DETERMINANTS OF CHEMOSENSITIVITY IN
TRANSITIONAL CELL CARCINOMA
OF THE BLADDER

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A Thesis submitted to the University of London for the degree of Master of Surgery

OCTOBER 1994
ABSTRACT

Transitional cell carcinoma of the bladder is sensitive to combination chemotherapy and response rates of up to 65% have been reported. However, 35% of cases do not respond and relapse is common. The reasons for treatment failure are unclear. One possible mechanism is the presence of innate drug resistance within tumours and another is potential variation in cytotoxic drug levels, resulting in underdosing of some patients. These two possibilities have been explored.

In this thesis two drug resistance mechanisms have been studied. Firstly expression of the enzyme Glutathione S-transferase \( \pi \), which conjugates cytotoxic agents to glutathione, has been measured in a series of tumours. Levels of enzyme expression have been related to outcome in a group of patients who went on to receive cisplatin based chemotherapy. No difference was demonstrated, in terms of enzyme expression between tumours that had responded to chemotherapy and those that had not.

Secondly a semi-quantitative, polymerase chain reaction based, assay has been developed in order to measure expression of the multidrug resistance gene (MDR1) at the mRNA level. This has involved amplifying the MDR1 gene relative to an internal reference gene, namely ribosomal RNA. Using this assay a 63 fold variation in MDR1 mRNA levels was demonstrated between tumours with a significantly higher level in poorly differentiated tumours.

A total of 57 pharmacokinetic studies were performed on 30 patients with transitional cell carcinoma receiving combination chemotherapy. Wide variations were observed in cytotoxic drug levels achieved and free cisplatin levels were
significantly higher in the group of patients demonstrating a clinical response to
treatment when compared to levels achieved in non-responders.

The studies performed in this thesis have revealed the limitations of current
cytotoxic dosing methods. The wide variation in MDR1 expression suggests that
classical multidrug resistance may be a significant mechanism in chemotherapy failure
in the treatment of transitional cell carcinoma of the bladder.
AUTHORS STATEMENT

This thesis represents the results of the author's work over a two year period. I was responsible for collecting all clinical material in the form of tumour biopsies and blood samples for pharmacokinetic studies. I was responsible for recruiting patients and obtaining all necessary clinical data.

In the laboratory I performed all the cisplatin analyses personally. Methotrexate analyses were performed by the department of toxicology at Newcastle General Hospital and vinblastine analyses were performed by Dr Aherne at the cancer research campaign laboratories at Sutton.

The MDR1 polymerase transcription assays described in chapter 3 were performed in conjunction with Steven Clifford who is a Ph.D student currently working in the cancer research unit in Newcastle. He kindly taught me the technique and I personally performed the entire assay in all its stages.

The immuno-histochemical techniques described were performed by Joan Vickers MLSO and the stained sections were interpreted by myself in conjunction with Dr Peter Birch MRCPath.

I have personally analyzed and discussed all the data included in this thesis.
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ACKNOWLEDGEMENTS

This thesis would not have been possible without the help and co-operation of a number of people to whom I would like to express my gratitude. Firstly I would like to thank Professor Neal for giving me the opportunity to carry out this work and for his support and guidance throughout the project. At the Cancer Research Unit I was supervised by Dr Herbie Newell in the pharmacokinetic studies and by Dr John Lunec in the molecular biology work and I am grateful to them both for help and advice. My particular thanks go to Steven Clifford who developed the MDR1 polymerase chain reaction based transcription assay described in this thesis and patiently taught me the technique.

No clinical and laboratory study can be undertaken without the goodwill of all patients and clinical staff involved. I would like to thank the chemotherapy team at Freeman Hospital namely, Sister Marsh, Mr Hall and Dr Roberts without whom the pharmacokinetic studies would not have been possible. Finally I would like to acknowledge the financial support of the North of England Cancer Research Campaign in funding all the work described in this thesis.
PUBLICATIONS ARISING FROM THIS THESIS


2. MDR1 gene expression in transitional cell carcinoma of the bladder.


CHAPTER 1: INTRODUCTION

1.1 EPIDEMIOLOGY AND NATURAL HISTORY OF BLADDER CANCER

The first author to report a bladder tumour was Fabricius Hildanus (1606-1627). This was a tumour removed incidentally at the time of lithotomy. Much later Chopart appreciated that not all bladder tumours had the same natural history when he reported that the bladder fungus (papillary tumour) behaved differently to the solid tumour (Chopart 1791).

Neoplasms of the urinary bladder are now known to be common in virtually every country where accurate cancer statistics are recorded. In England and Wales the Office of population censuses and surveys 1989, (published 1994) registered approximately 11,000 new cases of bladder cancer (8,009 male and 3,334 female). Bladder cancer accounted for approximately 5000 deaths in 1992 (3,299 males and 1,612 females).

The majority of bladder cancers seen in Western countries are transitional cell carcinomas, arising from the transitional urothelium lining the bladder. Squamous cell carcinomas are more common in Middle Eastern countries, such as Egypt, where bilharzia is endemic. Adenocarcinoma of the bladder is uncommon, accounting for less than 2% of cancers. Cancers may also arise from non epithelial elements such as sarcomas and lymphomas but these are rare. Table 1 illustrates the common types of epithelial bladder cancer.
Table 1.1 Classification of epithelial bladder tumours

1. Transitional cell carcinoma (>90%)
2. Squamous cell carcinoma (5%)
3. Adenocarcinoma (2%)
4. Mixed combination of transitional, squamous, adenocarcinoma
5. Miscellaneous small cell carcinoma
   spindle cell carcinoma
   carcinoid
6. Secondary carcinoma (local spread or distant metastasis)

Transitional cell carcinoma

Aetiology

Factors reported to be causally related to bladder cancer include cigarette smoking (Doll and Peto 1981), occupational exposure to chemicals (Cole et al., 1972), analgesic abuse (Piper et al., 1985), and exposure to cytotoxic chemotherapeutic agents; in particular cyclophosphamide. In the Western world cigarette smoking is the most significant risk factor in numerical terms. In general the interval from exposure to occupational carcinogens to the development of a tumour is approximately 25 years. The risk from exposure to environmental carcinogens may be modified according to host factors such as genetic polymorphisms. For example, deletion of the GSTM1 gene, whose product is the detoxifying enzyme Glutathione S-transferase
\( \mu \), has been found to be associated with an increased risk of bladder cancer (Daly et al., 1993).

Transitional cell carcinomas are particularly associated with cigarette smoking and exposure to industrial carcinogens. Carcinogens such as Benzo-a 4,5 pyrene oxide, a common carcinogen in cigarette smoke, can produce lesions in the genome of transitional epithelial cells, initiating carcinogenesis. Further evidence is the finding of carcinogenic substances in the urine of smokers such as tryptophan metabolites and \( \beta \) 2-Napthylamine (Kerr et al., 1965, Yamasaki et al. 1977).

It is certain that multiple lesions in the genome are required for malignant transformation of the cell and abnormalities in the form of allelic deletions in chromosomes 1, 5, 7, 9, 11, 17, 18 and 21 have all been reported in bladder cancer. There are several potential mechanisms for carcinogenesis in these tumours. Firstly the induction of oncogenes, such as the ras family located on chromosomes 1, 11 and 12. Secondly the inactivation of tumour suppressor genes such as p53 and the retinoblastoma gene. Normally tumour suppressor genes such as p53 will prevent replication in a cell with abnormal DNA and drive the cell into apoptosis. When inactivation occurs, for example due to a mutation, the cell continues to replicate. A third type of carcinogenic mechanism may be the amplification or over-expression of genes that encode for growth factors and their receptors which regulate growth and differentiation of cells e.g. Epidermal Growth Factor receptor (Neal et al., 1990).

The multistage theory of carcinogenesis has been applied to bladder cancer both in an experimental and clinical setting. This theory proposes that the process of carcinogenesis passes through sequential stages, namely initiation and promotion.
Exposure to an initiating factor such as a carcinogen results in damage to DNA. This in itself is not sufficient to result in a tumour developing unless the initiated cell population is further stimulated by a promoter substance. The promoter may or may not be carcinogenic. If it is, then it is termed a complete carcinogen.

More than 90% of bladder tumours in the United Kingdom are transitional cell carcinomas, arising from transitional urothelium. Their macroscopic appearance may be papillary, solid or mixed. Microscopically they have typically malignant features such as an increased number of cell layers, loss of polarity, abnormal maturation from basal to superficial layers, increased cytoplasmic to nuclear ratio and increased mitoses. Metaplasia is common in transitional cell carcinomas, giving rise to squamous and adenocarcinomatous elements. Alternatively squamous carcinomas and adenocarcinomas may arise de novo.

Carcinoma in Situ

Carcinoma in situ is a histological diagnosis consisting of the presence of poorly differentiated malignant urothelial cells confined to the urothelium, with an intact basement membrane. It represents a field change in the urothelium and is commonly multifocal. Some patients with this condition have a protracted clinical course without developing invasive bladder cancer but others will rapidly progress to muscle invasive disease (Althausen et al., 1976). Carcinoma in situ is an indicator of poor prognosis and may be seen adjacent to approximately 50% of poorly differentiated tumours.
Squamous cell carcinoma

This is defined as a pure squamous tumour without any other cellular pattern. It needs to be distinguished from transitional cell carcinomas with squamoid features. Squamous cell carcinoma tends to be secondary to chronic inflammation and subsequent metaplasia from a transitional type of epithelium to a squamous type. In the case of bilharzia this inflammation is in response to the parasite Schistosoma Haematobium which is the commonest cause of squamous cell carcinoma in the middle East (Lucas 1982). Tumours have no sex preponderence and tend to occur in younger patients than those developing transitional cell tumours. As the ova migrate through the bladder wall from the perivesical venous sinusoids, they excite an intense chronic inflammatory response. In Western countries the cause of the chronic inflammation is more likely to be chronic bacterial cystitis or bladder calculus.

Adenocarcinoma

Adenocarcinoma of the bladder may be primary or secondary in origin. Of the primary tumours approximately 30% arise from an embryonic remnant of columnar cells in the vault of the bladder adjacent to the obliterated urachal remnant and the remainder are secondary to glandular metaplasia. Secondary adenocarcinoma of the bladder is more common and may arise by direct local invasion from surrounding pelvic structures such as colon, uterus, ovary and prostate or by distant metastasis via a haematogenous route. The diagnosis of primary adenocarcinoma is therefore made after exclusion of an adenocarcinoma arising at another site.
**STAGE and GRADE**

All tumours may be assigned a stage and grade. Grade is assigned according to the degree of differentiation of the tumour cells (Koss 1974). One common method is to classify these into 3 groups:

- **G1**: well differentiated
- **G2**: moderately differentiated
- **G3**: poorly differentiated

Tumours are staged according to UICC criteria (1978) which combines both clinical and pathological criteria. The principles of this staging method are outlined in *Figure 1.1*

Bladder cancers are termed superficial or invasive according to whether muscle invasion has or has not occurred. This certainly is an over-simplification, as within the superficial group, Ta and T1 tumours behave very differently. Approximately 25% of poorly differentiated T1 tumours rapidly progress to muscle invasive disease and if associated with carcinoma in situ this figure rises to 50% (Anderstrom *et al.*, 1980). Accurate staging of bladder cancer is of crucial importance in planning treatment according to the predicted natural history of the tumour.

**PROGNOSIS**

Approximately 70-80% of patients with transitional cell carcinoma (TCC) of the bladder will present with low grade superficial tumours (Ta). The majority of patients will develop tumour recurrences (Althausen *et al.*, 1976). Most recurrences are new tumours arising from other areas of dysplastic urothelium but some may be
true recurrences from inadequate treatment or implantation (Page et al., 1978). These patients are effectively treated with endoscopic resection and have a 5 year survival of 85-90% (Heney et al., 1983).

In contrast, patients presenting with tumour invading into or through the muscle layer of the bladder wall (T2,3,4) have a worse prognosis. Most patients with invasive bladder cancer already have invasive disease at the time of diagnosis (Hopkins et al., 1983). Despite advances in surgical and radiotherapy techniques, there has been little change in survival rates over the past 40 years with less than half of patients with invasive bladder cancer living for 5 years or more after conventional therapy (Raghavan et al., 1990). As many as 50% of patients with muscle invasive disease have occult metastases at time of presentation and most of these patients develop clinically evident metastases within one year (Prout et al., 1979). Patients with metastatic disease rarely survive 2 years whereas patients with very limited regional lymph node disease have a better prognosis and as many as 30% may survive for 5 years following radical cystectomy and lymphadenectomy (Skinner and Lieskovsky, 1984).

In the knowledge that a significant proportion of patients with muscle invasive bladder cancer already have metastases at the time of presentation, trials of neoadjuvant chemotherapy alongside conventional treatment are presently being undertaken in an attempt to improve survival in patients with transitional cell carcinoma of the bladder.
**Figure 1.** TNM classification of bladder tumours (UICC, 1978)

- **T0**: No evidence of tumour
- **TIS**: Pre-invasive carcinoma (carcinoma in situ)
- **Ta**: Papillary non invasive carcinoma
- **T1**: Invasion into lamina propria, no mass palpable after resection
- **T2**: Invasion of superficial muscle, no mass palpable after resection
- **T3a**: Invasion of deep muscle, mass palpable after resection
- **T3b**: Invasion of deep muscle through to perivesical fat
- **T4a**: Invasion of prostate, uterus or vagina
- **T4b**: Tumour fixed to pelvic side wall or abdominal wall
- **TX**: Requirements to assess tumour cannot be met
1.2 SYSTEMIC CHEMOTHERAPY IN THE TREATMENT OF BLADDER CANCER

Over the past decade, many trials investigating the efficacy of different chemotherapeutic agents in TCC bladder have been reported. Patients with loco-regional recurrence or distant metastasis have been treated with systemic cytotoxics, occasionally with dramatic responses. Subsequently, chemotherapy has been used in an adjuvant setting for patients with lymph node involvement discovered at the time of cystectomy. More recently, combination chemotherapy has been used in a neoadjuvant setting prior to radical surgery or radiotherapy in an attempt to increase survival in patients with locally advanced disease. In order to discuss the development of chemotherapy it is important to understand the criteria used to grade response to treatment.

Response criteria

Early phase I and II chemotherapy trials are often difficult to interpret due to the lack of accurate response criteria (Yagoda A. 1980). In 1985, response criteria for urothelial tumours were outlined at the Consensus Development Conference on Guidelines for Clinical Research in Bladder Cancer (Van Oosterom et al., 1986) (Table 1.2).

Clinical complete response (cCR) is defined as the complete disappearance of all clinical, radiographic, cytologic and biochemical evidence of disease, including negative cystoscopy and biopsy. A pathologic complete response (pCR) requires the histologic confirmation of the absence of residual disease after laparotomy and
cystectomy, or adequate biopsy of sites of previous disease such as bone. A surgical complete response (CRs) is defined as the complete resection of all residual viable disease after chemotherapy induced remission. The clinical response prior to surgery may be cCR, partial remission (PR), or minor response (MR).

Table 1.2  Response criteria for urothelial tract tumours

<table>
<thead>
<tr>
<th><strong>Response</strong></th>
<th><strong>Criteria</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complete remission</strong></td>
<td>Clinical CR (cCR) : Complete disappearance of all clinical evidence of tumour on physical examination, cystoscopy + biopsy, CT scan and urine cytology for one month.</td>
</tr>
<tr>
<td></td>
<td>Pathological CR (pCR) : Adequate biopsy material indicating no evidence of disease by cystectomy or biopsy of sites of known previous disease.</td>
</tr>
<tr>
<td><strong>Partial remission</strong></td>
<td>Greater than 50% decrease on physical examination or radiography of the summed products of the perpendicular diameters of all measured lesions for 1 month. No simultaneous increase in size of any lesion or the appearance of any new lesions may occur.</td>
</tr>
<tr>
<td><strong>Minor response</strong></td>
<td>25-49% decrease in the summed products of measured lesions for 1 month.</td>
</tr>
<tr>
<td><strong>Stabilization</strong></td>
<td>Less than a 25% decrease or increase in the summed products of measured lesions for a minimum of 3 months.</td>
</tr>
<tr>
<td><strong>Progression</strong></td>
<td>Greater than 25% increase in the sum of all measured lesions for greater than 1 month, the appearance of new lesions, or a mixed response.</td>
</tr>
</tbody>
</table>

(Developments in Bladder Cancer  1986)
**Single agent trials**

A number of chemotherapeutic agents have been used in the treatment of TCC bladder. The most active drugs are cisplatin, methotrexate, doxorubicin and vinblastine. All of these drugs have been shown to induce remissions (Sternberg 1988, Yagoda 1987). The majority of responses are PR and last from 3-5 months. CR occurs rarely with single agent chemotherapy and almost never in cases of bone metastases or locoregional disease.

**Cisplatin**

Cisplatin (DDP) is a heavy metal, non-cell cycle dependent drug that functions as an alkylating agent by inhibiting DNA replication, probably by intrastrand cross linking. For bladder cancer, it is usually administered at a dose of 70 or 100 mg/m². Patients treated with single agent cisplatin have achieved overall response rates (CR + PR) from 9% to 65% at doses of cisplatin ranging from 50 to 100 mg/m2. *Table 1.3* outlines activity of cisplatin reported in 15 separate studies. The majority of responses were partial and occurred within 2 to 5 weeks following the initiation of therapy. Yagoda *et al.*, (1976) first reported a response rate of 35% in 23 patients with advanced bladder cancer, treated with single agent cisplatin at a dose of 1.25-1.6 mg/Kg every 3-4 weeks. This degree of activity has been confirmed in subsequent studies and pooled data from 15 trials gives an overall response rate of approximately 30% (*Table 1.3*). Although cisplatin was given at a dose of 70 mg/m2 in most of these studies, the optimal dose is not known.
Table 1.3  Single agent cisplatin activity.

<table>
<thead>
<tr>
<th>CR and PR</th>
<th>% CR and PR</th>
<th>Reference</th>
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<tbody>
<tr>
<td>8/23</td>
<td>35</td>
<td>Yagoda 1976</td>
</tr>
<tr>
<td>10/28</td>
<td>36</td>
<td>Yagoda 1979</td>
</tr>
<tr>
<td>19/51</td>
<td>37</td>
<td>Merrin 1979</td>
</tr>
<tr>
<td>4/8</td>
<td>50</td>
<td>Peters 1980</td>
</tr>
<tr>
<td>9/21</td>
<td>43</td>
<td>Herr 1980</td>
</tr>
<tr>
<td>7/27</td>
<td>26</td>
<td>Oliver 1981a</td>
</tr>
<tr>
<td>9/27</td>
<td>33</td>
<td>Soloway 1981</td>
</tr>
<tr>
<td>10/50</td>
<td>20</td>
<td>Soloway 1983</td>
</tr>
<tr>
<td>11/17</td>
<td>65</td>
<td>Fagg 1984</td>
</tr>
<tr>
<td>30/50</td>
<td>60</td>
<td>Raghavan 1985</td>
</tr>
<tr>
<td>17/48</td>
<td>35</td>
<td>Khandekar 1985</td>
</tr>
<tr>
<td>3/15</td>
<td>20</td>
<td>Oliver 1986</td>
</tr>
<tr>
<td>7/48</td>
<td>15</td>
<td>Troner 1987</td>
</tr>
<tr>
<td>17/55</td>
<td>31</td>
<td>Hillcoat 1989</td>
</tr>
<tr>
<td>10/110</td>
<td>9</td>
<td>Loehrer 1990</td>
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</table>

Total 171/578  30%

23
It remains unclear as to whether a dose response relationship exists for cisplatin, as may be the case for other solid tumours such as testicular or ovarian carcinomas. The wide range of response rates reported for single agents in these early studies may be due in part to differences in staging (particularly clinical versus pathological staging), schedule and dose differences and variability in the criteria of response. Modern trials tend to employ more stringent definitions of response than do trials reported in the 1970s and early 1980s.

**Methotrexate**

Methotrexate (MTX) is an anti-folate drug which is cell cycle and phase specific. It binds to the enzyme dihydrofolate reductase, inhibiting the synthesis of thymidylic acid and therefore of DNA and RNA. The affinity of the drug for dihydrofolate reductase is 100,000 times greater than that for folic acid and its binding is permanent. There is growing evidence that methotrexate may in fact be a pro-drug with most of its pharmacological activity arising from its polyglutamate derivatives. Early trials of MTX in bladder cancer used a variety of doses, schedules and routes of administration, including oral, intravenous, intravesical and intra-arterial (Yagoda 1976).

Using three different regimens consisting of methotrexate doses of 50mg i.v., 100mg i.v. and 200 mg i.m., with folinic acid rescue Turner (1981) reported response in 3/25, 12/23 and 8/16 respectively, for an overall response rate of 36% in 64 patients. Using a dose of 100 mg/m² weekly with leucovorin rescue, Oliver (1981b) reported responses in 0/6 patients with bone metastases, in 3/9 patients with clinically measurable metastases and in 4/16 patients with primary bladder lesions.
which had recurred after radiotherapy. Natale et al., (1981) reported 42 patients with measurable indicator lesions treated with either high dose methotrexate (250 mg/m²) or low dose methotrexate (0.5-1.0 mg/Kg weekly). Only one patient responded in the high dose group whereas 33% of patients receiving low dose methotrexate achieved partial responses. The optimal dose for methotrexate has yet to be determined but high dose treatment does not appear to confer any advantages over the response rates of approximately 30%, achieved with low dose treatment. Results from single agent studies are outlined in Table 1.4. The overall response rate is approximately 30%.

Table 1.4 Single agent Methotrexate activity.

<table>
<thead>
<tr>
<th>CR and PR</th>
<th>% CR and PR</th>
<th>Reference</th>
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<td>23/64</td>
<td>36</td>
<td>Turner 1981</td>
</tr>
<tr>
<td>7/31</td>
<td>23</td>
<td>Oliver 1981b</td>
</tr>
<tr>
<td>11/42</td>
<td>26</td>
<td>Natale 1981</td>
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</tbody>
</table>

Total 41/137  30%

Adriamycin

Adriamycin (doxorubicin) is an anthracycline antibiotic with multiple mechanisms of action, including the intercalation of DNA nucleotide pairs. It is maximally toxic in the S-phase but can effect all phases of the cell cycle. Adriamycin is most commonly prescribed at a dose of 30-75 mg/m². In early trials using a dose of 60-75 mg/m² every 3 weeks, remissions were achieved in 14 out of 39 patients (O'Bryan 1973). Subsequent studies have demonstrated response as a single agent from 5%-40% (Table 1.5). The most recent trial from the Eastern Cooperative Oncology Group
randomized 108 patients to either adriamycin or 5-Fluorouracil. The adriamycin group showed 5/45 responders (Knight et al., 1983). Overall, adriamycin produces a cumulative response rate of approximately 19%. One side effect which is particularly distressing to the patient is alopecia. Remissions are uncommon after 3 doses and the median duration of response is 3-4 months. In an attempt to reduce the cardiac toxicity of anthracyclines, analogues of adriamycin have been developed. Epirubicin, the 4' epimer of adriamycin has shown activity in the intravesical treatment of superficial bladder tumours and is now being used as a systemic agent in combination with cisplatin and methotrexate (EpiCM).

Table 1.5 Single agent Adriamycin activity

<table>
<thead>
<tr>
<th>CR and PR</th>
<th>% CR and PR</th>
<th>References</th>
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<tr>
<td>14/39</td>
<td>36</td>
<td>O'Bryan 1973</td>
</tr>
<tr>
<td>1/10</td>
<td>10</td>
<td>Merrin 1975</td>
</tr>
<tr>
<td>1/19</td>
<td>5</td>
<td>Weinstein 1976</td>
</tr>
<tr>
<td>5/35</td>
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<td>Yagoda 1977</td>
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<tr>
<td>11/65</td>
<td>17</td>
<td>O'Bryan 1977</td>
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<tr>
<td>6/15</td>
<td>40</td>
<td>Fossa 1981</td>
</tr>
<tr>
<td>8/41</td>
<td>20</td>
<td>Gagliano 1983</td>
</tr>
<tr>
<td>5/45</td>
<td>11</td>
<td>Knight 1983</td>
</tr>
</tbody>
</table>

Total 51/269 19%
Vinblastine

Vinblastine sulphate is a vinca alkaloid that inhibits mitosis by forcing the cell into cycle arrest in metaphase. A limited number of patients with bladder cancer have been treated with vinblastine as a single agent. Using doses of 0.1-0.15 mg/Kg, Blumenreich et al., (1982) obtained partial responses in 5 out of 28 patients, the majority of whom had received prior chemotherapy. In an European Organisation for Research into the Treatment of Cancer (EORTC) study vincristine given on a weekly schedule showed little antitumour efficacy and gave rise to considerable toxicity (Richards et al., 1983).

