INHERENT SENSITIVITY AND ACQUIRED RESISTANCE IN HUMAN TESTICULAR GERM CELL TUMOURS IN VITRO

Mary Claire Walker, B.Sc.

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Institute of Urology, University College London,

St Paul's Hospital, 24 Endell Street, London WC2H 9AE.

ABSTRACT

Advanced testicular germ cell tumours, in contrast to the majority of adult solid tumours, are curable using chemotherapy. The reasons for their sensitivity to chemotherapeutic drugs are unknown.

A model system is required to study the mechanisms. Ten continuous cell lines derived from human non-seminomatous testicular germ cell tumours, cultured under identical conditions, were characterized in terms of population doubling time, intermitotic time, cell loss rate, colony-forming efficiency, proportion of S-phase cells, DNA ploidy levels, isozyme pattern, tumorigenicity in nude mice and xenograft morphology.

To determine whether testicular tumour cell lines retain chemosensitivity *in vitro*, the responses of five testicular and five bladder tumour cell lines were compared. Using a colony-forming assay, the testicular tumour cell lines were, on average, five times more sensitive to cisplatin and adriamycin (comparing IC70s). Thus chemosensitivity is inherent to the cells, and is not due solely to humoral factors such as blood supply or immunogenicity.

One mechanism that may be involved is differential binding of drug to DNA. Binding of cisplatin was compared in a testicular and a bladder cell line using atomic absorption spectrophotometry. Identical amounts of cisplatin were bound, indicating that testicular tumour cells may be less able to repair damaged DNA. Activity of an enzyme involved in a specific DNA repair pathway, prevention of crosslink formation by chloroethylnitrosoureas, O^6-alkylguanine-DNA alkyltransferase, was measured.

Development of resistance to cisplatin is a major cause of treatment failure. One testicular and one bladder cell line with stable cisplatin resistance were developed by continuous exposure to increasing concentrations of cisplatin. The growth characteristics, isozyme pattern and chromosome composition of the cell lines were compared, and degree of cross-resistance to other anticancer agents was measured.

Thus in vitro model systems for studying inherent sensitivity and acquired drug resistance in testicular tumours were developed and characterized, and used to investigate two possible mechanisms. In the long term, understanding the mechanisms underlying differential sensitivity may result in more effective treatment for the resistant tumours.

In memory of my mother

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LIST OF ABBREVIATIONS

DAB 3,3'-diaminobenzidine tetrahydrochloride

DAPI 4',6-diamino-2-phenylindole dihydrochloride

dCTP deoxycytidine triphosphate

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

DTE dithioerythritol

EDTA ethylenediaminetetra-acetic acid, disodium salt

EMS ethyl methanesulfonate

HN2 nitrogen mustard

MMS methyl methanesulfonate

MNU N-nitroso-N-methylurea

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBSA Dulbecco's phosphate buffered saline A (8 g NaCl, 2.16 g $Na_2HPO_4.7H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl per litre ddH_2O, pH 7.4)

PCA perchloric acid

PMSF phenylmethylsulfonyl fluoride

RNA ribonucleic acid

SDS sodium dodecyl sulphate

SSC standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate)

TE Tris EDTA (Tris HCl 0.01 M, Na EDTA 0.001 M)

TIPNS triisopropylnaphthalene sulfonic acid

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CHAPTER 1. GENERAL INTRODUCTION

Major advances have been made in the treatment of metastatic testicular cancer over the last 30 years. Cisplatin-based combination chemotherapy now can cure approximately 80% of all patients with disseminated disease. This is in contrast to the majority of adult solid tumours which, once they have metastasized, are either difficult or impossible to cure.

In this study, I have used cell lines derived from non-seminomatous testicular germ cell tumours (NSGCTs) to study some of the mechanisms controlling sensitivity, and these have been compared with cell lines derived from transitional cell carcinomas of the bladder. Hence this chapter reviews the development of chemotherapy for metastatic testis tumours, and briefly describes the current status of bladder cancer chemotherapy.

TREATMENT OF TESTICULAR GERM CELL TUMOURS

Clinical staging and histopathological classification

The diagnosis of testicular germ cell tumour is usually made on histological examination of the primary tumour following orchidectomy. In order to determine the extent of disease, non-invasive investigations are carried out, including CT (computerised axial tomography) scans and measurement of tumour marker levels in serum (alphafetoprotein AFP and human chorionic gonadotrophin HCG) (reviewed by Peckham, 1988). Volume and extent of metastases and serum AFP and HCG levels are important prognostic factors, so accurate staging is required to enable the most appropriate treatment to be given. The Royal Marsden Hospital staging classification of testicular tumours is shown in Table 1.1.

Table 1.1. Royal Marsden Hospital staging classification of testicular tumours

Stage

I Lymphogram negative, marker negative, no evidence of metastases

II Lymphogram positive, metastases confined to abdominal nodes.
Three subgroups are recognised:
A. Maximum diameter of metastases < 2 cm
B. Maximum diameter of metastases 2-5 cm
C. Maximum diameter of metastases > 5 cm

III Involvement of supra- and infradiaphragmatic lymph nodes. No extralymphatic metastases.
Abdominal status - A, B, C as for Stage II

IV Extralymphatic metastases. Suffixes as follows: 0=lymphogram negative; A, B, C as for Stage II

Lung status
L1 ⩽ 3 metastases
L2 multiple metastases < 2 cm maximum diameter
L3 multiple metastases > 2 cm diameter

Liver status
H+ liver involvement

From: Peckham, 1988.

Table 1.2. A simplified histopathological classification of testicular germ cell tumours

British Testicular Tumour Panel	American (Mostofi, 1980)	histological appearance
seminoma	seminoma	composed of uniform, round or polygonal cells
malignant teratoma undifferentiated (MTU)	embryonal carcinoma	composed of malignant cells with no mature elements
malignant teratoma intermediate (MTI)	embryonal carcinoma with teratoma (teratocarcinoma)	composed of undifferentiated and differentiated tissue
teratoma differentiated (TD)	teratoma	composed of fully differentiated tissue such as muscle, bone, cartilage
malignant teratoma trophoblastic (MTT)	choriocarcinoma	composed of trophoblastic elements, and possibly MTI or MTU

For more detail see: Pugh, 1976; Mostofi, 1980; Damjanov, 1983.

Approximately 95% of testicular tumours are of germ cell origin, of which about 40% are seminoma, a further 20% are combined tumours consisting of seminoma plus NSGCT and 40% are NSGCTs (Anderson, 1986). The British and American pathological classification systems are described briefly in Table 1.2. NSGCTs can be subdivided into undifferentiated and differentiated tumours. Undifferentiated NSGCTs are called embryonal carcinomas in the American system. Some NSGCTs contain stem cells capable of differentiation into somatic or extra-embryonic tissues. Somatic differentiation gives rise to the mixture of tissues seen in teratomas, including muscle, cartilage and bone. Extra-embryonic tissues found in teratomas correspond to the chorionic membrane and the yolk sac. Trophoblastic elements (choriocarcinoma) may occur alone, or in combination with other tumour elements. These tumours are characterized by the presence of cytotrophoblast and syncytiotrophoblast, and all are associated with detectable HCG secretion. Their presence indicates the most aggressive, fast-growing type of NSGCT. Yolk sac carcinoma elements are found in about 40% of NSGCTs, and they are associated with elevated serum AFP levels (Damjanov, 1983; Grigor, 1986). Most male germ cell tumours arise in the testis, but they may occasionally be extra-gonadal in origin. Once a diagnosis has been made and the tumour staged, treatment proceeds as follows.

Stage I NSGCT

There are a number of treatment options for patients with clinical Stage I disease. Retroperitoneal lymph node dissection (RPLND) with or without adjuvant chemotherapy is common in the US, and gives a 3 year cure rate of 100%. In one series, 5% relapsed, but were successfully treated with chemotherapy (Lieskovsky et al, 1984). However, RPLND represents overtreatment of the majority of patients, since metastases

are likely to occur only in 20-30% of clinically Stage I patients. This operation also carries a high risk of ejaculatory failure and consequent infertility, an important factor given that relatively young men are being treated. However, recent improvements in the RPLND procedure have resulted in 90% of patients retaining normal ejaculation (Wise and Scardino, 1988).

In the UK, Peckham et al (1982) introduced orchidectomy alone followed by surveillance in 1979, replacing adjuvant radiotherapy to para-aortic lymph nodes, for the best prognosis Stage I patients (no evidence of metastases on CT scan, normal marker levels prior to orchidectomy). One or both of the serum markers HCG and AFP is elevated in 80-85% of patients with disseminated NSGCTs, providing an indicator of relapse (Loehrer et al, 1988). In a retrospective study of 259 patients treated with orchidectomy and surveillance, 70 (27%) relapsed with metastatic disease, 53 of these within the first 12 months after orchidectomy (Freedman et al, 1987). All but 3 patients relapsing were successfully treated with chemotherapy. The estimated 4 year survival rate is 98%, as good as previous results with radiotherapy or RPLND (Freedman et al, 1987). This study enabled the histological features which increase the risk of relapse to be identified (invasion of testicular veins, invasion of testicular lymphatics, absence of yolk sac elements and presence of undifferentiated cells) (Freedman et al, 1987). In a current trial, high-risk patients are given a short course of chemotherapy (BEP) following orchidectomy (Peckham, 1988).

The majority of Stage I patients (approximately 70%) who do not relapse are spared unnecessary treatment. The surveillance policy has been adopted in the UK, and by some specialised centres in the US and

Europe. A danger of surveillance is poor patient compliance. In a few cases in the US, loss to follow-up has resulted in patients presenting with advanced disease (Loehrer et al, 1988). Because of the risks associated with the surveillance policy, the modified RPLND remains the treatment of choice in the US (Donoghue et al, 1988).

Stages IIA and IIB NSGCT

The management of patients with Stage II NSGCT depends on the volume of disease. The treatment of patients with Stages IIA and IIB is controversial, the US favouring RPLND plus adjuvant chemotherapy, depending on the extent of disease (Skinner and Lieskovsky, 1988), the UK favouring chemotherapy alone (Peckham, 1988). Out of 54 Stage IIA and IIB patients treated with primary chemotherapy at the Royal Marsden Hospital between 1977 and 1984, 52 are alive without evidence of disease (96%) (Peckham and Hendry, 1985; Peckham, 1988). In a study of 87 patients with Stage IIA and IIB disease treated with RPLND and adjuvant chemotherapy, a 3 year cure rate of 94% was obtained (Lieskovsky et al, 1984). Comparing the results of these two studies, there is no apparent difference in survival between the US and the UK. Patients presenting with clinical Stage IIC NSGCT (metastases in abdominal lymph nodes, > 5 cm in diameter) receive chemotherapy, like patients with Stages III and IV.

Stages IIC, III and IV NSGCT

Chemotherapy of advanced, metastatic testicular NSGCT is one of the major successes in oncology. The development of combination chemotherapy for NSGCT over the last 30 years will be discussed later. Currently, the 5-year survival rate for all patients with Stages IIC, III and IV disease is about 80%. In a series of 320 patients treated at the Royal Marsden Hospital between 1976 and 1985, actuarial

survival at 5 years is 81% (Peckham, 1988). More than 90% of the relapses occurred during the first year following chemotherapy, so these figures are likely to represent long-term cures. Similar figures are obtained in the US (Einhorn, 1988).

Following chemotherapy for metastatic NSGCTs, a residual mass is visible on CT scan in about 25% of patients, particularly in those presenting with bulky disease, and resection of the mass is carried out to achieve a CR. In an analysis of 67 patients treated at the Charing Cross Hospital, histological examination showed that the residual mass contained fibrotic scar tissue in 27%, mature teratoma in 43% and undifferentiated malignant cells in 30% (Newlands and Reynolds, 1989). Patients with fibrotic or necrotic masses have a good prognosis and only 20% of patients with apparently mature teratoma relapse. However, less than half the patients with residual tumour remain disease-free following surgery. Hence the latter group are given further chemotherapy (Ellis and Sikora, 1987; Peckham, 1988).

Stage I seminoma

Approximately 70% of seminoma patients present with clinical Stage I disease (Einhorn, 1986). Seminomas are more radiosensitive than NSGCT and adjuvant radiotherapy to regional lymph nodes after orchidectomy produces a cure rate in excess of 90% (Einhorn, 1986; Peckham, 1988).

Stage II seminoma

Response rates of patients with Stage II seminoma (metastases in abdominal lymph nodes) depend on the volume of metastases. The preferred treatment for patients with Stages IIA and IIB seminoma is radiotherapy to the abdominal lymph nodes (Peckham, 1988). Stage IIC (lymph nodes > 5 cm in diameter) carries the worst prognosis, 22% of

Stage IIC patients (11/49) relapsing following surgery and radiotherapy, compared with 0% (0/6) for IIA and 6% (1/18) for IIB (Evensen et al, 1985). Because of the sensitivity of seminoma to platinum-based chemotherapy, Gregory and Peckham (1986) and Peckham (1988) recommend chemotherapy using single agent carboplatin following surgery as initial treatment for Stage IIC patients. Other clinicians prefer abdominal radiation, careful follow-up and chemotherapy only on relapse (Einhorn, 1986).

Stages III and IV seminoma

Patients rarely present with advanced metastatic seminoma, but these respond well to chemotherapy, both single-agent (cisplatin or carboplatin) and combination therapy. From a Royal Marsden Hospital series of 44 patients treated with cisplatin-containing combination chemotherapy or carboplatin as a single agent, 91% were alive and disease-free 12-73 months following treatment (Peckham et al, 1985).

DEVELOPMENT OF CHEMOTHERAPY FOR NSGCT

a. Single agent chemotherapy

Metastatic NSGCTs are sensitive to a wide range of chemotherapeutic drugs; all drugs adequately evaluated as single agents have shown some activity (Williams, 1977). In the mid-1950s, a number of agents began to be evaluated alone or in combination. The response rates of metastatic NSGCTs to various drugs as single agents, derived from pooled data, are shown in Table 1.3.

Table 1.3. Single agent responses in metastatic NSGCTs

	number of patients	response rate CR	PR
cisplatin	70	22%	44%
vinblastine	25	16%	36%
bleomycin	38	3%	28%
actinomycin-D	31	16%	36%
mithramycin	305	10%	27%
methotrexate	16	13%	33%
adriamycin	19	5%	65%

Abbreviations

CR: complete response, defined as disappearance of all measurable lesions for at least one month

PR: partial response, defined as at least a 50% reduction in size of all measurable lesions, for at least one month

From: Williams, 1977; Oliver, 1979

Blokhin et al (1958) found that testicular tumours were responsive to an alkylating agent, sarcolysin (now known as phenylalanine mustard or melphalan). 90% (38/42) of patients with advanced seminoma had objective responses to sarcolysin; 19 of these were 'alive and fit for work' 2 to 6 years after treatment. 11/16 patients with combined tumours (teratomas with seminomatous areas) also responded (Chebotareva, 1964).

The synthetic organic antibacterial compound nitrofurazone caused marked atrophy of normal testis when used to treat patients with prostatic carcinoma, but little or no cytotoxicity against the tumours. Thus it was used to treat 4 patients with seminoma, producing marked degeneration of the tumour cells and increased fibrosis (Friedgood et al, 1952). Nitrofurazone caused a reduction in the size of pulmonary metastases in a patient with metastatic teratocarcinoma, but also caused severe toxicity (Szczukowski et al, 1958).

Mithramycin was shown to cause dramatic regressions in the pulmonary metastases of two patients with embryonal cell carcinoma; one remaining disease-free for nearly 4 years (Curreri and Ansfield, 1960; Brown and Kennedy, 1965). Some clinicians considered it too toxic, and for several years in the 1960s it was withdrawn from clinical use (Kennedy, 1972). Although undoubtably active against testicular tumours, it is rarely used because of its toxicity.

Vinblastine caused significant regression of a metastatic embryonal cell carcinoma of the testis (Wright et al, 1962). An objective response rate of 52% was achieved amongst 21 patients with metastatic testicular tumours treated with vinblastine as a single agent (Samuels and Howe, 1970). Two of the four patients achieving a CR in this study

had received prior chemotherapy. One of the CRs survived at least 45 months after treatment (Samuels and Howe, 1970).

The earliest clinical trials using adriamycin as a single agent showed that it had activity against a number of solid tumours (Bonadonna et al, 1969), including testicular cancer (Bonadonna et al, 1970). The objective response rate (corresponding to PR) was 65% (2/4 seminomas, 5/10 embryonal carcinomas, 5/5 teratocarcinomas, 1/1 choriocarcinoma), but the responses were generally of short duration (1 to 12 months) (Monfardini et al, 1972). Summarizing data on 59 patients with testicular cancer treated by a number of groups, there was an overall response rate of 29%, and the duration of remission ranged from 2 to 18 months (Slavik, 1975).

Single agent activity of the antimetabolite class of compounds has not been studied extensively. Four out of 10 patients with metastatic embryonal cell carcinoma treated with methotrexate achieved CRs; all survived at least 5 years following treatment. In contrast, the mean survival of the non-responders was 4 months (Wyatt and McAninch, 1967). Nine out of 17 patients with bulky stage IV disease receiving methotrexate as first-line chemotherapy achieved partial remission (53%) (Smith et al, 1988). However, the duration of response was short (41-75 days), and only occurred in patients whose tumours contained trophoblastic elements (Smith et al, 1988). 5-fluorouracil (5-FU) has shown activity; one of two patients with embryonal cell carcinoma achieved an objective response following 5-FU treatment, but a patient with seminoma did not (Allaire et al, 1961).

In the early 1970s, bleomycin was shown to have single agent activity against metastatic testicular cancer (Yagoda et al, 1972; Blum et al, 1973). In a review of several studies on single agent bleomycin, in

patients with advanced tumours refractory to conventional treatment, 8/21 patients (38%) with embryonal carcinoma responded to bleomycin, and 2/8 patients (25%) with teratocarcinoma, but durations were short (2-4 and 1.5-2 months, respectively) (Blum et al, 1973). The most serious side-effect of bleomycin was decreased pulmonary function due to fibrosis. There was also skin and mucosal ulceration. However, it was not myelosuppressive (Yagoda et al, 1972; Bonadonna et al, 1972).

A major advance in the treatment of metastatic NSGCT was the demonstration of the antitumour activity of cisplatin. Responses were observed in 5 out of 21 pretreated patients and the major side effects were nephrotoxicity, severe nausea and vomiting and occasionally neurotoxicity, ototoxicity and anaemia (Rossof et al, 1972). However, cisplatin caused only mild myelosuppression which was transient and non-life-threatening. In the original study, the one patient with embryonal carcinoma of the testis did not respond to cisplatin (Rossof et al, 1972). Subsequently, responses were reported in 9/11 patients with testicular tumours refractory to conventional treatment including actinomycin-D and adriamycin. Three of these were complete regressions, lasting at least 5-11 months (Higby et al, 1974). Dose-escalation studies in the group of 45 patients showed that irreversible kidney damage occurred in 5/9 patients following a single dose of 100 mg/m^2 IV, and in 2/3 patients receiving 24 mg/m^2 daily for 5 days. Toxicity caused by 20 mg/m^2 daily for 5 days was considered acceptable (Higby et al, 1974).

The activity of VP-16 (etoposide) as a single agent in testicular tumours was demonstrated in 1977 (Cavalli et al, 1977; Newlands and Bagshawe, 1977; Fitzharris et al, 1980). Out of 24 evaluable patients, 3 achieved CR and 8 PR (overall response rate 46%). All except one had

received prior chemotherapy and/or radiotherapy and had relapsed. Single agent activity of VP-16 as a second-line agent was comparable with that of other drugs used as first-line agents (Fitzharris et al, 1980).

b. Development of combination chemotherapy

The first account of treatment of patients with metastatic testicular cancer used a combination of chlorambucil, methotrexate and actinomycin-D (Li et al, 1960). Of 23 patients treated, 12 (52%) showed objective responses, 7 of which showed a 'complete or nearly complete' response (30%). Three of these maintained their CR for 9-18 months. From 1955 onwards, Mackenzie (1966) treated patients with metastatic testis cancer using either actinomycin-D alone, or in combination with other agents including chlorambucil, methotrexate, mithramycin, vinblastine and vincristine. Overall, 24/154 patients (16%) were rendered free of disease, 7 of these surviving for at least 3 years.

Actinomycin-D alone or with chlorambucil and methotrexate became the standard treatment for disseminated testicular cancer throughout the 1960s (Williams and Einhorn, 1983). The complete response rate in early studies was 10-20%; half of these were cured, whereas the other half relapsed and ultimately died. Patients who relapsed after complete response tended to do so within 1 or 2 years of treatment (Einhorn, 1981), because testicular tumours are fast-growing and regrow soon after chemotherapy if they have not been completely eradicated. If a patient with disseminated testis cancer is still disease-free 2 years after chemotherapy, there is 99% probability of cure (Einhorn, 1981).

The lack of myelosuppression caused by bleomycin made it a useful agent for combination chemotherapy. Preclinical studies suggested synergism between vinblastine and bleomycin (Barranco and Humphrey, 1971), attributed to the ability of vinblastine to arrest cells in mitosis and of bleomycin to kill cells in mitosis (Einhorn, 1986). A combination of vinblastine and bleomycin was introduced at the M D Anderson Hospital and Tumor Institute in Texas (Samuels et al, 1975; 1976; 1979). Initial studies using VB-1 (see Table 1.4) in patients with Stage III teratocarcinomas resulted in 10/24 (42%) CRs. One patient relapsed, but the remainder survived at least 3 years and were considered cured (Samuels et al, 1976).

Table 1.4. Vinblastine and bleomycin protocols at M D Anderson Hospital

VB-1	Vinblastine 0.4 mg/kg total dose, days 1, 2 Bleomycin 30 units IM twice weekly x 10 weeks If a response was seen, further courses were given, as follows: Vinblastine 0.4 mg/kg total dose, days 1, 2 Bleomycin 30 units IM twice weekly x 5 weeks Repeat at 4-week intervals
VB-2	Bleomycin 30 units/l normal saline over 24h x 5 days Vinblastine 0.4 mg/kg days 5, 6
VB-3	Vinblastine 0.4-0.6 mg/kg days 1, 2 Bleomycin 30 units/l normal saline over 24h x 5 days (days 2-6) Courses repeated every 3-4 weeks x 3 or 4
VB-3 + cisplatin	Add cisplatin 100 mg/m^2 on recovery from myelosuppression x 2 at 7-day intervals

From: Carter, 1983.

In vitro data suggested that continuous exposure of cells to bleomycin markedly improved cell kill (Drewinko et al, 1972). Changing the method of administration of bleomycin from intermittent to continuous infusion increased the CR rate. The three recorded patients receiving VB-2 all responded, 1 of them achieving a CR (reviewed by Carter, 1983). VB-3 (vinblastine on days 1 and 2, bleomycin infusion days 2-6) increased the CR rate to 65% (59/91 evaluable patients), with a follow-up time in excess of 2 years (Samuels et al, 1979). These data were obtained in patients with a wide range of metastatic disease, from limited nodal involvement to metastases in liver and brain (Samuels et al, 1979). In a subgroup of patients with metastatic disease who had received prior radiotherapy, only 20% remained disease-free after 2 years. Increasing the dose of vinblastine increased the CR rate. Bleomycin caused interstitial pneumonitis in 2% of patients, but chest X-rays and gallium scans enabled early detection of this side effect, while it was still at a reversible stage (Samuels et al, 1979).

Reports of the marked single agent activity of cisplatin led to the development of the cisplatin-containing combination regimens. At the Memorial Sloan-Kettering Cancer Center (MSKCC) in New York a series of combination protocols was developed called the VAB program (see Table 1.5). This began in 1972 with a combination of vinblastine, actinomycin-D and bleomycin (VAB-I). VAB-I differed in dosage and method of administration from the Samuels protocol described above, and was not as successful; 22% CRs in 68 evaluable patients, 13% being long-term disease-free survivors (Golbey et al, 1979).

Table 1.5. Summary of MSKCC protocols VAB-I to VAB-VI

VAB-I

Vinblastine, 0.025-0.05 mg/kg
Actinomycin-D, 0.0075-0.015 mg/kg
Bleomycin, 0.4 mg/kg
All drugs given on days 1, 2, 3, 9, 10, 11 + weekly maintenance

VAB-II

Vinblastine, 0.06 mg/kg, day 1
Actinomycin-D, 0.02 mg/kg, day 1
Bleomycin, 0.5 mg/kg/day, days 1-7, continuous IV infusion
Cisplatin, 1 mg/kg, day 8
(Induction cycle repeated every 3-4 months; consolidation with vinblastine, actinomycin-D and bleomycin weekly; cisplatin replacing actinomycin-D every 3 weeks; maintenance after second induction cycle with chlorambucil, vinblastine and actinomycin-D every 3 weeks for 2-3 years)

VAB-III

Vinblastine, 4 mg/m^2, day 1
Actinomycin-D, 1 mg/m^2, day 1
Bleomycin, 20 mg/m^2, days 1-7, continuous IV infusion
Cisplatin, 120 mg/m^2, day 8, with prehydration and mannitol
Cyclophosphamide, 600 mg/m^2, day 1
(Induction cycle repeated after 5 months; consolidation every 3 weeks with vinblastine, adriamycin and chlorambucil alternating with vinblastine, actinomycin-D and chlorambucil, and with vinblastine, cisplatin and chlorambucil. Maintenance every 3 weeks for 2 years with vinblastine, actinomycin-D and chlorambucil)

VAB-IV

Similar to VAB-III, but induction cycles repeated at 16 weeks and 32 weeks instead of at 5 months. Consolidation phase shortened. Maintenance program same as VAB-III, for 2 years

VAB-V

Intensified version of VAB-IV for poor prognosis patients
Vinblastine, 8 mg/m^2, day 1
Actinomycin-D, 1.5 mg/m^2, day 1
Bleomycin, 30 mg, day 1
Bleomycin, 20 mg/m^2/day, days 1-6, continuous IV infusion
Cisplatin, 120 mg/m^2, days 7, 28 with mannitol-induced diuresis
Cyclophosphamide, 600 mg/m^2, days 1, 28
Adriamycin, 40 mg/m^2, day 28
(Induction cycle repeated at 15 and 30 weeks; consolidation every 3 weeks with vinblastine, actinomycin-D and bleomycin alternating with vinblastine, adriamycin and bleomycin. Maintenance every 3 weeks for a total of 2-2.5 years from the start of therapy, with vinblastine and actinomycin-D)

VAB-VI

Vinblastine, 4 mg/m^2, day 1
Actinomycin-D, 1 mg/m^2, day 1
Bleomycin, 30 units/m^2, IV push, day 1
Bleomycin, 20 units/m^2/day, days 1-3, continuous IV infusion
Cisplatin, 120 mg/m^2, day 4
Cyclophosphamide, 600 mg/m^2, day 1
(Induction cycle repeated every 3-4 weeks for 3-4 courses; no bleomycin in third cycle; no consolidation; no maintenance)

From: Golbey et al, 1979; Carter, 1983; Vugrin et al, 1983a; Seitz et al, 1988.

The VAB-II regimen, used between 1974 and 1976, resulted in 25/50 patients (50%) achieving CR, with a median duration of response of 13 months. Of these patients, 23 became clinically free of disease on chemotherapy alone, the other 2 patients had residual disease which was removed by surgery after chemotherapy. 11/50 (22%) were disease-free 3 years after treatment (Golbey et al, 1979; Carter, 1983). VAB-III, a complicated 7-drug regimen used from 1975 to 1977 resulted in a CR rate following surgical resection of residual disease of 60%. 45% were disease-free with a minimum follow-up of 2 years (Golbey et al, 1979; Williams and Einhorn, 1983). This protocol included high-dose cisplatin, with prehydration and mannitol diuresis to minimize renal toxicity, and cyclophosphamide and adriamycin, which both show single agent activity.

VAB-IV was similar to VAB-III, but increased the frequency of the induction phase. It was used between 1976 and 1978 and gave an initial CR rate of 80% for patients who were previously untreated, and a 68% disease-free survival at 1 year (Golbey et al, 1979; Vugrin et al, 1981). VAB-V was used for patients with poor prognosis, including 15 who had received prior chemotherapy, between 1977 and 1979. 18/38 patients (47%) achieved complete remission, either with chemotherapy alone, or chemotherapy plus surgery. 14 of these (37% overall) remained disease-free for a median follow-up of 4 years. However, toxicity was severe, especially mucositis after bleomycin infusion, and myelosuppression. Toxicity often caused a delay in induction cycles (12 months instead of 8), and 30% of patients actually refused chemotherapy (Vugrin et al, 1983a).

The most recent protocol, VAB-VI, introduced in 1979, has a high initial CR rate of 90%, and 80% disease-free survival at a median

follow-up of 2 years (Vugrin et al, 1983b). This durable CR rate, obtained in patients with disseminated tumours, is comparable with that currently obtained using other cisplatin-based regimens, such as PVB (Loehrer et al, 1988). The VAB-VI protocol differs from the earlier ones in some important respects; induction cycles are more frequent and maintenance therapy is abolished as unnecessary. The duration of therapy has decreased from 2-2.5 years to 12 months. The lower CR rates obtained using VAB-I to VAB-IV may have been due to the long intervals between high-dose cisplatin treatments (induction cycles repeated every 3-5 months, compared with every 3-4 weeks in VAB-VI) (Einhorn, 1988).

In 1974, as cisplatin was being introduced into the VAB protocol, it was also being incorporated into vinblastine plus bleomycin regimens at the Indiana University Hospital (PVB). In the first trial of PVB, from 1974 to 1976, 0.2 mg/kg vinblastine was given on days 1 and 2, with cisplatin and bleomycin doses as shown in Table 1.5. Vinblastine (0.3 mg/kg every 4 weeks) and Bacillus Calmette-Guerin were given as maintenance therapy for 2 years; patients were only given cisplatin in their fourth induction cycle if they had not already achieved a complete remission (Einhorn and Donoghue, 1977; Seitz et al, 1988). In this study, 33/47 (70%) patients achieved a CR, and a further 5 patients (11%) were rendered disease-free by post-chemotherapy surgical resection of tumour. Thirty patients (64%) survived, disease-free, for 5 years (Einhorn, 1981) and 28 (60%) for 10 years (Seitz et al, 1988).

Although this PVB regimen produced good therapeutic results, toxicity was a problem. Cisplatin nephrotoxicity was controlled using hydration with normal saline before and during the 5-day course. Bleomycin-

induced pulmonary fibrosis was rare, but the major toxicity was due to high-dose vinblastine - severe granulocytopenia and fever. In 1976, a randomized trial compared standard PVB with a PVB protocol containing low-dose vinblastine (0.3 mg/kg) in the induction program. In the same trial, the potential benefit of adding adriamycin to PVB in terms of increased CR rate and long-term survival was assessed (Einhorn and Williams, 1980; reviewed by Williams and Einhorn, 1983). The choice of adriamycin was based on a study of 10 patients with disseminated disease refractory to vinblastine and bleomycin who were treated with adriamycin and cisplatin. All responded objectively, with 1 CR and 9 PRs, and a median duration of remission of 5 months (Einhorn and Williams, 1978). However, addition of adriamycin to the PVB protocol did not increase the CR rate or the long-term survival rate (80% and 72% respectively) (Einhorn, 1981). Reduction of vinblastine dose did reduce the haematological toxicity (15% of patients developed granulocytopenic fever, compared with 38% on the original PVB regimen), without compromising the therapeutic efficacy (the CR rate following surgical resection was 82% with low-dose vinblastine and 81% with the original PVB) (Einhorn, 1981; Williams and Einhorn, 1983). The lower vinblastine dose was adopted for subsequent therapy and adriamycin was not included in later protocols. The extent of disease was the main prognostic variable. 30/31 (97%) patients with minimal metastatic disease achieved CR (Williams and Einhorn, 1983) compared with 50% of patients with massive pulmonary metastases and advanced abdominal disease (Einhorn, 1981).

The value of maintenance chemotherapy was assessed in a randomized trial of patients achieving CR after induction therapy (Einhorn, 1981). 12% of patients receiving vinblastine maintenance therapy

relapsed compared with 7% of patients not receiving maintenance. The results showed that maintenance therapy is unnecessary in disseminated testicular cancer treated with 4 courses of PVB.

Following the demonstration of the marked single agent activity of VP-16 in salvage therapy, its activity in combination regimens was investigated. The Indiana University group used it alone or in combination with cisplatin and adriamycin with or without bleomycin in salvage therapy for patients relapsing after PVB (Williams et al, 1980). The combination produced 14/33 CRs and 15/33 PRs. One third of patients remained disease-free for at least 6 to 23 months following treatment, a significant achievement for salvage chemotherapy. On the basis of these data, and those obtained by other groups, the role of VP-16 in first-line therapy was evaluated.

