MECHANISMS OF RESISTANCE TO CISPLATIN IN OVARIAN CANCER CELLS

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DECLARATION

I declare that this thesis was composed by myself and that the work presented has been carried out solely by me except where indicated in the text and below.

The characterisation of cell lines was performed in collaboration with the following: cytogenetic studies: Dr Gordon Lowther; Immunocytochemistry, Mr Gordon Wishart; Measurement of cell volume, Miss Patricia Thomson.

The development of the direct assay analysis of intrastrand platinum-DNA adducts was performed in collaboration with Mr J. Gilmour Morrison (HPLC, Department of Medical Oncology, University of Glasgow), Mr Keith McKay and Miss Kate Sampson (ICP-MS; Scottish Universities Research and Reactor Centre, East Kilbride). All other platinum analysis was by ICP-MS at the Scottish Universities Research and Reactor Centre. The work on differential platinum uptake was presented at the British Oncological Association Scientific Meeting, University of Bath, July 1991.

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DEDICATION

To Paul

ABSTRACT

Cisplatin is highly active against epithelial ovarian cancer; unfortunately the widespread emergence of clinical resistance to this drug has limited its overall therapeutic benefit. Many mechanisms have been proposed to explain the development of platinum resistance; these include differential platinum accumulation, intracellular inactivation of platinum by thiol-containing compounds, qualitative and quantitative differences in platinum-DNA adduct formation, and differential DNA repair capacity. The multidrug resistance P- glycoprotein efflux pump has also been implicated.

The availability of continuous ovarian cancer cell lines with induced platinum resistance provides a useful model for the study of this phenomenon in vitro. Three ovarian carcinoma continuous cell lines, A2780, 2780AD and 2780CP, were found to have a stable, 27-fold, range of cisplatin sensitivity, using a modified MTT chemosensitivity assay. This experimental model was used to investigate various theoretical mechanisms of platinum resistance.

The cytotoxic action of cisplatin is believed to be due to the formation of intrastrand platinum-DNA adducts. Investigation of these lesions is hampered by their extremely low intracellular levels (10^{-15} to 10^{-18} M). Indirect immunochemical methods, using polyclonal antibodies, have until recently been the only sufficiently sensitive technique. Inductively coupled plasma mass spectrometry (ICP-MS), a highly sensitive technique for platinum analysis, was used to develop a new assay for the direct measurement of intrastrand platinum-DNA adducts. Highly platinated calf thymus DNA was disaggregated enzymatically; nucleotides and platinum-containing oligonucleotides were separated by HPLC using an anion exchange column. Purified adduct standards were used to calibrate the chromatogram; platinum analysis of eluate

fractions revealed two peaks coinciding with the eluate positions of the two major intrastrand adducts.

Electrothermal vaporisation, a modification of ICP-MS, was used to improve analytical sensitivity prior to the direct investigation of intrastrand adducts in this cellular model of induced platinum resistance. Therapeutically relevant doses of cisplatin were used; absolute adduct levels, and the kinetics of their formation and repair, were measured. Technical problems encountered at the lower limits of sensitivity reduced results to a semiquantitative level. Further modifications are possible, however, to achieve greater accuracy.

Accumulation of platinum, both intracellular and DNA-bound, was measured by ICP-MS; a linear relationship was observed between intracellular platinum levels and chemosensitivity for all three cell lines. Platinum accumulation was reduced in both resistant sublines, although to a similar degree in each. Uptake did not appear to be energy-dependent; nor did the P glycoprotein efflux pump appear to be a significant factor.

Induced cisplatin resistance was found to be associated with elevated levels of reduced glutathione (GSH). The inhibitor of glutathione synthesis, buthionione sulfoxidine (BSO), was used to achieve up to 100% reduction in GSH levels; however no effect on cisplatin sensitivity was observed under these experimental conditions.

These experiments, using continuous cell lines with a high degree of induced cisplatin resistance, provide further evidence for the multifactorial nature of cisplatin resistance. Differential platinum accumulation may account for a significant proportion of observed cisplatin resistance; intracellular platinum inactivation by GSH did not appear relevant. A promising new assay for the direct measurement of intrastrand platinum-DNA adducts is described.

CHAPTER 1

INTRODUCTION

Ovarian cancer is now the commonest gynaecological cancer in both the U.K. and the U.S.A.; constituting about 25% of all gynaecologic cancers but accounting for 47% of all deaths from cancer of the genital tract (Barber, 1989). Worldwide, its incidence shows marked geographical variation, being five times more common in Scandinavian countries than in the Far East, with the U.K. ranking fairly high. Information from the West of Scotland Cancer Surveillance Unit show a crude prevalence for this region of 39.8 / 10^5 for cases diagnosed since 1985, and a corresponding incidence value of 20.5 / 10^5 in 1990. Since 1975 the incidence of this disease has risen slightly in the West of Scotland. Although ovarian cancer in this country represents only 4% of malignant disease by incidence, it is responsible for 6% of all female deaths (Sharp et al, 1989) with an overall five year survival of only 28%. This is largely due to its relatively late presentation, with only one third of all cases presenting whilst the disease is still localised and therefore resectable (Young, 1987). Epithelial ovarian cancer (EOC) comprises 90% of all malignant ovarian tumours (Fox, 1985) and is often the most refractory to treatment. A better understanding of the nature of this disease, and the underlying nature of its initial sensitivity and later resistance to chemotherapy, is essential for any further advances in its clinical management.