Table 1.6 Single agent Alkaloid activity

<table>
<thead>
<tr>
<th>Drug</th>
<th>CR and PR</th>
<th>% PR and CR</th>
<th>Reference</th>
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<tr>
<td>Vinblastine</td>
<td>5/28</td>
<td>18</td>
<td>Blumenreich 1982</td>
</tr>
<tr>
<td>Vincristine</td>
<td>3/37</td>
<td>8</td>
<td>Richards 1983</td>
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</table>

Other Drugs

Cyclophosphamide has been studied in older trials, with response rates of up to 52% being reported (Chun 1988). However its efficiency as a single agent appears to be considerably lower as demonstrated by Yagoda who reported just 2 partial responses in 26 patients (Yagoda et al., 1977). Mitomycin C has been reported to yield an overall response rate of 21% in trials completed before 1975. No recent trial has assessed its activity as a single agent using modern response criteria.
**Combination chemotherapy**

During the late 1970s and early 1980s, the possibility of improving response rates by the use of combination regimens was explored. Most of the combinations were cisplatin or methotrexate based, as these were considered the most active single chemotherapeutic agents in TCC. The addition of cyclophosphamide to cisplatin did not result in increased response rates over cisplatin alone (Soloway 1983). However the combination of cisplatin and adriamycin did increase the proportion of responses from 19% to 43%, when compared to cisplatin alone (Gagliano 1983). In 1977 the M D Anderson hospital advocated CISCA, a combination of cisplatin, adriamycin and cyclophosphamide (Sternberg 1977) with a response rate of 83%. As the series grew the response rate fell to 42% (Logothetis 1985). Two randomised studies have compared CISCA to cisplatin alone and although the proportion of CR was significantly greater in the CISCA group (Khandekar 1985), there was no significant difference in the overall response rate.

Although many studies have been performed using methotrexate in combination with other agents including vinblastine, adriamycin, cyclophosphamide and mitomycin, only one randomized trial against single agent methotrexate has been reported. This study compared methotrexate and cisplatin to methotrexate alone, reporting responses in 45% and 33% of cases respectively (Hillcoat et al., 1989).

Methotrexate combinations with drugs other than cisplatin have response rates from 31% to 39% and these results have 95% confidence limits which overlap response rates seen with methotrexate alone (Sternberg 1988, Yagoda 1987). The combination of methotrexate and cisplatin has resulted in response rates as high as 47% with a CR of 21% (Carmichael 1985). The overall response rate for
methotrexate and cisplatin for locally advanced disease is 64% in 160 patients. However of these, only 14% achieved CR.

Despite the lack of evidence supporting the use of many combination regimens, urologists and oncologists continued to use more intricate combinations of drugs in the hope that high response rates might lead to longer survival. The most active regimens were combined by the South West Oncology Group into a four drug regimen - MVAC (methotrexate, vinblastine, adriamycin and cisplatin). Soon after this the North Carolina Oncology Group developed a three drug regimen - CMV, (cisplatin, methotrexate and vinblastine). These two regimens are the most widely used today.

Table 1.7 Dose (mg/m²) and schedule of MVAC

<table>
<thead>
<tr>
<th>MVAC</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MVAC

In 1985 Sternberg reported the results of MVAC in a group of 24 patients (Sternberg 1985). She reported an overall response rate of 71% with a complete response rate of 50%. This series was updated in 1988 (Sternberg 1988) and with 83 patients
treated, 69% had responded and 37% had achieved a complete response. The median survival for patients with PR was 11 months, non responders survived a median of 7 months. The median duration of response for CR was 36 months and the 2 and 3 year survival rates for CR were 71% and 55% respectively. Toxicity was predominantly myelosuppression with 20% of cases requiring hospital admission with neutropenic sepsis and there were 4% treatment related deaths. Nephrotoxicity was seen to a lesser extent. Nausea, vomiting, alopecia and mucositis were common. The dosing schedule for MVAC and CMV is outlined in Table 1.7, and Table 1.8. A number of other groups have reported their experiences with the MVAC regimen. Tannock et al., (1989) treated 41 patients and noted 4 complete and 8 partial responses for an overall response rate of just 40% with marked toxicity. A report of the Japanese experience was closer to the Memorial results with a response rate of 57% in 58 patients. MVAC has been compared to cisplatin alone in phase III trials. The Intergroup trial (Loehrer 1990) compared single agent cisplatin at 70 mg/m² with the MVAC regimen and the response rate to cisplatin alone was 9% as compared to 33% for MVAC (p<0.001). However, the response rate for cisplatin alone (9%) is the lowest reported in any clinical trial.

Table 1.8 Dose (mg/m²) and schedule for CMV

<table>
<thead>
<tr>
<th>CMV</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CMV

This regimen was developed at Stanford University in 1985 and the North Carolina Oncology Group reported their initial results in 58 patients with metastatic disease (Harker et al., 1985). Twenty eight percent achieved a CR and a further 28% a PR, for a total response of 56%. Response rates were seen at all sites and the median duration of response was 9 months. Median survival for CR was 11 months and 7 months for PR. Although not as high as the overall response rates reported with MVAC this still represented a considerable improvement over single agent chemotherapy in terms of response. Two patients who were treated during the pilot experience at higher doses of methotrexate and vinblastine than those specified by the current protocol (40 and 5 mg/m², respectively) died as a result of sepsis. The doses of methotrexate and vinblastine were subsequently reduced and the protocol modified to include a requirement for creatinine clearance on day 8 of the regimen.

In a more recent study from the MRC (Medical Research Council) Urological working party, Fossa et al., (1992) reported a phase II study in patients with T3/T4 M0 disease treated with CMV. A 57% response rate was seen in 51 patients, which is similar to that of Harker et al. (1985) and a 45% response reported by Roberts et al. (1991) (CM only). Perhaps the most optimistic claim comes from Miller et al. (1993), who reported that with aggressive surgical resection of residual disease, overall CR can be achieved in 55% of patients with locally advanced disease.

However, with increasing experience it has become clear that a substantial proportion of tumours that respond to these combination regimens are destined to relapse and the percentage of patients actually cured is much lower than initially anticipated (Tannock et al., 1989). In patients with metastatic disease a small but
significant advantage can be conferred, in terms of survival, from the use of combination chemotherapy over single agent therapy (low dose cisplatin) (Loehrer et al., 1990). The question as to whether combination chemotherapy confers significant survival advantage in the neoadjuvant setting has yet to be answered. In order to define the role of chemotherapy in this setting more clearly, a large randomised controlled trial in being carried out under the auspices of the MRC and the EORTC. This trial will test the survival impact of 3 cycles of CMV on patients with locally invasive, non metastatic disease, prior to radical cystectomy or radiotherapy. Patients studied in this thesis are taken from this trial and a second trial of patients receiving either CMV or MV for metastatic disease.

1.3. DRUG RESISTANCE MECHANISMS

One potential mechanism for tumours failing to respond to chemotherapy is the presence of innate drug resistance. Development of acquired drug resistance to chemical agents was first recognised in 1948 when Sidney Farber described partial remission of childhood leukaemia with aminopterin. Drug resistance in the clinical setting may be defined as the ability of tumour cells to survive exposure to cytotoxic agents when administered at the maximum dose tolerated by normal tissues. This situation may arise as the result of metabolic variation in the handling of a prescribed drug, leading to increased breakdown or decreased activation. Secondly tumour cell growth kinetics may determine outcome. Treatment which reduces bulk disease but fails to eradicate residual disease may lead to the rapid regrowth of disease following chemotherapy. Thirdly resistance may be due to innate or acquired cellular drug
resistance mechanisms that enable tumour cells to survive exposure to otherwise lethal concentrations of cytotoxic agents.

1. CLASSICAL MULTIDRUG RESISTANCE (MDR)

Multidrug resistance is the phenomenon of broad cross resistance to a group of structurally and functionally distinct cytotoxic agents, which are derived from naturally occurring substances. Clinically this problem came to light in the 1960's when resistance was noted to the vinca alkaloids and the anthracycline antibiotics. Cells expressing this "Classical MDR" phenotype are cross resistant to doxorubicin, daunorubicin, colchicine, actinomycin D, vinblastine, vincristine, vindesine and etoposide. The molecular basis for this type of resistance is the result of reduced intracellular drug accumulation, due to the active pumping of drug out of the cell by a 170 kD membrane glycoprotein, otherwise known as the p-glycoprotein. This glycoprotein is the product of the MDR1 gene, located on the long arm of chromosome 7.

Different p-glycoprotein isoforms have been isolated and have been shown to be encoded by a family of closely related genes. They are referred to as pgp genes in hamsters and MDR genes in humans and mice. Two human genes, MDR1 and MDR2 (Roninson et al., 1986) and three rodent genes have been identified. In the mouse, these three genes are referred to as mdr1a, 1b (also known as mdr3 and mdr1) and mdr2 and in the hamster pgp1, pgp2 and pgp3. Mouse 1a, 1b, hamster pgp1 and human MDR1 are all associated with drug resistance, whereas mouse mdr2,
hamster pgp3 and human MDR2 are not. The function of the human MDR2 gene remains unknown. The two human isoforms of the gene show approximately 80% homology at the amino acid level which would suggest a gene duplication.

The first experimental observation of cross resistance was made by Kessel et al., (1968) in an anthracycline resistant tumour cell line that was also resistant to vinblastine. The membrane glycoprotein (P-gp) was subsequently identified in 1976 (Juliano and Ling 1976). The mechanism of P-gp mediated drug resistance has been fully reviewed by Endicott and Ling (1989). It is thought to act as a drug efflux pump with broad specificity for natural products such as the vinca alkaloids and anthracyclines. This pump is energy dependent and can be blocked by high doses of chemicals such as the calcium antagonist Verapamil (Bellamy et al.,1988). This potential for reversal of resistance has made the MDR phenomenon of particular clinical interest. However clinically the high doses of verapamil required are difficult to achieve.

Data are now emerging which suggest that the p-glycoprotein may have biological functions other than those involved in cytotoxic drug transport. These functions involve the ability of cells to invade and metastasise. A study in colon cancer (Weinstein et al.,1991) showed increasing numbers of cells at the invading edge of tumours with high metastatic potential. These observations may be important in interpreting p-glycoprotein positivity and clinical outcome.

In 1987 the coding sequence for MDR1 mRNA was established by Ueda et al. (1987). This gave rise to the production of relatively specific antibodies C219 and
MRK16 to the P-gp product. In addition, the development of the ultra-sensitive polymerase chain reaction (PCR) technique has led to a further expansion of the information on MDR1 expression at the mRNA level (Noonan et al., 1990). This latter technique provides a method for assaying MDR1 mRNA in tissues with very low levels of gene expression, such as bladder.

**P-Glycoprotein expression in normal tissues**

Normal cells that express p-glycoprotein are all epithelial with the exception of placental trophoblasts and a subset of capillary endothelial cells at the blood brain barrier. P-glycoprotein is expressed on the luminal surface of columnar epithelial cells throughout the gastrointestinal tract. It is also present in pancreatic and biliary ducts. In the kidney, distribution is restricted to the luminal pole of the proximal tubules (the site of tubular secretion). This distribution of p-glycoprotein resulted in the proposal that the normal physiological role for the glycoprotein was to inhibit absorption and facilitate excretion of potentially toxic dietary and environmental, naturally occurring, toxins. However this does not account for the high levels of expression found in adrenal cortex.

**P-glycoprotein expression in human tumours**

Expression of P-gp is commonly and readily detectable in epithelial tumours derived from tissues that normally express P-gp e.g. renal, colon, adrenal and pancreatic cancers. P-gp is also expressed by tumour cells derived from normal cells that do not express the MDR phenotype e.g. bladder tumours and sarcomas.

Fojo et al., (1987) measured MDR1 RNA levels in a series of human tumours...
and in normal tissues. They found that MDR1 was expressed at high levels in the kidney and at intermediate levels in the lung, liver, jejunum, colon and rectum. They also found high levels of expression in tumours from adrenal and colon. This supports the distribution of the p-glycoprotein product on immuno-histochemistry.

Goldstein et al., (1989) have also studied MDR1 RNA expression but in a wider range of tumours. High expressers were found to be adrenal, colonic, hepatic and renal tumours. They also demonstrated increased levels of MDR1 expression in tumours relapsing on chemotherapy.

**P-glycoprotein expression in bladder tumours**

Attempts to study p-glycoprotein expression in bladder tumours have been hampered by low levels of detection and the inability to quantitate levels of expression. Cordon Cardo (1991) was unable to detect the p-glycoprotein in normal bladder using 3 antibodies (HYB-241, HYB-612 and C219). However he was able to demonstrate positivity in 4 out of 10 bladder tumours. Goldstein et al., (1989) were able to detect low levels of MDR1 RNA in just 1 out of 6 bladder tumours. Benson et al (1991) studied P-gp expression in a range of bladder tumours using flow cytometry, based on the antibody C219. They reported low but variable levels of P-gp but more significantly, elevated levels of expression following chemotherapy. However we now know that endothelial cells and stromal cells may stain positively with C219 (Schlaifer et al 1990) and this must throw doubt on flow cytometric work and western blots based on antibodies used on tumour biopsies.

More sensitive techniques are now available and Noonan et al (1990) developed a polymerase chain reaction (PCR) based transcription assay and looked
at a range of tumours reported to have low or undetectable levels of MDR1 expression such as bladder. They showed that MDR1 expression at the mRNA level was measurable in tissues such as bladder and from cell line work showed that such low levels could give rise to the MDR phenotype.

In a study from Memorial Sloan Kettering, Petrylak et al., (1992) suggested that induction of the multidrug resistance phenotype contributes to clinical drug resistance in bladder cancer. They used the monoclonal antibody HYB 241 to demonstrate p-glycoprotein expression in tumours before and after treatment with combination chemotherapy. Prior to treatment 13% of the primary and none of the metastatic lesions expressed the p-glycoprotein whereas 32% (6/19) of the treated tumours and 55% (6/11) of the treated metastatic tumours stained positively.

To date, there has been little work on MDR1 expression in bladder tumours because of difficulty in quantifying low levels of expression and an assumption that these low levels would not be significant. However, bladder cancer cell line studies have shown that P-gp expression is proportional to degree of resistance to adriamycin and suggest that multidrug resistance is an important phenomenon in bladder cancer. This method of cellular drug resistance is of great potential interest in tumours treated with adriamycin and vinblastine, as is the case with urothelial cancer.
Other mechanisms are now known to exist which can lead to a pattern of multidrug resistance which do not involve P-gp or the MDR1 gene. Danks et al., (1987) demonstrated this in a leukaemic cell line and reported that there was no difference in terms of intracellular drug accumulation between parent and resistant line, thus excluding a P-gp like mechanism. The term atypical MDR was used to describe this phenotype and was later found to be due to altered expression of an enzyme, namely Topoisomerase II. There are 2 forms of this enzyme: the $\alpha$ form (170kD) and the $\beta$ form (180kD) and their function is to change the topology of DNA by cleaving open both strands and allowing the double helix to wind and unwind. In so doing the Topoisomerases permit the unlocking of DNA, the decatenation of circular interlocked DNA and reduction of supercoiling. The drugs known to interact with topoisomerase II and trap it in its DNA bound form include adriamycin, daunorubicin, mitoxanthrone and actinomycin D. Topoisomerase activity during proliferation correlates with sensitivity to intercalating agents (Davies S.M. et al., 1988) and cells with undetectable levels of Topoisomerase II are resistant to topoisomerase inhibitors. There has been very little work on Topoisomerase II mediated resistance in human tumours but it remains a potential mechanism for resistance to anthracyclines which are commonly used in the treatment of bladder cancer.
3. GLUTATHIONE S-TRANSFERASES

The cytosolic Glutathione S-Transferases (GST) are a family of widely distributed proteins that act both as enzymes in the detoxification of carcinogens and some drugs, by conjugation with glutathione and also as intracellular binding proteins. Cytosolic and microsomal forms exist and the cytosolic enzymes may be subdivided into 3 groups according to their isoelectric points $\alpha$, $\mu$ and $\pi$. The distribution of GSTs in different human tissues is not uniform. Certain forms with high activity in one organ may be present in lower activity or absent in other tissues. A frequent phenomenon is the elevated expression of GST $\pi$ in many human tumours.

GST $\pi$ expression has been linked to the development of drug resistance to both cisplatin (Batist et al., 1986) and adriamycin (Nakagawa et al., 1990) in cell line experiments. Recently, decreased GST $\pi$ expression has been postulated in drug sensitive human testicular germ cell tumours (Strohmeyer et al., 1992). GST $\pi$ expression has not been studied in bladder tumours to date. However overall GST activity has been found to be significantly elevated in a group of superficial tumours when compared to normal urothelium (Lafuente et al., 1989). This may have potential implications for intravesical chemotherapy used for superficial bladder tumours.

4. Glutathione

Glutathione is specifically implicated with resistance to cisplatin. Glutathione is the principal intracellular thiol and it has been postulated that by binding the electrophile, cisplatin it may decrease cisplatin cytotoxicity and nephrotoxicity. An important study
by Russo et al.,(1986) showed that a 50% increase in glutathione levels increased the cisplatin resistance of normal human lung fibroblasts by a factor of 1.4. In addition Mayer et al.,(1989) have reported depletion of kidney glutathione, by an inhibitor of its synthesis, buthionine-sulfoximine, decreased cisplatin toxicity but had no effect on tumour response in rats. Human tumour cell line work has been more encouraging. Hospers et al.,(1988) reported a 3.4 fold increase in glutathione levels in a cisplatin resistant human small cell lung carcinoma cell line and Hamilton et al.,(1985) reported a 3.2 fold increase in glutathione levels in a cisplatin resistant ovarian carcinoma cell line. The role of glutathione in mediating cisplatin resistance is thus unclear at present. Elevated levels of glutathione have been observed as part of a resistant phenotype but only when cells were selected by continuous exposure to cisplatin. There is no direct evidence that glutathione inactivates enough intracellular cisplatin to inactivate cisplatin and increases in glutathione levels may be a stress response to cisplatin.

5. *Di-hydrofolate reductase*

Methotrexate has been widely used in the treatment of bladder cancer, both at high and low dose. As mentioned earlier, methotrexate acts by inhibiting the enzyme dihydrofolate reductase (dHFR). Although there is no direct evidence for resistance in bladder tumours, amplification of the dHFR gene and consequently elevated levels of the enzyme have been described as a potential resistance mechanism in human tumours (Schweitzer 1990). For example in leukaemia patients post chemotherapy, dHFR amplification has been reported (Horns 1984). Cell line studies have suggested
other innate resistance mechanisms to methotrexate such as reduced folate transport and reduced polyglutamation (Schweitzer 1990), but this has not been investigated in a clinical setting.

6. DNA inducible genes and repair

The ability to repair DNA damage is one potential mechanism for resistance to cisplatin. Cisplatin acts by forming intra-strand crosslinks and increased ability to repair this damage has been shown to be associated with resistance (Masuda et al., 1990). Repairing DNA damage involves the cleavage of apurinic sites which are generated when alkylated damaged bases are removed from DNA. This cleavage is achieved by means of an enzyme, AP-endonuclease. This enzyme also removes fragmented deoxyribose groups at the 3' end of the DNA strand breaks induced by free radicals and it is a key enzyme in the multistep process of DNA excision repair and re-synthesis of intact DNA. Increased levels of activity of this enzyme is a potential mechanism of resistance to cytotoxics which damage DNA.

7. Metallothioneins

Metallothioneins (MTs) comprise a family of proteins involved in zinc homeostasis and in the detoxification of heavy metals. They have therefore been investigated in the context of cisplatin resistance. Although elevated levels of MT have been demonstrated in cisplatin resistant cell lines e.g. a 5 fold increase in MT level in a cisplatin resistant human lung small cell carcinoma cell line (Kelley 1988), MT
expression did not correlate with cisplatin resistance in cell lines obtained from patient's tumours that were refractory to cisplatin (Schilder 1988). This would tend to suggest that MT overexpression may not be causally related to cisplatin resistance and may be a stress response in cell lines repeatedly exposed to cisplatin. Metallthionein expression can be induced by the Harvey ras oncogene and this is a potential reason for drug resistance in some human cancers.

1.4. PHARMACOKINETICS OF DRUGS COMMONLY USED IN BLADDER CANCER

The delivery of an inadequate dose of cytotoxic agent is one potential reason for a tumour either failing to respond to initial therapy, or relapsing early due to the presence of residual disease. Optimum treatment with many drugs depends on achieving a plasma concentration that lies between specific limits set by a minimum effective concentration and a maximum safe concentration. There may be some dispute about the most appropriate values for these limits but concentrations lying above the maximum safe concentration are usually associated with toxicity whilst those lying below the minimum effective concentration are generally ineffective. This is especially important in the field of cytotoxic chemotherapy as toxicity can be lethal and the price of underdosage may be the missed opportunity of cure. In the case of most anti-cancer drugs the largest dose which the patient can reasonably tolerate is given. A proportion of patients will respond to this dose and the remainder will not. The relative insensitivity of most common cancers to these drugs means that a therapeutic serum concentration cannot be achieved.
Pharmacokinetics is concerned with the study of the time course of drug absorption, distribution, metabolism and excretion and with the relationship of these variables to the intensity and time course of therapeutic and adverse effects of drugs. These parameters may vary considerably between patients. Absorption is not a variable in the case of systemic chemotherapy as all drugs are given by the intravenous route, either as a bolus or as a continuous infusion.

Distribution
Following intravenous injection of a cytotoxic agent the drug becomes mixed in the plasma. It may bind to plasma proteins (e.g. cisplatin) and diffuse to a varying degree into red blood cells and extravascular tissues. The subsequent decline of these concentrations is due to the elimination of the drug from the body by processes such as renal excretion and biliary excretion. Distribution and elimination are often described by the use of models. The most frequently used models are the one and two compartment models. The one compartment model assumes that distribution is instantaneous, whereas the two compartment model assumes that there is a significant distribution phase followed by an elimination phase. The compartments have no physiological or anatomical meaning but are mathematical concepts.

The one compartment model adequately describes the changes with time of plasma concentration of drugs which rapidly distribute between plasma and tissue after administration. Elimination from the compartment follows first order kinetics, that is to say the rate of elimination is directly proportional to the amount of drug present. Another consequence of one compartmental model is that the area under the curve of the plasma concentration-time curve following intravenous administration is
a linear function of the administered dose.

The two compartment model is used for drugs where distribution between plasma and tissues is less rapid. The central compartment comprises plasma and tissues with the extracellular spaces of well perfused tissues such as heart, lung, liver and kidneys. The peripheral compartment comprises less well perfused tissues such as muscle, skin and fat. When a drug is given intravenously the level falls in a biphasic fashion. The initial fall represents distribution from the central compartment to the peripheral compartment \((\alpha)\). Once an equilibrium has been attained between peripheral and central compartments the elimination phase becomes evident \((\beta)\).

**Elimination**

The major routes of elimination are metabolism by the liver and excretion by the kidney. Factors such as hepatic blood flow and genetic differences in metabolic activity can influence the rate of metabolism of an individual drug. Genetic polymorphisms for enzymes involved in metabolism of cytotoxic agents therefore influence activity and excretion.

Two important concepts which help the understanding of elimination are half-life and clearance. The half-life is the time required for the plasma concentration to decrease by 50% after absorption and distribution are complete. Clearance is a term used for the rate at which elimination occurs from plasma. It may be expressed as total clearance from the body or more specifically by an individual organ e.g. renal clearance.
Cisplatin pharmacokinetics

Cisplatin is probably the most effective agent in the treatment of bladder cancer. The drug has been the subject of numerous pharmacological studies in animals and humans. Plasma and urinary levels of platinum have been measured by two techniques, namely atomic absorption spectrophotometry and high performance liquid chromatography. Subsequently it has become clear that cisplatin binds rapidly and irreversibly with plasma proteins and that this protein bound fraction has no cytotoxic activity (Takahashi et al., 1985). The free cisplatin is the active component in terms of both response and toxicity. It has been shown that less than 50% of the total drug administered is cleared by the kidney (Levi et al., 1982). Cisplatin binds rapidly to proteins and high levels of platinum complexes are deposited in tissues for long periods. The main toxicity from cisplatin is renal, although it can be reduced significantly by forced diuresis, allowing higher doses of cisplatin to be administered (Corden et al., 1985). Vermonken et al., (1982) studied free platinum levels in plasma ultrafiltrate of patients following bolus, 3 hour and 24 infusions. They showed that free platinum availability (measured as the area under the curve of a graph of serum concentration versus time) was the same, regardless of the mode of administration. Toxicity, in particular nausea, was reduced in patients who had longer infusions and lower peak cisplatin levels. It would therefore seem sensible to prescribe longer infusions and avoid toxicity. Reece et al., (1987) studied free cisplatin in patients with ovarian cancer and reported clearance of free cisplatin at 253 ml/min/m² and a plasma half life of 31.6 minutes which is in agreement with previous published series. Renal clearance exceeded creatinine clearance in all patients, which confirmed previous suggestions of active tubular secretion of cisplatin. A one
compartment model accurately described the observed free cisplatin level-time data during and after infusion in all patients. This is due to the very rapid distribution of free cisplatin from plasma into tissues as it becomes bound to protein.