VP-16 was substituted for vinblastine in PVB in an attempt to decrease the toxicity of this regimen. Investigators at the Royal Marsden Hospital showed that BEP was as effective as PVB but less toxic; 37/43 patients (86%) attained long-term complete responses (Peckham et al, 1983). The Southeastern Cancer Study Group (SECSG) in the US then compared BEP with PVB in a randomized prospective trial (Williams et al, 1987). The overall therapeutic results were similar (74% of patients treated with PVB achieved disease-free status compared with 83% treated with BEP - not significantly different), but BEP was regarded as superior to PVB because of its reduced toxicity. VP-16 is myelosuppressive, but it does not cause the other toxicities of vinblastine - with BEP there were fewer parasthesias, abdominal cramps and myalgias. In a subgroup of patients with poor prognosis (nodes > 10 cm in diameter, lung involvement, high AFP and HCG levels), 77% of patients became disease-free on BEP, compared with 61% on PVB,

suggesting that it has greater efficacy in this subgroup (Williams et al, 1987).

More aggressive chemotherapy regimens have been investigated for patients with poor prognosis. Precise definitions of this subgroup vary, making direct comparisons between different groups difficult, but large tumour volume and high serum marker levels are independent prognostic variables (MRC Working Party on testicular tumours, 1985). In order to increase survival in high risk patients, various protocol modifications have been studied. These include dose escalation, for example, Ozols et al (1983) doubled the dose of cisplatin to 200 mg/m^2 over 5 days. High-dose cisplatin with vinblastine, VP-16 and bleomycin (PVeBV) was used to treat poor prognosis patients. Cisplatin was administered in hypertonic saline, greatly reducing the nephrotoxicity and enabling the high dose to be used. Treatment was followed by autologous bone marrow transplantation (ABMT) in patients receiving 4 courses of high-dose chemotherapy (Ozols et al, 1983). However, toxicity was still severe, especially myelosupression and ototoxicity (Ozols et al, 1983). An actuarial 5-year survival rate of 78%, compared with 48% on PVB, was achieved (P value 0.06, not significantly different) (Ozols et al, 1988). Total doses of 1200 mg/m^2 VP-16 with 1500 mg/m^2 carboplatin were used in patients who had relapsed after conventional therapy. Even with autologous bone marrow support, bone marrow suppression was severe, 7 out of the first 33 patients treated dying of granulocytopenia and infection (reviewed by Horwich, 1989).

Table 1.6. Summary of current chemotherapy regimens for metastatic NSGCTs

VAB-VI

Cyclophosphamide, 600 mg/m^2, day 1
Vinblastine, 4 mg/m^2, day 1
Actinomycin-D, 1 mg/m^2, day 1
Bleomycin, 30 U/m^2, IV push, day 1
Bleomycin, 20 U/m^2/d x 24h infusion, days 1-3
Cisplatin, 120 mg/m^2, day 4
(Cycles repeated monthly; no bleomycin in third cycle)

PVB

Cisplatin, 20 mg/m^2, days 1-5
Vinblastine, 0.15 mg/kg, days 1, 2
Bleomycin, 30 U weekly x 12
(Cycles repeated every 3 weeks)

BEP

Cisplatin, 20 mg/m^2, days 1-5
Bleomycin, 30 U weekly x 12
Etoposide, 100mg/m^2, days 1-5
(Cycles repeated every 3 weeks)

EP

As BEP, omitting bleomycin

POMB-ACE

POMB
Vincristine, 1 mg/m^2, day 1
Methotrexate, 300mg/m^2, 12h infusion, day 1; folinic acid rescue, 15 mg 12-hourly x 4, days 2, 3
Bleomycin, 15 mg, 24h infusion, days 2, 3
Cisplatin, 12 mg/m^2, 12h infusion, day 4

ACE
Etoposide, 100 mg/m^2, days 1-5
Actinomycin-D, 0.5 mg IV, days 3, 4, 5
Cyclophosphamide, 500 mg/m^2, IV, day 5
(Sequence: 2 courses POMB followed by ACE, then POMB and ACE alternate until patient is in remission as measured by HCG and AFP levels, usually 3-5 courses of POMB. Interval between courses normally 9-11 days)

From: Seitz et al, 1988; Hitchins et al, 1989.

Another approach to intensification of treatment has been to reduce the interval between courses of chemotherapy. NSGCTs are rapidly proliferating tumours (Fossa et al, 1985) and grow back rapidly between chemotherapy cycles (Horwich, 1989). A combination of vincristine, cisplatin and bleomycin was used in patients with bulky abdominal or advanced metastatic disease (Wettlaufer et al, 1984). Because of the lack of myelosuppression, four 5-day courses of cisplatin-based chemotherapy could be given in less than a month. A complete clinical remission was achieved in 93% of patients; 83% remained in CR for at least 18 to 46 months (Wettlaufer et al, 1984). The POMB-ACE regimen used by the Charing Cross Hospital also has a high cisplatin dose rate. Furthermore, reducing the time between induction cycles greatly increased the efficacy of the VAB protocol, as discussed earlier. Intensive induction chemotherapy was used in a Royal Marsden Hospital study with poor prognosis patients. Four courses of bleomycin, vincristine and cisplatin (BOP) at 7-day intervals were followed by three courses of BEP or EP at 21-day intervals, for a total of 13 weeks of chemotherapy. Results were good - 85% of patients (23/27) were free of progressive disease at a median follow-up of 2 years. 20/27 (74%) of patients achieved CR following chemotherapy and surgery, none of whom relapsed during follow-up. However, 2 patients died of bleomycin pneumonitis (Horwich et al, 1989).

Regimens containing a large number of drugs have been used for high-risk patients. The POMB/ACE regimen was developed at the Charing Cross Hospital in 1977. Initial treatment with two courses of cisplatin, vincristine, methotrexate and bleomycin is followed by actinomycin D, etoposide and cyclophosphamide. POMB is then alternated with ACE until markers return to normal, then OMB alternates with ACE for a further

12 weeks. Overall, 89% of patients (171/193) with metastatic NSGCT are alive with a median follow-up of 4.1 years (Hitchins et al, 1989).

As well as improving survival in the group of patients with the worst prognosis, the current aim of chemotherapy for metastatic NSGCTs is to reduce toxicity for the patients in good prognosis groups. Ways of reducing toxicity include: dosage reduction, decreasing the number of cycles, using fewer drugs and less toxic drugs (Peckham, 1988). One approach being investigated is the substitution of carboplatin for cisplatin in combination chemotherapy. Carboplatin does not cause significant renal toxicity, neurotoxicity or otoxicity; its predominant side effect is myelosuppression. A current MRC randomised trial is comparing BEP with CEB (carboplatin, etoposide, bleomycin) in good prognosis patients (Horwich, 1989).

Another way of minimizing toxicity in good prognosis patients is by reducing the number of courses of chemotherapy. The SECSG began a trial in 1984 comparing the standard treatment (4 courses of BEP over 12 weeks) with 3 courses of BEP over 9 weeks. With only a short follow-up period, 3 courses gave equivalent results to 4 courses in patients with minimal or moderate extent disease, and reduced toxicity (Seitz et al, 1988). As discussed above, lowering the dose of vinblastine in the original PVB regimen decreased toxicity without affecting patient survival (Einhorn, 1981). A recent study also showed that reducing the dose of vinblastine or bleomycin in patients receiving PVB, when forced to because of excessive toxicity, did not compromise initial treatment response or subsequent survival, compared with patients receiving full-dose chemotherapy (Levi et al, 1989).

Reducing the number of drugs used is another way of reducing toxicity. A randomized study is being carried out by the EORTC comparing EP with BEP in good prognosis patients. Early results show that there was no difference in CR rate or continuous disease-free survival. Toxicity was reduced with EP, there was no lung or skin toxicity, and less myelosupression than with BEP (reviewed by Horwich, 1989).

TREATMENT OF BLADDER CANCER

Clinical staging and histopathological classification

Treatment and prognosis of bladder cancer is dependent on the stage of the disease. The UICC histopathological staging system for bladder cancer is shown in Table 1.7 (UICC, 1988). About 80% of patients present with papillary, superficial tumours (Ta, T1, Tis) and are treated with transurethral resection (TUR). In 50-70% of these patients single or multiple recurrences occur. In a small proportion of these cases (10-20%), the recurrences are invasive. Once the tumour has invaded the deep muscle layer surrounding the bladder (T3), or beyond, chances of cure diminish (Souhami and Tobias, 1986).

Table 1.7. UICC histopathological staging classification for bladder cancer

Stage	
Tis	Pre-invasive carcinoma (carcinoma-in-situ)
Ta	Papillary non-invasive carcinoma
T0	No evidence of primary tumour found on histological examination of specimen
T1	Tumour invading subepithelial connective tissue, but not extending beyond the lamina propria
T2	Tumour invading superficial muscle (not more than half way through muscle coat)
T3	Tumour invading deep muscle (more than half way through muscle coat, T3a; or with invasion of perivesical fat, T3b)
T4	Tumour with invasion of prostate, uterus, vagina, pelvic wall or abdominal wall
TX	The extent of invasion cannot be assessed

Stage grouping			
Stage 0	Tis	N0	M0
	Ta	N0	M0
Stage 1	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3a	N0	M0
	T3b	N0	M0
Stage IV	T4	N0	M0
	Any T	N1, N2, N3	M0
	Any T	Any N	M1

From: UICC, 1988.

CHEMOTHERAPY OF ADVANCED BLADDER CANCER

Advanced transitional cell carcinoma of the bladder does respond to chemotherapy, but responses are generally of a short duration. Once it has metastasized, cures are rarely achieved.

Pooling data from a large number of phase II trials indicate that cisplatin is the most active single agent, with an average response rate of 30% (CR + PR) (see table 1.8, and reviews by Harker and Torti, 1983; Scher, 1989). Cisplatin is commonly used at doses ranging from 70 to 100 mg/m^2, slightly lower than those used for NSGCTs. Generally, responses are partial and of short duration. Patients with metastatic bladder cancer, whose tumours progress on chemotherapy, rarely survive for a year. Median survival is 3-5 months (Yagoda, 1985).

Adriamycin is also an effective single agent in the treatment of bladder cancer. Cumulative data from the US National Cancer Institute (NCI) showed that, out of 136 patients evaluated, there were 40 objective responses, only 3 of which were CRs. The overall response rate in the 136 patients was 27%, and the duration of remission was 1-11+ months (Slavik et al, 1975).

Various combinations of drugs found to be active as single agents have been used to try and improve the rate and duration of responses (Harker and Torti, 1983). Some randomized trials have shown that combination chemotherapy increases the number of CRs compared with single agent therapy (for example Gagliano et al, 1983; Hillcoat et al, 1989) but other data does not support this (for example Soloway et al, 1983; reviewed by Scher, 1989). The increased initial CR rate for patients treated with cisplatin and methotrexate compared with cisplatin alone did not lead to increased survival (Hillcoat et al, 1989).

Table 1.8 Single agent responses in advanced bladder cancer

drug	number of patients	average % response (CR+PR)
cisplatin	320	30
adriamycin	248	17
methotrexate	236	29
mitomycin-c	42	13
vinblastine	38	16
VP-16	47	2
5-FU	105	15

From: Yagoda, 1988.

Three combinations commonly used are M-VAC (methotrexate, vinblastine, adriamycin, cisplatin), CMV (cisplatin, methotrexate, vinblastine) and CISCA (cisplatin, cyclophosphamide, adriamycin). M-VAC has greatly improved the initial response rate compared with single agent therapy in studies using a selected group of patients (Sternberg et al, 1988). Out of 132 patients, an initial response rate of 72% was achieved (CR + PR), with 36% CRs. Of these CRs, 11% required surgical resection of residual disease (Sternberg et al, 1989). 73% of CRs relapsed within 3 years, but 59% of CRs are surviving at 3 years. Overall, 20% of patients are still alive, and median survival was 13 months (Sternberg et al, 1989). However, using the same protocol, Tannock et al (1989) achieved a lower CR rate (10%) and reported a higher toxicity than

Sternberg's group. This reflects differences in the patients studied, for example, the majority of patients in the Tannock study (33/41) were being treated palliatively and post-chemotherapy surgery was not feasible.

Of patients receiving CMV, 28% achieved a CR, 43% of these remaining in remission 6 to 35 months after treatment (12% overall) (Harker et al, 1985). In a retrospective study of CISCA, an initial CR rate of 36% was obtained, 31% of these (12% overall) surviving longer than 4 years (Logothetis et al, 1989a; Scher, 1989).

Development of treatment schedules is continuing: in a recent study, an escalated M-VAC protocol was given with recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) to patients who had progressed on conventional chemotherapy. 4/22 (18%) of patients achieved CRs lasting 4-8 months, showing that responses are achievable even in this heavily-pretreated group (Logothetis et al, 1989b).

Thus combination chemotherapy does seem to increase the proportion of CRs, and increase the response durations, of patients with unresectable metastatic bladder cancer, compared with single agent therapy. However, the toxicity caused by combination chemotherapy is much greater than that associated with single agent therapy, so its use in predominantly elderly patients with advanced disease, for whom chemotherapy is largely palliative, is questionable.

Currently, the best treatment results in approximately 12% of patients with metastatic bladder cancer surviving up to 4 years. This contrasts with the treatment of metastatic testicular cancer, where currently more than 80% of patients are cured.

CHAPTER 2. MATERIALS AND METHODS

2.1. Continuous cell lines

The origins of the continuous cell lines used in this study are shown in Table 2.1. Details of any prior treatment (chemo- or radiotherapy) received by the patient are shown in Table 2.2. The cell lines were obtained from various sources. Tera I, Tera II and NEC-8 were obtained from Dr J Fogh (Memorial Sloan-Kettering Institute for Cancer Research, New York, USA); SuSa was supplied by Dr B Hogan (Imperial Cancer Research Fund, Mill Hill Laboratories, London, England); T3B1 was provided by Prof A M Neville (Ludwig Institute for Cancer Research, Sutton, England); 833K and 1618K were obtained from Dr N J Vogelzang (University of Chicago Medical Center, Chicago, USA); GH and HL were provided by Dr R Kurth (Paul-Ehrlich-Institut, Frankfurt, FRG); GCT27 was obtained from Dr M F Pera (Institute for Cancer Research, Sutton, England); RT112 and RT4 were established in our laboratory; T24 was provided by Dr C O'Toole (Cambridge, England) and was established by Dr J Bubenik (Charles University, Prague, Czechoslovakia); HT1197 and HT1376 were obtained from Prof S Rasheed (University of Southern California, Los Angeles, USA).

2.2. Cell culture

2.2.a. Routine maintenance of cell lines

All the cell lines were maintained under identical culture conditions as monolayers in 25cm^2 flasks (Nunc, Gibco Ltd, Paisley, Scotland) in RPMI 1640 medium (Gibco) supplemented with 5% heat-inactivated foetal calf serum and 2mM L-glutamine (Flow Laboratories, Rickmansworth, England) at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. During the course of this work, foetal calf serum from six different batches was used. Each was tested to ensure that the colony-forming

efficiencies of the cell lines were similar in each successive batch. Cells were subcultured using an aqueous solution of 0.05% trypsin (Difco 1:250, London, England) and 0.016% versene (ethylenediamine-tetra-acetic acid disodium salt) (BDH Ltd, Poole, England). All cell lines were used over a range of 10 *in vitro* passages to minimise any changes which might occur as a result of long-term culture.

2.2.b. Mycoplasma testing

Cell lines were screened every six months for mycoplasma (PPLO; pleuropneumonia-like organism) contamination by growing the cell lines on sterile microscope slides until semi-confluent, then staining with Hoechst 33258 (bisbenzimide; Sigma Chemical Co Ltd, Poole, England), as described (Freshney, 1987). Hoechst 33258 is a fluorescent dye which binds specifically to DNA. When examined under a fluorescence microscope, the nuclei of mycoplasma-free cells fluoresce brightly, with no background staining. In mycoplasma-contaminated cells, a particulate or filamentous extra-nuclear fluorescence is seen. This staining technique is not specific for mycoplasma - any DNA-containing infection such as certain viruses will also be visible.

2.2.c. Preparation of a single-cell suspension

When cells were required for further study, exponentially-growing cells were detached from flasks by incubating in trypsin/versene solution for a maximum of 5 minutes at 37 ^{o}C, and a single-cell suspension was produced by syringing twice through a 19 gauge needle. Viable cell numbers were estimated by counting the cells excluding trypan blue dye, using a haemocytometer (Freshney, 1983).

2.3. Characterization of testicular tumour cell lines

2.3.a. Tumorigenicity in nude mice

Exponentially-growing cells were enzymically detached, washed once in medium containing serum and twice in medium without serum. Approximately 10^7 cells in 0.1 ml unsupplemented medium were inoculated subcutaneously into one or both flanks of nude mice (at least three mice per cell line). The mice were examined weekly for evidence of tumour development, and killed by cervical dislocation when tumours had grown to 0.5-1 cm diameter. Tumours were excised, fixed in formol saline and processed using standard histological procedures. Paraffin sections were stained with haematoxylin and eosin and examined by an experienced histopathologist.

2.3.b. Isozyme analysis

Exponentially-growing cells were enzymically detached, washed once in medium containing 5% serum and once in PBSA, counted, then resuspended in PBSA at 10^7 /ml and transferred to 1 ml cryotubes (Nunc). Cells were centrifuged, the supernatant decanted and the pellets allowed to air-dry for 10 min before storage in liquid nitrogen until analysis. A minimum of 5 polymorphic enzymes were typed for each cell line using horizontal starch gel electrophoresis (Harris and Hopkinson, 1976; Freshney, 1987). A cell extract was prepared by thawing the frozen cell pellet, refreezing and rethawing it and pipetting the cell suspension using a Pasteur pipette to disrupt the cell membranes. The homogenate was transferred to Eppendorf tubes and centrifuged in a microcentrifuge for 2 minutes. The supernatant was stored at -70^{o}C until analysis.

Isozymes migrate at different rates depending on their net charge and the strength of the electrical field. The compositions of the buffers

used, and the electrohoresis conditions are specific for the isozymes being studied (see Harris and Hopkinson, 1976). Using GOTM (mitochondrial glutamic oxaloacetic transaminase) as an example, the cell extract was applied to the starch gel and electrophoresed at 5 V/cm for 17 hours at 4 °C. The electrophoresis buffer used was 0.2 M Na_2HPO_4, 0.03 M boric acid, pH 7.0. The stains used to visualize the bands are also specific for the isozymes being studied. GOTM catalyses the conversion of aspartate and 2-oxoglutarate to oxaloacetate and glutamate. The oxaloacetate produced reacts with the diazonium salt Fast Blue B to form a blue band which marks the position of GOTM on the gel. Therefore the gel was stained with 50 ml of 30 mM L-aspartic acid, 15 mM 2-oxoglutaric acid and 5 mg/ml Fast Blue B in 0.1 M Tris-HCl buffer, pH 7.4 at 37 °C (Davidson et al, 1970; Harris and Hopkinson, 1976). The other isozymes were stained using the methods described in Harris and Hopkinson (1976).

2.3.c. DNA analysis using locus-specific probes

Cell pellets were prepared as above (2.3.b) and stored in liquid nitrogen until analysis. High molecular weight DNA was prepared from the cell pellets, as previously described (Edwards et al, 1985). Pellets were thawed and lysed in 1 ml lysis buffer (Tris 0.01 M, EDTA 0.01 M, NaCl 0.15 M, pH 8.0 containing 0.5% SDS) in the presence of proteinase K (300 μg/ml). The mixture was transferred to an Eppendorf tube and an equal volume of TE-saturated phenol-chloroform-isoamyl alcohol (24:24:1) (TE is Tris-HCl 0.01 M at pH 8.0, Na EDTA 0.001 M; TE-saturated phenol is 3 ml TE to 10 ml phenol) was added. The mixture was vortexed, then centrifuged in a microcentrifuge for 1 minute. The DNA-containing aqueous phase was transferred to a fresh Eppendorf tube. Contaminating RNA was removed from the aqueous phase by

treatment with T_1 and T_2 RNAses (100 units of RNAse T_1, 5 units of RNAse T_2 for 45 minutes at 37°C) and DNA was purified using phenol-chloroform-isoamyl alcohol then chloroform alone, to remove any contaminating phenol. DNA was precipitated in ethanol containing 1% sodium acetate and rinsed in 70% ethanol. High molecular weight DNA was spooled out on a glass rod, dissolved in TE and stored at 4°C.

The probes λMS1 and λMS31, derived from locus-specific hypervariable sequences (Wong et al, 1987) were provided by ICI Diagnostics, Northwich, England, in the form of cut-out insert. Random oligonucleotide priming (Feinberg and Vogelstein, 1984) was used to label denatured probe with [α-^{32}P]dCTP (Amersham International PLC, Amersham, England). Random oligonucleotide priming is a method which enables DNA to be labelled to a high specific activity. In principle, this technique uses random hexamers of bases which, under the appropriate conditions, hybridize to single stranded DNA and act as primers for initiation of incorporation of bases, including [α-^{32}P]dCTP, into the intervening areas of single-stranded DNA by DNA polymerase I.

DNA from the cell pellets (5 μg) was digested with restriction endonuclease HinfI in buffer (0.015 M Tris HCl, 0.01 M $MgCl_2$, 0.1 M NaCl, 0.001 M DTE, pH 7.5) at 37°C (Anglian Biotechnology, Colchester, England) and electrophoresed for 24 h at 1.3 V/cm in a 0.8% agarose gel. Electrophoresis buffer was 0.04 M Tris, 0.005 M sodium acetate, 0.001 M EDTA, pH 7.6. The DNA was transferred to a Gene Screen Plus nylon filter (Du Pont, Stevenage, England) according to the method described by Perbal (1988). Hybridization of DNA on the filter with ^{32}P-labelled probe was carried out according to the pre-hybridization and hybridization conditions of Maniatis et al (1982).

Prehybridization was carried out in 6xSSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 5 x Denhardt's solution (consisting of 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA) and 100 µg/ml denatured salmon sperm DNA at 65 °C. Hybridization was carried out in a solution containing the constituents of the pre-hybridization solution, plus 0.01 M EDTA and labelled, denatured probe, at 65 °C. Sodium pyrophosphate (0.1%) was added to the pre-hybridization and hybridization solutions, and 10% dextran sulphate was added to the hybridization solution. Non-specific binding was removed by washing the filter in 2xSSC, 0.1% SDS at 65 °C, then in 0.2xSSC, 0.1% SDS at the same temperature. Autoradiographs were prepared by exposing the filters to Kodak X-Omat AR film overnight at -70 °C in the presence of intensifying screens.

2.3.d. Population doubling times

A single-cell suspension was plated into 3.5 cm petri dishes (Nunc) containing 3 ml prewarmed and gassed medium, at a range of cell densities (50, 100 and 200 x 10^3 cells/dish). After 48, 72 and 96 hours incubation, cells were enzymically detached and a single-cell suspension produced. Cells were diluted in a known volume of PBSA and the number of cells in two 0.5 ml aliquots of each sample estimated using a Coulter ZB counter (Coulter Electronics, Luton, England). The mean results from a minimum of two experiments were plotted on semilogarithmic graph paper to indicate the exponential part of the growth curve, and the PDT was calculated over the exponential part using the formula : $PDT = \ln 2 \times t / \ln(Nt/No)$, where t = time interval, No = number of cells at the start of t, and Nt = number of cells at the end of t.

2.3.e. Intermitotic times and cell death rates using time-lapse cinemicroscopy

Exponentially-growing cells in 5 cm plastic petri dishes (Nunc) were placed on an Olympus IMT inverted microscope fitted with a 37 ^{o}C moist chamber and 5% CO_2 supply. The microscope focus was adjusted, and a suitable field of cells was chosen. This consisted of a number of individual cells, or small groups, with spaces between them to allow cell number to increase with time. Cells were left for a few hours to allow the conditions to equilibrate. Photographs of a microscope field of cells under phase contrast optics were taken using a motor-driven cine camera (Bolex) linked to the microscope. The integral timer was set to take photographs at the rate of 15 frames an hour. A black and white film (Kodak AHU) was used, for optimal resolution. Films were analysed on an LW stop-action projector and cells were followed for two to four generations, as previously described (Riddle, 1979). Cells were followed by marking their divisions on paper overlaying the projected film and recording the frame numbers at which divisions occurred, and similarly for the daughter cells, and so on. From the frame numbers, the time between mitoses was calculated for individual cells. The mean intermitotic time for each cell line was calculated from all the individual cells. The numbers of cells dying in each generation and the numbers of cells failing to divide by the end of the film were also determined.

2.3.f. Colony-forming efficiency

A single-cell suspension was obtained and plated into 5 cm petri dishes (Nunc) containing 5 ml prewarmed and gassed medium, at a range of cell densities (400-6400 cells/dish), with three replicates per cell number. After 14 days incubation, colonies were fixed in methanol

and stained in 10% Giemsa (Gurrs Improved R66; BDH). Colonies consisting of more than 50 cells were counted using a binocular dissecting microscope and the colony-forming efficiency (CFE) determined using the formula : % CFE = mean number of colonies per dish x 100/number of viable cells seeded. The mean CFE was calculated from a minimum of 3 separate experiments.

2.3.g. DNA ploidy and proportion of 'S'-phase cells using flow cytometry

Exponentially-growing cells were enzymically detached and approximately 10^6 cells were resuspended in 4 ml medium containing 5% serum. A single-cell suspension was prepared by syringing through 19, 21 and 25 gauge needles, and filtering through 35 μm pore size polyester gauze. Chicken red blood cells (10^5) or human lymphocytes were added as a standard and cells were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Boehringer Mannheim, FRG) at a final concentration of 1 μg/ml in 0.2% Triton X-100. Cells (10^4) were analysed using a Becton Dickinson FACS Analyser (Becton Dickinson, Mountain View, California, USA). DNA histograms were plotted automatically and used to calculate the DNA ploidy levels, from the position of the G_1 peak of the aneuploid cells relative to the peak of the normal diploid lymphocytes or chicken red blood cells. Normal lymphocytes have a DNA index of 1.0; the chicken red blood cells have a DNA index of 0.33. Ploidy levels were expressed as a ratio of the normal diploid DNA content. The coefficient of variation (CV) for the G_1 peak was calculated using an on-line Consort 30 computer, and the data are derived from histograms with CVs of less than 5.

Percentage 'S'-phase cells were calculated as described (Baisch et al,

1976). In principle, the number of cells in the area under the curve between the G_1 and G_2+M peaks are measured. To do this, the mean number of cells per channel in a representative area of the 'S'-phase part of the histogram is multiplied by the number of channels separating the G_1 and G_2+M peaks. This is divided by the total number of cells in the histogram, and expressed as a percentage.

2.4. Cytotoxicity assays

2.4.a. Continuous exposure colony forming assay (Chapter 3, cisplatin and adriamycin sensitivity)

A single-cell suspension of exponentially-growing cells was plated into 5 cm petri dishes (Nunc) containing 5 ml prewarmed and gassed medium at a cell density (400-4000/dish, depending on the plating efficiency of the cell line) which would produce approximately 250 colonies in control dishes. Cells were incubated for 48 h, then the medium was removed and replaced with either fresh medium (five dishes of controls) or with medium containing a range of drug concentrations (three dishes per concentration). Cells were incubated at 37 ^{o}C for a further 6-19 days, depending on the growth rate of the cell line. After fixing and staining (2.3.f), colonies consisting of more than 50 cells were counted. The mean number of colonies at each drug concentration was expressed as a percentage of the controls. Results from a minimum of three separate experiments were combined and averaged, and graphs of percentage cell survival plotted against drug concentration on semi-logarithmic graph paper. Statistical analysis of the data was performed as described (2.9).

2.4.b. One hour exposure colony-forming assay (Chapter 7, levels of resistance in cisplatin-resistant cell lines)

The method for continuous exposure colony-forming assays was followed,

but after a 1 h exposure to a range of drug concentrations, medium was aspirated from all the dishes and the cells were washed three times with warmed (37 °C) medium without serum. Fresh complete medium was replaced in the dishes, which were incubated for a further 12 days before counting colonies. Percentage survival at each concentration was calculated relative to the controls. Statistical analysis was performed as described (2.9), and the results are the means of a minimum of 3 separate experiments.

2.4.c. One hour exposure transfer assay (Chapter 5, cisplatin/DNA binding)

Cell monolayers growing exponentially in 175 cm^2 flasks (Nunc) were exposed to a range of cisplatin concentrations for 1 h. Medium was aspirated and the cells rinsed with PBSA, enzymatically detached and resuspended in medium containing serum. The single-cell suspension was counted using a haemocytometer, and a known number of cells plated into 10 cm petri dishes (Nunc) containing 10 ml prewarmed and gassed medium. The number of cells plated depended on drug concentration and cell line used, ranging from 500/dish (RT112 controls) to 10^5/dish (RT112 and 833K following exposure to the highest cisplatin concentration). After 14 days incubation, colonies were counted and the mean number of colonies at each drug concentration expressed as a percentage of the controls (allowing for differences in cell numbers plated). Results are the means of 3 separate experiments. Statistical analysis was performed as described (2.9).

2.4.d. MTT assay (Chapter 6, MNU and mitozolomide sensitivity; Chapter 7, cross-resistance of cisplatin-resistant cell lines)

This is a rapid colorimetric assay using a tetrazolium dye (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma)

which is reduced by the mitochondrial dehydrogenases of viable cells to purple MTT-formazan (Mosmann, 1983). The assay measures the ability of drug-treated cells to reduce MTT relative to the ability of untreated cells to reduce MTT, and hence provides a measure of cell survival.

Preliminary experiments were carried out to determine the optimum MTT concentration and cell number for each cell line. The optimum combination of these parameters would result in an absorbance of 1-1.5 absorbance units in semi-confluent control wells 7 days later. To determine the optimum assay conditions, 96-well microwell plates (Nunc) were set up in triplicate, containing a range of cell numbers in 200 µl of medium in rows 2-12, and medium alone in row 1. After 7 days incubation at 37°C and 5% CO_2, 50 µl of a range of concentrations of MTT (Sigma), one concentration per plate, were added. The plates were incubated at 37°C for 3 h. Medium containing MTT was aspirated gently, taking care not to dislodge cells. DMSO (dimethyl sulfoxide; Sigma) (100 µl/well) was added to solubilize the purple MTT-formazan crystals, and the plates were shaken gently, to aid solubilization. Absorbance at 540 nm was measured using a Titertek Multiskan MCC spectrophotometer (Flow Laboratories), and the appropriate cell number and MTT concentration chosen from the blank-corrected absorbances. The MTT concentration used for all the cell lines was 800 µg/ml, but cell numbers used differed depending on the cell line.

For drug assays, exponentially-growing cells were enzymatically detached and 150 µl of a single-cell suspension at the predetermined optimum cell density was plated into rows 2-12 of a 96-well microwell plate (Nunc) using a multichannel pipette (Flow Laboratories). Medium alone (150 µl) was added to row 1 as a blank. After 24 hours

incubation at 37°C, 50 µl fresh medium was added to rows 1 and 2 (blanks and controls), or 50 µl of medium containing a range of drug concentrations (rows 3-12). After a further 6 days incubation, 50 µl of 4 mg/ml MTT (Sigma) dissolved in PBSA (final concentration 800 µg/ml) was added to every well and the plates incubated at 37°C for 3 hours. Medium containing MTT was aspirated and DMSO was added, as described above.

Absorbance at 540 nm was measured as above, and mean absorbances in drug-treated wells relative to the controls (row 2) were calculated using an on-line Amstrad PC1640 (Amstrad Consumer Electronics) and a program supplied by Flow Laboratories. The MTT program records the absorbance in every well, then subtracts the mean absorbance in the row of blanks (row 1) from the absorbances in rows 2-12. It then calculates the percentage absorbance in each of the drug-treated rows relative to the controls, taken as 100%, by dividing each mean treated value by the mean untreated (row 2) value and multiplying by 100. Graphs were plotted of each experiment, decreasing absorbance versus increasing drug concentration. IC50 values (ie. concentration of drug which reduces the absorbance to 50% of the control value) were calculated from the straight part of the graphs using the Oxstat program, as described (2.9). The mean IC50s and standard errors (SE)s of a minimum of 3 separate experiments were calculated. Statistical analysis was performed as described (2.9).

2.5. Chemotherapeutic drugs

Standard pharmaceutical preparations of chemotherapeutic drugs were used in these studies (described in Table 2.3). Initial solvents were used as recommended by the manufacturers, at the minimum concentration necessary to obtain solution. For drug assays, the highest

concentration of solvent used in the drug-treated dishes was used as the solvent control. Drugs were made up fresh each time, and used immediately. Exceptions were mitozolomide and carboplatin. Mitozolomide was provided as a 100 mg/ml solution in DMSO, and was stored at -20°C. It is stable under these conditions (G. Margison, personal communication). Carboplatin (10 mg; Sigma) was dissolved in 10 ml sterile water, and immediately frozen at -20°C in glass ampoules. An aliquot was taken out of the freezer and used immediately; unused drug was never refrozen. For development of cisplatin-resistant cell lines, small amounts of cisplatin were required often, so frozen stocks were prepared by dissolving the pharmaceutical preparation in sterile water (to give a final concentration of 1 mg/ml cisplatin in 0.9% sodium chloride), and freezing aliquots at -20°C.