Aetiology

Little is known about the aetiology of ovarian cancer, although a role for 'incessant' ovulation has been suggested owing to its link with low parity and oral contraceptive usage. A recent decline of 2% in its incidence in premenopausal women over the past

fifteen years (C. Gillies, West of Scotland Cancer Surveillance Unit, personal communication) may also be associated with the latter factor. Genetic factors account for a small (around 5%) proportion of all ovarian cancer cases (Lynch et al, 1990). There also appears to be a link with breast cancer, which may be mediated through nulliparity. More recent evidence, however, has implicated the involvement of the BRCA-1 gene, located on the long arm of chromosome 17 in the familial breast-ovarian cancer syndrome (Cannistra, 1993). The insidious nature of ovarian malignancy means that screening for the disease in its early stages (a combination of clinical assessment, pelvic ultrasonography and serum tumour markers has been used) is difficult and should be confined to a defined at risk population (Andolf, 1986).

Overall prognosis is associated most strongly with clinical stage at presentation; histological indices (type, grade, quantitative aspects) provide a more accurate individual assessment (Baak et al., 1988; Young, 1987). Meticulous surgical staging is essential in the clinical management of EOC, particularly since five-year survival in its advanced stages is of the order of only 25 - 30%.

Clinical management

A greater understanding of the disease and its natural history has led to improved survival figures in recent years. The mainstay of treatment remains surgery, which allows accurate staging and histopathologic assessment. A vertical incision is essential for adequate exposure and complete exploration of the abdomen and pelvis. For adjuvant therapy to be optimally effective, complete extirpation of tumour is desirable - chemotherapy is more likely to be effective if only micrometastases remain. In practise, optimal cytoreductive surgery entails a radical oophorectomy procedure (extended total abdominal hysterectomy with bilateral salpingo-oophorectomy, omentectomy and resection of any other macroscopic disease), aiming to leave residual disease no greater than 1.5 cm in diameter (Griffiths, 1975). Such surgery ideally should only be performed by experienced gynaecological

surgeons with adequate allocated operating time, when optimal cytoreduction is achievable in about 85% of cases with low morbidity (Hacker, 1989). It is likely, however, in most cases that the ultimate clinical outcome is related mainly to the inherent biological properties of the tumour. The beneficial effect of optimal cytoreductive surgery may therefore be due to its intrinsic surgical resectability (Hacker and van der Burg, 1993). A recent study comparing interventional cytoreductive surgery with further chemotherapy for patients with residual EOC greater than 1 cm following primary laparotomy showed clear survival benefits for the former group (van der Burg et al., 1993), providing further evidence for the importance of adequate surgical management of this disease.

In early stage EOC, adequate surgery alone may be curative. Most gynaecologic oncologists would, however, accept the rationale for adjunctive therapy for stage Ic and beyond. Adjunctive radiotherapy was used prior to the development of active agents for chemotherapy; nowadays it is recognised as useful only for resistant small volume residual disease, particularly if confined to the pelvis. Intraperitoneal radiotherapy, in the form of radioactive colloids such as gold (^{198}Au), phosphorus (^{32}P), or yttrium (^{90}Y), has recently been used as adjuvant or consolidation therapy in selected patients.

In advanced EOC, chemotherapy is clearly superior to radiotherapy for adjunctive treatment; response rate is variable, however, and can only be accurately assessed by second-look laparotomy. If a histological complete response (CR) is proven, five year survival is around 70%, compared with a figure of around 35% for non-histologically proven CR. It seems logical that the most complete response is achieved with the use of multiple agents in combination regimes. The most active single agent is cisplatinum (CDDP), which produces an overall clinical response rate of 50% (Wiltshaw, 1985) and a pathological CR of around 13%. There are as yet no published studies comparing single-agent CDDP with a combination regime including

CDDP, although an overview of all relevant studies is currently being conducted (MRC Gynaecological Working Party, 1990). The second generation platinum compound, carboplatin, also acts by platinum-DNA adduct formation, although it is less reactive and therefore larger doses are required for a similar clinical effect. The different toxicity profile of carboplatin compared with CDDP provides some therapeutic advantage, but a lack of long-term data has prevented its recommendation as a routine replacement for CDDP in chemotherapy regimes for the treatment of EOC (Advanced Ovarian Cancer Trialists Group, 1991; Vermorken et al., 1993).

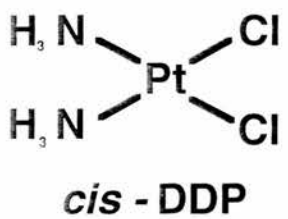


FIGURE 1: Molecular structure of CDDP

Cisplatin (CDDP)

CDDP is a neutral, square-planar coordination complex with two labile chloride groups and two relatively inert ammine ligands in the *cis* configuration (Figure 1). Its cytotoxic effect was discovered serendipitously (Rosenberg et al., 1965) in a classic experiment designed to investigate the effect of electromagnetic fields on bacterial growth. Bacteria were noted to grow in a filamentous fashion, up to three hundred times their normal length, when a low voltage was applied between two platinum electrodes. This effect, specifically inhibiting cell division, was later shown to be due to the electrolysis products cis-diamminetetrachloroplatinum (IV) and cis-diamminedichloroplatinum (II) (CDDP). Corresponding *trans* isomers acted merely as bacteriocides, with no effect on cell division. Such a specific action of CDDP to inhibit cell division, whilst allowing cellular growth to continue, implies interference with DNA replication whilst RNA and protein synthesis continue normally. Other

known DNA-damaging agents (UV- and X-irradiation, anti-tumour alkylating compounds) act in a similar fashion.