*Methotrexate pharmacokinetics*

Methotrexate can be given either orally, intramuscularly or by the intravenous route. At low doses, the elimination of methotrexate is best described by a two compartmental model. The initial phase of distribution has a t1/2 of approximately 0.75-1 hours and the second phase t1/2 of 2-3.5 hours, which is predominantly determined by renal elimination. A third phase may be seen in high dose methotrexate studies due to biliary excretion and subsequent reabsorption via the gut. This effect tends to occur 24 hours after administration of drug and at low doses levels are often unmeasurable at this time. Methotrexate enters into normal and malignant cells via an energy dependent carrier mediated transport process, as does folate. This transport process is saturable and large oral doses can be taken up more slowly. Elimination is predominantly renal and methotrexate undergoes active tubular secretion as well as filtration. Methotrexate may be metabolised by gut flora during enterohepatic circulation and polyglutamate derivatives may be synthesised in the liver. These metabolites are not significant in intravenous therapy but may be important with oral therapy.

Variation in pharmacokinetic handling of methotrexate is of particular relevance in high dose therapy. Toxicity and probably response is related to the time period above a certain concentration at which methotrexate and its metabolites are active. Methotrexate levels of >$10^7$M at 24 hours, >$10^8$M at 48 hours > $10^7$M at
72 hours after treatment are associated with increased toxicity (Stoller et al., 1977). In order to avoid toxicity drug level monitoring is an essential in high dosage. In cases where toxic levels are anticipated the use of leucovorin rescue is advocated (Ackland S. et al., 1987). Leucovorin acts by replenishing intracellular tetrahydrofolate pools; this reverses the action of methotrexate and as well as reducing toxicity. However, there is evidence that it may also reduce antitumour activity (Browman 1990).

**Vinblastine pharmacokinetics**

The pharmacokinetics of Vinblastine were first studied in detail by Owellen et al., (1977). At this time a radioimmunoassay had been developed that enabled measurement of radiolable excretion. Vinblastine has to be given by the intravenous route as it is highly irritant to mucous membranes. It may be given as a bolus or an infusion. Owellen reported a three compartmental model, with the following values; alpha phase $t_{1/2} = 3.9$ minutes, beta phase $t_{1/2} = 53$ minutes and gamma phase $t_{1/2} = 1173$ minutes. Subsequent authors have described vinblastine pharmacokinetics as essentially bicompartamental. Vinblastine is predominantly metabolised in the liver and excreted in the bile although some 5% is cleared by the kidney. Following a standard bolus dose, drug levels usually remain in the therapeutic range ($>1$ ng/ml) for approximately 48-72 hours.

Impaired liver function may result in altered pharmacokinetics and dose reduction has been recomended in patients with a low serum albumin concentration (Ratain et al., 1987a). The major metabolite of vinblastine is deacetylvinblastine
which is more active, on a weight basis than vinblastine.

A limited sampling model has been developed for vinblastine by Ratain et al., (1987b). They were able to calculate AUC from two time points at 10 hrs and 36 hrs post bolus dose and slightly more accurately from three time points at 1.5 hrs, 10 hrs and 36 hrs post bolus. This three point limited sampling model has been employed in this thesis in order to relate vinblastine AUC to toxicity and response.

**Normalisation of Drug Dosage**

All cytotoxic agents studied in this thesis, in common with most anti-cancer drugs, are prescribed according to a derived surface area. The use of a predicted surface area rather than body weight as a means of normalising drug dose has been established in oncology since the early 1960s. The aim of normalising drug dosage using surface area is to reduce the relative dose as body size increases. This should avoid overdosage whilst maintaining therapeutic activity. This is based on evidence from Fredreich et al., (1966) that the LD10 dose of anticancer drugs in various animal species correlated linearly with the maximum tolerated dose in humans if the doses were calculated on the basis of body surface area (mg/m^2) but that the correlation was non linear if mg/kg was used. The Dubois height-weight formula has become the standard means of predicting surface area (Dubois 1916). There are other formulae but the Dubois formula became the most commonly used. The classic Dubois formula is actually only based on a sample size of 9 individuals.

Studies by Collins et al., (1986) have shown that the area under the curve (AUC) is more closely correlated to drug toxicity across different species than the dose in mg/m^2. The AUC is calculated by plotting a graph of plasma level of the
drug versus time and measuring the area beneath the curve. It thus represents the product of concentration x time and is measured in units such as mg/ml.min. The current method of prescribing a fixed dose according to surface area with modifications according to toxicity, results in frequent reduction of dosage but rarely will doses be increased due to absence of toxicity. Thus, whereas over treatment is avoided, the risk of undertreatment remains.

1.5 AIMS OF THIS THESIS

It is clear from the existing literature that transitional cell carcinoma of the bladder is a chemosensitive tumour with response rates as high as 60% reported following treatment with combination chemotherapy. However the majority of patients relapse following treatment and 40% or more show no initial response. The overall aim of this study was to determine possible mechanisms for failure of response to combination chemotherapy in bladder cancer. The study was restricted by the number of patients receiving chemotherapy in the urology department at Freeman Hospital over a two year period and by the chemotherapy regimens prescribed. There are likely to be many different reasons for variations in response to treatment and two possibilities are firstly the presence of innate drug resistance mechanisms within tumours and secondly an inadequate dose of cytotoxic agent.
AIMS

1. To determine whether expression of the enzyme Glutathione S-transferase \( \pi \) correlated with response to cisplatin based chemotherapy. Cell line experiments would suggest that increased GST (\( \pi \)) levels would result in decreased response rates due to inactivation of cytotoxic agents by conjugation with glutathione.

2. To attempt to quantify expression of the MDR1 gene in a range of transitional cell carcinomas. This had proved impossible to date, prior to the studies reported here, due to low levels of expression and the absence of sufficiently sensitive techniques.

3. To perform pharmacokinetic studies on all patients receiving cisplatin, methotrexate and vinblastine chemotherapy in order to establish whether significant variation in AUC occurs and to relate any such variation in AUC for each drug (or time above a threshold drug level in the case of methotrexate) to response and toxicity data.
CHAPTER 2: GST π EXPRESSION IN TRANSITIONAL CELL CARCINOMA
AND CLINICAL RESPONSE TO CISPLATIN BASED COMBINATION
CHEMOTHERAPY.

AIM: To establish whether expression of the enzyme Glutathione S-transferase π (GSTπ) is related to response in patients with transitional cell carcinoma of the bladder receiving cisplatin based combination chemotherapy.

Interest in GST π as a marker for drug resistance has been generated by a number of reports showing that increased levels of GST π are found in drug resistant cells including a multidrug-resistant human breast cancer cell line and a non-small cell lung cancer cell line resistant to cisplatin (Batist et al., 1986, Cowan et al., 1986). The GSTs conjugate reduced glutathione to a wide variety of xenobiotics and this facilitates elimination of the latter, since the resulting metabolites are more water soluble. GSTs can also bind some lipophilic compounds and either prevent them interacting with DNA or act as carrier proteins, assisting biliary excretion. Because they interact with such a wide range of nucleophiles, it may be expected that elevation of GSTs can produce cytotoxic drug resistance in human tumours. The fact that GST π is the predominant GST isoenzyme found in human cancer tissue raises the possibility that this may be a clinically significant drug resistance mechanism. If GST π was the rate limiting enzyme for conjugation of intracellular cisplatin with glutathione, then increased levels of enzyme may give rise to increased levels of resistance.
Immuno-histochemical localisation of the GST π protein became possible with the production of specific antibodies. Campbell et al., (1991) studied GST π expression in a range of normal tissues and common human tumours. The presence of GST π was reported in highest levels in ductular cells of liver, pancreas, salivary glands and kidney. Of 39 different tumour types studied 31 demonstrated the protein, including bladder, however it was noted that the expression of GST π varied within tumour types. Tumours which are generally resistant to chemotherapy e.g. colonic carcinomas, tend to have high levels of GST π expression, whilst those which are responsive tend to have low levels e.g. tumours of head and neck (Clapper et al., 1987). There have been no immuno-histochemical studies in bladder tumours. However, a recent study from Newcastle demonstrated a four fold increase in mRNA levels for GST π in human bladder cancer, as compared to normal bladder (J Carmichael et al., unpublished observation). Total GST activity has been studied in bladder cancer (Lafuente et al., 1989) and a ten fold increase in overall GST activity in superficial tumours as compared to adjacent normal urothelium was reported.

In addition to a possible role in drug resistance, GST π has also been investigated as a marker of malignant transformation in tumours of colon (Kodate et al., 1986), breast (Cairns et al., 1992) and uterine cervix (Randall et al., 1990). It has been suggested that nuclear staining for GST π by immuno-histochemistry may be a marker for cervical intra-epithelial neoplasia (CIN), (Randall et al., 1990). All cases of CIN were reported as showing intense nuclear staining whereas cytoplasmic staining was seen in normal epithelium and all cases of neoplasia.

In transitional cell carcinoma of the bladder the success of treatment with cytotoxic
agents may be limited by intrinsic or acquired drug resistance. Levels of the detoxifying enzyme GST \(\pi\) may be a factor in conferring the resistant phenotype. For individual patients, knowledge of levels of expression may allow prediction of response to cisplatin based chemotherapy. The purpose of this study was to evaluate this hypothesis. The technique of immuno-histochemistry allows ready assessment of the distribution of GST \(\pi\) within bladder tumours at a cellular and subcellular level and does not require fresh or frozen tissue. The antibody used has been shown to work well on paraffin embedded archival tissue (Cairns et al., 1991), enabling material to be studied from patients who have already received cisplatin based combination chemotherapy and on whom, response data are available.

**MATERIALS AND METHODS**

A group of patients with bladder tumours was selected on the basis that all patients with invasive tumours had received systemic chemotherapy. These included patients with the following tumours: 6 normal biopsies, 23 superficial tumours (12 Ta, 11 T1) and 26 invasive tumours. Staging was according to UICC (1978) criteria (see chapter 1). All 26 invasive tumours had been treated by systemic chemotherapy; of these, 12 had exhibited a response to chemotherapy, whereas 14 had either shown no response or tumour progression. Patients had received cisplatin based combination chemotherapy (Table 2.1) and had been assessed by cystoscopy, biopsy and CT Scan before and after treatment. Responses had been graded CR - complete response (no pathological evidence of tumour on deep resection biopsy), PR -partial response, NR -no response and Prog - tumour progression.
**Table 2.1 - Chemotherapy Regimens**

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Total</th>
<th>Response</th>
<th>No Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate, Vinblastine, Adriamycin, Cisplatin (MVAC)</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Epirubicin, Cisplatin, Methotrexate (EPICM)</td>
<td>9</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Cisplatin, Methotrexate, (CM)</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Cisplatin, Methotrexate, Vinblastine, (CMV)</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

**Immunohistochemistry Methods**

Polyclonal GST π antisera (Novocastra Laboratories) were raised in rabbits using enzymes extracted from human spleen. Antiserum specificity and titre was assessed using both an enzyme linked immuno assay (ELISA) technique and immunoblotting. No cross reactions with basic and neutral forms of GST isolated from human liver, or with other cytosolic proteins, were seen at dilutions of 1:1,000 (ELISA, immunoblotting) and 1:100 (immuno-histochemistry).

Three μm paraffin wax sections were cut from tissue blocks and mounted on lysine coated slides. The sections were dried, dewaxed and rehydrated. After washing with water the sections were immersed in TRIS buffered saline for 5 minutes and then covered by normal swine serum diluted 1 in 5 with TRIS buffered saline. After 10 minutes, excess swine serum was removed and replaced by the primary anti GST antibody at a dilution of 1 in 200. After overnight incubation at 4°C, the sections were rinsed twice in TRIS buffered saline for 5 minutes and swine anti rabbit secondary antibody (Dako) at a dilution of 1 in 400 applied. Following incubation
for 30 minutes at room temperature, the sections were washed in TRIS buffered saline
and incubated in Avidin Biotin Complex for 30 mins. Sections were then rinsed for
10 mins in TRIS buffered saline and the peroxidase reaction developed using
diaminobenzidine solution with 1% hydrogen peroxide. Sections were incubated with
this solution for 2 mins and then thoroughly washed in water, counter stained with
Haematoxylin and mounted. Hepatic bile duct was used as a positive control, while
ommission of primary and secondary antibodies served as negative controls.

**Table 2.2 - Immuno-Histochemistry results**

<table>
<thead>
<tr>
<th>Positive Staining</th>
<th>Superficial TCC N=23</th>
<th>Invasive TCC N=26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>Nucleus</td>
<td>1</td>
<td>13</td>
</tr>
</tbody>
</table>

Nuclear Staining: Fisher's exact Test p=0.003

**Scoring**

Scoring was performed jointly by an experienced histopathologist and the author.

Tumours were scored according to whether staining was nuclear, cytoplasmic or both.

There was little variation in intensity of staining in the nucleus or cytoplasm. Where
nuclear staining occurred, it was present in greater than 50% of cells. Tumours were
scored either positive or negative. Statistical analysis was performed using Fisher’s
exact test.
Table 2.3 - Tumour Stage

<table>
<thead>
<tr>
<th>STAGE</th>
<th>Positive Nuclear Staining</th>
<th>Negative Nuclear Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>T1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>T2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>T3</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>T4</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

Immunohistochemistry

The GST \( \pi \) polyclonal antibody stained cytoplasm in normal urothelium and in all tumours. Figure 2.1 shows intense cytoplasmic staining in a well differentiated papillary, superficial bladder tumour. In addition to tumour the stroma was stained positively in all cases. This included smooth muscle, endothelial and inflammatory cells at variable intensity.

Nuclear staining was seen in one out of twenty three superficial tumours (9%) and in thirteen out of 26 invasive tumours (50%) (Table 2.2). Figure 2.2 demonstrates nuclear staining in a moderately differentiated tumour which is invading lamina propria. This difference was significant using Fisher's exact test \( p=0.003 \). There was no association between tumour stage and nuclear staining seen in the invasive tumour group. Carcinoma in situ was identified in three of the sections and there was evidence of nuclear staining in two of these cases. (Figure 2.3) shows intense nuclear staining in an area of carcinoma in situ. In addition, positive nuclear staining was seen in one case of dysplasia. There was no relationship between
nuclear staining and response to chemotherapy (Table 2.4).

**TABLE 2.4 - Response to Chemotherapy**

<table>
<thead>
<tr>
<th></th>
<th>Responders (12)</th>
<th>Non Responders (14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Nuclear</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>

Nuclear Staining: Fisher's Test $p = 0.23$
Figure 2.1

Cytoplasmic staining seen in a superficial, papillary tumour. (TaG1)

Magnified x250
Nuclear staining seen in a moderately differentiated tumour invading lamina propria (T1G2). Magnified x250.
Figure 2.3

Nuclear staining seen in an area of carcinoma in situ. Magnified x250
Fig 2.4 Glutathione Synthesis

- glutamate
- cysteine
- cysteine moieties

- gamma glutamate cysteine transferase
- gamma glutamate traspeptidase

- glutathione synthetase

- glutathione S-transferases
- conjugation with electrophiles

- non toxic thiol ethers
CHAPTER 3: QUANTIFICATION OF THE MDR1 GENE IN TRANSITIONAL CELL CARCINOMA OF THE BLADDER.

AIM

The aim of this study was to develop a polymerase chain reaction (PCR)-based transcription assay to map out the range of expression of the MDR1 gene at the mRNA level, in a group of bladder tumours. Levels of expression could then be related to pathological data, including stage, grade and survival.

The multiple drug resistance gene MDR1 encodes for a membrane glycoprotein which can actively "pump" structurally unrelated drugs out of cells, thus making an individual cell resistant to chemotherapy. The background to classical multiple drug resistance and p-glycoprotein distribution in a number of tissues has been explored in chapter 1. This method of drug resistance is of particular interest in bladder cancer as anthracyclines are commonly used, both systemically and intra-vesically. In addition the vinca alkaloid vinblastine is used in combination with other cytotoxic agents in the CMV and MVAC regimens. If there was evidence of high levels of MDR1 gene expression in some tumours then these tumours may be more likely to be resistant to anthracyclines and vinca alkaloids than tumours with low levels of expression. In addition, the possibility of reversing this type of resistance with drugs such as verapamil or cyclosporin has been proposed, although existing evidence would suggest that the side effects of the high doses of verapamil necessary to achieve
reversal of resistance in vitro prohibit its clinical use. It is hoped that the development of new reversal agents will overcome the problem of morbidity.

Several authors have attempted to study expression of the p-glycoprotein in bladder epithelium by immunohistochemistry. Initially, Cordon Cardo (1991) reported staining for the p-glycoprotein in 4 out of 10 tumours and the absence of staining in normal bladder, using a panel of antibodies. More recently Naito et al., (1992) studied p-glycoprotein expression in transitional cell carcinomas of the urinary tract using the antibody MRK16 on frozen tissue and also assessed chemosensitivity to adriamycin by a microtiter succinate dehydrogenase inhibition test applied to tumour explants. Of 19 bladder tumours, 6 (32%) showed staining for the p-glycoprotein and for the whole group of tumours there was a correlation between expression of the p-glycoprotein and the MDR phenotype. Immuno-histochemistry, however is not without its limitations. Tumours are scored either positive or negative depending on whether the p-glycoprotein is detected or not. It is likely that there is a range of expression rather than two discrete groups and therefore it makes more sense to try and quantify expression numerically. Another problem is the reproducibility of the immuno-histochemistry and the range of antibodies to the p-glycoprotein available is in part indicative of the problems encountered with their use. In our department we have studied 20 frozen bladder tumours with the antibody C219 and were unable to detect a single positively staining tumour (unpublished data).

Benson et al., (1991) studied 21 bladder tumours and 8 normal bladder biopsies using the monoclonal antibody C219, detecting staining by flow cytometry.
P-glycoprotein levels in tumours were measured as the percentage of cells expressing the protein. No p-glycoprotein was detected in normal bladder biopsies. Using these parameters they reported higher levels of expression in invasive bladder tumours and in patients who had received chemotherapy prior to biopsy. These results must be interpreted with caution as it is now well recognised that using C219, positive staining is also seen in lymphocytes and whereas this can be taken into account with immuno-histochemistry the flow cytometer cannot make the distinction. This criticism however does not apply to negative results, such as the absence of staining in normal bladder.

Another method of detecting MDR1 expression is to measure RNA levels. Goldstein (1989) measured MDR1 RNA in a range of tumours using Northern blotting and was able to detect low levels in just one out of six bladder tumours. This evidence supports the impression from immuno-histochemical studies that MDR1 expression in bladder tumours is at a low level compared to tissues such as kidney and colon. Thus it would appear that low levels of expression of the MDR1 gene make quantification at the protein level or message level very difficult without a method of amplification.

Noonan et al., (1990) addressed this problem of quantitative analysis of the MDR1 gene in human tumours with low levels of expression, by developing a polymerase chain reaction (PCR) based transcription assay. This basically involves measuring the expression of MDR1 mRNA within a tumour relative to an internal reference gene (in this case β2 microglobulin). The great appeal of this method is that it allows the quantification of expression as opposed to the crude positive /
negative type of result gained from immuno-histochemistry. In addition, only small amounts of material are required and the technique will tolerate partial degradation of tumour specimen. Noonan et al., (1990) studied a range of tumours, including two bladder tumours and were able to detect levels of MDR1 mRNA expression in tumours in which MDR1 expression had not been observed by standard assays.

IMMUNOHISTOCHEMICAL STUDY OF P-GLYCOPROTEIN EXPRESSION IN BLADDER CANCER

In an attempt to demonstrate p-glycoprotein expression in bladder tumours 20 tumours were stained with the monoclonal antibody C219 (CIS U.K.), which binds to an intracellular epitope in the C-terminal region of the p-glycoprotein molecule. Detection of the p-glycoprotein was reported in 4 of 10 bladder tumours by Cordon Cardo (1989) using a panel of antibodies including C219. If p-glycoprotein expression could be demonstrated then it would be possible to study MDR1 expression at the message level and the protein level.

Immunohistochemical method

Twenty frozen bladder tumours of varying stage and grade were studied. Ten tumours were superficial (Ta,T1) and 10 showed evidence of muscle invasion. Adrenal tissue was used as a positive control and negative controls consisted of sections from which antibody had been omitted. Tumour biopsies and adrenal tissue had been collected fresh and immediately snap frozen in Isopentane cooled in liquid nitrogen. Biopsies were then stored at -80°C.
Sections were air dried for 20 minutes prior to fixing in cold acetone for 10 minutes and transferred to Tris Buffered Saline (TBS). Excess buffer was then removed and sections transferred to normal rabbit serum for 10 minutes. C219 was applied at dilutions of 1:100, 1:50 and 1:20 and incubated overnight. Sections were subsequently washed with TBS and incubated with horse radish peroxidase diluted 1:20 and diaminobenzidine solution in the presence of hydrogen peroxide. After washing in running water and counterstaining with Carazzi's stain slides were cleared and mounted. All sections were then examined by myself in conjunction with Mary Robinson (Consultant Histopathologist).

Despite positive staining being observed in adrenal control tissue, there was no evidence of p-glycoprotein in any of the tumours studied. The same findings were achieved with C219 at dilutions of 1:100, 1:50 and 1:20. It was therefore concluded that the level of p-glycoprotein expression in these tumours was too low to be detected by immuno-histochemistry using C219. The finding of p-glycoprotein expression in 4 out of 10 bladder tumours (Cordon Cardo 1991) involved the use of a panel of antibodies. It is conceivable that other available antibodies to the p-glycoprotein which recognise different epitopes, may have demonstrated expression of the protein but C219 was the only antibody available to the department.

Due to the limitations of methods to detect MDR1 expression at the protein level and the very low levels of expression involved, we determined to attempt to measure MDR1 expression at the message level by developing a semi-quantitative polymerase chain reaction based transcription assay. The methodology for this assay is described below.
Figure 3.1 Summary of polymerase chain reaction transcription assay

RNA extraction → RNA → Reverse Transcriptase → cDNA → RNA

25 cycles
Polymerase Chain Reaction

↓ primers MDR B2M 18S

DNA Electrophoresis
separate products

measure activity with phosphorimager

Regression analysis
x coefficient

MDR/18S B2M/18S
PCR TRANSCRIPTION ASSAY TO MEASURE MDR1 EXPRESSION

PATIENTS AND METHODS

Tumour biopsies were collected from 32 patients undergoing either trans-urethral resection of tumour or cystectomy for transitional cell carcinoma of the bladder, at Freeman Hospital. Tumours were of varying stage, as shown in table 3.1.

Table 3.1 Stage of bladder tumours studied.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta</td>
<td>9</td>
</tr>
<tr>
<td>T1</td>
<td>6</td>
</tr>
<tr>
<td>T2</td>
<td>2</td>
</tr>
<tr>
<td>T3</td>
<td>9</td>
</tr>
<tr>
<td>T4</td>
<td>6</td>
</tr>
</tbody>
</table>

Biopsies were snap frozen in isopentane cooled in liquid nitrogen and stored at -80°C prior to analysis. Part of the same biopsy material was sent to the department of pathology for histology. The PCR transcription assay can be broken down into a series of steps, starting with extraction of RNA from biopsy tissue.
a) RNA extraction

b) Synthesizing complementary DNA (cDNA) by reverse transcription

c) Amplifying cDNA by PCR

d) Separating PCR products

e) Quantifying MDR1 gene product

These steps are illustrated graphically in Figure 3.1 and shall now be described in detail.

RNA Extraction

RNA was extracted using an adaptation of the RNAzol™ method (Chomczynski, 1989). All extractions are performed using RNA-free reagents and baked glassware.

1. Pre-cool 50ml Falcon tubes (Gibco BRL) on crushed ice. Homogenize tissue in liquid Nitrogen with a pestle and mortar and transfer to Falcon tube.

2. Add 5ml RNAzol (Biogenesis Ltd) to homogenate and agitate thoroughly. RNAzol is a mixture of phenol, guanidinium thiocyanate and β-mercaptoethanol.

3. Add 1 ml chloroform (BDH), shake and then centrifuge at 4000 rpm (Mistral 3000i) for 10 minutes. This separates the homogenate into two discrete phases; a lower blue phenol-chloroform phase and a colourless upper aqueous phase. The RNA lies in the upper aqueous phase and DNA and protein lies in the lower organic phase or at the interface.

4. Remove aqueous phase containing RNA and add 2 ml of 20 mM Tris-HCL (pH 7.4) buffer to the remaining organic phase. Recentrifuge and remove aqueous phase to obtain any remaining RNA.
5. Centrifuge combined aqueous phases at 4000 rpm for 5 mins and decant supernatant into a Corex tube. Add an equal volume of isopropanol which causes RNA precipitation, mix and place on ice for 15 mins. Centrifuge at 10,000 rpm for 30 minutes at 4°C resulting in the formation of a RNA pellet.

6. Remove supernatant and wash RNA pellet by adding 5 ml of 70% ethanol (reagent grade BDH) and wash the pellet by agitation. Remove ethanol and allow pellet to dry. Add 200 µl of RNAase free distilled water and transfer to a 1.5 ml microfuge tube.

7. Determine amount of RNA and purity by optical densitometry (Lambda II spectrometer, Perkin Elmer). Optical Density (O.D.) of 1 represents 40 µg/ml RNA. Pure RNA yields an O.D.260/O.D.280 ratio between 1.7 and 2.0.

