<table>
<thead>
<tr>
<th>Compound</th>
<th>10(\mu)M</th>
<th></th>
<th>20(\mu)M</th>
<th></th>
<th>50(\mu)M</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S I</td>
<td>S I</td>
<td>S I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vernolepin</td>
<td>73±4 85±3</td>
<td>59±2 62±2</td>
<td>48±2 38±3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estramustine</td>
<td>85±1 70±10</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>4</td>
<td>76±5 59±7</td>
<td>59±2 63±2</td>
<td>47±2 23±3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>- -</td>
<td>94±5 81±3</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>15</td>
<td>- -</td>
<td>94±5 85±3</td>
<td>112±4 127±1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>- -</td>
<td>85±3 90±2</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>20</td>
<td>- -</td>
<td>68±3 90±3</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>21a</td>
<td>- -</td>
<td>81±5 87±1</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
</tbody>
</table>

Dashes represent not tested.
Figure 3.38. Effect of short exposure to compound 4 (0.8 and 0.96 μM), followed by removal, on growth of HeLa S3 cells. Means of duplicate results.
Figure 3.39. Uptake of $^{86}\text{Rb}^+$ by HeLa S3 cells, in the absence (*) and presence (o) of ouabain (0.6 mM). Means of 3 determinations ± SEM.
Figure 3.40. Uptake of $^{86}$Rb$^+$ by HeLa S3 cells, in the absence (•) and presence (○) of vernolepin (100μM). Means of 3 determinations ± SEM.
Figure 3.41. Effect of vernolepin (■) and 4 (○) on $^{86}\text{Rb}^+$ uptake by HeLa S3 cells. Means of 3 determinations ± SEM.
Figure 3.42. Comparison of effect of 4 (*) and its parent lactone 7 (△) on $^{86}\text{Rb}^+$ uptake by HeLa S3 cells. Means of 3 determinations ± SEM.
Figure 3.43. Effect of 21a (○) and estramustine (●) on $^{86}\text{Rb}^+$ uptake by HeLa S3 cells. Means of 3 determinations ± SEM.
Estramustine and the epoxide compound 21a have much lower activity than vernolepin and 4, (Figure 3.43). ED$_{50}$ values for the compounds are given in Table 3.7.

Table 3.7. ED$_{50}$ values for inhibition of $^{86}$Rb$^+$ uptake by HeLa S3 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vernolepin</td>
<td>40.3±4.4</td>
</tr>
<tr>
<td>Estramustine</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4</td>
<td>12.2±0.2</td>
</tr>
<tr>
<td>7</td>
<td>&gt;100</td>
</tr>
<tr>
<td>21a</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Inhibition of the isolated enzyme by vernolepin follows a time-course similar to the effect on $^{86}$Rb$^+$ uptake, (Figure 3.44). A one hour pre-incubation with drugs was used to determine the relative activities of compounds, given in Table 3.8.
Figure 3.44. Effect of vernolepin (100μM) on Na\(^+\)/K\(^+\) ATPase activity with time. Means of 3 determinations ± SEM.
Table 3.8. Inhibition\(^a\) of Na\(^+\)/K\(^+\) ATPase by compounds at 100μM.
(1 hour pre-incubation).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ouabain Sensitive</th>
<th>Ouabain Insensitive</th>
<th>HeLa S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vernolepin</td>
<td>24±2</td>
<td>22±5</td>
<td>33±5</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>30</td>
<td>56±3</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>-</td>
<td>-</td>
<td>62</td>
</tr>
</tbody>
</table>

\(^a\) % Inhibition values are means of 3 (± s.e.m.) or two determinations. Dashes represent not tested.

3.4.3. Uptake and incorporation into macromolecules of [\(^3\)H]-Thymidine by HeLa S3 cells.

Inhibition of [\(^3\)H]-Thymidine uptake by elephantopin reaches a maximum within 30 minutes, (Figure 3.45) in accord with literature reports. \[^{43}\] The inhibition by compound 4 follows a similar time course, (Figure 3.46). Incubation periods of 30 minutes with drug were used to produce dose/inhibition curves of [\(^3\)H]-Thymidine uptake.

The inhibition observed with elephantopin is similar to that recorded elsewhere, (Figure 3.47).\[^{43}\] Estramustine is relatively inactive, (Figure 3.48). Compound 4 is the most active of the
Figure 3.45. Uptake of $[^3H]$-Thymidine by HeLa S3 cells in the absence (●) and presence (▲) of elephantopin (100μM). Means of duplicate results.
Figure 3.46. Uptake of $[^3\text{H}]$-Thymidine by HeLa S3 cells in the absence (•) and presence (▲) of 4 (100μM). Means of duplicate results.
Figure 3.47. Effect of elephantopin on [3H]-Thymidine uptake by HeLa S3 cells. Means of 3 determinations ± SEM.
Figure 3.48. Effect of estramustine on $[^3H]$-Thymidine uptake by HeLa S3 cells. Means of 3 determinations ± SEM.
alkylating compounds tested, with approximately ten-fold the activity of elephantopin, (Figure 3.49). While the thiophenol adduct of 4, compound 15 is also considerably more active than elephantopin, (Figure 3.50).

Testing of the lactone 7, to eliminate the possibility of inhibition due to the steroid nucleus itself, revealed activity similar to that of 15, (Figure 3.51). The activities of oestradiol and diethylstilboestrol, (Figure 3.52) confirmed this finding. Unrelated compounds such as benzocaine, dexamethazone and progesterone were also found to have activity in this system, (Figure 3.53, 3.54 and 3.55). The time course of inhibition by dexamethasone is similar to that of elephantopin and 4, (Figure 3.56).

IC₅₀ values for the compounds are given below in Table 3.9.

Table 3.9. IC₅₀ values for inhibition of [³H]-Thymidine uptake.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elephantopin</td>
<td>39.1±2.3</td>
</tr>
<tr>
<td>Estramustine</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Dexamethazone</td>
<td>25.4±5.8</td>
</tr>
<tr>
<td>Progesterone</td>
<td>6.6ᵇ</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>5.6±0.3</td>
</tr>
<tr>
<td>D.E.S.</td>
<td>3.3±0.2</td>
</tr>
<tr>
<td>Benzocaine</td>
<td>7.5ᵇ</td>
</tr>
<tr>
<td>4</td>
<td>4.0±1.0</td>
</tr>
<tr>
<td>7</td>
<td>14.1±4.0</td>
</tr>
<tr>
<td>15</td>
<td>17.3±1.0</td>
</tr>
</tbody>
</table>

ᵃ Values are means (4 or more determinations) ± SEM.ᵇ Figures are means of 2 determinations. D.E.S. = diethylstilboestrol
Figure 3.49. Effect of 4 on $[^3]H$-Thymidine uptake by HeLa S3 cells. Means of 6 determinations ± SEM.
Figure 3.50. Effect of 15 on [3H]-Thymidine uptake by HeLa S3 cells.

Means of 6 determinations ± SEM.
Figure 3.51. Effect of $Z$ on $[^3H]$-Thymidine uptake by HeLa S3 cells.

Means of 6 determinations ± SEM.
Figure 3.52. Effect of oestradiol and diethylstilboestrol on [3H]-Thymidine uptake by HeLa S3 cells. Means of 3 determinations ± SEM.
Figure 3.53. Effect of benzocaine on $[^3H]$-Thymidine uptake by HeLa S3 cells. Single determinations.
Figure 3.54. Effect of dexamethasone on $[^{3}H]$-Thymidine uptake.

