STUDY OF NOVEL DUAL TOPOISOMERASE POISONS AS POTENTIAL ANTI-CANCER DRUGS

By

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This thesis is dedicated to the Paul David Lewis whose love and encouragement will always be remembered and to Joyce Lewis whose strength has been an inspiration.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphocytic leukaemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>AR</td>
<td>Adriamycin resistance</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cam/CPT</td>
<td>Camptothecin</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation recruitment domain</td>
</tr>
<tr>
<td>CC</td>
<td>Cleavable complex</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>Cox</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CPT-11</td>
<td>Irinotecan</td>
</tr>
<tr>
<td>DACA</td>
<td>N-[2-(dimethylamino)ethyl]acridine-4-carboxamide</td>
</tr>
<tr>
<td>DPC</td>
<td>DNA protein crosslink</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxorubicin (adriamycin)</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxoide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FS</td>
<td>Farnesyltransferase inhibitors</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-flourouracil</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumours</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/Erk-kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple drug resistance</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane permeabilisation</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRP</td>
<td>Mulit-drug resistance associated protein</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS with 0.1% Tween 20.</td>
</tr>
<tr>
<td>Pgp</td>
<td>Permeability glycoprotein</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PIG</td>
<td>p53 inducible genes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>p-p53</td>
<td>Phosphorylated p53</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small cell lung cancer</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SN-38</td>
<td>7-ethyl-10-hydroxycamptothecin</td>
</tr>
<tr>
<td>TARDIS</td>
<td>Trapped in agarose DNA immunostaining</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with 0.1% Tween 20</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Topo</td>
<td>Topoisomerase</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labelling</td>
</tr>
<tr>
<td>VM-26</td>
<td>Teniposide</td>
</tr>
<tr>
<td>VP16</td>
<td>Etoposide</td>
</tr>
<tr>
<td>Z-VAD-FMK</td>
<td>N-Benzoxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone</td>
</tr>
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XR5944 and XR11576 are two potent DNA interactive agents, previously shown to be capable of stabilising topoisomerase I and II cleavable complexes in vitro. This thesis investigated the possibility that the mechanism(s) of action of these compounds may be unrelated to that of topo inhibition. These studies made use of a wide range of drug-sensitive and resistant cancer cell lines. It was demonstrated that both XR5944 and XR11576 retain potent cytotoxicity in cancer cell lines presenting with atypical drug resistance to single topoisomerase poisons. XR5944 showed less potency in cell lines expressing ABC- transporter proteins, but this may not be sufficient to compromise the activity of this potent antitumour agent in the clinical setting. The mechanism of XR11576 induced cytotoxicity was not affected by any of these transporters and from this and other observations it may possess a different mechanism of action from XR5944.

An apoptotic response was observed in XR5944 and XR11576 treated cells. Factors such as Bax and t-Bid were expressed in increasing amounts in response to treatment and implicated the mitochondrial route of apoptosis in these compounds’ mechanism of cell kill. Both XR5944 and XR11576 induced appreciable levels of DNA-protein crosslink formation and induced a p53 DNA damage response in drug treated cells. Differences in the timing of onset and extent of DNA damage and p53 induction were noticed between the two compounds. Overall, XR5944 was slower at causing these DNA interactive effects in line with the onset of cytotoxicity. However, this did not appear to pose any particular problems, such as scheduling of dosing, with regard to its in vivo activity, as shown by the work of others.
These findings suggest that both XR5944 and XR11576 promote potent cytotoxicity in cancer cell lines showing multiple mechanisms of drug resistance and, therefore, should be of use in the clinic for the treatment of drug resistant tumours. The data presented in this thesis suggest that neither agent exerts its cytotoxicity via a topo directed effect. Furthermore, some of the data generated suggests that these two compounds may differ from each other in their mechanisms of action.
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CHAPTER 1

Introduction
1 Introduction

1.1 Overview of cancer and cancer chemotherapy

In the year 2000, 6.2 million people died from cancer worldwide. Given the current trends in lifestyle behaviour such as smoking, heavy alcohol drinking and high exposure to the sun, this number is expected to reach 15 million by 2020 (Stewart and Kleihues, 2003). The principle approaches of cancer therapy are currently surgery, radiotherapy and chemotherapy. In the past, treatment has involved surgical removal of the neoplasm and this has been followed by the subsequent application of radiotherapy to kill any remaining cells. These treatments lead to a 40% cure rate of all cancer patients. Notably, this leaves 60% of cases resulting in mortality (Verweij and deJonge 2000). In most of the 60% of cases remaining, the cancer spreads, or has already spread, and results in metastases at different locations around the body. The disease becomes systemic and effective treatment is then required at more than one site. It is for this reason that the development of cancer chemotherapy over the last 60 years has produced significant advances in the field of cancer treatment as it provides a systemic mode of attacking both primary tumours and metastases.

Conventional cancer chemotherapy started with the observation that poisonous mustard gas used in the First World War suppressed bone marrow regeneration. In the Second World War research was escalated when mustard gas was a potential threat to allied forces. The alkylation chemistry of mustard compounds was discovered as early as 1946 but it was not until 1966 that the compounds’ ability to crosslink DNA was comprehensively shown (Kohn et al., 1966). Prior to the discovery of the mechanism of action of nitrogen mustards, these compounds were
used in clinical trials in lymphoma patients. Some tumours regressed but severe toxicity was a limiting factor (Goodman et al., 1946). However, these compounds showed for the first time that cancer could be treated by chemical means. This was the start of cancer chemotherapy which has greatly improved the outcome of many malignancies.

1.1.1 Aims of chemotherapy
Chemotherapy has a number of objectives, depending on the particular clinical scenario. Chemotherapy may be used to cure or to improve survival- in locally advanced or recurrent disease- but it may also be used to prolong life in terminal cases affording a good quality of life in both groups of patients. Adjuvant chemotherapy may prolong life even for people who are not terminally ill; Chemotherapy can be used as a stand-alone treatment in some cases where it has curative potential and also in addition to other treatment such as radiotherapy or surgery. The basis of chemotherapy has been the fractional kill hypothesis that states ‘that a given concentration of drug given for a defined period will kill a constant fraction of cells independent of the absolute number of cells’ (Chabner 1990a). Each cycle of treatment will therefore kill a remaining fraction of cells. The result of the treatment is dependent on dose and frequency of administration. Notably, this hypothesis has limitations. Solid tumours are especially heterogeneous and parts of the tumour will be anoxic and lacking in nutrients. For this reason, some of the cells will not be actively replicating and will therefore be refractory to cell cycle specific agents.

1.1.2 Combination Chemotherapy
Cancer chemotherapy has only limited success when a single agent is used as a treatment. Tumour cell populations are often heterogeneous and only some cells will be sensitive to a given agent. Resistant cells will survive treatment and continue to
proliferate resulting in a resistant population of cells. In this way, even tumours initially responsive to treatment acquire resistance to single agents over time.

Combination chemotherapy combines multiple drugs with different mechanisms of action. Tumour cells that are resistant to one agent may still be sensitive to another in a combination regimen. Moreover, greater toxicity to tumour cells should occur as cells are targeted by more than one approach simultaneously. Drug regimens can be designed accounting for known factors of resistance. Similarly, drugs can be combined with different side effects so that there is no cumulative adverse response or drug interactions. For example, agents which rely on renal excretion, may be more toxic when given in combination with others which also give rise to renal toxicity and hence, should be avoided (Kaufmann and Chabner, 2001).

Combinations of drugs have been used to successfully treat malignancies otherwise incurable by use of single agents. The approach of combination chemotherapy has dramatically improving survival rates in many cancers. These include acute lymphoid leukaemia (ALL) treated with a combination of vincristine, doxorubicin, prednisone, and L-asparaginase and has resulted in survival rates greater than 70%. Also testicular cancer has been effectively treated with a combination of bleomycin, cisplatin and vinblastine or etoposide resulting in a cure rate of over 80% (Kaufmann and Chabner, 2001, Einhorn 1990, McCaffrey and Bajorin, 1998).

1.1.3 Adjuvant Chemotherapy
Chemotherapy can also be used as an adjuvant particularly after the surgical removal of the main tumour in order to reduce the risk of recurrence. It is given as treatment
for micrometastatic disease where small tumours have already spread to other sites. Adjuvant chemotherapy has been shown to delay recurrence and prolong survival in breast, colorectal, cervical malignancies and also in osteosarcoma (Kaufmann and Chabner, 2001). For some time chemotherapy has been used in conjunction with radiotherapy in order to sensitise cells to the effects of radiation by inhibition of processes such as DNA repair. In two randomised trials in advanced cervical cancer patients, using radiotherapy with or without combination chemotherapy, survival was significantly increased with chemotherapy. Cumulative rates of 5-year survival were 67% for those who received chemotherapy and radiation and only 40% for those receiving radiotherapy alone (Morris et al., 1999; Rose et al., 1999).

In some cases, however, chemotherapy is used to prolong life or quality of life for a short duration rather than to elicit a cure. In these circumstances, the toxicities of chemotherapy have to be considered alongside any possible benefits.

Neo-adjuvant therapy has also proven successful. This is chemotherapy administered before surgery or irradiation in order to reduce the bulk of a tumour and improve the probability of complete surgical resection. It may also decrease local recurrence and allows organ preservation. This is of particular use in cancers of the head and neck, oesophagus and many other tumour types (Kaufmann and Chabner, 2001).

### 1.1.4 Currently Used Chemotherapeutics

A large number of chemotherapeutic agents are now used as part of cancer treatment. Topoisomerase poisons are amongst these agents and will be discussed in section 1.2.5.
1.1.4.1 Antifolates
In 1948 Farber et al., showed that aminopterin could inhibit the proliferation of leukaemia cells and cause remission in children with ALL. Aminopterin is an analogue of folic acid and binds tightly to dihydrofolate reductase (DHFR), a key enzyme in folate homeostasis. Inhibition of this enzyme leads to a decrease in the concentration of folates resulting in inhibition of purine and thymidylate synthesis that are essential in cellular replication. Methotrexate (MTX) is widely used in leukaemias, choriocarcinomas, head and neck, breast, bladder carcinomas and lymphomas (Messmann and Allegra, 2001). Other antifolate agents are also used in the treatment of rheumatoid arthritis, psoriasis and parasitic infections. Side effects include myelosuppression, mucositis of the gastro-intestinal tract and liver toxicity.

1.1.4.2 Antimetabolites
Analognues of nucleosides found in DNA and RNA have been used to successfully inhibit DNA and RNA synthesis and thereby cause the arrest of cellular proliferation. Cytidine analogues such as arabinosylcytosine (AraC) are effective anti-tumour agents as well as being anti-viral compounds. Other nucleoside analogues such as 6-thioguanine, is used in acute myeloid leukaemia (AML) and ALL. Fluorinated pyrimidines such as 5-flurouracil (5-FU) are potent antimetabolites that are incorporated into RNA and interfere with translation. 5-FU also inhibits thymidylate synthase (TS) arresting production of deoxythymidine triphosphate (dTTP) that is essential for DNA synthesis (Grem 2001). AraC is commonly used in combination with doxorubicin or daunorubicin in AML and as a second-line treatment in childhood ALL. Toxicities observed with treatment include gastrointestinal ulceration, myelosuppression, neurotoxicity, cardiotoxicity and dermatological and ocular disturbances.
1.1.4.3 Tubulin Interacting Agents

The vinca alkaloids are nitrogenous bases that can be derived from the periwinkle plant *Catharanthus roseus*. This group includes agents such as colchicines, vincristine and vinblastine. Vinca alkaloids induce cytotoxicity by binding tubulin in the intracellular matrix and so inhibiting microtubule formation. Therefore, the formation of the mitotic spindle is blocked and cells accumulate in mitosis. Vinca alkaloids are used in acute leukaemias and Hodgkin’s disease. Vincristine causes peripheral neurotoxicity as well as gastrointestinal, genitourinary, endocrine and cardiovascular disturbances. Vinblastine causes myelosuppression particularly neutropaenia (Rowinsky and Donehower, 2001).

Paclitaxel (Taxol™) is another compound that also binds tubulin but enhances formation of microtubules and prevents the re-use of the tubulin monomers. This compound was discovered as part of the National Cancer Institute drug screen in which thousands of plant extracts were screened for antitumour activity. Paclitaxel has been approved for use in a number of malignancies including chemotherapy-resistant ovarian cancer, advanced breast cancer and lung cancer. Side effects include neutropaenia, hypersensitivity reactions, peripheral neuropathy and cardiac rhythm disturbances.

1.1.4.4 Alkylating Agents

Chemotherapy started with the use of nitrogen mustard compounds and these were later found to behave as DNA alkylating agents. These agents substitute an alkyl group within the compound for a hydrogen atom on cellular DNA. This comes about following the formation of reactive intermediate compounds that attack the DNA at nucleophillic sites (Colvin and Chabner, 1990). Agents such as cyclophosphamide,
melphalan and nitrosoureas cause interstrand DNA cross-links that result in DNA damage and cell death (Figure 1).

![Figure 1 Alkylating Agents.](image)

DNA cross-linking (1) and Intercalation (2) by DNA targeting drugs such as alkylating agents. Crosslinking agents form covalent and hydrogen bonds between juxtapositional bases on different strands of DNA. Intercalating agents form similar bonds between bases adjacent to each other (Hurley 2002).

Cyclophosphamide is a widely used derivative of nitrogen mustard that undergoes metabolic activation via the cytochrome P450 system. It may be often combined with melphalan and carmustine for treatment in acute leukaemia and in autologous transplantation for lymphoma and breast cancers. Side effects include suppression of haematopoiesis, cardiac necrosis, veno-occlusive disease of the liver and haemorrhagic cystitis. The latter side effect can be largely overcome by the use of 'Mesna' that detoxifies the toxic metabolic by-product acrolein. Ifosamide, a structural analogue of cyclophosphamide, is used to treat germ-cell tumours, soft
tissue sarcomas and in some carcinomas of the cervix and uroendothelial tract (Nieto and Jones, 2002). Ifosfamide has similar side effects to cyclophosphamide, including encephalopathy, which may be severe.

1.1.4.5 Platinum Containing Agents
Platinum containing agents such as cisplatin bind DNA bases covalently and form adducts by inter and intra-strand crosslinks. If the adducts are not repaired DNA lesions are then produced resulting in cell death. Platinum agents such as cisplatin and carboplatin are used to treat numerous malignancies, resulting in significant cure rates in testicular cancer (Reed, 2001). They are also used in SCLC and ovarian cancer. Toxicities observed with treatment include vomiting, neurotoxicity and renal damage. Cisplatin induces free radical formation and depletion of antioxidant enzymes causing lipid peroxidation. This results in damage to the outer hairy cells of the cochlea and ototoxicity (Nieto and Jones, 2002).

1.1.5 New Targets & Approaches
The rationale for development of new chemotherapeutic agents has changed over the last two decades. Previously, mass-screening programmes had been the main approach to finding new agents and these compounds were then modified to create more effective, less toxic analogues. This strategy proved successful in part, with the discovery of compounds like camptothecin and subsequent development of less toxic analogues such as irinotecan. However, over the last two decades, research has been focussed on establishing new and more specific targets for chemotherapy and finding agents aimed at these targets. For example, tyrosine kinases have been targeted in chronic myeloid leukaemia (CML) where inhibition of the Bcr-Abl tyrosine kinase (a
product of the Philadelphia chromosome) by imatinib mesylate (Glivec™) has been shown to be effective. Glivec has also been approved for use in inoperable gastrointestinal stromal tumours (GIST) (Demetri, 2001). Another novel target is Human Epidermal growth factor Receptor 2 (HER2/c-erbB2) which has been shown to be an effective target in breast cancer. Trastuzumab (Herceptin™) is a monoclonal antibody that prevents HER2 binding and can mediate antibody-dependent cytotoxicity of tumour cells (Leyland-Jones 2002).

1.1.6 Matrix Metalloproteinase Inhibitors
Much interest has been shown in the development of anti-angiogenic agents such as Marimastat, a metalloproteinase inhibitor. Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes often overexpressed in tumours and are capable of degrading the extracellular matrix around a tumour thereby aiding cell migration and metastasis. Marimastat is one of a number of MMP inhibitors which have been tested in clinical trials. The results in a range of tumour types have been disappointing and none of these compounds have been licensed to date. Ultimately, if they have a role this is likely to be in combination with cytotoxic drugs.

1.1.7 Farnesyltransferase Inhibitors
Selective inhibitors of farnesyltransferase (FT), an enzyme responsible for the addition of a 15-carbon farnesyl group to key proteins such as Ras, Rho, PxF and lamins A and B are also in development. Farnesylation activates Ras and subsequently triggers the PI3 kinase/AKT and Raf/MEK/ERK pathways implicated in cell proliferation. R115777 (Zarnestra™) has shown activity in 75% of a large number of cell lines in vitro with mutations in K-ras being the main cause of resistance. Furthermore, a partial response was seen in 29% of 35 cases of acute
myeloid leukaemia (AML) (Karp et al., 2001). Numerous other FT inhibitors are currently in development (Haluska et al., 2002).

1.1.8 Antisense Therapy

Antisense therapy is designed to inhibit the production of certain proteins that give a survival advantage to cancer cells such as anti-apoptotic proteins, angiogenesis factors, and proteins involved in signalling pathways. The strategy of antisense based therapy operates via interfering with mRNA before protein production can take place (Figure 2).

![Antisense Therapy Diagram](image)

**Figure 2 Antisense Therapy.**

Oligonucleotides are specifically designed to bind to a target region on the mRNA and inhibit synthesis of new protein (Jansen and Zangemeister-Wittke, 2002).

Conventional therapy interferes non-specifically with DNA replication and RNA synthesis whereas antisense therapy prevents mRNA synthesis of a specific gene and does not interfere with any other region of DNA within the cell.
Small 18-21mer interfering RNAs have been designed with complementary sequences to RNA. These inhibit translation of the mRNA and promote RNA cleavage by RNase H (Jansen and Zangemeister-Wittke, 2002). Antisense RNAs have been designed for \textit{bcl-2}, an anti-apoptotic gene. An increase in efficacy of dacarbazine treated human tumour xenografts in mice was observed when it was used in combination with \textit{bcl-2} antisense (Jansen \textit{et al.}, 2000). A phase I/II trial in patients with malignant melanoma using a combination of dacarbazine and Genasense (BCL2 antisense oligonucleotide, Genta Inc, USA) showed antitumour responses in 6/14 patients (Jansen \textit{et al.}, 2000). A number of phase III trials incorporating Genasense with cytotoxic agents are currently being conducted.

1.1.9 \textit{Gene therapies using adenovirus viruses}

DNA viruses replicate using host cells and in some cases use the same proliferation pathways as cancer cells. Viruses that could selectively replicate in tumour cells and destroy them would prove a useful tool in cancer therapy. The Onyx-015 adenovirus was one of the first viruses to be developed for clinical use. The virus contains an 827 base-pair deletion in the E1B region of its genome rendering it incapable of blocking the p53 function of its host cell. In tumour cells however, a mutated or deleted p53 is often observed giving rise to non-functional p53, and in these cells the virus can replicate and kill the host cell. A phase II study was carried out in patients with recurrent carcinomas of the head and neck. Of 30 evaluated patients, 19 experienced a measurable response in conjunction with cisplatin-based chemotherapy (Ries and Korn, 2002). Phase III trials involving ONYX-015 are currently underway.
1.1.10 Pharmacogenomics
Response to chemotherapy may be influenced by gender, general medical condition and individual differences in genotypes affecting factors such as drug metabolism (Diasio 2001). Pharmacogenomics seeks to identify genetic differences between individuals that may influence the efficacy of specific treatments. Some specific pharmacogenetic variations have been identified as important in chemotherapy both in predicting positive outcomes and tolerance to specific agents.

Breast cancer patients that rapidly progress to metastatic disease and have a poor prognosis have been shown to have similar molecular 'signatures' when examined by microarray. Expression of genes regulating the cell cycle, metastasis, angiogenesis and invasiveness of breast cancer have been found to be consistent with poor prognosis (van’t Veer et al., 2002). This study was also able to identify those patients for whom no extra benefit could be gained from adjuvant chemotherapy. Moreover, carriers of \textit{BRCA1}, a gene identified in hereditary breast cancer, also appear to have similar signatures.

Genetic variations can also influence the toxicity of chemotherapy drugs. Decreased levels of dihydropyrimidine dehydrogenase (DPD) have been associated with severe toxicity with 5-FU treatment as this enzyme is responsible in part for its catabolism (Harris \textit{et al.}, 1991). Deficiencies in DPD have been attributed to familial genetic conditions (Diasio \textit{et al.}, 1988). These studies illustrate how the genetic make-up of the individual may affect the response to a specific chemotherapy agent. Pharmacogenomics aims to consider these variations when deciding on the appropriate treatment for the patient. This may be through administration of a different dosage of drug or an alternative agent. In the future a patients' genotype
may be a significant factor in deciding which treatment may be used. In the pre-
genomics era only single polymorphisms had been identified and it is possible that
multiple genetic variations may be influential in determining a therapeutic response
(Ulrich et al., 2003). Further research using microarray-based technology is needed to
fully understand all the genetic factors that are involved in response to cancer
chemotherapy.

1.1.11 The role of cancer chemotherapy
Treatments aimed at new targets, such as oncogenes, have in some cases shown
promising results in clinical trials focusing on specific certain types of malignancy.
However, as most human cancer is characterised by multiple and complex genetic
changes, the inactivation of a single genetic target may not prove a lasting or effective
strategy. The target gene has to be present and be an important part of neoplasia at
that stage of the tumour progression. Targeting an angiogenic factor may not be
effective in an early stage tumour. Furthermore, oncogene inactivation has resulted
in tumour regression in mice and animal models but this may represent an
oversimplification of the clinical scenario where more than one oncogene can be
activated. Moreover, the loss of an oncogene can be compensated for by other genes
(Felsher 2003). It is likely that chemotherapeutic cytotoxic drugs will continue to
play an important role in cancer therapy. One successful class of chemotherapeutic
agents are topoisomerase (topo) poisons which have proven to be effective cytotoxic
agents giving rise to tumour regression.
1.2 Topoisomerases (Topos)

Topos were first described by James Wang in 1971 as the *Escherichia coli* omega protein and it was demonstrated that this protein could relax negative supercoils *in vitro* (Wang, 1971). A role in DNA replication was postulated and subsequently confirmed (Champoux, 1981).

In mammals there are at least 5 known genes for topos. These encode three described groups of topos. The first group is that of topo I. Included in this group is the eukaryotic topo I enzyme (Wallis *et al.*, 1989, Hanai *et al.*, 1996). The second group is topo II and is further divided into α and β subclasses. Finally, topo III has been identified in yeast and humans and shows homology to *E.coli* topo I (Wallis *et al.*, 1989, Hanai *et al.*, 1996).

**Table 1: Topoisomerase groups**

(Nittis 1998, Christensen *et al.*, 2002)

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>Topo I</th>
<th>Topo II</th>
<th>Topo III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A – includes</td>
<td>α - expression peaks in mitosis</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><em>E.coli</em> topo I</td>
<td>β - present throughout cell cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1B – includes</td>
<td>Removal of positive and negative supercoils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse topo I</td>
<td>Removal of positive and negative supercoils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Action</td>
<td>Removal of negative supercoils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanism</td>
<td>Cleaves one strand of DNA to allow passage of another strand.</td>
<td>Cleaves both strands of DNA</td>
<td>Cleaves one strand of DNA.</td>
</tr>
</tbody>
</table>
1.2.1 Proteins

Type I topos are 100kDa monomer proteins encoded for by a single copy gene located on chromosome 20q12-13.2. They, like other topos, have a nuclear location and have a tyrosine residue in their catalytic site (Champoux, 1981).

Topo II is encoded for by the Top2 gene. Two subtypes of topo II – alpha and beta-have been described in eukaryotes, which are 170 and 180kDa, respectively. The subtypes show conservation throughout the amino acid sequence and only diverge at the carboxyl terminus (Austin et al., 1993). The mechanism of action of the two types is indistinguishable but differences in expression are seen. The alpha subtype is expressed more in proliferating cells whereas the beta type is seen in equal levels in proliferative and quiescent cells. Topo IIβ is essential for viability in yeast and mammalian development. mRNA levels for topo IIβ vary no more than two-fold during the cell cycle, whereas for topo IIα there is up to 10-fold increase seen in mRNA levels, accumulating during late S-phase and mitosis and then falling rapidly (Issacs et al., 1998, Holm et al., 1985).