Manufacturing cDNA

RNA, which has been extracted from biopsy material, is stored at -80°C prior to cDNA synthesis. This involves the creation of a single strand of DNA from the RNA using an RNA dependent DNA polymerase. The DNA copy is subsequently cleaned and checked for purity and the quantity of DNA is estimated by the incorporation of a trace, radiolabelled α-32P dATP, during synthesis. The DNA is complementary to mRNA hence its name. The method employed was the so called random primer extension method described by Noonan and Roninson (1988). This results in a single strand of cDNA, the second strand is manufactured in the first cycle of the PCR reaction.
Synthesis Reaction

1. Defrost 10µg RNA and add 109µl sterile distilled water and 3.5µl Ribonuclease (RNase) inhibitor (Gibco BRL). Agitate and then heat for 5 mins at 100°C in a heating block (Tecam Dri-block DB4, Techne).

2. Combine with the following components to give a total volume of 200µl

<table>
<thead>
<tr>
<th>µl/reaction</th>
<th>concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human placental RNase inhibitor (10 units/µl)</td>
<td>3.5</td>
</tr>
<tr>
<td>Pd(N)₉ Random Hexamers (0.09/µl)</td>
<td>10</td>
</tr>
<tr>
<td>dithiotheritol dTT (0.1M)</td>
<td>20</td>
</tr>
<tr>
<td>5 x reverse transcriptase (RT) buffer</td>
<td>40</td>
</tr>
<tr>
<td>dNTP mix (100mM, 25mM each dA,T,C,GTP)</td>
<td>8</td>
</tr>
<tr>
<td>M-MLV Reverse transcriptase (200units/µl)(Gibco)</td>
<td>6</td>
</tr>
</tbody>
</table>

3. Agitate and then incubate at 37°C for 90 mins in a water bath. After 90 mins terminate reaction by heating mixture to 100°C. This denatures the reverse transcriptase.

Purify cDNA

In order to clean the cDNA, unincorporated nucleotides and random hexamers must be removed. This is achieved by passing the cDNA product though pre-packed cDNA spun columns containing Sephacryl™ S-300 (Pharmacia).

1. Equilibrate columns in 1 x Tris EDTA buffer containing 0.1M NaCl. Resuspend Sephacryl gel in its own buffer, drain and add 2ml in above buffer.

2. Place drained column in a 15 ml Corex tube. Centrifuge at 400 x g for 2
mins. Place cDNA on top of column and recentrifuge as above with an eppendorf tube in place to collect the effluent. This will contain purified cDNA and buffer.

Assessing first strand synthesis

Having manufactured and cleaned the cDNA it is important to check both the efficiency of the reaction and to quantify the product. Synthesis of cDNA was performed in runs of 8 samples. In each run, one sample had radiolabelled dATP introduced into the cDNA. Radioactivity within the cDNA was then subsequently measured. The ratio between total activity and activity from the cDNA will represent the relative amount of dATP incorporated in the reaction and hence the efficiency. The amount of cDNA manufactured can be expressed as the same ratio multiplied by the total weight of initial free nucleotide present in the reaction mixture.

1. Replace 10μl of water with 10μl of α-32P dATP in reaction mixture
2. Manufacture cDNA as above. Dilute 10μl of product in 190 μl of 1 x TE buffer
3. Run sample though a Sephadex G 50 column (Pharmacia) and collect effluent. Add 100μl of buffer to the column and collect effluent a total of 8 times. Measure activity in the respective effluents using a scintillation counter (Wallac 1410, Pharmacia). Early samples will represent the higher molecular weight cDNA and later samples the smaller nucleotides.
POLYMERASE CHAIN REACTION

Principles

The polymerase chain reaction is a technique for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. The PCR method was devised and named by Mullis et al., (1987) although the principle had been described in detail by Kleppe et al., (1971) over a decade earlier. The use of PCR was limited until heat resistant DNA polymerases were discovered such as that of the organism Thermus Aquaticus and the development of automated thermal cyclers.

A double strand of DNA is made from a single strand of cDNA by the action of DNA polymerase, during the first cycle of the reaction. In subsequent cycles both strands of DNA are copied, after denaturation, by the action of two primers both of which are complimentary to opposite strands of the DNA (sense and antisense). The reaction requires deoxynucleotide precursors for the synthesis of DNA, heat stable DNA polymerase, oligonucleotide primers, template and buffer containing magnesium to enable binding of primer to the template. Initially synthesis will go beyond the sequence complimentary to the primer but with each cycle the amount of DNA in the region flanked by the primers will increase exponentially whereas longer sequences will only increase in a linear fashion.

After 25 cycles there is approximately a $1 \times 10^4$ fold increase in DNA. The products may then be separated and quantitated. The reaction takes place within a thermal cycler and the temperature determines the three different stages of the reaction. At 95°C the DNA is denatured into two separate single strands. Then at
50-55°C the oligonucleotide primers anneal to the DNA template and at 70-75°C DNA synthesis occurs. The temperature then returns to 95°C and the cycle repeats. PCR can be used in a number of settings but in this study it has been employed specifically for measuring the levels of expression of the MDR1 gene in bladder tumours.

In order to quantitate MDR1 gene product the methods described by Noonan et al., (1990) were employed. This involves amplifying a target gene, namely MDR1 together with an internal reference standard. Expression of the internal reference gene should be constant, as expression of the target gene is measured relative to the reference gene and expressed as a ratio. Noonan et al., (1990) chose $\beta$2-microglobulin as the reference gene, because it is ubiquitous in all epithelia and because it is also a membrane glycoprotein. However there is evidence for decreased expression of $\beta$2-microglobulin in poorly differentiated bladder tumours (Walton et al., 1986) and it was therefore decided to include a second internal reference gene and for this purpose 18s ribosomal RNA was chosen. Ribosomal RNA is commonly used as an internal control in other techniques in molecular biology such as Northern blots, to ensure equal loading of quantities of RNA. Ribosomal RNA levels have been shown to remain constant even when total RNA content varies (Hirsch 1967).

THE PCR TRANSCRIPTION ASSAY

Three sets of four serial dilutions of template cDNA (one each for MDR1, $\beta$2M and 18s rRNA) are amplified independently with specific oligonucleotide primers. Radiolabel is incorporated so that the relative amounts of product can be measured and expressed as a ratio.
PCR PRIMERS

It is possible to derive primers for the genes studied as they have all previously been sequenced; MDR1 (Chen et al., 1990), β2M (Gussow et al., 1987) and 18s (Gonzalez and Schmickel 1986). Primers used for MDR1 and β2M are the same as those used by Noonan et al., (1990). The primers for 18s rRNA were derived from the program for PCR-primer prediction described by Lowe et al., (1990). Primers were synthesized using the automated Applied Biosystems model 392 DNA/RNA synthesiser. Before use, primers are dried, resuspended in water, their concentration determined by ultraviolet spectrophotometry and diluted to a final concentration of 12pm/μl.

MDR1

(SN) strand-  CCCATCATGGCAATAGCAGG  (Residues 2596-2615)
ASN) strand  TCAATCTCTGCTCCTGA  (Residues 2733-2752)

β2M

SN strand-  ACCCCCACTGAAAAACATGA  (Residues 1544-1563)
ASN strand  ATCTTCAACCTCCATGATA  (Residues 2253-2262)

18s

SN strand-  ATGCTCTTAGCTGAGTGTCC  (Residues 763-782)
ASN strand  AACTACGACGGTATCTGATC  (Residues 1055-1074)

SN : sense  ASN : antisense
These primers result in base pair products of:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td>167 bp</td>
</tr>
<tr>
<td>β2M</td>
<td>120 bp</td>
</tr>
<tr>
<td>18s</td>
<td>311 bp</td>
</tr>
</tbody>
</table>

PCR reactions were set up with the following reagents:

- **10 x buffer**
- Taq Polymerase (5U/μl)
- dNTP (8.75mM-2.5mM TCG, 1.25 mM A)
- dATP P32 radiolabel 10 mCi/ml (Amersham)
- sterile distilled water
- ASN primer (12pm/μl)
- SN primer (12pm/μl)
- cDNA (at appropriate dilution)
- MgCl2

The optimal MgCl2 concentration for maximal yield of product varied and was found to lie at the indicated concentrations for individual primers shown above.

**Quantification of Product**

After 25 cycles of PCR have been completed, 10μl of product are taken and loaded into wells on a 12% polyacrylamide gel. Products are then separated accordingly by...
Figure 3.2 Autoradiograph demonstrating PCR products from serial dilutions of cDNA amplified with primers specific to MDR1 and β2 microglobulin.

Figure 3.3 “Rangefinder assay”
10 dilutions of cDNA are amplified using specific primers for MDR1, β2M and 18S. Those dilutions resulting in products falling on the exponential part of the curve are selected for subsequent assays for the individual sample.
electrophoresis at 100 V for 90 mins using a Tris/borate/EDTA. The gels were dried and products visualised using a Phosphorimagery (Molecular Dynamics). Before the Phoshorimagery became available products were identified by autoradiography and an example of an autoradiograph is demonstrated in (Figure 3.2). The phosphorimagery detects radioactivity within the PCR product and using "Imagequant" software this activity is measured in counts per minute for each band selected. A graph of activity in counts per minute versus cDNA dilution is then plotted as in Figure 3.3. It is apparent from this graph that there is a linear relationship between cDNA concentration and quantity of product (counts per minute) in the middle portion of the curve. There is a threshold phase representing insufficient template for an efficient reaction and a saturation phase when reaction constituents become exhausted.

**Determination of range of dilutions for amplification**

In order to measure MDR1 gene expression relative to a reference gene, reactions must be performed at cDNA dilutions which result in an increase of product which is linear when plotted. In order to ensure that this is indeed the case, an initial replicate is performed to determine this range of cDNA dilutions. The reaction is then repeated using these dilutions. Serial dilutions were performed in sterile distilled water.

The range of dilutions are: MDR $10^0$, $5 \times 10^1$, $10^1$, $5 \times 10^2$...$10^4$ $\mu l$

$\beta 2 M$ $5 \times 10^4$, $10^4$, $5 \times 10^2$...$5 \times 10^4$ $\mu l$

$18s$ $10^3$, $5 \times 10^2$, $10^2$...$10^7$ $\mu l$
An example of the so called range finder assay is shown in Figure 3.3. Four dilutions of cDNA resulting in products lying on the exponential part of the curve are selected for subsequent replicates. For each assay the same conditions as described above for the PCR reaction are repeated. The PCR transcription assay is performed 3 times for each tumour sample and the mean of the 3 assays calculated for the purposes of comparing MDR1 gene expression in individual tumours.

**Quantification of gene expression.**

From the graph of cDNA dilutions plotted against quantity of product measured in counts per minute (cpm), regression analysis is performed for the 4 points generated, (Quattro Pro, Borlands). In all cases the correlation coefficient was greater than 0.95, indicating a linear relationship between PCR product and cDNA dilution. Regression analysis also yields the gradient of the straight line generated and this is termed the X coefficient. X coefficients are generated for MDR1 and β2 microglobulin and these are expressed relative to the internal reference standard 18s as MDR/18s and β2M/18s respectively.

**ASSAY EFFICIENCY**

In order to validate results, the relative efficiency of the PCR amplification in different tissues was measured in three different tissue types and one drug resistant small cell lung cancer cell line to ensure that any variation in gene expression ratios was not a reflection of different amplification efficiencies.

The drug resistant cell line (H69/LX4) was supplied by Dr P Twentyman.
This line was derived by stepwise selection in Adriamycin (Twentyman et al., 1986) and subsequently cultured in RPMI 1640 medium (Northumbria biochemicals). After harvesting, RNA was extracted from the cell line using the RNAzol method as described earlier.

The relative maximum efficiency of a particular reaction was defined by the equation:

\[ P_n = P_{n-1} (1 + e) \]

- \( P \) = amount of product at the end of the \( n^\text{th} \) cycle
- \( n \) = cycle number
- \( e \) = efficiency

The figure \( n \) varies for each gene measured as the number of cycles performed before maximum efficiency is reached is different for each gene. It is clear from Table 3.2 that even though data are not complete on all tissues, the wide variation in MDR1 expression seen between tissues is not due to variation in amplification efficiency of the genes involved. There is a difference between the amplification efficiencies of the respective genes but this difference is constant. As the transcription assay is comparative, provided this difference remains constant it will not result in any difference in measured gene expression ratios between tumours.
Figure 3.4 MDR1/18S Expression in all tumours

Figure 3.5 B2M/18S Expression in all tumours
Table 3.2 Efficiency of amplification during PCR

<table>
<thead>
<tr>
<th>Tissue</th>
<th>MDR1/18S</th>
<th>Efficiency Ratio</th>
<th>MDR1/B2M</th>
<th>Efficiency Ratio</th>
<th>Efficiency Ratio</th>
<th>Efficiency Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69/LX4 cell line</td>
<td>2.8 x 10^3</td>
<td>1.14</td>
<td>.88</td>
<td>.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>2.0 x 10^4</td>
<td>1.19</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal bladder</td>
<td>3.9 x 10^3</td>
<td>1.16</td>
<td>.88</td>
<td>.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder Tumour</td>
<td>3.4 x 10^4</td>
<td>*</td>
<td>.94</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: result not available

RESULTS

MDR1 mRNA was detected in all bladder tumours studied as shown in Figure 3.4. The range of expression was 34 x 10^4 - 0.54 x 10^4. This represents a 63 fold variation in MDR1 gene expression. In comparison, adrenal tissue (which is known to have high levels of MDR1 expression), had an MDR1/18s ratio 27 fold greater than the mean level of expression in bladder tumours.

The mean coefficient of variation for MDR1 expression for repeat determination within a sample was 46% (range 4-83%). The 63 fold inter-tumour variation cannot therefore be due to differences between replicate assays alone although it may contribute to a lesser extent.

β2 Microglobulin was also detected in all tumours (Figure 3.5). However the range of expression for β2 microglobulin was considerably greater (68 x 10^4 - 0.37 x 10^4) which represents a 184 fold variation. The mean coefficient of variation for
β2M replicates on an individual sample was 43% (range 6-102%) which is similar to that for MDR1 expression.

Table 3.3 Tumour stage v mean MDR1 and β2 microglobulin expression

<table>
<thead>
<tr>
<th>Tumour Stage</th>
<th>Superficial (Ta, T1)</th>
<th>Invasive (T2, T3, T4)</th>
<th>* p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR/18s x 10^4</td>
<td>4.87</td>
<td>9.84</td>
<td>0.08</td>
</tr>
<tr>
<td>B2M/18S x 10^4</td>
<td>5.90</td>
<td>15.88</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* student t test

The difference in MDR1 expression between superficial and muscle invasive tumours is not significant (p=0.08). One tumour within the superficial group (T1G3) had a high level of MDR1 expression and this type of tumour often behaves as an invasive tumour, so that some authors feel it should no longer be classed as a superficial tumour (Abel et al., 1988). Perhaps a more representative method of analysing the two groups is to use tumour grade. Invasive tumours tend to be poorly differentiated as opposed to superficial tumours which tend to be well differentiated.
Figure 3.6 Relationship between MDR1 expression and patient survival.

- **1. High MDR1**
- **2. Low MDR1**

\[ p = 0.36 \]
Table 3.4 Tumour grade v mean MDR1 and B2 microglobulin expression

<table>
<thead>
<tr>
<th>Tumour Grade</th>
<th>G1 / G2</th>
<th>G3</th>
<th>* p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR/18s x 10^4</td>
<td>3.41</td>
<td>10.75</td>
<td>0.006</td>
</tr>
<tr>
<td>β2M/18s x 10^4</td>
<td>5.1</td>
<td>15.95</td>
<td>0.042</td>
</tr>
</tbody>
</table>

*student t test

When MDR1 expression is related to grade, a clear difference emerges between poorly differentiated and well differentiated tumours (p=0.006). A significant difference in β2 microglobulin levels between the two groups is also seen (p=0.042).

MDRI expression and response to chemotherapy

Of the 32 patients studied, 7 received chemotherapy regimens including drugs implicated in multidrug resistance. Response to chemotherapy was observed in 3 of the 7 patients but there was no overall difference between the responders and non responders in terms of MDR1 expression.

MDRI expression v Survival

In order to investigate a potential relationship between MDR1 expression and survival patients were split into two groups according to whether the MDR1/18s ratio was greater or less than the mean figure for all tumours (i.e. MDR/18s > 1 x 10^4 or MDR/18s < 9.99 x 10^4). Statistical analysis was performed by means of the log
The survival data is complicated by the fact that not all patients will have received the same form of treatment, for example some muscle invasive tumours will have been treated by radical cystectomy and some by radical radiotherapy. In addition the follow up time is variable and short.

Patients whose tumours had a higher MDR1 expression did not have a significantly shorter survival period (p=0.36). The median survival for patients with high MDR1 expression was 25 months compared to 36 months in those patients with low levels of expression. The degree of differentiation of bladder tumours is recognised as a prognostic indicator, with poorly differentiated tumours having a poor outlook and as a relationship between MDR1 expression and tumour grade has previously been demonstrated a poor prognosis would therefore be expected in tumours with high levels of MDR1 expression. This is not the case in the patients studied but the analysis is limited by small numbers and a short follow up period.
IMMUNOHISTOCHEMICAL STUDY OF P53 OVEREXPRESSION IN RELATION TO MDR1 mRNA EXPRESSION

Mutation of the tumour suppressor gene p53 has been linked with upregulation of the MDR1 gene by binding to the promoter region (Chin et al. 1992). The protein product of the tumour suppressor gene p53 is a nuclear phosphoprotein, normally expressed at undetectable levels in human cells and serves to regulate cell growth and division. Mutation of the p53 gene results in an increased half life of the protein and is an indicator of poor prognosis in bladder cancer (Wright et al. 1991). Mutation of the p53 protein product is therefore associated with overexpression which can be detected by immunohistochemistry.

In order to investigate a potential relationship between MDR1 expression and p53 overexpression in bladder tumours, immuno-histochemistry was performed on sections from all bladder tumours for which MDR1 data were available.

IMMUNOHISTOCHEMICAL METHOD

P53 expression was determined using a polyclonal antibody CM1 (Novocastra Laboratories) on paraffin embedded sections. Parraffin wax sections (4μ) of formol sublimate fixed tissue were cut and then dried for 30 minutes. Following dewaxing and hydration, sections were immersed in Tris Buffered Saline (TBS) for 2 minutes. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide. Sections were then incubated in diluted normal swine serum for 10 minutes. A standard avidin-biotin complex (ABC) technique was used and sections
were incubated in polyclonal CM1, diluted 1:500 and incubated in biotinylated swine anti rabbit immunoglobulin (1:400) in TBS for 30 minutes. CM1 stains both the wild type and mutant form of p53 (Midgley et al. 1992). Positive control material was a formol sublimate fixed adenocarcinoma of the colon and a positively staining bladder tumour. Negative controls were performed by omitting the primary antibody in each case.

Tumours were scored positive or negative according to the presence of discrete nuclear staining. The degree of staining was scored as positive (+), strongly positive (++) or absent (-). Scoring was performed jointly by a Consultant Histopathologist (Dr Mary Robinson) and myself.

RESULTS

Expression of the p53 protein was detected in 11 of 26 (44%) tumours for which material was available. Tumours were grouped as either p53 positive or p53 negative as demonstrated in figure 3.7. There was no significant difference in terms of MDR/18S levels between tumour which overexpressed the p53 protein and those that did not. There was also no increase in MDR/18S levels in those tumours scored (++) as opposed to those scored (+).
Figure 3.7 MDR1 expression in bladder tumours in relationship to p53 status, as determined by immuno-histochemistry.
Figure 3.8

Nuclear staining for the p53 protein in a poorly differentiated bladder tumour, treated with the polyclonal antibody CM1. Magnified x 250.
CHAPTER 4: PHARMACOKINETIC DETERMINANTS OF CISPLATIN ACTIVITY AND TOXICITY IN TRANSITIONAL CELL CARCINOMA OF THE BLADDER.

AIMS

1. To measure free cisplatin levels in all patients receiving cisplatin based combination chemotherapy for transitional cell carcinoma of the bladder.

2. To determine the variation in free cisplatin AUC (area under the curve of plasma levels v time) achieved in all patients when prescribed according to surface area alone.

3. To relate any variation in AUC observed to toxicity and tumour response.

4. To attempt to develop a limited sampling model for calculating free cisplatin AUC.

Introduction

Dose Response

The pharmacokinetics and pharmacodynamics of cisplatin have been discussed in chapter 1, along with response rates of cisplatin either as a single agent or in combination with other cytotoxic agents. There has been little work on the relationship between cisplatin dose and response in bladder cancer and interpretation of clinical studies is made difficult by the wide variety of dose schedules that have been employed. In the majority of reported studies cisplatin has been administered at a dose of 70 mg/m². Other schedules with doses of 50, 100 and 120 mg/m² have resulted in similar proportions of responses (30-40%) (Yagoda 1987, Scher 1985,
A more quantitative measure of exposure to drug is the AUC, that is to say the area beneath the curve of plasma levels of cisplatin plotted against time, in units of mg/ml.min. Now that the method exists for measuring free cisplatin the AUC for individual patients at set doses of drug can be calculated and related to response and toxicity. This has not yet been carried out for bladder cancer. An even more sensitive technique is the measurement of platinum adducts, which reflects the amount of platinum active at an intracellular level. Reed et al., (1987) have reported a weak correlation between white blood cell platinum adducts and tumour response in patients receiving platinum based chemotherapy for ovarian cancer. Although there was initially some doubt concerning the methodology, other authors have now confirmed this correlation and in addition, established a correlation between free cisplatin AUC and adduct kinetics in a range of solid tumours (Schellens et al., 1993). Several randomised trials of platinum-dose response have been reported but the results are difficult to interpret because cisplatin was often given in combination with another drug. Thus in the treatment of testicular cancer, 120 mg/m² cisplatin was superior, in terms of response, to 15 mg/m² per day for 5 days, when dose and schedule of vinblastine and bleomycin were held constant (Samson et al., 1984). Again in testicular cancer, 40 mg/m² cisplatin per day for 5 days was better than 20 mg/m² per day for 5 days, but in this trial the group receiving high dose cisplatin also received etoposide (Ozols et al., 1988). Survival amongst patients with ovarian cancer treated with 100 mg/m² alone was compared with that among patients treated with 20 mg/m² cisplatin in combination with low dose chlorambucil; the high dose resulted in longer survival amongst patients with stage 3, but not
stage 4 disease (Wiltshaw et al., 1986). In a retrospective review of the dose intensity of various chemotherapy regimens used in the treatment of ovarian cancer, cisplatin was reported to be the only drug whose relative dose intensity correlated significantly with clinical response and survival (Levin 1987).

All the tumours mentioned so far have a high degree of sensitivity to cisplatin, however the dose response relationship for cisplatin is less well established in tumours with a lesser degree of sensitivity. In the treatment of recurrent cervical cancer, Bonomi et al., (1985) found a statistically significant advantage for cisplatin given at a dose of 100mg/m² versus 50 mg/m² in terms of response rate, but not in terms of survival. Forastiere et al., (1982) reported responses in 4 of 19 patients with metastatic breast cancer treated with cisplatin 120 mg/m² compared with no responses in 18 patients treated with cisplatin 60 mg/m². The same group reported a 73% response rate to a fractionated dose of 200 mg/m² in 22 patients with advanced head and neck cancer, 4 of whom had not responded at standard dose.

There are sufficient data to suggest a dose-response relationship for cisplatin in tumours with proven sensitivity, so it would seem likely that the same might be the case for bladder cancer.

Dosing according to surface area is based on empirical observations of interspecies differences for a number of different drugs. However the AUC for a drug has been shown to be more closely correlated with drug toxicity than the dose /m² (Collins et al., 1986). In the case of cisplatin it is the free component which is active so it is the free cisplatin AUC that is of most interest. As mentioned earlier
there are no reports in the literature looking at a potential relationship between free cisplatin AUC and response or toxicity in bladder cancer, indeed there are very few clinical studies in which free cisplatin has been measured. In a small study of 11 patients with assorted malignancies, Desoize et al., (1991) reported no response to treatment in patients with low free cisplatin AUC, suggesting a possible AUC response relationship. Higher dosage of cisplatin will lead to higher plasma levels (Himmelstein et al., 1981) but not in all cases as will become clear later in this chapter.

If there was a relationship between AUC and response in bladder cancer, which is sensitive to single agent cisplatin in 30-40% of patients, then an argument could be made for tailoring dose according to AUC as determined from pharmacokinetic studies on individual patients. It may be that patients who fail to respond to treatment, or relapse early have actually had an inadequate dose of drug. This hypothesis will be investigated in this chapter.

Toxicity
Cisplatin is not only one of the most potent cytotoxic agents used in the treatment of solid tumours it can also be one of the most toxic. Phase I clinical trials identified dose dependent nephrotoxicity as the most serious side effect of the drug, occurring with increasing frequency in doses greater than 50 mg/m² (Rozencweig et al., 1977). Initially, dose fractionation was employed in an attempt to reduce toxicity. Later prolonged infusion times were introduced and this considerably reduced toxicity (Bozzino et al.,1981). The total free cisplatin AUC may be the same for a rapid one
hour infusion as for a longer 24 hour infusion (Vermonken et al., 1982), yet the degree of toxicity is significantly reduced by the use of longer infusions (Reece et al., 1989). This suggests that these toxic effects may be due to peak levels rather than AUC. Nephrotoxicity may be further reduced by a combination of pre-hydration and forced diuresis and Corden et al., (1985) maintain that doses up to 200 mg/m² can be tolerated with minimal toxicity using this method. However, despite adequate pre-hydration and forced diuresis, some patients will still develop nephrotoxicity. This has been shown to be dose related by Campbell et al., (1983), who also demonstrated that patients who develop renal impairment have significantly higher total plasma platinum levels than those who do not. In addition to reducing the risk of serious nephrotoxicity the use of longer infusions also decreases the incidence of nausea and vomiting (Reece et al., 1989), suggesting that this toxicity is also related to peak levels. Nausea and vomiting were universal when cisplatin was given as a bolus in doses greater than 20 mg/m², however longer infusions and the routine use of modern anti-emetics such as ondansetron have made what was a major clinical problem, uncommon.