2.6. Cisplatin/DNA binding (Chapter 5)

2.6a. Cisplatin exposure

Cells growing exponentially as semi-confluent monolayers in 175 cm^2 flasks (Nunc) were incubated for 1 hour at 37°C in 10, 25 and 50 μM cisplatin (approximately 3.3, 8.3 and 16.7 $\mu g/ml$) (eight flasks per cisplatin concentration). Medium was poured out of the flasks, cells were washed with 10 ml PBSA and harvested enzymically (2.2.a). Cells from 8 flasks were pooled and pellets consisting of 1-1.5 x 10^8 cells/cisplatin concentration were stored in 50 ml centrifuge tubes (Falcon Labware, Marathon Laboratory Supplies, London, England), in liquid nitrogen. Cytotoxicity at these cisplatin concentrations was assessed in a parallel experiment using one flask for each cisplatin concentration and one control flask incubated in fresh medium for 1 hour at 37°C, as described (2.4.c).

2.6.b. DNA extraction

DNA was extracted as described (Pera et al, 1981). In summary, frozen cell pellets in 50 ml polypropylene centrifuge tubes (Falcon Labware) were thawed and lysed into a solution consisting of 6% w/v p-aminosalicylic acid (BDH), 1% w/v sodium chloride (BDH), 1% w/v triisopropylnaphthalene sulphonic acid (sodium salt) (TIPNS) (Kodak), 6% v/v butan-2-ol (BDH) (1 ml lysis solution/2 x 10^7 cells). Usually 10^8 cells were lysed into 5 ml of this solution. TIPNS is a nuclease inhibitor which stops DNA being degraded. Tubes were shaken until a homogeneous suspension was obtained, then an equal volume of a phenol-based deproteinising solution containing 500 g phenol (BDH), 75 ml m-cresol (Sigma), 0.5 g 8-hydroxyquinoline (BDH) and 55 ml water was added. Tubes were shaken until a milky emulsion was produced, then centrifuged at 4000 rpm, 4 ^{o}C for 30 minutes to separate the DNA-containing aqueous layer from the protein-containing phenol layer. A Pasteur pipette with a U-shaped tip was used to separate the viscous aqueous layer from the phenol layer. The aqueous layer was transferred to a 50 ml conical flask.

2-ethoxyethanol (BDH) (twice the original volume, ie 10 ml) was gently layered on top of the aqueous layer, and the flask was gently swirled to precipitate the DNA. The DNA was wound onto a spatula, removed from the 2-ethoxyethanol, and rinsed three times in absolute ethanol (BDH). DNA was dissolved in 0.5 volume (2.5 ml) double distilled water (ddw), 20 µl 1% RNAse (250 U of T1 and 10 U of T2) (Sigma) was added and the DNA incubated at 37 ^{o}C for 10 min. Proteinase K (100 µl, 1 mg/ml) was added to remove RNAse and any contaminating protein and the mixture was incubated for a further 15 min. The DNA was purified using a phenol extraction. Phenol reagent (3 ml) was added to the DNA solution in a polypropylene tube, the tube was shaken, then

centrifuged at 1000-1500 rpm. The aqueous layer was transferred to a fresh tube and sodium acetate (1%) (250 µl of 10% sodium acetate solution) was added to the aqueous phase. Addition of salt is necessary to precipitate DNA from ethanol. An overlayer of absolute ethanol was added, and the tube was inverted gently to mix. The DNA was hooked out of the tube and rinsed twice in ethanol, then twice in diethyl ether. The DNA was placed in a preweighed Eppendorf microfuge tube. The DNA was dried in a vacuum desiccator with sodium hydroxide desiccant overnight.

Microfuge tubes were weighed and the weight of DNA calculated. DNA samples were dissolved in concentrated hydrochloric acid to a concentration of 30 mg/ml. The lids were fastened and held securely in place while the tubes were incubated at 56 ^{o}C in a waterbath for 1 h, to hydrolyse the DNA. Samples were centrifuged in an Eppendorf microfuge for 5-10 min. Samples (5 µl) were diluted 800-fold in ddw (4 ml) and their absorbance at 260 nm was measured using a Beckman u.v. spectrophotometer, to assess the purity of the DNA. Diluted hydrochloric acid was used as a control. Percentage purity was calculated relative to an absorbance of 1 mg/ml fully hydrolysed DNA of 26 absorbance units.

2.6.c. Measurement of the amount of platinum bound to DNA

A Perkin Elmer atomic absorption spectrophotometer (model 306) with a Perkin Elmer HGA72 graphite cell atomizer was used in these experiments. The graphite furnace was initially heated to 100 ^{o}C for 15 seconds to dry the sample; this was followed by 1 minute of thermal destruction at 1400 ^{o}C, then 8 seconds of atomization at 2600 ^{o}C. Absorption at 266 nm was measured. A standard curve for platinum absorption was obtained using a range of concentrations of platinum

chloride diluted in 1M hydrochloric acid. The amount of platinum contained in 10 μl-50 μl aliquots of DNA samples was calculated from the standard curve and the purity of the DNA, and expressed as nmoles platinum/g DNA. Results are the means of 4 separate experiments. Statistical analysis of the data was carried out as described (2.9).

2.7. Measurement of O^6-alkylguanine-DNA alkyltransferase (ATase) levels in testicular and bladder cell lines

2.7.a. Preparation of cells

Exponentially-growing cells were harvested enzymatically and rapidly resuspended in medium containing serum in order to inactivate trypsin. Cells were washed twice in cold (4 °C) PBSA, counted, and pellets containing 2×10^7 cells were stored in liquid nitrogen.

2.7.b. Preparation of cell extracts

The frozen cell pellets were thawed into 1 ml buffer I, consisting of 50mM Tris, 1mM EDTA, 3mM dithiothreitol at pH 8.3 and stored on ice. Cells were sonicated twice for 10 sec; firstly with 12 μm and secondly with 18 μm peak-to-peak distance, with cooling on ice in between. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Sigma) (0.01 vol or 10 μl of 8.7 mg/ml PMSF in ethanol) was added immediately after the second sonication. The sonicates were transferred into Eppendorf tubes and centrifuged at 16000 rpm, 4 °C for 10 min. The supernatants were transferred to fresh Eppendorf tubes on ice.

2.7.c. Measurement of ATase levels

Substrate (100 μl of 100 μg/ml ^{3}H-MNU-methylated-DNA in buffer I, containing approximately 10000 cpm, 6% of this in O^6-methylguanine) was placed at the bottom of scintillation counter mini-vials (Sterilin Ltd, Teddington, England), and a range of volumes of cell supernatants

were added (3 per sample). Tubes were vortexed briefly. Positive and negative controls were included: the positive control was a range of volumes (10, 20, 40, 60, 80 and 100 μl) of a semi-purified extract of E. coli harbouring the plasmid p061 which contains the E. coli ogt ATase gene. The extract was diluted 1:200 in buffer I containing 1 mg/ml bovine serum albumin (BSA). Buffer I was added as necessary, to make the volumes in all the tubes up to 400 μl. The negative control was substrate and 400 μl buffer I only. Tubes were vortexed and incubated at 37°C for 2 h in a waterbath.

BSA solution (100 μl of 10 mg/ml BSA in buffer I, as a co-precipitant) and perchloric acid (PCA) (200 μl of 4M PCA) were added simultaneously to every tube, to precipitate protein and DNA. After the addition of 2 ml of 1M PCA, the mixture was heated at 75°C for 45 min. This completely hydrolyses residual substrate DNA, but not protein, to acid-soluble material. After centrifugation at 3000 rpm for 10 min, the supernatant was aspirated, the precipitate washed with 4 ml of 1M PCA and resuspended in 300 μl of 0.01M sodium hydroxide. Ecoscint A scintillation fluid (3 ml) (Mensura Technology Ltd, Wigan, Lancs) was added and the amount of radioactivity in the total protein measured using an LKB 1211 Minibeta liquid scintillation counter.

Protein content of the cell extracts was estimated using the Bradford assay (Bradford, 1976). This is based on the change in the peak absorbance of Coomassie Blue dye from 465 to 595 nm when it binds to protein in acidic solution. The kit form, Biorad reagent (Biorad, Watford, England), was used. A range of concentrations of BSA (in duplicate) in Biorad reagent were read at 595 nm and used to plot a standard curve. The absorbances of the cell extracts in Biorad reagent (in triplicate) were measured and their protein concentrations

calculated from the standard curve. Cell extracts were diluted as necessary, so that they fell on the straight part of the standard curve. The amount of ATase was calculated: a graph of cpm in protein was plotted against volume of extract, negative control values providing the background level, and ATase activity was calculated from the slope of the straight line. This was converted to fmol ATase/ml by dividing by 14 (a factor derived from the specific activity of ^{3}H-MNU-methylated-DNA [20 Ci/mmol] and the counting efficiency [31%]), and to fmol ATase/mg protein by dividing by the protein content of the extract. The results are the mean values obtained from two or three separate experiments for each cell line, each individual value being effectively the mean of triplicate estimations.

2.7.d. Measurement of cytotoxicity of methylating and chloroethylating agents

The cytotoxicities of one monofunctional methylating agent (N-nitroso-N-methylurea, MNU) (Sigma) and one crosslinking chloroethylating agent (mitozolomide) (May and Baker, Dagenham, England) against the bladder cell lines RT112, RT112-CP, RT4, HT1376, HT1197 and MGH-U1 and the testicular cell lines SuSa, SuSa-CP, 1618K, Tera II, GH and GCT27, were compared using the MTT assay, as described (2.4.d). Statistical analysis was performed, as described (2.9).

2.8. Development and characterization of cisplatin-resistant testicular and bladder cell lines

2.8.a. Development of cisplatin-resistant cell lines

i. RT112

The RT112 bladder cancer cell line, growing exponentially as a monolayer in 25 cm^{2} flasks (Gibco), was exposed to 80 ng/ml cisplatin. This concentration of cisplatin kills 30% of RT112 cells in

a continuous exposure colony-forming assay. The flask was subcultured 4 days later, and the cells were incubated in fresh medium containing the same cisplatin concentration (80 ng/ml). Two days later, when the cells were again apparently growing exponentially, fresh medium containing 100 ng/ml cisplatin was added. Cells were subcultured again after another 2 days, and 4 days later fresh medium containing 130 ng/ml cisplatin was added. A similar pattern of increasing cisplatin concentration was continued, and after a month of continuous exposure, RT112 cells were growing in 300 ng/ml cisplatin. Treatment was continued for a total of 14 months, and at the end of this time RT112 cells were able to grow through 3.5 μg/ml cisplatin.

ii. SuSa

The SuSa NSGCT cell line, growing exponentially as a monolayer in 25 cm^2 flasks (Gibco), was exposed to 50 ng/ml cisplatin. This concentration kills 90% of SuSa cells in a continuous exposure colony-forming assay. It was chosen because of the ease with which induction of resistance was initiated in RT112. SuSa cells were subcultured at a ratio of 1:2 after 2 days, and incubated in fresh medium containing the same cisplatin concentration. They were subcultured again (1:2) 6 days later, and the cisplatin concentration was increased to 62.5 ng/ml after a further 8 days. After a month of continuous exposure, SuSa cells were growing in 75 ng/ml cisplatin. Treatment was continued for a total of 11 months, and at the end of this time SuSa cells were able to grow through 300 ng/ml cisplatin.

Decisions about when and how much to increase the cisplatin concentration were based on the microscopic appearance of the cells. If large numbers were lifting off, the flask would be replenished with fresh medium at the same concentration, whereas if the cells appeared

to be tolerating the drug, the concentration would be increased by a factor of a maximum of 30%. The stepwise increases in cisplatin concentration for RT112 (A) and SuSa (B) are shown diagrammatically in Figure 2.1. Aliquots of cells were periodically frozen in liquid nitrogen to provide a stock in case of contamination.

Following continuous exposure to cisplatin, the resulting sublines (designated RT112-CP and SuSa-CP) were maintained for 3 months in the absence of drug, and their levels of resistance were determined at monthly intervals. The sensitivities of parent and resistant cell lines were compared using a 1 hour exposure colony-forming assay, as described (2.4.b). There was no change in response during the three months in which the sublines were tested.

Parent and resistant cell lines were regularly screened for mycoplasma contamination, using Hoechst 33258, as described (2.2.b). They were consistently negative. Following development of resistance, RT112-CP and SuSa-CP were used over a restricted range of 10 passages. The population doubling times, intermitotic times, cell death rates and colony-forming efficiencies of parent and resistant cell lines were measured, as described (2.3.d, 2.3.e and 2.3.f).

2.8.b. Percentage DNA-synthesising cells, using bromodeoxyuridine (BrdUrd) staining

BrdUrd is an analogue of thymidine which can be incorporated into DNA during S-phase of the cell cycle. The percentage BrdUrd-stained cells in RT112, RT112-CP, SuSa and SuSa-CP were determined by exposing cells growing exponentially on microscope slides to 10 μM 5-bromo-2'-deoxyuridine (BrdUrd) (Sigma) in serum-free medium for 30 min at 37 °C. Slides were rinsed twice in PBSA, pH 7.4, then fixed in a 1:1 mixture of methanol and ethanol for 1 hour. Slides were stored in 70% ethanol

until immunocytochemical staining with an anti-BrdUrd monoclonal antibody (Gratzner, 1982) was performed as follows.

1. Slides were treated with 0.5% hydrogen peroxide (H_2O_2) (Merck) in methanol for 30 min, to inhibit endogenous peroxidase.

2. In order for anti-BrdUrd antibody to bind to BrdUrd in DNA, the DNA must be partially denatured to obtain single-stranded regions. Slides were treated with prewarmed 1 M HCl (Merck) at 60 °C for 8 min. They were then rinsed 3 times in PBSA, pH 7.2 and the area to be stained was marked on the slide using a diamond.

3. Mouse anti-human anti-BrdUrd antibody (Becton Dickinson, Mountain View, California, USA) was diluted 1:100 in 20% normal rabbit serum (DAKO-immunoglobulins, Denmark) in PBSA. Diluted antibody (250 µl) was added dropwise to slides lying inside a moisture chamber, and left for 30 min. Slides were washed 3 times in PBSA before the second antibody was added.

4. Rabbit anti-mouse IgG conjugated to peroxidase (Dakopatts, Hamburg, FRG) was diluted 1:75 in 0.6% BSA (bovine serum albumin) (Janssen, Beerse, Belgium) in PBSA. Second antibody (250 µl) was added to slides in the moisture chamber for 30 min. Slides were washed 3 times in PBSA.

5. 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) was used to visualize the BrdUrd. When exposed to peroxidase, a brown precipitate is produced. DAB was dissolved in Tris (Merck) buffer, pH7.6, at 0.5 mg/ml. Tris buffer was composed of 6.1 g Tris dissolved in 50 ml ddH_2O, to which 37 ml 1N HCl was added, and the total volume was made up to 1 l with ddH_2O.

6. After 5 min in DAB, the slides were rinsed in running water, dehydrated in graded alcohols (50%, 70%, 95%, 100%), rinsed in xylene and mounted with DePeX and a coverslip.

Known positive slides (tissue sections which had stained positively after incorporation of BrdUrd from an earlier batch) were included as controls when new batches of BrdUrd or anti-BrdUrd antibody were used. Negative controls were always included. These were 1) DAB alone, no first or second antibody, and 2) DAB and second antibody, no first antibody. These were consistently negative. Using phase-contrast microscopy, the proportion of positively-stained nuclei was obtained by counting a total of 1000 cells/slide on duplicate slides. The experiment was repeated at least twice, and the mean and SE for each cell line calculated.

2.8.c. Karyotypic analysis of parent and resistant cell lines

Exponentially-growing cells in 25 cm^2 flasks were incubated in colchicine (50 µl of a 100 µg/ml solution was added to 5 ml of medium in a flask) at 37°C for 1 hour. This arrests cells in metaphase and causes chromosome condensation. Cells were rinsed in prewarmed hypotonic salt solution ('hypo', consisting of 0.3% KCl, 0.02% EDTA, 0.48% HEPES at pH7.4) and incubated in 3 ml 'hypo' for 20 min at 37°C. Cells were pipetted to dislodge them, then 'hypo' and cells were centrifuged at 1000 rpm for 5 min. The supernatant was aspirated gently and the cell pellet was resuspended in about 200 µl 'hypo'. Fixative (a 3:1 mixture of methanol and glacial acetic acid) was added dropwise, with constant mixing. A total of approximately 3 ml of fixative was added to each pellet. Cells were centrifuged at 1000 rpm for 5 min, fixative aspirated and the cells were resuspended in fresh fixative so that the suspension had the cloudy appearance of frosted glass. Microscope slides were cooled at -20°C and 2 or 3 drops of cell suspension were dropped from a height of 6-12 inches. Slides were stored for a week before staining.

Prior to orcein-staining and Giemsa-banding (G-banding), the chromosome spreads were soaked overnight in SSC, dehydrated in 50%, 95% and 100% ethanol and air dried. For orcein-staining, slides were soaked in 2% orcein solution in 45% acetic acid for 1 hour then taken through graded alcohols, immersed in xylene and mounted with DePeX and a coverslip. Modal chromosome numbers were obtained by counting 15 orcein-stained metaphases from each cell line. For G-banding, slides were soaked in 1% trypsin solution in 0.9% saline for 5 minutes at 15 ^{o}C. Trypsinization is required to partially digest the chromosomal proteins. Slides were rinsed in saline, then transferred to 20% Giemsa solution in SSC at pH 6.8 for 5 minutes. Slides were washed in SSC, then air dried. Slides were immersed in xylene and mounted with DePeX and a coverslip. Metaphase spreads in which the chromosomes were clearly banded and not overlapping were photographed. Chromosomes from at least four G-banded metaphases for each cell line were cut out of the photographs, sorted on the basis of their size and banding pattern and pasted onto sheets of white cardboard. Chromosome rearrangements were marked on the sheets. Chromosome rearrangements, losses and gains in the four metaphases of each cell line were recorded.

2.8.d. Induction of micronuclei by cisplatin in parent and resistant cell lines

Micronuclei are either whole chromosomes or acentric chromosomal fragments which are not incorporated into daughter nuclei when a cell divides. Their presence indicates that damage to DNA has occurred. In order to measure micronucleus production in the parent and cisplatin-resistant cell lines following cisplatin exposure, a single-cell suspension of exponentially-growing cells (4×10^5 RT112, RT112-CP, SuSa-CP and 6×10^5 SuSa) was plated into 3.5 cm petri dishes (Nunc),

containing a sterile coverslip and 3 ml prewarmed and gassed medium. After 48 h incubation at 37°C, medium was removed and replaced with medium containing a range of cisplatin concentrations, or medium alone (controls). After a 1 h exposure to cisplatin, cells were washed three times with medium, then incubated in fresh medium for a further 48, 72, 96 or 120 hours. At each time point, two dishes per concentration and control were rinsed in PBSA, fixed for 10 min in methanol-glacial acetic acid (3:1 v/v), rinsed again then air-dried. Micronuclei were stained with Hoechst 33258 (Sigma) (1 μg/ml for 5 min) and counted using a Zeiss fluorescence microscope.

The criteria for identifying micronuclei described by Heddle et al (1981) were used. Briefly, micronuclei were scored if they were: (a) adjacent to, but not touching, the nucleus; (b) stained with less or equal intensity relative to the nucleus, and (c) their diameter was less than one third that of the nucleus. Cells with extensive nuclear fragmentation were not scored. Coverslips were randomized and counted blind. At least 1000 nuclei were scored per coverslip, from at least five different areas of the coverslip, taken at random. The number of micronuclei per thousand nuclei was expressed as a percentage and the mean value for the two coverslips per concentration or control calculated. Percentage micronuclei in control coverslips (background micronucleus production) were subtracted from treated values. The results are the means of 2-4 separate experiments for each cell line.

2.8.e. Cross-resistance studies on cisplatin-resistant cell lines

The responses of parental and resistant cell lines to the inducing agent, cisplatin, and to carboplatin, adriamycin, methotrexate, VP-16 and bleomycin (see Table 2.3.) were compared using the MTT assay, as described (2.4.d). Results are derived from a minimum of 3 separate

experiments; statistical analysis was performed as described (2.9). Levels of cross-resistance (resistance ratios) to each drug were obtained by dividing the IC50 for the resistant subline by the IC50 for the parental line.

2.9. Statistical analysis

Data obtained from cytotoxicity assays (2.4.a to 2.4.d) were handled in the same way. Dose-response curves were plotted for each individual experiment on semi-logarithmic graph paper. Points which lay on the straight part of the curve (judged by eye) were used for linear regression analysis of each experiment using the Oxstat II program on an Amstrad PC1640. IC50, IC70 or IC90 values were obtained from the linear regression analysis, and the means and standard errors (SE) of a minimum of 3 separate experiments calculated. These are presented in tabular form. For the colony-forming assays, graphs were plotted of mean survival (±SE) against drug concentration. SEs were omitted from Figure 1, Chapter 3, to avoid overcrowding the graphs. For the MTT assays, data are presented in tabular form only.

For comparison of drug sensitivity data obtained from testis cell lines with data obtained from bladder cell lines (in Chapters 3 and 6), a non-parametric test, the Mann-Whitney U test, was used, because the data did not appear to be normally distributed. Similarly, for comparison of a number of growth characteristics between testis and bladder cell lines (in Chapter 4), the Mann-Whitney U test was used. However, where the data was assumed to be normally distributed, for example comparing IC70s between individual cell lines (in Chapters 5 and 7), the means and standard deviations (SDs) of the replicate experiments were compared using a Student's unpaired t test.

Table 2.1. Origin of continuous cell lines

CELL LINE	TUMOUR OF ORIGIN	HISTOLOGY OF TUMOUR	PREVIOUS TREATMENT	YEAR OF ESTABLISHMENT	REFERENCE
Non-seminomatous testicular germ cell tumours					
SuSa	primary, testis	terato-carcinoma	none	1976	Hogan et al, 1977
TeraI	lung metastasis	embryonal carcinoma	RT,CT	1970	Fogh and Trempe, 1975
TeraII	lung metastasis	embryonal carcinoma	RT,CT	1971	Fogh and Trempe, 1975
NEC-8	primary, testis	embryonal carcinoma	none	1977	Yamamoto et al, 1979
T3B1	NR	embryonal carcinoma, YST	NR	NR	Cotte et al, 1981
833K	abdominal metastasis	teratoma, seminoma, EC, chorio-carcinoma	CT	1975	Bronson et al, 1980
1618K	primary, retro-peritoneum	embryonal carcinoma	CT	1981	Vogelzang et al, 1983
GH	primary, testis	NR	none	1979	Loewer et al, 1981
HL	primary, testis	NR	none	NR	Loewer et al, 1981
GCT27	primary, testis	terato-carcinoma	none	NR	Pera et al, 1987a

CELL LINE	TUMOUR OF ORIGIN	HISTOLOGY OF TUMOUR	PREVIOUS TREATMENT	YEAR OF ESTABLISHMENT	REFERENCE
Transitional cell carcinomas of the bladder					
RT112	primary, bladder	stage NR grade G2	none	1973	Masters et al, 1986
RT4	recurrence, bladder	stage T2 grade G1	198 Au grains	1967	Rigby and Franks, 1970
T24	recurrence, bladder	stage NR grade G3	none	1970	Bubenik et al, 1973
HT1197	recurrence, bladder	stage T2+ grade G4	none	1972	Rasheed et al, 1977
HT1376	primary, bladder	stage T2+ grade G3	none	1973	Rasheed et al, 1977

Abbreviations:

CT, chemotherapy
RT, radiotherapy
EC, embryonal carcinoma
YST, yolk sac tumour
NR, not recorded

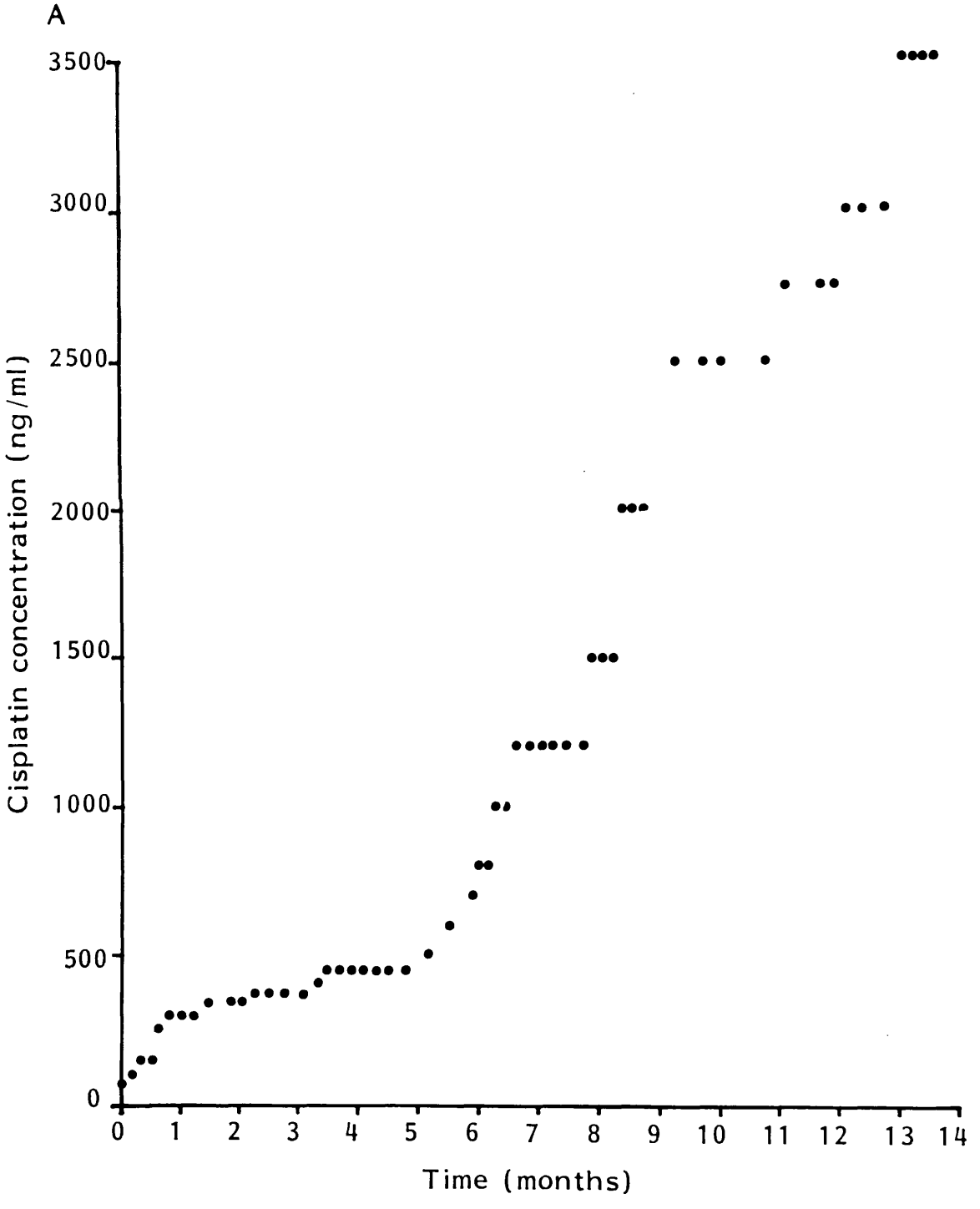
Table 2.2. Details of treatment given to the patient before the tumour sample used to establish the cell line was removed.

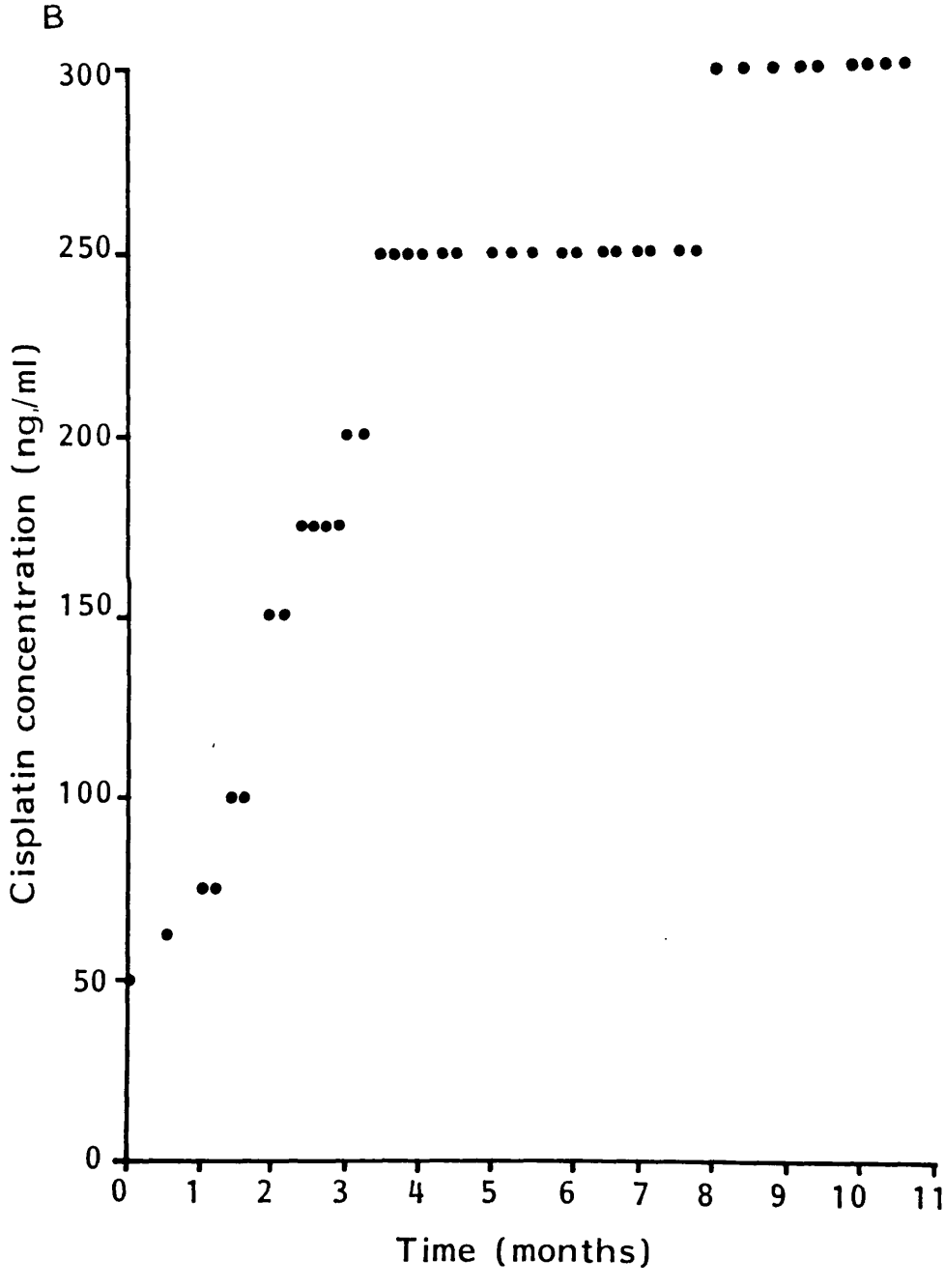
CELL LINE	PRIOR TREATMENT AND TIME BEFORE RESECTION, WHERE RECORDED
Tera I	radiotherapy: 3000 rads to paraaortic region, presumably including area of lung from which cell line was derived chemotherapy: 5-fluorouracil, methotrexate, vincristine, Actinomycin D, cyclophosphamide, vinblastine, mithramycin
Tera II	radiotherapy: 3000 rads, Co 60 source, abdomen and leg (i.e. not directed at lung metastasis from which the cell line is derived) chemotherapy: 5-fluorouracil, methotrexate, vincristine, Actinomycin D, cyclophosphamide, vinblastine
833K	chemotherapy: methotrexate, Actinomycin D, cyclophosphamide, approximately 2 months prior to removal of tumour sample at autopsy
1618K	chemotherapy: 4 cycles of vinblastine, cisplatin, bleomycin, and 5 cycles of cisplatin, bleomycin, adriamycin, VP-16, followed by resection of residual thoracic masses, from which the cell line was established.
RT4	radioactive gold grains implanted into the urothelium two years earlier

Table 2.3. Chemotherapeutic drugs used, together with their sources and initial solvents

drug	initial solvent	source
cisplatin	water	Farmitalia Carlo Erba, St Albans, England
adriamycin (doxorubicin)	PBSA	Farmitalia Carlo Erba, St Albans, England
methotrexate	PBSA	Lederle Laboratories Div, Gosport, England
VP-16 (etoposide)	medium	Bristol-Myers Pharmaceuticals, Slough, England
carboplatin	water	Sigma Chemical Co Ltd, Poole, England
bleomycin	PBSA	Lundbeck Ltd, Luton, England
mitozolomide	DMSO	May and Baker Ltd, Dagenham, England
N-nitroso-N-methylurea (MNU)	DMSO	Sigma Chemical Co Ltd, Poole, England

Figure 2.1. Development of resistant lines: RT112-CP from RT112 (A), and SuSa-CP from SuSa (B). Dots represent addition of fresh medium containing cisplatin.