Topo III was identified as a 73kDa protein in 1982 and is the least described of the topos. Digate and Marians (1988), purified the enzyme from a topo I deficient strain of E.coli, and showed that it could potently decatenate and separate pBR322 DNA replication in vitro. Soon after the TOP3 was identified as a gene required to suppress recombination in yeast (Wallis et al., 1989). Eukaryotic topo III is homologous to bacterial topos I and II and has been classed with type I topos. At least two known human genes encode for topo type III including TOP3 located on chromosome 17p11.2-12 (Hanai et al., 1996).
1.2.2 Functions of Topos

1.2.2.1 Transcription
During transcription positive supercoils accumulate in front of the DNA polymerases as they move along the opened double helix. Negative supercoils form behind the polymerase and positive ones in front (Figure 3). This places torsional strain on the unreplicated template and would arrest further transcription if the supercoils were not removed. Topo I removes the supercoiling by cleaving one of the strands and allowing it to rotate, thereby removing the strain, before resealing it in an unstrained conformation. Topo II activity cleaves both DNA strands.

![Diagram of supercoiling during transcription](image)

**Figure 3 Supercoiling during transcription**
*Positive supercoils accumulated in front of the DNA polymerase as the strands are forced to twist round each other. Negative ones accumulate behind the enzyme to compensate for this (Adapted from Weaver 1999).*

A further role of topo I may be in selective transcriptional activation. *In vitro* studies have shown that Dr2 protein, also called hTopo I, is part of the TFIID complex and specifically interacts with TATA box binding protein (TBP). The absence of hTopo I represses basal level transcription (Merino *et al.*, 1993). Notably, topo I was also found to interact with human transcription factor IIIC and promoted accurate
termination and transcription re-initiation by RNA polymerase III (Wang and Roeder, 1998). It is also significant that whilst the binding of the enzyme is needed for transcriptional activation, its relaxation activity is required for transcriptional elongation (Pommier et al., 1998).

1.2.2.2 Cell Replication
Topos have been shown to be an essential factor for successful DNA replication. In line with this, Uemura and Yanagida., (1984), demonstrated that topo 1A and 1B yeast mutants grew poorly. In addition, topo I has been shown to be a prerequisite for embryonic development in many organisms such as Drosophila (Lee et al., 1993). Topo II function is needed in both prokaryotic and eukaryotic cells (Nitiss, 1998). Without topo II yeast plasmids are fully replicated but accumulate as catenated dimers (DiNardo et al., 1984).

1.2.2.3 Genome Stability
Topos have been implicated in genome stability and suppression of mitotic recombination. Inactivating any of the three topos in yeast may result in a hyper-recombinant phenotype. For example, the removal of topo III in yeast resulted in a hyper-recombinogenic strain that grew 50% more slowly than other isogenic strains (Wallis et al., 1989). Moreover, topo III may also be related to the genetic instability associated with the disease Ataxia Telangiectasia (AT). In affected individuals, AT gives rise to an increased sensitivity to radiation. Chromosomal abnormalities caused by radiation damage are not corrected and the cells continue to replicate with
abnormalities present. Importantly, antisense constructs of the truncated TOP3 gene can correct genetic instability in AT cells (Fritz et al., 1997).

1.2.2.4 Mechanism of action of topos

1.2.2.4.1 Binding
The main function of topos is to remove negative supercoils. This is carried out by the covalent attachment of the topo to the 5' end of the DNA being replicated. Topo binding sequences vary between organisms and type of topo (Stevnser et al., 1989, Andersen et al., 1985).

1.2.2.4.2 Transesterification
Following the binding of the topo to the 5' terminal, its' tyrosine hydroxyl group forms a phosphodiester bond with the 3' phosphate on DNA (Champoux, 1981). Strand scission occurs as the formation of the phosphodiester bond liberates the 5' hydroxyl, thus generating a strand break (Lynn et al., 1989). Topo I activity gives rise to a single strand break (Figure 4) whereas topo II cleaves both strands of DNA (Figure 5).

1.2.2.4.3 Single Strand Passage
Relaxation of the strain in the DNA is relieved by topos rotating the DNA strands. However, the degree to which the strands are allowed to rotate has been debated and not been resolved (reviewed by Pommier et al., 1998).

1.2.2.4.4 DNA Religation
Ligation of the two DNA strands is completed once they have rotated sufficiently to release the supercoiling. The reaction is once again a transesterification reaction. The exposed 5' hydroxyl group of the cleaved DNA acts as a nucleophile, attacking the phosphodiester bond between a tyrosine residue on the enzyme and the DNA. This releases the DNA from the topo and allows the re-formation of the phosphodiester
bond between the de-oxyribonucleotides. However, another mechanism is possible where a single stranded DNA segment containing a 5' hydroxyl group acts as the nucleophile and releases the DNA from the enzyme (Figure 4). The segment can then bond the original DNA which will result in strand exchange or recombination (Pommier et al., 1998).
Figure 4 Mechanism of action of topo I

The tyrosine of DNA topo I acts as a nucleophile and a transesterification reaction takes place between the hydroxyl on the enzyme and the phosphate of the sugar-phosphate backbone of the DNA. This subsequently causes strand cleavage and the strands are then free to rotate and release the accumulated supercoils whilst being held in close proximity to each other by the topo. The active site tyrosines are in equilibrium with DNA between cleaved and uncleaved sites. ATP binding causes the enzyme to undergo a significant conformational change forcing parts of the enzyme away from each other to open a gap large enough for the DNA duplex to pass through. Religation takes place as the 3' strand ending acts as a nucleophile and breaks the ester bond between the tyrosine and the DNA thus ligating the strands back together (adapted from Pommier et al., 1998.)
Figure 5 Topo II Mechanism of Action

1. DNA polymerase moves along template and causes the accumulation of positive and negative supercoils. DNA topo attracted.
2. Topo II bonds with sugar phosphate back bone by transesterification
3. ATP hydrolysis drives conformational change in topo allowing cleavage of the DNA and movement of strands to release supercoils
4. Topo ligates strands together and allows polymerase to continue replication.
1.2.3 Topoisomerases as therapeutic targets in cancer

Topoisomerases (topos) are essential DNA interacting enzymes that can be targeted in cancer chemotherapy. Altered levels of topos are seen in certain tumours. Husain et al., (1994) showed that colorectal tumours had a 5-35 fold increase in topo I protein and likewise, prostate cancers showed a 2-10 fold increase in comparison with normal tissue. This was accompanied by an 11-40 fold and 4-26 fold increase in catalytic activity in colon and prostate tumours, respectively. The increased expression of topo I was attributed to an increase in transcription or increased mRNA stability as mRNA levels were shown to be above that of normal tissue. Elevated topo I activity has also been found in cervical carcinoma compared to normal cervix. Treatment with a topo I poison can, therefore, be an effective mode of treatment and has been found to sensitise cells to radiotherapy (Chen et al., 2000). Moreover, increased topo I levels have been shown in mature residual teratoma which is usually refractory to chemotherapy so a topo I directed therapy may be beneficially incorporated into treatment (Berney et al., 2002).

Topo IIα expression is also increased in various malignancies and is often reflective of proliferative activity (Provencio et al., 2003; Skotheim et al., 2003). A study investigating topo IIα expression in breast cancer using immunohistochemistry showed no expression in non-malignant cases, whereas expression was correlated with proliferation and malignancy (Dingemans et al., 1998). A similar study in hepatocellular carcinoma confirmed that a more aggressive phenotype is often associated with higher topo IIα levels (Watanuki et al., 2002). A recent report by Geurin et al. (2003) identified topo II gene amplification as a frequent event in childhood ALL. Although a correlation with response to anthracycline or topo
directed therapies was not made, those cases that showed a low level of \textit{TOP2A} gene amplification were strongly associated with high risk and glucocorticoid resistance and poor outcome (Geurin \textit{et al.} 2003).

It can, therefore, be concluded that levels of topos, both at the gene and protein level may be relevant in the management of some human malignancies.

\textbf{1.2.4 Topoisomerase poisons}

\textbf{1.2.4.1 History of Development}
Podophyllotoxins have been used in folklore medicine since the first millennium and have been isolated from various members of the \textit{podophyllum} family for the treatment of a number of diseases, in particular condylomata acuminata and genital warts (Leiter \textit{et al.}, 1950). However, it was not until the 1950s that crude fractionation of the original compound podophyllin lead to the identification of four active compounds (Leiter \textit{et al.}, 1950). Of these compounds podophyllotoxin, \(\alpha\)-peltatin and \(\beta\)-peltatin were found to be effective against sarcomas, acute stem cell leukaemia and mammary adenocarcinomas transplanted into mice (Greenspan \textit{et al.}, 1950). Subsequently, etoposide (VP16) and camptothecin were synthesised in 1966 and teniposide (VM-26) in 1967 (reviewed in Takimoto and Thomas,, 2001).

Clinical trials in the 1970s demonstrated the antineoplastic effects of etoposide and teniposide in numerous malignancies such as AML, SCLC, gastric, breast and ovarian cancers (Radice \textit{et al.}, 1979). Etoposide was the first compound to be recognised as a topo poison and was licensed in 1983 for clinical use. It was not until 1984, however, that its mechanism of action was identified. A similar history precedes the use of camptothecin (CPT) derivatives. It was isolated from the Chinese tree, \textit{Camptotheca}
acuminata, over thirty years ago but it was only later that its topo poisoning activity became known after it had been tested in clinical trials (detailed below).

1.2.5 Currently used topo-directed drugs

1.2.5.1 Topo I targeting drugs

CPT was the first topo I poison to be identified and was discovered in 1966 during a National Cancer Institute mass screen of natural products. It entered clinical trials in the 1970s but toxicity was too great for the trials to continue. Later, in the 1980s its mechanism of action was identified and subsequent work led to the development of less toxic analogues irinotecan (CPT-11) and topotecan (Table 2). These compounds have a 5-ring structure with a chiral centre located on C-20 on the terminal lactone ring and exhibit stereospecific activity. The S-isomer is up to 100 times more biologically active than the R-isomer form. Notably, these compounds are unstable in aqueous solution because of the lactone ring. At physiological pH the less active carboxylate form is favoured whereas at higher pH, the more active form is favoured which is of significance in the bladder where it causes severe haemorrhagic cystitis.

CPT and its derivatives are thought to bind DNA at topo I cleavage sites, generally a T↓G/A site (Fukasawa et al., 1998,) and trap the enzyme whilst it is held in a complex with the DNA (the cleavable complex formation) (Jaxel et al., 1991). When these complexes are encountered by a replication fork, this may result in arrest in DNA replication and repair, and ultimately cell death (Figure 7). The main side effects of these agents are neutropaenia, nausea, diarrhoea (which may be severe and dose-limiting), fatigue, alopecia, skin rash and mucositis.
Figure 6: Structure of Camptothecin and analogues

![Camptothecin Structure](image)

Table 2: Side groups for CPT derivatives

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camptothecin</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>SN-38</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>CH₃CH₂</td>
</tr>
<tr>
<td>Topotecan</td>
<td>H</td>
<td>OH</td>
<td>(CH₃)₂NHCH₂</td>
<td>H</td>
</tr>
<tr>
<td>9-Aminocamptothecin</td>
<td>H</td>
<td>H</td>
<td>NH₂</td>
<td>H</td>
</tr>
<tr>
<td>9-Nitrocamptothecin</td>
<td>H</td>
<td>H</td>
<td>NO₂</td>
<td>H</td>
</tr>
<tr>
<td>Irinotecan (CPT-11)</td>
<td>H</td>
<td></td>
<td></td>
<td>CH₃CH₂</td>
</tr>
</tbody>
</table>
Figure 7: Cleavable complex stabilisation by topo-poisons

Topo covalently binds to DNA and forms the cleavable complex (CC). Topos cause the cleavage of one (topo I) or both (topo II) DNA strands. After allowing the DNA to unravel and releasing the torsional strain caused by DNA polymerase, topos ligate the DNA and disassociate from the DNA.

1.2.5.1.1 Irinotecan (CPT-11, Campto®)
Irinotecan is a pro-drug and needs biological activation to SN-38 (7-ethyl-10-hydroxycamptothecin) resulting in a 1000-fold increase in potency compared to irinotecan (Kawato et al., 1991). This reaction is catalysed by irinotecan carboxylesterase, found in the liver and serum (Kaneda et al., 1990). Variation in expression of this converting enzyme is observed between patients and this may influence the apparent inter-individual sensitivity to irinotecan (Van Ark-Otte et al., 1991).
1998). In 1996 irinotecan was approved in the US for use in cases of advanced colorectal cancer refractory to 5-FU. Japan has subsequently licensed the drug for use in other malignancies including gastric, ovarian and small cell lung cancers (Takimoto and Thomas, 2001). The main dose limiting toxicity is profuse diarrhoea that can be potentially life threatening and requires supportive treatment with high doses of loperamide or similar agents.

1.2.5.1.2 Topotecan (TPT, Hycamtin®)
Topotecan is a water soluble derivative of CPT. It was licensed in the US in 1996 as a second line treatment in advanced ovarian cancer and in 1998 as a second-line treatment for small cell lung cancer (SCLC). It is most commonly used in combination with platinum containing agents, as it appears to work synergistically by inhibiting the removal of platinum adducts (Takimoto et al., 1998). The main dose limiting toxicity is myelosuppression.

1.2.5.1.3 Other Derivatives
Other derivatives of CPT are currently in development. DX8951f is a water soluble compound exhibiting better topo I inhibition than topotecan or SN-38. Karenitecan (BNP1350) is another derivative of CPT that is highly lipophillic and is more stable than other analogues. For this reason it can be taken orally. It has also been shown that the carboxylate and lactone forms of the compound bind DNA without topo I. However, the influence this has on the compound’s toxicity has yet to be determined (Hausheer et al., 2001, Van Hattum et al., 2002). NB-506 has been shown to be one of the most potent topo I poisons, showing significant antitumour activity in vitro and in vivo (Yoshinari et al., 1995). NB-506 shows a different cleavage site to
camptothecin and analogues, with enhanced cleavage being seen at a C/T\rightarrow G site (Fukasawa et al., 1998). During the development of NB-506 the synthetic derivative J-107088 was discovered. J-107088 was shown to possess very strong binding to topo I and potent \textit{in vivo} antitumour efficacy in human tumour xenografts in nude mice models (Yoshinari et al., 1999).

1.2.6 \textit{Topo II targeting drugs}

Many of the topo anticancer agents approved for clinical use target type II topos. These agents include the anthracyclines doxorubicin, daunorubicin, idarubicin, and epirubicin and other classes of agents such as etoposide, teniposide and mitoxantrone. These drugs have curative potential for several haematological malignancies, such as leukaemia and also show activity in a variety of solid tumours (Table 3).

1.2.6.1 \textit{Etoposide & Teniposide}

Etoposide (VP16) was the first anticancer drug shown to work by poisoning topo II and soon after the closely related analogue teniposide (VM-26) was also developed. VP16 and VM-26 are compounds of the class epipodophyllotoxins. Both agents are used against a wide variety of neoplasms (Table 3).

Etoposide produces a 70% response in SCLC patients when used in combination with carboplatin or cisplatin. It is also an important agent in the preparation of patients for bone marrow transplants (Idhe 1992). VP-16 is used as a front line agent in SCLC, germ-cell tumours and Kaposi's sarcoma (Belani et al., 1994). VM-26 is used in drug
combination for treatment of poor prognosis ALL cases. It is also a second line drug in advanced childhood lymphoblastic lymphoma and other non-Hodgkin’s lymphomas as well as many other types of adult and paediatric tumours, such as sarcomas (Muggia 1994).

Epipodophyllotoxins stabilise topo II cleavable complexes with DNA and mediate chromosomal breakages by decreasing religation during replication. However, if religation does occur it may be between different sections (non-homologous DNA strand repair) of the DNA resulting in gene translocations and oncogenesis. This is seen after VP-16 and VM-26 treatment and can give rise to secondary leukaemias in some cases. Cases of secondary AML have been identified in patients previously treated with epipodophyllotoxins (Felix et al., 1995a). Translocations of the \textit{bcr} locus of the \textit{MLL} gene are the most commonly identified after VP16 and VM26 treatments particularly at chromosome band \textit{11q23} and \textit{21q22} which correspond to topo II cleavage sites (Felix et al., 1995b). Incidence rates range between 2 and 12\% of patients treated with epipodophyllotoxins and the mean latency period is 2 years. This risk is further increased when radiotherapy is used in conjunction with etoposide (Hawkins et al., 1992). It is possible that other genetic changes may take place in order for oncogenesis to occur, although these changes have not yet been identified (Felix 1998).
Table 3 Clinical Use of topo II poisoning agents

(Hande 1998, Kellner et al., 2000)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Trade Name(s)</th>
<th>Major Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aclarubicin</td>
<td>Aclacinomycin A</td>
<td>Acute Myeloid Leukaemia</td>
</tr>
<tr>
<td>Amsacrine</td>
<td>acridinyl anisidine</td>
<td>Acute Leukaemia</td>
</tr>
<tr>
<td></td>
<td>m-AMSA</td>
<td></td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Cerubidine</td>
<td>Acute lymphoid leukaemia*</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Adriamycin</td>
<td>Lymphomas*</td>
</tr>
<tr>
<td></td>
<td>Rubex</td>
<td>Breast Cancer*</td>
</tr>
<tr>
<td></td>
<td>Doxil</td>
<td>Sarcomas, * Kaposi's sarcoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leukaemias, ovarian cancer</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Vepesid</td>
<td>Testicular Cancer*</td>
</tr>
<tr>
<td></td>
<td>Etopophos</td>
<td>Small cell lung cancer*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymphomas</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ewing's sarcoma, Kaposi's sarcoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ovarian cancer</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>Pharmorubicin</td>
<td>Adjuvant breast cancer</td>
</tr>
<tr>
<td></td>
<td>PFS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ellence</td>
<td></td>
</tr>
<tr>
<td>Idarubicin</td>
<td>Idamycin</td>
<td>Acute myelogenous leukaemia</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Novantrone</td>
<td>Acute Leukaemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast Cancer</td>
</tr>
<tr>
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<td></td>
<td>Ovarian Cancer</td>
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<tr>
<td></td>
<td></td>
<td>Lymphomas</td>
</tr>
<tr>
<td>Teniposide</td>
<td>Vumon</td>
<td>Poor prognosis acute lymphoblastic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>leukaemia*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small cell lung cancer</td>
</tr>
</tbody>
</table>
1.2.6.2 Doxorubicin & Daunorubicin

Figure 9: Doxorubicin.

Doxorubicin (Adriamycin) and daunorubicin are members of the anthracycline family of anticancer drugs. Daunorubicin (Figure 10) was the first anthracycline to be developed but is used less widely than Doxorubicin which was developed slightly later.

As Table 3 shows, doxorubicin is used to treat a variety of cancers, generally in combination regimes. Combinations such as doxorubicin, bleomycin, vinblastine and dacarbazine is standard therapy for advanced Hodgkin’s disease with about 80% of patients achieving complete remission and 60% are cured (Hande 1998). Unfortunately, both daunorubicin and doxorubicin have serious toxicities such as myelosuppression, alopecia and mucositis following drug administration. Irreversible cardiac damage is seen with doxorubicin administration, particularly when the cumulative dose exceeds 700mg/m². This is possibly related to free-radical damage that cardiac muscle cannot repair. Pericarditis may also occur although this is less common (Doroshow 2001).

Figure 10 Daunorubicin
1.2.6.3 Mitoxantrone

Figure 11: Mitoxantrone

Mitoxantrone (MTX) is an anthraquinone compound that is less toxic than doxorubicin and daunorubicin and has therefore been incorporated into combinations of drugs in place of doxorubicin. It is used particularly in cases where there is thought to be a significant risk from the toxicities of doxorubicin or daunorubicin. MTX is also used in the treatment of leukaemia and breast cancers. However, some research indicates that MTX may also display slightly less anti-tumour activity (Hande 1998).

1.2.6.4 Other Agents in Development

ER-37328 is a novel carbazole that inhibits topo II activity through stabilisation of the topo II cleavable complexes and has been shown to have potent antitumour activity greater than that of etoposide in vitro and in vivo (Nakamura et al., 2002). Importantly, ER-37328 showed strong tumour regression in mice that received colon 38 tumour cells to the liver as colon cancers whereas both etoposide and doxorubicin only exhibited growth inhibitory activity (Nakamura et al., 2003).

Another novel topo II agent, which targets topo IIβ, is XK469. As most single topo II directed agents mediate their cytotoxic effects by preferential targeting of topo IIα, XK469 represents a new class of topo II poison. XK469 has been shown to modulate topo IIα levels. Pre-treatment with XK469 in cells that express very low levels of topo IIα was shown to induce expression of topo IIα within 24h. This effect
sensitised cells to VP16 through upregulation of the target enzyme, topo IIα. Similar findings were observed in cells obtained from CML patients with undetectable levels of topo IIα where treatment with XK469 induced expression and gave rise to sensitisation to VP16 (Mensah-Osman et al., 2002). Therefore, this agent may be of use in tumours expressing low levels of topo IIα when used in combination with topo II poisons.

1.2.7 Dual Topo Inhibitors

1.2.7.1 Rationale for developing dual poisons
Topo I and II are considered clinically relevant targets for cancer chemotherapy as both are important for DNA replication and transcription. Resistance to topo poisons has been linked to decreased expression of the topo target (see section 311.2.3). The decrease in one topo can often be compensated for by increased levels of another. In order to overcome this, inhibition of topos I and II by sequential or simultaneous use of topo I and II poisons has been explored. Pre-clinical research showed sequential administration of CPT-11 and doxorubicin, potentiated cytotoxicity in human tumour xenografts in nude mice (Kim et al., 1992). Similarly, sequential treatment of topotecan followed by etoposide, a topo II poison, resulted in a synergistic response both in vitro and in vivo in human colon cancer xenograft models (Whitacre et al., 1997). Other studies have demonstrated that sequential treatment with a topo I and II poison may be of benefit in cancer treatment whereas simultaneous application of the same drug regimens is not as effective (Chen et al., 2002, Kaufmann 1991).

Clinical trials with sequential application of topo I and II poisons have also been carried out. A phase I clinical trial of sequential CPT-11 and etoposide in metastatic non-small cell lung cancer showed disappointing results with considerable toxicity
and limited efficacy (Ando et al., 1997). However, a phase II trial, again in metastatic non-small cell lung cancer, using the same regimen at different doses showed an overall response rate of 21% with acceptable toxicity (Oshita et al., 1997). Other clinical trials using topotecan or irinotecan and etoposide have shown good responses but with severe side effects (Herben et al., 1997, Hammond et al., 1998, Oshita et al., 1997). A more positive result was seen in a phase I trial of sequentially administered doxorubicin and topotecan in refractory solid tumours. 6 out of 17 patients had a partial response and overall the toxicity (chiefly neutropaenia) was manageable (Seiden et al., 2002).

Overall, it appears that sequential treatment with topo I and II poisons can be advantageous over single agents, however toxicity does appear to be increased. This may be because of other mechanisms of cytotoxicity caused by topo poisons such as free radical generation and covalent binding to DNA which may limit the therapeutic window to a critical degree (Vasey and Kaye, 1997).

Selectively targeting topo I and II, without the complication of other cytotoxic mechanisms, may result in improved antitumour activity with limited toxicity. It is hoped that this can be achieved by the use of single agents that can stabilise topo I and II cleavable complexes. Currently, dual poisons are in development which inhibit topos I and II simultaneously. It is hoped that these agents will have increased efficacy without some of the more serious toxicities. Some of these agents are discussed below.
1.2.7.1 **Intoplicine (RP60475)**

Intoplicine was one of the first anticancer agents found to inhibit the relaxation and decatenation activities of topo I and II (Riou *et al.*, 1993). Intoplicine mediates topo I and II induced DNA single-strand and double stranded breaks and its antitumour activity has been attributed to topo inhibition (Poddevin *et al.*, 1993, Riou *et al.*, 1993). When tested *in vitro*, it was found to have a broad spectrum of activity particularly against cell lines derived from the breast, non-small cell lung cancer and the ovaries (Eckardt *et al.*, 1994). Phase I trials indicated that myelosuppression would not be the main dose limiting toxicity but intoplicine has also been shown to cause significant liver toxicity (Abigerdes *et al.*, 1996, Van Gijn *et al.*, 1999).