For a detailed account of toxicities encountered in phase I, II and phase III trials in man the reader is referred to Von Hoff and Rosenberg (1979). Ototoxicity has also been reported since initial trials, often complicating the course of patients experiencing nephrotoxicity. Myelosuppression has become more frequent as more intensive regimens have been developed and may be particularly severe in patients who have received prior chemotherapy. Allergic reactions and neurotoxic manifestations have been reported in a very small number of cases but are not generally a problem. Of greater significance is electrolyte disturbance, particularly
**FIGURE 4.1 Chemotherapy regimens**

CMV (Cisplatin 100mg/m²) 3 cycles

CMV (Cisplatin 70mg/m²) up to 6 cycles

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 8</th>
<th>DAY 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>CIS</td>
<td>MTX</td>
<td>REPEAT</td>
</tr>
<tr>
<td>VIN</td>
<td>VIN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EPICM (Cisplatin 70mg/m²) up to 6 cycles

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>DAY 8</th>
<th>DAY 15</th>
<th>DAY 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPI</td>
<td>MTX</td>
<td>MTX</td>
<td>REPEAT</td>
</tr>
<tr>
<td>CIS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CMEPI (Cisplatin 70mg/m²) up to 3 cycles

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPI</td>
<td>CIS</td>
<td>REPEAT</td>
</tr>
<tr>
<td>MTX</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CIS : cisplatin  
MTX : methotrexate  
VIN : vinblastine  
EPI : epirubicin
hypomagnesaemia which may be symptomatic. This may be due in part to a direct

toxic effect of cisplatin on renal tubules and in part due to side effects from diuretic
drugs.

There is then a great potential for toxicity, which is dose dependent,
particularly at higher cisplatin doses. As with response data there has been very
little work on the relationship between free cisplatin AUC and toxicity in patients
receiving cisplatin and data are only available for dose toxicity. Perhaps the best
study was that by Campbell et al., (1982) who studied 77 patients with several
different types of carcinoma receiving cisplatin by infusion at doses ranging from 40-
100 mg/m². They found that patients receiving a higher dose of cisplatin had higher
plasma platinum levels and a greater incidence of nephrotoxicity. AUC was not
measured but it is very likely that AUC was also related to nephrotoxicity. If there
was significant variation in cisplatin AUC between patients using current methods
of dosage, then it may be possible to correlate AUC and toxicity and to determine
the degree of toxicity that would be acceptable or necessary for tumour response.

Patients and Methods
Pharmacokinetic studies were performed on all patients receiving cisplatin based
combination chemotherapy for transitional cell carcinoma of the bladder at the
Freeman Hospital between January 1992 and October 1993. This involved 4
different chemotherapy regimens which are listed in table 4.1. The treatment
schedules are outlined in Figure 4.1. Wherever possible, studies were performed on
more than one cycle per patient. A total of 57 studies were carried out on 30
patients.
Figure 4.2a

Stage of bladder tumour in study patients

Figure 4.2b

Grade of bladder tumour in study patients
Figure 4.2c

Distribution of metastases in study group

Metastases (36.0%)

No Metastases (64.0%)
Table 4.1 Chemotherapy regimens for patients studied

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Dose (mg/m²)</th>
<th>No Patients</th>
<th>No cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>cisplatin 100</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>CMV</td>
<td>cisplatin 70</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>EPICM</td>
<td>cisplatin 70</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>CMEPI</td>
<td>cisplatin 70</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

It was necessary to study patients receiving different regimens as the number of patients treated with any one schedule was relatively small. All cycles were included in the analysis of both toxicity and pharmacokinetic variation. Response data analysis was restricted to patients receiving CMV chemotherapy who had completed their treatment and in whom pharmacokinetic data were complete, i.e. 17 patients.

The stage, grade and metastatic status of tumours treated is represented in Figure 4.2a, 4.2b and 4.3c. All the patients receiving CMV with the higher dose of cisplatin (100 mg/m²) were taking part in a European study of neoadjuvant chemotherapy, in which they received three cycles of chemotherapy prior to definitive treatment in the form of radical cystectomy or radical radiotherapy. Patients who received CMV with cisplatin at a dose of 70 mg/m² were taking part in a trial of CMV versus MV for locally advanced or metastatic bladder cancer.

Those patients receiving EPICM had locally advanced and metastatic disease and were not in a trial. Patients receiving CMEPI were part of a pilot study for the treatment of poorly differentiated microinvasive tumours (T1G3) with a combination
of systemic chemotherapy and intravesical treatment.

Eligibility criteria for the four protocols are listed in Table 4.2. No age limit was specified provided patients were mentally and physically able to undergo treatment and follow up. Informed consent was obtained from all patients and ethical committee approval granted.

Table 4.2 Eligibility criteria

<table>
<thead>
<tr>
<th></th>
<th>GFR* ml/min</th>
<th>WBC x10⁹/L</th>
<th>PLAT x10⁹/L</th>
<th>TUMOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV (100)</td>
<td>&gt; 60</td>
<td>&gt; 3.5</td>
<td>&gt; 100</td>
<td>T2, T3, T4a, N0, M0</td>
</tr>
<tr>
<td>CMV (70)</td>
<td>&gt; 50</td>
<td>&gt; 3.5</td>
<td>&gt; 100</td>
<td>T4b, N+, M+</td>
</tr>
<tr>
<td>EPICM</td>
<td>&gt; 45</td>
<td>&gt; 3.5</td>
<td>&gt; 120</td>
<td>T3, T4a, T4b, M+</td>
</tr>
<tr>
<td>CMEPI</td>
<td>&gt; 60</td>
<td>&gt; 3.5</td>
<td>&gt; 100</td>
<td>T1G3, N0, M0</td>
</tr>
</tbody>
</table>

(* GFR determined by Cockcroft formula)

Cockcroft Formula

This is a formula for the calculation of creatinine clearance from serum creatinine. It was described by Cockcroft and Gault (1976) and involves the calculation of creatinine clearance with the following equation:

\[
GFR = \frac{(140-\text{age}) \times \text{wt kg}}{72 \times \text{creat (mg/100ml)}}
\]

\[\text{GFR} = \frac{(140-\text{age}) \times \text{wt kg}}{72 \times \text{creat (mg/100ml)}} (15\% \text{ less in females)}
\]

The above formula gave a correlation coefficient between predicted and measured creatinine clearance of 0.83, in the original paper.
Cisplatin administration

Cisplatin dose was calculated according to the surface area of the patient. Patients were pre-hydrated with 1L normal saline prior to treatment and if urine output fell below 100 ml/hr then 100 ml of 20% mannitol was given. The cisplatin infusion was run over 4 hours using a programmed infusion pump. In the 16 hours post infusion patients received a further 2L normal saline supplemented with 20 mEq KCl and 1 gm of MgSO₄.

Sample collection

Four ml of venous blood were taken prior to the start of the 4 hour cisplatin infusion and at predetermined times thereafter at: 15, 30, 60, 120, 180, 240, 255, 270, 285, 300, 360 and 1440 minutes. Samples were placed in cooled heparinised bottles and immediately centrifuged for 5 minutes at 3,000 rpm in a cooled centrifuge (Mistral 3000i) at 4°C. Plasma from each sample was then aspirated part of which (1.5 ml) was stored at -80°C and part (1 ml) centrifuged again, using a Centrifree micropartition system (Amicon) at 2,000 x gravity for 10 minutes. This resulted in an ultrafiltrate droplet (MW 30,000) of approximately 30 μl. The ultrafiltrate was then frozen and stored at -80°C prior to analysis.

All patients had their GFR (glomerular filtration rate) measured on the day of the cisplatin infusion, according to ⁵¹Cr-EDTA clearance using a two sample calculation, as routinely used by the Department of Medical Physics at the Freeman Hospital.
Methodology for the analysis of cisplatin in whole plasma and plasma ultrafiltrate.

An atomic absorption spectrophotometer (Philips PU 9100X) was used in order to measure the concentration of platinum in all samples, including both ultrafiltrates and plasma samples. The ultrafiltrate represents the "free" cisplatin, in other words cisplatin which is not bound to plasma proteins, as opposed to the total plasma cisplatin which represents the sum of the free and the protein bound components. The amount which is protein bound can be deduced by simply subtracting the free from the total.

Atomic absorption spectrophotometry is one of the most sensitive instrument used in the detection of platinum. Samples are vapourised by carbon rod atomization in a graphite cuvette (so called flameless technique). The atomised vapour so formed contains free atoms of platinum which, when illuminated by a light source that radiates light with the frequency characteristics of that element (266 nm for platinum) absorbs a photon of a wavelength corresponding to its atomic absorption spectrum, thus exciting it. The amount of absorption is a function of the concentration of platinum in a fixed volume (10 or 20 μl) of aspirate and is measured by photomultipliers which detect the amount of light absorbed. This figure for light absorption is then converted into a platinum concentration by reference to a calibration graph calculated from 3 different platinum standards of pre-determined concentration.

A three stage heating program was used for the ultrafiltrate, consisting of drying at 100°C for 60 seconds, ashing at 1,100°C and atomising at 2,500°C. For the protein bound samples the drying temperature was reduced to 90°C. The ramp rate
of the program was 200°C /sec. An Argon flow rate of 4 L/min was maintained at all stages except atomization in order to prevent combustion of the graphite cuvette. Two analyses were performed on all samples and the mean recorded.

*Preparation of cisplatin quality assurance controls and calibration standards*

A quality assurance control sample at a concentration of 1 µg/ml was included in every run of patient samples analysed to ensure reproducibility. Limits accepted for the control were 0.9 -1.1 µg/ml. The lower limit of detection was 0.2 µg/ml which is equivalent to 2 x background and the assay was sensitive to within 0.1 µg/ml. The controls were made in one batch from a stock cisplatin solution prepared as described below. The coefficient of variation for the assay, calculated from 40 repeat measurements of the same 1µg/ml standard was 17.2 %.

1. Dissolve a fixed amount of cisplatin in distilled water.
   
   e.g. 10 mg in 20 mls = 500 µg/ml

2. Agitate for 1 minute to ensure complete dissolution.

3. Take 50 µl of solution (25 µg) and add to 25 mls phosphate buffered saline (PBS) for ultrafiltrate controls and to 25 mls plasma for plasma controls.

4. Store as 1 ml aliquots at -20°C

Calibration standards were made at 1, 2 and 4 µg/ml in a similar fashion. 200 µl of 500 µg/ml cisplatin solution (100 µg) were added to 25 mls of PBS or to 25 mls plasma giving rise to a 4 µg/ml solution which was serially diluted to 2 µg/ml and 1 µg/ml respectively. All samples and controls were diluted 1:20 in 0.1 M HCl for
Figure 4.3 Free and protein bound cisplatin levels in a patient receiving 100 mg/m² cisplatin by 4 hour infusion.
Data analysis

Plasma concentrations of free and bound cisplatin were analysed by a non-compartmental pharmacokinetic analysis in which the AUC of free or bound cisplatin was calculated by the trapezoidal rule. To assist in pharmacokinetic calculations and to allow comparison of free and bound cisplatin data, concentrations are expressed as \( \mu g/ml \) cisplatin equivalents. In an attempt to establish a limited sampling model, linear regression analysis was used to investigate relationships between the free cisplatin AUC and plateau level (mean of 1-4 hr concentrations), end infusion free cisplatin and 24 hr protein bound cisplatin. The limited sampling model generated was tested using measurements of predictive performance as described by Sheiner and Beale (1981). "Minitab" statistical software was used for unpaired, two tailed \( t \) tests in order to assess response and toxicity. GFRs performed at The Freeman Hospital Department of Medical Physics were validated using NIRAS software (Medical Physics) and all volumes of distribution lay between 15 and 25 L.

RESULTS

Figure 4.3 shows a graph of free and protein bound cisplatin concentrations in a patient receiving CMV (cisplatin at a dose of 100 mg/m\(^2\)). After the patient had been prehydrated, the infusion was started at time 0, finishing at 4 hours. The ultrafiltrable or free cisplatin concentration reaches a peak at 60 minutes, forming a steady plateau thereafter. When the infusion stopped the free cisplatin fell rapidly.
and in some cases was unmeasurable at 300 minutes. Cisplatin binds rapidly to plasma proteins and hence the protein bound component rises steadily, reaching a peak at approximately 240 minutes. The protein bound component has a much longer half life and at 24 hours has hardly fallen.

The free cisplatin AUC is derived by calculating the area below the curve produced by plotting the free cisplatin level against time. It is the free cisplatin that is of most interest, as it represents the active component. Other figures used in later analysis are:

a) the end infusion cisplatin concentration, that is to say the free cisplatin at 240 minutes when the infusion is stopped.

b) the mean plateau cisplatin, a mean generated from the free cisplatin level at 120, 180 and 240 minutes.

c) the 24 hour protein bound cisplatin.

<table>
<thead>
<tr>
<th>Dose</th>
<th>mean AUC</th>
<th>median</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 mg/m²</td>
<td>0.21</td>
<td>0.20</td>
<td>0.07</td>
</tr>
<tr>
<td>100 mg/m²</td>
<td>0.34</td>
<td>0.33</td>
<td>0.06</td>
</tr>
</tbody>
</table>

student t test p=0.00001

Mann-Whitney p < 0.01
Figure 4.4 Range of free cisplatin AUCs observed in all cycles studied.
It would be expected that a higher free cisplatin AUC would result from a higher dose of drug and this is indeed the case, despite the overlap between the ranges of distribution, as indicated in Figure 4.4. The difference between the two groups is highly significant.

There was considerable inter-patient variation in free cisplatin AUC, both in the 70 mg/m² group and the 100 mg/m² group. This is represented in Figure 4.4 and Table 4.3, in which overlap is clearly seen between the two groups, with some patients at a lower dose of cisplatin actually achieving a higher free cisplatin AUC than those at a higher dose. The highest recorded AUC was in fact in a patient dosed at 70 mg/m², who had impaired renal function with a GFR of 45 ml/min.

Table 4.4 Inter and Intra patient variation in free cisplatin AUC

<table>
<thead>
<tr>
<th>VARIATION</th>
<th>Mean AUC coefficient of variation (70 mg.m²)</th>
<th>Mean AUC coefficient of variation (100 mg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter-patient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cycles (n=56)</td>
<td>33%</td>
<td>22%</td>
</tr>
<tr>
<td>Intra-patient (n=19)</td>
<td>18%</td>
<td>10%</td>
</tr>
<tr>
<td>Inter-patient (n=19)</td>
<td>22%</td>
<td>13%</td>
</tr>
</tbody>
</table>

To further investigate the reasons for the variability in cisplatin AUC values, the inter and intra-patient variation in cisplatin AUC values were compared (Table 4.4). This has been performed for all patients and for 19 patients who were studied on more than 1 cycle (3 cycles n=6, 2 cycles n=13). The mean intra-patient
coefficient of variation was 18% (SD 9.4) for the 70 mg/m² group and 10% (S.D. 5.8) for the 100 mg/m² group, as compared to 22% and 13% for inter-patient variation in the same group of patients. This indicates that the wide distribution of AUCs is due in part to variation in pharmacokinetics within individual patients over successive cycles of treatment in addition to variation between patients.

Response - AUC relationships

Response data were analysed on all patients treated with CMV. Patients receiving the regimens EPICM or CMEPI were not included as epirubicin levels were not measured as part of this project. Data for methotrexate and vinblastine are discussed in later chapters. Not all the patients receiving CMV chemotherapy completed their treatment; 4 patients withdrew from the trial and 1 patient died from a gastrointestinal bleed before tumour response had been assessed. This left a total of 17 patients in whom data were complete for correlation of response and first course free cisplatin AUC. Response was assessed according to criteria mentioned in the introduction chapter. These data were obtained from the trial records and the consultants who determined whether a tumour had responded or not had no knowledge of the pharmacokinetic data. Patients were placed in one of two groups - responders or non responders. The responder group will have had tumours which either showed a complete pathological response or a reduction of tumour volume by 50% or more, according to examination under anaesthesia and computerised axial tomographic scan (CAT scan). The examination under anaesthesia is performed with the patient paralysed and anaesthetised so that the abdominal wall is relaxed. An
Figure 4.5 Relationship between first cycle free cisplatin AUC and response in patients with transitional cell carcinoma of the bladder treated with CMV. (student t test)
assessment of tumour size is the made by bimanual palpation of the tumour. It can be difficult to assess 50% reduction in a three dimensional tumour in the curved wall of a distensible organ and CAT scan interpretation can be complicated by blood clot and oedema following transurethral resection. The non-responders will have either shown no change or progression of their tumours. Of the 17 patients, 11 (65%) demonstrated tumour response and 6 (35%) did not. Of the responders 3 were complete, with no evidence of tumour on biopsy. Due to the low numbers involved the analysis performed is of a univariate type (student t test).

Figure 4.5 shows the response data plotted against first cycle free cisplatin AUC. The responders had a higher mean free AUC and this difference was significant (p=0.018), suggesting an AUC response relationship for cisplatin in this small group of patients. When the same analysis is performed for the second cycle free cisplatin the difference between responders and non-responders is no longer significant at the 5% level (p=0.13). However, second cycle data were only available on 19 patients which probably accounts for this higher p value. The three complete responders had first cycle AUCs of 0.28, 0.23 and 0.30 mg/ml.min respectively. Two tumours demonstrated progression whilst on treatment and the first cycle AUCs for these patients were both 0.15 mg/ml.min.

Response - Dose relationship

Another way of analysing the data is to compare response rates in high dose and low dose treatment groups according to dose rather than AUC. We know from the variation within the dosage groups studied that dose is not an accurate reflection of
the actual exposure to drug as determined by AUC, yet there was a significant difference in mean AUC between the high and low dose groups. Table 4.5 shows response rates in the two groups.

Using Fisher’s exact test the difference in response rates is not found to be significant whereas the difference in response rates compared to AUC is significant. This discrepancy is probably due to the overlap in AUC seen between the two groups which is not distinguished by using dose as a measure of drug exposure.

Table 4.5 Dose Response relationship for cisplatin

<table>
<thead>
<tr>
<th></th>
<th>Cisplatin 70 mg/m²</th>
<th>Cisplatin 100 mg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>No Response</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Fisher’s exact test p=0.25

Toxicity - AUC relationships

Unlike response data, which were limited, toxicity data were available for all cycles studied. As discussed earlier, common toxicities following cisplatin therapy are nephrotoxicity and myelosuppression. The relationship of severity of these side effects to free cisplatin AUC has been correlated in both a qualitative and quantitative fashion. In order to define haematological and renal toxicity the World Health Organisation scoring system has been used (Table 4.6). Any patient who
Figure 4.6  Timing of platlet and WBC nadir in a patient following 3 cycles of CMV for transitional cell carcinoma of the bladder.
scored one or more in any category was defined as having suffered toxicity. The nadir for WBC and platlet count following chemotherapy was found to be 14 days post cisplatin, although this calculation was limited by the timing of blood samples (day 8, 14 and 21). This was observed by plotting blood counts against time as in Figure 4.6.

### Table 4.6 W.H.O. Recommendations for grading of toxicity

<table>
<thead>
<tr>
<th>Grade</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10⁹/l)</td>
<td>&gt; 4.0</td>
<td>3-3.9</td>
<td>2-2.9</td>
<td>1-1.9</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Platlets (10⁹/l)</td>
<td>&gt; 100</td>
<td>75-99</td>
<td>50-74</td>
<td>25-49</td>
<td>&lt; 25</td>
</tr>
<tr>
<td>Creatinine (male)</td>
<td>&lt; 138</td>
<td>138-275</td>
<td>275-550</td>
<td>550-1100</td>
<td>&gt; 1100</td>
</tr>
<tr>
<td>Creatinine (female)</td>
<td>&lt; 113</td>
<td>113-225</td>
<td>225-450</td>
<td>450-900</td>
<td>&gt; 900</td>
</tr>
</tbody>
</table>

(Creatinine normal range for Freeman labs is male < 110 and female < 90)

Of 29 first cycle studies, toxicity was encountered in 18 patients as defined by the above criteria (62%). The difference in free cisplatin AUC between toxic and non-toxic patients responders and non-responders is demonstrated graphically in Figure 4.7. This difference is highly statistically significant (p=0.005). When comparing toxicity following the second cycle of chemotherapy the difference between the two groups is reduced to a level of marginal significance (p=0.08).

In an attempt to correlate AUC and haematological toxicity in a more quantitative fashion, the % fall in WBC and platelets were calculated (i.e. Pretreatment - Nadir divided by Pretreatment x 100. Figure 4.8. shows these data
Figure 4.8 Relationship between free cisplatin AUC and % fall in total WBC in all patients studied.
graphically and it is apparent that there is no obvious relationship between AUC and the suppression of WBC. There was also no demonstrable relationship between free cisplatin and thrombocytopenia.

Toxicity - Dose Relationship

As with response data, toxicity data may also be compared for the high dose and low dose group. The same criteria for toxicity were applied (WHO score > 1) and the results are demonstrated in Table 4.7.

Table 4.7 Toxicity - Dose relationship for cisplatin. (CMV only)

<table>
<thead>
<tr>
<th></th>
<th>Cisplatin 70 mg/m²</th>
<th>Cisplatin 100 mg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxicity</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>No Toxicity</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

Fisher's exact test p=0.05

The difference between the two cisplatin dose groups in terms of toxicity is significant as compared to the difference in terms of response which was not. The level of significance is not as great as that for AUC, as would be expected considering the variation in AUC between dosage groups is not taken into account when comparing dose alone.

It may be argued that in order to establish an AUC for cisplatin sufficient to obtain response a degree of toxicity is inevitable, as rapidly proliferating cells are killed. In order to explore this possibility response and toxicity data were compared
in the CMV patients.

Table 4.8 Response \( v \) WBC Toxicity in CMV patients

<table>
<thead>
<tr>
<th>W.H.O. score</th>
<th>Toxicity = 1</th>
<th>Toxicity = 2-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxicity</td>
<td>Toxic</td>
<td>Non Toxic</td>
</tr>
<tr>
<td>Response</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>No Response</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Fisher's test: \( p=0.27 \) \( p=0.48 \)

The difference between responders and non-responders, in terms of toxicity, is not significant when taking a cut off at a WHO score of 1 (\( p=0.27 \)), or a WHO score of 2 (\( p=0.48 \)).

The development of a limited sampling strategy for the evaluation of cisplatin pharmacokinetics

All free cisplatin AUCs in this study were generated by plotting free cisplatin concentrations from multiple samples against time and calculating the area under the ensuing curve by the trapezoidal method. This involved taking a total of 12 samples from each patient. Clearly this would be difficult to arrange as a routine clinical test in patients receiving cisplatin chemotherapy and therefore the possibility of developing a limited blood sampling model was explored.

After approximately 60 minutes, the free cisplatin concentration reaches a
Figure 4.9 Relationship between free cisplatin AUC and cisplatin plateaux concentrations in patients with transitional cell bladder cancer treated with cisplatin.
plateaux which is maintained until the infusion is stopped (see Figure 4.3). The
plateaux free cisplatin concentration was calculated as the mean of the levels
measured at 120, 180 and 240 minutes and the relationship between the plateaux and
the plateaux studied. Figure 4.9 shows mean the mean plateau free cisplatin
congestion plotted against AUC for all patients in all cycles. Using linear
regression analysis, this yields a correlation coefficient of 0.86. Taking this
approach one step further the relationship between the cisplatin concentration at the
end of the infusion and free cisplatin AUC was also studied Figure 4.10 The single
sample regression analysis gives a correlation coefficient value of 0.82, indicating
only a small fall in the significance of the correlation and a single sample model,
such as this, would be applicable clinically.

In order to measure the predictive performance of this sigle sample limited
sampling model, methods described by Sheiner and Beale (1981) were employed.
This involves calculating the mean squared prediction error (measure of precision)
and the mean prediction error (measure of bias) for the proposed prediction model.
These quantities are easily measured and are a more accurate reflection of the
performance of a prediction of measurement method than computing the correlation
coefficient.

To derive the limited sampling model, linear regression analysis was
performed on the end infusion free cisplatin levels v free cisplatin AUC data on half
of the series of cisplatin cycles. The series was split into two groups chronologically
and a limited sampling model derived on the first 28 cycles tested on the subsequent
28 cycles.
Figure 4.10  Relationship between free cisplatin AUC and the end of infusion free cisplatin concentration in patients with bladder cancer treated with cisplatin.
model: \( \text{free AUC} = 0.196 \times \text{End Infusion free cisplatin} + 0.041 \)

\( r=0.82 \)

(Precision) \( \text{root mean squared error} = 14\% \ (95\% \text{ c.i.} = 8-17) \)

(Bias) \( \text{mean prediction error} = +1.2\% \ (95\% \text{ c.i.} = -4.3-6.7) \)

Thus the limited sampling model is accurate to within 13\% (95\% c.i. 9-16\%) and the estimate is unbiased as demonstrated by a mean prediction error of only 1.2\% (95\% c.i. -4.3-6.7).