Mode of action of CDDP

Considerable evidence now exists to support the interaction of CDDP with cellular DNA as critical for its cytotoxicity. In aqueous solution a variety of partially and fully hydrolysed species are formed (Figure 2); in addition, loss of a proton from the aquated species, with substitution of chloride by water, allows the formation of various hydroxy species. The *cis* configuration is retained throughout these reactions. In extracellular fluid the concentration of chloride ion is sufficiently high to maintain CDDP in its neutral (dichloro-) form. However, once inside the cell, the intracellular chloride level declines sharply, with consequent promotion of hydrolysis of the labile CDDP chloride ligands. This aquated species reacts subsequently with a variety of intracellular components, including DNA.

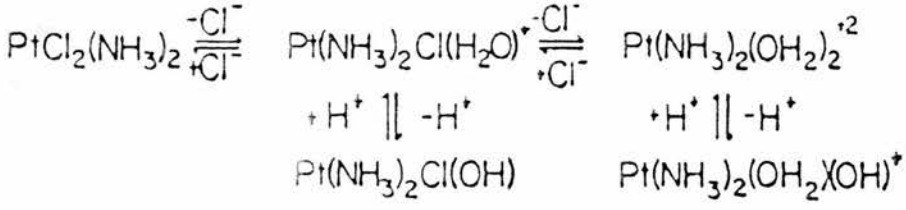


FIGURE 2: Hydrolysis Reactions of CDDP (from Pinto and Lippard 1985)

Further evidence that CDDP is a DNA-damaging agent has been supplied by studies of prophage induction (Pinto and Lippard, 1985), showing that lysogeny can be indirectly induced in non-lysogenic *Eschericia coli* by conjugation with a CDDP-treated lysogenic strain. Since bacterial conjugation implies transfer of DNA alone, it follows that this effect is due to platinum adducted to donor prophage DNA. Other studies have shown that CDDP can inhibit DNA replication even at subtoxic doses

and after removal of the drug. It is not yet certain whether the antitumour efficacy of CDDP is a consequence of impaired DNA replication or transcription (Donahue et al., 1990). Initially its effect appeared to be due to inactivation of DNA as a template in replication, rather than to interference with enzymes involved in DNA synthesis, as CDDP will only inhibit DNA polymerase activity at very high doses (Harder et al., 1986). More recent work (Sorenson and Eastman, 1988a and 1988b), using CDDP-treated murine leukaemia (L1210) cells, has shown that DNA replication actually continues for some time after CDDP treatment, even when cell division has halted. Flow cytometry was used to demonstrate arrest in the G2 stage of cell division, the duration of which was proportional to the amount of CDDP used. At low CDDP concentration, G2 arrest was transient, and cells ultimately survived exposure. At higher doses of CDDP, G2 arrest was less readily reversible, after which time cellular disintegration was observed, any recovery of growth originating from a limited number of surviving cells. Analysis of DNA damage in these cells merely demonstrated the occurrence of significant numbers of double-strand breaks at high levels of CDDP, apparently the first sign of cell death. The lack of evidence for single-strand DNA breaks in G2-arrested cells fails to confirm a role for post-replication repair in this phase of the cell cycle. Sorenson and Eastman have therefore suggested that inhibition of transcription is more critical to the toxic action of CDDP than is inhibition of DNA synthesis. At high levels of CDDP it is possible that repair processes for recovery of transcription are overwhelmed. thus leading to cell death.

CDDP in ovarian cancer

The activity of CDDP in EOC was first reported in 1976 (Wiltshaw et al., 1976); since then many studies have confirmed CDDP to be the drug of first choice in advanced EOC. A major problem, however, has been the emergence of clinical resistance: primary response rates of 80 - 90% are generally achieved (Sutton et al., 1989) but the impact of such chemotherapy on long-term survival, particularly in

advanced disease, is unfortunately minimal, owing to the widespread emergence of CDDP resistance. Improved management of the toxic side-effects of CDDP has recently allowed dose escalation to overcome some of this resistance, although neurological side-effects then became dose-limiting (Ozols, 1989). A retrospective analysis of relative CDDP dose intensity in thirty three randomised trials (Levin and Hryniuk, 1987; Kaye et al., 1992) also demonstrated the importance of received CDDP dose intensity to survival. Ultimately, however, ovarian cancer becomes refractory to further dose escalation, owing to the increasing proportion of resistant tumour cells.

In an attempt to circumvent this major clinical problem of platinum resistance, a fuller understanding of the mechanism of action of CDDP is required, particularly at the level of its interaction with cellular DNA. It is likely that CDDP resistance is multifactorial in origin: different experimental models have incriminated different mechanisms for this phenomenon.