B
300
250
200
150
100
50
0
Cisplatin concentration (ng/ml)
0
1
2
3
4
5
6
7
8
9
10
11
Time (months)

CHAPTER 3. DIFFERENTIAL SENSITIVITIES OF HUMAN TESTICULAR AND BLADDER TUMOUR CELL LINES TO CISPLATIN AND ADRIAMYCIN

INTRODUCTION

Metastatic testicular germ cell tumours are cured in approximately 80% of patients using combination chemotherapy (reviewed in Chapter 1, General Introduction). In contrast, most other 'solid' tumours in adults show intrinsic or acquired resistance and are difficult or impossible to cure. The mechanisms underlying the sensitivity of testicular tumours to chemotherapy are not understood. In order to investigate the biochemical basis of drug sensitivity relevant model systems were required.

A number of potential model systems were available, including the human tumour colony-forming assay, xenografts and continuous cell lines. Assays have been developed which enable fresh human tumours to be grown *in vitro*, known as human tumour colony-forming assays (Hamburger and Salmon, 1977; Courtenay and Mills, 1978). However, there are numerous problems associated with growing colony-forming cells from biopsies, using them for chemosensitivity assays, and interpreting the data (discussed by Selby et al, 1983). Another model is human tumour xenografts in immunodeficient animals. A number of testicular xenografts were available (for example, Selby et al, 1979; Cotte et al, 1982). However, there are several advantages to using continuous cell lines rather than xenografts as a model system. There are more testis cell lines available, they are easier and cheaper to maintain and use, experimental animals are not required, and results are obtained more quickly. For these reasons, I first tried to establish that cell lines from human tumours retain drug sensitivity *in vitro*.

A possible criticism of using cell lines is that they do not retain the properties of their tumours of origin. However, this study (see Chapter 4), and others (Andrews et al, 1983; Vogelzang et al, 1983; Pera et al, 1987a) have demonstrated that testicular tumour cell lines produce tumours in immunodeficient animals which morphologically resemble NSGCTs, providing evidence that at least some of their biological characteristics are retained despite long periods in culture.

When this study began in 1984 there were no data published about the chemosensitivity of human testicular tumour cell lines. To determine whether continuous cell lines derived from testicular tumours retain their hypersensitivity in vitro, the sensitivities of five non-seminomatous testicular tumour cell lines (SuSa, Tera II, 833K, NEC-8, T3B1) to cisplatin and adriamycin were compared with those of five bladder cancer cell lines (RT4, RT112, HT1376, HT1197, T24). Bladder cancer was chosen for comparison because it is another cisplatin-sensitive urological tumour, but one which is usually incurable due to the presence or development of drug resistance.

Cisplatin was chosen because it is the most active single agent in the treatment of both testicular and bladder cancer (Oliver, 1984; Harker and Torti, 1983), and forms the basis of combination chemotherapy protocols (reviewed in Chapter 1). Adriamycin is active as a single agent against testicular and bladder cancer (Slavik, 1975), and is currently included in the M-VAC and CISCA protocols for treatment of metastatic bladder cancer (Sternberg et al, 1989; Logothetis et al, 1989a).

RESULTS

The responses of the testicular and bladder tumour cell lines to a range of concentrations of cisplatin and adriamycin were compared using a colony-forming assay, as described (Chapter 2, Section 4.a). Dose-response curves, plotted as described in Chapter 2, Section 9, are shown in Figure 3.1 A and B. In order to compare the responses of the cell lines, a point which lay on the straight part of the survival curve, in this case an IC70 (concentration required to reduce colony forming ability by 70%), was used. IC70s for each individual experiment were obtained using regression analysis and these were combined to obtain the mean IC70 for each cell line, as described in Chapter 2, Section 9. The resulting means and standard errors (SEs) are shown in Table 3.1. Subsequently, the responses of a further three testicular tumour cell lines (1618K, GH and GCT27) to cisplatin, were obtained using the same method (see Table 3.1)

In order to assess the overall difference in sensitivity between the bladder and testicular tumour cell lines, their IC70s were analysed in a Mann-Whitney U test (see Chapter 2, Section 9). A non-parametric test was chosen because the IC70s did not appear to have a normal distribution. For example, the NEC-8 testis tumour cell line has a cisplatin IC70 of 161 ng/ml, which is considerably higher than the other testis cell lines, and falls within the range of the bladder cell lines. Similarly with adriamycin, one testis cell line, T3B1, has a similar response to the bladder cell lines. Comparing the responses to cisplatin of the eight testis cell lines and the five bladder cell lines, the testis cell lines are significantly more sensitive than the bladder cell lines ($P<0.02$). Comparing the responses to adriamycin of the five testis and five bladder tumour cell lines, the testis cell

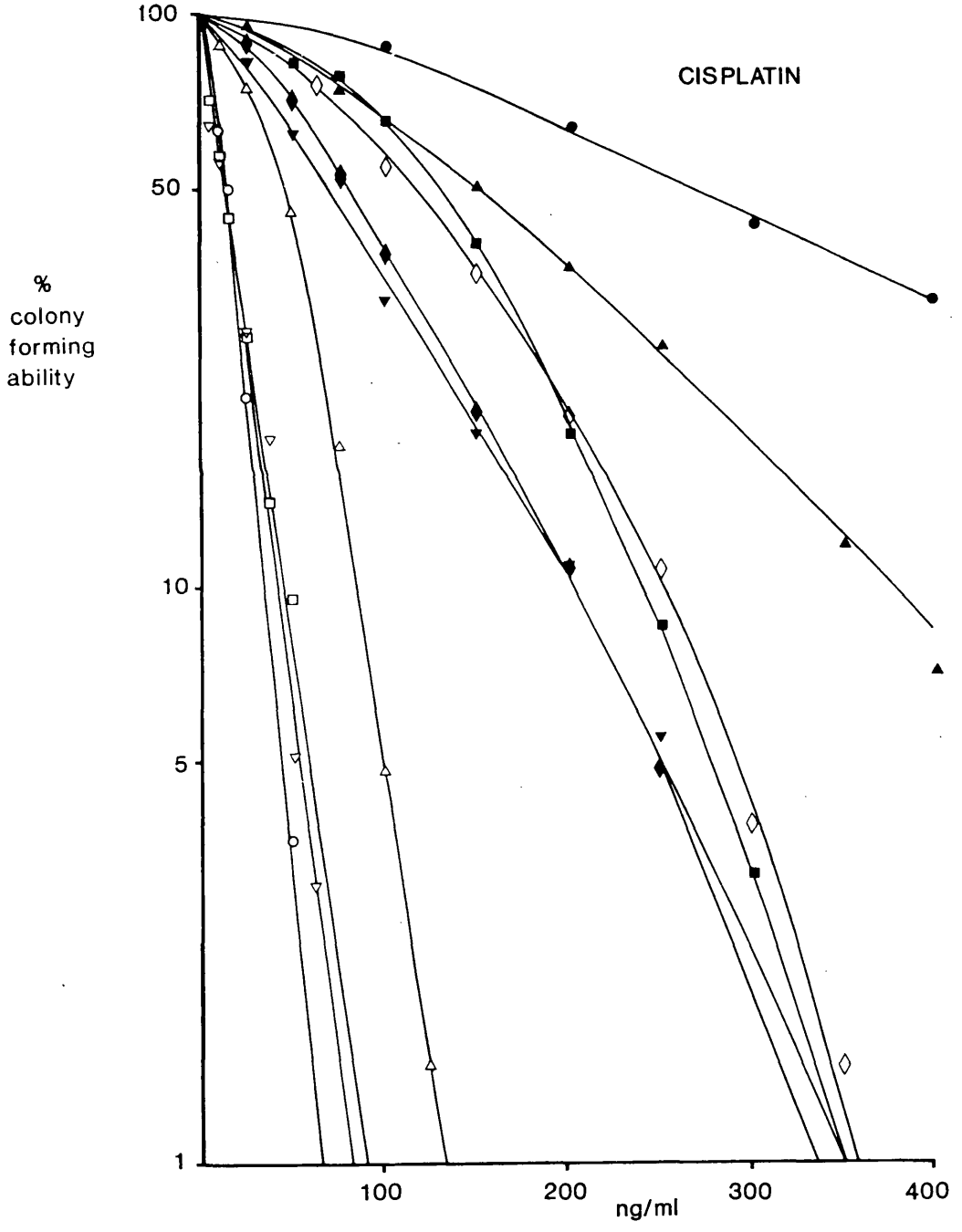
lines are again significantly more sensitive than the bladder cell lines ($P<0.05$).

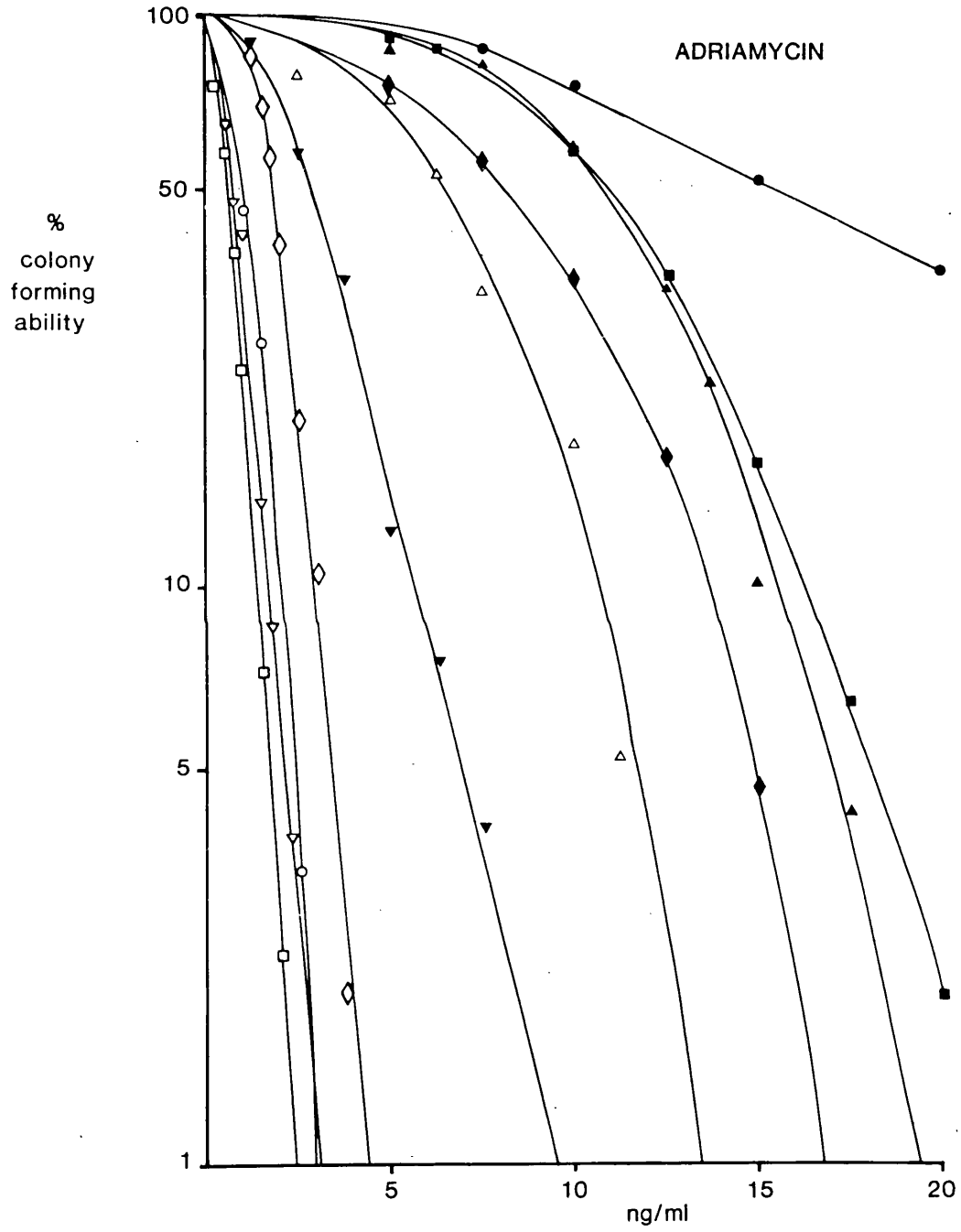
Table 3.1. Concentrations of cisplatin and adriamycin required to reduce colony-forming ability by 70% (IC70) in testicular and bladder tumour cell lines.

Cell line	cisplatin IC70 ±SE (ng/ml)	adriamycin IC70 ±SE (ng/ml)
Testis		
SuSa	23.6±3.4	0.82±0.03
Tera II	18.7±2.5	1.07±0.07
NEC-8	160.8±17.6	2.12±0.03
T3B1	60.2±4.2	7.24±0.09
833K	21.0±1.3	1.16±0.17
1618K	42.2±5.4	ND
GH	23.3±0.9	ND
GCT27	20.6±2.5	ND
Bladder		
RT112	214.3±11.5	12.2±0.3
RT4	430.7±18.3	18.6±0.9
T24	159.4±6.2	12.3±0.9
HT1197	111.6±3.1	3.6±0.4
HT1376	117.9±4.7	10.5±0.7
Range		
Testis	18.7-160.8	0.82-7.24
Bladder	111.6-430.7	3.60-18.6

Abbreviation: ND, not done

Figure 3.1. Dose-response curves of the 5 testicular and 5 bladder tumour cell lines to cisplatin (A) and adriamycin (B). Cell lines are represented by the following symbols: testicular - SuSa (□), Tera II (○), NEC-8 (◊), T3B1 (△), 833K (▽); bladder - RT112 (▲), RT4 (●), T24 (■), HT1197 (▼), HT1376 (♦). Standard errors for individual points are not shown to prevent overcrowding the graphs. The extent of variation between experiments is shown in Table 3.1.




ADRIAMYCIN
100
50
10
5
1
% colony forming ability
5
10
15
20
ng/ml

DISCUSSION

This study shows that cell lines derived from testicular germ cell tumours are hypersensitive to cisplatin and adriamycin, reflecting the clinical responsiveness of NSGCTs to chemotherapy. This is an important observation because it implies that the sensitivity of NSGCTs is not due to humoral factors such as blood supply or immunogenicity, as suggested by Oliver (1985). It is concluded that the chemosensitivity of testis cancer is due to inherent biochemical differences between testis tumour cells and cells derived from less responsive tumour types.

Since this work began, two other studies (Oosterhuis et al, 1984; Pera et al, 1987b) have indicated that testicular tumour cells *in vitro* are hypersensitive to anticancer agents. Cell lines derived from murine and human embryonal carcinomas were compared with cell lines derived from other murine and human tumours, including colorectal, breast and bladder cancer (Oosterhuis et al, 1984). Inhibition of colony formation by a range of cisplatin concentrations was compared in five of the human cell lines. The two testis tumour cell lines studied (Tera I and 2102Ep) were more sensitive to inhibition of colony formation by cisplatin than a melanoma, a colon and a bladder carcinoma cell line (T24) (Oosterhuis et al, 1984). The cisplatin sensitivities of three human testicular tumour cell lines were measured in a colony-forming assay (Pera et al, 1987b). Two of the three testis cell lines were more sensitive to cisplatin than normal human fibroblasts (Pera et al, 1987b).

Extending the study described in this chapter, the sensitivities of five human testicular tumour cell lines and four human bladder cancer cell lines to gamma-radiation, and of two testis and two bladder cell

lines to u.v. radiation were measured (Parris et al, 1988; Parris, 1989). The testis cell lines were more sensitive to gamma- and u.v. radiation than the bladder cell lines. Further evidence for the hypersensitivity of testis tumour cell lines comes from our recent experiments comparing the sensitivities of six testis and five bladder tumour cell lines to twelve different chemotherapeutic drugs, including vinblastine, VP-16, bleomycin, methotrexate and 5-fluorouracil. On average, the testis cell lines were more sensitive to all the drugs than the bladder cell lines, comparing IC50s in an MTT assay (Coren et al, 1990). Thus our data, together with those of Oosterhuis et al (1984) and Pera et al (1987b), indicate that testicular tumour cell lines are hypersensitive to anticancer agents.

Cell lines derived from transitional cell carcinomas of the bladder have been used throughout these studies as a basis for comparison. Metastatic bladder cancer is a tumour type which does respond to cisplatin-based chemotherapy (see Chapter 1, General Introduction), but responses occur in a smaller proportion of patients than NSGCTs, and are generally short-term. A greater differential sensitivity might have been observed between testis tumour cell lines and cell lines derived from relatively unresponsive tumours such as colon or kidney cancer. However, showing a differential sensitivity *in vitro* between two responsive tumour types, which differ in their curability *in vivo*, provides stronger evidence for the suitability of this model system for investigating drug sensitivity.

There are other sensitive tumour types besides NSGCTs, for example certain lymphomas and leukemias which have a high response rate to chemotherapy and are often cured (see Carter et al, 1981). Furthermore, small cell lung cancer (SCLC), although rarely curable

once advanced, responds initially in at least 50% of patients. Further work is required to determine whether panels of cell lines derived from these responsive tumour types are also hypersensitive to chemotherapeutic drugs compared with the more resistant tumour types. If so, this would provide further evidence that cell lines derived from sensitive tumour types retain their hypersensitivity in vitro, and it is not just a property of testis tumour cell lines. Recently, the National Cancer Institute in the US has begun to use panels of cell lines derived from different types of human tumours as a preliminary screen for compounds with antitumour activity, providing further support for the use of continuous cell lines as a model system for human cancer (Paull et al, 1989).

It has been suggested that chemotherapeutic drugs may cure testicular tumours by inducing cell differentiation. Differentiated teratoma, but not EC, is found in the metastases of some patients whose primary tumours had contained both types of cells. This finding has become more common since the introduction of chemotherapy (Oosterhuis et al, 1983). To assess whether exposure to cisplatin caused induction of differentiation in EC cells in vitro, human and murine EC cells were exposed to non-cytotoxic concentrations of cisplatin and their morphology and antigen expression studied (Oosterhuis et al, 1984). Expression of cell-surface differentiation antigens (stage-specific embryonic antigens SSEA-1 and SSEA-3; H2) was unchanged in human and murine EC cells following cisplatin exposure. This in vitro finding supports the generally-accepted view that the presence of residual differentiated teratoma following chemotherapy in vivo is due to the differential sensitivity of EC cells resulting in the preferential killing of these cells by chemotherapeutic drugs, and not to induction

of differentiation in the EC cells by the drugs (Oosterhuis et al, 1983). From my own data, there was no evidence for morphological differentiation in drug-treated testis cells. Furthermore, if differentiation is associated with a slowing-down of proliferation, there was no evidence for this in colonies formed following treatment with cisplatin - cells which survived grew and formed colonies equal in size to the controls.

Previously, cell lines derived from patients with the heritable, cancer-prone syndromes xeroderma pigmentosum (XP), ataxia-telangiectasia (AT) and Fanconi's anemia (FA) have been used to study the mechanisms involved in their sensitivity. Some of these mechanisms may also be implicated in the differential sensitivity of testis and bladder tumour cell lines. AT cells are amongst the most radiosensitive of all human cells to gamma-radiation. In normal cells, exposure to ionizing radiation results in inhibition of DNA synthesis, which is believed to allow time for repair of DNA damage to occur. AT cells, however, fail to inhibit DNA synthesis following exposure to radiation, which might contribute to their radiosensitivity. Having demonstrated that testis tumour cell lines were intermediate in sensitivity to gamma-radiation relative to a normal fibroblast cell line (MRC5-V1) and an AT cell line (AT5BIVA), Parris et al (1988) measured inhibition of DNA synthesis following gamma-radiation in three testis, three bladder and the MRC5-V1 and AT5BIVA cell lines. There was a pronounced inhibition of DNA synthesis in the normal fibroblasts and a similar degree of inhibition was observed in two of the bladder cell lines. In contrast, the AT cells failed to inhibit DNA synthesis altogether. The other bladder cell line and the testis cell lines showed a slightly reduced ability to inhibit DNA synthesis, but this was not as marked as in the AT cells. There was no apparent

correlation within the testis and bladder tumour cell lines between radiosensitivity and inhibition of DNA synthesis.

Fibroblasts derived from patients with the inherited disease XP are hypersensitive to u.v. radiation. There are two groups of XP cells with different u.v. sensitivities in vitro. The most sensitive XP cells are defective in DNA excision repair (summarized in Lehmann et al, 1977). The second group, termed XP 'variant' cells are also hypersensitive to u.v. radiation compared with normal fibroblasts, but to a lesser extent than the excision repair-deficient XP cells. XP 'variant' cells are defective in post-replication repair (Lehmann et al, 1977). The testicular tumour cell lines studied were as sensitive to u.v. radiation as the most u.v. sensitive XP cells, i.e. those which have a defect in excision repair. The bladder cancer cell lines had a similar u.v. response to normal human fibroblasts (Parris, 1989). Thus the response of the two testis cell lines may be controlled by a defect in excision repair. This could be assessed by comparing unscheduled DNA synthesis in the testicular and bladder tumour cell lines, using the method described by Bootsma et al (1970).

Of the eight testicular tumour cell lines whose response to cisplatin was studied, one, NEC-8, was considerably more resistant than the others to cisplatin, showing a response which was within the range of the bladder cancer cell lines. Conversely, another of the testis tumour cell lines, T3B1, showed a response to adriamycin which was within the range of the bladder cancer cell lines. The finding that not all testis tumour cell lines are hypersensitive to chemotherapeutic drugs might appear to bring their use as a model system into question. Alternatively, it may simply reflect the clinical situation, where a proportion of NSGCT patients are not cured

by chemotherapy, due either to intrinsic or acquired resistance. Furthermore, the finding that one testis tumour cell line is resistant to cisplatin and another is resistant to adriamycin, implies that individual tumours may have different patterns of response to different agents, and a tumour which does not respond to cisplatin, for instance, might respond to adriamycin or other chemotherapeutic drugs.

However, NEC-8 and T3B1, the testis tumour cell lines which are the most resistant to cisplatin and adriamycin, are also the two mycoplasma-contaminated cell lines, so it is possible that their increased resistance might be due to mycoplasma contamination. Both cisplatin and adriamycin bind to DNA, and mycoplasma contain DNA, so their presence in the cytoplasm of the cells might provide competition for cisplatin or adriamycin which would otherwise have been binding to the DNA of the tumour cell. However, the finding that one cell line is particularly resistant to cisplatin and the other to adriamycin argues against their resistance being simply due to mycoplasma contamination.

It is possible that the differential sensitivity of the bladder and testis cell lines to cisplatin and adriamycin might be influenced by the treatment given to the patient from whom they were derived, prior to their establishment in culture. There are examples of cases where a cell line derived from a patient after chemotherapy is more resistant than a cell line established from the same patient before chemotherapy (for examples see Wolf et al, 1987; de Vries et al, 1989). None of the bladder cell lines were derived from patients who had received prior chemotherapy, however the patient from whom the RT4 bladder cancer cell line was derived had received local radiotherapy to her tumour two years previously. RT4 is the bladder cell line which is most

resistant to cisplatin and adriamycin and this may be due to the development of cross-resistance to chemotherapeutic drugs following radiotherapy. The gamma-radiation responsiveness of this cell line was not assessed, but if it is also more resistant to gamma-radiation than the other bladder cell lines, this would support the suggestion that its prior treatment has influenced its response as a cell line.

Of the testis tumour cell lines, three of the eight whose sensitivities to cisplatin were measured were established from patients who had previously received chemotherapy. Only one germ cell tumour cell line, 1618K, was derived from a patient who had received cisplatin-based combination chemotherapy (see Table 2.2), and it was the most resistant to cisplatin, if the two mycoplasma-contaminated cell lines are excluded. The patients from whom Tera II and 833K were derived had also received prior chemotherapy, but not adriamycin or cisplatin.

In conclusion, the finding that a panel of testicular tumour cell lines are, on average, hypersensitive to two chemotherapeutic drugs relative to a panel of cell lines derived from a less curable type of tumour supports their use as a model system for studying the molecular basis of drug sensitivity.

CHAPTER 4. BIOLOGICAL CHARACTERISTICS OF TESTICULAR TUMOUR CELL LINES AND COMPARISON WITH BLADDER CANCER CELL LINES

INTRODUCTION

Having shown that testis tumour cell lines are hypersensitive to cisplatin and adriamycin *in vitro*, reflecting the curability of such tumours in patients, I then studied certain biological characteristics of ten continuous cell lines derived from human NSGCTs. The cell lines were transplanted into nude mice to investigate their tumorigenicity and determine whether the morphology of the xenografts corresponded to that of NSGCT. Isozyme analysis was used to confirm a human origin for the cell lines and to determine whether each cell line was derived from a different individual.

There is an association between short volume doubling times in tumours and high response rates to chemotherapy (Skipper and Perry, 1970; Shackney et al, 1978). Therefore, to determine whether there was an association between growth rates and drug sensitivities *in vitro*, some of the growth properties of the testicular and bladder tumour cell lines, including population doubling times, 'S'-phase fractions by flow cytometry, intermitotic times, cell death rates and colony-forming efficiencies, were measured. Similarly, DNA ploidy was compared.

There have been a number of studies describing the characteristics of human NSGCT cell lines (see reviews by Andrews, 1988; Andrews et al, 1987). These studies did not standardize culture conditions, in contrast to my work. Furthermore, most of these studies were concerned with differentiation of testis tumour cells *in vitro*, and not their sensitivity to drugs.

RESULTS

1. Tumorigenicity in nude mice

The ability of the testis cell lines to form tumours in nude mice and their resulting morphology are summarized in Table 4.1. Five of the eight testis cell lines studied were tumorigenic, with differing 'take' rates and latency times. Haematoxylin and eosin stained sections of the xenografts were examined by a histopathologist specializing in urological cancers, who described them all as epithelial tumours, consistent with an origin from testicular tumours. Some of the xenografts showed evidence of differentiation, with areas resembling seminoma in T3B1, HL and GCT27, and yolk sac tumour in T3B1.

2. Isozyme and DNA analysis

The isozyme profiles of seven of the testis cell lines differed at one or more of the loci studied (shown in Table 4.2), indicating that each is derived from a different individual. The profiles also differed from that of HeLa, indicating that the cells are not cross-contaminated with this cell line. The isozyme profiles of NEC-8, HL and GH were identical at the loci studied, so in order to determine whether these cell lines were cross-contaminated, DNA analysis was carried out. DNA analysis is based on the 'DNA fingerprint' technique established by Jeffreys et al (1985a and b). Probes which recognise highly polymorphic regions of DNA (locus-specific minisatellite probes) have been cloned (Wong et al, 1987). The probability of two unrelated individuals having the same DNA banding pattern at the locus recognised by λMS31 is approximately 2 in 10^4, assuming no mutations have occurred (Wong et al, 1987), whereas the probability of them having the same isozyme patterns at the 13 loci commonly used is about 2.5 in 100 (Povey et al, 1976). Hybridization of DNA from GH, HL and

NEC-8 with the λMS31 probe (Figure 4.1) showed that NEC-8 was distinct from HL and GH, but the latter two cell lines had identical bands, showing that cross-contamination had occurred.

3. Growth characteristics

The population doubling times (PDTs), intermitotic times (IMTs), cell death rates, colony-forming efficiencies (CFEs) and proportion of 'S'-phase cells in the testicular and bladder cell lines are shown in Table 4.3. PDTs ranged from 18 to 38 hours in the testicular cell lines, compared with 21 to 61 hours in the bladder cell lines. Thus there is extensive overlap between the two groups of cells with respect to their PDTs. The mean PDT for the testis lines is 26.6±2.2 hours (mean±SE), while for the bladder lines the mean PDT is 34.3±7.3 hours.

IMTs were obtained using time-lapse cinemicroscopy. IMTs ranged from 19 to 32 hours, with a mean of 24.4±1.4 hours, in the testis lines, and from 17 to 47 hours, with a mean of 30.6±7.0 hours, in the bladder lines. Thus there was extensive overlap between the two groups of cell lines. Cell death rates, obtained from the proportion of cells dying during the first two generations of cells analysed for IMTs, ranged from 2% to 47% for the testis cell lines, and 0.5% to 36% for the bladder cell lines. Again there was overlap between the testis and bladder cell lines with respect to cell death rate.

Both groups of cell lines had a wide range of CFEs. These ranged from 6% to 48%, with a mean of 16±5% in the testis cell lines, and from 7% to 87%, with a mean of 39±15% in the bladder cell lines. The proportion of 'S'-phase cells, studied at the same time as DNA ploidy using flow cytometry, was difficult to assess in the testis and

bladder cell lines. The values obtained were only rough approximations, and ranged from 38% to 52% in the testis lines, and from 6% to 38% in the bladder lines. Although the testis cell lines, on average, have higher 'S'-phase fractions, the unreliability of the data means that this finding should be interpreted with caution.

In order to find out whether there was a relationship between chemosensitivity and growth rate in testicular and bladder tumour cells lines, the results presented in this chapter were combined with the data on cisplatin and adriamycin sensitivity from Chapter 3. These are presented together in Table 4.4 and are discussed later.

4. DNA ploidy

Ploidy is expressed as the DNA index, ie. the amount of DNA in each of the cell lines relative to the normal diploid content, taken as a value of 1. Ploidy levels ranged from 1.1 to 1.7 in the testis cell lines, and from 1.1 to 2.4 in the bladder cell lines. The mean DNA index for the testis cell lines was 1.33±0.08, and for the bladder lines, 1.78±0.20. Again there is overlap, RT112 having a similar ploidy level to the majority of the testicular cell lines, and 1618K having a similar ploidy level to the majority of the bladder cell lines.

5. Mycoplasma screening

Eight of the testis cell lines, and all the bladder cell lines, were consistently negative for mycoplasma contamination. However, two of the testis cell lines, T3B1 and NEC-8, were intermittently positive and consequently were examined in less detail in order to avoid possible contamination of other cell lines.

6. Morphology of testis cell lines in vitro

The morphologies of the testis lines, growing under the same culture conditions in 25 cm^2 flasks, were examined under phase contrast microscopy.

SuSa: polygonal cells, tending to form colonies with very tightly-packed cells in the centre. If cultures are left without passaging, the centres of colonies tend to lift off as hollow spheres or cylinders and float away in the medium.

Tera I: sparse, spindle-shaped cells, difficult to maintain, and would not form colonies on plastic.

Tera II: immediately after passaging cultures consist mainly of spindle-shaped cells; as they become more confluent, areas of tightly-packed cuboidal cells predominate.

NEC-8: polygonal, evenly-spaced cells

T3B1: polygonal, regularly-packed cells, tending to form more tightly-packed colonies as they become confluent.

833K: polygonal cells, evenly-packed colonies.

1618K: large, evenly-packed cuboidal cells in the centres of colonies, surrounded by loosely-packed, spindle-shaped cells.

GH/HL: polygonal cells forming discrete colonies.

GCT27: Most cells are cuboidal, with cytoplasmic projections, while some are long and slender. All have prominent nucleoli.

Table 4.1. Tumorigenicity and xenograft histopathology of the testis tumour cell lines

cell line	number of mice	number of tumours and time from inoculation to resection	xenograft histopathology
TeraII	3M, 3F	1 (4 months)	Undifferentiated epithelial tumour
T3B1	3F	3 (3-5 weeks)	Poorly-differentiated epithelial tumour with a high mitotic rate. Some areas consist of cells with regular nuclei and distinct nucleoli, resembling seminoma. Other areas have a microcystic pattern, resembling yolk sac elements.
1618K	4M	3 (3-4 months)	Undifferentiated, anaplastic epithelial tumour resembling teratoma. Cells have a high mitotic rate, with pleiomorphic and hyperchromatic nuclei.
HL/GH	3F, 5M	1 (2 months)	Very anaplastic, epithelial tumour consisting of cells with regular nuclei and distinct nucleoli, resembling seminoma
GCT27	3F, 3M	1 (9 months)	Undifferentiated epithelial tumour. Some areas consist of cells arranged in sheets with scattered multinucleated giant cells, resembling seminoma. Other areas consist of cells with pleiomorphic nuclei, resembling undifferentiated teratoma.
833K	3F, 6M	0	
NEC-8	3F	0	
SuSa	3F, 2M	0	

Abbreviations: F, female M, male

Table 4.2. Isozyme profiles of the testicular tumour cell lines

Cell line	enzyme loci								
	PGM1	PGM3	GOTm	GOTs	ESD	ADA	ACP1	GLO	PGP
Tera I	1	ba	ND	ND	2	1	ND	2-1	ND
Tera II	1	a	ND	ND	2	2	ND	1	ND
SuSa	1	a	ND	ND	2-1	1	ND	2	ND
NEC-8	1	a	ND	ND	1	1	ND	2	ND
T3B1	1	a	ND	ND	1	1	ND	1	ND
833K	1	ba b>a	1	ND	1	1	A	1	ND
1618K	2-1	a	1	ND	1	1	BA	2	ND
HL	1	a	1	ND	1	1	BA	2	ND
GH	1	a	1	1	1	1	ND	2	1
GCT27	1	ND	0	0	ND	1	ND	2	1

Abbreviations:

PGM1 and 3: first and third loci of phosphoglucomutase; GOTm and s: mitochondrial and soluble glutamate-oxaloacetate transaminase; ESD: esterase D; ADA: adenine deaminase; ACP1: first locus of acid phosphatase; GLO: glyoxalase; PGP phosphoglycolate phosphatase; ND: not done.