In order to further simplify the limited sampling model a relationship was sought between 24 hour protein bound cisplatin and free cisplatin AUC. Fournier et al (1988) had demonstrated a relationship between free cisplatin AUC and total platinum measurement 24 hr after intravenous bolus of cisplatin \( r=0.70, p<0.001 \). However from this study we were unable to demonstrate a relationship for these patients and the correlation coefficient between total cisplatin levels and free cisplatin was low at \( r=0.39 \). The reason for this is unclear but may be in part related to variation in plasma protein levels of patients with advanced malignancy.

An investigation of the accuracy of the Cockcroft formula in predicting glomerular filtrate rates.

All the protocols for chemotherapy regimens used in the patients studied required the calculation of GFR by the Cockcroft formula (Cockcroft and Gault 1976). This
The formula was first described in 1976 and involves the calculation of GFR from the serum creatinine and the patient's age and weight as shown below.

\[
\text{male } \text{GFR} = 1.23 \times \left( 140 - \text{age} \right) \times \text{weight (kg)} / \text{serum creatinine}
\]

\[
\text{female } \text{GFR} = 1.05 \times \left( 140 - \text{age} \right) \times \text{weight (kg)} / \text{serum creatinine}
\]

Blood samples were taken prior to \(^{51}\text{Cr-EDTA}\) and at 120 and 240 minutes. The EDTA GFR results were validated using NIRAS software (Medical Physics) and in all cases except two the volume of distribution lay between 15 and 22 L. These two GFRs were not used in subsequent analysis. This range was taken from (Ladeegaard-Pedersen and Engell 1972) who calculated the normal distribution of volume of distribution for \(^{51}\text{Cr-EDTA}\) in humans. However the accuracy of this formula for calculating GFR in patients has been questioned and McDermott \textit{et al} (1987) demonstrated a correlation coefficient of just 0.40 between creatinine clearance and a clearance derived from the Cockcroft formula. The average difference between the predicted and measured creatinine clearance was 25.3%. There is also evidence that any measure of glomerular filtration based on serum creatinine in patients receiving cisplatin chemotherapy is flawed. Daugaard \textit{et al}., (1988) studied the effects of cisplatin on glomerular function measuring GFR by \(^{51}\text{Cr-EDTA}\) clearance and creatinine clearance. They found that during treatment there was no correlation between \(^{51}\text{Cr-EDTA}\) and creatinine clearance and suggested that this may be due to the significant fall in serum creatinine which was observed during treatment.
Figure 4.11  Relationship between GFR calculated by the Cockcroft formula and that measured by $^{51}$Cr EDTA clearance.
Figure 4.11 shows the GFR calculated by the Cockcroft formula, plotted against the $^{51}$Cr-EDTA GFR. Linear regression analysis yields a correlation coefficient of $r=0.70$. In order to more accurately test the predictive accuracy of the Cockcroft formula the methods described by Sheiner and Beale were applied in the same manner as with the limited sampling model for calculating free cisplatin AUC. To test the Cockcroft formula it was possible to use data from all patients as the equation was pre-determined. $^{51}$Cr-EDTA is the "true" GFR and the Cockcroft formula derived value, the predicted GFR.

(Precision) root mean squared error = 33 % (95% c.i. =25,38)
(Bias) mean prediction error = -23 % (95% c.i. =-17,-29)

The use of the Cockcroft formula would therefore appear to result in an underestimate of the true GFR as calculated by $^{51}$Cr-EDTA clearance, by on average 23 % with an accuracy in the range 25-38%. This degree of inaccuracy is similar to that reported by McDermott et al (1987). It would appear that the role of the Cockcroft formula is of limited use in predicting GFR especially in patients receiving cisplatin chemotherapy when the serum creatinine is likely to fall during treatment.
CHAPTER 5: PHARMACOKINETIC DETERMINANTS OF METHOTREXATE ACTIVITY AND TOXICITY IN TRANSITIONAL CELL CARCINOMA OF THE BLADDER.

AIMS

1. To measure methotrexate levels in all patients with transitional cell carcinoma of the bladder receiving methotrexate.

2. To determine the variation in methotrexate AUC (area under the curve of methotrexate levels vs time) in all patients studied.

3. To relate any variation in methotrexate AUC observed to response and toxicity.

4. To determine whether routine folinic acid rescue is warranted in patients receiving methotrexate at a dose of 30 mg/m².

The pharmacokinetics of methotrexate have been outlined in chapter 1. Following intravenous administration at low dose a biphasic elimination is seen whereas at high doses a third phase may be evident due to biliary excretion. The first phase is due to the distribution of methotrexate into body fluids and the second phase reflects renal clearance. Approximately 50% of the drug is protein bound and at low doses metabolism does not occur to a significant extent.

Dose Response

As discussed in Chapter 1, the anti-metabolite methotrexate was one of the first cytotoxic agents used in the treatment of bladder cancer and as a single agent,
response rates of approximately 30% were achieved (varying from 18-38%). Early trials of methotrexate resulted in a variety of doses, schedules and routes of administration including oral, intramuscular, intra-arterial and intravenous. Methotrexate was first studied in bladder cancer by Burn et al. (1966) who gave methotrexate by intra-arterial infusion at 40-50 mg over 24 hours and observed responses in 8 of 12 patients. Hall et al. (1974) reported a 26% response rate in 42 patients with advanced disease treated with 50-100 mg methotrexate intravenously every two weeks. High dose methotrexate was made possible by the advent of folinic acid rescue (Levitt 1973). Folinic acid acid (leucovorin) is converted to 5-methyltetrahydrofolate, which is the predominant reduced folate in the body and simply replenishes depleted reduced folate pools. As yet, the optimal dosage regimen for methotrexate in the treatment of bladder cancer is not known because there has been no study which has randomised low dose (30-40 mg/m²) versus high dose methotrexate (100-200 mg/m²). The only randomised trial in the literature of high dose versus low dose methotrexate has been carried out in another chemosensitive tumour, squamous cell head and neck cancer and no benefit was seen in the high dose group (Woods et al., 1981). Increased survival rates have been reported in patients with osteosarcoma treated at higher doses (Jaffe et al. 1971) but there has been no randomised prospective study to compare different regimens.

Encouraging results have been reported by treating muscle invasive bladder cancer by extensive local resection and high dose methotrexate (2g over 6 hr infusion). Two year survival rates were reported as 60% (Hall et al. 1984) and 90% (Soquet et al. 1981). Response rates cannot be assessed as the primary tumour was resected, but the survival figures are better than would be anticipated from muscle
invasive disease. The appeal of this form of treatment is that the bladder is conserved.

*In vitro* experiments have established that a threshold concentration is required for methotrexate cytotoxicity and that cell kill increases with subsequent increases in concentration and duration of exposure (Pinedo et al 1977). On this basis it would be expected that a dose-response relationship would exist for methotrexate in chemosensitive tumours. Although there have been no randomised trials of high dose versus low dose methotrexate in bladder cancer, it is possible to compare response rates reported from different regimens.

Turner *et al*., (1983) treated patients with advanced bladder cancer using three separate regimens; 50 mg every two weeks, 100 mg every two weeks and 200 mg with leucovorin rescue every two weeks. They suggested a dose response relationship as the response rates for the three regimens were; 13%, 50% and 54% respectively. Oliver *et al*., (1984) have also reported good response rates with a high dose regimens. Patients with either locoregional bladder cancer or metastatic disease were treated with 200 mg/m² methotrexate and leucovorin rescue. Of 21 patients with metastatic disease 43% showed a response and of 32 patients with locoregional disease 28% showed a response. However, not all studies support a dose response relationship, Natale *et al* (1981) reported 42 patients treated with either high dose methotrexate (250 mg/m²) every 2 weeks, or low dose methotrexate (0.5-1.0 mg/kg weekly) as single agent treatment. One patient in the high dose group (n=9) achieved a partial response lasting 5 months whereas 33% of patients in the low dose
group (n=33) achieved a partial response. However it is difficult to draw conclusions from this study as the timing as well as the dose differs between the two groups.

In a further attempt to demonstrate an advantage for high dose therapy in the treatment of bladder cancer, Brausi et al., (1990) studied the dose-response relationship for methotrexate in combination with cisplatin in murine bladder cancer. The tumour model used was MTB2 which is histologically similar to human transitional cell carcinoma. Measurable tumours were grown in 123 mice and treated with cisplatin alone or cisplatin and methotrexate at increasing doses up to 80 mg. The combination of cisplatin with methotrexate 50 mg (with leucovorin rescue) along with cisplatin alone were the most active regimens. No statistically significant difference was observed between high dose and low dose methotrexate therapy.

Although the advent of pharmacokinetic monitoring along with folinic acid rescue has led to the common usage of high dose methotrexate there is very little evidence to suggest a dose-response relationship. Folinic acid rescue may in fact decrease the antitumour effect of methotrexate at low doses by the early reverse of methotrexate cytotoxicity, as demonstrated in a study of head and neck tumours treated with weekly methotrexate 40 mg/m² for 8 weeks with or without leucovorin. Browman et al., (1990). Browman randomosed 61 patients with locally advanced head and neck cancer to receive either methotrexate (40 mg/m²) with folinic acid rescue or methotrexate with placebo. Of the patients receiving folinic acid 5 (17.2%) were responders, compared with 11 responders (36.7%) in the group receiving
placebo. There was also a significantly higher incidence of toxicity in the group receiving placebo.

Despite an extensive literature on the pharmacology of methotrexate, recommended schedules are based on empiricism and not on a knowledge of the dependence of the cytostatic effect of the drug on concentration and exposure time. It is impossible to relate response data from published series of bladder tumours treated with methotrexate to either AUC or time above a threshold concentration for methotrexate activity.

Data exist from other malignancies to suggest a wide variation in serum methotrexate levels when prescribed by surface area. Evans et al. (1986) monitored methotrexate levels in 108 children receiving high dose treatment for acute lymphocytic leukaemia. Variability between patients in methotrexate clearance resulted in steady state serum concentrations that ranged from 9.3 to 25.4 $\mu$M. This variation was clinically significant as patients with median methotrexate concentrations of 16 $\mu$M had a lower probability of remaining in remission. It is likely that a similar variation would be seen in patients receiving methotrexate for bladder cancer and this may of clinical significance.

*Dose-Toxicity*

High dose methotrexate therapy is not without complications. Common toxicities encountered are myelosuppression and mucositis. In its most extreme form toxicity can be fatal. Stoller et al. (1977) demonstrated the potential benefits of
pharmacokinetic monitoring of methotrexate levels in order to prevent toxicity by the administration of leucovorin rescue. Methotrexate plasma clearance was studied on 395 courses of high dose methotrexate infusions in 78 patients. Forty-eight hour levels of methotrexate greater than $9 \times 10^7$ M were associated with a high frequency of toxicity. Those patients with 48 hour methotrexate levels <$9 \times 10^7$ M who did not experience toxicity had a faster clearance rate than those who did. This important study allowed the identification of patients likely to experience toxicity by the measurement of serum methotrexate levels at 48 hours. Although an increase in creatinine may be an early sign of impending toxicity, in Stoller’s series this would only have identified half of the toxic patients, suggesting that individual differences in tubular function or drug metabolism might account for delayed excretion and toxicity. Having identified patients who are likely to experience toxicity, folinic acid rescue may be given and it has been recommended that this be continued until methotrexate levels fall below $1 \times 10^4$ M (Jolivet et al., 1983).

**PATIENTS and METHODS**

Pharmacokinetic studies were performed on the same group of patients described in Chapter 4, with the exception of patients receiving EPICM. This latter group received a higher dose of methotrexate (40 mg/m$^2$) and as epirubicin levels were not studied this group could not be included in the analysis of the response data. Response data were therefore restricted to those patients receiving CMV chemotherapy who had completed their treatment and for whom pharmacokinetic data were available. A total of 40 studies were performed in 23 patients. Details concerning the different regimens, eligibility criteria and types of tumours treated are
all included in Chapter 4 and are summarised in Table 4.1. Wherever possible pharmacokinetic studies were performed on more than one cycle for each individual patient in order to determine intra-patient variation.

**Table 5.1 Chemotherapy regimens of patients studied.**

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Dose (mg/m²)</th>
<th>No Patients</th>
<th>No cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>cisplatin 100 methotrexate 30 vinblastine 4</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>CMV</td>
<td>cisplatin 70 methotrexate 30 vinblastine 4</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>CMEPI</td>
<td>cisplatin 70 methotrexate 30 epirubicin 80*</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* intravesical epirubicin 80 mg

**Pharmacokinetic methodology**

Four ml of venous blood were taken prior to administration of the methotrexate bolus and at 15, 30, 45, 60, 120, 240, 360, 600, 1440 and 2880 minutes post bolus. All samples were taken from the opposite arm to that which had been used for administering the methotrexate. Samples were placed in cooled heparinised bottles and immediately centrifuged for 5 minutes at 3,000 rpm in a cooled centrifuge (Mistral 3000i) at 4°C. Plasma was then aspirated and stored at -80°C prior to analysis. Glomerular filtration rates were measured by ⁵¹Cr-EDTA clearance as...
Figure 5.1 Relationship between methotrexate AUC calculated by the trapezoidal model and AUC calculated by a 2 compartmental model.
described in Chapter 4.

Methodology for the analysis of methotrexate in plasma

Plasma samples were analyzed at Newcastle General Hospital in the Department of Toxicology. An immunoassay kit (EMIT, Syva U.K.) was used as by Oellerich (1980). The assay was run on a Roche MIRA analyser set to detect methotrexate concentrations in the range 0.1-2.0 μM and samples with levels above 2.0 μM were diluted in saline and reassayed. The lower detection limit was 0.1 μM. For each batch analyzed, a quality control was included. The coefficient of variation for a single sample (concentration 1.21 μM) was 4.8%, over a total of 16 assays and that of a second sample (concentration 0.99 μM) was 4.1 % over 13 assays.

For each pharmacokinetic study the AUC was determined using the linear trapezoidal method. There was a good correlation between AUC calculated by the linear trapezoidal method and AUC calculated by fitting a 2 compartmental model to the data using a non-linear least squares analysis and a weighting of 1/y (Graphpad intermediate) (Figure 5.1).

From the fitted biexponential equation:

\[ C_t = A e^{-\alpha t} + B e^{-\beta t} \]

Where \( C_t \) is the concentration at time \( t \), \( A \) and \( B \) are concentration constants and \( \alpha \) and \( \beta \) are the first-order rate constants for the distribution and elimination phases.

The AUC from zero to infinity was calculated as:

\[ \text{AUC} = \frac{A}{\alpha} + \frac{B}{\beta} \]
Figure 5.2 Median serum methotrexate levels following a bolus dose of 30 mg/m².
Methotrexate clearance was calculated as \( \text{Dose} / \text{AUC} \).

There were 2 cycles where the correlation between AUC from the trapezoidal model and the 2 compartmental model was poor as can be seen from the graph. In one of these cases the data set was incomplete and in the other the methotrexate clearance as calculated by the trapezoidal rule was low.

The time periods over which plasma methotrexate levels were greater than 1.0 \( \mu \text{M} \) and 0.1 \( \mu \text{M} \) thresholds were also calculated for each patient using Graphpad software. These time periods and the methotrexate level at 24 hrs, along with AUC, were subsequently related to toxicity and response data. Statistical analysis was performed using the Minitab program. For the purpose of calculating results the trapezoidal AUCs were used.

**RESULTS**

Forty methotrexate pharmacokinetic studies were performed in 23 patients. Four of these analyses were performed on patients who had received a reduced dose of methotrexate according to protocol, due to either leucopenia or a low GFR, and these modified courses (50\% or 75\% of full dose) were omitted from subsequent analyses. *Figure 5.2* shows a graph of median plasma methotrexate levels (\( \mu \text{M} \)) *versus* time for all patients. After the bolus administration the plasma methotrexate level decreases exponentially. The median figures for the parameters measured are demonstrated below.
Figure 5.3  Distribution of methotrexate AUC values in all patients treated at a dose of 30 mg/m².
Table 5.2 Methotrexate Pharmacokinetics in all cycles studied.

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (mg/ml.min)</td>
<td>0.46</td>
<td>0.21 - 1.02</td>
</tr>
<tr>
<td>Time &gt; 1.0 μM (mins)</td>
<td>330</td>
<td>58 - 651</td>
</tr>
<tr>
<td>Time &gt; 0.1 μM (mins)</td>
<td>1217</td>
<td>378 - 3878</td>
</tr>
<tr>
<td>24 hr Mtx (μM)</td>
<td>0.1</td>
<td>0 - 0.6</td>
</tr>
</tbody>
</table>

The plasma methotrexate level at 48 hours was only measurable in 7 cycles. In 5 of these it was 0.1 μM and in 2 cases 0.2 μM. The 5-fold variation in methotrexate AUC seen in all cycles is demonstrated graphically in Figure 5.3. It is clear from Figure 5.3 that there is a wide variation in methotrexate levels between patients. The contribution of intra-patient variation to the overall distribution is demonstrated in Table 5.3.

Table 5.3 Inter and Intra patient variation in plasma methotrexate AUC

<table>
<thead>
<tr>
<th></th>
<th>Mean Methotrexate AUC coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter- patient cycles all courses (n=36)</td>
<td>41%</td>
</tr>
<tr>
<td>Inter-patient first course cycles (n=23)</td>
<td>45%</td>
</tr>
<tr>
<td>Intra-patient cycles (n=12)</td>
<td>23%</td>
</tr>
<tr>
<td>Inter-patient cycles (n=12)</td>
<td>39%</td>
</tr>
</tbody>
</table>
Figure 5.4 Relationship between methotrexate clearance and GFR.
There is little difference between the coefficient of variation for methotrexate AUC calculated for all courses of treatment when compared to that for the first cycle only. However, in 12 patients, pharmacokinetic data are available for more than one cycle and these would suggest that intra-patient variation in methotrexate AUC may be significant. In this group the intra patient mean coefficient of variation in methotrexate AUC was 23% which accounts for a large component of the interpatient variation seen (39%).

Methotrexate is predominantly cleared by glomerular filtration and the rate of clearance determines the AUC. Clearance is calculated as dose/AUC as discussed earlier. The correlation between methotrexate clearance and GFR is shown in Figure 5.4 (r=0.59). This confirms that GFR (as calculated by $^{51}$Cr EDTA clearance) is the major determinant of methotrexate clearance and hence AUC. In a proportion of patients the GFR decreased with subsequent cycles of chemotherapy and this is likely to account for a large component of the intra patient variation as well as the interpatient variation.

Relationship between methotrexate pharmacokinetics and response

In order to determine whether variation in tumour response was related to methotrexate AUC, univariate analysis was performed, comparing mean methotrexate AUCs in patients who demonstrated a response to treatment to AUCs in non-responders (response criteria as defined in chapter 4). All patients included in the
Figure 5.5 Relationship between first cycle methotrexate AUC and response in patients with transitional cell carcinoma of the bladder treated with CMV. (student t test)
analysis completed all cycles of chemotherapy at full dose. As shown Figure 5.5, there was no relationship between response and methotrexate AUC (student t test, \( p = 0.45 \)).

An alternative pharmacokinetic parameter to AUC for methotrexate is the time of exposure to a level of methotrexate likely to have cytotoxic activity. In this respect, the time period for which methotrexate concentrations were >1.0 \( \mu \text{M} \), >0.1 \( \mu \text{M} \) and the concentration at 24 hours were compared in responding versus non-responding patients.

\[
\begin{array}{|c|c|c|}
\hline
 & \text{Responders} & \text{Non Responders} & \text{p value*} \\
\hline
\text{Mean time > 1\muM} & 343 & 333 & 0.9 \\
\text{Mean time > 0.1 \muM} & 1860 & 1260 & 0.53 \\
\text{Methotrexate 24 hr(\muM)} & 0.15 & 0.12 & 0.23 \\
\hline
\end{array}
\]

*student t test

Using univariate analysis there was no significant difference between responders and non-responders in time above a threshold concentration of either \( 1\mu \text{M} \) or \( 0.1\mu \text{M} \), between responders and non responders. Similarly, no difference was seen between the two groups in terms of 24 hr methotrexate levels.
Figure 5.6 Relationship between first cycle methotrexate AUC and toxicity (haematological and renal) in patients with transitional cell carcinoma of the bladder treated with CMV. (student t test)
Relationship between methotrexate pharmacokinetics and toxicity.

The commonest toxicities for methotrexate are myelosuppression and nephrotoxicity and mucositis. These were graded as in Chapter 4, according to the W.H.O. scoring system and related to methotrexate AUC. Mucositis was not scored prospectively and it is impossible to quantify in a retrospective fashion. There was no significant relationship between first cycle AUC and toxicity as shown in Figure 5.6 (student t test, p=0.3).

Methotrexate activity is related to the period of time that serum levels are elevated above a threshold. Toxicity has therefore also been related to the time period during which methotrexate levels were greater than 1\(\mu\)M and 0.1\(\mu\)M (Tables 5.5 and 5.6).

Table 5.5 WBC Toxicity relative to mean time (minutes) methotrexate > 1\(\mu\)M and >0.1\(\mu\)M (1st cycle data n=22).

<table>
<thead>
<tr>
<th>Score</th>
<th>W.H.O. ≥1 (n=14)</th>
<th>W.H.O. ≥2 (n=12)</th>
<th>W.H.O. ≥3 (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxicity</td>
<td>T</td>
<td>NT</td>
<td>P*</td>
</tr>
<tr>
<td>Time(min) &gt;1(\mu)M</td>
<td>370</td>
<td>216</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>336</td>
<td>230</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>396</td>
<td>252</td>
<td>0.03</td>
</tr>
<tr>
<td>Time(min) &gt;0.1(\mu)M</td>
<td>1491</td>
<td>975</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1421</td>
<td>963</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1813</td>
<td>993</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*student t test
Table 5.6 Nephrotoxicity relative to mean time (minutes) methotrexate > 1μM and
>0.1 μM.

<table>
<thead>
<tr>
<th>Score</th>
<th>W.H.O. ≥1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxicity</td>
<td>T</td>
</tr>
<tr>
<td>Time (min)</td>
<td>397</td>
</tr>
<tr>
<td>&gt;1μM</td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>1599</td>
</tr>
<tr>
<td>&gt;0.1μM</td>
<td></td>
</tr>
</tbody>
</table>

Using these parameters it becomes clear that exposure to methotrexate levels
>1μM for longer periods results in significantly greater toxicity both in terms of
myelosuppression and nephrotoxicity (Table 5.5 and 5.6). This difference in time of
exposure to methotrexate is no longer significant at the 5% level in terms of toxicity
when the threshold is taken at 0.1μM. Myelosuppression grade W.H.O.≥3 occurred
in 7 patients and these patients had significantly longer periods of exposure to
methotrexate at both 1μM (p=0.03) and 0.1μM (p=0.02).
CHAPTER 6: THE PHARMACOKINETICS OF VINBLASTINE AND THEIR RELATIONSHIP TO TUMOUR RESPONSE AND TOXICITY IN PATIENTS WITH TRANSITIONAL CELL CARCINOMA OF THE BLADDER.

AIMS

1. To measure vinblastine levels in all patients receiving vinblastine as part or combination chemotherapy for transitional cell carcinoma of the bladder.

2. To determine the variation in vinblastine AUC (area beneath the curve of vinblastine levels v time), as determined by a limited sampling model, in all patients studied.

3. To relate any potential variation to response and toxicity.

In comparison to cisplatin and methotrexate there is very little literature on vinblastine in the treatment of transitional cell carcinoma of the bladder. There are only two papers published on single agent vinblastine chemotherapy in bladder cancer and these quote response rates of 8% (Richards et al., 1983) and 18% (Blumenreich et al., 1982), respectively.

Vinblastine is cleared by the liver and hepatic dysfunction can cause variations in drug levels. Ratain et al., (1987) demonstrated significant inter-patient differences in vinblastine pharmacokinetics when administered either as a bolus or as a continuous infusion. They suggested that these differences were due to variations in serum albumin, which is not uncommon in patients with malignancy, but were unable
to show a relationship between serum alkaline phosphatase and vinblastine clearance as has been reported for vincristine (Van den Berg et al., 1982). Van den Berg had shown that vinblastine clearance was slower in patients with elevated alkaline phosphatase levels.

There is no evidence for a vinblastine dose response relationship in bladder cancer but Lu et al., (1983) have demonstrated improved clinical response in patients with advanced breast cancer to be associated with slow clearance of vinblastine. They went on to suggest that a prolonged infusion of vinblastine may be more appropriate than a bolus dose, as vinblastine is rapidly cleared from the plasma, metabolised and binds extensively to platelets. There is no reason to suggest that a dose response relationship should not exist for bladder cancer, as in order to reduce effectively the number of tumour cells, sufficient to show a clinical response, the tumour cells must be exposed to an adequate concentration of drug for an optimal duration. It is likely that variation in vinblastine clearance in bladder cancer patients will be of the same degree as in other tumours types, resulting in underdosing of some patients and overdosing of others.