Multidrug resistance

Resistance of cultured carcinoma cells to a variety of unrelated antineoplastic drugs that are natural products (e.g. doxorubicin, daunorubicin, etoposide, vincristine, vinblastine) has been found in many systems to be due to the overexpression of a plasma membrane glycoprotein (P-glycoprotein). This appears to act as a drug efflux pump, maintaining low intracellular levels of cytotoxic drugs in cells which express the multidrug resistance (mdr) phenotype. P-glycoprotein overexpression is uncommon in previously untreated disease, but is frequently found in patients who have been treated with any of the above-mentioned drugs (Lazo and Bahnson, 1989). The mdr phenotype is not normally considered typical of CDDP resistance. Mdr gene amplification in ovarian cancer specimens has been assessed in a number of studies; (Bourhis et al., 1989 and Bell et al., 1985) and found to be relatively uncommon. In EOC amplification and overexpression of the mdr gene was only found in cell lines

derived from patients with clinical multidrug resistance, including to adriamycin (Onishi et al., 1989).

Platinum resistance in ovarian cancer

Reduced accumulation of platinum has, however, been a consistent finding in CDDP-resistant cell lines of various origins, despite the lack of evidence for the involvement of P-glycoprotein. The exact mechanism whereby platinum enters cells has not yet been elucidated, but alterations in transport across the cell membrane, possibly mediated by cyclic AMP, have been implicated (Mann et al., 1991). Improved platinum uptake into cancer cells offers clear potential for improving the clinical responsiveness of the tumour to CDDP.

Once inside the cell, inactivation of intracellular platinum by the electrophilic thiol compounds glutathione (GSH) and metallothionein (MT) may also contribute to the emergence of platinum resistance. An association between cellular GSH levels and CDDP resistance has been found in many cell lines but it is not clear whether this reflects intracellular inactivation or a general stress response following CDDP treatment. Little actual evidence exists to support a significant reaction between intracellular GSH and CDDP; similarly there is little to suggest a causative link between overexpression of MT and CDDP resistance (Andrews and Howell, 1990). The availability of compounds such as buthionine-S-R-sulfoximine (BSO) to reduce cellular GSH levels has invoked the possibility of modulating tumour response to CDDP therapy, both *in vitro* and *in vivo*.

It is now widely acknowledged that the critical cytotoxic target for CDDP is cellular DNA, through the formation of platinum-DNA (Pt-DNA) adducts. Differences in the rate of adduct formation or the ability of cells to repair CDDP-induced DNA damage may thus be important determinants of cellular sensitivity to CDDP. Indeed, cell lines derived from patients with defective DNA repair mechanisms are known to be highly

sensitive to CDDP (Pera et al., 1987). The development of indirect immunochemical techniques for the accurate and sensitive quantitation of these intrastrand Pt-DNA adducts (Fichtinger-Schepman et al., 1985b and Eastman, 1987) has stimulated much recent interest in the relationship between such adducts and the cytotoxic action of CDDP. The competitive enzyme-linked immunosorbent assay (ELISA) of Fichtinger-Schepman and colleagues, which uses polyclonal antisera to specific synthetic Pt-DNA adducts, appears to give the most sensitive and consistent results. Unfortunately, however, these antibodies are scarce and are not commercially available; in addition, the use of specific antisera against previously recognised adducts may conceal the presence of other minor, but potentially significant lesions.

Aims of thesis

The aims of this thesis can be summarised as follows:

1 To develop a method for the direct detection of platinum-DNA adducts.

2 To determine levels of the major platinum-DNA adducts in a model of CDDP resistance in ovarian cancer *in vitro*.

3 To investigate whether a change in platinum accumulation could account for CDDP resistance.

4 To attempt to sensitise ovarian cancer cells to CDDP by manipulation of the cellular glutathione status.

Layout of thesis

Chapters 2 - 6 are self-contained, with introduction, materials and methods, results and discussion sections.

This thesis attempts to investigate further the mechanisms of platinum resistance in ovarian cancer cells, using a series of three human ovarian cancer continuous cell

lines. The parental cell line was originally derived from a patient with untreated ovarian cancer; its full characterisation has never been officially published. Platinum resistance in one subline had been derived by continuous culture in the presence of CDDP, and in the other had been noted incidentally in a subline with induced resistance to adriamycin. The two CDDP-resistant sublines were thus believed to reflect both induced and intrinsic mechanisms for cellular CDDP resistance. In Chapter 2 these three cell lines, used throughout this work, are further characterised. A modified MTT chemosensitivity assay for assessing cellular sensitivity to CDDP *in vitro* is described, and a broad range of CDDP resistance established for these cell lines. Immunohistochemistry for P-glycoprotein expression demonstrated the lack of relevance for the multidrug resistance phenotype in CDDP resistance.

The availability of an innovative and highly sensitive method for platinum analysis, inductively coupled plasma mass spectrometry (ICP-MS), led to the first major aim of this work: to develop a new assay for the direct analysis of platinum-DNA adducts. Such a direct assay would enable the detection of previously unrecognised Pt-DNA lesions and the corroboration of findings from other studies using the ELISA method for their analysis. Chapter 3 describes the development of the direct assay for measuring the two major intrastrand Pt-DNA adducts, Pt-GG and Pt-AG, using enzymatic disaggregation of highly platinated calf thymus DNA followed by anion-exchange chromatography for the separation of nucleotides and Pt-oligonucleotides, and ICP-MS for the detection of platinum peaks in elution fractions. Having established the elution positions of the two major intrastrand adducts, in Chapter 4 adduct formation and repair is assessed, and related to CDDP-resistance, in the three ovarian cancer cell lines, following treatment with CDDP. The potential of this assay for the direct measurement of these adducts in biological systems is evaluated.