Table 4.3. Growth characteristics of the testicular and bladder tumour cell lines

Cell line	PDT ±SE (h)	IMT ±SE (h)	% cell death	% CFE ±SE	%'S'-phase cells±SE	DNA index
Testis						
SuSa	27.7±1.3	28.5±1.2	7	5.6±0.9	48±4	1.1
Tera I	38.4±1.3	31.7±1.6	7	-	ND	ND
Tera II	30.8±0.9	23.1±0.7 *	9 *	5.8±1.1	43±7	1.3
NEC-8	17.7	22.4±1.1	20	26.1±4.3	ND	ND
T3B1	18.4	19.2±0.6	28	48.3±4.2	ND	ND
833K	23.2±1.0	19.7±0.7	2	13.0±2.0	44±3	1.3
1618K	32.1±1.8	22.8±0.9	9	9.6±1.8	40	1.7
HL	24.5±2.1	20.1±0.8	21	11.1±2.5	38	1.2
GH	25.2±0.6	24.1±1.2	37	12.2±3.5	42±2	1.3
GCT27	25.7±1.9	28.5±1.1	47	9.0±2.1	52	1.3
Bladder						
RT112	21.6±0.1	21.0±1.1	2	55.3±6.4	28±3	1.1
RT4	37	-	-	6.9±0.6	22±2	1.8
T24	21	17	0.5	87.0±10.3	6±1	1.8
HT1197	61	47	36	9.1±0.8	38	1.8
HT1376	31	37.5±1.5	3	38.3±2.0	30	2.4

Abbreviations:

PDT, population doubling time
CFE, colony-forming efficiency
ND, not done
SE, standard error
IMT, intermitotic time

*
IMT and cell death data derived from one generation only; Tera I did not form colonies on plastic; RT4 forms such tightly-packed colonies that IMT and % cell death could not be obtained.
Bladder PDT (RT4, T24, HT1197, HT1376), IMT and cell death (T24, HT1197) data were obtained from Masters et al (1986), and information about inter-experimental variation was not available.
DNA ploidy levels are the means of two separate experiments; the maximum variation between experiments was ±0.1, so SEs are not shown.
Because of the difficulty in measuring proportion of 'S'-phase cells, some of the 'S'-phase data is derived from one experiment only.
Data on HL is not included in calculations of mean values in the text.

Table 4.4. Growth characteristics and cisplatin and adriamycin sensitivities of testis and bladder tumour cell lines.

Cell line	PDT (h)	IMT (h)	%cell death	CP IC70 (ng/ml)	ADR IC70 (ng/ml)	%CFE	%'S' phase	DNA index
Testis								
SuSa	28	28	7	24	0.8	6	48	1.1
Tera I	38	32	7	ND	ND	-	ND	ND
Tera II	31	23	9	19	1.1	6	43	1.3
NEC-8	18	22	20	161	2.1	26	ND	ND
T3B1	18	19	28	60	7.2	48	ND	ND
833K	23	20	2	21	1.2	13	44	1.3
1618K	32	23	9	42	ND	10	40	1.7
GH	25	24	37	23	ND	12	42	1.3
GCT27	26	28	47	21	ND	9	52	1.3
Bladder								
RT112	22	21	2	214	12	55	28	1.1
RT4	37	-	-	431	19	7	22	1.8
T24	21	17	0.5	159	12	87	6	1.8
HT1197	61	47	36	112	4	9	38	1.8
HT1376	31	37	3	118	10	38	30	2.4

Abbreviations:

PDT, population doubling time
IMT, intermitotic time
CP, cisplatin
ADR, adriamycin
IC70, concentration required to inhibit cell survival by 70%
CFE, colony forming efficiency
ND, not done

Figure 4.1. Autoradiograph of GH, HL, NEC-8 and HeLa DNA hybridized with λMS31 hypervariable probe.

DISCUSSION

The ability of cells of a continuous cell line to form tumours when inoculated subcutaneously into immunodeficient animals is evidence for their continued tumorigenicity. However, the 'take' rate varies, and some well-characterized tumour cell lines are unable to produce tumours on xenotransplantation (Freshney, 1983; Masters et al, 1986). For instance, five cross-contaminated sublines of the bladder cell line T24, which had been growing in different laboratories for long periods, differed in their tumorigenicity (Masters et al, 1986). It is possible that the immunogenicity of the cells, especially cell-surface antigen expression, may change depending on the culture conditions or the time in culture. Although lacking in T-cells, congenitally athymic nude mice do have natural killer cells, which might enable them to recognise some human tumour cells as foreign, and reject them (Hildemann et al, 1981).

In the present study, five of the eight cell lines examined produced tumours, with differing take rates. Tera II and HL/GH had very low take rates, only 1/6 and 1/8 nude mice producing tumours, respectively. Tera II has been xenografted previously, and take rates on s.c. inoculation into nude mide ranged from 1/7 (Jewett, 1978) to 3/3 (Andrews et al, 1984), reflecting the differences that can be obtained with the same cell line. T3B1 had a high take rate in the present study, all the mice producing tumours within 3-5 weeks of inoculation. Tumours developed in 3/4 mice inoculated with 1618K in the present study, and reached approximately 1 cm diameter within 4 months of inoculation. This is a higher take rate than that described previously (Vogelzang et al, 1983), in which 4/12 athymic mice produced tumours within 50 days of inoculation. Xenografts were produced in 1/6 mice inoculated with GCT27 in the present study, with

a long latency period (9 months). In an earlier study, 30% of nude mice formed tumours when inoculated with GCT27, with a median latency period of 14 weeks (Pera et al, 1987a). Using nude mice given whole body irradiation immediately before tumour inoculation, a higher take rate (62%) and shorter latency period (5 weeks) were obtained for GCT27 (Schlappack et al, 1988), indicating that immunosuppression of nude mice may facilitate xenograft production.

The xenografts produced in the present study retained some of the histological features associated with NSGCTs. Previous studies have shown that, comparing the histology of xenografts derived from cell lines with the histology of the original tumours, many testis cell lines have lost their capacity for differentiation (see review by Andrews et al, 1987). Most xenografts consist of cells with the morphology of undifferentiated embryonal carcinoma cells (EC cells). Tera II, GCT27 and 1618K are exceptions - they show some somatic differentiation (Andrews et al, 1984; 1987; Pera et al, 1987a). For example, GCT27 was derived from a tumour containing undifferentiated, differentiated, yolk sac and seminomatous elements (Pera et al, 87a; Schlappack et al, 1988; see Table 2.1). The xenograft obtained in the present study consisted of areas of undifferentiated and seminomatous elements, but no evidence of somatic differentiation. Early-passage cultures (ie. less than 15 passages) of GCT27 produced xenografts with evidence of differentiation - consisting of EC cells with somatic elements including squamous, cuboidal and glandular epithelia, bone, cartilage and cells staining for AFP and HCG (Pera et al, 1987a). Cells from later passages produced xenografts consisting solely of monomorphic cells resembling EC (Pera et al, 1989). This suggests that the number of passages, or the time in culture, can influence extent

of differentiation of xenografts. The GCT27 cells in our laboratory are used between passages 58 and 67, which may explain why, like Pera et al (1989), the xenograft produced in the present study shows little evidence of differentiation.

Although differentiation has been described in one xenograft derived from Tera II (Andrews et al, 1983), xenografts produced from this cell line usually consist of EC cells (Jewett, 1978; Andrews et al, 1984). The histological appearance of the xenograft produced in the present study correlates with the latter description. The one tumour produced from Tera II which contained differentiated elements, described by Andrews et al (1983), was re-established in culture, producing the cell line designated NTera2. Clonal lines were derived from NTera2, and xenografts of these clones consist of EC cells plus various somatic tissues, mainly neural, but also muscular and glandular structures, showing that cell lines, originally derived from Tera II, have retained the capacity for somatic differentiation (Andrews et al, 1984).

The morphology of the xenografts produced by 1618K in an earlier study depended on whether the cells had previously been growing in the presence or absence of a feeder layer. Cells growing on mitomycin c-treated human embryonic lung fibroblasts produced tumours in athymic mice which consisted of EC cells plus well-differentiated epithelial cells arranged in glands (Vogelzang et al, 1983). In contrast, 1618K cells growing in the absence of feeders produced xenografts consisting of pure EC (Vogelzang et al, 1983). The tumours produced by 1618K, also growing without feeders, in the present study, consisted of undifferentiated epithelial cells resembling teratoma, which is consistent with the EC of the American classification system. This

suggests that the growth conditions immediately prior to inoculation of cell lines into nude mice can affect the extent of differentiation of the resulting tumours. Growth on feeder cells may enable testis tumour cell lines to retain more of the differentiated features of their tumours of origin, which are then expressed when the cells are xenografted. Establishment and maintenance of future testis tumour cell lines on feeder layers may increase the chances of developing cell lines which are useful for studies of differentiation and embryonic development.

Of the three cell lines which were non-tumorigenic in this study, SuSa previously did not produce tumours in nude mice soon after its establishment (Hogan et al, 1977). 833K produced tumours in nude mice (Andrews et al, 1980; 1987), as did NEC-8, following inoculation into the cheek pouches of immunosuppressed hamsters, and i.p. into irradiated nude mice (Yamamoto et al, 1979; Motoyama et al, 1987). As in the present study, no tumours were produced when NEC-8 was inoculated s.c. into unirradiated nude mice (Motoyama et al, 1987). If prior irradiation had been given in this study to immunosuppress the animals, take rates might have been higher, and cell lines such as NEC-8 and 833K which were not tumorigenic in this study, but have been in others, might have produced tumours.

The sex of the host animal may contribute to tumorigenicity. Initially, female nude mice were used in this study, but subsequently males were used, in the belief that, since the cell lines were testicular in origin, male hormones might facilitate the growth of xenografts. Furthermore, Jewett (1978) used male nude mice for xenografting fresh testis tumours to avoid problems with the HY antigen system, so again, immunogenicity may be involved. However, in

the present study, the results are inconclusive; only two tumorigenic lines were inoculated into both males and females, and one of these grew in a male and the other in a female.

Isozyme analysis is used to identify cross-contamination between cell lines and to indicate the species of origin. The phenotypes of several polymorphic enzymes have been shown to be stable when comparing human tumour cell lines with normal cells from the patient of origin (Wright et al, 1979). Furthermore, human tumour cell lines grown for many years in different laboratories have stable phenotypes (Povey et al, 1973). DNA analysis using locus-specific hypervariable probes is a more powerful technique for distinguishing between individuals than isozyme analysis, and is replacing isozyme analysis as a method for distinguishing human cell lines. In this study, a combination of isozyme and DNA analysis shows that, except for GH and HL which were established in the same laboratory, each of the testis cell lines is derived from a different individual, and they are not cross-contaminated. The identities of the bladder cell lines were previously established (Masters et al, 1986; 1988). As yet, it is not known whether the cross-contamination of GH and HL occurred before or after arrival in our laboratory, but the originators are sending me further cells to check this point. Previously, five human bladder cancer cell lines, claimed to be distinct, were shown to have been cross-contaminated in other laboratories with T24 (Masters et al, 1986; 1988).

Because of the apparent clinical association between fast growth rate and curability of tumours (Skipper and Perry, 1970; Shackney et al, 1978), I investigated whether a similar correlation existed between growth kinetics and chemosensitivity in the testis and bladder cell

lines. Comparing PDTs, there is overlap between the two groups of cell lines: from 18 to 38 hours for the testicular cell lines, and 21 to 61 hours for the bladder cell lines. Because PDT only gives a measure of the overall growth rate, and does not assess the doubling times of individual cells, nor takes account of the natural cell death rate, IMTs were studied using time-lapse cinemicroscopy. This gives a precise value for the time individual cells take to divide, and follows them through successive divisions. IMTs were calculated from cells followed for 2, 3 or 4 generations, except for NEC-8, which was only followed for 1 generation. They range from 19 to 32 h for the testis lines, and from 17 to 47 h for the bladder lines. Thus, like the result for PDT, there is no clear-cut difference between the testicular and bladder cell lines which might account for their differential chemosensitivity.

Overall, there is a good correlation between the two measures of growth rate (PDT and IMT) in the testis and bladder tumour cell lines. Comparing PDT and IMT in individual cell lines, however, they differ in some cases. For example, several of the cell lines have faster IMTs than PDTs. This would be expected in cell lines which have a high cell death rate, because PDT only assesses the overall doubling time of the whole population, and takes no account of cell loss. This is demonstrated by the bladder cell line HT1197. However, there are other cases where a high cell death rate is not associated with a difference between PDT and IMT, such as GCT27. In this cell line, IMT is longer than PDT, which is an unexpected result. The reasons are unknown, but may be due to the conditions under which the cell lines were grown for the two assays. For the PDT assay, cells were plated at relatively high initial densities and PDT was measured between the two time points on a growth curve which gave the fastest increase in cell

numbers. Thus this method of measuring growth rate selects for the time at which the cells are fastest-growing. In contrast, cells were plated at lower densities for the measurement of IMT. In order to be able to follow cells using time-lapse cinemicroscopy, there must be gaps between individual cells or small groups of cells, which they can divide and move into during the course of photographing them. Thus if they are density-dependent, cells might be growing more slowly during IMT measurement. Furthermore, the cells which are followed are not selected for fast growth, and IMT measurements commonly include cells with IMTs of 50 to 60 hours.

Relating growth rate to chemosensitivity in individual cell lines, the bladder cancer cell lines RT112 and T24 have similar PDTs and IMTs to the testicular tumour cell lines SuSa and 833K, but RT112 and T24 are 7-10 times more resistant to cisplatin, and 10-15 times more resistant to adriamycin, comparing IC70s. Within the two groups of cell lines there is also no association between growth rate and sensitivity. For example, HT1197 is the slowest-growing of all the bladder cell lines, with the longest PDT and IMT, yet it is the most sensitive to cisplatin and adriamycin. Within the testicular cell lines, T3B1 is one of the fastest-growing, with a PDT of 18 hours and an IMT of 19 hours, yet it is the least sensitive of the testis cell lines to cisplatin.

Differences in cell death rates between different tumour types might contribute towards curability. High cell loss rates have been associated with high proliferation rates (Malaise et al, 1973). According to the stem cell theory for tumour cell growth, if a tumour *in vivo* has a high rate of loss of the differentiated 'end' cells, this will increase the net proportion of stem cells, and make the

tumour more difficult to cure. Cell death rates ranged from 2% to 47% for the testicular cell lines, and from 0.5% to 36% for the bladder cell lines. There was no correlation between cell death rate and the growth rates of the cell lines, measured as PDT or IMT. Thus there is no evidence to support the findings of Malaise et al (1973). Cell death rates are difficult to calculate accurately, because some of the slower-growing cells in the population will not be included if they have not divided by the end of the film, increasing the apparent proportion of dead cells in later generations. In order to minimize this error, cell death was calculated from the percentage of cells dying as a proportion of the total number of cells analysed in the first and second generations.

If cells are differentiating in culture, this might be seen as an increasing proportion of slow-growing cells or cells which fail to divide. The proportions of first and second generation cells which had not divided before the end of the film were analysed for each cell line. Three cell lines had apparently high proportions of such cells: Tera I and GH (testis) and HT1376 (bladder). However, when individual cells which failed to divide by the end of the film were analysed, very few, if any, had been followed long enough that their times without dividing exceeded the maximum IMT of the cells which had divided. Thus there was no evidence for a subpopulation of 'differentiated' cells with very long IMTs, or which failed to divide, in any of the cell lines. In order to study whether, as has been suggested, chemotherapy induces the differentiation of testis tumour cells, it would be necessary to treat cells with a low concentration of drug e.g. an IC10 concentration, wash it off and follow the cells using time lapse cinemicroscopy. If a subpopulation was observed which

did not die, but divided very slowly or not at all, there would be evidence for drug-induced differentiation. It would be necessary to follow the cells for a longer time than was done in this study.

There is a wide variation in the CFEs of the testis and bladder cell lines. CFEs are dependent on culture conditions - for instance, when testing batches of serum before buying a new batch, some are unable to support colony formation by the testis and bladder cell lines tested. Serum concentration may also affect CFEs. We use 5% serum, which is a lower concentration than is commonly used for growth of human testis tumour cells, and may reduce their CFEs. Thus culture conditions can cause variation in CFEs, which we minimize by growing bladder and testis cell lines under identical conditions, but I feel it would be unwise to attempt to correlate CFEs with any of the other growth characteristics. In spite of changes in culture conditions, the CFE of GCT27 (9%) in the present study is identical to that originally described (Pera et al, 1987a). Then, the cells were growing in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 1 μg/ml hydrocortisone.

'S'-phase fractions of the bladder cell lines had been measured previously using the labelling index (LI) method. [^{3}H]thymidine uptake into DNA-synthesising cells is measured following a 15 minute exposure (Masters et al, 1986). 'S'-phase values obtained using the two methods differed. For example, T24 had an LI of 51%, but an 'S'-phase fraction of 6% using flow cytometry. T24 is a fast-growing cell line and on this basis a relatively high S-phase would have been expected. The reasons for the low result on three separate occasions using flow cytometry are unknown. If the S-phase cells of T24 were particularly sensitive to trypsinization relative to cells at other phases of the

cell cycle, this might account for the observed low S-phase value. To study this point further, it will be necessary to measure the S-phase fractions of the testis and bladder cell lines using alternative methods, such as [^{3}H]thymidine uptake or BrdUrd uptake.

DNA ploidy levels measure the amount of DNA in cells of a tumour or a cell line. The amount of DNA ranged from 1.1 to 1.7 times the normal diploid level in the testis cells, and from 1.1 to 2.4 times the normal diploid level in the bladder cells. Again, there is overlap, RT112 having a similar level to most of the testis lines, and 1618K having a similar level to most of the bladder lines. The amount of DNA present in the nucleus of a cell may influence sensitivity to drugs such as cisplatin and adriamycin which bind to DNA. At the same cisplatin concentration, a cell which has less DNA may be more sensitive to the cytotoxic effects of cisplatin than a cell with more DNA, because the damage might be more concentrated over a smaller amount of DNA, and less easily repaired. This does not seem to be contributing to differential sensitivity, because of the overlap between the two types of cells: for example, the RT112 bladder cells have a lower ploidy level than five testis cell lines, but are more resistant to cisplatin than any of these testis lines.

Cultures of the testicular cell lines at high cell density all have a typical epithelial appearance, but within this, each of the cell lines has a characteristic morphology. In areas of lower cell density, some of the cell lines take on a more fibroblastoid appearance. This behaviour is typical of epithelial cells, and not specific to testis tumour cells. Morphology of tumour cell lines is highly dependent on culture conditions (Freshney, 1983). Despite this, the testis cell lines in our laboratory retain the morphological features described in

the literature. SuSa cells showed a tendency for colonies to pile up in the centre and form chains of epithelial vesicles which floated off into the medium (Hogan et al, 1977). When sectioned and stained, these were composed of cells resembling the undifferentiated epithelial cells of the original tumour (Hogan et al, 1977). SuSa cells in our laboratory showed a similar tendency, but this was minimized by regular passaging and addition of fresh medium. Tera I and Tera II are the least epithelioid of all the lines. Tera II was previously described as consisting of elongated cells which formed monolayers with smooth, well-defined cell boundaries (Andrews et al, 1980). Under our culture conditions, Tera II cells, when sparse, are elongated, but with increasing time after passage, form areas of tightly-packed, cuboidal cells. Clones of the subline NTera2 (described above) can be induced to differentiate by exposure to retinoic acid. Cells have the morphological appearance of neurons and express neurofilament polypeptides (reviewed by Andrews, 1988). In another testis cell line, 2102Ep, morphological differentiation can be induced simply by changing the culture conditions (Andrews, 1988). Furthermore, a clonal line derived from GCT27 spontaneously differentiates in the absence of feeder cells into cells with features similar to extraembryonic endoderm, and neurons. However, morphological differentiation in vitro rarely occurs to the extent to which it is seen in NSGCTs, or in some xenografts. This may be due to the selection process which occurs when a cell line is established in vitro; cells which are able to grow on plastic may not be the multipotential stem cells, or it may be due to culture conditions; growth as a monolayer may not facilitate differentiation, and 3-D contacts may be required.

In summary, I have shown that human testicular tumour cell lines are hypersensitive to cisplatin and adriamycin compared with human bladder

cancer cell lines. In spite of an *in vivo* correlation between fast growth rate and chemosensitivity, no such relationship was observed in this study, measuring growth rate as PDT or IMT. Xenografts of the testicular tumour cell lines retained some of the histological features associated with NSGCTs, and isozyme analysis confirmed their human origin. Since the differential sensitivity of the testis and bladder cell lines was not due to differences in growth rate, other potential factors controlling sensitivity were studied. Firstly, the amount of cisplatin binding to its target, DNA, was compared in testis and bladder tumour cell lines.

CHAPTER 5. COMPARISON OF CISPLATIN BINDING TO DNA IN TESTICULAR AND BLADDER TUMOUR CELL LINES

INTRODUCTION

The aim of this study was to find out whether differences in the amount of drug binding to its target might contribute to differential sensitivity of testicular and bladder tumour cells. The work presented in Chapters 3 and 4 showed that testis tumour cell lines are hypersensitive to cisplatin and adriamycin compared with bladder cancer cell lines and that they provide a model system with which to investigate the mechanisms controlling differential sensitivity.

Cisplatin is believed to exert its cytotoxic action through binding to DNA (Roberts and Thomson, 1979). Previous studies had examined the relationship between cisplatin-DNA binding and survival in human cells (for example Fraval et al, 1978; Pera et al, 1981; reviewed by Roberts et al, 1986a). These studies indicated that some cell types are more sensitive than others to DNA-bound cisplatin and that this may be due to differences in their DNA repair capacities. However, when this study began, there were no data published comparing cisplatin-DNA binding in testicular tumour cells with other cell types.

The amount of platinum bound to DNA in the 833K testicular cell line and the RT112 bladder cell line after a 1 hour exposure to a range of cisplatin concentrations was measured using atomic absorption spectrophotometry (AAS) (see Chapter 2, Section 6). Cell survival was assessed at the same cisplatin concentrations and under the same conditions, using a colony-forming assay (see Chapter 2, Sections 4.c and 6).

RESULTS

The amounts of platinum (Pt) bound to DNA (expressed as nmoles Pt/g DNA) in 833K and RT112 cells are shown in Figure 5.1. Results are the means and SEs of 4 separate experiments. The amount of platinum bound increases linearly with increasing cisplatin concentration (10, 25 and 50 μM, ie 3, 7.5 and 15 μg/ml). At the same cisplatin concentrations, similar amounts of platinum are bound to DNA in 833K and RT112 cells (Figure 5.1 and Table 5.1). Combining data from all the points, 2.9 ± 0.2 nmol Pt are bound/g DNA/μM cisplatin solution after a 1 hour exposure.

Dose-response curves of 833K and RT112 in a colony-forming assay carried out in parallel to the binding assays are shown in Figure 5.2. In this assay, there is a 2.8-fold difference in cisplatin sensitivity between 833K and RT112, comparing mean IC90s (cisplatin concentration required to reduce survival to 10% of the control level). The mean IC90 for 833K was 8.7 μM (2.6 μg/ml) and for RT112 was 23.3 μM (7.0 μg/ml). The mean IC90s are significantly different when compared using a Student's unpaired t test ($P<0.002$). For 833K, the amount of platinum bound to DNA at an IC90 cisplatin concentration is approximately 26 nmol Pt/g DNA, whereas for RT112 it is 67 nmol Pt/g DNA, giving a 2.6-fold difference in the amount of cisplatin-DNA binding at the same level of cell kill.

Table 5.1. Platinum binding and survival in 833K and RT112 cells.

	cisplatin concentration (μM)	cisplatin concentration (μg/ml)	nmol Pt bound/ g DNA ± SE	% cell survival ± SE
833K	10	3	36.2 ± 2.2	10.9 ± 4.4
	25	7.5	59.2 ± 10.2	0.61 ± 0.32
	50	15	135.3 ± 19.5	0.004 ± 0.002
RT112	10	3	31.1 ± 10.5	47.3 ± 4.7
	25	7.5	65.3 ± 18.9	7.0 ± 3.2
	50	15	138.0 ± 32.5	1.0 ± 0.5

Figure 5.1. Amount of platinum bound to DNA in 833K (▽) and RT112 (▲) cells, expressed as nmoles Pt/g DNA. The points show the means ± SEs of 4 separate experiments.

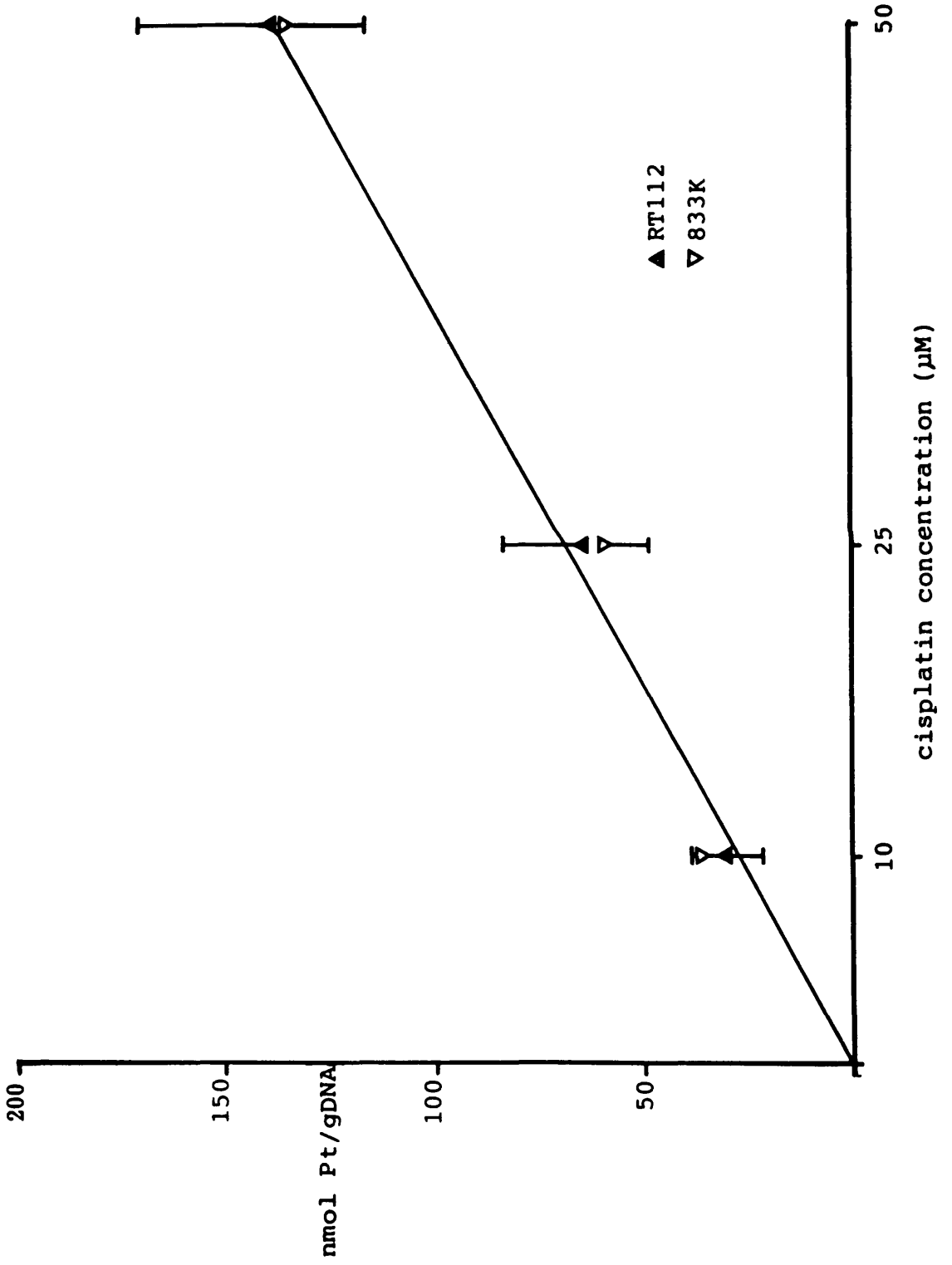
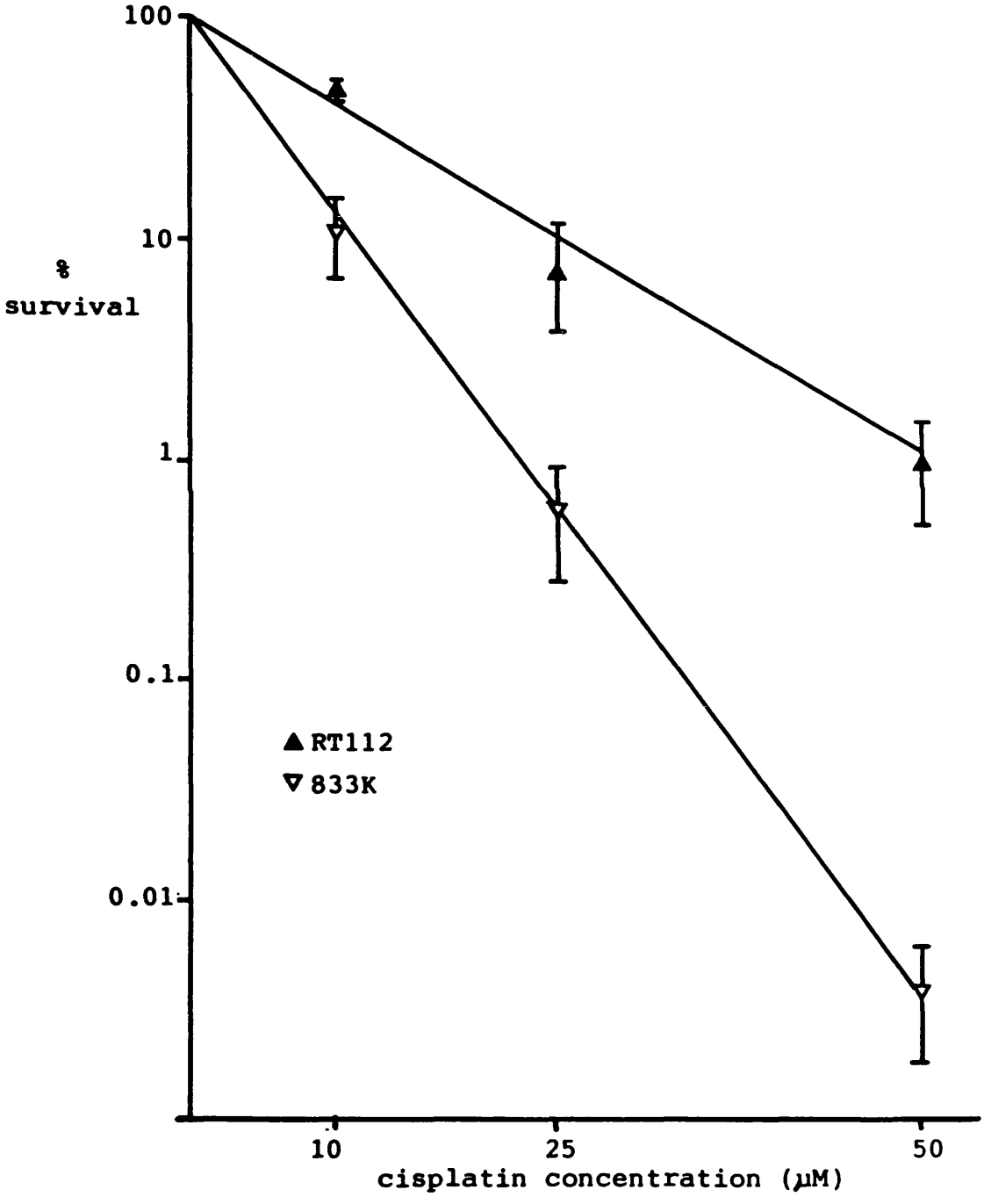


Figure 5.2. Clonogenic survival of 833K (▽) and RT112 (▲) cells following a 1 hour exposure to 10, 25 and 50 μM cisplatin. The points show the means ± SEs of 3 separate experiments.