Means of 6 determinations ± SEM.
Figure 3.55. Effect of progesterone on [3H]-Thymidine uptake. Single determinations.
Figure 3.56. Uptake of $[^3H]$-Thymidine by HeLa S3 cells in the absence (△) and presence (○) of dexamethasone (10μM). Values represent ± SEM. n = 3.
Inhibition of $[^3\text{H}]$-thymidine incorporation was apparent only at high concentrations of elephantopin and 4, (Figures 3.57 and 3.58). Dexamethazone did not inhibit $[^3\text{H}]$-thymidine incorporation at doses up to 200μM. This compound is reported to inhibit incorporation at concentrations below 1μM, using a different assay system, however. [125]

IC$_{50}$ values for inhibition of $[^3\text{H}]$-thymidine incorporation into macromolecules are given below in Table 3.10.

Table 3.10. IC$_{50}$ values for inhibition of $[^3\text{H}]$-thymidine incorporation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (μM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elephantopin</td>
<td>67.7±2.</td>
</tr>
<tr>
<td>4</td>
<td>77.7±7</td>
</tr>
<tr>
<td>Dexamethazone</td>
<td>&gt;&gt;200</td>
</tr>
</tbody>
</table>

$^a$ Values are means (3 or more determinations) ± SEM.

3.4.4. Inhibition of mitochondrial respirations and effects on lysosomal membrane stability.

Elephantopin and 4 inhibit state 3 respiration of rat liver homogenates, at 100μM, (Table 3.11). State 4 respiration is largely unaffected at this dose. Oestradiol causes inhibition of approximately the same degree.

Pre-incubation of homogenate with Elephantopin and 4 for 2 hours at 4°C did not greatly increase the extent of inhibition, (Table 3.11).
Figure 3.57. Effect of elephantopin on [$^3$H]-Thymidine incorporation into acid insoluble material by HeLa S3 cells. Means of determinations ± SEM. n = 4.
Figure 3.58. Effect of 4 on $[^3H]$-Thymidine incorporation into acid insoluble material by HeLa S3 cells. Means of 4 determinations ± SEM.
Table 3.11. **Inhibition of rat liver mitochondrial respiration by compounds at 100μM**.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Control</th>
<th>State 4</th>
<th>State 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elephantopin</td>
<td>95.9±7.5 (102.8±0.5)</td>
<td>71.9±6.2 (60.6±5.2)</td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>101.2±4.4</td>
<td></td>
<td>75.2±1.6</td>
</tr>
<tr>
<td>4</td>
<td>111.9±7.5 (80.8±9.0)</td>
<td>65.9±1.2 (56.2±1.0)</td>
<td></td>
</tr>
</tbody>
</table>

\[a\] values represent means (3 determinations) ± SEM. Figures in parentheses are results obtained after 2 hour pre-incubation periods at 4°C. Control values for oxygen consumption were 45.5±2.0, and 210.7±8.0 n atoms O minute⁻¹ for state 4 and state 3 respiration respectively.
Cells treated with 4, examined by scanning and transmission electron microscopy, exhibit contraction of cytoplasm, as shown in Figures 3.59a and 3.59b. Compound 4 produces progressive vacuolation and disintegration of the cytoplasm, while the nucleus appears relatively intact, (Figures 3.60 a-d). Production of vacuoles was apparent in all cells treated with 4, but not those treated with elephantopin. The possibility that 4 destabilises lysosomal membranes, releasing hydrolase enzymes was investigated using rat liver microsomal preparations.

Compound 4 destabilises rat liver lysosomes to a considerable extent, (Figure 3.61). The lactone 7 in contrast, stabilises the lysosomal preparation. The sesquiterpene compounds, elephantopin and vernolepin stabilise lysosomal membranes, in line with previous reports. [59]

Compound 4 does not affect the activity of the lysosomal marker enzyme aryl sulphatase, (Figure 3.62). Elephantopin and vernolepin inhibit this enzyme slightly as previously reported, as does the lactone 7.

3.4.5 Cytotoxicity Towards Microorganisms

Estramustine, elephantopin and the \(\alpha\)-methylene lactone 4 did not inhibit the growth of *Escherichia coli*, *Penicillium crysogenum* or *Candida albicans* at concentrations up to 50\(\mu\)g.ml\(^{-1}\) (i.e. \(>100\mu\)M). These results are in accord with those reported for sesquiterpene compounds against *Staphylococcus aureus* and *Bacillus subtilis* where minimal inhibitory concentration values of 25-100\(\mu\)g.ml\(^{-1}\) were recorded. [61]
Figure 3.59. Scanning electron micrographs of untreated cells (a), and cells treated with 4 at 1.0 µM for 24 hours (b).
Figure 3.60 a-d. Transmission electron micrographs of cells from the same sample treated with 4 (1.0 μM) for 24 hours.
Figure 3.60 continued.
Figure 3.61. Effect of steroidal and sesquiterpene α-methylene lactones on release of the lysosomal enzyme aryl sulphatase. Results are expressed as percentage values of enzyme released from triton X-100 treated liver cytosol. Means of 6 determinations ± SEM. El = elephantopin, Ver = vernolepin. Compounds at 100 μM.
Figure 3.62. Effect of steroidal and sesquiterpene lactones on aryl sulphatase activity. Results expressed as percentage of control activity in triton X-100 treated liver cytosols. Means of 3 determinations ± SEM. El = elephantopin, Ver = vernolepin. Compounds at 100 μM.
4. DISCUSSION
4.1 Steroidal Alkylating Agents Selectively Toxic to Oestrogen-Responsive Tumours: The Assay Systems

The assay procedures employed were designed to detect compounds which exhibit selectivity of action towards oestrogen responsive tumours. Initially, cytotoxicity was determined using the HeLa S3 cell line. Selectivity of action was assessed from two in vitro assays; 1) Receptor binding to oestrogen receptors in the rat uterine cytosols, and 2) Cytotoxicity against cell-lines having oestrogen receptors.

4.1.1 Cytotoxicity:

HeLa S3 cells were used to assess cytotoxicity, as the biochemistry of this cell line has been studied extensively. [126,127] The cells are susceptible to the α-methylene lactone containing elephantopin and vernolepin and indeed have previously been used to test the cytotoxicity of sesquiterpene α-methylene lactones. [43] The Cancer Chemotherapy National Service Center (CCNSC) protocol to assess cytotoxicity was followed. The drug contact time, of 48 hours, is approximately twice the cell cycle time of HeLa S3 cells. Any drug effect specific for a particular phase of the cell-cycle will thus be allowed expression.