Chapter 5 describes the use of ICP-MS as an analytical technique to assess the relationship between differential platinum uptake and Pt-DNA binding and the degree

of CDDP resistance in the two resistant sublines. A relationship between platinum accumulation and CDDP resistance was found at both the cellular and the nuclear level.

Finally, in Chapter 6 the role of intracellular GSH content, and its relationship with CDDP resistance was examined in the three ovarian cancer cell lines. The capacity of BSO, in the reduction of cellular GSH levels, and its consequent potential for therapeutic modulation of the response of ovarian cancer cells to CDDP was explored, and found to be minimal.

Chapter 7 discusses the general findings of this thesis with respect to the multifactorial nature of CDDP resistance in ovarian cancer cells. The development of more active analogues of CDDP, in the light of improved understanding of the mechanisms of platinum resistance, and the use of resistance modulators to improve clinical responsiveness are some possible clinical applications of this work.

In summary, the work described in this thesis provides further characterisation of three ovarian cancer continuous cell lines with a range of CDDP resistance induced *in vitro*. A novel and highly sensitive technique for the analysis of platinum (ICP-MS), potentially capable of the direct evaluation of Pt-DNA interactions, was assessed. A direct assay for the estimation of the two major intrastrand Pt-DNA adducts in biological systems was developed and appraised. Other putative mechanisms for platinum resistance -differential platinum accumulation and intracellular inactivation by GSH- were investigated in this experimental model.

CHAPTER 2

GENERAL METHODS AND EXPERIMENTAL METHODS FOR THE STUDY OF PLATINUM RESISTANCE

INTRODUCTION

CDDP and its analogues are rapidly becoming the most widely used cytotoxic agents available today for use against solid tumours. The emergence of clinical platinum resistance, however, is a major obstacle to improved longterm patient survival. An important aim of this thesis was to characterise further the nature of this platinum resistance. Theoretical mechanisms of platinum resistance have been deduced from animal and human tumour models; an appropriate study system is essential for meaningful conclusions to be drawn.

Experimental models

The phenomenon of acquired drug resistance has been extensively studied in many tissue culture systems. Extrapolation of such models to the clinical situation has traditionally been more difficult (Wolf et al., 1987). Early work with primary culture of tumour biopsies demonstrated evidence of intrinsic cellular variations in chemosensitivity: a major potential clinical application being the development of predictive assays for chemotherapy using individual tumour specimens. Clonogenic assays,which test the ability of tumour stem cells to reproduce and form a colony of cells after chemotherapy,have been largely used for this work. Observations of the mathematical characteristics of cell survival curves (log cell survival versus dose) yield theoretical information on cellular sensitivity and resistance (Freshney & Dandy, 1983). Such assays, using bilayer semisolid agar with enriched media, were

developed (Hamburger & Salmon, 1977; Courtenay et al., 1978) on the basis that semisolid medium suppresses the growth of most normal cells. Technical problems with these assays in primary culture include low colony forming efficiencies, poor predictive value of chemosensitivity *in vitro* and a lack of good evidence for *in vitro / in vivo* correlation in prospective trials. Increasing scepticism has thus been generated about their value (von Hoff, 1983) particularly as the low plating efficiencies cited suggest that colony growth in suspension only occurs after some highly selective process. In addition it is not possible to vary the duration of drug exposure, thus limiting the experimental value of this system. Some of these problems, however, can be overcome by the use of various complex tissue culture techniques (Ajani et al., 1987, Balconi et al., 1988; Stampfer et al., 1980). Such primary cultures of EOC cells probably provide the model most closely related to the situation *in vivo* for the study of chemosensitivity and resistance.

Freshly derived ovarian cancer cell lines combine these advantages with those of reproducibility and availability. Unfortunately the success rate for the establishment of such freshly derived cell lines is only around 1 -10% (Nio et al. 1989; Langdon et al., 1988). Xenografts, established from primary ovarian tumours in nude mice, can be used as an experimental model system (Massaza et al., 1989; Balkwill et al., 1990). These display similarities in histology, immunocytochemistry, hormone responsiveness, and DNA / chromosome content to the original tumours. Clinical correlations of chemosensitivity are generally good, but some important differences exist, such as faster growth rate, lower metabolic potential, inappropriateness of site and blood supply . In addition, such models are expensive and not amenable to rapid chemosensitivity screening.

The recent establishment of various continuous ovarian cancer cell lines has greatly facilitated both the evaluation of cytotoxic drugs and the investigation of drug resistance. Such cell lines can be easily grown, and are both reproducible and

convenient. However they represent a selected subpopulation of cells and thus may not accurately reflect the true cellular heterogeneity of the original tumour (Balconi et al., 1988). Alterations in growth characteristics and pharmacokinetics with increasing culture duration (Hill, 1983) and contamination by better established continuous lines may also occur. Cell lines with specific resistance to certain agents have been produced by their selection *in vitro* in increasing drug concentrations; such paired sensitive and resistant cell lines offer an attractive model for the study of drug resistance, despite obvious disadvantages. Resistance observed between such pairs of cell lines is frequently far higher than is clinically relevant. This may, however, facilitate the demonstration of contributory factors in acquired drug resistance. Continuous EOC cell lines have been established from the same patient both before and after chemotherapy (Langdon et al., 1988 and Hamilton et al., 1989), providing an alternative model for the study of acquired drug resistance. Heterogeneity in the chemosensitivity of ovarian tumours has also been observed in primary culture (Balconi et al., 1988 and Rotman et al., 1988), thus demonstrating a probable role for intrinsic drug resistance.