DISCUSSION

Cell survival and binding of cisplatin to DNA were measured in a testicular and a bladder tumour cell line immediately after a 1 hour exposure to a range of concentrations of cisplatin. The testicular tumour cells were more sensitive to cisplatin, but at the same drug concentrations, similar amounts of platinum were bound in the two cell lines, showing that differences in the initial levels of binding did not account for the difference in response. Comparing the amount bound at equitoxic concentrations, an IC90 dose, approximately 2.6-fold less drug bound to the DNA was required to kill the testicular tumour cells.

At the same time as I obtained these data, Pera et al (1987b) also showed that testicular tumour cell lines are hypersensitive to a given initial amount of cisplatin-DNA binding compared with other cell types. Platinum binding was measured using the same method, AAS. Binding and survival were measured in three human testis tumour cell lines and fibroblasts derived from a patient with the inherited syndrome Fanconi's anemia, which like XP fibroblasts, are hypersensitive to cisplatin. Two of the three testis tumour cell lines, and the FA cells, were more sensitive to cisplatin-induced DNA damage than other cell types, including normal human fibroblasts (Pera et al, 1981) and Walker rat carcinoma cells (Roberts et al, 1986b). Thus these data and the results presented in this chapter show that cells derived from testicular germ cell tumours are more sensitive to a given amount of initial binding of cisplatin to DNA than cells derived from other tumour types, and from normal tissue.

While the overall levels of binding were the same in the testis and bladder cell lines, this study gives no information about where the

drug is bound - it may be binding to areas which are important for survival in testis cells to a greater extent than bladder cells. Chromatin structure will contribute to the accessibiity of the DNA to cisplatin and, furthermore, to repair enzymes. Regions of DNA important for survival may be better protected in bladder cells than testis cells. However, the finding that the same amounts of cisplatin were bound to DNA in bladder and testis tumour cell lines suggested that the mechanism(s) controlling differential sensitivity are operating after this stage, and that ability to repair DNA damage was an important area for subsequent study.

There is evidence for selective repair of key areas of DNA following damage. In human and rodent cells treated with u.v. radiation, pyrimidine dimers are removed more rapidly from the dihydrofolate reductase gene than from the DNA as a whole, indicating that there is preferential repair of active genes (Bohr, 1987). It is possible that bladder cancer cells may be able to repair areas important for survival faster or more accurately than testis tumour cells. If DNA repair over the whole genome was measured using conventional techniques, differences in ability to repair specific genes would not be picked up. Alternatively, some cell types may be better able to tolerate lesions in their DNA and replicate past them, subsequently repairing them by post-replication repair (van den Berg and Roberts, 1975; Fox, 1984). XP 'variant' cells are an example of such a cell type: they are intermediate in u.v. sensitivity relative to excision repair-deficient XP cells and normal fibroblasts. Since the testis cell lines were as sensitive to u.v. as the most sensitive, excision repair-deficient XP cells, evidence so far suggests that they are more likely to be excision repair-deficient than post-replication repair-

deficient. We have undertaken studies on DNA repair in bladder and testis cells and these are discussed in Chapter 8, General Discussion.

In contrast, if the amount of cisplatin bound to DNA had been greater in the sensitive testis tumour cell line than the resistant bladder cell line, this finding would have suggested that the mechanism(s) controlling sensitivity were operating before the drug had bound to its target. In this case, important areas for further study would have been uptake of cisplatin and binding to other intracellular molecules besides DNA - particularly glutathione (GSH) and metallothionein. Binding of cisplatin to GSH or metallothionein would inactivate the drug and prevent it from binding bifunctionally to DNA. Increased levels of GSH and metallothionein have been implicated as resistance mechanisms in cell lines in which cisplatin resistance has been induced *in vitro* (Hamilton et al, 1985; Andrews et al, 1986; Teicher et al, 1987). However, although these factors have not been investigated directly, the data presented here suggest that they do not contribute to the differential sensitivity of testicular and bladder tumour cells, since at the same cisplatin concentrations the same amounts are bound to DNA.

AAS has been criticized as a method for measuring cisplatin binding because it requires relatively high concentrations of cisplatin to be used for the binding to be measurable. However, in this study, the two lower concentrations used (10 and 25 μM cisplatin, equivalent to 3 and 7.5 μg/ml) gave levels of cytotoxicity commonly measured in colony-forming assays. Thus this criticism is not valid. Another way of measuring the amount of cisplatin bound to DNA is to incubate cells in medium containing labelled drug ([^{195m}Pt]cisplatin), extract the DNA and measure the amount of label in the DNA by scintillation counting.

We used this method to compare binding in two bladder cancer cell lines, RT112 and T24 (Bedford et al, 1987a). Roughly similar values for binding of cisplatin to DNA in RT112 cells were obtained using the two different methods. Using [^{195m}Pt]cisplatin, a value for binding of 1.9 nmol Pt/g DNA/μM was obtained, compared with a value of 2.9 nmol Pt/g DNA/μM obtained using AAS.

A third method which also gives a value for the initial amount of cisplatin bound to DNA is measurement of specific cisplatin-DNA adducts using antibodies raised against them, in a competitive ELISA assay (Fichtinger-Schepman et al, 1985). The amounts of four specific Pt-DNA adducts were measured in two testis (833K and SuSa) and one bladder (RT112) cell line in order to study intrastrand crosslinking (Bedford et al, 1988; discussed in Chapter 8, General Discussion). Binding was measured following exposure to a single cisplatin concentration, 16.7 μM (5 μg/ml). In this case, binding at 16.7 μM cisplatin did not correlate with my atomic absorption data presented here, or with cytotoxicity. Comparing initial binding levels, 833K bound twice as much platinum as SuSa, and RT112 was intermediate between these two. However, the total amount bound was obtained by adding the amounts present as the four different adducts, so any errors associated with measurement of each of the adducts would have been compounded. Furthermore, the linear relationship between cisplatin concentration and DNA binding adds support for the AAS data, whereas the ELISA data is derived from a single cisplatin concentration. However, despite the different methods used and possibilities for error, the absolute levels of platinum binding were of the same order: 57 nmol Pt/g DNA for 833K and 33 nmol Pt/g DNA for RT112 using the ELISA assay, compared with 46 nmol Pt/g DNA for 833K and RT112 using AAS, at a cisplatin concentration of 16.7 μM.

Data from a number of other model systems also suggest that cell types which are hypersensitive to drugs and radiation have similar levels of initial cisplatin-DNA binding compared with more resistant cells. Comparing survival of fibroblasts from an individual with the ionising radiation-sensitive syndrome XP with normal human foetal lung fibroblasts, at a given level of DNA binding the XP cells were four-fold more sensitive (Fraval et al, 1978). A marked difference in cytotoxicity relative to the amount of platinum bound was also observed in the Walker 256 rat carcinoma model. The wild-type (WS) cells are extremely sensitive to bifunctional agents (Roberts et al, 1984). Identical amounts of platinum (10 nmol Pt/g DNA) were bound to DNA in both cell lines immediately after a 1 hour exposure to 5 μM cisplatin. This resulted in survivals of 60% in the Walker resistant cells and 0.01% in the Walker sensitive cells (Roberts et al, 1986a).

Thus I have shown that testis tumour cell lines are hypersensitive to cisplatin relative to bladder cancer cell lines and that this is not due to differences in the amount of cisplatin binding to its target in 833K and RT112. This finding suggests that the mechanisms controlling sensitivity in testis cells operate after cisplatin has bound to DNA, and may involve differences in DNA repair capacity.

CHAPTER 6. O^6-ALKYLGUANINE-DNA ALKYLTRANSFERASE ACTIVITY IN HUMAN TESTICULAR AND BLADDER TUMOUR CELL LINES

INTRODUCTION

In order to determine whether testis and bladder tumour cell lines differ in their ability to repair the DNA lesion O^6-alkylguanine, levels of the specific enzyme which removes this adduct, O^6-alkylguanine-DNA alkyltransferase (ATase), were measured. The sensitivities of the bladder and testicular tumour cell lines to nitrosoureas were also assessed. ATase repairs DNA damage caused by methylating and chloroethylating antitumour agents such as procarbazine, BCNU and CCNU and thus plays a role in determining the response of tumour cells to these agents (reviewed by Yarosh, 1985; Fox and Roberts, 1987; Margison and O'Connor, 1989).

Removal of O^6-alkylguanine from DNA was demonstrated in E. coli (Lawley and Orr, 1970) and mammalian cell extracts (Pegg and Hui, 1978) and the repair mechanism has since been elucidated. In both prokaryotes and eukaryotes, an alkyl group is transferred from the O^6 position of guanine to a cysteine residue in the active site of the ATase. Each enzyme molecule can remove one alkyl group and is itself inactivated in a 'suicide' reaction (Olsson and Lindahl, 1980). In E. coli, methylating agents such as MNNG trigger an adaptive response, increasing transcription of the ATase gene *ada* (reviewed by Lindahl et al, 1988). Less is known about regulation of the mammalian enzyme, but it is inducible in some cells (Laval and Laval, 1984; reviewed by Saffhill et al, 1985; Yarosh, 1985). The E. coli *ada* gene has been cloned by several groups. When transfected into a number of different mammalian cell types which lack ATase, it confers resistance to the cytotoxic and mutagenic effects of alkylating agents such as

methylnitrosourea (MNU) and chloroethylnitrosoureas (CNUs) (reviewed by Margison and O'Connor, 1989).

In vivo, ATase levels vary widely between species, between individuals, and between tissues (reviewed by D'Incalci et al, 1988). ATase is present in all normal human tissues studied (see table in D'Incalci et al, 1988), levels ranging from 67 fmol/mg protein in brain to 485 fmol/mg protein in liver (Gerson et al, 1986). Human tumours contain ATase, but the levels frequently differ from the corresponding normal tissues (reviewed by Yarosh, 1985; D'Incalci et al, 1988). For example, a two-fold higher level of ATase activity was found in a human bladder tumour compared with normal bladder cells from the same patient (290 fmol/mg protein in the tumour, 140 fmol/mg protein in normal tissue) (Myrnes et al, 1984). In contrast, some tumours have significantly less activity than normal tissues, a factor which might be exploited by treating such patients with CNUs. However, data from two studies showed that only 3 out of 24 (Myrnes et al, 1984) and 2 out of 6 (Kyrtopoulos et al, 1984) tumours had significantly lower ATase activity than the normal tissue.

Continuous cell lines also vary in their ATase activity. Cells which lack ATase have been designated Mer^-. The Mer^- phenotype was originally defined by the specific inability of cells to reactivate MNNG-damaged adenovirus 5 (Day et al, 1980a) but now applies generally to cells which are deficient in repair of O^6-alkylguanine. The Mer^- phenotype has been found in about 20% of human tumour cell lines studied (Day et al, 1983). Strains of the hamster-derived cell lines V79 and CHO, and of the human cell line HeLa, are usually Mer^-. V79 cells express ATase at a level of 2-4 fmol/mg protein. Viral (SV40) transformation of Mer^+ human fibroblast cell lines often produces Mer^-

cells, which are more sensitive to killing by MNNG than their untransformed parental lines (Day et al, 1980b). Since human tumours appear to be Mer^+, it is possible that ATase can be lost during establishment of cell lines *in vitro* (Fox and Roberts, 1987).

Mer^+ cell lines are commonly resistant to the cytotoxic effects of mono- and bifunctional methylating and chloroethylating agents. The ATase enzyme is thought to reduce the cytotoxicity of agents such as the clinically-used CNUs by removing an alkyl group from the O^6 position of guanine before interstrand crosslinks can be formed (Kohn, 1977; Erickson et al, 1980a; 1980b). Evidence that alkylation at the O^6 position of guanine is cytotoxic comes from studies in which the endogenous ATase is depleted, either by prior exposure of cells to non-toxic doses of alkylating agents, or by exposure to O^6-methylguanine. These pre-treatments increase the sensitivity of the cells to subsequent treatment with alkylating agents (Gibson et al, 1986; Gerson et al, 1988). Further evidence that O^6-alkylguanine in DNA is cytotoxic comes from transfection of the bacterial *ada* gene, or its truncated derivatives, into various Mer^- mammalian cells. Clones with high ATase activity are more resistant to MNU and chlorozotocin, but not to MMS or HN2. MNU and chlorozotocin produce high levels of O^6-methylguanine and O^6-chloroethylguanine, respectively. In contrast, MMS and HN2 cause very little O^6-substitution (see review by Fox and Roberts, 1987). These data provide evidence that O^6-methylguanine is a cytotoxic lesion in mammalian cells.

MNU was chosen for this study because it is a monofunctional methylating agent, producing O^6-methylguanine, but not crosslinks, in DNA. It is a potent mutagen, teratogen and clastogen. MNU underwent

clinical trials in the USSR starting in 1966 and objective responses were observed in 38% of patients (47/122), particularly patients with lung cancer and Hodgkin's disease (Emanuel et al, 1974).

Mitozolomide (M-B 39565) (Stevens et al, 1984) is believed to be a prodrug for a chloroethyltriazene, which acts like a CNU (Gibson et al, 1984). It is believed that CNUs kill cells by initially forming a mono-adduct, O^6-chloroethylguanine, which then forms a crosslink with the cytosine group on the opposite strand of DNA (see review by Margison and O'Connor, 1989). Mitozolomide is highly active against a range of murine tumour model systems (Hickman et al, 1985), and against human tumour xenografts growing s.c. in nude mice (Fodstad et al, 1985). On the basis of the preclinical results, it underwent clinical trial against a number of different solid tumours, in the belief that it might be more effective than the CNUs currently used clinically. Partial responses were obtained in phase II trials of mitozolomide against small cell lung cancer, melanoma and ovarian cancer (Harding et al, 1988; 1989), but no responses were obtained among 15 patients with metastatic bladder cancer (Blackledge et al, 1989). The major side effect was severe, delayed thrombocytopenia which, together with its disappointing lack of clinical activity, resulted in it being withdrawn from clinical trial (G. Blackledge, personal communication).

In vitro, mitozolomide produced a 7-fold greater cytotoxicity (comparing IC50s) in Mer$^-$ compared with Mer$^+$ cells, which correlated with the presence of DNA interstrand crosslinking in Mer$^-$, but not Mer$^+$, cells (Gibson et al, 1984; 1985a). It was chosen for this study because its cytotoxicty is believed to be solely due to chloroethylation of guanine and crosslink formation, and not

carbamoylation, in contrast to other clinically-used CNUs such as CCNU (Horgan et al, 1983; Sariban et al, 1984). Differential cytotoxicity of carbamoylating CNUs against Mer^- cells relative to Mer^+ cells is lower than non-carbamoylating CNUs, due to the sensitivity of both types of cells to killing by carbamoylation (Sariban et al, 1984).

The responses of five testicular and five bladder cancer cell lines, and the cisplatin-resistant sublines SuSa-CP and RT112-CP (described in Chapter 7), to a monofunctional methylating agent (MNU) and a crosslinking chloroethylating agent (mitozolomide) were assessed using the MTT assay, as described (Chapter 2, Section 4.d). ATase levels in the cell lines were measured (see Chapter 2, Section 7 for methods), to determine whether ATase level correlates with nitrosourea sensitivity in these testicular and bladder tumour cells.

RESULTS

Sensitivities of the testis and bladder cell lines to MNU and mitozolomide (expressed as IC50s), and ATase levels (expressed as fmol ATase/mg protein) are shown in Table 6.1.

The testicular cell lines were more sensitive to MNU and mitozolomide than the bladder cell lines. Comparing the means of all the IC50s, the testis cell lines were 3.8-fold more sensitive to MNU, and 4.1-fold more sensitive to mitozolomide, than the bladder cell lines. The means are significantly different ($P<0.01$) for both MNU and mitozolomide, using a Mann-Whitney U test (Chapter 2, Section 9). IC50 concentrations of MNU ranged from 1.2 to 28 μg/ml for testis, and 54 to 67 μg/ml for bladder cell lines. IC50 concentrations of mitozolomide ranged from 0.3 to 3.1 μg/ml for testis, and 4.5 to 9.9 μg/ml for bladder cell lines. Thus there was no overlap in IC50s between the testis and bladder cell lines.

ATase activity in 4 out of 5 bladder cancer cell lines ranged from 279 to 603 fmol/mg protein, higher than any of the testis cell lines. In contrast, one bladder cell line, HT1197, had an ATase level (11 fmol/mg) which was lower than all the testis tumour cell lines except GCT27 (3.3 fmol/mg). ATase activity in the other 4 testicular tumour cell lines ranged from 39 to 206 fmol/mg protein.

Relating IC50s to ATase levels in the bladder cancer cell lines, excluding HT1197 there is some correlation between sensitivity and ATase level. For example, HT1376 has the second highest ATase level of all the cell lines, and is the most resistant to mitozolomide and the second most resistant to MNU. In contrast, HT1197 has a low ATase level but is relatively resistant to MNU and mitozolomide. RT112 and

HT1197 have almost identical IC50s to both MNU and mitozolomide, but RT112 has 387 fmol ATase/mg protein, compared with 11 fmol/mg in HT1197.

Relating IC50s to ATase levels in the testicular tumour cell lines, there is some correlation between sensitivity and ATase level. For example, 1618K has the (joint) highest ATase level of all the testis cell lines, and is the most resistant of all the testis cell lines to MNU and mitozolomide. GCT27 has the lowest ATase level, and is the most sensitive to both agents. Between these extremes, the other testis cell lines also show some correlation. However, anomalies exist; for example, 1618K and Tera II have similar IC50s to MNU and mitozolomide, but 1618K has 206 fmol/mg ATase, compared with 39 fmol/mg in Tera II.

Relating sensitivity and ATase levels across the two groups of cells, GCT27 (testis) and HT1197 (bladder) have similar ATase levels (3.3 and 11 fmol/mg protein), but GCT27 is 50-fold more sensitive to MNU, and 14-fold more sensitive to mitozolomide, than HT1197. Four of the testis cell lines have higher ATase levels than HT1197, but they are 2-6 times more sensitive to MNU and mitozolomide, comparing IC50s. Ignoring HT1197 and GCT27, the two testis cell lines with the highest ATase levels, 1618K and SuSa, have approximately 25 % less ATase than RT4, the bladder cell line with the lowest ATase level, but the bladder cell line is 2-5 times more resistant to MNU and 5-6 times more resistant to mitozolomide, than 1618K and SuSa.

Comparing cisplatin-resistant SuSa-CP and RT112-CP with their sensitive parental lines, SuSa-CP has a significantly higher level of ATase than SuSa (470 compared with 206 fmol/mg protein) ($P<0.01$ in a Student's unpaired t-test). There is also a difference in sensitivity:

comparing IC50s, SuSa-CP is 2.4-fold more resistant to MNU, and 3.1-fold more resistant to mitozolomide than the SuSa parental line. RT112 and RT112-CP have similar ATase levels, and similar sensitivity to MNU. However, RT112-CP is 2.3-fold more resistant to mitozolomide than the parental line.

Table 6.1. ATase activity and sensitivity to MNU and mitozolomide

Cell line	ATase level ±SE (fmol/mg protein)	MNU IC50±SE (μg/ml)	mitozolomide IC50±SE (μg/ml)
Testis			
SuSa	206 ± 27	12.2 ± 3.4	1.34 ± 0.18
Tera II	39 ± 23	26.1 ± 8.1	1.15 ± 0.22
GH	106 ± 15	13.1 ± 1.3	0.68 ± 0.16
1618K	206 ± 24	28.1 ± 2.8	1.82 ± 0.31
GCT27	3.3 ± 0.7	1.18 ± 0.21	0.33 ± 0.06
SuSa-CP	470 ± 20	29.0 ± 1.8	4.19 ± 0.50
Bladder			
RT112	387 ± 9	56.9 ± 4.1	4.46 ± 0.35
RT4	279 ± 1	66.7 ± 2.0	8.84 ± 0.44
HT1376	509 ± 67	65.6 ± 3.1	9.94 ± 1.88
HT1197	11 ± 9	59.6 ± 4.0	4.49 ± 0.49
MGH-U1	603 ± 58	53.9 ± 4.9	5.27 ± 0.50
RT112-CP	301 ± 75	67.3 ± 4.9	10.49 ± 0.64
Testis			
Mean		16.1	1.06
Range		1.2-28.1	0.33-1.82
Bladder			
Mean		60.5	6.6
Range		53.9-66.7	4.46-9.94

DISCUSSION

The data obtained in this study suggest that ATase levels do not account for the differences in sensitivity to CNUs between bladder and testicular tumour cell lines. For example, HT1197 (bladder) and GCT27 (testis) contain similar ATase levels, but have a 50-fold difference in sensitivity to MNU and a 14-fold difference in sensitivity to mitozolomide.

ATase activity does correlate with CNU cytotoxicity in human and murine cell lines in some studies (Day et al, 1980b; Scudiero et al, 1984; Bodell et al, 1986; Jelinek et al, 1988; Smith and Brent, 1989) but not others (Samson and Linn, 1987; Maynard et al, 1989). There are several possible mechanisms besides ATase level which could explain differential sensitivity to CNUs. These include differences in uptake of drug and binding to intracellular molecules other than DNA, such as glutathione (GSH). Uptake and binding to GSH have not been measured, so they are discussed in relation to differential sensitivity in general in Chapter 8, General Discussion.

If ATase levels alone are not enough to account for the differential sensitivity of testis and bladder cells, it is possible that other repair mechanisms capable of removing O^6-lesions exist. For example, V79 and V79/79 Chinese hamster cell lines, which lack ATase activity, can remove O^6-n-butylguanine from DNA (Boyle et al, 1987). This is probably by nucleotide excision repair, the process which recognises and removes bulky adducts including u.v.-induced pyrimidine dimers. In certain cases, the smaller methyl group can also be removed by an excision repair process. Following MNU treatment, the V79/79 strain can remove O^6-methylguanine from its DNA, but V79 cells cannot (Wild

et al, 1983; Boyle et al, 1987). Thus the substrate specificity of the non-ATase repair mechanism appears to vary between these cell lines.

This study confirms others (Day et al, 1980b; Gibson et al, 1985a) which show that monofunctional methylating agents are cytotoxic. Cell kill by MNU appears to be due to different mechanisms in different cell types. For example, exposure of Mer^- HeLa cells to MNU in early S-phase caused inhibition of DNA synthesis in the second post-treatment S-phase. This implies that, while the O^6 lesions did not block replication in the first post-treatment S-phase, they did in the second. If the lesions were recognised by an endonuclease which excised the DNA close to the lesion, in the next round of DNA synthesis double strand breaks could be produced (reviewed by Fox and Roberts, 1987). In contrast, V79 cells, which are also Mer^-, but considerably more resistant to MNU, may lack the ability to incise DNA close to O^6 lesions, so reduced numbers of double strand breaks form. Similar events might be responsible for the difference in sensitivity between the HT1197 and GCT27 cell lines, which also have very low levels of ATase. The GCT27 cells may respond like the Mer^- HeLa cells described above, causing double strand breaks and cytotoxicity, while the HT1197 cells may respond like the V79 cells, causing considerably fewer double strand breaks and less cytotoxicity.

Another factor that might contribute to differential sensitivity is the existence of different mechanisms of cell killing by CNUs besides production of O^6-lesions. Alkylating agents produce at least thirteen different lesions in DNA, in varying proportions, most nitrogen and oxygen atoms being possible sites of attack (Margison and O'Connor, 1989). Besides O^6-alkylguanine, other lesions produced in isolated DNA include N7-methylguanine and N3-methyladenine (reviewed by Margison

and O'Connor, 1989). For methylating agents, O^6-methylguanine and N7-methylguanine (the major product) do not block DNA synthesis, however N3-methyladenine does (Margison and O'Connor, 1989). Thus formation and repair of lesions other than O^6-methylguanine may contribute to sensitivity in some cell types. One mechanism of repair which might be involved is via DNA glycosylases, enzymes involved in repair of certain N-alkylated purines. If the testis cells are deficient in any of the stages involved in excision repair, and if this is an important protective mechanism for testis and bladder cell lines exposed to alkylating agents, this might account for their greater sensitivity to MNU and mitozolomide.

Several studies have shown that pretreatment of Mer^+ cells with O^6-methylguanine sensitizes them to killing by nitrosoureas (Dolan et al, 1988; Gerson et al, 1988). In contrast, pretreatment of Mer^+ Raji cells with O^6-methylguanine reduces the level of ATase, but does not sensitize the cells to the cytotoxic effects of MNNG, BCNU or CCNU. This implies that O^6 lesions are not the cytotoxic lesion in these cells, and another binding site is responsible for cell killing (Karran and Williams, 1985). Another explanation would be that the O^6 lesions are cytotoxic, and although the cells are Mer^+, a different repair mechanism, possibly an excision repair mechanism, which is not affected by depletion of ATase, is being used to remove the O^6 lesions. Following on from the conclusions of Karran and Williams (1985), if lesions other than those at O^6 of guanine are responsible for cytotoxicity in the bladder and testis cell lines studied here, differential sensitivity of bladder and testis cells might be due to differences in the formation and repair of lesions other than O^6-alkylguanine. It is also possible that the differential sensitivity may be due to a combination of effects and not to a single factor.

An alternative explanation for CNU resistance in cells with low ATase activity is that some cells may be better able to tolerate or bypass the O^6-alkylguanine lesion in their DNA than others (Fox and Roberts, 1987). Another factor which may influence the response of cells to CNUs is the accessibility of the O^6-alkyl group to ATase. The arrangement of DNA and histone proteins in chromatin may make the lesion inaccessible in cell lines, such as the testis lines, which have relatively high ATase levels but are sensitive to MNU and mitozolomide.

Ability of cells to resynthesise ATase may play a role in determining sensitivity to MNU. The ATase assay measures the level of ATase in untreated cells. However, the MTT assay assesses the cytotoxicity of MNU and mitozolomide over 6 days' incubation in drug. In theory, since MNU does not crosslink DNA, the cells could resynthesise ATase and use it to continue removing O^6 lesions. In contrast, following mitozolomide treatment, crosslink formation reaches a peak about 12 hours after treatment (Gibson et al, 1984), preventing ATase removing the methyl group. Hence resynthesis of ATase is unlikely to contribute to protection from mitozolomide cytotoxicity. A cell line which has a high initial level of ATase, but a low resynthesis capacity, might appear more sensitive to MNU than a cell line with the same initial level, but a high resynthesis capacity. Hence differences in ability to resynthesise ATase following MNU exposure might contribute to differential sensitivity of bladder and testis cells. However, a factor arguing against resynthesis as an important determinant of sensitivity is the short half-life of MNU and mitozolomide in medium at $37^{o}C$ (approximately 1 hour for mitozolomide (Brindley et al, 1987)). If ATase is the protective mechanism, initial levels are

likely to be an important factor in determining response, and not ability to resynthesise it in the next round of protein synthesis. The ability of surviving cells to resynthesise ATase following MNU exposure could be measured using uptake of [^{35}S]methionine to label all newly-synthesised proteins. Gel electrophoresis could be used to separate the proteins and autoradiography to check whether label was incorporated into a band corresponding to ATase.

Cisplatin is believed to bind predominantly to the N7 position of guanine, and to form DNA inter- and intrastrand crosslinks and DNA-protein crosslinks. No correlation has been found between the Mer phenotype and the extent of cisplatin-induced DNA interstrand crosslinking in human tumour cell lines, suggesting that ATase is not involved in repair of cisplatin-induced lesions (Laurent et al, 1981; Gibson et al, 1985b). In vitro, there is evidence for ATase activity being increased by exposure of cells to cisplatin. The H4 rat hepatoma cell line was incubated in an IC50 concentration of cisplatin for 1 hour; ATase was measured 48 hours later, and an approximate 4-fold increase in the number of ATase molecules per cell was observed (Lefebvre and Laval, 1986). In the present study, there was no evidence for increased ATase level in the subline of the RT112 bladder cell line which had been exposed continuously to cisplatin for a year, and had stable, induced cisplatin resistance (RT112-CP). MNU sensitivity was unchanged, but RT112-CP was 2.3-fold more resistant than RT112 to mitozolomide. This suggests that RT112-CP has acquired resistance to crosslinking alkylating agents, but not monofunctional alkylating agents, since sensitivity to MNU is not affected. This result implies that the resistance is not mediated by ATase, since ATase would be expected to increase resistance to both MNU and

mitozolomide. It might be due to an enhanced ability to repair crosslinks by an alternative mechanism, although this is not supported by our data on crosslink removal by RT112 and RT112-CP following cisplatin exposure (Bedford et al, 1987b).

The cisplatin-resistant testicular cell line, SuSa-CP, had a higher ATase level than the parental line, and was cross-resistant to both MNU and mitozolomide. This implies that the increased ATase may be contributing to MNU and mitozolomide resistance in SuSa-CP. The level of ATase in the SuSa-CP cells was comparable with the levels in some bladder cell lines. However, the SuSa-CP cells remained 2-fold more sensitive to MNU and mitozolomide than the bladder cell lines with comparable ATase levels.

The finding that the sensitivity of testis tumour cell lines to MNU and mitozolomide is not due to the lack of the specific repair enzyme O^6-alkylguanine-DNA alkyltransferase does not rule out the involvement of repair mechanisms in determining their sensitivity to anticancer agents. The SuSa testicular cell line is deficient in repair of DNA inter- and intrastrand crosslinks caused by cisplatin whereas the 833K testis and RT112 bladder cell lines are proficient in repair of these lesions (Bedford et al, 1988). Further studies on the repair capacity of the testis and bladder cell lines are currently being undertaken, using a plasmid-repair assay (described in Debenham et al, 1987).

CHAPTER 7. DEVELOPMENT AND CHARACTERIZATION OF CISPLATIN-RESISTANT HUMAN TESTICULAR AND BLADDER TUMOUR CELL LINES

INTRODUCTION

Cisplatin is the most active single agent in the treatment of metastatic testicular germ cell tumours and transitional cell carcinomas of the bladder (see Chapter 1, General Introduction). 20% of patients with metastatic testis cancer and most of those with metastatic bladder cancer die of disease uncontrolled by cisplatin-containing combination chemotherapy. Cisplatin-resistant cell lines have been developed from many types of tumour to provide model systems for identifying the mechanisms involved and methods of overcoming resistance. However, cisplatin-resistant human bladder and testicular tumour cell lines have not previously been described.

Testis tumour cell lines are, on average, more sensitive to cisplatin than bladder cancer cell lines (Chapter 3), providing a model system for studying the mechanisms controlling intrinsic sensitivity. However, it is not known whether the mechanisms controlling acquired drug resistance are the reverse of those underlying drug sensitivity, or are unrelated. To compare acquired drug resistance with intrinsic drug sensitivity, cisplatin-resistant bladder and testicular tumour cell lines were developed and characterized.

RESULTS

Development of resistance

After 14 months exposure to gradually increasing concentrations of drug, the resulting RT112 subline, designated RT112-CP, was able to grow continuously in 3.5 μg/ml cisplatin. After 11 months exposure to increasing concentrations of drug, SuSa-CP was able to grow in 300 ng/ml cisplatin.

Cytotoxicity assays

Dose response curves for the parent and resistant cell lines are shown in Figure 7.1. The mean concentrations of drug required to reduce colony forming ability by 70% (IC70) are shown in Table 7.1. Comparing IC70s, RT112-CP was 4-fold more resistant to cisplatin than RT112, and SuSa-CP was 4-fold more resistant than SuSa. Comparing the mean IC70s in a Student's unpaired t test, they are significantly different ($P<0.001$) for both RT112-CP versus RT112 and SuSa-CP versus SuSa. These levels of resistance were stable when RT112-CP and SuSa-CP were maintained in the absence of cisplatin for three months.

Characteristics of parent and resistant cell lines

The population doubling times (PDTs), colony-forming efficiencies (CFEs), intermitotic times (IMTs) and proportion of DNA-synthesising cells using BrdUrd uptake, in parent and resistant cell lines are shown in Table 7.1. Comparing RT112 and RT112-CP, PDT, IMT and CFE were similar, but the proportion of BrdUrd-positive cells was significantly increased ($P<0.05$ using a Student's unpaired t test). Comparing SuSa and SuSa-CP, PDT was similar, but IMT, CFE and proportion of BrdUrd-positive cells were significantly different (see Table 7.1 for P values). CFE increased, whereas IMT and proportion of BrdUrd-stained cells decreased.

Isozyme analysis

The isozyme profiles of SuSa and SuSa-CP were identical, but those of RT112 and RT112-CP differed (Table 7.2). The most striking change was the appearance of PGM1 in RT112-CP; this was not detected in the parental line (Figure 7.2). Both cell lines were heterozygous for esterase D, but the relative proportions of the isozymes had altered, such that the product of the ESD*1 allele was predominant in RT112, but in RT112-CP the product of ESD*2 was predominant (Figure 7.3). Other isozyme patterns, including the uncommon heterozygous pattern of GOTm, were unchanged.

DNA analysis

Because of the changes in isozyme profile in RT112-CP, a further check on its identity was carried out using two hypervariable DNA probes, λMS1 and pλg3. RT112 and RT112-CP gave identical heterozygous patterns using these probes, confirming their common origin. Figure 7.4 shows the autoradiograph of RT112 and RT112-CP DNA hybridized with λMS1 probe.