The HeLa S3 system should only detect general cytotoxicity, not potential selectivity. Results indicate that HeLa S3 cells neither contain oestrogen receptors, nor respond to oestrogens in the growth
medium, confirming previous work. The HeLa S3 line has been reported to contain glucocorticoid receptors, but not oestrogen receptors. [125]

Some use of the MCF-7 (human breast cancer) and GH3 (rat pituitary tumour), oestrogen receptor bearing cell lines was made. These cells allow the study of oestrogens and anti-oestrogens. The MCF-7 cell line is one of the best in vitro model systems of an oestrogen-responsive mammary carcinoma presently available. [105,128] This cell line is employed by a number of groups investigating cytotoxic-linked oestrogens. [93,129] Oestrogen receptors have been identified and characterised, and importantly the receptor mechanism is functional. [128] Growth of MCF-7 cells is reported to be stimulated by the presence of oestrogens but inhibited by anti-oestrogens. [105,118] Progesterone receptors have also been identified and are oestrogen inducible. [105,128]

However, the MCF-7 line contains human RNA tumour virus particles. [130] This meant it could not be routinely used in our laboratories. Some experiments were performed by the author during a visit to I.C.I. Pharmaceuticals Divisions, Alderley Park. A modified assay, using greater numbers of cells in the initial innoculum was employed. This resulted in higher IC₅₀ values, thus the IC₅₀ for elephantopin against MCF-7 was approximately 5-10 x that obtained against HeLa S3. The effect of changes in innoculum number of cells was confirmed using HeLa S3 cells. From the result it is apparent that initial cell number in the assay has a critical effect on the IC₅₀ value.
Increasing cell numbers produces a corresponding increase in the level of drug required for cytostatic activity. Account of this effect must therefore be taken in evaluating the results of the MCF-7 assay.

As an alternative the GH3 line was used. Growth of these cells was inhibited by elephantopin and vernolepin with the same potency as shown towards HeLa S3. However, assays using GH3 cells employed a longer drug contact time, taking account of their slower growth rate. Like the MCF-7 cell the GH3 cell contains functional oestrogen receptors as evidenced by the oestrogen-induced synthesis of prolactin and of progesterone receptors. [109] However, unlike MCF-7 cells growth of this line is not stimulated by oestrogens. [109] On the other hand GH3 cells will concentrate oestradiol from medium supplemented with the $[^{3}H]$-labelled hormone. [77]

Although the majority of the cytotoxicity studies were performed on HeLa S3 cells, use was made of the oestrogen receptor containing lines. This gave a cross section of in vitro model systems. If any selectivity is evident it might be expected to show up as differing activities towards the various cell types.

4.1.2 Oestrogen receptor binding studies

Affinity for oestrogen receptors in rat uterine cytosols was used as an indirect indicator of specificity in the oestrogen-linked compounds. The assay is widely used as a model system in
evaluating the potential specificity of cytotoxic-linked oestrogens. [93,101] Comparison with test drugs and standard oestrogens is therefore possible.

Relatively small amounts of test compound are necessary in this assay. In contrast testing in animal systems, such as DMBA rat tumours [131], requires quantities in the gramme range. It has the further advantage that quantities of receptor containing cytosol can be prepared easily. As an alternative and for comparison, receptor containing cytosols from cell cultures were also used. However, this requires production of large numbers of cells and for this reason use of GH3 cell cytosols was limited. Receptor binding in cytosols from GH3 was assessed to determine if the specificity of the receptors from pituitary cells was equivalent to rat uterine receptors.

One disadvantage of the receptor-binding system is that it does not distinguish between oestrogenic and anti-oestrogenic compounds. [132] Any anti-oestrogenic properties of oestrogen-linked drugs will not therefore be detected from this assay alone. On the other hand the test system does allow determination of the potential of the compounds for alkylating the oestrogen receptor. This technique is valuable for three reasons;

1) It gives an indication of alkylating activity against cellular nucleophiles other than the 'model' thiol compounds, glutathione and cysteine.
2) Information may be gained on the structure of the receptor binding site.

3) It may provide information on the mode of action of the compounds on receptor bearing cells.
4.2 Cytotoxicity and Potential Selectivity of Steroidal Alkylating Agents

4.2.1 D-ring steroidal α-methylene δ-lactones

The synthesis of the D-ring lactone, estrolactone 1 and its open ring form estrolic acid 2 was first described by Westerfeld. [133] The biological properties of the two compounds were studied in the course of research on the metabolism of oestrone. [134] The lactone 1 is reported to have an oestrogenic potency one-sixteenth, and the acid 2 one-fifteenth, of the parent oestrone, as determined by the Allen/Doisy test. [134,135] The compounds apparently stimulate pituitary hormone secretion to a greater degree than can be accounted for by oestrogenic activity. [134,135]

The activity of these compounds as oestrogens prompted us to synthesise and study α-methylene-δ-lactone derivatives of the parent lactone, [136] as potential cytotoxic oestrogens. Since estrolactone 1 is oestrogenic, albeit mildly, the α-methylene
derivatives might be reasonably expected to have some affinity for oestrogen receptors, and hence be selectively toxic towards receptor-bearing cells. A series of these compounds of general structure 3 were synthesized in the Organic Chemistry Laboratory of this department by L. Chagonda. [137]

![Chemical Structure 3](image)

It is apparent from the effects of the compounds on HeLa S3 growth that the \( \alpha \)-methylene-lactone function plays an integral role in their cytotoxic activity. Thus the compounds 4, 5, and 6 with activity in the 1 \( \mu \)M range have greatly increased activity over

![Chemical Structures 4-10](image)
their parent lactones 7, 8 and 9 which require in excess of 20 μM for activity. Furthermore compound 10, the α-methyl analogue of 6, has one-nineteenth the activity of the α-methylene compound 4. The necessity of the α-methylene lactone group in cytotoxic activity is well documented. [37] Thus of two A-ring cholesterol derivatives (Fig. 4.1) only b is cytotoxic. [87] Sesquiterpene compounds show a similar dependence on the presence of an α-methylene lactone function for cytotoxicity. [37] Reduction of elephantopin to tetrahydroelephantopin or hexahydroelephantopin, results in loss of activity. [37]
The importance of the α-methylene lactone group is further underlined by the markedly reduced activity observed in those compounds in which the methylene group is substituted. Compounds 11, 12, 13, and 14 are only weakly cytotoxic relative to the unsubstituted compounds 4, 5, and 6. Steric and/or electronic effects may be responsible for the lack of activity of the
substituted compounds. This effect has also been observed in a series of alkoxy-substituted α-methylene-γ-lactones. [46]

An apparent anomaly is the thiophenol adduct, 15. This compound exhibits considerable cytotoxic activity, having approximately half the activity of the parent compound 4. The reasons for this are obscure. The compound may have activity in its own right. Alternatively it may undergo metabolic conversion in the cells to regenerate the free methylene function. This conversion might be possible by sulphoxidation and elimination or S-dealkylation followed by elimination, [138] such as occurs with compounds like azathioprin. [138] Enzyme systems capable of both conversions have been characterised. [138] Regeneration of the α-methylene group may release thiophenol, which is itself cytotoxic. [139] Another possibility is that the thiophenol adduct might undergo an exchange reaction with a more nucleophilic thiol group, such as might be found in the active site of enzymes. [140] Exchange reactions with essential thiols would have the same end result as the Michael addition in alkylating these thiols.
The IC₅₀ values obtained for vernolepin and elephantopin on HeLa S3 cells agree closely with literature values for activities on these and other cell lines. [37,43] The steroidal compounds tested have activities in the same range as these standard compounds but are more active than the drug estramustine which is used in treatment of prostate cancer [141]

The steroidal compounds seem sufficiently cytotoxic to exhibit anti-tumour activity and therefore fulfil one of the criteria necessary for a useful anti-cancer agent.