1.2.7.2 **TAS-103**

![TAS-103](image)

**Figure 12 Tas-103**

Tas-103 (Figure 12) is described as a dual topo I and II targeted drug. However, it is not a strict dual topo poison as it only affects topo I as a secondary effect due to the intercalation of the DNA at high concentrations and effects on DNA topology. It has been shown to catalytically inhibit topo II and block the religation of DNA strands in topo II cleavable complexes (Wilson *et al.*, 1999, Fortune *et al.*, 1999). P-glycoprotein (Pgp) and multidrug resistance protein (MRP), which are possible causes of resistant phenotypes, do not recognise Tas-103 as a substrate (Minderman *et al.*, 2000).
Another dual inhibitor that is under development is F11782 (Kruczynski et al., 2000). This pentafluorinated epipodophyllloid (Figure 13) is less potent \textit{in vitro} than other dual topo poisons but displays promising results \textit{in vivo} in human tumour xenografts (Kruczynski et al., 2002). The drug inhibits the catalytic activity of both topo I and II. However F11782 also appears to have a unique mechanism of action. It is possible that F11782 can induce a conformational change in the topo II reaction cycle such that it resembles the ATP-bound form of the enzyme, resulting in an inhibition of DNA binding. The ability of F11782 to form complexes in an ATP-independent fashion sets this agent apart from other agents in this class (Jensen et al., 2003). It is a potent inhibitor of nucleotide excision repair and could be useful in potentiating the cytotoxicity of DNA cross-linking agents such as cisplatin (Barret et al., 2002a). Synergistic effects have been demonstrated in A549 human non-small cell lung cancer cells and GCT27 human testicular teratoma cells, with cisplatin, mitomycin C, etoposide and doxorubicin (Barret et al., 2002b).
1.2.7.4 DACA

Figure 14 DACA

DACA (XR5000) was originally described as an intercalating topo poison which primarily stabilises cleavage complexes in topo IIα cleavable complexes primarily, and also some topo IIβ and topo I complexes at high concentrations (Bridewell et al., 1999). Subsequently, the involvement of topo IIβ and topo I in the mechanism of action of DACA become less certain (Padget et al., 2000; Denny and Baguley 2003). In studies using a cell-free system the drug concentration needed for suppression of topo II was shown to be higher than that for topo I. Direct inhibition of topo II by ICRF-187 did not significantly reduce the cytotoxic properties of DACA in vitro which would be expected if its main mechanism of action was mediated by topo II (Pastwa et al., 1998). However, RNA synthesis is inhibited at lower concentrations of DACA than other topo II poisons suggesting another mechanism of cell kill. DACA is still active in Pgp-expressing P388 cells and in Jurkat cells that exhibit lower expression levels of topo II. In 3 phase II studies involving ovary, glioblastoma and non-small cell lung cancer treatment with DACA showed a number of minor responses but the overall response was not sufficient to warrant further studies (Denny and Baguley 2003).

1.2.7.5 XR5944 & XR11576

These compounds were originally described as dual topo I and II poisons as they stabilise both topo I and II cleavable complexes using the Trapped In Agarose DNA...
Immunostaining (TARDIS) assay (Jobson et al., 2002). Both compounds have recently entered clinical trials in Europe.

**Figure 15 XR5944**

XR5944 is a *bis*-phenazine that stabilises DNA complexes with topo I and II. It has shown good efficacy against implanted human tumour xenografts in nude mice and appears to be more potent than Tas-103. The efficacy of XR5944 was shown to be compromised by the multidrug resistance proteins Pgp and MRP. However, it continued to show efficacy in cells with atypical resistance such as changes in topos (Stewart et al., 2001). Recent research using mutant yeast strains suggests XR5944 may have a novel mechanism of action in addition to that of inhibiting topos. Studies have shown that sensitivity to XR5944 is conferred by mutations in ribosomal proteins, RNA processing proteins or RNA polymerases (Fleming et al., 2003). It is unclear at this stage whether stabilisation of cleavable complexes occurs as a result of RNA interference or is responsible for genomic errors in RNA processing proteins.

**Figure 16 XR11576.**
In preliminary studies, XR11576 was shown to be a potent inhibitor of purified topo I and IIα (Mistry et al., 2002). Like XR5944, it stabilises DNA-topo cleavable complexes. As yet, no resistance has been observed in Pgp or MRP expressing cell lines and XR11576 has shown high potency against a panel of murine and human cancer cell lines (Mistry et al., 2002) and efficacy when administered orally or intravenously against a number of human tumour xenografts in nude mice.
1.3 Hypothesis:

It is suggested that XR5944 and XR11576 will be more effective than agents that target just one form of topo, in promoting cytotoxicity of cancer cells due to their dual activity against topos I and II. Additionally, that they will be effective in cancer cells with resistance to single agents as they are able to target more than one topo simultaneously. As a consequence of this, they will induce greater levels of DNA damaging and cell killing effects than single topo poisons.

1.4 Outline of study

This hypothesis was tested by examining the cytotoxicity of XR5944 and XR11576, two novel dual topo I and II poisons, in comparison with currently used single acting agents such as CPT and VP16. This was initially assessed by cytotoxicity testing for XR5944 and XR11576. The potency of the drugs was determined when presented with a variety of clinically relevant resistance mechanisms. These included ABC-transporter proteins, which have been implicated in the efflux of drugs from cancer cells, and also atypical drug resistance as a result of decreased expression of one or more of the target topo enzymes.

Secondly, the nature of the interaction of the drugs with cellular DNA was also examined and the extent of the resulting DNA damage assessed. It has been reported that numerous topo poisons work by stabilising DNA-topo interactions and prevent the disassociation of the enzyme and thereby creating an obstruction to replication complexes. The extent of DNA-topo interactions was examined and also the subsequent DNA damage caused by the arrest of DNA replication.
Topo poisons have been shown to cause cell cycle perturbations and cell death by activation of apoptotic pathways. Therefore, cell cycle patterns were examined before and after treatment with topo poisons and any disturbances noted. Furthermore, expression of proteins such as p53 that are activated in response to DNA damage was explored. p53 expression is induced in response to DNA damage and transactivates other proteins resulting in DNA repair, cell cycle arrest and apoptosis.

Finally, detection of factors involved in apoptotic pathways leading to cell death was carried out. Expression of apoptotic proteins such as Bcl-2 family members and cleaved PARP was detected by Western blotting. Caspase activation was also measured by flow cytometry and Western blotting.
CHAPTER 2

Materials and Methods
2 Materials and Methods

2.1 Chemical and Cytotoxic Drugs

All chemicals and reagents were of the purest form available and of analytical grade. Etoposide (VP-16), camptothecin and doxorubicin were obtained from Sigma Aldrich (Poole). XR5944, XR11576 and XR9576 were all provided by Xenova PLC (Slough). Staurosporine was obtained from Alexis Corporation (Nottingham). Doxorubicin was made up in sterile distilled water and stored as frozen stock solutions in aliquots. All other compounds were made up in DMSO and stored as frozen aliquots prior to use.

2.2 Cell Culture

All cell culture materials were purchased from Sigma Aldrich (Poole, UK) unless stated otherwise. Foetal calf serum (FCS) was purchased from Invitrogen, Paisley and heat inactivated at 55°C for 30 mins. Human ovarian carcinoma PEO1 cells (obtained from Professor Fran Balkwill, ICRF, London), HL60 human promyelocytic leukaemia and K562 human chronic myelogenous leukaemia cells (both obtained from Jean Sargent, Pembury, Kent) were grown in RPMI-1640 Hepes modified media with 2mM glutamine, 10% FCS and antibiotics (500IU of Penicillin, 50µg/ml neomycin, 100µg/ml streptomycin) at 37°C/5%CO₂ in a humidifying incubator. MCF-7 and MDA-MB231 human breast adenocarcinoma cells (both obtained from ECAAC) were grown under the same conditions with DMEM Hepes modified media with 2mM glutamine, 10% FCS and antibiotics as before.

Drug-resistant cell lines with acquired resistance to topoisomerase inhibitory agents were generated by growth in increasing increments of the appropriate selecting agent...
on successive passages (by Dr Helen Coley, University of Surrey). PEO1 cells with VP16 resistance, designated as PEO1VP16R, were maintained in 0.2\(\mu\)M VP16, with the camptothecin-resistant variant PEO1CamR being maintained at 3\(\mu\)M camptothecin. VP16 resistant MB-231 cells, designated MB231VP16R, were maintained in 1\(\mu\)M VP16. K562AR and HL60AR were maintained in the presence of 100\(\mu\)M and 50\(\mu\)M doxorubicin, respectively. Cells were grown in drug free media for at least one passage before experimental use.

CHO-K1 and mutant hypersensitive derivatives of the parental line Chinese hamster ovary CHO line (obtained from Dr Jeff Cummings, ICRF, Edinburgh) were grown in Ham's F10 modified media with 2\(\mu\)M glutamine, 10\% FCS and antibiotics as before, under the previously described culture conditions. Mutant hypersensitive cell lines ADR-1, ADR-3 and ADR-6 were originally isolated from parental CHO-K1 treated with point mutagen ethyl methanesulphonate for 24 h (Robson et al., 1987, Davies et al., 1990). ADR-R cells were obtained by exposure of CHO-K1 cells to increasing concentrations of doxorubicin over several months (Hoban et al., 1992). Cells were routinely tested for mycoplasma using a PCR based method and only cells found to be negative were used for experiments.

2.3 MTT cytotoxicity testing

Colorimetric 96-well based assays using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Aldrich, Poole, UK) were carried to determine cell growth in the presence of various anti-cancer agents. The MTT assay was performed as described by Mosmann (1983) and modified by Twentyman and Luscombe (1987). Tumour cells in suspension were seeded at between 1 and 3 \(\times\) 10^4
cells/ml depending on cell doubling times, into a 96 well plate, left for 24 h at 37°C/5% CO₂, and then 50μl of the appropriate drug dilution in increasing amounts, or control medium was added. Trays were incubated for a period allowing an increase of 10-20 times the original cell number in untreated control cell wells. This was followed by the addition of 125μg of MTT dissolved in 25μl of PBS. After 4h of incubation at 37°C the media was either removed by careful aspiration in the case of monolayer cultures, or following centrifugation of the plates at 1000g for 5mins. The resulting tetrazolium crystals were resuspended in 200μl of DMSO and dissolved by careful agitation for 5 mins. The resulting absorbances were read at 540nm using a Multiskan RC spectrophotometer (Labsystems, UK) with Genesis 3.05 Software (Life Sciences, UK). Cell viability was assessed based on mean absorbance values at a particular drug concentration expressed as a fraction of the mean control well absorbance values. The IC₅₀ concentration was determined by finding the drug concentration at which 50% of the cells were viable relative to the untreated control wells.

2.4 RT-PCR

2.4.1 RNA Extraction

Total RNA was extracted from cells using 1ml Triazol nucleic acid extractor (Invitrogen, Paisley,) per 5-10 × 10⁶ cells. This was left to incubate at room temperature for 5mins to allow complete disassociation of nucleoprotein complexes. Subsequently, 0.2mls chloroform for each ml of Triazol was added to the suspension and mixed vigorously for 15secs and further incubated for 2-3mins at room temperature. Samples were then centrifuged at 16,000g at 4°C on a Eppendorf
centrifuge 5415R for 15mins and separated into a lower red phenol-chloroform phase and a colourless upper aqueous phase which was decanted into clean tubes. 0.5ml of isopropanol per 1ml of Triazol used for initial homogenisation, was added and mixed by inversion. Samples were incubated at room temperature for 10mins then centrifuged at 16,000g for 10 mins at 4 °C. The RNA precipitate formed a gel-like pellet, which was washed in 75% ethanol and re-dissolved in RNase-free water.

2.4.2 cDNA Synthesis

cDNA was synthesised with 500ng of total cellular RNA quantified with an RNA quantitation kit (Sigma Aldrich, Poole). 50ng random hexamers, 10nM mixed dNTPs, 0.1pM MgCl₂, 200 units of Superscript II® (Invitrogen, Paisley, UK), and 2µl of buffer (containing 50mM Tris-HCl (pH 8.3) 7mM MgCl₂, 40mM KCl, 10mM DTT, 0.1 mg/ml BSA, 0.025mM oligo (dT)₅₀, 0.25mM poly(A)₄₀₀ and 0.01% NP-40.®), and 40 units recombinant ribonuclease inhibitor was added. The reaction mix was left at room temperature for 10mins, followed by 50mins at 42°C. The reaction was terminated by incubation at 70°C for 10mins. 2 units of RNase H were added to the final mix to remove RNA and left to incubate at 37°C for 20mins.

2.4.3 PCR

Two µl of cDNA was added to 5µl of buffer containing 500mM KCl, 100mM Tris-HCl (pH 9.0 at 25°C) and 1.0% Triton® X-100., 25mM MgCl₂, 10nM mixed dNTPs, 1 µl of each sense and antisense primer (10µM). 2 units of Taq polymerase (Promega, Southampton) was used to amplify the target regions. Oligonucleotide sequences for the MDR1 and MRP genes were taken from Noonan et al., (1990). Topoisomerase primers were designed using Pubmed and Genosys Oligomail and were as follows.
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For human derived cell lines, primers for:

**MRP** sense TCTCCCGACATGACCGAGGCT, antisense
GAATATGCCCGACTTCTTTCC, product size 140bp.

**Pgp** sense CCCATCATTGCAATAGCAGG, antisense
GTTCAAACCTTCTGCTCCTGA, product size 167bp.

**Topo I** sense GCCAGTATTTCAAAAGCCC, antisense
TTGATGATTATATCCTCGGG, product size: 254 bp.

**Topo II** sense ACCATTGCAGCCTGTA, antisense GCTCTTCCCCATATTATCC,
product size 210bp.

**Topo II**β sense ACAGGTGGTGCTATG, antisense GTTTCACCTGACACC
product size 205bp.

**Beta globulin** sense ACCCCCACTGAAAAAGATGA, antisense
ATCTTCAACCTCCATGATG, product size 120bp.

For hamster derived CHO cell lines primer sequences were as follows, for topo I
sense AAAGGTGAGAAAGACTGGC, antisense CTCTAAGAGAAACAGCAACCC,
product size 232 bp, for topo IIα sense GGTACTATTGAAGAGCTGGC, antisense
TCAGTACCATTCAACATGCG, product size 163 bp, for topo IIβ sense
AGAAGAGCATATGATTTGCG, antisense TCATGAATAACTTTGAGGCG,
product size 158 bp, for beta actin sense TTCTATGAGCTGAGTCTCCC, antisense
CTGAATACACACTCCAGGCG, product size 203 bp.

PCR using a Techne Touchgene PCR machine was performed with an initial
denaturing of 10 mins at 72°C followed by 35 cycles of PCR of 45 seconds at 94°C
for the elongation step, 45 seconds at 54°C and 90 seconds at 72°C. This was
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followed by 10mins incubation at 72°C. PCR using topo primers was carried out in the same manner as described above except for the annealing temperature which was raised to 58°C.

RT-PCR was also carried out on cellular RNA extracted from CHO-K1 and mutant hypersensitive cell lines in exponential growth. 35 cycles of PCR were performed using oligonucleotide primers designed for amplification of hamster beta-actin as a ubiquitously expressed house-keeping gene, topoisomerase I and topoisomerase IIα and IIβ. PCR products were run on 1.2% agarose gels at 100V containing 0.2μg/ml of ethidium bromide (Sigma Aldrich, Poole) for 1h.

2.5 Western blot analysis

2.5.1 Membrane Preparations

Crude cell membrane preparations were made from cells by lysing cells with 250μl of lysis buffer containing 1mM Phenylmethlysulphonyl fluoride (PMSF), 1mM sodium vanadate (NaVO₄), 10μg of aprotinin and leupeptin, 150mM NaCl, in 10mM Tris buffer (pH 7.4). The cells were left to lyse for 30mins on ice and then sonicated at 18 set for 10 seconds. A 10 minute centrifugation at 450g was carried out, followed by a further ultraspin of 60,000g for 60mins using a Beckman L7-65 ultracentrifuge, both of which were at 4°C. The pellet was finally resuspended in 200μl of freshly made lysis buffer with 0.2% SDS.
2.5.2 Cytosolic Preparations

Cytosolic preparations were made from cells by lysing cells using 250μl of hypotonic lysis buffer containing 1mM PMSF, 1mM NaVO₄, 10μg of aprotinin and leupeptin, 150mM NaCl, in 2mM Tris buffer (pH 7.5). The cells were left to lyse for 30mins and then sonicated at 18 set for 10 seconds using a MSE Soniprep 150. A 10 minute centrifugation at 60g was performed at 4°C. The pellet was finally resuspended in 200μl of freshly made lysis buffer with 0.2% SDS.

Whole cell lysates prepared for apoptosis experiments were treated at an IC₈₀ concentration calculated after 72 h drug exposure. PEO1 cells were treated with 5μM VP16, 100nM XR5944 or 200nM XR11576 for 16-48 h. Staurosporine was used as a positive control by treating cells with a concentration of 1μM for 48 h. Detection of p53 and p21^{WAF-1/Cip1} was carried out on MCF7 cells were treated with an IC₅₀ concentration (based on MTT data obtained following 72h drug exposure) for 16-48h. The concentrations used were as follows, doxorubicin 225nM, XR5944 30nM and XR11576 60nM. A positive control was used to detect phosphorylated p53. This was obtained by treating MCF7 cells with 65μM of VP16 (Autogen Technical Support, Calne, UK).

2.5.3 Nuclear Preparations

Trypsinised cells were washed in PBS and then lysed in 200μl of nuclear buffer (30mM Tris (pH 7.5) 1.5mM MgCl₂, 10mM KCl, 1mM Phenylmethylsulphonyl fluoride (PMSF), 50μg/μl leupeptin and aprotinin, 1% Triton X-100 v/v). Samples were vortexed and left on ice for 5 mins. Samples were then centrifuged at 16,000g...
for 1.5 mins. The supernatant (cytosolic fraction) was incubated with 0.25 mg/ml RNase, 0.22% SDS and 1.2 mM DTT. The pelleted nuclei were resuspended in 0.2% SDS, 10 mM DTT, 50 mM Tris (pH 7.5), 5 mM MgCl$_2$, 1 mM PMSF, 50 µg/µl leupeptin and aprotinin. Both fractions were incubated at 4°C for 60 mins with constant rotation and vortexed every 15 mins.

### 2.5.4 PARP Lysates

Monolayers of cells were scraped and washed in cold PBS. Pelleted cells were then resuspended in 250 µl of extraction buffer (62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS). Samples were sonicated on ice for 20 secs at 18 set.

### 2.5.5 Western Blotting

Protein concentrations were determined based on the Lowry method using the Bio-Rad DC protein assay detection kit.

Western blotting reagents were purchased from Invitrogen (Paisley). 40 µg of protein were run with 5 µl of loading buffer (0.5 M Tris, 4% SDS, 2% DTT, 1% Glycerol v/v, 5 mM PMSF, 0.5 µg Bromophenol blue, (pH 8.1)) on SDS Bis-Tris gels in MES buffer (50 mM morpholinoethane sulphonic acid, 50 mM Tris, 0.1% SDS, 1 mM EDTA (pH 7.3)) for 45 mins at 200 V. Gels were blotted for 2 h onto nitrocellulose membrane in transfer buffer (25 mM Bicine, 25 mM Bis-tris, 1 mM EDTA, 0.05 mM chlorobutanol (pH 7.2)) at 30 V and blocked for at least 1 h in milk block (1% milk, 1% Bovine serum albumin, 0.1% Tween 20 in PBS). Membranes were gently rotated with the appropriate primary antibody (Table 4) with an overnight incubation at 4°C. Membranes were washed in PBS with 1% Tween 20 (PBST) for up to 30 mins and
then incubated with secondary horseradish peroxidase (HRP) linked antibodies for one hour with constant rotation. An anti-goat HRP-conjugated antibody (Dako, Cambridgshire, UK) was used at a 1 in 1000 concentration. Anti-rabbit and anti-mouse HRP-conjugated antibodies (Sigma, Poole, UK) were used at 1 in 3000. The membranes were washed again in PBST. Signals were detected using chemiluminescence reagents (Supersignal, Pierce).

### Table 4 Primary Antibodies

<table>
<thead>
<tr>
<th>% Acrylamide in SDS Page Gel</th>
<th>Sample Preparation</th>
<th>Primary Antibody &amp; Dilution</th>
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</thead>
<tbody>
<tr>
<td>10</td>
<td>Membrane</td>
<td>MRP (C-20), Santa Cruz 1:100</td>
</tr>
<tr>
<td>10</td>
<td>Membrane</td>
<td>MDR1 (H241), Santa Cruz 1:100</td>
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<tr>
<td>3-8</td>
<td>Nuclear</td>
<td>Topo I (sc-5342), Santa Cruz 1:100</td>
</tr>
<tr>
<td>3-7</td>
<td>Nuclear</td>
<td>Topo IIα (sc-5346), Santa Cruz 1:100</td>
</tr>
<tr>
<td>3-8</td>
<td>Nuclear</td>
<td>Topo IIβ (sc-13059), Santa Cruz 1:100</td>
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<tr>
<td>10</td>
<td>PARP lysates</td>
<td>PARP (C-210) Alexis 1:500</td>
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<tr>
<td>12</td>
<td>Whole cell lysate</td>
<td>BID (R&amp;D Systems) 1µg/ml</td>
</tr>
<tr>
<td>12</td>
<td>Whole cell lysate</td>
<td>Bax (P-19) Santa Cruz 1:100</td>
</tr>
<tr>
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<td>P53 (DO-1) Santa Cruz 1:1000</td>
</tr>
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<td>10</td>
<td>Whole cell lysate</td>
<td>Phosphorylated p53 (sc-7997) Santa Cruz 1:200</td>
</tr>
<tr>
<td>4-12</td>
<td>Whole cell lysate</td>
<td>P21 (sc-397G) Santa Cruz 1:100</td>
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<tr>
<td>12</td>
<td>Whole cell lysate</td>
<td>Bcl-XL (H-5) Santa Cruz 1:100</td>
</tr>
<tr>
<td>12</td>
<td>Whole cell lysate</td>
<td>Bcl-2 (C-2) Santa Cruz 1:100</td>
</tr>
<tr>
<td>10</td>
<td>Whole cell lysate</td>
<td>Caspase-8 (Ab-3) Oncogene 1:100</td>
</tr>
<tr>
<td>Various</td>
<td>Whole cell Lysate or Nuclear</td>
<td>Actin (Ab-1) Oncogene 1:10 000</td>
</tr>
</tbody>
</table>
Chapter 2 Materials and Methods

2.6 DNA Protein Cross-Links

The method used was modified from that described by Zhitkovich & Costa (1992). Cells were seeded at $3 \times 10^4$ cells/ml into 80cm$^2$ flasks and left for 4h at 37°C/5% CO$_2$. DNA was labelled with 0.5mCi/ml of media of tritiated thymidine (specific activity 20 Ci/mmol) for 48h after which time the media was removed and replaced with fresh. After 2h, cells were treated with drugs and left at 37°C. Samples for the 4h time point were treated with the IC$_{50}$ concentration of drug obtained after 24h drug exposure using the MTT assay (VP16 24µM, XR5944 200nM or XR11576 300nM). 24, 48 and 72 hour samples were treated with VP16 0.5µM, XR5944 7nM or XR11576 30nM which were approximately twenty five per cent (or less in the case of VP16), of the IC$_{50}$ concentration obtained following 72h continuous drug exposure using the MTT assay. Control flasks were treated with media alone. To harvest, cells were removed from the surface of the flasks by scraping and the resulting slurry was then centrifuged, washed twice in PBS and an aliquot taken to determine cell number using a counting chamber. An equal number of cells were lysed in 1.5% SDS, 20mM Tris-HCl and 1mM PMSF for each sample compared with control cells. To detect crosslinks, samples were heated to 65°C and vortexed. DNA-protein complexes were resuspended in 500µl of 200mM KCl in 20mM Tris (pH 7.5) by passing the sample through 1000µl pipette 5 times. After cooling on ice for 5mins the samples were centrifuged at 4°C for 5mins at 8000g. Each pellet was then resuspended in 1ml of 100mM KCl in 20mM Tris (pH 7.5) and passed through a pipette 5 times. After heating for 10mins at 65°C to fully dissolve the precipitate, samples were left on ice for 5mins and centrifuged at 8000g. The samples were washed twice more in the same manner in 100mM KCl in 20mM Tris. Finally, the pellet was resuspended in 1
ml of water and this was added to 4mls Optiphase Safe scintillant. (Fisher Scientific, Loughborough) disintegrations per minute (DPM) were counted over one minute and treated samples compared with control untreated samples.