Ovarian cancer continuous cell lines

A panel of three related continuous EOC cell lines was used throughout this work: A2780 was originally derived from an untreated patient with ovarian cancer (Eva et al., 1982). Its adriamycin-resistant subline, 2780AD, was produced by the culture of the parental cell line in increasing concentrations of adriamycin until significant adriamycin resistance was achieved. This subline was also found to display a moderate degree of cross-resistance to CDDP; resistance to both agents was stable in drugfree tissue culture conditions for around six weeks. A CDDP-resistant subline, 2780CP, had been produced in similar fashion by stepwise selection in increasing concentrations of CDDP.

Chemosensitivity was determined *in vitro* by a tetrazolium dye based microtitration assay, developed and characterised within the Department of Medical Oncology (Plumb et al., 1989). This assay was shown to give identical results to a standard clonogenic assay and allowed large numbers of simultaneous chemosensitivity assays to be performed under standardised conditions, whereas these cell lines have poor plating efficiencies in clonogenic assays (about 15%) and only produce very small colonies.

This chapter aims to characterise further the cell line A2780, and its resistant sublines 2780AD and 2780CP, and also gives details of general methods used throughout the thesis.

MATERIALS AND METHODS

Cell Lines

All three human ovarian carcinoma continuous cell lines (A2780, 2780AD and 2780CP) were obtained from Dr R. F. Ozols (Fox Chase Cancer Centre, Philadelphia, U.S.A.). 2780AD was known to express high levels of P glycoprotein (Sugawara et al., 1988).

Cell Culture

All cell lines were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Northumbria Biologicals, Cramlington, Northumberland, U.K.) prepared from 10 x concentrated stock solutions. All medium was supplemented with glutamine (2 mM) and foetal calf serum (10%), buffered by the addition of sodium bicarbonate (0.075%) and equilibrated with CO_2 (2% in air). No antibiotics were used in routine maintenance.

The cell lines all grew as adherent monolayer cultures, and were maintained in the exponential phase of growth in 75 cm^2-flasks (J. Bibby Science Products Ltd, Staffordshire, U.K.) or in 25 cm^2 flasks (Nunclon, Life Technologies Ltd, Paisley, U.K.). Cells were subcultured once a week. The cell monolayer was exposed to trypsin (0.25%, Life Technologies Ltd, Paisley, U.K.) in the presence of ethylenediaminetetraacetic acid (EDTA, 1 mM, BDH, Poole,U.K.) in Ca^{++} and Mg^{++} free phosphate buffered saline (Dulbecco's A, PBS) for 30 seconds. This solution was then removed and the monolayer incubated for 5 minutes, at 37° C. The trypsin was then inactivated and the cells collected by resuspension in 10 ml of culture medium. Cell number was determined with an electronic counter (Coulter Model ZBI, Coulter Electronics Ltd, Luton, Bedfordshire, U.K.), and cells reseeded into new flasks at a density of 5 x 10^5/ 25 cm^2 flask or 2 x 10^6/ 75 cm^2 flask.. Flasks were then

equilibrated with CO_2 (2% in air) and sealed prior to incubation. Cultures were fed with new medium at two or three day intervals.

Resistance was maintained in the adriamycin-resistant subline, 2780AD, by its continuous maintenance in a low concentration of adriamycin (2 μM, in standard culture medium). For the CDDP-resistant subline, 2780CP, resistance was maintained by its intermittent exposure to low doses of CDDP in standard culture medium. CDDP resistance was found to be stable for up to six months in drug-free medium. Both cell lines were cultured in drug-free medium for at least five days prior to experimentation.

Mycoplasma Testing

All cell lines were examined monthly for mycoplasma contamination by the Hoechst staining method.

Maintenance of Cell Stocks

Stocks of all cell lines were held in liquid nitrogen.In general, cells were maintained in culture for three months and then replaced with fresh cells from stock. This aimed to reduce variability caused by maintenance in continuous culture, and also to minimise the risk of cross-contamination.

Monolayer cultures were harvested with trypsin, collected into a sterile universal container (Bibby Sterilin Ltd., Stone, Staffordshire, U.K.) and centrifuged for 5 minutes at 200g. The resulting cell pellet was then resuspended in culture medium containing dimethyl sulphoxide (10%, Merck, Thornliebank, Glasgow, U.K.) at an approximate density of 5 x 10^6/ ml. This suspension was then stored in aliquots in 1 ml cryotubes (Nunclon, Life Technologies Ltd., Paisley, U.K.) and frozen at a rate of 1°C per minute in an insulate container in a freezer at -70° C. After at least four hours the cells were transferred into liquid nitrogen.

When required, an ampoule was removed into warm water (37° C). The cell suspension was then transferred into a 25 cm^2 flask and 5 ml culture medium added. The cells were equilibrated with CO_2 (2%), sealed and incubated at 37°C for 24 hours. The medium was then changed and the cells allowed to grow. At least two passages were performed prior to use in experiments.