On the other hand, oestrogen-receptor binding results with the D-rin compounds proved disappointing. The most cytotoxic compound, the 3-acetoxy derivative 5, had an affinity for the oestrogen receptor approximately one-thousand times less than that of the synthetic oestrogen, DES. DES was found to have an ED₅₀ of approximately 5 nM in displacing [³H]-oestradiol from the receptor, in agreement with other reports. [120,121]
Poor receptor affinity in 4 and 5 was not unexpected since a 3-hydroxyl function is believed to be necessary for binding. [142,143] The poor binding observed with the 3-hydroxy compound, 6, was unexpected. It was felt that the poor binding properties could be due to reaction of the α-methylene function with thiols present in the cytosol, thereby preventing access to the binding sites. The uterine cytosols were found to contain 0.2 mM thiols in addition to the dithiothreitol added to prevent their oxidation. This is in the same range as that determined by Katzenellenbogen. [101] To test whether cytosolic thiols were interfering, cytosols were treated with bromoacetone-dimethylacetal as described by Katzenellenbogen. [101] This removed measurable thiols but reduced specific binding by only 15%. In addition, the affinity of DES for the receptor was unaffected (ED₅₀ in treated cytosol 4.0 nM, compared to 5.5 nM in untreated cytosols).

Treatment of cytosols with bromoacetone-dimethylacetal did not significantly improve the receptor binding of the 3-hydroxy-analogue 6. The lack of binding observed for this compound must therefore be caused by the D-ring lactone structure. This view was supported by experiments which demonstrated the poor receptor binding of estrolactone 1 and estrolic acid 2, although as discussed previously these compounds are oestrogenic.

Estrolic acid 2 has greater oestrogenic potency than the lactone 1. [132,133] However, this difference in oestrogenic potency is not due to enhanced receptor affinity, c.f. Figure 3.24. Of course the binding
assay only measures affinity for the receptor and not efficacy. Possibly the difference is due to the ability of the estrolic acid-receptor complex to induce an oestrogenic response in target cells. The greater effect observed on pituitary secretion of estrolic acid and estrolactone is apparently not caused by preferential binding to pituitary receptors. As evidenced from binding studies to GH3 cell receptors.

The relatively poor binding affinity of the D-ring compounds is reflected in their lack of selectivity against oestrogen receptor bearing cell lines. The IC50 values for cytostatic activity against the oestrogen receptor bearing GH3 cells are in the same range as those for the HeLa S3 line. The compounds also have comparable activities relative to each other on GH3 cells indicating a common mode of action on the two cell lines.

A similar result was observed with the MCF-7, mammary carcinoma cell line. The steroid compounds showed no better activity than the unselective elephantopin. Dose/response curves against the MCF-7 cells were shifted to the right relative to those for HeLa S3 and GH3 cells. However, this was as a result of the slightly different assay protocol used, i.e. increased numbers of cells.

Three types of interaction with the receptor are involved in oestrogen binding: [142]

1) Hydrogen bonding with the terminal oxygen groups.
2) π-complex formation with the aromatic A-ring.
3) Hydrophobic interactions with the steroid skeleton, which are believed to be the most influential on receptor binding.

The low receptor affinity observed for the D-ring lactone compounds is probably as a result of the disruption of these interactions. In order to progress with this series of compounds we need to know how closely they fit the criteria for oestrogen receptor activation.

A very important property of oestrogenic molecules is the distance between the terminal oxygens. In DES the terminal oxygens are 12.1Å apart. DES has a slightly higher affinity for the oestrogen receptor than oestradiol in which the oxygens are 10.9Å apart. The 17 OH of oestradiol is believed to hydrogen bond a molecule of water during receptor binding. [144] This increases the distance between terminal oxygens from 10.9 to 12.1Å, as for DES. The distance between the terminal oxygens of the D-ring compounds is similar to that for DES. (Figure 4.2)

It is thus apparent that simple intramolecular distance between terminal oxygen groups is not sufficient to provide compounds recognisable to the receptor. Other factors must be considered. Thus the carbonyl oxygens of D-ring lactones cannot hydrogen bond to the same extent as a hydroxyl group, since they act only as hydrogen acceptors. This is shown also with oestrone, which binds to a lesser degree than oestradiol. [143] The orientation of the terminal oxygen functions is important. [145,146] 17α-oestradiol has only one tenth
Figure 4.2. Molecular models of estrolactone and diethylstilboestrol (DES) showing similarities between intramolecular distance and positioning of terminal oxygens.
the binding affinity of the 17 β-isomer. [142] Reduced hydrogen bonding at the 3-position would also occur in the 3-substituted compounds 4 and 5 and their corresponding lactones 7 and 8. The importance of a free 3-OH is confirmed by the weak binding of 3-methoxyoestradiol compared with oestradiol itself. [143]

Hydrogen bonding of the 3-hydroxyl grouping in 6, estrolactone and estrolic acid may also be affected by their D-ring structures. The 3-hydroxyl of oestrone is more acidic than that of oestradiol by 0.10 pK units. [144] This is believed to be caused by the long range electrophilic effect of the 17-keto group. [144] As a result the 3-hydroxyl of oestrone acts only as a hydrogen donor, while that of oestradiol acts as both donor and acceptor. [144]

Obviously changes in the overall conformation of the steroid will affect the relative positions of the oxygens, and so affect their binding. Similarly effects on hydrophobic bonding and π-complex formation will occur when there is a change in the molecular conformation. Introduction of the lactone group into the D-ring may cause an alteration in this overall conformation of the steroid via conformational transmission. [144,145] In the two natural oestrogens oestradiol and oestrone, the commonest form has the B-ring in the 7α, 8β-half chair conformation. [144] In this structure the C(9), C(10), C(5) and C(6) atoms are in the same plane. [144] The overall effect is to produce a molecule which is almost planar between the A and B rings, [144,146]. Figure 4.3. Disruption of this conformation may reduce both hydrogen bonding of the 3-hydroxyl and π-complex formation.
Figure 4.3. Bay regions of oestradiol where substitution may alter conformation, and the conformation of the oestradiol molecule.

with the A ring, as well as hydrophobic interactions with the other rings. Exocyclic interactions between rings, particularly in the A/C and B/D bay regions, profoundly affects the overall steroid conformation. [144,145]

It is worth noting that of the D-ring structures tested, only one compound, 16 displayed receptor affinity, displacing 100% of

$[^3]$H oestradiol at 2.5 μM. This compound lacked an exocyclic α,β-unsaturated group and was therefore non-toxic against HeLa S3.
Furthermore it had no demonstrable oestrogenic properties when assayed on MCF-7 cells. Over the range of doses used it neither inhibited nor stimulated MCF-7 growth, in medium devoid of oestrogen. The position of the phenyl ring would seem important for receptor affinity since the unsaturated derivative $^{17}$ displayed no receptor binding activity at 2.5 $\mu$M. This compound was also inactive in cytotoxicity assays against HeLa S3 cells. It will be interesting to determine why $^{17}$ should have oestrogen receptor affinity.

From the results it is clear that introduction of an $\alpha$-methylene lactone group into one of the oestrogen rings is counter-productive. The function produces too great a disruption of the oestrogen structure thereby causing a loss of receptor binding activity and hence of potential selectivity.