2.7 Comet Assay

The comet assay is designed to detect single strand breaks under alkaline conditions (Figure 17). MB231 cells were treated with IC_{50} concentrations obtained after 24h drug exposure of VP16, XR5944 or XR11576 for 4h at 37°C/5 % CO_2 (VP16 24μM, XR5944 200nM, XR11576 300nM). Cells were scraped, washed in PBS and embedded in an agarose sandwich on warmed glass slides pre-coated with 1% agarose. The sandwich consisted of a first layer of 5% agarose, second layer 1.7% agarose with mixed with cells, final layer of 0.8% agarose. Slides were submerged in cold lysis buffer (2.5M NaCl, 100mM EDTA disodium salt, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 8.0) for one hour at 4°C and protected from light. Under red light, slides were transferred to a horizontal electrophoresis tank, covered in running buffer (0.3 M NaOH, 1 mM EDTA pH 13), and incubated at 10°C for 40 mins to allow unwinding of the DNA. Electrophoresis was then carried out at 0.8V/cm and 300 mA for 36mins. After electrophoresis, slides were neutralised with Tris buffer (0.5M p.H 7.5) and stained with ethidium bromide (20ng/ml). Images were visualised by fluorescent microscopy using a Leitz Laborlux S microscope. DNA damage was expressed as comet tail length (μm) using the Komet 3.1 image analysis program (Kinetic Imaging, Liverpool). Comet tail length was measured from the edge of each nucleus to the end of the tail. 50 comets were counted as a data point, 25 from two duplicate slides.
Figure 17 Comet Assay

Pre-treated cells are embedded in an agarose sandwich and then lysed. DNA is left to unwind and then migrates with the application of an electric current. DNA is visualised with propidium iodide staining and viewed under UV light. Damaged DNA is carried further than intact DNA giving the appearance of a 'comet tail' behind the main nucleus of the cell. The Comet tail length can be measured quantitatively as a measure of DNA damage.
2.8 **Microscopic Analysis Using Hoechst 33258**

2.5 x 10^4 cells/ml were grown in 8-well chamber slides overnight at 37°C/5%CO_2_. Drug was then added to wells in duplicate. After incubation with drug, non-adherent cells were removed and centrifuged. The chamber was then removed from the slide and the slide gently washed in PBS. Cells were fixed in 3% formaldehyde in PBS at 4°C for at least 24h. Cells were stained in 100nM bis-benzimide trihydrochloride (Hoechst 33258) (Sigma, Poole, UK) in PBS for 10mins, washed briefly in PBS and viewed under UV light (>420nm emission, 364nm excitation).

2.9 **Flow Cytometry**

2.9.1 **Propidium Iodide Staining**

4 x 10^4 cells/ml were left to adhere in flasks for 4-6h. Cells were then treated with drug and harvested at the appropriate time. Cells were removed by trypsinisation, centrifuged and the pelleted cells washed in PBS before resuspension in 2mls 70% ethanol in PBS, added whilst vortexing. Samples were left at 4°C for at least 24h. After washing in PBS, cells were stained in 33μg/ml propidium iodide (Sigma, Poole, UK) and 1mg/ml Ribonuclease A (Sigma, Poole, UK) for at least 30mins at 37°C in the dark. Fluorescence >575nm versus light scatter was measured with an excitation of 488nm on a Beckman-Coulter Epics XL™.

2.9.2 **TUNEL Assay**

The terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labeling (TUNEL) assay is designed to measure fragmented DNA cleaved as part of the apoptotic response. Terminal Deoxynucleotidyl transferase (TdT) labels the 3'-OH
termini of cleaved DNA with modified nucleotides (dUTPs). These nucleotides are then conjugated with FITC and detected by flow cytometry (excitation 488nm, FL1 525nm). FITC fluorescence increases proportionately to the increase in damaged DNA.

4 × 10⁴ cells/ml were left to adhere in flasks for 4-6h. Cells were then treated with drug and harvested at the appropriate time. Cells were washed in cold PBS and fixed in 1% formaldehyde for 15mins on ice. Cells were then centrifuged and resuspended in cold 70% ethanol and stored at -20°C for at least 24h. Cells were analysed for apoptosis using a TUNEL Apop-Tag kit (Flowgen, Leicestershire). Cells were centrifuged and the pellet equilibrated in the manufacturers buffer. The cells were centrifuged again and the pellet resuspended in fluorescein-12 dUTP and TdT containing solution for at least 30mins at 37°C with occasional vortexing and protection from light. The reaction was terminated by adding the manufacturers stop wash buffer reagent. Samples were analysed on a Beckman-Coulter Epics XL flow cytometer (excitation 488nM, fluorescence >525nM) and a Zeiss LSM 510 Meta confocal microscope.

2.9.3 Annexin V staining

An Annexin V-FITC conjugated apoptosis detection kit was used as described by the manufacturers protocol (Oncogene, CN Biosciences, Beeston, UK). 1 × 10⁶ cells were harvested by trypsinisation, washed twice in cold PBS and resuspended in 500µl of the manufacturers binding buffer. 1.25µl of FITC conjugated Annexin V was added per sample giving a final concentration of approximately 2µg/ml. Samples were incubated in the dark for 15mins at room temperature and then centrifuged.
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Cells were resuspended in 500μl of cold binding buffer with 0.6µg/ml of propidium iodide. Samples were analysed by flow cytometry.
CHAPTER 3

Activity of XR5944 and XR11576 in sensitive and topoisomerase-drug resistant cancer cell lines
3 Activity of XR5944 and XR11576 in drug sensitive and topoisomerase- drug resistant cancer cell lines

3.1 Introduction

Topo poisons are an effective group of antitumour agents that display a wide spectrum of antitumour activity as discussed in Chapter 1. However, their efficacy can be affected by the expression of a variety of cellular proteins such as multidrug resistance protein (MRP) and P-glycoprotein (Pgp). Furthermore, other factors contribute to topo drug resistance such as changes in expression of apoptogenic proteins, altered drug metabolism and decreased expression of the drug's target (reviewed in Larsen and Skladanowski, 1998).

3.1.1 Mechanisms of Resistance- ABC Transporters

ATP binding cassette (ABC) transporters play an important role in protecting the body from cytotoxins in some areas such as the brain, testis and placenta (see Table 5). Multidrug-resistance (MDR) in tumour cells is associated with an increase in transmembrane ABC transporter expression. These transporters actively secrete administered cancer drugs such as doxorubicin and Taxol into the gastrointestinal tract and reduce their efficacy (Sparreboom et al., 1997). Moreover, they are responsible for the export of chemotherapeutic agents from tumour cells and prevent cytotoxicity as the drug is actively transported from the cancer cells.
**Table 5 ABC Transporters**
(Tan et al., 2000, Thomas and Coley, 2003)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size</th>
<th>Location</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pgp</td>
<td>170kDa</td>
<td>Colon, small intestine, adrenal and hepatic tissues. Blood brain barrier.</td>
<td>Anthracyclines, taxanes, teniposide, vinblastine, vincristine, topotecan, etoposide, mitoxantrone,</td>
</tr>
<tr>
<td>MRP</td>
<td>190kDa</td>
<td>Plasma membrane, endoplasmic reticulum.</td>
<td>Anthracyclines, methotrexate, vincristine, etoposide</td>
</tr>
<tr>
<td>BCRP</td>
<td>72kDa</td>
<td>Placental syncytiotrophoblasts, blood-brain and blood-testes barriers, some breast cancer cell lines.</td>
<td>Anthracyclines, Mitoxantrone, Topotecan, SN-38</td>
</tr>
</tbody>
</table>

### 3.1.1.1 Pgp

P-glycoprotein (P for permeability) is a 170kDa membrane glycoprotein, and was the first ATP-binding cassette protein (ABC protein) to be described. The protein is encoded on the *MDR-1* gene located on chromosome 7 (Weinstein et al., 1990). Pgp function requires ATP and its depletion causes the accumulation of substrates within the cell (see Table 5). One of the significant characteristics of Pgp is its broad recognition of substrates. It has been suggested that several hundred compounds could be recognised by Pgp, nearly all of which are hydrophobic (Litman et al., 2001).

Pgp is expressed heterogeneously in normal tissues and in particularly high levels in the endothelium of the blood-brain barriers, adrenal cortex and proximal renal tubules (see Table 5). Increased Pgp expression is seen in a variety of cancers such as leukaemias, breast tumours and Kaposis sarcomas where it is thought to be
responsible for decreased intracellular drug accumulation (Weinstein et al., 1990). In a study of paediatric tumours, Pgp expression correlated with an increase in the probability of relapse after a clinical response to treatment whereas lack of Pgp expression correlated with better responses and increased survival (Chan et al., 1990).

In another study investigating acute myeloid leukaemia (AML) an 81% complete remission rate was seen for Pgp negative cases compared with 12% for Pgp positive cases (Leith et al., 1999).

3.1.1.1.1 Pgp Modulators

Drug efflux mediated by Pgp can be modulated by agents that block membrane transport. These modulating agents, therefore, potentiate the cytotoxicity of anticancer compounds by allowing their intracellular accumulation. Membrane active agents such as verapamil, cyclosporin A and calmodulin antagonists modulate Pgp but have also shown unacceptable toxicity in clinical trials due to the high concentration of compound necessary to inhibit Pgp. For example, cardiac arrhythmias were noted in the majority of patients treated with verapamil, the first compound to achieve a reversal of multi-drug resistance due to Pgp expression (Durie and Dalton 1988, Dalton et al., 1989). In general, severe side effects are noted when these modulators are used in combination with anticancer drugs as a result of altered pharmacokinetics of the latter. Consequently, dose-reduction is often necessary and this results in a reduction in efficacy of the anticancer agents (reviewed in Sikic et al., 1997).

More recently, other modulators including R101933 (laniquidar) and XR9576 (tariquidar) have been developed which inhibit Pgp more effectively and do not appear to affect cytotoxic drug metabolism as their predecessors do (reviewed by Thomas and Coley, 2003). XR9576 is an anthranilamide derivative that has been shown to sensitise Pgp expressing cell lines to etoposide, doxorubicin and vincristine.
Phase I trials with XR9576 in healthy volunteers indicated the compound is well tolerated and causes sustainable Pgp inhibition for at least 24h (Stewart et al., 2000). To date, phase II and III clinical trials have been conducted in ovarian and lung cancer with inconclusive results.

3.1.1.2 MRP

Multi-drug resistance associated protein 1 (MRP-1) is a 190kDa membrane protein encoded on chromosome 16 and shares 15% amino acid homology with Pgp (Cole et al., 1992). MRP is ATP dependent and is overexpressed in many non-Pgp expressing MDR cancer cell line models (Loe et al., 1996). It is found on both intracellular membranes such as the Golgi apparatus and the basolateral membrane of mucosal cells. MRP is responsible for both the inability to accumulate drugs within cells by secreting them into the interstitium and the blood, and also the sequestration of drugs away from intracellular targets (Gottesman et al., 2002). MRP expression has been shown to be significant in cases of non-small cell and small cell lung cancer where MRP levels correlated with resistance to certain chemotherapeutic agents (Wright et al., 1998). Increased MRP expression has also been reported in chronic lymphocytic leukaemia in 10-34% cases (Gottesman et al., 2002).

There are 6 other proteins currently described in the MRP family that also possess transport function. Currently, MRP1 is thought to be the most clinically relevant MRP protein in human cancer. In humans, mutations of the MRP2 gene are thought to contribute to Dubin-Johnson syndrome, an autosomal recessive disorder resulting in hyperbiliarinemia (Kartenback et al., 1996). MRP4 and MRP5 can transport antiviral cyclic nucleosides but not anticancer agents (Reid et al., 2003).
3.1.1.3 BCRP
Recently another transporter, breast cancer resistance protein (BCRP or ABCG2) has been described by Doyle et al., (1998) in anthracycline resistant MCF-7 breast cells. BCRP has been found to be a member of the ATP-binding cassette family. Expression of BCRP is seen particularly in the epithelium of the small intestine, colon, liver canicular membrane, and in the ducts and lobules of the breast. Furthermore, it has been found in the placental syncytiotrophoblasts, blood-brain and blood-testis barriers but the role of BCRP here is less clear (Maliepaard et al., 2001, Allen and Schinkel., 2002). BCRP may also contribute to resistance to topo inhibiting drugs in some cell lines. These include mitoxantrone resistant breast, colon, gastric and fibrosarcoma cell lines (Ross et al., 1999).

It has been demonstrated that BCRP functions more like Pgp than MRP as it is not affected by depletion of cellular glutathione and is able to transport drugs without drug-conjugate co-factors (Chen et al., 1990). Similar to Pgp, BCRP limits the re-uptake of drugs from the intestine and displays other protective functions such as in the maternal-foetal barrier (Maliepaard et al., 2001).

3.1.2 Mechanisms of Resistance – Atypical resistance
Atypical resistance is the term used to describe drug resistance that cannot be attributed to the presence of ABC-transporters but is due to changes in the target of the particular drug given as treatment. In the case of topo poisons, changes in topo expression levels, mutations in topos, or changes in the location of the topo can confer atypical resistance (Beck et al., 1987, Chen and Beck, 1993).

3.1.2.1 Topo expression and drug resistance
Several in vitro studies have shown that sensitivity to topo poisons is linked to expression levels of the putative topo target. Decreased expression of one type of
topo results in less intracellular target for a topo-directed agent. Doxorubicin resistance in SCLC cells has been ascribed to decreased topo IIα levels as a result of a reduction in gene copy number (Withoff et al., 1996). The resistance of many leukaemia cell lines to anthracyclines and epipodophyllotoxins has been associated with decreased protein expression and sometimes a decrease in topo II enzyme activity, including decreased expression of topo IIβ in ALL cell lines (Brown et al., 1995). Moreover, resistance to topo I directed agents has been linked to decreased expression of their cellular target. Resistance to the camptothecin analogue CPT-11 in human non-small cell lung cancer cells has been attributed to a 75% decrease in topo I levels (Kanzawa et al., 1990).

Clinical resistance to topo poisons in solid tumours has also been linked to decreased expression of topos. Topo IIα and IIβ protein expression levels were found to be lower in bladder cancer samples after unsuccessful treatment with epirubicin compared with samples from patients who responded to treatment. Furthermore, topo II expression levels in all bladder tumours were shown to be lower than that of normal bladder sections (Davies et al., 1996). Similar findings have been observed in nephroblastoma and ovarian cancer samples after unsuccessful treatment with other topo poisons (Dingemans et al., 1998). This may be due to an alternative topo compensating for the loss of function caused by decreased expression of the target topo. Compensation by another topo reduces the efficacy of a topo poison as there is no overall loss of topo function and so tumour cells will continue to proliferate. This has led to the development of dual topo poisons where topos I and II are targeted simultaneously to prevent any compensation of one topo form for another.
3.1.2.2 Topo Mutations

As with other enzyme-targeting drugs, topo inhibitors are designed to bind to specific regions of the target protein. Therefore, mutations in the enzyme may inhibit the interaction of the drug with the topo. Mutations in topo I can render cells resistant to camptothecin (Rubin et al., 1994, Chang et al., 2002). In particular, changes in amino acid residues 361, 362, 363 or 364 in the DNA binding region of topo I or in the active site at tyrosine 723 can reduce sensitivity in vitro (Larsen and Skladanowski, 1998). Moreover, substitution of arginine for alanine in the N-terminal of topo I results in camptothecin resistance. This substitution has no effect on the efficacy of netropsin which also inhibits topo I. These findings, however, suggest mutations in topo I arise specifically in response to the topo I poison that is encountered and may be different according to which topo poison is used (Knab et al., 1995).

In addition, in some cell lines topo II gene mutations have been suggested to be responsible for reduced sensitivity to topo inhibitors (Lee et al., 1992). In another report several cancer cell lines with resistance to teniposide were also found to be cross resistant to etoposide. Furthermore, these cells expressed topo IIα with point mutations in the ATP binding site or near the active site tyrosine (Patel and Fisher 1993, Bugg et al., 1991). Etoposide resistant leukaemic cells exhibiting cross resistance to several topo II inhibitors carry multiple mutations in the TOP2 gene. These changes are near the catalytic tyrosine 804 site (Patel and Fisher, 1993). Furthermore, in teniposide resistant cells a base substitution of arginine with glutamine at position 449 in the ATP binding domain has been noted. Similarly, substitution of arginine for lysine at position 486 renders cells resistant to amsacrine and this is also seen in tumour biopsies following treatment with etoposide (Hinds et al., 1991, Kubo et al., 1996). This illustrates that mutations may occur in the catalytic...
site or elsewhere in the enzyme such as in ATP or DNA binding regions and confer drug resistance (Larsen and Skladanowski, 1998). However, other studies on clinical samples have not identified topo mutations after unsuccessful treatment with topo poisons suggesting that other factors also contribute to topo drug resistance (Danks et al., 1993).

3.1.2.3 Subcellular localisation of topos
A shift in topo localisation from the nucleus to the cytoplasm reduces the formation of cleavable complexes and confers resistance to topo poisons that stabilise cleavable complexes. This shift has been observed in several topo II drug resistant cell lines. For example, topo IIα has been shown to translocate from the nucleus to the cytoplasm in cells treated sequentially with topotecan and etoposide with mitoxantrone (Chen et al., 2002). Translocation of topo I has been reported less frequently. High concentrations of camptothecin have been shown to cause a translocation of topo I from the nucleus to the cytoplasm in CPT resistant lines. However, this translocation may not be relevant to cellular resistance as sensitivity to camptothecin was shown not to be affected (Buckwalter et al., 1996).
3.2 Aims

- To characterise the VP16 and camptothecin resistant cell line models used in this study by investigating ABC transporter and topo expression, in order to ascertain their suitability for assessing the activities of XR5944 and XR11576.

- To determine the efficacy of XR5944 and XR11576 when presented with overexpressed ABC transporters and decreased expression of topos.

- To confirm that XR5944 is a substrate for Pgp by demonstrating that Pgp inhibition with a MDR modulator reverses resistance to XR5944.

- Initial experiments showed that XR5944 was slower than XR11576 to produce cytotoxicity and so determination of time-dependent cytotoxicity was investigated.
3.3 Materials and Methods

Chapter 2 describes general methodology.

3.3.1 Cell Lines

A description of cell line models used in this part of the study is given in Chapter 2. K562, K562AR, HL60 and HL60AR human leukaemia cells were cultured as described in Materials and Methods. K562 cells were originally derived from a CML patient in blast crisis and have a translocation of chromosomes 15 and 17 and have been identified as highly undifferentiated erythroleukaemic cells of the granulocytic series (Lozzio and Lozzio, 1975, Klein et al., 1976). K562AR doxorubicin resistant cells were used as an example of Pgp expressing cells and HL60AR doxorubicin resistant cells were used as an example of a MRP expressing line.

T8 cells (provided by Professor Jan Schellens, Netherlands Cancer Institute, Amsterdam, NL) resistant to topotecan were used as BCRP expressing cells, and Igrov-1 cells as a known cell line that does not express BCRP (Maliepaard et al., 1999).

3.3.2 Cytotoxicity and duration of exposure experiments

Experiments were carried out on MB231 cells to determine the effect of duration of exposure on the cytotoxicity of XR5944 and XR11576 using the MTT assay. Cells were exposed to concentrations of XR5944 or XR11576 for a duration of 1-72 h after which time the media was removed and replaced with fresh, drug-free media. Time points were selected in order to assess the effects of short (1-24 h) and long (24-72 h) drug exposure on cytotoxicity. The assay was terminated 72 h after the addition of the drugs.
3.3.3 Pgp Modulation

In order to ascertain whether resistance to topo poisons was due to the presence of Pgp, the MTT assay was performed on K562 and K562AR cells exposed to XR5944 or XR11576 with or without simultaneous treatment with 100nM XR9576 for 72 h.

3.3.4 Western Blotting Modifications

Detection of topo proteins was carried out using electrophoresis on 3-8% tris-acetate gels with tris-acetate running buffer (Invitrogen, Paisley) containing 50mM Tris Base, 50mM Tricine and 0.1% SDS, pH 7.24. 40μg of protein from cell preparations was electrophoresed for 1.5h at 150V. Protein was blotted onto nitrocellulose membranes for 2h at 30V. Membranes were blocked for 1h in 1% BSA, 1% milk in TBST and exposed overnight to antibody at 4°C. Chemiluminescence was used to detect levels of protein expression as stated in Materials and Methods.
3.4 Results

3.4.1 Expression of Pgp and MRP proteins

Figure 18 Pgp and MRP are not overexpressed in human tumour cell lines with acquired resistance to topo poisons.

Membrane preparations were obtained from cells and subjected to SDS-page electrophoresis on 10% acrylamide gels. Proteins were blotted onto nitrocellulose membranes which were then probed with anti-Pgp (H241 Santa Cruz) or anti-MRP (C20 Santa Cruz) and HRP linked anti-rabbit (Sigma) or goat secondary antibody (Dako).

K562AR and HL60AR cells express Pgp and MRP respectively and were used as positive controls (Figure 18). All the other cell lines examined did not express Pgp or MRP.

3.4.2 Expression of BCRP Protein

Data obtained in our laboratory have shown that drug resistant cell lines PEO1CamR, PEO1VP16R and MB231VP16R do not overexpress BCRP (data not shown).
3.4.3 Expression of topos

Figure 19 Pgp and MRP RNA expression is unaltered in topo-drug resistant human tumour cell lines

RT-PCR was performed using oligonucleotide primers specific for the ABC transporters on total cellular RNA from parental and resistant cells as described in Materials and Methods. K562AR cells alone expressed Pgp RNA. All the tested cell lines expressed MRP at RNA level. Beta globulin was used to confirm equal RNA concentrations between samples.

Pgp RNA expression correlated with protein expression illustrated in Figure 18. Notably, MRP expression differed with RNA levels indicating a role for RNA processing or protein degradation downstream of RNA production resulting in selective expression of MRP in HL60AR cells.
Figure 20 Altered topo protein expression in topo-drug resistant human tumour cell lines

Nuclear and cytoplasmic extracts were obtained from exponentially growing cells and subjected to Western blotting as described in Materials and Methods. Proteins were blotted onto nitrocellulose membranes and probed with anti-topo I, IIα or IIβ antibodies (Santa Cruz) Western blotting was carried out on cell lysates obtained on 3 separate occasions. Results shown below are representative of one set of lysates. Topo protein expression in resistant cell lines were compared qualitatively with parental cell lines. Beta actin expression confirmed equal protein concentrations between samples.

PEO1CamR cells expressed less topo I protein than the parental PEO1 line. A small decrease in topo IIα protein was also detected in PEO1VP16R and PEO1CamR cells in comparison with parental cells. Topo IIβ expression was similar between parental and resistant PEO1 cells. A decreased expression of all three topo proteins was detected in MB231VP16R cells in comparison with parental MB231 cells (Figure 20).
3.4.4 Expression of Topo RNA

Figure 21 Altered topo RNA expression in VP16 resistant human tumour cell line MB231VP16R

Total cellular RNA was extracted from exponentially growing cells and subjected to RT-PCR as described in materials and methods. Topo RNA levels were assessed on 4 separate occasions. Beta globulin expression confirmed equal RNA concentrations between samples.

PEO1 cells expressed similar RNA levels of topo IIα and IIβ in comparison to resistant PEO1 derived cell lines, PEO1CamR and PEO1VP16R. A modest decrease in topo I was seen for PEO1CamR. Topo RNA expression in PEO1 and PEO1 resistant cell lines did not correlate with protein levels (Figure 20). MB231VP16R cells expressed less topo IIα and IIβ RNA than MB231 cells. Topo I RNA expression was very low in comparison to the parental line. MB231 and MB231VP16R RNA levels were consistent with topo protein levels (Figure 20).
3.4.5 Cytotoxicity Testing in human cancer cell lines.