Myelosuppression and neurotoxicity are the commonest types of vinblastine toxicity encountered, although they are not as severe as with other vinca alkaloids e.g. vincristine. Ratain and Vogelzang (1987a), having observed increased cytotoxicity from prolonged exposure of solid tumour cell lines to vinblastine, performed a Phase I study of prolonged infusion vinblastine in patients with a range of solid tumours. They demonstrated a significant relationship between steady state plasma vinblastine levels and myelosuppression and went on to recommend
prospective monitoring of vinblastine levels, with dose adjustment to avoid toxicity.

Ratain and Vogelzang (1987b) developed a limited sampling model in order to make monitoring of vinblastine levels more practical. They performed detailed pharmacokinetic analyses using 16 time points on 30 patients treated with a bolus dose of vinblastine (3 mg/m²). An equation for calculating the total AUC was developed by multiple linear regression analysis, using the first 15 patients as a training set.

The equation is as follows:

\[
AUC \text{ (ng/ml.hr)} = 38 \times C_{10hr} + 73.8 \times C_{36hr} - 12.9
\]

where \( C_{10} \) and \( C_{36} \) represent the serum vinblastine concentration at 10 hours and 36 hours respectively. This model was validated on the other 15 patients \((r=0.94)\) and the mean predictive error was 13%. The equation was further altered to include a vinblastine level at 1.5 hours. This improved the correlation coefficient to \( r=0.95 \). This was the equation used in the present study as a limited sampling model for vinblastine AUC:

\[
AUC \text{ (ng/ml.hr)} = 5.0 \times C_{1.5hr} + 19.0 \times C_{10hr} + 81.9 \times C_{36hr} - 18.0
\]

From the literature available, there would appear to be evidence for inter-patient variation in vinblastine clearance, probably related to hepatic function (Ratain et al., 1987a, Van den Berg et al., 1982). Such variation may result in avoidable toxicity in some patients and the underdosing of others. If this is the case in bladder cancer, there may be a relationship between vinblastine AUC, toxicity, and response.
Figure 6.1  Distribution of vinblastine AUC values in all patients treated at a dose of 4 mg/m².
In this chapter this hypothesis has been explored.

**METHODS**

Thirty seven pharmacokinetic studies were performed on 22 patients receiving vinblastine as part of CMV combination chemotherapy over a 22 month period at The Freeman hospital (*Table 6.1*). All patients received the same dose of vinblastine (4 mg/m²). The distribution of patients according to stage and grade of their tumours is outlined in Chapter 4.

*Table 6.1 Chemotherapy regimens studied*

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Vinblastine</th>
<th>No patients</th>
<th>No cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV (100 mg/m²)</td>
<td>4 mg/m²</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>CMV (70 mg/m²)</td>
<td>4 mg/m²</td>
<td>11</td>
<td>20</td>
</tr>
</tbody>
</table>

*Sampling and pharmacokinetic calculations.*

Four mls of venous blood were taken prior to the vinblastine bolus and at 1.5, 10 and 36 hours. Samples were taken from the opposite arm to that which received the bolus, were placed in cooled heparinised bottles and centrifuged for 5 minutes at 3,000 rpm in a cooled centrifuge (Mistral 3000i). Plasma was aspirated from each sample and stored at -80°C. Vinblastine levels were measured by Dr Wynne Aherne
Figure 6.2 Relationship between vinblastine AUC and serum albumin in patients receiving CMV for bladder cancer.
at the Institute of Cancer Research, Sutton, Surrey, using a radioimmunoassay described by Teale, Clough and Marks (1977). The sensitivity of the assay was 0.5 ng/ml and addition of less than 50μl of normal human plasma did not effect the binding to antibody, although 100μl did. Therefore all samples were assayed at three dilutions (1/2, 1/4 and 1/10), equivalent to the addition of 50, 25 and 10μl of plasma, and the results reported as the mean of the three dilutions. The limit of detection was 1ng/ml. The assay variation demonstrated a coefficient of variation of 10-15% and recovery of drug from plasma was 96% at 20 ng/ml. In each assay the effect of normal plasma was checked and a recovery sample (vinblastine "spiked" into normal plasma) was included. The batch of antiserum used was R57/1 and used at a dilution of 1/200. A non-equilibrium assay was used i.e. antibody and standards were incubated at 4°C for 1 hour and for 0.5 hours following the addition of radiolabel (approximately 1500cpm in the total tubes). Results were evaluated using RIASMART (Canberra Packard) data processing with a 4 parameter logistic plot. Vinblastine AUCs were then calculated using the limited sampling model (Ratain and Vogelzang 1987) described in the introduction to this chapter.

RESULTS

It is clear that there is a wide variation in vinblastine AUC following a 4 mg/m² and this is shown graphically in Figure 6.1. One patient achieved an AUC three times higher than the median figure. This patient had hepatic metastases and deranged liver function tests with an alanine transaminase of 83 ng/ml (normal 0-17 ng/ml) and serum albumin of 38 g/l (normal range 38-48 g/l). Figure 6.2 shows vinblastine
AUC plotted against serum albumin. There was no overall correlation between vinblastine AUC and serum albumin or alanine trasaminase, another indicator of hepatic function.

Table 6.2 Variation in Vinblastine AUC

<table>
<thead>
<tr>
<th>Median AUC (ng/ml.hr)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>462</td>
<td>152-1521</td>
</tr>
</tbody>
</table>

Data were available for more than one cycle in 11 patients (3 cycles n=4, 2 cycles n=7). The mean intra-patient variation was 17% as compared to 44% between individual patients, on the first course of treatment.

Table 6.3 Inter-patient and intra-patient variation in Vinblastine AUC

<table>
<thead>
<tr>
<th></th>
<th>Mean coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter patient variation</td>
<td>44 %</td>
</tr>
<tr>
<td>Intra patient variation</td>
<td>17 %</td>
</tr>
</tbody>
</table>

Vinblastine Pharmacokinetics-Response relationships.

Response data were available for 16 patients who had completed their respective chemotherapy courses and for whom pharmacokinetic data were available. As with cisplatin and methotrexate analysis, patients were placed in two groups, responders
Figure 6.3  Relationship between first cycle vinblastine AUC and response in patients with transitional cell carcinoma of the bladder receiving CMV (student t test)
and non-responders. Figure 6.3 shows the first cycle vinblastine AUCs in responders and non-responders. There is no significant difference between the two groups using a two sided unpaired t test (p=0.65). When performing the same statistical analysis on second cycle AUCs again there is no statistical difference between the two groups (p=0.45).

Unlike cisplatin there would appear to be no dose response relationship for vinblastine in this small series, using univariate analysis.

Vinblastine pharmacokinetics-Toxicity relationships.

Toxicity was defined according to W.H.O. criteria as mentioned in chapter 4. In the case of vinblastine, nephrotoxicity is not relevant as the drug is not nephrotoxic and toxicity is therefore confined to leucopenia and thrombocytopenia. Figure 6.4 shows first cycle vinblastine AUCs in patients that had WHO grade 1 toxicity or greater and in those who had no toxicity. Although the mean vinblastine AUC in the toxic group is higher than the mean of those with no toxicity, the difference is not significant (student’s t test p=0.19). Similarly, when applying the same statistical analysis to the second cycle vinblastine AUC, the difference between the two groups is not significant (p=0.29).
Figure 6.4  Relationship between first cycle vinblastine AUC and haematological toxicity in patients with transitional cell carcinoma of the bladder receiving CMV (student t test)
CHAPTER 7 : DISCUSSION

7.1 GST \( \pi \) Expression as a predictor of response to chemotherapy in bladder cancer.

Tumour GST \( \pi \) expression does not appear to be related to drug resistance in this series of patients receiving systemic chemotherapy. The enzyme was widely distributed in normal and malignant tissue. The only notable difference between tumours was in the distribution of the staining. However, there was no difference in staining pattern between those tumours responding to chemotherapy and those not responding to treatment (Table 2.4). Nuclear staining was seen in 7 out of 12 responders and 6 out of 14 non responders. Our results do not support previous \textit{in vitro} work, predominantly carried out in drug resistant cell lines, suggesting GST \( \pi \) may act as a modulator of response to systemic chemotherapy. Cell lines have certain advantages as a model for tumours, in that there is no contamination from stroma and there is no heterogeneity, as seen in most human tumours. However cell lines are raised in an artificial environment and cells will inevitably be selected that preferentially survive in the culture medium used. In the case of drug resistant cell lines this problem is amplified so the cell line may not reflect mechanisms within human tumours. It may be that elevated levels of GST \( \pi \) are seen in resistant cell lines as a stress response to exposure to cisplatin.

Although immuno-histochemistry cannot measure the functional activity of the enzyme it would appear unlikely that GSTs and in particular GST \( \pi \) are the rate limiting step in the conjugation of cisplatin electrophiles to glutathione, based on the widespread cytoplamic staining seen in all tumours and the absence of a relationship
between nuclear staining and response. The wide distribution of enzyme in stroma and normal urothelium should be of interest to those working with enzyme assays or blotting techniques using whole tissue extracts. In such studies stromal contamination may account for a significant proportion of overall activity.

If GST π is not a regulator of detoxification by conjugation, then perhaps the total cellular glutathione level is. Figure 2.4 shows the pathway for glutathione synthesis. Glutathione is either synthesized de novo from glutamate and cysteine or recycled from cysteine moieties in the cell membrane. There is growing evidence from ovarian cancer cell line work that high levels of resistance to cisplatin in human ovarian cancer are associated with a marked increase in glutathione synthesis. The drug buthionine-(SR)-sulfoximine (BSO) inhibits gamma glutamyl synthetase, a key enzyme in glutathione synthesis (see fig 2.4). If this drug markedly potentiates cytotoxicity of an anti-cancer drug, this is indirect evidence for a role of glutathione in the protection against that particular agent. Depletion of glutathione levels, using BSO, has been shown to increase sensitivity of ovarian cancer cells to cisplatin and melphalan (Mistry et al., 1991). In the same study there was no correlation between overall GST activity and cisplatin cytotoxicity. Glutathione is synthesized intracellularly by the successive actions of γ-glutamylcysteine synthetase and glutathione synthetase. Gamma glutamyl cysteine synthetase is the rate limiting enzyme in glutathione production and γ-glutamyl transpeptidase, is a key catalyst. Godwin et al., (1992) have measured glutathione levels in cisplatin resistant ovarian cancer cell lines and found a 13-50 fold elevation, as compared to non resistant parent cell lines. They also found resistance to be associated with enhanced expression of mRNAs for the enzymes γ-glutamyl transpeptidase and γ-glutamyl cysteine synthetase.
(again, overall GST activity was not effected). It would be interesting to measure glutathione levels in cisplatin resistant bladder cancer cell lines and to compare this with the parent cell line. It may be possible to measure glutathione levels in bladder tumours before and after cisplatin therapy to see if levels are elevated in resistant tumours as in ovary.

The significance of nuclear as opposed to cytoplasmic staining is unclear. Only 1 of 23 superficial tumours studied demonstrated nuclear staining as opposed to 13 of 26 invasive tumours (p=0.003). The solitary superficial tumour with nuclear staining was invading lamina propria (stage T1). Nuclear staining has been reported previously in cervical intraepithelial neoplasia (Shiratori et al., 1987) and it was proposed that this may be a marker of malignant transformation. This phenomenon was further supported by Randall et al., (1990) who, using the same antibody as was employed in this study, reported intense nuclear staining in all grades of cervical intra-epithelial neoplasia and less intense nuclear staining in invasive cervical carcinomas. This is of particular interest with regard to carcinoma in situ. Of the 3 cases with carcinoma in situ, 2 showed intense nuclear staining (Figure 2.3) and this finding would support a potential role for nuclear distribution of GST π as a marker of malignant transformation.

The GSTs are predominantly cytosolic enzymes and one can only speculate as to the role of GST π within the nucleus. Initially nuclear expression of GSTs was thought to be an artefact, but now that it has been consistently observed using a range of antisera in several different tissues it is recognised as a genuine phenomenon. Ketterrer et al., (1987) suggested that nuclear GSTs may play a role in the breakdown of peroxidised DNA and increased levels of the enzyme may reflect DNA damage.
The GSTs have been most widely studied in the rat in which a particular GST YbYb, present in the nucleus, is able to bind steroid hormones (Homma et al., 1985). Such observations raise the possibility of a similar role in bladder cancer but there is no direct evidence.

In summary, GST π does not appear to have any potential role as a marker of drug resistance in this small group of bladder tumours. The finding of nuclear staining in areas of carcinoma in situ and invasive tumours was of interest and may support a role for nuclear GST π as a marker of malignant transformation. Ovarian cancer cell line work would suggest that cellular glutathione levels may be a predictor of resistance to cisplatin based chemotherapy rather than the GSTs and this should be the next area for research into cisplatin based resistance in bladder cancer, both in cell lines and tumour biopsies.

7.2 Quantification of MDR1 expression at the mRNA level by RT PCR.

The main aim of this study was to develop a quantitative method of measuring MDR1 mRNA in bladder tumours, having failed to demonstrate p-glycoprotein at the protein level, using the antibody C219, and this has been achieved. The wide range of MDR1 expression (63 fold) may have implications for the chemotherapeutic treatment of these tumours because drugs implicated in classical drug resistance are used in the treatment of both superficial and invasive tumours. The assay has the practical advantages of requiring only small quantities of tissue (10 μg RNA) and does not require the addition of an external reference standard.
This variation in MDR1 expression is unlikely to be artefactual as the assay has been validated in a number of different ways. The mean coefficient of variation of 46% demonstrates that the assay is repeatable and that the degree of variation observed is not due solely to inter assay changes. Although the PCR amplification efficiency varies, with 18S ribosomal RNA the most efficient, the amplification efficiencies of the respective genes are constant in a range of different tissues and in a drug resistant cell line as shown in Table.3.2.

The pattern of distribution of MDR1 mRNA within different tissues was also in agreement with previous immuno-histochemical and RNA studies. Adrenal tissue was studied as it is recognised to be one of several tissues with high levels of MDR1 expression and would therefore be useful to compare to bladder tumour MDR1 expression. The resulting MDR1/18s ratio for adrenal tissue was $2.00 \times 10^4$ which is 27 fold greater than the mean figure for all bladder tumour studied. The drug resistant small cell lung cancer cell line studied had an MDR/18s ratio of $2.8 \times 10^3$ in comparison.

Another potential cause for artefact might be contamination of biopsy material with normal bladder. This is unlikely for several reasons. Firstly in previous studies MDR1 expression has been undetectable in normal bladder both at the protein and RNA level. In the one normal bladder sample studied by the PCR transcription assay, a level of expression similar to that of one of the invasive bladder tumours was recorded. However this sample was a full thickness biopsy taken from a patient undergoing an enterocystoplasty for idiopathic detrusor instability and may not be representative on the grounds that the bladder may not be normal and the biopsy included fat, muscle and urothelium. Secondly, any potential contamination will be
greatest in the superficial tumour group and it was this group that had the lower level of MDR1 expression. Clearly, a greater number of normal bladder biopsies need to be studied by this assay technique in order to determine MDR1 expression in normal detrusor muscle and normal urothelium.

The increased levels of MDR1 expression in high grade tumours is likely to be due to altered levels of gene transcription. In a recent study within the department, MDR1 mRNA expression was measured in a drug resistant bladder cancer cell line (KK47/ADM) and its parent cell line (KK47) by both Northern blotting and the PCR transcription assay (S.C.Clifford unpublished data). Both techniques showed a 55 fold increase in MDR1 mRNA expression in the resistant cell line, (which further validates the assay). Southern blot analysis of MDR1 gene copy numbers revealed a 5 fold increase in MDR1 DNA in the resistant cell line as compared to the parent line. It is therefore apparent that the 55 fold increase in MDR1 mRNA observed is predominantly due to increased transcription. Although this finding is from the artificial environment of a cell line it may reflect similar mechanisms for increased expression in bladder tumours.

Loss of transcriptional control is associated with de-differentiation in poorly differentiated tumours. It is possible that such alterations in transcriptional control could be mediated by the activation of oncogenes or inactivaton of tumour suppressor genes, which are both more common in high grade tumours.

These potential mechanisms for controlling MDR1 transcription are not proven but recently the tumour suppressor gene p53 has been implicated. This is a tumour
suppressor gene whose protein product can prevent tumour cell replication by recognising abnormal DNA and either prevent cell replication, allowing time for DNA repair or initiate apoptosis (Lane 1992). Mutations of this gene result in a non functional mutated protein product. Mutations are common in solid tumours (Levine et al. 1991) and may be present in as many as a third of high grade bladder tumours (J.Lunec unpublished data). Chin et al (1992) reported that mutant p53 specifically stimulated the MDR1 promoter and wild type p53 exerted specific repression of activity. This may be a potential explanation for increased MDR1 transcription in high grade tumours.

In an attempt to investigate this hypothesis, p53 expression was studied at the protein level but no relationship was demonstrated between MDR1/18S levels and p53 status (Figure 3.7). It is now apparent that mutation of the p53 gene is only one of several mechanisms for prolonging the half life of the protein and hence its detectability by immuno-histochemistry. The p53 protein can be stabilised by the action of the viral gene products of SV40 (large T antigen), the adenovirus E1B protein and the papillomavirus E6 protein. In addition there is evidence to suggest that p53 stability is increased by the cellular protein MDM2 (murine double minute 2) (Finlay 1993). Therefore it is no longer true to say that positive staining represents gene mutation. In order to investigate a potential relationship between MDR1 expression and p53 mutation in bladder cancer, it will be necessary to sequence this same group of tumours for p53 mutations.

The ultimate test of the relevance of this assay to the clinical situation is
whether measuring MDR1 mRNA expression is a true reflection of what is happening at the protein level and whether the levels detected are of clinical significance. It is the p-glycoprotein that is responsible for the MDR phenotype, actively pumping drugs out of cells. Immuno-histochemistry or Western blotting would be the obvious way to measure the p-glycoprotein product but unfortunately currently available antibodies will only work on frozen tissue and this is not available as all biopsy material available was used in RNA extraction. There is indirect evidence from other studies that MDR1 RNA levels reflect p-glycoprotein levels (Fojo et al., 1987) which makes significant post transcriptional regulation of expression unlikely.

There is no definitive study to suggest a level of MDR1 mRNA expression which is necessary for the MDR phenotype, however it is possible to compare the levels measured in bladder cancer with those from other studies. Noonan et al., (1990) measured MDR1 mRNA levels in KB carcinoma cell lines with different degrees of resistance and related MDR1 mRNA expression to resistance to vinblastine and adriamycin. The range of MDR1 levels was 14 - 68 times lower than that seen in adrenal tissue and this distribution is similar to that reported for bladder tumours in this chapter. Noonan et al., stated that a 4.7 fold increase in MDR1 mRNA levels within the cell line studied resulted in a 5.25 and 2.9 fold increase respectively in drug resistance. This provides indirect evidence that the level of MDR1 expression seen in bladder tumours is clinically significant.

The relationship between MDR1 expression and stage and grade of bladder tumour is suggestive of a role as a prognostic indicator independent of response to
chemotherapy. This is indeed the case for breast cancer (Verelle et al., 1991) and neuroblastoma (Chan et al., 1991) where overexpression of the p-glycoprotein was significantly related to poor prognosis and survival in untreated tumours. As mentioned earlier, survival analysis in the study group of bladder tumours is complicated by the different treatments and follow up periods and these variables may account for the lack of significant association demonstrated between MDR1 mRNA expression and survival (p=0.36). Similarly no relationship was demonstrated between MDR1 mRNA expression and tumour progression.

**β2 microglobulin expression**

β2 Microglobulin would appear to be a poor reference standard, as there was a 140 fold variation in levels of expression, relative to 18s in all tumours. There was no correlation between β2 microglobulin expression and MDR1 expression (correlation coefficient r=0.13) but there was a stage and grade relationship. β2 microglobulin levels were significantly higher in poorly differentiated tumours (p=0.042). This differs from a previous immuno-histochemical study reporting down regulation of β2-microglobulin in poorly differentiated transitional cell carcinomas of the bladder (Walton et al., 1986). This report of deletion is in keeping with the deletion of other class I MHC antigens in malignancy such as blood group antigens. Coon et al., (1980) reported that lack of detection of the ABH blood group antigens was common in bladder tumours and predicted a poor prognosis with a higher incidence of local recurrence and muscle invasion.

The findings of this study where β2 microglobulin was measured at the
message level therefore contradict previous work at the protein level. A possible explanation for this could be post transcriptional modification and it would be interesting to study expression at the protein level relative to the message level. The wide variation in $\beta_2$-microglobulin levels observed raises questions as to the validity of previous studies in which $\beta_2$-microglobulin was used as an internal reference standard (Noonan et al., 1988). It is unlikely that this degree of variation is specific to bladder tumours and potential variations need to be excluded in the tissues previously studied.

The use of $\beta_2$ microglobulin as an internal reference standard must be questioned and certainly for bladder tumours would seem inappropriate. The development of the PCR transcription assay described in this chapter has made possible the quantitation of MDR1 mRNA expression in bladder tumours. A wide range of MDR1 expression has been demonstrated with a significantly higher level of expression in poorly differentiated tumours. These tumours may therefore be less likely to respond to chemotherapy with drugs associated with multidrug resistance. This assay can now be used to relate MDR1 expression to response of tumours to chemotherapy and investigate a predictive role.

7.3 The pharmacokinetics of cisplatin in relation to response and toxicity.

The proportion of responders to CMV chemotherapy (47% partial and 18% complete, for a total response of 65%) is similar to other reported series (see introduction chapter). It would appear that a free cisplatin AUC response relationship
exists for cisplatin in the treatment of bladder cancer, when first cycle data are analysed. Such a relationship has already been established for other chemosensitive tumours in terms of dose but not AUC, for example ovarian carcinomas (Levin 1987). Whether this response rate translates into survival advantage or not will become apparent as follow up data become available. The difference in free cisplatin AUC between responders and non-responders is reduced when second cycle data are analysed (p=0.13). The reason for this is unclear but it may be a reflection of the smaller sample size and the intra patient variation in AUC.

There is very little literature with which to compare the data, in terms of dose response or AUC response. No trial of 70 mg/m² v 100 mg/m² cisplatin therapy in bladder cancer has been undertaken although response rates are similar in separate series using either low dose or high dose therapy in combination with other drugs. Indirect evidence for a relationship between dose and response in bladder cancer comes from a study of 54 patients receiving MVAC chemotherapy (Igawa et al., 1992). They reported that reduced dose of drug was significantly related to failure of response but it is unclear from the results which drugs were reduced and to what level.

The limitations of relating dose of drug to response and toxicity are exposed by the two fold variation seen in both high and low dose groups. When response was studied in relation to dose, no significant relationship was demonstrated. However the relationship between free cisplatin AUC and response was significant. Similarly the level of significance for difference in dose between patients experiencing toxicity was less than that demonstrated when comparing free cisplatin AUC.
Another factor which is associated with poor outcome is the presence of distant metastases (Sternberg et al., 1988). Of the 17 patients on whom response data were available a total of 6 had evidence of distant metastatic disease and of these 3 (50%) showed partial response. This compares with a response rate of 65% for the whole group. The first cycle free cisplatin AUCs for those patients with metastatic disease showing evidence of response were 0.21, 0.17, and 0.20 mg/ml.min respectively as compared to 0.20, 0.15 and 0.15 mg/ml.min in those with no evidence of response. The numbers are too small to draw any conclusions but perhaps it is not surprising that response rates in patients with metastatic disease are less when they receive low dose cisplatin (70 mg/m²), often have a poor performance status and a greater tumour bulk.

The AUC toxicity relationship follows a similar pattern to that demonstrated for response. The difference between first cycle free cisplatin AUC in patients with and without toxicity is significant whereas the difference between the two groups for second cycle AUC is only weakly significant (p=0.08). In ovarian cancer Rankin et al., (1992) have shown that in order to achieve an AUC of free carboplatin sufficient to obtain response, a degree of toxicity was inevitable. In other words it is not possible to have a significant cytotoxic effect from carboplatin without also killing rapidly proliferating haemopoietic cells (at least grade 1 leucopenia). In patients treated with CMV, toxicity was seen in 10 out of 11 responders but was also seen in 4 out of 6 non-responders resulting in an insignificant difference (p=0.27). The difference between the two groups remained insignificant when increasing the cut off point for determining toxicity (Table 4.8). Quantitative analysis comparing the
mean percentage fall in WBC of responders and non-responders also showed no significant difference (p=0.75). Four patients receiving CMV chemotherapy (cisplatin at a dose of 100 mg/m²) were withdrawn from the neoadjuvant trial after only one cycle, due to nephrotoxicity as defined by protocol (GFR less than 50 ml/min as calculated by the Cockcroft formula). This may have resulted in an element of bias as, bearing in mind the fact that the Cockcroft formula persistently underestimates the true GFR, these patients may not have had as significant a fall in renal function as was thought. This may have contributed to a lack of association between toxicity and response as these patients could obviously not be included in the response data. In addition a persistent underestimation of GFR will result in some patients being denied chemotherapy when in fact renal function is adequate to tolerate drugs cleared by the kidney. Creatinine clearance has been shown to be a poor measure of glomerular function in patients receiving cisplatin (Daugaard et al., 1988). They reported a close correlation between \(^{51}Cr\)-EDTA clearance and creatinine clearance before and 3 months following cisplatin chemotherapy but during treatment there was no such correlation. Serum creatinine levels decreased significantly during treatment which they attributed to muscle wasting. The Cockcroft estimation of GFR would therefore seem a poor method of assessing GFR in patients receiving cisplatin chemotherapy and \(^{51}Cr\)-EDTA should be regarded as the method of choice.