Accordingly it was necessary to determine if the $\alpha$-methylene lactone function could be attached to oestrogens via an exocyclic linkage without serious reduction of receptor binding affinity. Exocyclic linking of $\alpha$-methylene-$\gamma$-lactones to steroids has been reported previously. [86] This involved reaction of ethyl $\alpha$-(bromomethyl) acrylate with the appropriate steroidal ketone, in this case cholesterol derivatives, by a Reformatsky-type reaction. For our purposes, however, the choice of the position on the oestrogen molecule for substitution was constrained by effects on receptor binding and by the chemistry of the reaction.
4.2.2 16-(α-methylene-γ-lactone) substituted oestrogen derivatives

The substitution of cytotoxic groups on the oestrogen skeleton affects receptor affinity in two ways: through effects on the hydrophobic character of the molecule and by steric hindrance. The cytotoxic group should be as lipophilic as possible. [141] The oestrogen receptor binding site is strongly lipophilic, [141] and additions of polar substituents, especially to the B and C rings are poorly tolerated, [143] although polar groups are tolerated to a greater degree in the D-ring, at the 16 position. [143]

Steric hindrance causes a reduction in oestrogen receptor affinity because of the strain it exerts on the receptor protein during binding. [142] Binding of oestrogens to their receptor is not by a simple diffusion process of the oestrogen into the binding site. [142] This is confirmed by the low association rate of the oestrogen-receptor complex which is much lower than would be expected for a diffusion dependent process. [142] It is believed that the binding process involves a conformational change in the receptor protein, to accommodate the steroid molecule. [142] The process is thus analogous to the induced-fit interaction of some enzymes with their substrates. [140]

Mapping of those positions on oestrogens which tolerate substitutions has been attempted by various groups. [142] Derivatisation of the 3- and 17-hydroxyl groups has been well documented, and produces a marked reduction in binding. [142] This effect has been observed with the nitrogen mustard compounds such as
estramustine. Addition of a methyl group at the 1, 2, 6α, 15α, 15β and 18 positions causes a significant reduction in binding. (Figure 4.4). Methyl additions at the 7α, 11β and 17α positions are better tolerated. Thus for example the steroidal alkylator ORG 4333 (11β-chloromethylestradiol) [123,148] exhibits receptor affinity in the same range as oestradiol; the methyl substitution at the 7α-position of oestradiol affords an increase in receptor binding over the parent compounds. [142] This is ascribed to the increased lipophilicity resulting from the addition. [142]

It was decided to synthesise and investigate the properties of oestrogens substituted at the 16-positions with an α-methyl-γ-lactone
group. 16-Substituted oestrogens do retain considerable receptor binding capacity. [149] Thus oestriol binds to the oestrogen receptor with an affinity similar to that of oestradiol. [143, 149] Also a series of 16-iodinated oestrogens have been produced which have receptor affinities in the same range as the parent oestrogens. [150] The synthesis of the 16-(α-methylene-γ-lactones) was accomplished by G.J. Edge in the Organic Chemistry Laboratory of this department [151].

Three compounds incorporating an α-methylene-γ-lactone ring were synthesised, namely 18, 19 and 20. The compounds were subsequently

\[
\begin{align*}
&18 \quad R = H \\
&19 \quad R = CH_3 \\
&20
\end{align*}
\]
assayed for cytotoxicity against HeLa S3 cells. The 16-spiro compound 20 proved the most cytotoxic of the three, followed by 18 and 19. The relatively poor activity of 19 against HeLa S3 cells is surprising, since in the D-ring series the 3-methoxy compound 4 is more active than the 3-hydroxy 6. The observed enhancement of reactivity of 20 over 18 may be due to interaction with the 17-OH, as with the hydroxyl adjacent to the methylene group seen in vernolepin. [37] The α-methylene-γ-lactone compounds exhibit considerably lower cytotoxicity (in region 10-20 μM) than the D-ring α-methylene-δ-lactones (activity in range 0.1-1 μM).

Receptor binding activity of the two compounds with a 3-hydroxy function, 18 and 20 was determined. Compound 20 has a binding affinity in the same range as estramustine (ED₅₀ ~ 3.4 μM). This result is disappointing since the compound is the nearest in structure to oestradiol, having a 17-hydroxy group. The spiro group is possibly too bulky, and too closely associated with the steroid skeleton. This may affect the hydrogen bonding capacity of the 17-hydroxyl. Alternatively the spiro function may affect the conformation of the oestradiol molecule, or the group may simply be too large to be accommodated by the receptor binding site at that point.

Evaluation of 18 showed significant binding, (ED₅₀ = 265 nM) more than tenfold that of 20 (ED₅₀ = 3400 nM). Unfortunately testing with different samples of 18 revealed a binding affinity considerably less than that for compound 20. The samples provided were prepared by crystallisation on a milligramme scale from a single mother
liqueour. Four isomers of \(\text{18}\) are possible owing to chiral centres at C16 and the exocyclic carbon forming part of the lactone ring (Figure 4.5).

![Figure 4.5 Chiral centres at the 16 position of compound 18.](image)

Fractional crystallisation may have given rise to either different mixtures or different single isomers. This was not detected from their spectroscopic properties, but the problem is currently under investigation in the Organic Chemistry Laboratory. The first sample of \(\text{18}\) produced may therefore consist mainly of the isomer which exhibits the better receptor-binding properties. The stereochemistry of substituents at the 16-position is known to be important. 16\(\alpha\)-Iodo oestrogens have lower affinity for the receptor than their 16\(\beta\) isomers.\[150\] Similarly the 16\(\alpha\)- and 16\(\beta\)-isomers of oestriol exhibit differences in receptor affinity, the 16\(\beta\) isomer having greater affinity.\[143\]

Compounds \(\text{18}\) and \(\text{20}\) displayed no selectivity against the ER+ GH\(_3\) cells. Cytotoxic doses were in the same range as for HeLa S3 cells.
The ED$_{50}$ value for 18 against MCF-7 cells was similar to that for the HeLa S3 line. However, a sample of 20 was not available for testing against the MCF-7 cell line.

Since dose-response curves for cytotoxic activity against MCF-7 are shifted to the right, and on comparison with results for elephantopin it is apparent that 18 exhibits some selectivity for this oestrogen-responsive cell line. Thus the IC$_{50}$ ratio MCF-7/HeLa for elephantopin is ~8.0 whereas for 18 is ~1.0 (Table 4.1).

This group of compounds therefore, whilst not highly cytotoxic, do seem to have the potential to provide useful drugs. However, any further advances in this area must await the difficult chemical task of separating the various isomers.

Table 4.1. IC$_{50}$ ratios, MCF-7/HeLa S3, GH$_3$/HeLa S3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ratio IC$_{50}$ v MCF-7</th>
<th>IC$_{50}$ v HeLa S3</th>
<th>Ratio IC$_{50}$ v GH$_3$</th>
<th>IC$_{50}$ v HeLa S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-ring α-methylene lactone 5</td>
<td>7.2</td>
<td></td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Elephantopin</td>
<td>8.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-α-methylene-γ-lactone substituted, 18</td>
<td>0.85</td>
<td></td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>Vernolepin</td>
<td>-</td>
<td></td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>
4.2.3 17α-Epoxyethano-compounds

A series of oestrogen epoxide derivatives were prepared by J.C. Gill in the Organic Chemistry Laboratory at this Department. The carcinogenic properties of these compounds, as potential oestrogen metabolites, is currently being investigated by Dr. Grover's group at the Cancer Research Institute Laboratories. In addition to their carcinogenic properties however, these epoxides are of interest as possible target selective agents against cancers of the breast. The epoxide alkylating function is cytotoxic, as well as carcinogenic. [62] Further it is less bulky than the α-methylene-γ-lactone functions, and might therefore disrupt receptor binding to a lesser degree.