3.4.5.1 Efficacy of XR5944 and XR11576 in MDR cell lines.

Table 6: Mean IC_{50} concentrations (nM) after 72h drug exposure for parental and drug resistant cell lines with and without XR9576.
(Standard Deviation), - not tested. Concentrations given below are the mean of four separate experiments).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Doxorubicin</th>
<th>XR5944</th>
<th>+XR9576</th>
<th>XR11576</th>
<th>+XR9576</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>1200 (200)</td>
<td>0.8 (0.12)</td>
<td>1.4 (1.0)</td>
<td>22.4 (10.1)</td>
<td>27.1 (13.9)</td>
</tr>
<tr>
<td>K562AR</td>
<td>8000 (1100)</td>
<td>33.8 (13.1)</td>
<td>2.7 (1.4)</td>
<td>48.8 (2.9)</td>
<td>32.8 (17.2)</td>
</tr>
<tr>
<td>HL60</td>
<td>40.0 (10)</td>
<td>6.0 (4.0)</td>
<td>-</td>
<td>65.0 (10)</td>
<td>-</td>
</tr>
<tr>
<td>HL60AR</td>
<td>300(50)</td>
<td>150 (50)</td>
<td>-</td>
<td>50.0 (15)</td>
<td>-</td>
</tr>
</tbody>
</table>

Human cancer cell lines expressing Pgp or MRP and their parental cell lines were exposed to 72 h of continuous drug exposure and subjected to cytotoxicity testing (Appendix I illustrates a typical MTT data set). K562AR cells showed approximately 7-fold resistance to doxorubicin, 42-fold cross resistance to XR5944 and low/residual (2-fold) cross resistance to XR11576 (Table 6). Pgp modulation with XR9576 did not alter sensitivity to XR5944 or XR11576 in K562 cells (Figure 22, Figure 24). However, in K562AR cells, additional treatment with XR9576 reversed resistance and rendered the cells equally sensitive to XR5944 as the parental K562 cells.

HL60AR cells showed 7.5-fold resistance to doxorubicin compared to parental HL60 cells (Table 6) with substantial cross-resistance to XR5944 of 25-fold and no cross resistance to XR11576. IC_{50} concentrations between HL60 cells and HL60AR were not significantly different with XR11576 treatment (P>0.05 using a two-tailed student T-test).
Figure 22 XR9576 does not affect the cytotoxicity of XR5944 in K562 cells using the MTT assay with 72 h drug exposure

![Graph showing the effect of XR5944 and XR9576 on cell viability in K562 cells.]

Figure 23 XR9576 sensitises K562AR cells to XR5944 using the MTT assay with 72 h drug exposure

![Graph showing the effect of XR5944 and XR9576 on cell viability in K562AR cells.]

The cytotoxicity of XR5944 was unaltered in K562 cells using a combination treatment with XR9576. Sensitivity to XR5944 was significantly less in K562AR cells without additional treatment with XR9576. There was no significant difference between parental K562 cells treated with XR5944 and K562AR cells treated with XR5944 and XR9576 (P>0.05 using a one-way ANOVA).
Figure 24 XR9576 does not affect the cytotoxicity of XR11576 in K562 cells using the MTT assay with 72 h drug exposure

Figure 25 XR9576 does not affect the cytotoxicity of XR11576 in K562AR cells using the MTT assay with 72 h drug exposure

XR9576 did not affect the cytotoxicity of XR11576 in K562 or K562AR cells. (P>0.05 with additional treatment using XR9576).
3.4.5.2 The cytotoxicity of XR5944 and XR11576 was not affected by alterations in cellular topo levels

Table 7 Mean IC\textsubscript{50} concentrations for parental and drug resistant human cell lines using the MTT assay with 72 h drug exposure (Standard deviation). Concentrations are given in nM except for *VP16 which is given in \muM. P values are bases on a two-tailed student t-test comparing resistant cell lines with the parental cell line. Concentrations shown below are the mean of four separate experiments. (-fold)indicates level of resistance to inducing drug.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Camptothecin</th>
<th>VP-16*</th>
<th>XR5944</th>
<th>XR11576</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO1</td>
<td>5.2 (0.79)</td>
<td>0.16 (0.07)</td>
<td>16.9 (4.1)</td>
<td>45.3 (2.0)</td>
</tr>
<tr>
<td>PEO1CamR (3-fold)</td>
<td>15.3 (0.5)</td>
<td>0.65 (0.25)</td>
<td>12.7 (5.7)</td>
<td>66.2 (12.1)</td>
</tr>
<tr>
<td>PEO1VP16R (10-fold)</td>
<td>5.9 (0.9)</td>
<td>1.70 (0.19)</td>
<td>17.1 (2.6)</td>
<td>48.8 (5.5)</td>
</tr>
<tr>
<td>MB231</td>
<td>610 (40)</td>
<td>11.0 (2.7)</td>
<td>28 (2.4)</td>
<td>126 (13.7)</td>
</tr>
<tr>
<td>MB231VPR (6-fold)</td>
<td>1290 (200)</td>
<td>65.0 (5.0)</td>
<td>30 (4.1)</td>
<td>135 (8.7)</td>
</tr>
</tbody>
</table>
3.4.6 Characterisation of CHO Cell Lines Expressing Altered Topo Levels

3.4.6.1 Expression of Pgp in CHO cell lines

Figure 26 ADR-R cells express Pgp

Membrane preparations were obtained from cells and subjected to SDS-page electrophoresis. Proteins were blotted onto nitrocellulose membranes and probed with anti-Pgp (H241 Santa Cruz) and HRP conjugated anti-rabbit secondary antibody (Sigma). K562AR cells were used as a positive control. Signals were detected using chemiluminescence. ADR-R was found to express Pgp (Figure 26), whilst all other cell lines did not. This was in agreement with published data (Cummings et al., 1996).
3.4.6.2 Expression of topo RNA in hamster cell lines

Figure 27 Altered topo RNA expression in mutant hypersensitive ADR cell lines in comparison to CHO-K1 parental cells

Total cellular RNA was extracted from exponentially growing cells and subjected to RT-PCR as described in materials and methods section. A small increase in topo IIα RNA in ADR-6, ADR-3, and ADR-1 cells was observed. An increase in topo IIβ at RNA level was observed in all mutant hypersensitive cells compared with CHO-K1 cells.
Table 8: Topo protein expression in CHO-K1 and hypersensitive cells (Taken from Cummings et al., 1996)

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>PROTEIN EXPRESSION LEVELS</th>
<th>T1</th>
<th>TII α</th>
<th>TIIIβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ADR-1</td>
<td>1</td>
<td>5↑</td>
<td>2↑</td>
<td></td>
</tr>
<tr>
<td>ADR-3</td>
<td>1</td>
<td>3↓</td>
<td>2.5↑</td>
<td></td>
</tr>
<tr>
<td>ADR-6</td>
<td>1.5↑</td>
<td>2↓</td>
<td>2↑</td>
<td></td>
</tr>
<tr>
<td>ADR-R</td>
<td>2↑</td>
<td>2↓</td>
<td>2.5↑</td>
<td></td>
</tr>
</tbody>
</table>

↑ indicated level of expression compared with parental CHO-K1 cells.

ADR-R cells also overexpress Pgp.

Western blotting was not possible on these cell lines due to the lack of commercially available antibodies that recognise hamster topos. RT-PCR only detected subtle changes in topo RNA expression and were different to topo protein expression previously described (Cummings et al., 1996).
3.4.6.3 Cytotoxicity Testing

Table 9: Mean IC$_{50}$ values obtained for CHO-K1 and mutant hypersensitive cells after 72 h of drug exposure.

Concentrations are given in nM and are the mean of four experiments. (Standard deviation)

$P$ values were calculated using a two-tailed student t-test comparing IC$_{50}$ values obtained for mutant hypersensitive concentrations with those for parental CHO-K1.

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>XR5944</th>
<th>XR11576</th>
<th>DOXORUBICIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-KI</td>
<td>89 (10)</td>
<td>40 (5.3)</td>
<td>666 (117.1)</td>
</tr>
<tr>
<td>(Parental line)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adr-1</td>
<td>116 (28.8)</td>
<td>66.6 (18.9)</td>
<td>690 (50.0)</td>
</tr>
<tr>
<td></td>
<td>$P = &gt; 0.05$</td>
<td>$P = &gt; 0.05$</td>
<td></td>
</tr>
<tr>
<td>Adr-3</td>
<td>380 (14.1)</td>
<td>248 (29.4)</td>
<td>835 (5.1)</td>
</tr>
<tr>
<td></td>
<td>$P = 0.007$</td>
<td>$P = 0.0002$</td>
<td>$P = &gt; 0.05$</td>
</tr>
<tr>
<td>Adr-6</td>
<td>126 (35.3)</td>
<td>220 (21.6)</td>
<td>550 (69.7)</td>
</tr>
<tr>
<td></td>
<td>$P = &gt;0.05$</td>
<td>$P = 0.0004$</td>
<td>$P = &gt; 0.05$</td>
</tr>
<tr>
<td>ADR-R</td>
<td>237 (28.2)</td>
<td>190 (16.2)</td>
<td>888 (89.1)</td>
</tr>
<tr>
<td></td>
<td>$P = 0.002$</td>
<td>$P = 0.004$</td>
<td>$P = &gt; 0.05$</td>
</tr>
</tbody>
</table>

Cytotoxicity testing showed that sensitivity to doxorubicin treatment in this panel of cell lines was similar between CHO mutant variants and the parental cell line. ADR-3 cells were consistently the least sensitive to both XR5944 and XR11576. This cell line expressed the lowest level of topo IIα protein (Table 8). Conversely, ADR-1 was the cell line showing most sensitivity to XR5944 and XR11576 and was comparable to parental CHO-K1 cells. ADR-1 cells had the highest level of topo IIα in the panel. ADR-6 and ADR-R showed similar sensitivity to XR11576 and showed a very similar profile of topo protein levels (Table 8). In contrast, for ADR-6 and ADR-R cells a difference in sensitivity to XR5944 was observed which may be due to the overexpression of Pgp in the latter. Thus, for this cell line panel there was some suggestion that levels of topo IIα may correlate with sensitivity to either XR11576 and/or XR5944.
3.4.7 Cytotoxicity of XR5944 and XR11576 after different drug exposure times

Figure 28 IC$_{50}$ values obtained following 1-72h drug exposure

To determine the effect of duration of drug exposure on cytotoxicity, exponentially growing MB231 cells were exposed to XR5944 and XR11576 for increasing amounts of time up to 72 h. Time points were chosen to allow for the effects of potentially rapidly acting compounds as well as those requiring a longer duration of exposure. Drug exposure of 10 h or more reduced the IC$_{50}$ concentration of XR11576 by 50% compared with concentrations at 1-8 hour exposure. Drug exposure to XR5944 was necessary for at least 24 h in order for the IC$_{50}$ concentration to be reduced by 50%.
3.5 Discussion

Topo-directed anti-cancer drugs have proven clinical utility, however, resistance to these agents is well documented. Underlying mechanisms have included overexpression of ABC-transporter proteins and changes in expression of target topoisomerase enzymes (Evans et al., 1994, Husain et al., 1994, Dingemans et al., 1998). The efficacy of XR5944 and XR11576 was evaluated in tumor cell lines presenting with these resistance mechanisms in order to estimate their usefulness in tumor cells refractory to currently used topo poisons.

Significant cross resistance to XR5944 (42-fold) in Pgp-expressing K562AR cells was observed. However, these cells were only weakly cross resistant to XR11576 (approx. 2-fold). To confirm that cross-resistance to XR5944 was due to the overexpression of Pgp in K562AR cells, the Pgp specific modulator XR9576 was used. XR9576 reversed resistance to XR5944 and as expected, had no effect on the cytotoxicity of XR11576. These data are in agreement with ex-vivo and in vitro experiments that show XR5944, but not XR11576, is transported by Pgp (Mistry et al., 2002, Stewart et al., 2001). Pgp modulation by XR9576 has also been shown to increase the sensitivity of Pgp expressing cells to other topo poisons such as doxorubicin and VP16 (Mistry et al., 2001, Cree et al., 2003).

XR5944 was also affected by the expression of MRP in HL60AR cells which showed 8-fold resistance to doxorubicin, 25-fold resistance to XR5944 but no cross resistance to XR11576 (Table 6). In the clinic tumors may present with expression of ABC-transporters, however, given the high potency of XR5944, this should not pose any
significant problem. RT-PCR was not able to distinguish between MRP RNA expression in the parental and resistant lines as MRP was ubiquitously expressed and did not correlate with protein levels (Figure 19, Figure 18). Other studies have demonstrated disparity between RNA and protein expression of MRP which may be due to downstream processing of RNA resulting in selective expression of the protein (Yanagisawa et al., 1999).

Resistance to topo poisons has also been attributed to alterations in topo expression levels. CHO-K1 cells and mutant hypersensitive derivatives with varying expression of topos were tested for sensitivity to currently used topo poisons as well as XR5944 and XR11576. Due to the species difference an overall reduction in sensitivity to topo poisons was observed compared with human derived cell lines. Currently used topo poisons have been developed to interact with the human form of the enzyme which show significant differences in DNA sequence compared to the rodent enzymes. The CHO cell lines were characterised following culture under our laboratory conditions. It was not possible to detect topos in CHO lines using Western blotting as the commercially available antibodies we used recognise human epitopes and not those of the hamster. Previously published data was obtained using 'in-house' derived antibodies which were not available to us (Cummings et al., 1996). Further work could include assessing the catalytic activity of the topos in these cell lines as this would further demonstrate any differences in topo activity between cell lines.

RNA levels in the CHO lines were measured by RT-PCR as part of this study. RNA expression of topo IIβ correlated with published protein expression levels whereas the correlation of topo I and IIα RNA was not so apparent. Topo I RNA levels were
similar between cell lines whereas increased protein expression was seen in ADR-6 and ADR-R cells. There were some difficulties encountered when assessing topo I RT-PCR reactions. Different primer sets were tried as well as modifications to PCR conditions. Topo I RNA expression was very similar between cell lines under these different conditions. There was discordance between protein and RNA estimations for topo IIα and β. Table 8 indicates a lower level of topo IIα protein in ADR-3, ADR-6 and ADR-R than RNA levels suggest. ADR-1 cells showed an increase in Topo IIα protein and mRNA.

The cytotoxicity of XR5944 and XR11576 was shown to vary throughout the panel of CHO cell lines. ADR-3 was the least sensitive to both XR11576 and to XR5944. In addition to expressing the lowest level of topo IIα, this cell line exhibited different morphology and cell doubling times to the rest of the panel. ADR-3 cells were larger with irregular shape with a prolonged doubling time. ADR-3, ADR-6 and ADR-R cells showed increased resistance to XR11576 of between 4- and 5- fold. These cell lines all showed decreased topo IIα protein expression compared with CHO-K1 cells. ADR-1 cells showed the highest level of topo IIα and this cell line was the most sensitive of the mutant cell lines to both XR5944 and XR11576. Although, overall levels of topo IIα protein appeared to correlate with sensitivity to both agents, there were other differences apparent in the cell line panel relating to expression of the other topos. Moreover, there may have been other factors (not hitherto reported) contributing to the spectrum of sensitivity to XR5944 and XR11576, such as changes in DNA repair capacity.
Chapter 3

Activity of XR5944 and XR11576 in sensitive and drug resistant cancer cell lines

The human cancer cell lines with acquired resistance to VP16 and camptothecin provide a better insight into the relevance of alterations in topo levels in human tumours. Human tumour cell lines did not possess any of the topo changes encountered in the CHO lines. None of the resistant human tumour cell lines showed increases in topos relative to their parental counterpart. MBVP16R cells had 6-fold acquired resistance to VP16, and showed decreased levels of all three topos measured. This cell line showed no cross resistance to XR5944 or XR11576. PEO1VP16R cells (10-fold resistance to VP16) and PEO1CamR cells (3-fold resistance to camptothecin) showed no downregulation in topos at RNA level but a small decrease in topo IIα protein (Figure 20). However, this did not manifest in altered sensitivity to XR5944 or XR11576. Any change in RNA may have been below the limits of detection of this method. Real time PCR would elucidate this further and measure gene copy number, providing a more quantitative determination of changes in topo gene expression. It can be concluded, that in the human cancer cell lines with resistance to topo poisons used in these experiments, no change in sensitivity was observed to the dual topo poisons XR5944 or XR11576. These findings are suggestive of alternative mechanisms of action for these agents. It is reasonable to suggest that XR5944 and XR11576 could be effective for use in the clinic where treatment using topo poisons has failed due to atypical resistance on the basis of these data. Moreover, given the potential novel mechanism(s) of action XR5944 and XR11576 may possess, these agents may prove useful agents for many pretreated tumours and also in first line therapy.

In conclusion, XR5944, in line with other topo poisons, is less effective in cell lines expressing MRP or Pgp although it retains potent cytotoxicity. XR5944 mediated
Activity of XR5944 and XR11576 in sensitive and drug resistant cancer cell lines

cytotoxicity showed a more gradual onset compared with XR11576 (Figure 28). XR5944 and XR11576 have been described as novel dual topo poisons and yet neither agent is affected by changes in expression of topos. It is also possible that XR5944 and XR11576 are able to promote cytotoxicity via routes that do not involve topos as they are unaffected by changes in expression levels of topos seen in the human tumour cell line panel. The association of the cytotoxicity of these agents with topo IIα, as seen for the CHO cell line panel, would appear to contradict the latter data. However, there may be other factors underlying the spectrum of sensitivity seen throughout that particular cell line panel.

The potent cytotoxicity of XR5944 has a slightly slower onset than that seen for XR11576. This was a feature that distinguished one compound from the other and may point to differing mechanisms of action. Recently, Fleming et al., (2003), have demonstrated that XR5944 may have effects on RNA processing and this may also contribute to mechanism of action of XR5944.

These studies set out to identify if XR5944 and XR11576 behaved in a similar way to standard topo interactive agents, as the data presented in this present chapter suggest they may not. In the following chapter, the mechanism of action of both XR5944 and XR11576 was further investigated by examining their DNA-interactive effects.
CHAPTER 4

DNA INTERACTIVE EFFECTS OF XR5944 AND XR11576
4 DNA-interactive effects of XR5944 and XR11576 in vitro

4.1 Introduction

Currently used topo poisons inhibit topo action by at least three identified mechanisms. Firstly, drugs such as doxorubicin and daunorubicin intercalate DNA and obstruct DNA unwinding that is due to the activity of topos (Figure 29 a). DNA intercalation may cause modifications to the structure of DNA and prevent topos from accessing the DNA. As a result, levels of topo-DNA interactions fall especially at high doses of drug (Pommier et al., 2001).

A second mechanism of action of topo poisons is to stabilise DNA-topo interactions in the cleavable complex formation. Drugs such as camptothecin, targeting topo I, interact with both the DNA and the topo, holding the complex together and preventing disassociation (Jaxel et al., 1989). DNA damage occurs when a replication fork collides with a cleavable complex and can result in single and double strand breaks. Other agents targeting topo II, such as VP16 and VM-26 prevent religation of DNA strands in the cleavable complex (Kohn et al., 2000). As the DNA is already in the stabilised cleaved form when it interacts with these agents this results in the double stranded DNA damage (Figure 29b). In addition, some topo-interactive compounds actively inhibit the topo enzyme before it can interact with DNA. Therefore, DNA accumulates in a supercoiled formation and replication ceases (Figure 29c). Agents such as ICRF159, ICRF187 and ICRF 193 directly inhibit topo II by this mechanism.
Transcription generates positive supercoiling in the template ahead of pol II with negative supercoiling in the DNA behind this. In living cells the supercoiled state of DNA has been shown to be modulated by topos (see Chapter 1). Some recent reports also ascribe a decrease in RNA polymerase (pol II) dependent transcription activity in cells treated with either topo I or topo II targeted drugs (Collins et al., 2001, Mondal et al., 2003). It has been hypothesised by Mondal et al., (2003) that as topo II poisons, such as VP16, have an effect on the G1 phase of the cell cycle (in addition to the effects seen for on the G2 phase, discussed further in Chapter 5) this is consistent with a transcription function for topo II.

**Figure 29 Mechanism of action of topoisomerase poisons**

(a) Drugs can intercalate DNA and force the arrest of topos' action in unravelling of DNA. DNA replication then halts. (b) Drugs can stabilise DNA-topo cleavable complexes which prevents further topo action. (c) Drugs can actively inhibit the topo before interaction with DNA. This is usually through binding of the active site.
Three mechanisms of topo inhibition illustrated in Figure 29 all cause DNA damage. However, intercalation and the stabilisation of cleavable complexes result in more rapid damage as the DNA is already in the process of being replicated and may be single stranded. The resulting DNA damage promotes recruitment of specific DNA damage response proteins such as p53 leading to transactivation of genes such as $p21^{WAF1/Cip1}$, the protein product of which inhibits DNA synthesis via cyclin-dependent kinases (discussed in more detail in Chapter 5). If the DNA lesion cannot be repaired then the cell will abort replication altogether and undergo apoptosis.

In this study, the effects of XR5944 and XR11576 on DNA were examined in comparison with VP-16. Recently, XR5944 and XR11576 have been shown to induce cleavable complex formation using the trapped in agarose DNA immunostaining (TARDIS) assay. In this procedure cells are embedded in agarose, lysed and the topos covalently bound to DNA then detected by immunofluorescence. Cleavable complex formation with topos I, IIα, and IIβ have been detected in a time and concentration dependent manner with both XR5944 and XR11576 using this technique (Jobson et al., 2002). The DNA-damaging effects of XR5944 and XR11576 were explored in our cell line models by looking at single strand breakage and DNA protein crosslinks (as an index of cleavable complex formation). The experiments incorporated VP-16 alongside the two test agents to allow comparison with a classical topo-interactive agent.
4.2 Aims

The aims of this part of the study were to explore the putative mechanism of action of XR5944 and XR11576 as topo-inhibitory agents and to look for possible differences between them. In order to do this we examined the ability of these agents to form DNA-protein crosslinks as an indicator of cleavable complex formation, strongly associated with topo-inhibitory drug activity. In addition, we used the Comet assay in order to give an indication of the DNA damaging effects of XR5944 and XR11576 as putative DNA interactive agents.

4.3 Materials and Methods

A detailed methodology has been described in Chapter 2.

4.3.1 The Comet Assay

The Comet assay is a modification of Rydberg and Johanson’s (1978) assay which directly quantifies DNA damage in individual cells in agarose in mid-alkaline conditions. Ostling and Johanson (1984) developed a microgel electrophoresis technique in which cells are lysed in high salt and detergent conditions and the DNA is then free to migrate towards the anode. Damaged DNA migrates further than intact DNA and migration can be quantified by staining with ethidium bromide and the intensity of the fluorescence then measured using a microscope photometer. The alkaline Comet assay is performed at pH >13 and causes the separation of double stranded DNA. The assay, therefore, detects single strand breaks which are induced at orders of magnitudes greater than that of double strand breaks by most genotoxic agents (Tice, 1995). Other modifications of the method under neutral pH conditions also allow for detection of double strand breaks.
This technique was carried out in the Section of Molecular Carcinogenesis, Institute of Cancer Research, Sutton, headed by Professor David Phillips. The Comet assay was carried out using MDA-MB231 breast cancer cells treated at equicytotoxic concentrations of the compound indicated for a period of 4h. The drug doses used were IC$_{50}$ concentrations derived from cytotoxicity testing that used a 24h drug exposure. At least 40 comets were counted from each slide and each sample was prepared in duplicate. A total of 5 separate experiments were performed.

**Figure 30 Images of Comets as seen by fluorescent microscopy.**  
(*x 400 magnification*)

![Healthy Cell](image)

![Cell with Single Strand DNA breaks](image)

![Apoptotic Cell](image)

### 4.3.2 DNA-Protein Crosslinks

DNA-proteins cross-links (DPCs) may be produced by suspected carcinogens such as UV, alkylating agents, formaldehyde and by certain anticancer agents such as topoisomerase poisons. The presence of DPCs may result in consequential DNA damage, cell death and loss of genetic material. Some cancer cells are particularly
susceptible to genetic changes as they have a decreased capacity to repair damaged DNA due to mutations in DNA repair proteins. In these cells, mutations involved in oncogenesis may occur and be passed on to daughter cells at an accelerated rate in comparison to normal cells.

DNA damage caused by doxorubicin and ellipticine shown to be detectable by full deproteinisation using the DNA alkaline elution assay in the 1970s (Ross et al., 1978). These data suggested that all strand breaks were protein associated. However, the role of topo II in the compounds’ mechanism of action was not discovered until later. Currently used topoisomerase poisons such as camptothecin, VP16 and teniposide have been shown to give rise to DPCs (Covey et al., 1989, Kerrigan et al., 1987). However, non-intercalative topo poisons such as F11782, which do not stabilise cleavable complexes, do not produce DPCs.

The method used to measure DPCs in the present study was based on that described by Zhitkovich & Costa (1992) using [³H] Thymidine to label DNA and a process of SDS-KCl precipitation. Tritium-labelled SDS-K⁺ precipitable material was quantified using a beta-radiation counter. DPCs were converted to the DPC coefficient, calculated as the ratio of SDS-precipitable DNA in drug treated cells to that in control, untreated cells.