Measurement of cell volume

Cell size was determined when necessary using an electronic particle counter (Model Industrial D; Coulter Electronics Ltd., Luton, Bedfordshire, U.K.). Calibration for cell size was performed with standard polystyrene divinyl benzene latex beads (P.D.V.B.Latex, Coulter Electronics Ltd., Luton, Bedfordshire, U.K.), using a half-count technique (Section 6.1, Reference Manual for the Coulter Counter Model D, issue A: 1984). The threshold (t), attenuation (A) and aperture (I) were determined for a given size of latex beads. The calibration constant (K_d) was calculated from the following equation:

$$K_d = \frac{d}{\sqrt[3]{t \times I \times A}}$$

where d is the known mean diameter of the P.D.V.B. particles in μm.

Cell size was calculated by the determination of t, A, and I for each cell line. The mean diameter of a single cell was then derived from the above formula and the volume of a cell, assuming a spherical shape, calculated.

Determination of doubling time

The doubling time of each cell line was determined from cell counts of cells grown in optimal culture conditions. For this, cells were seeded in 1 ml of culture medium into 24-well plates (Nunclon, Life Technologies Ltd., Paisley, U.K.) at a density of 10^4 cells per well and grown at 37°C in a humidified atmosphere of 2% CO_2 in air.

From day 2 onwards cells in three wells were trypsinised and counted daily as described. Medium in all other wells was replaced daily. Cell doubling time was calculated from the shape of the exponential growth phase obtained from a plot of the logarithm of cell number against time.

Chromosomal analysis

For chromosomal analysis of all three cell lines, cells were grown in 25 cm^2 flasks in Ham's F10 medium (Gibco, Paisley, U.K.), supplemented with foetal calf serum (10%), newborn calf serum (10%) and penicillin / streptomycin solution (1%).

Subconfluent cells in the exponential phase of growth were incubated for 2.5 hours with colcemid (Gibco, Paisley, U.K.) at 37° C; the cells were then detached using trypsin / versene (0.25% trypsin; 0.5 g/l versene) and centrifuged. After resuspension in hypotonic solution (0.0375M KCl), the cells were recentrifuged and fixed by the dropwise addition on methanol: glacial acetic acid (3:1). Microscopic slides were prepared by spreading several drops of cell suspension in fresh fixative onto grease-free, wet, ice-cold slides, and allowing them to air-dry after the addition of a few drops of fixative. When dry, the slides were G-banded by pre-treatment with trypsin (0.1% Difco trypsin in Sorensen's buffer). Metaphase spreads were counted and analysed under oil immersion (x100) microscopy.

Chemosensitivity assay

Drug sensitivity was determined by a tetrazolium based microtitration assay (Mosmann, 1983) which was further developed and optimised in the Department (Plumb et al., 1989). In this assay cells in the exponential phase of growth are exposed to drug for a fixed time period, and then allowed to grow in drug-free medium for 2 - 3 doublings. Final cell number is determined indirectly by the reduction of tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Company, Poole, U.K.), by live, but not dead, cells

to a water-insoluble purple formazan product. Under appropriate conditions the absorbance of the reduced dye at 570 nm is proportional to the biochemically viable cell number. This modified MTT assay has been proven to give results comparable to those obtained in a standard clonogenic assay for both adherent and non-adherent cell lines (Plumb et al., 1989).

Determination of the optimal concentration of MTT

Since MTT itself can be cytotoxic to cells, the optimal concentration for maximal MTT-formazan and minimal toxicity was determined for each cell line.

Cells were plated out into 96-well microtitre plates (12 x 8 wells, Linbro, ICN Biomedicals Ltd., High Wycombe, Buckinghamshire, U.K.) in 200 µl of medium at a concentration of 1 x 10^4 cells per well and grown at 37° C for 2 - 3 days in a humidified atmosphere of 2% CO_2 in air. The first and last columns of 8 wells on the plate acted as controls, containing medium alone. Medium was then removed and replaced with 200 µl medium containing HEPES buffer (Life Technologies Ltd., Paisley, U.K.,10 mM). MTT to a final concentration ranging between 0.1 and 1.0 ng / ml was then added to the middle ten columns, and the plates incubated in darkness in otherwise standard conditions for four hours. All wells were then aspirated and the resulting MTT-formazan crystals dissolved in 200 µl dimethyl sulphoxide, with the addition of 25 µl of Sorensen's glycine buffer (glycine 0.1M, NaCl 0.1M, pH 10.5).

Absorbance at a wavelength of 570 nm was then measured using an ELISA microplate reader (Bio-rad Model 3550, Bio-rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.). The outer columns of the plate, containing no cells, were used as blanks for the plate reader.

Estimations of chemosensitivity

CDDP was obtained as a non-sterile powder (Sigma Chemical Company, Poole, U.K.) and prepared as a stock solution at 1 mg / ml in PBS, filter sterilised (pore size 0.22 μm, Millex-GS, Millipore Ltd., Watford, Hertfordshire, U.K.) and stored at 4° C in darkness for up to 2 months.