Two 17α-epoxyethano-diastereomers 21a and 21b have been examined to date. The stereochemistry is assigned tentatively by

![Figure 4.5](image_url)

J.C. Gill on the basis of mechanistic studies

As discussed previously, substitutions at the 17α position are tolerated well. Introduction of a methyl group at this position in oestradiol reduces receptor affinity by only 13%. [142] The synthetic
oestrogen 17α-ethinyl-oestradiol has a higher receptor binding activity than oestradiol. [143] The two epoxides are possible metabolites of ethinyloestradiol, and may retain some of the binding properties of the parent compounds.

The epoxides exhibit cytostatic activity against HeLa S3 cells in the same range as that for the 16-(α-methylene-γ-lactone) oestrogens, but lower than that for the D-ring compounds. The epoxides are also cytotoxic since at higher doses they reduce cell numbers below inoculum levels. Isomer a (IC₅₀ ~ 6.5 μM) is approximately three times as active against HeLa S3 cells as b (IC₅₀ ~ 18.6 μM). The difference in activity must therefore be due either to a difference in reactivity or to the stereochemistry of a which may be selective for some essential cellular nucleophile.

Both isomers show an affinity for the oestrogen receptor, a having approximately tenfold the binding activity of b (ED₅₀ a = 79, b = 724 nM). Isomer a is by far the most active of the compounds tested to date. It has approximately one-seventh the activity of oestradiol but is more active than oestrone.

The difference in binding affinity of the two isomers is presumably caused by some steric effect in the receptor binding site. The epoxide group of a apparently disrupts hydrogen bonding of the 17β-hydroxyl to a lesser extent that b.
The preferred conformation of \( a \) in which the methylene group of the epoxide is remote from the ring junction (X) has the epoxide oxygen **anti** to the 17\( \beta \)-OH. Conversely the equivalent conformation of \( b \) (\( Y \)) has the epoxide oxygen **syn** to the 17\( \beta \)-OH. This may explain the differential hydrogen bonding and binding observed. (Figure 4.6)

The compounds showed no selectivity against the ER\(^{+} \) and GH\(_{3} \) cells. However, the 16-substituted compound \( 18 \) also displayed a lack of selectivity against GH\(_{3} \) cells, but was selective against MCF-7 cells. (Table 3.3, page 93) The epoxides were prepared subsequent to the testing of the D-ring and 16-substituted compounds against MCF-7 cells. They have therefore not as yet been assayed on the MCF-7 cell line. If their receptor binding activity is reflected in their selectivity towards oestrogen responsive cells, the 17\( \alpha \)-epoxides should show considerable activity against the MCF-7 line and possibly tumours with ER\(^{+} \) cells.

4.2.4 Receptor alkylation

Both the 16-\( \alpha \)-methylene-\( \gamma \)-lactones and the 17-epoxyethano-compounds display some degree of irreversible binding to the oestrogen receptor. This was of the same order as reported for the hexestrol-2,3-oxidopropyl ether [101] and also for ORG 4333 [123] which

\[ \text{Hexestrol-2,3-oxidopropyl ether} \]

\[ \text{ORG 4333} \]

Figure 4.6
Figure 4.6. Molecular models showing preferred conformations of \(21a\) (X) and \(21b\) (Y) in which the methylene groups are remote from the ring junction.
was used as a standard in our assays. Both of these latter compounds have been designed as potential affinity labels for the oestrogen-receptor. Previous studies have reported almost total irreversible binding of nitrogen mustard derivatives of DES and oestrone. Previous studies have reported almost total irreversible binding of nitrogen mustard derivatives of DES and oestrone. However, the assay system used, unlike that employed in the present study, did not preclude denaturation of the receptor.

The degree of irreversible binding of the test compounds is apparently related to their affinities for the receptor. Saturation levels of compounds were used, but their dissociation rates from the receptor may differ. Compounds forming relatively stable complexes would thus be more likely to form covalent links. In the case of the epoxides the greater degree of irreversible binding observed for 21a may be a combination of its greater affinity and its greater reactivity.
4.3. **Final Comments on Cytotoxicity and Selectivity**

The 17α-epoxyethano-oestrogen 21a, on the basis of its affinity for the oestrogen receptor, appears the most promising as a drug selective for breast neoplasms. The compound, together with its isomer 21b, has cytotoxic activity similar to that reported for cytotoxic-linked oestrogens presently under investigation. This is rather weak compared to the natural products investigated, but the reduced electrophilic activity may be an advantage in allowing the compound to reach the target cells before non-specific reactions inactivate it. The potential for selectivity of 21a could be better evaluated from its cytotoxicity towards MCF7 cells, and in vivo testing on DMBA-induced mammary adenocarcinomas in rats.

The 16-(α-methylene-γ-lactone) substituted oestrogens 18 and 20 have lower affinity for the oestrogen receptor than the epoxides. However, 18 was selectively cytotoxic towards MCF-7 cells, and this activity may be improved by separation of the isomer with maximum receptor binding activity.
The oestrogen-linked alkylating drugs, including those studied in this work, form covalent links with the oestrogen receptor to some degree. This property may greatly reduce the effectiveness of this class of drug against ER+ tumours which are not growth responsive to oestrogens.

It is probable that more molecules of drug are necessary to kill the target cell than there are receptors present. To accumulate to lethal levels therefore, an oestrogen-linked drug should dissociate from the receptor to react with essential cellular nucleophiles, leaving the receptor free to bind another molecule of drug. [149] There is considerable evidence to suggest such recycling of oestrogen receptors occurs in the presence of oestrogens. [153] Indeed receptor recycling has been proposed as a possible mechanism for modulating the target cell response to oestrogen. [153]

Drugs which are believed to act primarily via alkylation of DNA, such as the epoxides and nitrogen mustards, may be particularly vulnerable to loss of cytotoxic activity by irreversible binding to the oestrogen receptor. There is also evidence to suggest that the steroid carrier may hinder interaction with DNA. [106] To overcome this the use of 'spacer' chains attaching the alkylating group to the oestrogen nucleus has been proposed. [142] It was hoped that this would also reduce covalent binding to the receptor, by distanci
nucleophiles. [142] However, affinity chromatographic work has demonstrated that a chain of at least 14 atoms attached at the 7α or 17α positions is necessary to penetrate the receptor. [142] Addition of a chain of this length results in significant loss of binding activity. [142]

Attachment of the alkylating moiety to the steroid carrier by a labile linkage, is an alternative approach but his type of drug will suffer from the disadvantage of non specific hydrolysis of the linkage before reaching the target cells. [154]
4.4 Mechanisms of Cytotoxicity

4.4.1 Reaction with thiols

The D-ring steroidal α-methylene-δ-lactones react rapidly with glutathione as observed for the sesquiterpene lactones elephantopin and vernolepin. In comparison the 16-(α-methylene-γ-lactone) oestrogens, the two 17α-epoxyethano oestrogens and estramustine react only extremely slowly.

As a general trend, ability to react with glutathione, (vernolepin > elephantopin > the D-ring α-methylene lactones (5, 4 > 6) >> 16α-methylene-γ-lactones, 17α-epoxides, estramustine), is reflected in the cytotoxicity of the compounds (5 > elephantopin > 4 > 6 > vernolepin, 16α-methylene-γ-lactones, 17α-epoxides, estramustine).