DPCs were measured in exponentially growing MDA-MB231 cells. Cells were exposed to drugs for 4h and were treated with an acute IC₅₀ concentration of drug (derived from MTT assays which used 24h, as opposed to continuous, drug exposure followed by termination of the assay at 72h). The doses used were for VP16 24μM,
XR5944 200nM and XR11576 300nM. 24-72h samples were treated at a lower concentration (XR5944 7nM, XR11576 30nM VP16 0.5µM) in order to maintain cell viability whilst initiating crosslinks over a longer time period. At high drug concentrations cells were too degraded to detect crosslinks after 24h. The concentration used for times 24-72h was 25% of the IC₅₀ concentration of XR5944 and XR11576, using a 72h exposure. VP16 treated cells were treated with a dose somewhat below this as otherwise the cells were too damaged at a higher concentration to measure DPCs.
4.4 Results

4.4.1 Comet Assay

The mean comet tail length was determined for 80 comets per experimental treatment. Histograms showing the frequency of distribution from a typical data set from one experiment are illustrated in Figure 31. The mean comet tail length was calculated for three treatments in comparison with the control (Table 10). After 4h of treatment VP16 and XR11576 treatment produced measurable comet tails whereas the effect for XR5944 was reduced and more inconsistent.

Figure 31: Frequency distribution of a typical data set of comets measured in μm, in drug treated and control MB231 cells after 4h
Table 10: Median values for all experiments using the 75 percentile of data sets
(Data set comprises of two slides with 40 comets counted on each, data shows comet tail length in μm))

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>CONTROL</th>
<th>VP-16</th>
<th>XR5944</th>
<th>XR11576</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>50</td>
<td>60</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>19</td>
<td>12*</td>
<td>23.5</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>60</td>
<td>51</td>
<td>57.5</td>
</tr>
</tbody>
</table>

A Mann-Whitney U test was carried out to see if the median comet lengths differed significantly from the controls at the 95% confidence level (1 and 2-tailed test). All drug-treated cells differed significantly from their respective controls giving p<0.001, apart from value marked *.
4.4.2 DNA-Protein Crosslinks (DPCs)

The mean DPC coefficients are shown in Table 11 and Table 12. Measurable DPC formation was observed in VP16 and XR11576 treated cells after 4h of treatment. XR5944 did not induce significant DPC formation compared with untreated control cells using a two-way ANOVA.

The DPC coefficient increased in VP16 treated cells from 1.9 to 3.4 between times 24 and 48h, with further increase to 5.7 at 72h. All DPC coefficients were significantly different from the control. No significant levels of DPCs were detected in XR5944 treated cells until 48h where the coefficient was 5.7. At 72h this increased to 11.1 and exceeded the levels achieved for both VP16 and XR11576 treated cells. DPCs in XR11576 treated cells were greatest at 24h with a DPC coefficient of 7.9. This decreased to 3.7 at 48 and 72h. The DPC coefficients for VP16 and XR11576 were all statistically significantly different from the control (P<0.05). These data are presented in Figure 32.
Table 11: DPC formation in MB231 cells treated with an acute IC\textsubscript{50} dose of drug for 4 h.

\[N = 4, \text{DPM} - \text{Disintegrations per minute. DPC} - \text{DNA protein crosslink. Significance was calculated using a one-way, two-sided ANOVA comparing the log mean of drug treated samples with the untreated control. SD = standard deviation.}\]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean (DPM)</th>
<th>DPC Coefficient</th>
<th>Mean Log Value</th>
<th>Log SD</th>
<th>Significance compared with untreated control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>114</td>
<td>1</td>
<td>2.0357</td>
<td>0.1620</td>
<td></td>
</tr>
<tr>
<td>VP16 24h</td>
<td>245</td>
<td>2.1</td>
<td>2.4345</td>
<td>0.0838</td>
<td>P &lt; 0.05 *</td>
</tr>
<tr>
<td>XR5944 24h</td>
<td>162</td>
<td>1.4</td>
<td>2.1371</td>
<td>0.3265</td>
<td>P &gt; 0.05 NS</td>
</tr>
<tr>
<td>XR11576 24h</td>
<td>547</td>
<td>4.8</td>
<td>2.7162</td>
<td>0.1650</td>
<td>P &lt; 0.001 ***</td>
</tr>
</tbody>
</table>

P values were <0.05, <0.01 and < 0.01 when the log drug treated DPC coefficient differed from the untreated control DPC coefficient by 0.3, 0.43 and 0.634 respectively.

Table 12: DPC formation in MB231 cells treated at 25% of the IC\textsubscript{50} concentration of each drug 24-72h.

\[DPM - \text{Disintegrations per minute. DPC} - \text{DNA protein crosslink. Significance was calculated using a one-way, two-sided ANOVA comparing the log mean of drug treated samples with the untreated control. N=>5} \]

\[SD = \text{standard deviation.}\]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean (DPM)</th>
<th>DPC Coefficient</th>
<th>Mean Log Value</th>
<th>Log SD</th>
<th>Significance compared with untreated control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>924</td>
<td>1</td>
<td>2.863</td>
<td>0.362</td>
<td></td>
</tr>
<tr>
<td>VP16 24h</td>
<td>1727</td>
<td>1.9</td>
<td>3.2</td>
<td>0.473</td>
<td>P &lt; 0.05 *</td>
</tr>
<tr>
<td>XR5944 24h</td>
<td>1124</td>
<td>1.2</td>
<td>2.783</td>
<td>0.725</td>
<td>P &gt; 0.05 NS</td>
</tr>
<tr>
<td>XR11576 24h</td>
<td>7261</td>
<td>7.9</td>
<td>3.416</td>
<td>0.7</td>
<td>P &lt; 0.001 ***</td>
</tr>
<tr>
<td>VP16 48h</td>
<td>3153</td>
<td>3.4</td>
<td>3.43</td>
<td>0.294</td>
<td>P &lt; 0.001 ***</td>
</tr>
<tr>
<td>XR5944 48h</td>
<td>5273</td>
<td>5.7</td>
<td>3.464</td>
<td>0.518</td>
<td>P &lt; 0.001 ***</td>
</tr>
<tr>
<td>XR11576 48h</td>
<td>3427</td>
<td>3.7</td>
<td>3.434</td>
<td>0.32</td>
<td>P &lt; 0.001 ***</td>
</tr>
<tr>
<td>VP16 72h</td>
<td>5240</td>
<td>5.7</td>
<td>3.675</td>
<td>0.155</td>
<td>P &lt; 0.001 ***</td>
</tr>
<tr>
<td>XR5944 72h</td>
<td>10264</td>
<td>11.1</td>
<td>3.938</td>
<td>0.293</td>
<td>P &lt; 0.001 ***</td>
</tr>
<tr>
<td>XR11576 72h</td>
<td>3406</td>
<td>3.7</td>
<td>3.418</td>
<td>0.39</td>
<td>P &lt; 0.001 ***</td>
</tr>
</tbody>
</table>

P values were <0.05, < 0.01 and P = < 0.001 when the log DPC coefficient for drug treated cells differed from the log control value of 2.86 by more than 0.102, 0.135 and 0.75 respectively.
Figure 32 DPC formation in MB231 cells after 4-72 hours of drug treatment
4.5 Discussion

XR5944 and XR11576 have been described as DNA-interactive agents that act through stabilisation of topo I and II cleavable complexes (Stewart et al., 2001, Mistry et al., 2002). Stabilisation of cleavable complexes results in DNA damage. In these experiments the DNA interactive effects were measured by examining single stranded breaks in MDA-MB231 cells after 4h of drug exposure using the Comet assay. VP16, XR5944 and XR11576 all produced single strand breaks (SSB) within 4h of drug treatment. XR5944 treatment produced the shortest median comet tail length. This is unlikely to be due to cellular uptake differences as cells were treated at equipotent doses. The MDA-MB231 cells used in this assay do not overexpress ABC transporters and so should accumulate the compounds with approximately the same efficiency (Chapter 3).

Comet tail length measurements used in these experiments provide an insight into the extent of the DNA damage caused by drug-DNA interactions. However, tail length can be influenced by image analysis resolution and can be sensitive to changes in drug concentration (Fairbaim et al., 1995). An alternative parameter to assess in the Comet assay is tail moment which is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both DNA size (in the tail length) and also the number of fragments (represented in the intensity). This allows for more elaborate statistical methods to be used when comparing treatments where only small changes are observed.

DPCs were also measured to provide more detail into DNA-interactive effects of the compounds. XR5944 did not produce significant levels DPCs within 4h whereas
levels detected in XR11576 and VP16 treated cells from 4h onwards reached statistical significance. Drug treatment times were extended using lower concentrations compared with the Comet assay so that cells could be incubated with drug for longer without extensive cell kill. This was best achieved using 25% of the IC\textsubscript{50} concentration obtained using a 72h drug exposure. XR5944 was slower to produce DNA-protein interactions and it also produces less DNA strand scission than XR11576, as deduced from the Comet data. This suggests XR5944 is slower at inducing cleavable complex formation than XR11576 (Jobson \textit{et al.}, 2002, Jobson \textit{et al.}, 2003). However, after 48h statistically significant levels of DNA-protein interactions were detected in XR5944 treated cells which far exceeded those seen for XR11576 or VP16 treated cells. This suggests that XR5944 does cause cleavable complex formation as part of its mechanism of action. However, similar to its peak of cytotoxicity, DPC formation does not happen as rapidly as for XR11576 (Chapter 3). Yang \textit{et al.}, (2003), have demonstrated that XR5944 is a more potent intercalator of DNA than XR11576 and that its linker chain can interact with the major groove of DNA. Furthermore, XR5944 is a \textit{bis}-intercalator, reacting with DNA in two places whereas XR11576 has only one possible site for intercalation. The increased DNA intercalation may account for the extensive DPC formation observed in XR5944 treated cells after 48h as it is possible more drug-DNA interactions were occurring compared with XR11576. However, further DNA binding studies would elucidate this further.

The profile of DPC induction in VP16 treated cells increased over time and peaked at 72 h. Greatest DPC formation was observed at 24h in XR11576 treated cells and then levels of DPCs started to decrease. The decrease in DPCs formation may have been
due to decreased cellular replication in response to XR11576 treatment resulting in less topo-DNA interactions. In addition, there may have been some repair of these DNA-drug adducts over time. All three agents examined had different profiles of DPC induction in MDA-MB231 cells. This suggests that the compounds used in these experiments exhibit different time-dependent effects on the stabilisation of cleavable complexes within cells. As already suggested, these differences are unlikely to be due to variation in transport across the cell membrane although cellular uptake was not measured. XR5944 may cause other intracellular effects within the cell as part of its mechanism of action which may occur prior to cleavable complex formation. RNA processing proteins have been shown to be affected by XR5944 treatment and it is possible that RNA effects may occur before stabilisation of cleavable complex formation as it is more abundant in the cell than DNA (Fleming et al., 2003). Further work is needed to elucidate the effects of XR5944 further as the precise effects on RNA processing are not currently known.
CHAPTER 5

Effects of XR5944 and XR11576 on the cell cycle and interactions with p53
5 Effects of XR5944 and XR11576 on the cell cycle and interactions with p53

5.1 Introduction

5.1.1 The Cell Cycle

Eukaryotic cells undergo a predetermined cycle of cell synthesis and division. The cell cycle can be divided into two parts interphase and mitosis. Interphase can be further divided into G1, S-phase, and G2 phase. DNA is replicated during S-phase and the cell actually divides during mitosis. However, in the absence of growth factors or nutrients, or when cell overcrowding occurs (such as in in vitro culture conditions), the cells will become quiescent. This state is referred to as G0. At this stage cells have a lowered rate of protein synthesis and have increased resistance to surviving stress. They can re-enter the cell cycle when conditions improve, however, or undergo cell death (sub G1) if unfavourable conditions persist. The length of the whole cycle varies considerably between cell types. Typically, actively growing human cells complete the process in 24 hours whereas yeast cells complete it in 90-120 minutes (Pelczar et al., 1993).

During the growth stages of the cell cycle, synthesis of cellular components such as ribosomes, membranes, mitochondria and endoplasmic reticulum occurs. However, chromosome replication only takes place during S-phase. Cells will arrest at the G1/S checkpoint after DNA damage to prevent replication of mutated DNA. This arrest is mediated by the inactivation of cyclin dependent kinases (CDKs) and cyclin complexes by p21WAF1/CIP1 in response to p53 transactivation. The G2/M (mitosis) transition is regulated by P34cdc2 and cyclin B. The cell cycle can also be stopped
here in response to DNA damage by inactivation of cdc2 by activation of the PI-3K family of proteins (Stewart and Pietenpol, 2001).

5.1.1.1 Cell cycle changes following treatment with topo directed agents
The cell cycle may be affected by topo poisons as a consequence of their DNA damaging effects. Topo II poisons primarily cause an accumulation in G2/M whereas topo I poisons tend to exhibit more of an effect in S-phase (Smith et al.1994, Ling et al., 1996, Cliby et al., 2002). As with other cytotoxic drugs the efficacy of topo poisons can be affected by changes in the cell cycle. The cytotoxic effect of etoposide has been shown to be significantly greater in cells that are in S or G2 phase, compared with those in G1. This effect coincides with the cyclical increase in topo IIα throughout the cycle that peaks at the end of S-phase (Chresta et al., 1992).

5.1.2 p53 and the cell cycle
p53 (TP53) is a tumour suppresser gene which is to be found to be mutated in over fifty per cent of cancers (Vogelstein 1990). p53 is actively involved in cellular growth arrest and apoptosis. Levels of p53 increase rapidly after DNA damage and this leads to transcriptional activation of p21WAF1/Cip1 (El-Deiry et al., 1993). The cell cycle is arrested as p21WAF1/Cip1 binds and inhibits CDKs that are responsible for phosphorylating the retinoblastoma protein, Rb. Rb is progressively phosphorylated during the cell cycle (Figure 33) with maximal phosphorylation being reached in late G1 and maintained until mitosis. In its hypophosphorylated state Rb sequesters E2F, a key transcription factor, and also recruits histone deacetylase which promotes nucleosome compaction (Sionov and Haupt, 1999). Nucleosome compaction and sequestration of E2F stops transcription and prevents the progression of the cell into S-phase where DNA is synthesised.
Following DNA damage, p53 upregulation causes cells to arrest in both G1 and G2/M phases of the cell cycle (Agarwal et al., 1995, Stewart et al., 1995). Initially, p53 gives rise to transcriptional activation of $p21^{WAF1/Cip1}$ via a response element in its promoter and increased expression then mediates a cell cycle arrest. $p21^{WAF1/Cip1}$ is a cyclin dependent kinase (CDK) inhibitor that selectively inhibits CDKs responsible for the G1/S and G2/M transitions of the cell cycle such as Cdk2, Cdk3, Cdk4 and Cdk6 (Harper et al., 1995, Agarwal et al., 1995). The mechanisms by which p53 can induce a G2 arrest has been shown to be attributed to transcriptional induction of gadd45 and 14-3-3δ as well as $p21^{WAF1/Cip1}$ (reviewed by Taylor and Stark 2001). GADD45 contributes to G2 arrest by dissociating CDK1 from its partner subunit cyclin B1. 14-3-3δ then sequesters CDK1/cyclinB1 in the cytoplasm preventing entry into mitosis (Chan et al., 1999). Clifford et al., (2003) also showed that downregulation of CDK1 and cyclin B1 is a major mechanism used by p53 to stabilize G2 arrest.

p53 is also instrumental in the induction of the apoptotic pathway leading to cell death (see chapter 6).
Figure 33: p53 and the cell cycle
(Modified from Sionov and Haupt, 1999, Bullock and Fersht, 2001)
p53 transcriptionally activates p21, an inhibitor of cyclin dependent kinases (CDKs) which, under normal conditions, are responsible for the progression of the cell cycle. Inhibition of CDKs causes cell cycle arrest in G1, G2 and M phases of the cell cycle. Inhibition of CDKs 4 and 6 stop the phosphorylation of the Rb protein and so Rb remains bound to E2F, a transcription factor, and prevents further transcription.
5.1.3 Post translational modifications to p53

The p53 gene encodes for a 393-amino acid protein, p53, that has three domains: a transactivation domain regulating the ability of p53 to act as a transcription factor, a central domain required for DNA binding, and a basic carboxy terminus for nuclear localization, oligomerisation and DNA binding (Stewart and Pietenpol, 2001, Hupp et al., 1992). Phosphorylation of p53 is thought to be important for the stabilisation and activation of the protein. Studies have shown there are eight phosphorylation sites in the transactivation domain (serines 6,9,15,20,33,37 and 46 and threonine 18). There are also five phosphorylation sites in the carboxy terminal oligomerisation domain (serines 315, 371, 376, 378, and 392). Phosphorylation at the transactivation domain may disrupt MDM2 binding to p53 and so is thought to stabilise p53. MDM2 negatively regulates p53 by direct binding and dampening of p53 activity in normal, unstressed cells, as well as by increasing p53 degradation through ubiquination (Woods and Vousden, 2001).

Phosphorylation of p53 at the carboxy terminal regulates nuclear localisation and transactivation (Hupp et al., 1992, Hecker et al., 1996). Increased phosphorylation at serine 15 results in increased acetylation (Lambert et al., 1998). Furthermore, acetylation of the p53 protein at the carboxy terminus is proposed to increase sequence specific binding by p53 (Stewart and Pietenpol, 2001).

5.1.3.1.1 p53 and chemosensitivity

p53 status may also be an important determinant in chemosensitivity to anticancer drugs. Loss of wild-type p53 function results in aggressive tumour phenotypes that are often resistant to ionising radiation and chemotherapy. This can occur
as a result of single points mutation in *TP53* resulting a mutated protein. Alternatively, disruptions in p53 regulatory pathways, such as overexpression of MDM2 frequently seen in soft tissue sarcomas, can contribute to decreased p53 function or complete cessation of p53 protein expression by its increased degradation (Oliner *et al.*, 1992).

Large-scale studies employing several tumour cell line models have shown that loss of wild-type p53 results in cancer cells with resistance to standard anticancer drugs (O'Connor *et al.*, 1997). Wu and El-Deiry (1996) demonstrated that sensitivity to doxorubicin, carboplatin and etoposide were all affected by loss of p53 mediated by the loss of cell cycle regulation by p21<sup>WAF1/Cip1</sup>. Loss of p53 also resulted in 100-fold resistance to Taxol in the same cell lines. Furthermore, other studies examining p53 status in tumour biopsies have also shown that loss of wild-type p53 gives rise to increased chemotherapy resistance (Vogt *et al.*, 2002, Kersemaekers *et al.*, 2002).

Restoration of wild-type p53 has been shown to sensitisce cancer cells to chemotherapeutic agents in *vitro*. Transfection of soft tissue sarcoma cells carrying a mutated p53, with wild-type p53 was found to increase the efficacy of doxorubicin (Zhan *et al.*, 2001). Furthermore, sensitivity to other chemotherapeutic agents such as 5-FU and vincristine has also been demonstrated in response to transfection with wild-type p53 (Li *et al.*, 2004).

### 5.1.4 p53 and Topoisomerases

The action of p53 may also be mediated in part by its interaction with topos. Topo I and topo IIα both directly interact with p53 (Gobert *et al.*, 1996, Hochhauser *et al.*, 1999). Topo I is recruited in response to DNA damage and this is conditional on the
presence of p53. p53 null cells do not recruit topo I and cells with mutant p53 are less effective in recruiting topo I (Mao et al., 2000). Topo I and p53 interaction may result in transcription of apoptogenic genes which would contribute to the apoptotic response of p53 (Soe et al., 2002). However, interaction with mutant p53 by topo I may also result in constitutive association between the proteins resulting in the increased likelihood of illegitimate recombination which may be of importance in tumourgenesis (Gobert et al., 1999). Further research is required to elucidate the precise mechanism of action.

Topo II may also be involved in the action of p53. It has been suggested that topoisomerase IIα may be one of the downstream targets for p53-dependent regulation of cell cycle progression (Wang et al., 1997, Sandri et al., 1996). Transcription from the topoisomerase IIα promoter decreases 15-fold on expression of wild type p53 suggesting that p53 acts as a transcriptional repressor. Mutant p53 exhibits much weaker repression (Wang et al., 1997). Inactivation of p53 would contribute to failure of cells to recognise cell cycle checkpoints and therefore accelerated cell proliferation. This would indicate that p53 activation in apoptosis would cause direct downregulation of topoisomerase IIα (Sandri et al., 1996).
5.2 Materials and Methods Modifications

5.2.1 Western Blotting

Western blots were performed as described in Chapter 2. These experiments used MCF-7 breast cancer cells which were prepared as whole cell lysates following drug treatment. Cells in monolayer culture at early exponential growth phase were treated with IC\textsubscript{50} concentrations achieved with continuous exposure over 72h of doxorubicin (225nM), XR5944 (30nM) or XR11576 (60nM). Doxorubicin was used as a reference compound. 40µg of lysate protein was subjected to western blotting using 10% bis-tris gels for p53 and phosphorylated p53 (p-p53) and 4-12% Bis-tris gels for p21\textsuperscript{WAF1/CIP1}. Nitrocellulose membranes were probed with either anti-p53 antibody (sc-126, Santa Cruz), phosphorylated p53 (sc-7997, Santa Cruz), p21\textsuperscript{WAF1/CIP1} (sc-397G, Santa Cruz) or actin (Ab-1, Oncogene Corp.) and a secondary HRP-linked antibody (Sigma). Signals were detected using chemiluminescence. To prevent cleavage of phosphate groups membranes used for phosphorylated p53 detection were blocked and the primary antibody diluted in 10% BSA in TBST with 1mM sodium ortho-vanadate. The secondary HRP-linked antibody was diluted in 10% BSA in TBST.

5.2.2 Flow Cytometry

Exponentially growing MCF7 cells were treated with IC\textsubscript{50} concentrations obtained after 72h drug exposure of doxorubicin (225nM), XR5944 (30nM) or XR11576 (60nM). Cells were resuspended in PBS and treated with 33µg/ml propidium iodide and 1mg/ml RNase A for at least 30 minutes at 37°C and protected from light.
Samples were analysed on a Beckman-Coulter Epics XL ™ flow cytometer (excitation 488nM, fluorescence >575nM).
5.3 Results

5.3.1 Expression of p53 and p21\(^{\text{WAF1/Cip1}}\)

Figure 34: p53 and p21\(^{\text{WAF1/Cip1}}\) expression is induced in MCF-7 cells after treatment with topo poisons

Cells were treated \(IC_{50}\) concentrations (obtained from MTT chemosensitivity testing using 72h continuous exposure) of VP16, XR5944 or XR11576 for 16-48h. Beta actin expression confirmed equal protein concentrations between samples.

Doxorubicin induced expression of p53 within 16h of drug exposure after which time expression started to decrease. p21\(^{\text{WAF1/Cip1}}\) expression was also induced within 16h and was greatest after 24h. p53 and p21\(^{\text{WAF1/Cip1}}\) expression increased with time of XR5944 exposure from 16 to 48h. p53 expression was consistent over 16-48 h with XR11576 treatment, whereas p21\(^{\text{WAF1/Cip1}}\) expression followed a very similar pattern as for Doxorubicin treatment. p53 and p21\(^{\text{WAF1/Cip1}}\) expression were barely detected in the negative control.
Figure 35 p53 is phosphorylated at Serine 392 in response to treatment with topo poisons

Whole cell lysates were prepared as described in the Materials and Methods chapter. MCF7 cells treated with 65μM VP16 for 6h were used as a positive control (Control +ve). Untreated MCF7 cells were used as a negative control (Control -ve). Other cells were treated at IC_{50} concentrations (obtained after 72h drug exposure) of VP16, XR5944 or XR11576 for 16-48h.

Phosphorylated p53 (p-p53) expression increased proportionately from 16 to 48h with doxorubicin and XR5944 treatment (Figure 35). XR11576 induced p-p53 expression at 16h. Levels of p-p53 peaked at 24h and slightly decreased at 48h. A low, residual level of p-p53 expression was detected in the negative control.