Karyotypic analysis

Karyotypes differed between parent and resistant cell lines. The modal number for RT112 was 47, but for RT112-CP was 43 (Table 7.3). Of the 10 marker chromosomes in RT112, only 3 were retained in RT112-CP; 6 new ones were gained and 5 parental markers were replaced by normal chromosomes in RT112-CP. No homogeneously staining regions or double minutes were seen (Figure 7.5). The karyotypes of both RT112 and RT112-CP were consistent in all the cells analysed. To confirm that the changes were not simply due to repeated passaging, RT112 was subcultured until its passage number reached that of the resistant subline. The karyotype of RT112 was stable over the number of passages

that RT112-CP had undergone while in cisplatin. Karyotypic changes in SuSa-CP were not as extensive as in RT112-CP. The modal numbers of SuSa and SuSa-CP were similar, and they had several markers in common (Table 7.3). RT112-CP and SuSa-CP did not have any markers in common.

Micronucleus production

Preliminary experiments showed that micronucleus production in these cell lines reached a peak 72 hours after a 1 hour cisplatin exposure (see Table 7.4). Increasing the cisplatin concentration led to increased production of micronuclei (Table 7.4). Comparing the numbers of micronuclei induced 72 hours after exposure to equimolar cisplatin concentrations (8 μg/ml for RT112 and RT112-CP; 2 μg/ml for SuSa and SuSa-CP), there were 4-fold fewer micronuclei in the resistant lines compared with their parental lines (Table 7.5).

Cross-resistance

IC50s (drug concentration causing a 50% decrease in optical density relative to the controls) for cisplatin, carboplatin, VP-16, bleomycin, methotrexate and adriamycin against RT112, RT112-CP, SuSa and SuSa-CP using the MTT assay, are shown in Table 7.6. Relative resistance (IC50 of resistant line/IC50 of parent line) is also shown in Table 7.6. RT112-CP and SuSa-CP had different levels of resistance to cisplatin in this assay compared with the colony-forming assay: RT112-CP 10-fold and SuSa-CP 2-fold. This is probably due to the different exposure time (6 days in the MTT assay, 1h in the colony-forming assay), different PDTs (SuSa and SuSa-CP are significantly slower than RT112 and RT112-CP), and the fact that the MTT assay measures cytostasis as well as cytotoxicity. Similar levels of cross-resistance to the cisplatin analogue, carboplatin (RT112: 10-fold,

SuSa: 2-fold), were observed. There was also a high degree of cross-resistance to the unrelated compound, methotrexate (RT112-CP 7-fold, SuSa-CP 3-fold). Adriamycin was the only drug studied to which there was no cross-resistance.

Table 7.1. Cisplatin sensitivity and growth characteristics of parent and resistant cell lines.

	Cell line			
	RT112	RT112-CP	SuSa	SuSa-CP
IC70 ±SE (μg/ml)	4.7 ±0.4	19.1 ±0.9 (P<0.001)	1.1 ±0.1	4.6 ±0.2 (P<0.001)
PDT ±SE (h)	21.6 ± 0.1	21.5 ± 1.5	27.7 ± 1.3	29.8 ± 0.8
IMT ±SE (h)	21.0 ± 1.1	22.4 ± 0.5	28.5 ± 1.2	19.3 ± 0.5 (P<0.001)
CFE ±SE (%)	55.3 ±6.4	51.0 ±3.5	7.6 ±2.0	16.6 ±2.1 (P<0.02)
Proportion of BrdUrd-stained cells ± SE (%)	31.6 ± 2.1	41.4 ± 2.0 (P<0.05)	64.5 ± 1.2	49.5 ± 3.3 (P<0.05)

IC70s were calculated from linear regression analysis of individual experiments (3-5 experiments for each cell line)

Statistical significance was assessed using a Student's unpaired t test. P values are shown for statistically significant differences only

Abbreviations:

PDT, population doubling time
CFE, colony forming efficiency
IMT, intermitotic time

Table 7.2. Isozyme profiles of parent and resistant cell lines.

Cell line	Enzyme loci								
	PGM1	PGM3	GOTm	GOTs	ESD	ADA	ACP1	GLO	PGP
RT112	0	ba	2-1	1	2-1 (1>2)	1	B	2-1	1
RT112-CP	1	ba (b>a)	2-1	1	2-1 (2>1)	1	B	2-1	1
SuSa	1	a	ND	ND	2-1	1	ND	2	ND
SuSa-CP	1	a	1	1	2-1	1	ND	2	1

Abbreviations:

PGM1 and 3, first and third loci of phosphoglucomutase: GOTm and s, mitochondrial and soluble glutamate-oxaloacetate transaminase: ESD, esterase D: ADA, adenosine deaminase: ACP1, first locus of acid phosphatase: GLO, glyoxalase: PGP, phosphoglycolate phosphatase: ND, not done.

Table 7.3. Chromosome rearrangements, monosomies and trisomies in parent and resistant cell lines

	RT112	RT112-CP	SuSa	SuSa-CP
modal no	47	43	53	52
chromosome				
1		del 1p	1p-,1q+ 1q-	1p-,1q+ 1q-
2				
3	3p-	3p-		
4		-4		
5			5p+	5p+
6	6q+		+6q+	+6q+
7			7q-	7q-
8	i8q	i8q		
9	9q+			+9
10			+10	
11		11p+		
12	12q+		+12,+i12p +?12q-	2xi12p 12p-
13	-13	13q-	13q+	-13,13q+
14	14p+			-14
15		15p+	-15,15q+	-15,-15
16		16p+		
17	17p+			
18		-18,-18	+18	-18
19		-19		
20	+20	20p+	+20	
21	i21q	i21q	-21	
22				
X				Xp+
Y	-	-		
unidentified markers	1 (?del13q)	1 (?3q-)	1 (?8q-)	3 (?8q-)

Abbreviations:

del, deletion
p, p arm of chromosome (short arm)
q, q arm of chromosome (long arm)
i, isochromosome
+, an extra copy of the chromosome is present
-, the chromosome is absent
p+ (or q+), the p (or q) arm of the chromosome has an addition
p- (or q-), the p (or q) arm of the chromosome has a deletion

Table 7.4. Increase in micronucleus production with time following cisplatin exposure

cell line	cisplatin conc, μg/ml	hours following cisplatin exposure 48	72	96	120
RT112	2	1.6±0.4	2.0±0.4	1.6±0.3	0.9±0.5
	4	2.8±0.1	3.1±0.8	2.8±1.1	2.1±0.6
	8	3.6±0.9	5.3±0.5	4.3±0.8	3.7±0.9
RT112-CP	8	1.1±0.4	1.3±0.4	1.5±0.6	0.9±0.4
	16	1.5±0.4	2.9±0.8	3.0±0.1	3.2±0.5
	32	2.3±0.6	4.1±0.2	4.6±0.2	4.7±1.1
SuSa	0.5	1.5±0.1	3.4±0.4	1.3±0.3	2.6±0.2
	1	2.3±0.7	5.3±0.4	2.8±0.6	2.8±0.3
	2	2.9±0.4	8.3±0.3	4.7±0.8	3.0±0.3
SuSa-CP	2	1.8±0.3	1.8±0.02	0.6±0.2	0.6±0.6
	4	3.7±1.2	2.9±0.6	2.4±0.4	0.7±1.0
	8	3.7±0.1	4.6±0.7	3.9±0.3	1.9±0.1

Abbreviations:

% MN, percentage of cells with micronuclei, minus the background level (background level in untreated cells is: RT112, 0.8±0.2; RT112-CP, 1.8±0.2; SuSa, 4.8±0.7; SuSa-CP, 3.4±0.4)

Table 7.5. Micronucleus production 72 hours following cisplatin exposure

RT112		RT112-CP	
cisplatin conc, μg/ml	% MN±SE	cisplatin conc, μg/ml	% MN±SE
2	2.0±0.4	8	1.3±0.4
4	3.1±0.8	16	2.9±0.8
8	5.3±0.5	32	4.1±0.2

SuSa		SuSa-CP	
cisplatin conc, μg/ml	% MN±SE	cisplatin conc, μg/ml	% MN±SE
0.5	3.4±0.4	2	1.8±0.02
1	5.3±0.4	4	2.9±0.6
2	8.3±0.3	8	4.6±0.7

Abbreviations:

conc, concentration

% MN, percentage of cells with micronuclei minus background (background levels: RT112 0.8±0.2; RT112-CP 1.8±0.2; SuSa 4.8±0.7; SuSa-CP 3.4±0.4)

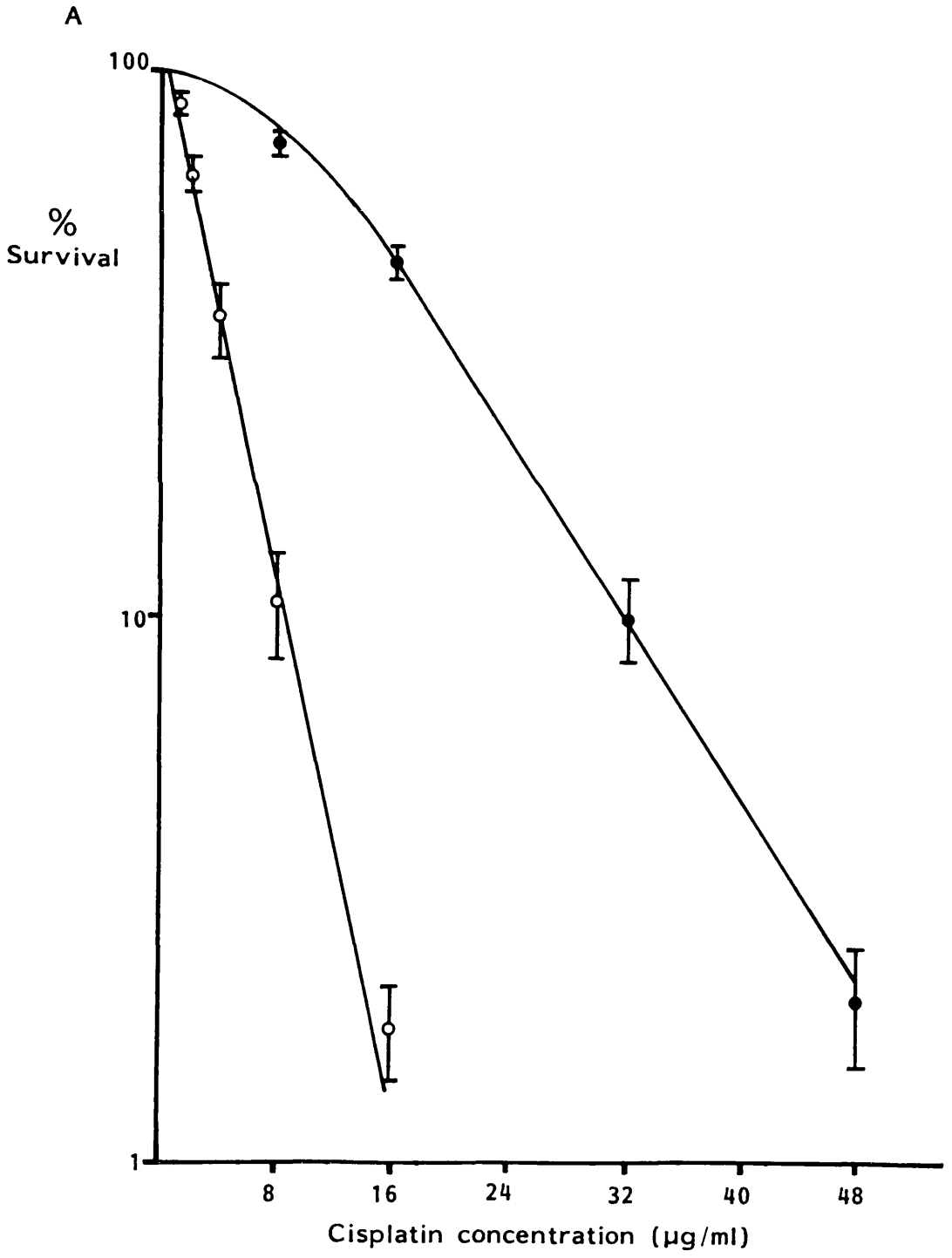
Table 7.6. IC50s and resistance ratios of parent and resistant lines.

Drug	Cell line					
	RT112	RT112-CP	Ratio	SuSa	SuSa-CP	Ratio
cisplatin	64 ±11	638 ±144	10.0	41± 8	87 ± 12	2.1
carboplatin	1800±400	18100±2100	9.9	500 ± 55	1000 ± 100	2.0
methotrexate	1.1 ± 0.1	7.3 ± 0.7	6.6	8.6 ± 0.6	25.6 ± 7.1	3.0
bleomycin	49 ±11	135 ±29	2.8	6.8 ±0.6	7.9 ±1.3	1.2
VP-16	185 ±20	346 ±57	1.9	11.3±1.7	20.5 ±2.3	1.8
adriamycin	12.3 ±0.9	12.6 ±0.2	1.0	1.7 ±0.2	1.6 ±0.1	1.0

Abbreviation:

Resistance ratio, IC50 of resistant line/IC50 of parent line

Figure 7.1. Dose-response curves of RT112 and RT112-CP (A), SuSa and SuSa-CP (B), to cisplatin. Cell lines are represented by the following symbols: RT112 (O), RT112-CP (●), SuSa (□), SuSa-CP (■). The points are the means ± SEs.



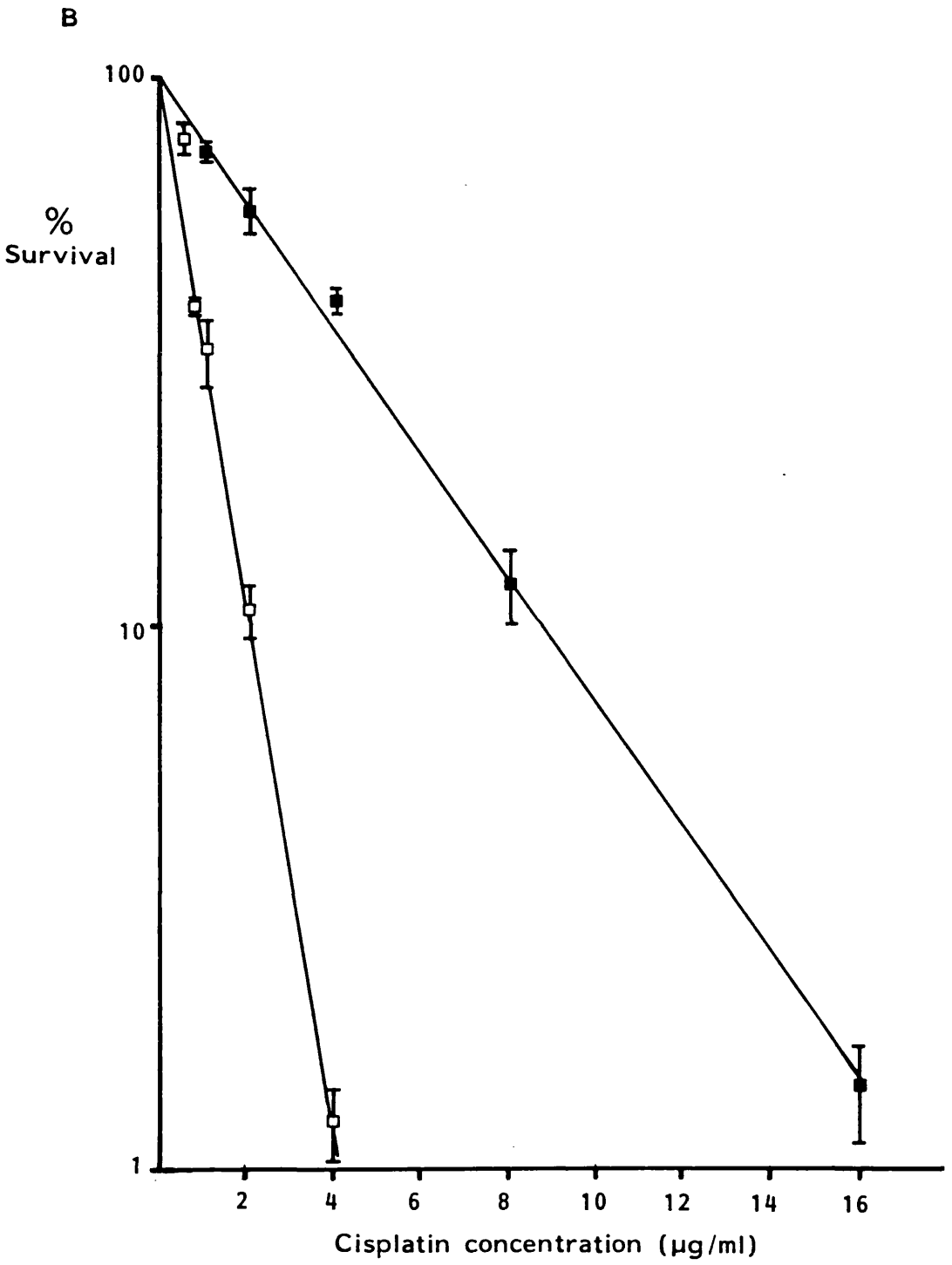

B
100
%
Survival
10
1
2
4
6
8
10
12
14
16
Cisplatin concentration (µg/ml)

Figure 7.2. Starch gel stained for PGM1 showing the presence of the isozyme in RT112-CP, and its absence from the parental line RT112.

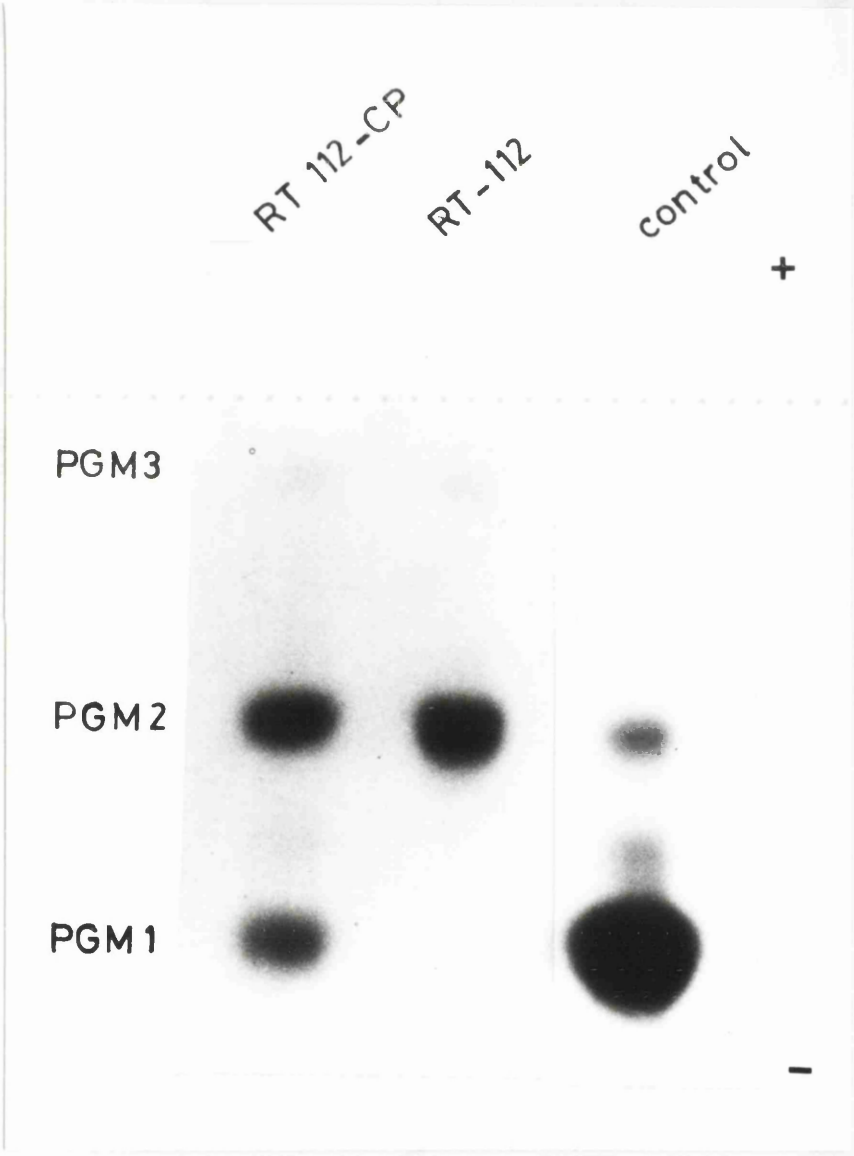


Figure 7.3. Starch gel stained for ESD showing predominance of ESD*1 in RT112, and ESD*2 in RT112-CP.

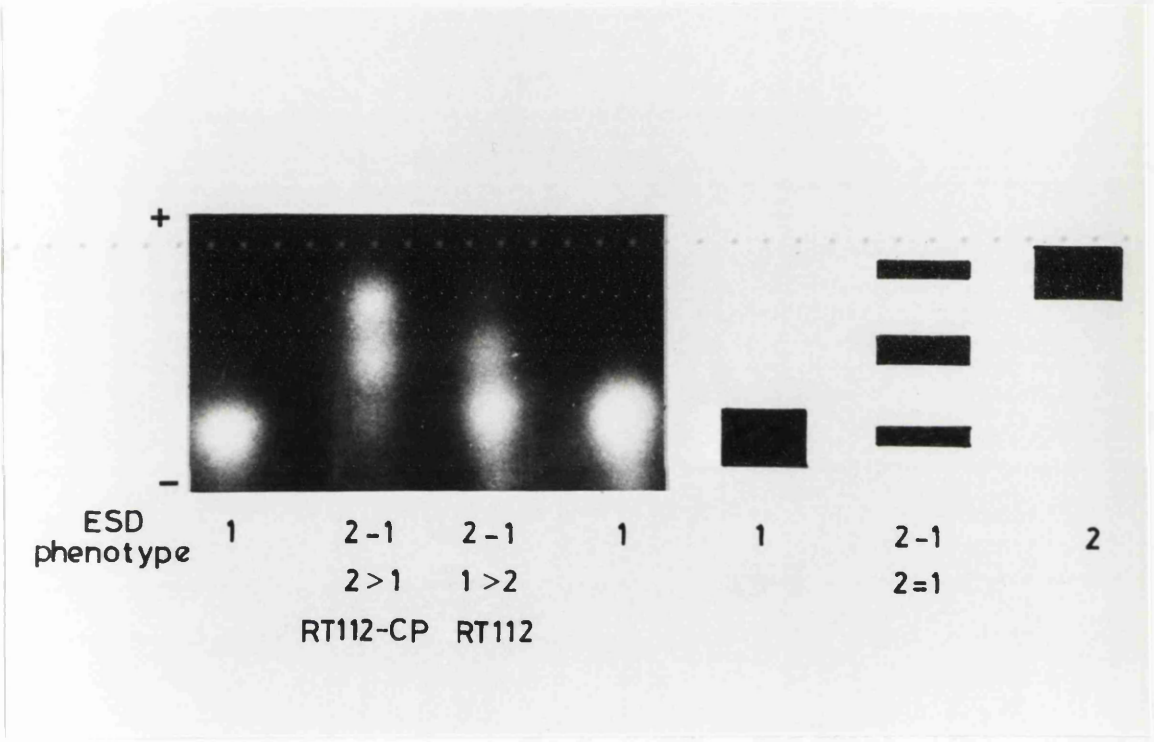


Figure 7.4. Autoradiograph of RT112 and RT112-CP DNA hybridized with λMS1 hypervariable probe.

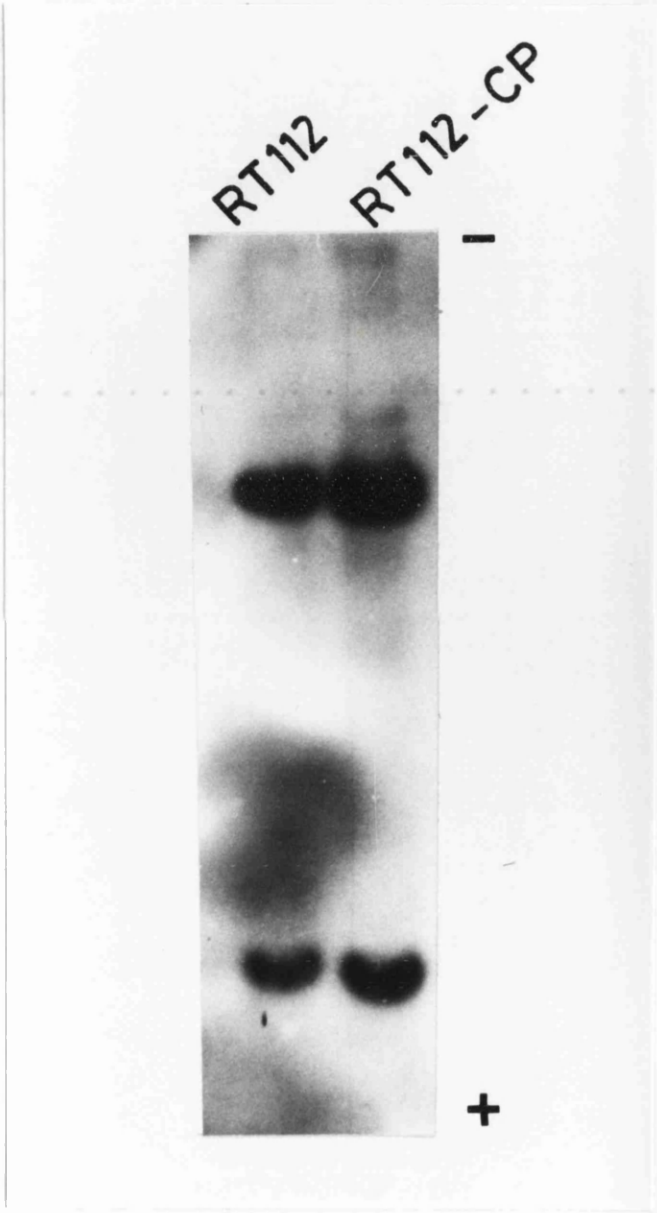
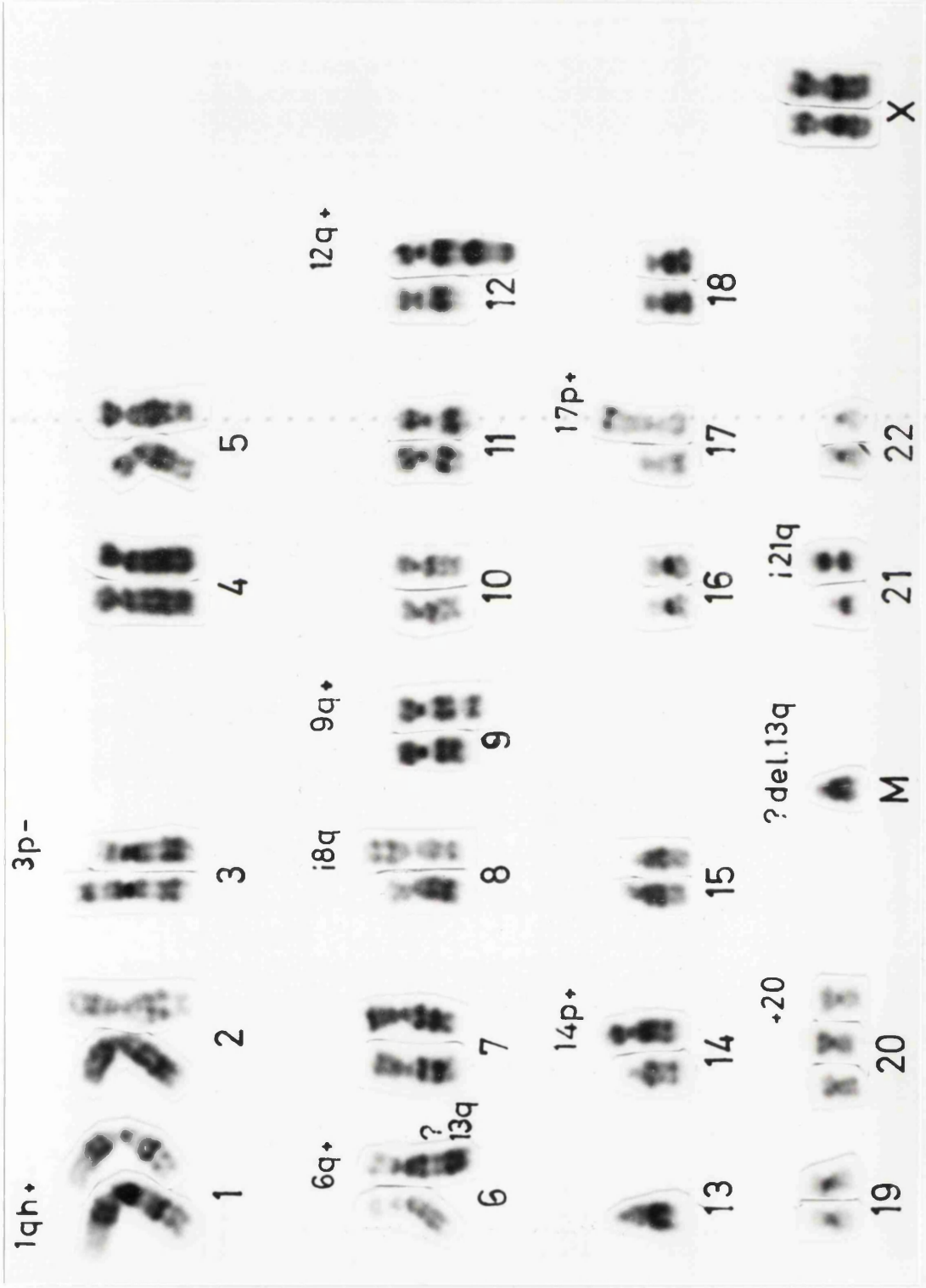
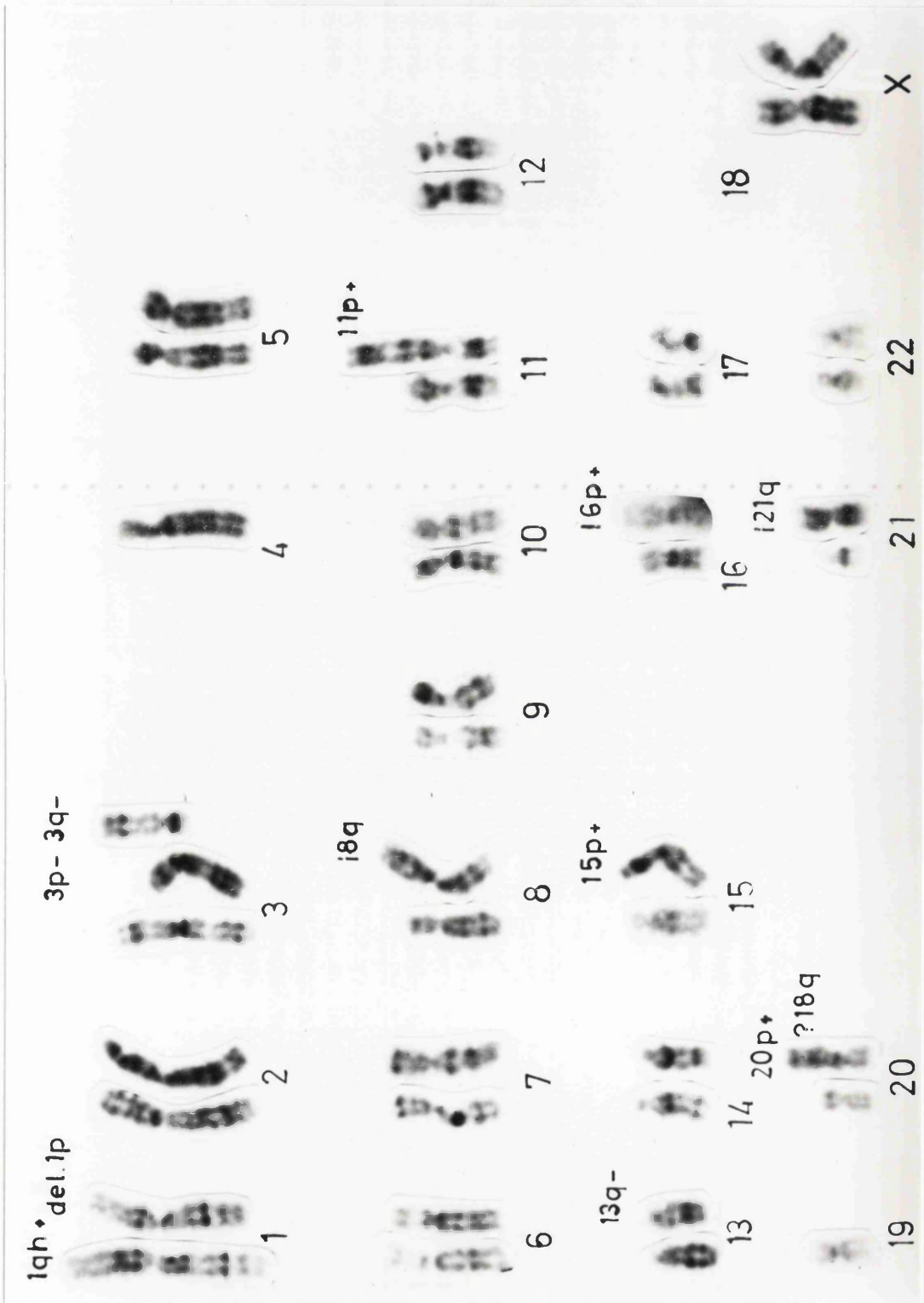


Figure 7.5. Karyotype of RT112 (A) and RT112-CP (B).