The reduced reactivity of the 3-hydroxy derivative 6 relative to the acetoxy 4 and the methoxy 5 may be due to lower solubility or to long range electronic effects of the C(3) substituents. Such effects have been reported in oestrogens [141]

All of the alkylating compounds tested reduce thiol levels in HeLa S3 cells. The D-ring α-methylene compound 4 is the most active. The 16-(α-methylene-γ-lactone) substituted oestrogens, the epoxides and estramustine reduce cellular thiols to a lesser degree. Reduction of thiols is dose-dependent, for example treatment of cells with 10 μM and 50 μM concentrations of 4 reduces cellular thiols by 38% and 64% respectively. The observed depletion of thiols applies to both cell surface and intracellular thiols. It is thus apparent
the compounds can penetrate the plasma membrane. The potent cytotoxic effect of the D-ring compounds may be due in part to increased cellular uptake. This contrasts with the sesquiterpene compound vernolepin. Vernolepin is less active than the D-ring \( \alpha \)-methylene lactones in reducing intracellular thiols, despite its greater reactivity with glutathione. Vernolepin also reacts more readily with glutathione than elephantopin but is less cytotoxic. These observations are believed to be due to the reduced lipophilicity of vernolepin relative to elephantopin. [37]

Time course experiments with vernolepin demonstrate that reduction of thiols reaches an equilibrium with approximately 61\% \(-\text{SH}\) remaining one hour or less after addition of drug. Vernolepin, at a dose which produces approximately 100\% inhibition of growth, does not significantly further reduce thiols from this value, even after 24 hours exposure. The rapidity of thiol depletion is paralleled in the cytostatic activity of the D-ring compounds. Growth inhibition below control levels is apparent 3 hours after exposure. The inhibition is only partly reversible by removal of drug and increases with longer exposure times. A similar effect has been observed with a sesquiterpene compound. [124] It seems that the effect of the D-ring compounds cannot be completely overcome by cellular repair mechanisms even after short exposure times.

Cytotoxicity of the D-ring compounds is reduced by the addition of mM levels of glutathione to the growth media at the same time as the drug.
This effect is presumably due to the formation of glutathione adducts. However, the protection afforded by glutathione is not total, even in the presence of a thousand-fold excess over the D-ring steroid. This may relate to the activity of the thiophenol substituted compound 15 (Table 3.1, page 88).

In spite of the effort put into this work by various laboratories, the toxicity of the α-methylene lactones is not considered to be as a result of intracellular glutathione depletion, though glutathione is involved in a number of cellular functions. Indeed cells may remain viable for some time despite almost complete depletion of glutathione. [124] However, reduction of glutathione levels does potentiate the cytotoxic effects of compounds such as vernolepin and elephantopin on treated cells. [124]

One of the main roles for glutathione is in detoxification, by removal of both endogenous toxins and toxic drugs such as the alkylating agents. Glutathione reacts with toxic electrophiles to form thioether adducts which are actively removed from the cell by a translocase system. [155] Depletion of intracellular glutathione therefore increases the vulnerability of cells to sulphhydryl reactive compounds such as the α-methylene lactones.

Accordingly the effects of the steroidal α-methylene lactones on a number of biochemical systems containing thiol species were investigated in an attempt to determine their possible modes of action. This has further implications since recently it has been
suggested that other alkylating agents may owe their antitumour
properties in part to alkylation of membrane nucleophiles. [156]
which may be targets rather than, or in addition to DNA.

4.4.2 [3H]-Thymidine uptake and incorporation into macromolecules

Intact membrane sulphydryl groups are required in a variety of
membrane transport mechanisms. [53] Alkylation or oxidation of these
groups reduces transport capacity. It is not surprising then that
sesquiterpene lactone alkylating compounds are reported to affect
uptake of a number of molecules by cells in culture. [43]

The D-ring α-methylene steroidal compound 4 is approximately
tenfold more potent in inhibiting thymidine uptake than elephantopin.
However, the parent lactone of 4 (compound 7) has activity in the
same range as 4. This finding suggests that alkylating ability may be
an unimportant factor.

Uptake of thymidine by cells in culture is a carrier-mediated
process. [157] Two distinct transport systems have been identified;
a high affinity active transport system with a narrow substrate
specificity, and a low affinity uniport system with broad substrate
specificity. [157] Both of these types of transport system are
susceptible to changes in the fluidity of the membrane, [158] such
as might be caused by the addition of lipophilic materials. Consequently
a series of compounds were tested to determine if alkylating activity
was a necessary prerequisite for inhibitory activity.
An inhibition of thymidine uptake was observed for the series of unrelated compounds such as benzocaine, dexamethazone, oestradiol. It is likely therefore that the observed inhibition with the α-methylene lactones is a result of a non-specific action on membrane fluidity. Inhibition of uridine and amino acid uptake by sesquiterpene α-methylene lactones in HeLa S3 cells is reputed to occur at relatively high concentrations [43], and this may also be due to non-specific membrane effects.

The effects of compounds on DNA synthesis was evaluated by their ability to inhibit incorporation of $[^3H]$-thymidine into acid insoluble material. The method used was a modification of that described by Plageman. [159] In contrast to the uptake studies, of the compounds tested only the α-methylene lactones 4 and elephantopin, inhibited thymidine incorporation, though this required very high doses in accordance with previous work on elephantopin. [43] We were unable to detect any effect of the glucocorticoid dexamethazone which reportedly inhibits thymidine incorporation [125] though dexamethazone did inhibit uptake of thymidine.

The depression of DNA synthesis reported with α-methylene lactone sesquiterpenoids [30,43] may be due to inhibition of DNA polymerases II and III which are known to be susceptible to thiol reagents. Inhibition of DNA synthesis is reportedly not caused by reduction of thymidine kinase activity. [44]
The sesquiterpene lactones do not react directly with DNA, [30,58] and it has not been possible to show any reaction of the steroidal \(\alpha\)-methylene lactones with guanosine \textit{in vitro}. However, a direct action on DNA following metabolic activation has been proposed. [44] Certainly damage to the DNA template by high levels of vernolepin and elephantopin has been shown. Reduction in the size of DNA fragments in HeLa S3 cells has been reported and is attributed to excision repair. [44] However, DNA endonuclease activity in Ehrlich ascites is depressed by sesquiterpenes, suggesting the reduction in DNA fragment size may be due to some other effect. [30]

The high doses required for inhibition of thymidine incorporation into macromolecules could be due to the short assay time used, relative to the cytotoxicity studies. On the other hand thiol depletion doses occur within this time scale. The rapidity with which the \(\alpha\)-methylene lactone compounds reduce cellular thiol levels suggests that if direct effects on DNA occur they may be later events.

4.4.3 \textbf{Effects on Na\textsuperscript{+}/K\textsuperscript{+} ATPase}

The activity of Na\textsuperscript{+}/K\textsuperscript{+} ATPase in HeLa S3 cells in culture was assessed by their ability to accumulate the potassium congener \(\textsuperscript{86}\text{Rb}\textsuperscript{+}\). This process reaches equilibrium after approximately 3 hours exposure to the isotope. That \(\textsuperscript{86}\text{Rb}\textsuperscript{+}\) uptake is mediated by a Na\textsuperscript{+}/K\textsuperscript{+} pump is evident from its abolition by ouabain.
Inhibition of $^{86}\text{Rb}^+$ uptake by the compounds tested follows the rank order of their cytotoxic potency and their ability to deplete cellular thiols. The D-ring $\alpha$-methylene compound $4$ is three-fold more active against the transport system than vernolepin which is more active than estramustine or the 16$\alpha$-epoxides $21a$ and $21b$. The effect on $^{86}\text{Rb}^+$ accumulation is caused predominantly by the alkylating activity of the compounds. This is demonstrated by the low activity of the D-ring lactone lacking an exocyclic methylene function $7$. Further the inhibition by vernolepin reaches a maximum in less than 30 minutes. Maximum depletion of cellular thiols occurs over a similar range. $\text{Na}^+/\text{K}^+$ ATPase has a number of essential thiols, some of which are located in shielded hydrophobic subunits. [160,161] Reaction with these shielded thiols is enhanced in alkylating agents which have hydrophobic structures.