The range of distribution for free cisplatin AUC was considerable. In the 70 mg/m² group the median was 0.21 mg.ml/min with a 2.4 fold variation and in the 100 mg/m² the median was 0.34 mg.ml/min with a 2 fold variation (Figure 4.4). In the knowledge that patients with a low first cycle free cisplatin AUC are less likely to respond to treatment this variation is clinically significant. The reason for this
variation did not appear to be related to GFR as there was no correlation between free cisplatin AUC and GFR (correlation co-efficient r=0.44).

A potential cause for this variation may be the method of calculation of dose according to surface area. There are potential limitations of this method which have been touched upon in chapter 1. Growchow et al., (1990) have examined clinical and pharmacokinetic data from 306 patients receiving 9 anti-neoplastic drugs and attempted to relate variation in pharmacokinetic parameters (plasma clearance rate, volume of distribution and AUC) to variability in body weight, height and predicted surface area. Of 96 correlation coefficients calculated, in only 5 did any of the measures of body size explain greater than 50% of the pharmacokinetics. Surface area would therefore not appear to be a good predictor of pharmacokinetic variation between different sized patients. The clearance of a drug is determined by the volume of distribution (α phase) and its elimination (β phase). Surface area measurements can only hope to estimate volume of distribution changes within a population but do not take account of variation in rates of drug elimination. In fact surface area can also be a poor measure of volume of distribution and part of the explanation for this may be variation between patients in body composition i.e. fatness, which may significantly alter the volume of distribution of a drug. Another potential variation in volume of distribution may be in effusions e.g. ascites which may be difficult to quantify.

In order to ensure that a patient receives an optimal dose of cytotoxic agent the AUC for that drug must be known. If this is measured for the first cycle of treatment then subsequent cycles can be adapted to ensure the AUC desired, so called adaptive control. In the case of carboplatin it is possible to calculate the AUC of
carboplatin received in a retrospective fashion, provided that the GFR and dose of carboplatin are known (Calvert et al., 1989). The same method is not applicable to cisplatin as it is not exclusively cleared by filtration as is the case for carboplatin. Therefore in order to calculate the AUC for free cisplatin, ultrafiltrate measurements need to undertaken. A method for making this a more practical proposition is the development of a limited sampling model for cisplatin. When the free cisplatin AUC was known for the first cycle, subsequent doses could be modified accordingly. A large component of the AUC is generated by the plateau phase of the infusion (Figure 4.3) and a mean cisplatin level for this plateau correlates with final AUC (r=0.86) (Figure 4.8). In order to produce a model which was based on one single sample, the end infusion free cisplatin level was correlated with AUC (r=0.82) (Figure 4.9). The use of a single sample has obvious practical benefits over taking a mean of the plateau, although inevitably the correlation is not as close. The data from all cisplatin cycles studied was split into two groups on a chronological basis. The equation for the line derived by linear regression of free cisplatin AUC v End infusion free cisplatin was derived from the first group and tested for predictive accuracy on the second group. The precision of the model is represented by the root mean squared error at 13% and the bias or predictive error is +1.2%. By using this model the calculated free cisplatin AUC will therefore be unbiased and the accuracy will on average be 13%. The use of this single sample method would allow the detection of patients receiving very low or high AUCs and permit the clinician to modify the subsequent dose, bearing in mind the fact that the variation in free cisplatin AUC in both the low and high dose cisplatin groups varied by over 200%.

The situation is complicated by the fact that a significant part of the variation
in free cisplatin observed was intra-patient. Thus in order to allow for this cisplatin
would have to be given over a much longer infusion e.g. 24 hours and dose modified
according to an AUC calculated from an early sample such as 4 hours.

Unfortunately the free cisplatin AUC required in order to achieve response in
bladder cancer is not known and so at this stage it would not be possible to
recommend a figure for optimum AUC. However a limited sampling model such as
this might enable a prospective trial of free cisplatin AUCs to try and achieve this
goal. From the data in this study an AUC in the region of 0.3 mg.ml/min would
seem appropriate. It may be possible in the future define a therapeutic window for
cisplatin in the treatment of bladder cancer within which chances of response are
maximised at acceptable levels of toxicity.

In order to further simplify the limited sampling model by the omission of the
ultrafiltrate step, the 24 hour protein bound cisplatin level was correlated with free
cisplatin AUC. This would have the practical benefits of not requiring an ultrafiltrate
and also would allow for a greater potential margin for error in the timing of the
sample as the protein bound cisplatin remains relatively constant over 48 hours. The
correlation is shown in Figure 4.10. Clearly the 24 hour protein bound cisplatin level
is not suitable for a limited sampling model with a coefficient of $r=0.39$. This may
be due in part to variations in plasma protein levels which is not an uncommon
finding in patients with malignancy.

In conclusion, this study has demonstrated considerable variation in free
cisplatin AUC. This variation results in a significant difference (using univariate
analysis) between responders and non-responders in terms of first cycle free cisplatin
AUC. The logical progression from this finding is the establishment of a prospective
study to determine optimal free cisplatin AUC in the treatment of bladder cancer and a limited sampling model would be of great practical benefit.

7.4 The pharmacokinetics of methotrexate in relation to toxicity and response.

This study has demonstrated a wide variation in methotrexate AUC when prescribed according to surface area with a range of 0.21-1.02 mg/ml.min, which represents a 5-fold variation. As mentioned earlier there are no studies in the literature of variability of methotrexate AUC in bladder cancer, but studies from other malignancies have suggested similar variability which may account for variation in response (Evans et al., 1986). For example Pearson et al., (1991) demonstrated a 3-fold variation in methotrexate AUC when given by the intra muscular route at a dose of 20 mg/m² in the treatment of acute lymphoblastic leukaemia.

The overall inter-patient variation in methotrexate AUC seen (41%) was shown by studying subsequent cycles in the same patient, to be due to a combination of inter and intra patient variation. The relationship between methotrexate clearance and GFR is well established and of particular significance in the treatment of bladder cancer where renal function may be impaired by ureteric obstruction.

The highest methotrexate AUC (1.02 mg/ml.min) occurred in a patient who had previously undergone cystectomy and urinary diversion in the form of an ileal conduit. High serum methotrexate levels in patients with ileal conduits have
previously been reported (Fossa 1990) and are said to result from reabsorption of methotrexate through the ileal conduit mucosa. This is a particular risk in patients with long conduits and those which have been created within a 2 year period prior to chemotherapy. The patient described above also had impaired renal function, with a GFR of 46 ml/min, which fell to 38 ml/min post treatment. It is therefore difficult to separate the two independent causes of an elevated serum methotrexate level but both factors may have contributed.

The limitations of prescribing by surface area alone have been discussed in chapter 4 and clearly the same arguments apply here. The calculation of dose by surface area assumes a volume of distribution that is proportional to the size of the patient, which is not necessarily the case, and makes no allowance for pharmacokinetic variables in drug clearance.

*Methotrexate pharmacokinetic-response relationships*

Despite the wide variation in methotrexate AUC demonstrated, there was no apparent difference between responders and non responders in terms of AUC, time >1μM, time >0.1μM or 24 hour methotrexate concentration. The most likely reason for this is the low serum methotrexate level achieved when prescribed at 30 mg/m² and the reversal of any potential action at 24 hours by the routine administration of folinic acid rescue. The level of serum methotrexate required for cytotoxicity and the time for which it must be maintained in bladder cancer patients are not known. Indirect evidence can be taken from Chabner et al., (1972) who studied methotrexate concentrations necessary for the *in vivo* inhibition of DNA synthesis in normal mouse
bone marrow cells, intestinal cells and ascitic tumour cells. These workers demonstrated that, over a wide range of methotrexate doses, recovery of benign or malignant cells, in terms of the ability to incorporate tritiated uridine, was only seen when the methotrexate concentration fell below $10^4$ M. Above this concentration there was an irreversible loss of DNA synthesis.

The peak methotrexate levels recorded in this study ranged from 6.8-2.3 x $10^4\mu$M and these levels are at 15 minutes post bolus, rapidly decaying to lower levels.

There is growing evidence that methotrexate may be a pro-drug, as discussed in Chapter 1, with many of its pharmacological actions resulting from its polyglutamate metabolites. Methotrexate polyglutamate synthesis increases with drug concentration and time of exposure. In human breast cancer cells polyglutamation only occurs after incubation with methotrexate for 6 hours at $2\mu$M (Jolivet et al., 1982). This profile is achieved with high dose methotrexate but not with low doses such as $30\text{mg/m}^2$ used in this study. This is further evidence to support the suggestion that the lack of a relationship between plasma concentrations and response in this study may be due to inadequate methotrexate levels.

Any potentially cytotoxic levels of enzyme inhibition achieved are in any case circumvented by the routine administration of folinic acid (15mg q.d.s.).

If the hypothesis is correct that the patients studied did not receive a cytotoxic dose of methotrexate is correct then it is hard to explain why the only study of high dose versus low dose methotrexate failed to demonstrate a significant difference.
between the two groups in terms of response (Natale et al., 1981). In this non-randomised study 9 patients with metastatic bladder cancer received high dose methotrexate (250 mg/m²) with folinic acid rescue and 36 received low dose methotrexate (0.5-1.0 mg/kg). The response rate in the low dose group was 30% as compared to 11% in the high dose group. It would be interesting to repeat the study in a randomised prospective trial with pharmacokinetic monitoring.

Underdosing with methotrexate may not only run the risk of a reduced response rates but may also induce drug resistance. Schimke (1984) postulated that if sufficient concentrations of methotrexate are not used, or if drug levels fluctuate above and below an effective cytotoxic concentration, over-replication of DNA, gene amplification and resistance can be generated. Pre and post-chemotherapy tumour biopsies are available for a number of these patients and it would be interesting to see if there is evidence of thymidylate synthase or dihydrofolate reductase gene amplification.

*Methotrexate pharmacokinetics - toxicity relationships and the role of folinic acid rescue.*

The advent of high dose methotrexate therapy stimulated the introduction of plasma methotrexate assays for use in identifying patients likely to suffer from toxicity and who would therefore benefit from folinic acid rescue (Stoller et al., 1977). Methotrexate levels associated with an increased risk of toxicity were > 10⁻³M at 24 hours, > 10⁻⁴M at 48 hours and > 10⁻⁷M at 72 hours. On this basis folinic acid rescue was recommended until the serum methotrexate level fell below 5 x 10⁻⁴M.
In the group of patients reported in this chapter studied the median methotrexate level at 24 hours was $0.1 \times 10^3 \text{M}$ (range $0-0.6 \times 10^3 \text{M}$). This would suggest that not one single patient actually required folinic acid rescue at 24 hours when methotrexate is administered at 30 mg/m$^3$. Similarly, at 48 hours methotrexate was detectable in only 6 of 41 cycles; in 4 cases the level was $1 \times 10^7 \text{M}$ and in the other two cases $2 \times 10^7 \text{M}$.

Despite the low dose of methotrexate and hence low plasma concentrations, it was possible to demonstrate a difference in terms of time of exposure to methotrexate levels greater than $1 \mu\text{M}$ and $0.1 \mu\text{M}$ between patients who did or did not experience myelosuppression or nephrotoxicity. However toxicity was not a clinical problem in any of the patients studied in that no patients suffered significant sepsis or mouth ulcers.

The routine use of folinic acid rescue in patients receiving low dose methotrexate should be questioned on two accounts. Firstly, there is the real possibility of reversing any potential antitumour action of the drug and secondly, the methotrexate concentrations achieved should not cause toxicity requiring rescue. There would also be a financial saving from halting the routine prescription of folinic acid. Folinic acid was originally introduced to improve the therapeutic index of methotrexate at high dosage and was not intended for routine use at low dose. The practice of prescribing folinic acid rescue with low dose methotrexate evolved as clinicians attempted to avoid the need for methotrexate assays in outpatient treatment.

This study strongly suggests that it would be safe to prescribe low dose
methotrexate without folinic acid rescue despite the 5-fold variation in AUC observed. If an individual patient has a particularly low GFR then it may be prudent to either reduce the methotrexate dose or measure 24 hour methotrexate levels to determine whether folinic acid rescue is appropriate. Patients with ileal conduits are also at risk of methotrexate toxicity.

This study has demonstrated that there is a wide variation (5 fold) in methotrexate AUC when prescribed according to surface area alone. This variation did not result in any response relationship that could be detected by univariate analysis. However the methotrexate exposure achieved may have been below the cytotoxic threshold for malignant cells. A relationship between methotrexate exposure and toxicity was found but toxicity was not a clinical problem. The levels of methotrexate measured in patients at 30 mg/m² would not appear to warrant the routine use of folinic acid rescue.

3.5 The pharmacokinetics of vinblastine in relation to response and toxicity.

The pharmacokinetic studies described in this chapter have shown a wide variation in vinblastine AUC, following a fixed dose of 4 mg/m². The variation was ten fold and even when one outlier is excluded, the variation is five fold.

Inter-patient and intra-patient variability in vinblastine pharmacokinetics have previously been studied by Ratain et al., (1987a) in a series of 24 patients. They demonstrated a correlation between vinblastine clearance and serum albumin but not with other markers of hepatic function e.g. bilirubin or alkaline phosphatase. They
also studied the renal clearance of vinblastine which accounted for approximately 8.5% of total clearance and was correlated with GFR (r=0.8). In this study, the wide variation in vinblastine AUC could not be explained by variation in hepatic function. Figure 6.2 shows a poor correlation between serum albumin and vinblastine AUC (r=0.35). This may be partly explained by the fact that only four patients had serum albumin levels outside of the normal range. Of these four patients, two had the highest recorded AUCs. In the 24 patients studied by Ratain (1987a) a larger proportion of patients had deranged liver function tests.

It is unlikely that variation in renal function is a significant factor in determining AUC as such a small proportion of vinblastine clearance (8.5%) takes place by this route. It is more likely that the cause for this variation is the method of dose calculation by surface area, the limitations of which have been discussed in both chapters 4 and 5.

Despite these variations in vinblastine AUC there was no significant difference in AUC between patients where tumours had shown a response and those where no response was observed. Vinblastine is one of the least active drugs in the treatment of bladder cancer and the highest response rate reported from single agent therapy is 18 % (Blumenreich 1982), whereas cisplatin can produce response rates of 60% as a single agent (Raghavan 1985). It may be that a higher dose of vinblastine is required to see significant responses and that AUCs achieved in this study are not in a therapeutic range.

In terms of toxicity there was a small difference in mean vinblastine AUC
between those experiencing WHO grade 1 toxicity (556 ng/ml.hr) and those who did not (449 ng/ml.hr), but this was not significant \((p=0.19)\). If the series had been larger this difference may have reached a significant level, as there is evidence for a relationship between pharmacokinetics and toxicity from previous studies (Ratain and Vogelzang 1986). The fact that no toxicity-response relationship was seen over the range of vinblastine AUCs reported here further supports the suggestion that the vinblastine AUCs achieved in this study are not in a therapeutic range. If drug levels are not sufficient to result in WHO grade 1 toxicity, it is difficult to believe that they are sufficient to produce antitumour activity.

From the data obtained in this study it would seem unlikely that vinblastine AUCs achieved at the dose of 4 mg/m² are sufficient to produce tumour response or toxicity. In order to determine the optimal AUC for vinblastine a single agent study needs to be carried out comparing high and low AUC therapy.
CHAPTER 8: CONCLUSIONS AND FUTURE AREAS OF RESEARCH

In the preceding chapters the potential relationships between tumour response, innate drug resistance mechanisms and the pharmacokinetics of commonly used cytotoxic agents have been investigated and discussed. No significant relationship was demonstrated between response and MDR1 or GST π expression.

In terms of pharmacokinetic determinants, the only significant relationship demonstrated was that between first cycle free cisplatin AUC (area under the curve of plasma levels v time) and tumour response. This was despite the wide variation in AUCs achieved in a clinical setting. The reasons behind these variations have been discussed in the respective chapters but involve the same broad principles. The AUC achieved will depend on the volume of distribution and rate of clearance of drug in each individual patient. Surface area is a poor predictor of volume of distribution and prescribing by surface area alone will not take into account variation in clearance rates.

It is difficult to draw conclusions using univariate analysis in terms of response relationships from these variations in AUCs. In order to clearly define factors affecting the outcome of patients with urothelial cancer following treatment with CMV a multivariate analysis is required. This has been performed using dose rather than AUC for MVAC with both univariate and multivariate analysis (Igawa et al., 1992). Using univariate analysis both dose of drug and metastatic status were significant predictors of response and using multivariate analysis (logistic regression) the p values for metastatic status and dose of drug were 0.068 and 0.082 respectively. It should be noted that low drug dosage was defined as those patients who had not
received full doses of cytotoxic drugs and does not take into account any potential variation in AUC at full dose.

The number of patients accrued in this study precludes the use of multivariate analysis such as Cox’s proportional hazards model. However it is possible to perform a logistic regression. This involves dividing patients into two groups for each independent variable e.g. for free cisplatin AUC, the patient will have either received more or less than the mean AUC taken from all patients studied. Logistic regression analysis then determines the probability of that variable determining response as defined by the Odds Ratio along with a "p" value to determine statistical significance. An Odds Ratio of 1 implies no increase in risk of treatment failure whereas an Odds Ratio of 2 would indicate a twofold increased risk of treatment failure with respect to that particular variable.

The table below demonstrates the results of a logistic regression (Minitab) performed on patients from this study. The confidence limits generated make the "p" values statistically invalid and in the case of the cisplatin patient data, the confidence interval was so wide that the software was unable to perform the calculation. This reflects the difficulty of attempting multivariate analysis with such a small number of patients.
Logistic regression analysis for factors determining response in patients with transitional cell carcinoma of the bladder receiving CMV.

<table>
<thead>
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<th>Variable</th>
<th>Odds Ratio</th>
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<th>p value</th>
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<td>0.08, 51</td>
<td>0.65</td>
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<td>% fall WBC</td>
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<td>0.96, 1.01</td>
<td>0.75</td>
</tr>
</tbody>
</table>

N/A : not applicable

For many of the cytotoxic agents used in the treatment of bladder cancer the optimal AUC is not known. In addition many of the commonly used regimens have evolved without the benefits of randomised trials and certainly without the knowledge of variation in AUCs of drugs in patients treated. There is a strong case for performing single agent trials guided by AUC for commonly used agents in order to establish the optimal AUC. This is at present being carried out for ovarian cancer, with a study of high versus low cisplatin AUC. It would be interesting to perform the same study for bladder cancer and then to compare optimal single agent cisplatin therapy with combination chemotherapy. The only study to attempt this in the literature was of single agent cisplatin (prescribed according to surface area) versus CMV (Loehr et al., 1990). They reported a response rate of 9% for single agent cisplatin as against 33% for combination chemotherapy. This response rate for
single agent cisplatin is the lowest found in the literature and may not represent the true picture.

From the univariate analyses (Student t test) presented in earlier chapters the only significant relationship at the 5% limit demonstrated was that between cisplatin AUC and response. No such relationship was found for methotrexate or vinblastine. This reinforces the view expressed in the introduction to this thesis that cisplatin is the most active cytotoxic agent in the treatment of transitional cell carcinoma of the bladder. Although methotrexate has been shown to be active in bladder cancer the levels achieved in this group of study patients may be insufficient to be therapeutic. Vinblastine is the least active of cytotoxic agents used in the treatment of bladder cancer and it is doubtful whether it contributes significantly to response rates following CMV combination chemotherapy.

The variations in the AUCs of cytotoxic agents observed has added significance in terms of drug resistance. If insufficient drug levels are achieved to kill all or even a majority of tumour cells, those cells remaining are more likely to acquire drug resistance mechanisms and so compromise subsequent cytotoxic chemotherapy.

If the hypothesis that cisplatin is the most significant cytotoxic drug in the treatment of bladder cancer is correct, then cisplatin drug resistance mechanisms assume particular importance. The glutathione S-transferases were discussed in chapter 2. It would appear that expression of the enzyme GST π does not predictor
cisplatin resistance and based on evidence from ovarian tumours, glutathione levels may be of greater significance and this is an area that warrants further research.

Classical multidrug resistance may be a significant mechanism of resistance in the treatment of transitional cell carcinoma of the bladder, bearing in mind the 63 fold variation within bladder tumours demonstrated. Its clinical significance is likely to be more applicable to anthracycline based chemotherapy both systemically and intra-vesically. If this is found to be the case, there remains the possibility of reversal of resistance using drugs such as calcium antagonists. Although the variation of MDR1 expression in tumours has been studied in this thesis the levels of expression in normal bladder need to be determined both in urothelium and muscle in order to give a complete picture and to answer questions of potential tumour biopsy contamination with normal tissue.

The question as to why such variation exists remains unanswered, other than to say that it most likely occurs at the transcription level. Potential mechanisms for altering transcription of the MDR1 gene need to be explored. The most obvious candidate is the tumour suppressor gene p53 as mutation of this gene has already been shown to result in increased levels of transcription in cell line work. Mutations can only be detected by direct sequencing of tumour DNA. Other tumour suppressor genes and oncogenes could also be investigated in this context.
Conclusion

In conclusion, the initial hypothesis that failure of response to combination chemotherapy in the treatment of transitional cell carcinoma may be related to inadequate cytotoxic drug levels or innate drug resistance mechanisms has not been proven. Although first cycle free cisplatin AUC levels were significantly higher (using a univariate analysis) in patients whose tumours demonstrated a response, a multivariate analysis would be required to confirm this as a significant factor independent of tumour burden and patient performance status. As is often the case, this thesis has raised more questions than answers. However, a number of interesting observations have been made which require further investigation.

1. This thesis has revealed the limitations of calculating dose of cytotoxic drugs by surface area alone. This method of calculating dose may result in patients failing to achieve therapeutic levels, particularly in the case of cisplatin. The therapeutic AUC for the commonly used cytotoxic agents in bladder cancer needs to be determined. The routine use of folinic acid in patients receiving low dose methotrexate must be questioned, as in this study no single patient achieved levels at which folinic acid rescue was originally intended.

2. The significance of drug resistance mechanisms in bladder cancer remains unclear. Expression of the enzyme GST π does not appear to determine tumour response to chemotherapy. This study has demonstrated that MDR1 expression is quantifiable. Although there was a 63 fold variation in range of MDR1 expression,
no relationship in terms of tumour response has been demonstrated.

It is hoped that in the future, cure rates in the treatment of bladder cancer may be improved by achieving therapeutic AUCs for cytotoxic agent and with the knowledge of cellular drug resistance mechanisms, methods of reversing this resistance may be developed.
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APPENDIX

STATISTICAL METHODS

I am grateful to Dr Peter Kelly from the University of Newcastle upon Tyne department of Statistics for help and advice regarding all the statistical methods employed in this thesis.

For the purpose of description, data was reported as either means with standard deviations or medians with ranges where appropriate.

Comparison between two groups.
For comparison of two groups of data either the Student t test was used or the Mann Whitney test. The Mann Whitney test was used to compare median values where the distribution was not normal. Where the distribution was normal, the Student t test was used to compare mean values. As the patient data was not paired, the unpaired t test was chosen.

Multivariate analysis.
In order to analyze all the pharmacokinetic data, Logistic regression was used (MINITAB statistical software, department of Medical Statistics, University of Newcastle upon Tyne.) The number of individual patients studied was too small to generate any meaningful statistical data, as is demonstrated by the confidence intervals (Chapter 6).
Survival data.
Survival data, in relationship to MDR1 gene expression, was analyzed by means of the Logrank test. This is a useful method for comparing the survival method of two or more groups and testing whether they are significantly different.

Correlation Coefficients.
In order to determine a potential association between two sets of data Spearman’s correlation coefficient was used. The correlation coefficient defined as $r$ gives a value of 1 if all points lie on a straight line regardless of slope.

$$r = \frac{\text{covariance of } x \text{ and } y}{\sqrt{\text{variance of } x \times \text{variance of } y}}$$

Fisher’s exact test.
In comparing 2 groups in a 2 x 2 contingency table Fisher’s exact test was used, where any of the expected levels were less than 5 in total. This was applicable in the analysis of the staining patterns of the immunohistochemistry studies.

Test of a predictive model.
In order to measure the predictive performance of the single sample limited sampling model to calculate free cisplatin AUC, methods described by Sheiner and Beale (1981) were employed. This involves calculating the mean squared prediction error (measure of precision) and the mean prediction error (measure of bias) for the proposed prediction model. These quantities are easily measured and are a more accurate reflection of the performance of a prediction of measurement method than
computing the correlation coefficient.

Coefficient of Variation

The coefficient of variation was used to compare variability in a mean value. This coefficient is the standard deviation of the distribution expressed as a percentage of the mean of the distribution. i.e. $\text{Coefficient of Variation} = \frac{\text{standard deviation}}{\text{mean}} \times 100$