A



B


1qh+ del.1p
3p- 3q-
i8q
15p+
16p+
11p+
13q-
20p+
?18q
i21q
1
2
3
4
5
6
7
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22
X

DISCUSSION

Cisplatin is the most effective single agent in the treatment of testicular and bladder cancer, but development of resistance is a major cause of treatment failure. In order to develop a model of acquired resistance to cisplatin, a bladder and a testicular cell line were continuously exposed to the drug for periods of 14 and 11 months. They are the first reported cisplatin-resistant cell lines derived *in vitro* from these tumour types.

Isozyme profiles were compared to demonstrate a common origin for the parent and resistant cell lines. The identical isozyme profiles of SuSa and SuSa-CP confirm their common origin. The uncommon phenotype of GOTm seen in both RT112 and RT112-CP, and the identical patterns seen in these two cell lines using the hypervariable locus-specific probes λMS1 and pλg3, are conclusive evidence for their origin from the same individual. The changes seen in PGM1 and ESD between RT112 and RT112-CP could be due to the selection of a subpopulation of cells with a different isozyme pattern during long-term culture in cisplatin. However, karyotypic analysis did not provide evidence for a subpopulation in RT112 with gross chromosomal changes. One of the probes used for DNA analysis, λMS1, is derived from chromosome 1p, and the PGM1 locus is also on 1p. Since both RT112 and RT112-CP are heterozygous for λMS1, there is no evidence for gross deletion of 1p to account for the lack of expression of PGM1 in the parent line. However, karyotypic analysis showed that there was a deletion of 1p in RT112-CP in the region of the PGM1 locus (1p21-22). Rearrangement in this area may have altered expression of the PGM1 gene. It seems likely that PGM1 expression can occasionally "switch off" in cultured cells, as in the erythroleukaemia line K562 (Povey et al, 1980).

Disappearance of the 6q+ marker, which is probably a 6/13 translocation, and appearance of the 13q- marker in RT112-CP may account for the change in ESD expression. There was no evidence of homogeneously staining regions or double minutes, and there was no marker common to RT112-CP and SuSa-CP which might account for their increased resistance.

Stable cisplatin resistance has been induced in human cell lines derived from other histological types of tumour, including head and neck (Frei et al, 1985; Teicher et al, 1986), ovarian (Andrews et al, 1985; Kikuchi et al, 1986; Behrens et al, 1987; Kuppen et al, 1988; Andrews et al, 1989) and lung (Hong et al, 1988; Hospers et al, 1988) cancers and a Burkitt lymphoma (Teicher et al, 1986). Studies on mechanisms of resistance in these cell lines show that it is multifactorial. The SCC-25/CP cell line (derived from a squamous cell carcinoma of the tongue) has decreased cisplatin uptake, increased protein sulfhydryl (metallothionein) content, increased activity of an enzyme (glutathione-S-transferase) involved in glutathione metabolism, and decreased levels of DNA cross-linking (Teicher et al, 1987). However, glutathione levels are unchanged (Teicher et al, 1987). There is evidence that different mechanisms contribute to resistance in different cell lines. For example, within the ovarian cell lines, glutathione content is increased in the resistant subline 2780CPR (Hamilton et al, 1985), but is unchanged in 2008/DDP and COLO/DDP (Andrews et al, 1985).

Initial studies on resistance in an RT112-CP subline (undertaken when cells were able to grow continuously in 2.5 μg/ml cisplatin) have shown that there is no difference in cisplatin uptake between RT112 and RT112-CP (Bedford et al, 1987b). There are only minor differences

in the amounts of cisplatin binding to DNA, and the peak levels of interstrand crosslinking of DNA by cisplatin are comparable. However, glutathione content is increased in this RT112-CP subline, and there is increased activity of two enzymes involved in glutathione metabolism (glutathione reductase and glutathione peroxidase) which may, at least partially, explain its increased resistance (Bedford et al, 1987b).

In the majority of cisplatin-resistant human cell lines resistance has been developed by prolonged, continuous exposure to drug. This does not resemble the clinical situation, where patients are treated intermittently with chemotherapy. Thus the resulting mechanisms of resistance in cell lines may differ from those which occur clinically and this is a limitation to the use of these cell lines in mechanistic studies.

There was no obvious association between the growth properties of the cell lines and the development of resistance. For example, PDTs were similar in both sets of parent and resistant cell lines. IMT decreased in SuSa-CP, but not RT112-CP. CFE increased in SuSa-CP, but not RT112-CP. The proportion of BrdUrd-stained cells increased in RT112-CP, but decreased in SuSa-CP. Published data on the relationship between growth rates and the development of resistance to cisplatin are also inconsistent. In two non-small cell (Hong et al, 1988) and one small cell lung cancer cell lines (Hospers et al, 1988) cisplatin-resistance development was associated with an approximately 2-fold slower population doubling time. In contrast, PDTs were similar in other cisplatin-resistant cell lines (Behrens et al, 1987; Kuppen et al, 1988).

The micronucleus assay has been used to study chromosomal damage

caused by a number of anticancer agents, including cisplatin (Bonatti et al, 1983; Kliesch and Adler, 1987). Cisplatin is believed to exert its cytotoxic effect through interaction with DNA (Roberts and Thomson, 1979). It causes chromosomal abberations (van den Berg and Roberts, 1975; Turnbull et al, 1979) and can cause mutations to 6-thioguanine resistance (Plooy and Lohman, 1979), but the precise nature of the cytotoxic lesion is not known. In this study, the micronucleus assay was used to investigate the extent of cisplatin-induced DNA damage in sensitive and resistant cell lines. It has been suggested that a similarity in dose dependency between the lethal effects and the induction of micronuclei provides evidence that chromosomal damage is the main cause of death for cells treated with cisplatin (Bonatti et al, 1983). However, despite seeing a similar relationship between micronucleus production and cytotoxicity, because of the practical limitations of the assay (low incidence of micronuclei relative to cytotoxicity and lack of mechanistic information obtained), there is insufficient evidence for a causal link.

Patterns of cross-resistance are similar in RT112-CP and SuSa-CP. In common with other studies (Behrens et al, 1987; Hong et al, 1988), both were cross-resistant to carboplatin to the same extent as cisplatin. They were also highly cross-resistant to methotrexate, a drug with no structural or functional similarity to cisplatin. A high level of cross-resistance to methotrexate was also seen in the squamous cell carcinoma cell line SCC25/CP (Teicher et al, 1986; Rosowsky et al, 1987). Neither RT112-CP nor SuSa-CP were cross-resistant to adriamycin. Some of the other cisplatin-resistant cell lines also lack cross-resistance, or are collaterally sensitive, to

adriamycin (Teicher et al, 1986; Behrens et al, 1987; Hong et al, 1988).

GENERAL DISCUSSION

This study shows that cell lines derived from testicular tumours are hypersensitive to cisplatin and adriamycin compared with bladder cancer cell lines. This finding is important because it provides evidence that inherent biochemical differences within the testis tumour cells control sensitivity and not, as previously suggested (Oliver, 1985), humoral factors such as differences in blood supply or immunogenicity. This finding also indicates that testis tumour cell lines provide a representative model system for studies on the intrinsic biochemical mechanisms of differential sensitivity.

Many theories have been put forward to explain the differences in response between different tumour types. The first part of the Discussion will consider stem cell fraction, tumour growth rate, proportion of cycling cells, mutation frequency and normal tissue sensitivity.

Stem cell model

According to the tumour stem cell model, continued growth of a tumour depends on the self-renewal capacity of a sub-population of cells, the stem cells (Lajtha, 1979). Cure of a tumour requires eradication of all stem cells and the probability of curing a tumour using chemo- or radiotherapy is inversely proportional to the number of stem cells. Thus, testis tumours might be curable because they contain a lower proportion of stem cells than other tumour types, including bladder cancer.

Assessing the proportion of stem cells in a tumour is difficult because there is no direct means of identification. One indirect method is the human tumour colony-forming assay, as described by

Hamburger and Salmon (1977) and Courtenay and Mills (1978). Data obtained in such assays indicates that there is little difference between testis and bladder tumours in their ability to form colonies in soft agar. Comparing data from several studies, plating efficiencies ranged from 0.0016% to 0.05% for testis (Sarosdy et al, 1982; Foster et al, 1983; Hashimura et al, 1984) and from 0.001% to 1.7% for bladder tumours (Sarosdy et al, 1982; Hashimura et al, 1984; Kovnat et al, 1984; Mackillop et al, 1985). Thus there is a range of values for clonogenic cell fraction of human testis and bladder tumours, and no clear evidence for differences which might relate to tumour curability.

However, there are theoretical and technical problems associated with the human tumour colony-forming assay. Firstly, a 'clonogenic' cell is not necessarily synonymous with a stem cell. Transitional cells may retain the capacity to undergo several divisions in vitro, and only six doublings may be required to produce a colony consisting of more than 50 cells. Hence in vitro assays do not distinguish between stem cells and transitional cells with a high proliferative potential. Secondly, clumping of cells when attempting to produce a single-cell suspension can produce artefactual colonies. Thirdly, some cells will be damaged in the preparation of single-cell suspensions. Fourthly, the assay conditions may be sub-optimal, and only a subpopulation of stem cells and/or transitional cells is able to grow (Selby et al, 1983; Chin et al, 1986).

Another indirect measure of stem cell fraction can be obtained from the colony-forming efficiencies (CFEs) of continuous cell lines. CFEs ranged from 6 to 48% in my series of testis cell lines, and from 7 to 87% in the bladder cell lines. Although overall the testis lines have

lower CFEs, the difference is not statistically significant. Thus, assuming the proportion of colony-forming cells *in vitro* is equivalent to that of stem cells *in vivo*, there is little indication that the testicular cell lines have lower stem cell fractions than the bladder cell lines. The major problem with using cell lines for this comparison is that they are a subpopulation of the original tumour, selected for their ability to grow as a monolayer on plastic. This may select for relatively fast-growing cells, or even for stem cells. Some of the technical problems associated with the human tumour colony-forming assay may also apply - for example, the clonogenic cells of the testis cell lines may be more sensitive to enzymatic disaggregation than those of the bladder cell lines, or testis clonogenic cells might be damaged more than non-clonogenic cells, so their CFEs would be reduced.

However, disregarding the problems of quantifying stem cells, I have shown that the colony-forming cells of testicular cell lines are more sensitive to cisplatin and adriamycin than the colony-forming cells of bladder cell lines. Thus if clonogenic cells in cell lines do correspond to stem cells in tumours, I have shown that testis tumour stem cells are intrinsically more sensitive to cisplatin and adriamycin than bladder tumour stem cells. This work was extended by Parris et al (1988) who showed that the clonogenic cells of testis cell lines were more sensitive to gamma-radiation than the clonogenic cells of bladder cell lines. If this is extrapolated to the clinical situation, the probability of eradicating the last stem cell by chemo- or radiotherapy, and hence curing the tumour, is greater for testis than bladder tumours due to their greater inherent sensitivity rather than differences in the proportion of stem cells.

Tumour growth rate

Cure rates are higher in rapidly-growing tumours (Breur, 1966; Skipper and Perry, 1970; Shackney et al, 1978) and testicular tumours are fast-growing (Collins et al, 1956; Breur, 1966; Shackney et al, 1978). In a review of the literature on tumour doubling times, Shackney et al (1978) found that testicular tumours had the shortest doubling times of 9 solid tumours studied, with a mean doubling time of 21 days. Non-Hodgkin's lymphoma had a doubling time of 25 days, whereas adenocarcinomas of the lung and colon had mean doubling times greater than 70 days. Testis cancer and non-Hodgkin's lymphoma are more responsive than adenocarcinomas of lung and colon.

I measured the growth rates (PDTs) of testis and bladder cell lines. There was overlap, with PDTs ranging from 18 h to 38 h in ten testis cell lines, and from 21 h to 61 h in five bladder cell lines. Comparing cell lines with similar growth rates, for example 833K (23 h) and RT112 (22 h), there was a 10-fold difference in sensitivity to cisplatin and adriamycin. Thus, for the cell lines, there is no correlation between growth rate and chemo- or radiosensitivity (Walker et al, 1987; Parris et al, 1988). Similarly, the mean intermitotic times of the testicular and bladder cell lines, which assess the time taken for individual cells to divide, did not correlate with their chemo- or radiosensitivity.

Attempts to correlate tumour volume doubling time with response to chemotherapy do not take account of the rates of growth and loss of individual cells. Cell loss is an important factor in the growth rate of a tumour (Steel, 1968). Cell loss in human tumours is difficult to measure, but a value can be obtained knowing the actual doubling time and the potential doubling time of the tumour (Steel, 1968).

Cell loss in the testis and bladder cell lines was estimated from the proportion of cells dying while being followed by time-lapse cinemicroscopy for the calculation of intermitotic times. There was a wide range of cell death rates in testis and bladder cell lines. Despite the *in vivo* correlation between fast growth rate and high cell loss rate, no such correlation was seen in the cell lines. However, if the *in vivo* relationship is due to decreasing nutrient supply as cells get further away from blood vessels, then a similar correlation *in vitro* would not be expected, because these conditions do not apply. Cell lines which have a slow population doubling time, but a high proportion of S-phase cells, might be predicted to have a high cell loss rate, as is seen in the HT1197 bladder cell line (Masters et al, 1986). However, no such correlations were seen in the testis cell lines - for example, Tera II had a relatively long population doubling time and a high S-phase fraction, but a low death rate.

Cell cycle effects

The proportion of cells which are actively cycling is another measure of tumour growth and predictor of response. Labelling index (LI) gives a guide to the proliferative activity of cells (Aherne et al, 1977). Proliferation rate was measured in tumour-bearing testicles perfused with ^{3}H-thymidine, following orchidectomy (Rabes, 1987). Cross-sections of an advanced stage embryonal carcinoma showed areas of proliferation alternating with areas of necrosis throughout the entire tumour. A LI of greater than 80% was found in the proliferating areas. In seminomas, the proliferating cells were mainly at the well-vascularized boundary of the tumour, where the LI was > 40%. This experiment provides evidence that testis tumours contain a high proportion of cycling cells, which could contribute to their

sensitivity. Again though, my clonogenic assay data indicate that the proliferating cells of testis tumours are more sensitive to drugs than the proliferating cells of bladder tumours.

Flow cytometry has also been used to assess the proliferative activity of testis tumours. The proportion of S-phase cells in the aneuploid cell fraction was high, ranging from 22% to 51% in 7 out of 8 tumours (Fossa et al, 1985). In contrast, normal diploid testis cells had a much lower S-phase fraction (4%) (Fossa et al, 1985), providing evidence that testicular tumours have a high proportion of actively-cycling cells, and that normal testis has a lower proportion of cycling cells than testis tumours. This may help to explain why chemotherapy kills testis tumour cells but spares normal testis cells, and some patients regain their fertility following treatment. If the normal testis contains a higher proportion of quiescent (G_0) cells, these may survive chemotherapy. The mean value for S-phase fraction of human bladder tumours, measured *in situ* using BrdUrd infusion, was 18%, with a range of 14-28% for five patients with G3 tumours (Nemoto et al, 1990). However, a limitation of these methods is that they do not give information about the kinetics of the 'stem' cells (or cells with self-renewal capacity) because it is not possible to distinguish them from the other tumour cells (Tannock, 1987).

In contrast to the data showing that testicular tumours are fast-growing, and correlating their fast growth rate with curability, there is also evidence that fast growth rate is associated with poor prognosis. The proliferation index (PI) was measured in patients with bulky metastatic testicular tumours, which carry a poor prognosis (Sledge et al, 1988). PI is the ratio of the number of cells in the G_2+M peak to the number of cells in the G_1 peak. Measurements were

made on the primary testicular tumour and all patients subsequently received similar cisplatin-based combination chemotherapy regimens. Patients whose tumours had a high proliferation rate had a significantly shorter mean survival than patients whose tumours had low proliferation rates. Testicular tumour proliferation has also been assessed by measuring the rate of rise of tumour marker production before chemotherapy in patients with metastatic testis tumours (Price et al, 1988). In patients with elevated HCG, there was a significant negative correlation between rate of HCG production doubling time and survival. These results show that rapid proliferation rates in testis cancer are associated with decreased curability. A cycle of chemotherapy kills a proportion of the cells, and a fast-growing tumour would be better able to repopulate in the time between courses than a slow-growing tumour.

Mutation frequency

Goldie and Coldman (1979) proposed that curability depends on the mutation rate of the tumour cells. A tumour with a high mutation rate would develop resistance earlier than a tumour with a low mutation rate. Goldie and Coldman (1983; 1984) expanded their original hypothesis to encompass tumour growth rate and stem cell theory. They calculate that if the probability of self-renewal (PSR) is 1, then it would only take 36 doublings for a single cell to increase to 10^{11} cells, whereas if the PSR is 0.51, 1500 doublings would be required to reach the same number of cells. Assuming that mutation occurs at a similar rate at each cell division, at a given size the tumour with the lower PSR is more likely to have developed resistance. Therefore another possible explanation for the apparent lack of mutation to drug resistance in testis cancer is that these tumours have a high PSR. Nevertheless, this still does not explain the hypersensitivity of

their clonogenic cells in vitro to cisplatin and other anticancer agents.

A corollary of the Goldie-Coldman hypothesis is that bladder tumours may be more resistant than testis tumours because they have a higher mutation rate. The spontaneous and induced mutation rates at the HGPRT locus were measured in three testis and three bladder cancer cell lines (Parris, 1989). There was a range of spontaneous mutation rates in both cell types with overlap between them. There was also a range of induced mutation rates, obtained both with the classical inducing agent EMS, and with cisplatin. Thus when mutation induction by a chemotherapeutic drug was assessed, there was no evidence from this study for a difference in spontaneous or induced mutation rates between bladder and testis tumour cell lines.

Tissue-specificity

The Goldie-Coldman hypothesis makes the assumption that tumour cells are initially sensitive. However, this may not be true for primary human tumours (van Putten, 1984). Intrinsic differences in sensitivity exist between tumours derived from different tissues (Mackillop, 1986). There are considerable differences in radiation sensitivity between cell lines derived from different tumour types, which correlate with the clinical radiation sensitivity of the tumour of origin (Arlett and Harcourt, 1980; Fertil and Malaise, 1985; Parris et al, 1988; Carmichael et al, 1989). There are also considerable differences in chemosensitivity between cell lines derived from tumour types with differing chemosensitivity in vivo (see Chapter 3; Walker et al, 1987; Coren et al, 1990). Furthermore, the correlation between radiation sensitivity and chemosensitivity of different classes of human tumour suggests that intrinsic chemo- and radiosensitivity may

have a common basis, and this may depend on the efficiency of the cellular repair processes (Mackillop, 1986). Preliminary data on DNA repair in the testis and bladder cell lines suggests that there is a repair defect in the SuSa testicular cell line. These data are discussed later in this chapter.

Normal tissues such as bone marrow and testis are particularly sensitive to chemotherapeutic drugs and irradiation. Bone marrow toxicity is the major dose-limiting toxicity for most chemotherapeutic drugs (see table in Carter et al, 1981). Testicular tumours are very responsive, as are a number of tumours of the haemopoietic system, including Hodgkin's disease, non-Hodgkin's lymphoma and ALL (Carter et al, 1981). This implies that certain tumours may be sensitive because they are derived from normal tissues which are themselves sensitive.

The mechanisms controlling sensitivity of normal tissues may also contribute to the sensitivity of tumours derived from them. The sensitivity of normal human testis to chemotherapy depends on the drugs used. Alkylating agents, such as chlorambucil or cyclophosphamide, cause complete azoospermia at high doses, implying that the normal stem cells are killed (reviewed by Gradishar and Schilsky, 1988). Treatment of Hodgkin's disease patients with MOPP or MVPP regimens containing alkylating agents commonly causes permanent azoospermia (Whitehead et al, 1982). In contrast, m-AMSA only causes temporary testis toxicity, indicating that it kills cells at later stages of spermatogenesis, but not stem cells (da Cunha et al, 1982). Patients treated with cisplatin-based chemotherapy initially become azoospermic, but spermatogenesis recovers in many patients within about 18 months (see editorial by Schilsky, 1989). Interpretation of data is difficult because there is a high incidence of oligospermia in

testis cancer patients before therapy is initiated, suggesting that the disease itself may have an inhibitory effect on spermatogenesis (Schilsky, 1989). However, in one study 46% of patients cured of metastatic testicular cancer regained their pretreatment sperm counts by five years after treatment (Hansen et al, 1989). This indicated that some of the normal stem cells survived, eventually repopulated the germinal epithelium and enabled spermatogenesis to resume.

Therapeutic index

Since the testicular tumour patients described by Hansen et al (1989) were cured, all the tumour stem cells must have been eradicated. This indicates a differential sensitivity between normal stem cells and tumour stem cells in testis. There are a number of factors which might contribute to this. These are a) existence of a blood-testis barrier, b) regulation of spermatogenesis, c) the process of malignant transformation, d) ability to differentiate and e) the stage of spermatogenesis from which testis tumours arise.

Firstly, the presence of the blood-testis barrier may contribute to differential sensitivity of normal and neoplastic testis. The normal testis stem cells in the remaining testis may be protected by the blood-testis barrier, while the tumour stem cells are not, since these are normally only present in metastases outside the barrier when chemotherapy begins, the primary tumour having previously been removed surgically.

Secondly, the regulation of spermatogenesis may make the normal stem cells more resistant than the tumour stem cells. In primates only about half the stem spermatogonia divide at each cycle of the germinal epithelium (reviewed by de Rooij, 1983). The remainder stay in a G /G
1 o

phase, and these might be less sensitive to cytotoxic drugs active against cycling cells. If the cells were damaged, by chemotherapy or radiation, the long periods between divisions would allow extra time for repair before they proliferated and repopulated the germinal epithelium. There is evidence that, the longer cells stay in G_1/G_o, the longer it takes them to respond to stimulation (de Rooij, 1983), which might account for the long delay before spermatogenesis is restored following chemotherapy.

Thirdly, normal stem cells may survive despite eradication of testicular tumour stem cells because one of the steps involved in malignant transformation confers sensitivity. However, *in vitro* transformation of normal cells has been found to increase their radio- (Arlett et al, 1988; Sklar, 1988a) and chemoresistance (Sklar, 1988b). Transformation of mouse NIH 3T3 cells with activated *ras* oncogenes resulted in a 4 to 8-fold increase in cisplatin resistance (Sklar, 1988b). Transfection of RT112, T24 and HT1376 bladder cell lines with SV40 (pSV3neo) did not alter their radiation response (Parris et al, 1989).

The curability of germ cell tumours might be related to their ability to differentiate. In a proportion of patients treated with chemotherapy, masses remain which consist solely of mature, differentiated teratoma (Oosterhuis et al, 1983; Loehrer et al, 1986). Prior to the introduction of cisplatin-based combination chemotherapy, the incidence of mature teratoma alone in metastases was extremely rare (Smithers, 1969; Pugh, 1976). This suggests either that chemotherapeutic drugs induce testis tumour cells to differentiate, or that the undifferentiated cells are more sensitive and are killed, leaving the differentiated cells behind (Hong et al, 1977; Oosterhuis

et al, 1983). The balance of evidence favours the latter hypothesis. Metastases from pure EC rarely if ever produce mature teratoma after chemotherapy, but become necrotic or fibrotic (Oosterhuis et al, 1983). The same pattern is seen in untreated tumours; only if differentiated tissue is seen in the primary tumour is it observed in the metastases. These data indicate that chemotherapy does not induce differentiation of undifferentiated (EC) cells, and this is not a factor contributing to curability.

Testis tumour stem cells might arise from a stage in spermatogenesis which is more sensitive to chemotherapy and radiation than normal stem cells and this extra sensitivity is retained in the tumour cells. The precise stage from which testicular tumours are derived is unknown. There is evidence for their origin from a germ cell, and the pre-malignant disease, carcinoma-in-situ, is widely believed to be the precursor of germ cell tumours (Skakkebaek et al, 1987). There is evidence that they are derived from a pre-meiotic stage of spermatogenesis: 14/15 testicular tumour cell lines studied contained an X and a Y chromosome (Wang et al, 1981). Furthermore, karyotypic analyses of NSGCTs have shown that they are commonly hyperdiploid (Rigby, 1968; Martineau, 1969; Atkin, 1973; Delozier-Blanchet et al, 1987; Sledge et al, 1987; Oosterhuis et al, 1989) with no haploid cells (Rigby, 1968).

Evidence that different stages of spermatogenesis differ in their sensitivity to drugs and radiation comes partly from studies which show that spermatogenesis is restored at different times following therapy (see above). Other studies on men have shown that differentiating spermatogonia are very sensitive to radiation and chemotherapeutic drugs, but stem cells, spermatocytes and spermatids

are relatively resistant (Rowley et al, 1974; Oakberg, 1984; Meistrich, 1986). Studies on rodents have also shown that cells at different stages of spermatogenesis differ in their radio- and chemosensitivity (Craig et al, 1961; Partington et al, 1964; Fox and Fox, 1967). It is tempting to speculate that, in men, testis tumours might be derived from differentiating spermatogonia and retain the sensitivity of these cells.

Spermatogenesis involves mitotic and meiotic cell division, followed by differentiation into highly specialized cells with limited metabolic functions. It is believed that stages of spermatogenesis after the meiotic division are less able to repair damaged DNA as cytoplasm is lost from the cells (reviewed by Moore, 1986). On this basis, spermatocytes and spermatids would have less repair capacity than stem cells and differentiating spermatogonia and the former would be predicted to be more sensitive. Thus the experimental evidence that differentiating spermatogonia are the most sensitive phase of spermatogenesis does not correlate with DNA repair ability as proposed by Moore (1986).

The sensitivities of normal rat germ cells at different stages of spermatogenesis to gamma-radiation have been related to their ability to repair DNA double-strand breaks (Coogan and Rosenblum, 1988). The most sensitive cell types, spermatogonia and preleptotene spermatocytes, were virtually unable to repair double-strand breaks 45 minutes after exposure to 6000 rads. The most resistant cell type, pachytene spermatocytes, was only able to repair 26% of double-strand breaks after 45 minutes. In contrast, in another study, Chinese hamster ovary cells repaired 80% of double-strand breaks 30 minutes after exposure to 10000 rads (Weibezahn et al, 1985). Furthermore,

normal human fibroblasts repaired double-strand breaks within 1 h of exposure to 5000 or 10000 rads, and almost 100% repair had occurred by 4 h (Woods et al, 1982). It is difficult to make direct comparisons between cells derived from different species, but it appears that normal rat testis cells may be deficient in repair of DNA double-strand breaks at certain stages of spermatogenesis, including the differentiating spermatogonia, and this may contribute to their sensitivity.

As well as the physiological factors contributing to testicular tumour curability, there are a number of biochemical mechanisms involved in differential sensitivity which are generally studied *in vitro*, and several of which have been studied in the testis and bladder cancer cell lines.

Mechanisms of drug resistance

i) Drug uptake

More drug may be taken up into testis tumour cells than bladder tumour cells, which would contribute to their sensitivity *in vitro*. Uptake of cisplatin correlates with cytotoxicity in some cell lines with different intrinsic sensitivities to cisplatin (Eichholtz-Wirth and Hietel, 1986; Bedford et al, 1988) but not others (Bedford et al, 1987a). We showed that uptake of [^{195m}Pt]cisplatin correlated with cytotoxicity in SuSa, 833K and RT112 cells (Bedford et al, 1988). Decreased uptake often correlates with increased resistance in cell lines with cisplatin resistance induced *in vitro* (Richon et al, 1987; Teicher et al, 1987; Kraker and Moore, 1988; Andrews et al, 1988; Andrews et al, 1989). Even though uptake can differ between cell lines, the finding that the same amounts of cisplatin can reach, and

bind to, DNA of testis and bladder tumour cells (Chapter 5) implies that uptake is not an important factor in determining differential sensitivity.

ii) Glutathione levels

Differences in glutathione levels might contribute to intrinsic differential sensitivity to cisplatin. Glutathione detoxifies several anticancer agents, either by binding to them and inactivating them, or by scavenging free radicals (see review by Arrick and Nathan, 1984). We compared the expression of two classes of GST (pi and alpha) in three bladder and four testis cell lines but no correlation was seen between GST expression and response (Davies et al, submitted). Evidence that glutathione level influences response in some cell lines, but not others, comes from studies which use buthionine sulfoximine (BSO) to deplete cells of glutathione. BSO treatment sensitizes some cells to cisplatin (Hamilton et al, 1985; Hromas et al, 1987), but not others (Andrews et al, 1985; 1986; Richon et al, 1987; Smith and Brock, 1988). Thus there is evidence that glutathione level may influence response to cisplatin in the testis and bladder cell lines. However, more data on glutathione levels, both in our cell lines, and in samples of fresh tumours, is required before the role of glutathione in chemosensitivity can be fully understood.

iii) DNA repair

I investigated a particular aspect of DNA repair in testis and bladder cancer cell lines; the removal of an alkyl group from the O^6 position of guanine by ATase. ATase levels and response to a monofunctional and a bifunctional alkylating agent were measured. Within the testicular cell lines, there was some correlation between ATase level and sensitivity to MNU and mitozolomide. However, comparing the two groups

of cells, the resistance of the bladder cell lines was not solely due to higher ATase levels, since one of the bladder cell lines, HT1197, had a very low ATase level, but was relatively resistant to these drugs. This raises the possibility that the bladder cells have another repair pathway which can remove the O^6-alkyl lesions, but which is lacking, or less efficient, in testis cells.

A correlation between the number of cisplatin-induced interstrand crosslinks and cytotoxicity has been made in some studies (Zwelling et al, 1979; Erickson et al, 1981) but not others (Strandberg et al, 1982; Rawlings and Roberts, 1986). We investigated the repair of inter- and intrastrand crosslinks in bladder and testicular tumour cell lines (Bedford et al, 1988). There was no detectable repair of inter- or intrastrand crosslinks in a sensitive testis line (SuSa) up to 24 h after cisplatin exposure. However, 50% of crosslinks were removed from the DNA of the bladder cell line RT112 and the other testis cell line, 833K. Thus there is no clear correlation between repair of inter- and intrastrand crosslinks and sensitivity in these testis and bladder cancer cell lines, and more work is required to clarify their relationship.

These data do not provide information about repair in specific areas of the genome, which can differ (Bohr, 1987). Resistant cells might be better able than sensitive cells to repair sequences which are important for survival. It would be interesting to find out whether the mechanisms underlying resistance in RT112-CP and SuSa-CP are the same. It is possible that the mechanisms involved in a subline derived from an initially sensitive, repair-deficient cell line will differ from those involved in a subline derived from an initially repair-proficient cell line. For example, DNA repair genes which are not

expressed in SuSa cells might be expressed in SuSa-CP, whereas they are already active in RT112 cells, and resistance in these cells may be due to other mechanisms, such as increased levels of glutathione and enzymes involved in its metabolism. Recent data has shown that SuSa-CP is unable to repair cisplatin-damaged plasmid DNA, but is able to repair u.v.-damaged plasmid whereas the parental line is deficient in repair of both types of adduct.

However, such studies do not give any information about the quality of repair in cell lines with differential cisplatin sensitivity; if it is an error-prone process even cells which are able to repair may subsequently be killed. There may also be differences in tolerance levels between cell lines: resistant cells may be better able to tolerate lesions remaining in the DNA and replicate past them, subsequently repairing them by post-replication repair. DNA repair efficiency is heterogeneous within the genome: resistant cells may be better able to selectively repair essential genomic regions. Ability to repair lesions may reflect the ability of cells to inhibit their DNA synthesis step, allowing more time for repair before the next round of DNA replication.

The finding that the testicular tumour cell lines are hypersensitive to a range of agents with differing modes of action (Walker et al, 1987; Parris et al, 1988; Coren et al, 1990) suggests either that they are defective in one step in repair which is common to all the agents, or that they are deficient in a large number of different mechanisms. To test the first possibility, the ability of the testis and bladder cell lines to repair a single double-strand break in plasmid DNA is currently being assessed in our laboratory using an assay developed by John Thacker (Debenham et al, 1987).

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