The activity of the nitrogen mustard estramustine is similar to that reported for nitrogen mustard itself. [54] The $\text{Na}^+/\text{K}^+$ ATPase of the PC 6 A mouse plasmacytoma cell line is inhibited by the action of nitrogen mustard, [54] but the monofunctional analogue (2-chloroethyl) dimethylamine is inactive. [54] The difunctional compounds are presumed to cross link parts of the enzyme protein. [54] The activity of the oestrogen alkylating compounds may therefore indicate a different, more specific site of action. For example binding and subsequent alkylation of the enzymic $\text{K}^+$ site. This idea may merit further investigation since attempts to prepare compounds with this property have been made in an effort to probe the active site of the enzyme. [161]
Within the group of alkylating oestrogens containing a monofunctional alkylating moiety, differences are apparent. Thus the α-methylene lactone compounds 4 and vernolepin have considerably steeper dose/response curves than the epoxides which behave in a similar manner to estramustine.

Inhibition of the isolated enzyme from HeLa S3 cells and of commercial enzyme preparations by vernolepin and the D-ring α-methylene lactone 4 was less easy to demonstrate than $^{86}$Rb$^+$ uptake inhibition. This difference has also been reported for the effect of nitrogen mustard on isolated enzyme preparations from PC6 A cells. [54] Indeed nitrogen mustard produced a 43.4% inhibition of Na$^+$/K$^+$ ATPase, but only at millimolar concentrations, five-fold higher than that used for vernolepin on the isolated HeLa S3 enzyme. The time-course of enzyme inhibition of vernolepin is similar to that for $^{86}$Rb$^+$ uptake by HeLa S3 cells. This further supports the view that the effect of these compounds on $^{86}$Rb$^+$ accumulation is caused by a direct action on the enzyme itself. The α-methylene lactone compounds 4 and vernolepin exhibited approximately equal inhibition of the ouabain sensitive and insensitive enzyme preparations.

The importance of thiol groups for enzyme activity was confirmed by the inhibition observed with the sulphhydryl reagent N-ethylmaleimide. This compound produced approximately two-fold greater inhibition of the enzymes than 4 or vernolepin.

Interest in the Na$^+$/K$^+$ ATPase system as a possible site of action for the anti-tumour alkylating drugs is comparatively recent. [156]
Previously it was considered that the number of enzyme molecules was too great in comparison to the levels of drug present under physiological conditions. [156] However, cells of the HeLa line have only approximately $10^6$ Na$^+$/K$^+$ ATPase molecules per cell and comparatively small loss of Na$^+$/K$^+$ pumping capacity would alter the flux of other essential ions, such as Ca$^{2+}$, across the cell membrane. [156] Consequently cross linking of the enzyme by a number of difunctional alkylating compounds, and subsequent imbalance of ions has been reported. [54,55] Disruption of ion balance in cells would be sufficient to produce all of the various effects reported for alkylating drugs on cells by cascade effects on both protein and DNA synthesis. [57,162,163]

4.4.4 Effects on oxidative phosphorylation

The D-ring α-methylene compound 4 and elephantopin have no significant effect on basal (state 4) respiration in mouse liver mitochondria, up to 100 μM. Pretreatment of the preparation with test compounds produced a similar result. ATP-stimulated (state 3) respiration is inhibited by both compounds to approximately the same degree at 100 μM, although oestradiol produces a similar inhibition. Pretreatment for 2 hours at 4°C marginally increases the inhibitory activity of 4 and elephantopin.

The results suggest the compounds act as inhibitors of respiration, rather than as uncoupling agents. The site of inhibition may be the ATPase complex of the mitochondria, or some transport mechanism. Inhibition of ADP uptake by the mitochondria would produce a similar
effect on respiration. [164] As with the effects observed on thymidine transport the greater part of the activity appears to be independent of the α-methylene lactone function. Respiratory activity in mitochondria is sensitive to alterations in the fluidity of the two membranes, which have a higher proportion of unsaturated phospholipids than other cellular membranes. [165]

Inhibition of respiration has been reported for the sesquiterpene α-methylene lactones both in vivo and in vitro. [59] Some reports indicate that stimulation of state 4 respiration occurs with some of these compounds, indicating they may act as uncoupling agents. [59] The doses used for in vitro testing were of the same level as those described in the present experiments.

4.4.5 Lysosomal membranes

The effects of the α-methylene lactones elephantopin and the α-methylene lactone steroidal compound 4 on cell ultrastructure was studied by scanning and transmission electron microscopy. The most notable change in the cells, treated with 4, was the disruption of the cytoplasm, eventually leading to total disintegration. This observation suggested that breakdown of lysosomal membranes may occur in cells treated with 4 and the released enzymes then destroy the cells. The effects of several compounds on the stability of lysosomes prepared from rat liver were therefore studied.
Compound 4 does indeed destabilise lysosomal membranes, as assessed by release of the enzyme aryl sulphatase. This activity of 4 is only apparent at the relatively high concentration of 100 μM; no effect was observed at concentrations of 1 μM or 10 μM. This property is presumed to be due to the alkylating function since the parent lactone 2 has by contrast a slight stabilising effect on the lysosomes. By contrast, the sesquiterpenes α-methylene lactones, vernolepin and elephantopin, were found to stabilise lysosomal membranes, as has been previously reported. [59] The anti-inflammatory activity of these compounds has been partly ascribed to this property. [59,60]

Elephantopin and vernolepin inhibit aryl sulphatase activity. This effect is believed to be due to alkylation. [59] The inhibition observed for the lactone 7 was slightly higher than that for the two sesquiterpenes. On the other hand the methylene lactone 4 has no inhibitory effect on aryl sulphatase.
4.5. Overview of Mechanisms of Cytotoxicity

All of the compounds tested perturb membrane functions in HeLa S3 cells and in isolated membrane preparations, to some degree. Thymidine uptake, oxidative phosphorylation and lysosomal membrane stability are all affected. However, the results from these assay systems must be interpreted with caution; since at the concentrations used the alkylating activity of the compounds may be masked by their intrinsic hydrophobicity, which also perturbs membrane function.

This is apparently not the case with the inhibition of Na\(^+\)/K\(^+\) ATPase of HeLa S3 cells observed for the \(\alpha\)-methylene lactone compounds. In this system the inhibition of the membrane ion pump is predominantly due to alkylating activity, for vernolepin and the steroidal \(\alpha\)-methylene lactone compound 4 at least. The relatively low activity of the epoxide 21a and nitrogen mustard compound estramustine may indicate they have a different site of action.

Alkylating agents of all classes have been found to inhibit a variety of cellular functions. To date it has not been possible to determine unequivocally which is their primary site of action, if indeed there is only one. However, continued investigation of the mode of action of the alkylating drugs may still prove productive in contributing to design of improved drugs.
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