Figure 36 (overleaf) shows the changes in cell cycle patterns that arise from treatment with topo drugs. Doxorubicin treatment resulted in slightly more cells in G2/M phase whereas treatment with both XR5944 and XR11576 resulted in more cells in the G1 phase of the cell cycle compared with an untreated control. XR11576 treated cells also had a higher percentage of cells in G2/M than the control (Table 13).
5.3.2 Cell Cycle Profiles measured by flow cytometry

Figure 36: XR5944 and XR11576 cause different cell cycle effects to doxorubicin in MCF7 cells

(a) Control cells  (b) Doxorubicin

(c) XR5944  (d) XR11576

Table 13 Percentage of cells in stages of the cell cycle.
Mean values are shown below (n=3) (Standard Deviation)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24h</td>
<td>63.7 (3.5)</td>
<td>15.5 (2.3)</td>
<td>17.1 (1.1)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>24h</td>
<td>60.1 (3.8)</td>
<td>5.7 (3.5)</td>
<td>28.8 (1.5)</td>
</tr>
<tr>
<td>XR5944</td>
<td>24h</td>
<td>69.1 (6.0)</td>
<td>14.4 (1.3)</td>
<td>13.7 (2.3)</td>
</tr>
<tr>
<td>XR11576</td>
<td>24h</td>
<td>68.5 (5.4)</td>
<td>6.0 (1.2)</td>
<td>22.8 (1.3)</td>
</tr>
</tbody>
</table>
5.4 Discussion

In response to DNA damage, increased expression of proteins such as p53 is induced. p53, in its role as a transcription factor, then causes the transcription of other p53 inducible genes (PIGs) such as \( p21^{WAF1/Cip1} \) resulting in the initiation of DNA repair pathways or apoptosis. In the last chapter XR5944 and XR11576 were shown to interact with DNA. Following this, DNA interaction and damage leads to increased p53 expression and downstream effectors in tumour cells treated with these 2 agents. The duration and extent of p53 induction differed for XR11576 and XR5944. Doxorubicin induced a strong p53 response, peaking at 16h after which time levels of p53 started to decrease. XR5944 treatment resulted in less induction of p53 expression than for doxorubicin or XR11576 at 16h, relative to control cells. In line with this observation, Comet and DPC data (Chapter 4) suggested that XR5944 does not produce immediate effects on DNA in contrast to VP-16 or XR11576. However, p53 expression levels increased over time and at 48h high levels were seen in XR5944 treated cells. XR11576 treatment gave rise to a marked increase in p53 expression from 16h and this was maintained over the experimental period.

In this part of the study, expression of \( p21^{WAF1/Cip1} \) and phosphorylated p53 (p-p53) were largely in parallel. The increase in phosphorylated p53 at serine 392 is indicative of increased transactivation. Phosphorylated p53 expression in doxorubicin treated cells increased from 16 to 72 h. This was correlated with an increase in \( p21^{WAF1/Cip1} \) expression which was greatest at 24h in doxorubicin treated cells, at which time the greatest proportion of cells in G2/M was observed (30% of cells). This effect was also seen in XR5944 and XR11576 treated cells where \( p21^{WAF1/Cip1} \) and phosphorylated p53 increased in a time dependent manner. In the MCF-7 cell line increased levels of phosphorylated p53 at serine
392 and p21WAF1/Cip1 expression correlated with a trend towards cell accumulating in the
G2/M phase of the cell cycle. In the present experiments, p21WAF1/Cip1 would appear to
have contributed to the increase in cells in G2/M phase.

XR5944 and XR11576 did not dramatically alter the cell cycle effects on cells treated at an
IC$_{50}$ concentration for 24h. However, a slightly greater number of cells accumulated in G1
and G2/M phase compared with the untreated control. Inhibition of both topo I and II by
XR5944 and XR11576 would be expected to cause an accumulation of cells in G1 and
G2/M phase as topo I inhibition has been shown to cause an accumulation of cells in G1,
and topo II inhibition results in a G2/M block (Cliby et al., 2002, Smith et al., 1994).

Doxorubicin caused a marked accumulation of cells in G2/M, consistent with a topo II
poison. Any changes in the cell cycle after drug treatment may have been more apparent if
the cells had been treated with a higher concentration of drug or the cells had been
synchronised prior to drug treatment. Other work has demonstrated that XR5944 does
cause a significant accumulation of synchronised cells in G1 and G2/M and a decrease of
cells in S-phase (Freathy et al., 2003, Sappal et al., 2004). Furthermore, recent work
carried out in our laboratories using higher drug concentrations has shown that XR11576
treatment of PEO1 cells gave rise to a substantial G2M block at 48h. In the same
experiments, XR5944 treatment failed to induce any particular cell cycle perturbation,
using an equicytotoxic dose (Coley HM, personal communication). These data further
demonstrate differences that exist between XR5944 and XR11576.

XR5944 was slower at inducing DNA damage detected by p53 than XR11576 and
doxorubicin in agreement with Comet data. p53, phosphorylation of p53 at serine 392 and
p21WAF1/Cip1 expression were all less in XR5944 treated cells than in XR11576 and
doxorubicin treated cells. These data also correlate with the observed time-dependency of XR5944 induced cytotoxicity discussed in Chapter 3. It can, therefore, be concluded that XR5944 and XR11576 show differing DNA interaction both in terms of the timing and extent of the damage caused.

Cells have the ability to recover from DNA damage even after the initiation of p53 and may not undergo cell death. To determine whether the DNA damage caused by XR5944 and XR11576 was lethal to cells, the nature and extent of apoptosis was investigated in drug treated cells.
CHAPTER 6

Apoptosis in response to treatment with XR5944 and XR11576
6  **Apoptosis in response to treatment with XR5944 and XR11576**

The term apoptosis was first used by Kerr *et al.*, (1972) to describe a cell death morphologically different from necrosis. Some of the characteristics of apoptosis and necrosis are described below in Table 14. Early research on apoptosis was concentrated on the development of the nematode *Caenorhabditis elegans*. In this organism the *CED* genes were shown to be responsible for the death of specific cells during development (Ellis *et al.*, 1991). Corresponding genes have since been found in human systems and have also been identified as being important in physiological processes.

**Table 14 Characteristics of apoptosis and necrosis**

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single cells</td>
<td>Groups of cells</td>
</tr>
<tr>
<td>Cell Shrinkage</td>
<td>Swelling of organelles and rupture of intracellular membranes</td>
</tr>
<tr>
<td>Nuclear and cytoplasmic fragmentation</td>
<td>Release of cell contents by cell lysis</td>
</tr>
<tr>
<td>Plasma membrane blebbing with</td>
<td>Plasma membrane blebbing and eventual rupture</td>
</tr>
<tr>
<td>maintenance of integrity</td>
<td></td>
</tr>
<tr>
<td>Formation of apoptotic bodies</td>
<td></td>
</tr>
<tr>
<td>High molecular weight DNA fragmentation (50-300kbp)</td>
<td>Random DNA fragmentation</td>
</tr>
</tbody>
</table>

Apoptotic cells typically undergo morphological changes outlined in Table 14. One of the earliest changes is the movement of phosphatidyl serine from the inner surface to the outer surface of the cell membrane. This is thought to be a signal to surrounding cells that the cell is dying and its contents are then taken up by neighbouring cells or phagocytes (Raff 1998).
Apoptosis is critical in cellular development, maturation of immune cells and cellular responses to chemical compounds. It can also occur in response to the disruption of cell-matrix interactions and is then termed 'anoikis' (Frisch and Francis 1994). The molecular mechanisms of anoikis are still incompletely understood but non-receptor kinases and caspases are thought to involved (Grossman et al., 2001).

Apoptosis has been shown to play a central role in human disease. Caspases are key proteases involved in implementing the apoptotic cascade and have been shown to mediate cell death in neurones after strokes. Inhibition of caspases can reduce neurone damage in animal models (Barinaga 1998). Similarly in Alzheimers patients neurones have been identified with characteristics of apoptotic cells. Moreover, apoptosis evading mechanisms have been identified in cancer cells which include overexpression of anti-apoptotic factors and decreased expression of apoptogenic proteins. These mechanisms are thought to contribute to tumorigenesis and are discussed further in 6.1.1.1.

6.1.1 Mechanisms

The two most described routes to apoptosis are the mitochondrial and death-receptor pathways. These pathways include mechanisms by which signals from one can interact with the other and bring about amplification of the apoptotic response. This has been described as ‘cross-talk.’ Both pathways involve action of caspases and converge by the activation of effector (executioner) caspases –3, -6 and –7.
6.1.1.1 Caspases

Caspases are a family of proteases that all have cysteine in their active site and cleave their target proteins at specific aspartic acids. Each is inactive in its proform but is activated by either the interaction of another protein, or in most cases, by another caspase, contributing to an apoptotic cascade. Caspases such as caspases —2, —8, -9 and —10 all have caspase activation recruitment domains (CARD) by which they can bind and activate other caspases.

Caspase-8 can be the result of ligand-receptor interaction but it is also observed in the mitochondrial pathway (detailed below). Activated caspase-8 then activates other effector caspases such as caspases —3,-6 and —7. Caspase —8 also cleaves Bid, a member of the Bcl-2 apoptogenic family described in 6.1.2.1, generating a carboxy-terminal fragment that can induce release of mitochondrial cytochrome c. Caspase —3 acts in a like manner on Bcl-2 and Bcl-xL to release their active apoptogenic fragment and inhibit their anti-apoptosis functions (Cheng et al., 1997). Caspase —3 also cleaves the inhibitor of caspase-activated DNase (CAD) allowing it to enter the nucleus of the cell where it degrades internucleosomal DNA (Lechardeur et al., 2000).

It is the action of the caspases that brings about the morphological breakdown that is so evident in apoptosis. Caspase —6 cleaves lamins which are the main constituents of the nuclear lamina. Moreover, caspase-3 interacts with structural components of the cell such as gelsolin and inhibits enzymes involved in DNA repair such as PARP (Slee et al., 2001).
6.1.1.2 Fas-Ligand

Apoptosis can be initiated by the contact-dependent interaction of extracellular death ligands to unique sensors called death receptors on the cell surface, such as Fas (APO1/CD95). The binding of Fas ligand causes its receptor to change conformation and in turn interact with adapter proteins such as FADD/MORT-1/FLICE and Daxx (Kischkel et al., 1995, Muzio et al., 1996). These adapter proteins then cause the activation of pro-caspase-8 and Jun N-terminal kinase which are important components of the apoptosis cascade that eventually leads to cell death (Jones et al., 1998).

Death receptor induced apoptosis has an important role in three main areas: i) T-cell and natural killer cell mediated death of targets such as virally infected cells and cancer cells ii) the killing of inflammatory cells iii) peripheral deletion of activated T-cells at the end of an immune response (Ashkenazi and Dixit, 1998). Other ligands include TRAIL (TNF-related apoptosis inducing ligand) and TWEAK (TNF weak inducer of apoptosis) (Grell et al., 1999). Death receptors are all part of the TNF receptor family and are thought to work in the same way as the Fas-ligand. Some research suggests that in the case of TRAIL, the mitochondrial route to apoptosis may also be initiated (Hersey and Zang, 2001).

6.1.1.3 Mitochondrial Route

An alternative apoptotic pathway is initiated by activation of p53 following DNA damage. In this case cytochrome c is released into the cell from the intermembrane space of the mitochondria and then binds at least two cytosolic proteins: apoptosis activating factor 1 (APAF1) and APAF3. APAF1 then binds pro-caspase-9 resulting
in its activation and forms an 'apoptosome.' Activated caspase -9 subsequently cleaves caspase-3 (see Figure 37). It is unclear how exactly the membrane of the mitochondria is permeabilised allowing cytochrome c release but it is thought that a pore is formed.

The mitochondrial route to apoptosis also engages other factors such as Apoptosis Inducing Factor (AIF), a 57kDa flavoprotein that induces nuclear changes and also has oxidoreductase properties in healthy cells. AIF is thought to aid release of cytochrome c and pro-caspase-9. Bax and Bid are two other proapoptotic factors important in the mitochondria. Both factors locate to the mitochondria after apoptosis begins. Bax associates with the membranes of the mitochondria and is thought to be part of the pore (Crompton 2000). Bid is cleaved by caspase-8 into 15 and 8 kDa fragments, of which the 15kDa fragment binds tightly to the mitochondria in the presence of Bax. Bid may be involved in connecting the death receptor and mitochondrial pathways as it is cleaved as part of the death receptor pathway and also locates to the mitochondria where it causes cytochrome c release (Yin 2000) (see Figure 37). This can be important in cells such as hepatocytes where effector caspases are not always efficiently activated by caspase-8 after death receptor aggregation. In this case, Bid triggers the mitochondrial route and so augments the apoptotic response.
Figure 37 Apoptotic Pathways
(Yin, 2000, Hersey and Zang, 2001)

Fas interaction with adapter proteins results in the activation of caspase-8. Caspase-8 causes the cleavage of Bid which relocates to the mitochondria and aids cytochrome c release in conjunction with other proteins such as Bax. Cytochrome c release can also be the result of p53 activation. Effector caspases are subsequently activated and result in the morphological changes evident in apoptosis.
6.1.2 Genes & Regulation

6.1.2.1 Bcl-family members

The bcl-2 gene encodes for a 26-kDa intra-cellular membrane associated protein and is homologous to other genes such as bcl-xl and bax. Proteins in the Bcl-2 family all share one of four conserved domains (BH1-4). Bcl-2 is associated with the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope in healthy cells whereas Bcl-XL only becomes associated with the mitochondrial in response to apoptosis. It has been suggested that the Bcl-2 family of proteins act as a barrier to cytochrome c release from the mitochondria and their decreased expression is associated with apoptosis (Rossè et al., 1998).

The bcl-2 gene was first identified as a translocation in human lymphoma and later its expression was confirmed in all haematopoietic cells (Korsmeyer 1992). Bcl-2 inhibits premature cell death that occurs during development or in response to stimuli. Deregulation of bcl-2 in cancer appears to contribute to the resistance of cells to chemotherapeutic drugs although the distinct biological effects of bcl-2 may differ depending on the anticancer agent is used (Miyashita and Reed, 1993, Del Bufalo et al., 2002).

6.1.2.2 p53 in apoptosis

Another key regulatory protein in apoptosis is p53 which has been discussed in the previous chapter. Some of the possible mechanisms of p53-induced apoptosis are shown in Figure 38. p53 transcriptionally activates apoptogenic factors such as Bax. However, this event is unlikely to be the sole mechanism of p53-induced apoptosis as the apoptotic function of p53 is not affected in bax deficient mice (Knudson et al., 2001). p53-mediated apoptosis is proposed to be mediated by the
transactivation of other apoptosis factors such as Fas/APO1, KILLER/DR5, IGF-BP3, AIP, Pidd and the p53 inducible genes (PIGs). For example, overexpression of the p53 transgene causes transcriptional induction of the TRAIL receptor DR5 (Takimoto and el-Diery 2000). Furthermore, a p53 responsive element has been identified in the Fas promoter and increased Fas expression is not observed in mutant p53 cell lines in response to apoptosis (Bennett et al., 1998).

p53 also transactivates IGF-BP3 and induces apoptosis by blocking IGF-1 survival signalling to the IGF-1 receptor (Sionov and Haupt, 1999). Furthermore, p53 activates genes in response to oxidative stress such as PIG3 and PIG8 (Polyak et al., 1997). This may contribute to mitochondrial apoptosis but induction of these genes is not sufficient to induce apoptosis alone.

Another potential mechanism by which p53 promotes cell death is by interaction with ASPP proteins (apoptosis stimulating proteins of p53) or (ankyrin repeat, SH3 domain and proline rich domain containing proteins). These proteins augment the expression of apoptogenic genes such as Bax (Slee and Lu, 2003). Interestingly, mutations in p53 have been shown to affect interaction with ASPP proteins and thereby limit the induction of apoptosis, possibly contributing to tumorigenesis (Iwabuchi et al., 1994).
**Figure 38: p53 mediated apoptotic signalling**

*p53* can transactivate apoptotic genes by both sequence specific and sequence independent pathways. Apoptosis induction can be attributed to activation of pro-apoptosis factors such as Fas, Bax, PIG3 and PIG8 and also by inhibition of antiapoptotic proteins such as Bcl-2 or blocking survival pathways such as IGF-1R. (Stewart and Pietenpol., 2001).
6.1.2.3 PARP
Poly (ADP-ribose) polymerase (PARP) ADP ribosylates nuclear proteins in response to DNA damage (D'Amours et al., 1999). This facilitates DNA repair by increasing the availability of the DNA to different repair proteins by ribosylation of histones and therefore decondensing of chromatin structure (D'Amours et al., 1999). Hyperactivation of PARP has been associated with inflammation, diabetes, shock and neuronal death (Chiarugi 2002). PARP activity may also lead to necrosis. PARP uses NAD\(^+\) as a substrate so on activation, PARP reduces NAD\(^+\) levels within the cell. It is thought that this has a detrimental effect on glycolysis and mitochondrial respiration leading to decreased ATP production and cellular dysfunction. Other cellular enzymes try to replace NAD\(^+\) but in doing so require ATP and so have the net effect of worsening the metabolic state of the cell and ultimately this can lead to necrosis (Berger, 1985).

PARP does not always prevent NAD\(^+\) reduction after DNA damage and PARP over expression can induce death by mechanisms unrelated to NAD\(^+\) or increased consumption or ATP (Van Gool et al., 1997). There is also evidence PARP is involved in apoptosis in most cells and PARP-null cells have been found to be resistant to certain types of cell death (Herceg and Wang, 2001). During apoptosis, caspase-3 and caspase-7 amongst other proteins, cleave PARP in order to prevent PARP induced necrosis or an inflammatory response.

6.1.3 Apoptosis in Chemotherapy
Apoptosis is considered the primary mechanism by which cells die in response to chemotherapeutics although other pathways may also be important. Resistance to chemotherapy can be acquired by the increased expression of anti-apoptotic proteins.
such as Bcl-2. A notable feature in follicular B-cell lymphoma is the chromosomal translocation of t(14:18) which couples the bcl-2 gene to an immunoglobulin heavy chain locus, leading to increased expression of Bcl-2 (McDonnell et al., 1989). Bcl-2 also contributes to tumourogenesis in acute promyelocytic leukaemia by interaction with the oncoprotein c-MYC or the PML-RARα receptor fusion protein, formed by the fusion of the PML (promyelocytic leukaemia) and RAR (retinoic acid receptor) genes (Reed et al., 1988, Strasser et al., 1990). An increased level of Bcl-2 has been associated with poor response to chemotherapy and is predictive of shorter, disease free survival (Campos et al., 1993).

Human melanomas and some cell lines show high levels of FLIPs (FADD-like interleukin-1 β-converting enzyme-like protease-inhibitory proteins). These proteins inhibit apoptosis induction via the death receptor pathway and contribute to resistance to apoptosis in cancer cell lines (Igney and Krammer, 2002). Alternatively, resistance can be acquired by inactivation of apoptogenic genes such as bax. Bax is mutated in certain types of cancer and reduced bax expression is associated with poor response to chemotherapy and shorter survival (Krajewski et al., 1995). Moreover, metastatic melanomas often do not express APAF1, an integral part of the apoptosome, and often fail to respond to chemotherapy (Soengas et al., 2001). Resistance to chemotherapy has also been attributed to decreased expression of caspases and death receptors (Mese et al., 2000).

6.1.4 Topo Poisons and Apoptosis

Topo poisons have been shown to cause apoptosis in cancer cells primarily by the mitochondrial pathway, although the Fas pathway may also be activated in some cells.
Doxorubicin-induced apoptosis in Fas-expressing lymphoid cells has been attributed to increased expression of the receptor in response to drug treatment (Fulda et al., 2000). However, camptothecin, doxorubicin and etoposide treatment in HT29 colon cells also caused an increase in Fas, but yet Fas inhibition did not protect cells from drug induced apoptosis, suggesting that the mitochondrial pathway may also be involved (Shao et al., 2001). Mitochondrial permeability and the expression of apoptogenic factors have also been demonstrated in response to treatment with topo poisons (Ferraro et al., 2000, Karpinich et al., 2002). Proapoptotic proteins Bak, Bax and Bik are activated in response to doxorubicin treatment (Panaretakis et al., 2002). Furthermore, bax deficient mouse embryo fibroblasts show resistance to doxorubicin supporting a central role for the mitochondrial route in the induction of apoptosis by topo poisons.
6.2 Aims

- To determine whether XR5944 and XR11576 cause apoptosis in cancer cell lines.
- To determine any differences in timing or mechanisms of cell death between XR5944 and XR11576.

The mitochondrial pathway has been identified as the main mechanism by which topo poisons cause apoptosis (Sordet et al., 2003). Therefore, evidence of this route will be sought to determine the induction of apoptosis by XR11576 and XR5944.
6.3 Materials and Methods

As described in Materials and Methods.

6.3.1 Active caspase -3 and caspase -9 assays

A PE-conjugated active caspase-3 antibody kit was obtained from BD Biosciences (Oxford). Cells were harvested by trypsinisation, washed in PBS and resuspended in 500μl of cytofix/cytoperm manufacturers solution. Samples were left on ice for 20 mins. Cells were centrifuged and pelleted cells resuspended in 100μl of perm/wash manufacturers buffer and 20μl of antibody solution containing monoclonal rabbit anti caspase-3 in phosphate buffer (pH 7.2) with 500mM NaCl, 0.09% (w/v) sodium azide and 0.2 % (w/v) BSA. Samples were left at room temperature for at least 30mins. Finally, cells were centrifuged, washed in perm/wash buffer, and resuspended in 500μl of perm/wash buffer for analysis by flow cytometry. Active caspase-9 was detected and measured in a similar manner using a using a kit containing a FITC-conjugated anti-caspase 9 antibody obtained from Flowgen (Leicestershire).

6.3.2 Chromatin Staining with H33258

PEO1 cells were seeded at 1 × 10^4 cells/ml onto a 8-chamber microscope slide and left to adhere for 46h at 37°C/5 % CO₂. Cells were treated at two concentrations for 48h, equivalent to IC₅₀ concentrations obtained using the MTT assay with 24h drug exposure (VP16 21μM, XR5944 200nM, XR11576 300nM) or an IC₅₀ concentration obtained after 72h of drug exposure (VP16 0.5μM, XR5944 10.5nM, XR11576 48nM) after which time the chamber was removed and the cells fixed in 3% v/v formaldehyde in PBS. Cells were stained with H33258 at 100nM in PBS, washed in
PBS and mounted with an anti-fade mounting solution (Invitrogen, Paisley). Cells were viewed under a fluorescent microscope.
6.4 Results

6.4.1 Annexin V labelling of phosphatidyl serine

Figure 39 Phosphatidyl serine is displaced in response to topodirected drug treatment

FL3 on the Y-axis shows log propidium iodide (PI) fluorescence. FL1 on the X-axis shows log FITC (Annexin V) fluorescence. Healthy PEO1 cells and those in early apoptosis maintain membrane integrity and do not take up PI. Cells undergoing apoptosis at any stage, stain positively for Annexin V. Late stage apoptosis results in the degradation of the cell membrane and therefore stain positively for PI as well as Annexin V. UL - upper left quadrant, undefined morphology, UR - upper right, late apoptotic or necrotic, LL - lower left, viable, LR - lower right, early apoptotic.
Table 15: Proportion of cells in apoptotic and viable quadrants after Annexin V staining

Values are given as percentages of gated cells after 48h drug exposure.

<table>
<thead>
<tr>
<th></th>
<th>UL</th>
<th>UR</th>
<th>LL</th>
<th>LR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Other</td>
<td>Late apoptotic or necrotic</td>
<td>Viable</td>
<td>Early apoptotic</td>
</tr>
<tr>
<td>Control</td>
<td>8.5</td>
<td>2.0</td>
<td>88.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0</td>
<td>3.4</td>
<td>9.6</td>
<td>88.0</td>
</tr>
<tr>
<td>VP16</td>
<td>27.8</td>
<td>13.0</td>
<td>51.1</td>
<td>8.1</td>
</tr>
<tr>
<td>XR5944</td>
<td>12.1</td>
<td>8.0</td>
<td>75.6</td>
<td>3.3</td>
</tr>
<tr>
<td>XR11576</td>
<td>24.8</td>
<td>65.8</td>
<td>8.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Untreated control cells had over 88% of cells in the viable lower, left quadrant (Table 15). Staurosporine treated cells were used as a positive control. Most of these cells were in early or late stage apoptosis. XR11576 produced the strongest apoptotic response. The greatest proportion of cells were in late apoptosis and only a small fraction were viable (Table 15). VP16 drug exposure resulted in over 50% of the cells being viable, and over 20% in apoptosis. XR5944 treatment resulted in less apoptotic cells within the time frame examined as most of the cells were still viable at 48h.
6.4.2 Active caspase-8 expression

Figure 40: Caspase-8 is cleaved in response to treatment with XR11576 and XR5944

Whole cell lysates of PEO1 control and drug treated cells were prepared as described in Materials and Methods and subjected to Western blotting. Nitrocellulose membranes were probed with anti-caspase-8 monoclonal antibody (Oncogene) and an anti-mouse HRP conjugated secondary antibody (Sigma).

Caspase-8 cleavage and activation was observed in all drug treated samples after 16-48h of drug exposure. The extent of caspase-8 activation appeared similar for both XR5944 and XR11576, and at a consistent level over 16-48h. A small amount of cleaved caspase-8 was detected in the control, untreated cells indicating a small level of background apoptosis, probably due to the level of confluence of the cells in culture.

Actin expression was also determined (see Figure 42).
6.4.3 Bid and Bax expression

Figure 41: Bax and t-Bid is expressed in response to XR11576 and XR5944 treatment

Whole cell lysates of control and drug treated cells were subjected to Western blotting as previously described. Nitrocellulose membranes were probed with anti-Bax (Ab-5, Santa Cruz) or anti-Bid (R & D Systems) antibodies, followed by an anti-rabbit or goat HRP conjugated secondary antibody (Sigma or Dako).

Exposure to any of the drugs induced Bax and t-BID expression. Overall, Bax expression was greater in XR11576 treated samples (Figure 41). VP16 treatment resulted in increasing t-Bid expression in a time-dependent manner. Likewise, taking into account the relative levels of bid, levels of the truncated protein were similar for XR11576 and XR5944 treated cells. Actin expression was also determined and can be seen overleaf.
6.4.4 Bcl-\text{XL} and Bcl-2 expression
Figure 42: Bcl-\text{x} and Bcl-2 expression decreases in response to
exposure to XR11576 and XR5944

Whole cell lysates of control and drug treated cells were prepared as described in
Materials and Methods. Nitrocellulose membranes were probed with anti-Bcl-\text{x} or
an anti-Bcl-2 (Santa Cruz) and an anti-mouse HRP conjugated secondary antibody.
Protein concentrations were confirmed by Actin detection.

An inverse correlation was observed between Bcl-\text{XL} expression and the duration of
drug exposure. XR11576 exposure resulted in the most rapid decrease in Bcl-\text{XL}
levels, but overall results were similar for all treatments. Likewise, the Bcl-2
expression levels followed a very similar pattern.
6.4.5 PARP Cleavage

Figure 43 PARP is cleaved in response to treatment with topo-directed agents

PARP cell lysates were prepared as described in Materials and Methods. 40µg of protein was electrophoresed on SDS page gels and subjected to western blotting. Mouse anti-PARP (Alexis) was used as a primary antibody followed by an anti-mouse HRP-conjugated secondary (Sigma). Signals were detected by chemiluminescence.

The 85kDa cleaved PARP band and parental 116kDa band were detected in all drug treated cells. Levels of cleaved PARP increased over time of exposure and were greatest at 72h. PARP cleavage was not detected in untreated control cells. Only the cleaved band was detected in staurosporine treated cells, used as a positive control.
6.4.6 Activation of caspase-3 and caspase-9

Figure 44: Caspase-3 is activated in response to XR5944 and XR11576

PEO1 ovarian carcinoma cells were treated with IC$_{80}$ concentrations (obtained following MTT testing with 72h of drug exposure) of VP16, XR5944 or XR11576 for 48h. Staurosporine was used as a positive control. Cells were harvested by trypsination and labelled with PE-conjugated rabbit anti-active caspase-3. Samples were analysed on a Beckman-Coulter Epics XL ™ using FL2 (fluorescence >575nM versus light scatter was measured with an excitation of 488nM). M1 – caspase-3 M2-activated caspase-3 within gated region. Data shown are representative of results obtained from repeat experiments (n=2). Percentages shown are gated cells with caspase-3 (first value) and activated caspase-3 (second value).
Figure 45: Caspase-9 is activated in response to XR5944 and XR11576

PEO1 cells were treated with ICₘₙ concentration of VP16, XR5944 or XR11576 for 48h. Staurosporine was used as a positive control. (Data not shown) Cells were harvested by trypsinization and labelled with PE-conjugated anti-active caspase-9.

Samples were analysed on a Beckman-Coulter Epics XL™ on FL1 (fluorescence >575nM versus light scatter was measured with an excitation of 488nM). Data shown are representative of results obtained from repeat experiments (n=2). Percentages shown are per cent of cells with activated caspase-9.
Caspases -3 and -9 were activated in response to drug treatment in PEO1 carcinoma cells. Caspase-3 activation appeared more complete for XR11576 than XR5944 treatment, but may reflect the temporal differences in cytotoxicity previously observed. The extent of caspase-9 activation seemed very similar for XR11576 and XR5944 treatments.
6.4.7 Evidence of apoptotic morphology with XR5944 and XR11576

Figure 46 and Figure 47 illustrate a typical sample of cells treated with two concentrations of drug compared to untreated control cells.

Cells were stained with a nuclear chromatin dye H33258. Untreated control cells had regularly shaped nuclei with some mitotic cells present. VP16 treated cells had brighter, irregular shaped nuclei showing extensive damage, characteristic of apoptotic cells, at both concentrations. Apoptotic bodies showing small, condensed nuclei, indicative of late stage apoptosis were also present. XR5944 treated cells appeared unchanged from controls at the lower concentration, without mitotic cells present. At higher XR5944 concentrations, cells showed nuclear compaction, characteristic of apoptosis. Nuclei appeared damage in XR11576 treated cells at a lower concentration and most cells were fully apoptotic at the higher concentration.
Figure 46 PEO1 cells stained with chromatin H33258 stain.
Cells were incubated for 48h with media alone or with low concentrations of VP16, XR5944 or XR11576
N-normal, M-mitotic, A-apoptotic, AB-apoptotic body. Duplicates are shown to illustrate consistent typical fields of view.
Figure 47 PEO1 cells stained with chromatin H33258 stain.
Cells were incubated for 48h with media alone or with high concentrations of VP16, XR5944 or XR11576
N - normal, M - mitotic, A - apoptotic, AB - apoptotic body. Duplicates are shown to illustrate consistant typical fields of view.
6.4.8 TUNEL Assay

Figure 48: DNA fragmentation in response to XR5944 and XR11576 treatment, measured by the TUNEL assay

PEO1 cells were treated with an IC$_{50}$ concentration of drug (obtained from MTT data with 72h drug exposure) for 48h. Staurosporine at 1uM was used as a positive control.
Table 16: Proportion of gated cells shown to be TUNEL positive following treatment with XR5944 and XR11576
Data shown represents a typical set of data.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of cells within marker region i.e. TUNEL positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>15.3</td>
</tr>
<tr>
<td>VP16</td>
<td>13.0</td>
</tr>
<tr>
<td>XR5944</td>
<td>3.6</td>
</tr>
<tr>
<td>XR11576</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Flow cytometric detection of dUTP labelled cleaved DNA at the 3'–OH terminal showed VP16 and XR11576 treated cells had more fragmented DNA, characteristic of apoptosis, than untreated control cells (Figure 48).

Microscopic analysis of TUNEL samples illustrated the different effects of each drug on the cells (Figure 49, overleaf). Staurosporine, VP16 and XR11576 treatment resulted in many cells exhibiting green FITC staining. Cells appeared swollen and some apoptotic bodies were also observed in VP16 treated cells, exemplifying late stage apoptosis. XR5944 treated cells had more regular morphology and greater PI staining. XR11576 showed greatest FITC labelling and cellular morphology was indicative of early apoptosis.
Figure 49: Confocal microscope pictures of drug treated and control cells after 48h using the TUNEL assay.

Red indicates propidium iodide staining. Green indicates FITC labelling. ×400 magnification. A = apoptotic, AB = apoptotic body, N = normal,

Control  Staurosporine

VP16  XR5944

XR11576
6.5 Discussion

Treatment with XR5944 and XRl 1576 resulted in PEO1 cells undergoing an apoptotic response. Evidence of apoptosis was detected by the movement of phosphatidyl serine to the outside of the cellular membrane, DNA fragmentation, activation of caspases, changes in expression of pro-and anti-apoptotic factors and morphological changes.

The extent of apoptosis observed following treatment with XR5944 and with XRl 1576 appeared different, within the time window observed. XR5944 produced an apparently weaker apoptotic response according to the Annexin V and TUNEL assays. These observations may, in part, be explained by the previously observed slower onset of XR5944 mediated cytotoxicity (Chapter 3). Therefore, it is possible that differences in the apoptotic response were due to experimental design.

However, some of the data obtained in this part of the study show little difference between XRl 1576 and XR5944 treatments. Bcl-2 expression decreased over time and in a similar manner with all drug treatments. Activation of caspase-8 was very similar for both drug treatments. The lack of differences between treatments and these apoptotic factors may have been in part due to the high drug concentrations used to promote apoptosis. However, the significance of these data are not entirely clear when compared with other data generated in this study.

Overall, data have shown clear evidence for the activation of the mitochondrial apoptotic pathway. Increased t-Bid and Bax expression were evidence of mitochondrial pore formation. Previous reports have indicated that Bax protein is
either unchanged or increased and Bcl2 underexpressed in cells treated with topo poisons (Kluza et al. 2000). Our data are in line with these observations and the decreased expression of Bcl-2 and Bcl-XL was suggestive of changes in mitochondrial integrity. These anti-apoptosis factors are degraded in response to apoptotic stimuli and Bcl-2 contributes to mitochondrial membrane permeabilisation (MMP) (Rossè et al., 1998). In response to MMP, caspase-9 is cleaved as part of the apoptosome and then activates executioner caspases -3,-6 and -7. In these experiments, activation of both caspase-9 and caspase-3 was detected after treatment of tumour cells with XR5944 and with XR11576. PARP cleavage was also observed after 24h of treatment with either drug. The extent of PARP cleavage and caspase-3 activation in XR5944 treated cells was somewhat less than that seen for XR11576 and probably represented the slight delay seen in onset of cytotoxicity by the former. These parameters are associated with a later stage of apoptosis and represented the degradation of 'death substrates'.

Further work could include examining other mechanisms of cell death such as necrosis. Necrosis has been observed in etoposide treated cells under ATP depleted conditions (Eguchi et al., 1997). Apoptosis can be inhibited by a reduction in intracellular ATP and if XR5944 continued to cause cell death under ATP depleted conditions, it could be concluded that XR5944 was causing cell death by necrotic pathways (Shimizu et al., 1996). The Annexin V method used in these experiments does not differentiate between late state apoptosis and necrosis and so some of the cells that were detected as apoptotic may have been necrotic.
In conclusion, an apoptotic response was observed in tumour cells treated with either XR11576 or XR5944. Evidence of activation of the mitochondrial pathway of apoptosis was observed, in agreement with other data obtained using topo poisons (Ferraro et al., 2000, Karpinich et al., 2002). It is important to note, however, that these molecular changes are also known to occur in cancer cells treated with other classes of chemotherapeutic agents. Subtle differences in the apoptotic response to XR5944 and XR11576 were seen.
CHAPTER 7

Final Discussion
7 Final Discussion

XR5944 and XR11576 originated from a drug development programme designed to target topoisomerases I and II. A predecessor of that research programme was DACA, originally described as a dual topo I and II poison. Phase II studies involving DACA showed some minor responses in recurrent glioblastoma multiforme, advanced ovarian cancer and metastatic colorectal cancer (Twelves et al., 2002, Caponigro et al., 2002). Moreover, 4 out of 12 NSCLC patients achieved disease stabilisation in another phase II study but severe toxicity was documented (Dittrich et al., 2003). However, the overall objective response rate of these trials was considered insufficient to warrant further studies. The precise target of DACA had been the subject of some speculation (see 1.2.7.4) The conclusion of various studies is that topo II\(\alpha\) represents the putative target of DACA as topo II\(\beta\) cleavable complexes are only stabilised at high concentrations (Padget et al., 2000).

XR5944 and XR11576 have also been described as dual topo I and II poisons (Stewart et al., 2001, Mistry et al., 2002). In contrast to this, some of the data generated in the present study show that the potency of XR5944 and XR11576 were not affected by changes in topo expression in human cancer cell lines (discussed in Chapter 3). We show data that indicate XR5944 and XR11576 are distinctly different from agents such as camptothecin and VP16 whose potencies are affected by decreased expression of topo I and II, respectively. This could be attributed to the dual stabilisation of topos I and II of XR11576 and XR5944, resulting in highly effective cytotoxicity. However, some decrease in cytotoxicity of the latter would be expected in cell lines exhibiting decreased expression of topo I and II simultaneously.
The work in the present study provides evidence that in the MBVP16R cell line, where there is marked downregulation of all 3 classes of topoisomerases, XR11576 and XR5944 retain potent cytotoxicity.

XR5944 and XR11576 both caused DNA damage and DNA-protein crosslinks, consistent with cleavable complex formation by a topo poison. However, these effects may be attributable to their ability to act as intercalative agents. When comparing the activities of these 2 agents relative to each other a number of differences were seen when assessing the DNA interaction. Despite the delay in onset of DPCs caused by XR5944, once initiated, greater DPC formation was observed following XR5944 treatment compared with treatment of cells with either XR11576 or VP16. DNA damage is an expected effect of topo inhibition as cleavable complex stabilisation results in DNA breakage. However, we suggest that the DNA damaging effects we are able to demonstrate by the present study occur as a result of other aspects of the mechanisms of action of these compounds.

The late onset of XR5944's activity was reflected in nearly all of the experiments presented in this thesis. The cytotoxicity of XR5944 was shown to increase up to 24h of drug exposure in comparison with 10h of exposure required using XR11576 for equivalent cytotoxicity. Furthermore, measurement of apoptotic endpoints confirmed XR5944 was slower in causing cell death than XR11576. However, there appeared to be no differences in the nature or extent of the apoptosis seen using equicytotoxic doses of either of these two agents. Notably, XR5944 was consistently more potent than XR11576 in the human cancer cell lines tested throughout this project, in line with previously published data (Mistry et al., 2002, Stewart et al., 2001).
It could be suggested that XR5944, and indeed XR11576, has other effects on the cell, independent of topo interactions, before topo-dependent effects such as DPC formation and G2 accumulation of cells take place. Recent work has demonstrated that XR5944 specifically may have effects on RNA processing (Fleming et al., 2003).

Cellular and molecular differences were observed in cells exposed to XR5944 and XR11576 throughout this study. As already mentioned, the timing of cytotoxicity, the profile of DPC formation and cell cycle changes were clearly different between compounds. XR5944 is also a substrate for ABC-transporter proteins whereas XR11576 is not. The differences observed after XR5944 and XR11576 treatment may be due to structural differences between the compounds. For example, XR5944 is more likely than XR11576 to cause pi-orbital interactions with DNA as it is a bis-phenazine with more aromatic rings than the latter (Yang et al., 2003). Moreover, the two sites of aromatic rings in XR5944 give rise to two possible sites for the compound to bind with DNA, whereas XR11576 only has one potential site. Some interactions and hydrogen bonding would also be possible between amine groups of the side chains of both XR5944 and XR11576, and DNA. However, more DNA interactions would be likely with XR5944 as there are more amide groups present. The greater number of potential DNA interactions with XR5944 may also account for the substantial DPC formation observed in cells following treatment, exceeding the level of formation seen for XR11576 and VP16. Similarly, some of the increased potency of XR5944, in comparison with XR11576, may be attributed to increased DNA interactions. However, the late onset of XR5944 DPC formation and cytotoxicity is surprising given the increased likelihood of DNA interactions. It
Chapter 7  Discussion and future work

L J Lewis

appears XR11576 causes DNA interactive effects before XR5944 despite being a less potent intercalator, for reasons that remain unclear.

*In vivo* findings have also demonstrated differences between XR5944 and XR11576. Tumour regression was observed in 8/10 mice transplanted with HT29 colon carcinomas treated with XR5944 (15 mg/kg i.v q4d × 3). A weight loss of only 4-5% was observed indicating that XR5944 was well tolerated. This was in comparison to Tas-103 which caused significant toxicity and death in treated animals with a schedule of 40mg/kg, q4d × 2 or 5mg/kg, qd × 5 in similar experiments (Stewart *et al.*, 2001). Moreover, treatment with XR11576 also resulted in tumour regression and was well tolerated but slightly higher body weight loss of 12% was observed with a schedule of 52.5mg/kg i.v q7d × 3 (Mistry *et al.*, 2002). These data suggest that XR5944 and XR11576 possess potent *in vivo* antitumour acitivity.

In conclusion, XR5944 and XR11576 are potent cytotoxic agents capable of causing cancer cell death *in vitro* with XR5944 at lower concentrations than some currently used topo poisons such as VP16. Although they were originally thought to behave as topo poisons, data shown in this thesis suggests this is not the case. Both compounds appear to be unrecognised by atypical drug resistance mechanisms. Furthermore, work in our laboratories has shown that generating *in vitro* cell line models with acquired resistance to XR5944 is virtually impossible whilst cell lines with acquired resistance to XR11576, VP16 or camptothecin is achievable (Coley, personal communication). Therefore, XR5944 in particular, should show efficacy in the clinic both as a potent cytotoxic compound and as a second line agent when resistance to other topo poisons has been acquired.
7.1 Future Work

The panel of Chinese hamster ovary (CHO) cell lines used to analyse the effects of altered topo expression on the potency of XR5944 and XR11576 were inconclusive and the human cell line models used did not include a cell line that lacked total expression of one type of particular topo. Therefore, the effect of individual topo expression on the efficacy of XR5944 and XR11576 cytotoxicity could be examined by using antisense-RNA techniques to inhibit all production of one type of topo in a cell line model. It would not be possible to inhibit all topo expression as these cells would not be viable. Therefore, separate inhibition of topo I, IIA and IIB would indicate the preference of the compounds for each topo. Similarly, pre-treatment with catalytic inhibitors before cytotoxicity testing would show how integral topos are in these compounds' toxicity as inhibition of topos would limit cleavable complex formation.

DNA damage could be more thoroughly examined by various experiments. Free radical formation has been shown to be important in topo II poison mediated DNA damage and this could be investigated by pre-treating cells with D,L-buthionine-S,R-sulphoxime (BSO) in order to deplete cellular glutathione levels followed by drug treatment (Drew and Miners, 1984). Under normal conditions, glutathione will limit free radical damage. If cells were rendered more sensitive to drug treatment by reduction in glutathione levels, it can be concluded that free radical formation is an important part of the compounds cytotoxicity.

As already mentioned, the involvement of necrosis in the mechanism of cell kill caused by XR5944 and XR11576 could be determined by causing ATP depletion in
cells and observing any difference in cell death. This could be achieved by incubating cells with glucose free media and oligomycin, an inhibitor of mitochondrial ATPase, and carrying out a MTT assay. The media would have to be replaced with fresh media with glucose in order for mitochondrial function to be restored for the termination of the assay (Eguchi et al., 1997). If cell death was inhibited under ATP depleted conditions, then it could be concluded that the compounds were causing cell death by apoptotic pathways. AIF and ceramide involvement in apoptosis could also be measured to investigate alternative apoptotic pathways. AIF expression could be measured by Western blotting and ceramide levels detected by a diacylglycerol kinase assay which measures total cellular ceramide levels (Preiss et al., 1986). Furthermore, an increase in FasL mRNA is seen when ceramide accumulates. This could be measured by RT-PCR before and after drug treatment.

Cytotoxic DNA lesions can lead to the initiation of DNA damage signalling pathways, cell cycle arrest, repair of the lesions, or apoptosis. Most of these responses were investigated during this project. However, the repair of the lesions caused by XR5944 and XR11576 was not examined. This may be a valuable area to investigate as repaired lesions often contain mutations and these may cause secondary cancers years after the initial chemotherapy (Baguley and Ferguson 1998). This has been particularly noted after VP16 treatment where the incidence of AML is abnormally high (Felix 1998).

**Concluding Remarks**

XR5944 and XR11576 are both currently in early phase clinical trials in Europe. It may be speculated that these agents could have pronounced side effects due to their...
high potency and effect on proliferating cells. However, in vivo findings have not demonstrated particularly severe side effects with treatment. This is in contrast to simultaneous treatment with topo I and II poisons where significant toxicity has been observed (Ando et al., 1997, Hammond et al., 1998, Herben et al., 1997). Given the potency of these compounds in vitro and the promising in vivo findings, the results of the clinical trials are, therefore, awaited with anticipation.
CHAPTER 8

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APPENDIX
Appendix: Cytotoxicity Testing

The following results illustrate an example of MTT cytotoxicity testing. Each drug dilution is tested in quadruplicate. The mean is taken of these absorbance readings which are given as optical units at a wavelength of 540nm. The mean value is compared with the mean untreated control and presented as a percentage of growth compared with the control. IC\textsubscript{50} values are calculated as the drug concentration resulting in 50% growth inhibition compared with the untreated control cells.

Table 17 MTT Cytotoxicity Testing for K562 Cells.
Cells were treated with a range of dilutions of XR5944 for 72 hours. Half the samples were treated concomitantly with XR9576.

<table>
<thead>
<tr>
<th>Concentration of XR5944 (nM)</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
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<tbody>
<tr>
<td>Reading 1</td>
<td>1.036</td>
<td>0.946</td>
<td>0.839</td>
<td>0.59</td>
<td>0.553</td>
<td>0.495</td>
<td>0.481</td>
<td>0.233</td>
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<tr>
<td>Reading 2</td>
<td>1.109</td>
<td>1.064</td>
<td>0.734</td>
<td>0.623</td>
<td>0.525</td>
<td>0.519</td>
<td>0.482</td>
<td>0.395</td>
</tr>
<tr>
<td>Reading 3</td>
<td>1.123</td>
<td>0.85</td>
<td>0.749</td>
<td>0.28</td>
<td>0.594</td>
<td>0.361</td>
<td>0.545</td>
<td>0.375</td>
</tr>
<tr>
<td>Reading 4</td>
<td>1.07</td>
<td>0.916</td>
<td>0.735</td>
<td>0.608</td>
<td>0.53</td>
<td>0.423</td>
<td>0.469</td>
<td>0.397</td>
</tr>
<tr>
<td>Mean</td>
<td>1.086</td>
<td>0.969</td>
<td>0.764</td>
<td>0.525</td>
<td>0.551</td>
<td>0.450</td>
<td>0.494</td>
<td>0.350</td>
</tr>
<tr>
<td>% of control</td>
<td>86.35</td>
<td>77.15</td>
<td>60.85</td>
<td>41.82</td>
<td>43.83</td>
<td>35.79</td>
<td>39.35</td>
<td>27.87</td>
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<tr>
<td>S.E.M</td>
<td>1.57</td>
<td>2.61</td>
<td>2.01</td>
<td>6.66</td>
<td>1.26</td>
<td>2.87</td>
<td>1.37</td>
<td>3.15</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>XR5944 &amp; XR9576</th>
<th>Concentration of XR5944 (nM)</th>
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<th>0.2</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
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<tbody>
<tr>
<td>Reading 1</td>
<td>1.069</td>
<td>0.984</td>
<td>0.613</td>
<td>0.696</td>
<td>0.409</td>
<td>0.405</td>
<td>0.449</td>
<td>0.295</td>
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<td>Reading 2</td>
<td>0.831</td>
<td>0.908</td>
<td>0.874</td>
<td>0.541</td>
<td>0.551</td>
<td>0.443</td>
<td>0.453</td>
<td>0.44</td>
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<tr>
<td>Reading 3</td>
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<td>0.893</td>
<td>0.564</td>
<td>0.492</td>
<td>0.576</td>
<td>0.533</td>
<td>0.414</td>
<td>0.488</td>
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<tr>
<td>Reading 4</td>
<td>0.8</td>
<td>0.849</td>
<td>0.559</td>
<td>0.485</td>
<td>0.544</td>
<td>0.315</td>
<td>0.461</td>
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<tr>
<td>Mean</td>
<td>0.877</td>
<td>0.909</td>
<td>0.603</td>
<td>0.529</td>
<td>0.520</td>
<td>0.424</td>
<td>0.444</td>
<td>0.421</td>
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<tr>
<td>% of control</td>
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<td>80.36</td>
<td>53.30</td>
<td>46.75</td>
<td>46.00</td>
<td>37.51</td>
<td>39.30</td>
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</tr>
<tr>
<td>S.E.M</td>
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<td>2.37</td>
<td>2.28</td>
<td>3.33</td>
<td>4.00</td>
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<tr>
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