For analysis of the sensitivity of each cell line to CDDP, cells were plated out into 96-well microtitre plates as described above, at a concentration of 5 x 10^2 to 1 x 10^3 cells per well and grown for 2 - 3 days under standard conditions. The first and last columns of the plate again contained medium alone. Cells were then exposed to drug for either 4 or 24 hours. A serial dilution (usually 1:4) of CDDP was prepared to give a range of 8 concentrations such that the ID_{50} of the cell line was in the middle of the range. 4 or 8 replicate wells were used for each drug exposure, and cells in the 2nd and 11th columns fed with drug-free medium only. Following CDDP exposure, medium and drug was removed by needle aspiration and cells fed daily with drug-free medium for 3 days. All wells were then fed with 200 μl of fresh medium buffered with HEPES buffer (10mM), and MTT (at optimal concentration) as described above,and plates incubated for 4 hours in darkness under otherwise standard conditions. Medium and MTT was then removed, formazan crystals dissolved in dimethyl sulphoxide / Sorensen's glycine buffer, and absorbance at 570 nm measured as described above. The outer 2 columns, containing no cells were used as a blank for the plate reader. Columns 2 and 11, containing cells not exposed to CDDP, were used to obtain the absorbance value for untreated cells. The absorbance values per CDDP exposure thus obtained were plotted as the percentage of that for untreated cells against a logarithmic scale of drug concentration. The ID_{50} value of CDDP for each cell line was defined as the concentration of CDDP required to obtain an absorbance value of 50% of that of untreated cells.

Conditions were optimised such that cells underwent at least 2 population doublings during the period between CDDP removal and estimation of viable cell number. Absorbance was shown to be linear with viable cell number in the range used for these experiments. To determine the stability of induced resistance to CDDP *in vitro*, some experiments were performed over increasing passage number over a period of six months.

Immunohistochemistry for P glycoprotein

To determine the contribution of the multidrug resistance phenotype (mdr) to CDDP resistance induced in these cell lines, immunohistochemistry using the C219 monoclonal antibody to P glycoprotein (CIS U.K. Ltd., High Wycombe, Buckinghamshire) was used at a final concentration of 10 μg / ml. Cytospin preparations of each cell line were made by mechanical removal from monolayer growth on 75 cm^2 flasks, centrifugation of the resulting cell suspension at 200g for five minutes, and resuspension in PBS. Aliquots (0.5 ml) were then pelleted onto clean glass microscope slides in a cytospin centrifuge (Shandon). The resulting cytospin preparations were then fixed in acetone at room temperature.

After washing in Tris saline (pH 7.6, 5 minutes), slides were stained using an indirect immuno-alkaline phosphatase technique, incubating first with specific primary mouse monoclonal antibody (C219) for 2 hours. This and all subsequent incubations were performed at room temperature in a humidified container. All antibody dilutions were carried out in Tris buffer (pH 7.6). After a further wash in Tris saline for 5 minutes, 100 μl of a second antibody (rabbit anti-mouse immunoglobulin, conjugated to alkaline phosphatase; Dako, High Wycombe, Buckinghamshire, U.K.) was applied at a concentration of 1:20 in normal human serum (50%, filtered) for 45 minutes. Slides were then washed again in Tris saline for 5 minutes and incubated with alkaline phosphate substrate solution, 100 μl per slide, for 30 minutes to produce a red reaction in cells which express P glycoprotein. Slides were counterstained with

Haematoxylin (15 seconds) and submerged in Scott's Tap Water Substitute for 20 seconds before mounting.

Cytospin preparations of a continuous small cell carcinoma of lung cell line, (H69LX10), known to express strongly P glycoprotein (Plumb et al, 1990), were used as positive controls. An irrelevant monoclonal antibody (Clonab LN-C, Biotest, U.K.) was substituted for the primary antibody to provide negative controls.

RESULTS

Morphological characteristics

All three cell lines were found to be morphologically similar at subconfluence, with A2780 cells appearing the most heterogeneous, 2780AD cells less so, and 2780CP cells larger and more like typical epithelial cells (Plate 1) under standard culture conditions.

Growth characteristics

Typical values for mean cell doubling times, cell volume and ploidy are shown in Table 1. Each growth experiment was performed at least twice in triplicate.

For chromosomal analysis, typical metaphase spreads for each cell line are shown in Plates 2, 3 and 4. All three cell lines were found to have a basically diploid chromosome number, with a variety of deletions, duplications and translocations being observed in the resistant sublines (Table 2).

Cell Line	Mean Doubling Time (h)	Mean Cell Volume (μm3)	Chromosome Number
A2780	26.4	508	Diploid
2780AD	29.4	767	Diploid
2780CP	22.5	612	Diploid

TABLE 1: Growth characteristics of ovarian cancer continuous cell lines under standard tissue culture conditions.

Cell Line	Ploidy	Observed karyotypic abnormalities	
A2780	46XX	$6q^+$	
2780AD	47XX	$1p^+$ (1/7) translocation $1q^-$ $2q^-$ $4q^-$	$6q^+$ $9q^+$ $12p^+$ $14q^-$ 18^- $21q^+$
2780CP	46XX	1q duplication $6q^-$ $7q^+$	$13p^+$ $20q^-$

TABLE 2: Karyotypic abnormalities displayed by ovarian cancer cell lines. (p: short arm of chromosome; q: long arm of chromosome; $^+$ denotes additional chromosomal material, $^-$ denotes deletion of chromosomal material)

Optimal concentration of MTT for use in the chemosensitivity assay

MTT was not found to have significant toxicity in any of the three ovarian cell lines, to a maximum concentration of 1.0 mg / ml (Figure 1). This concentration of MTT was therefore used throughout all subsequent chemosensitivity assays.

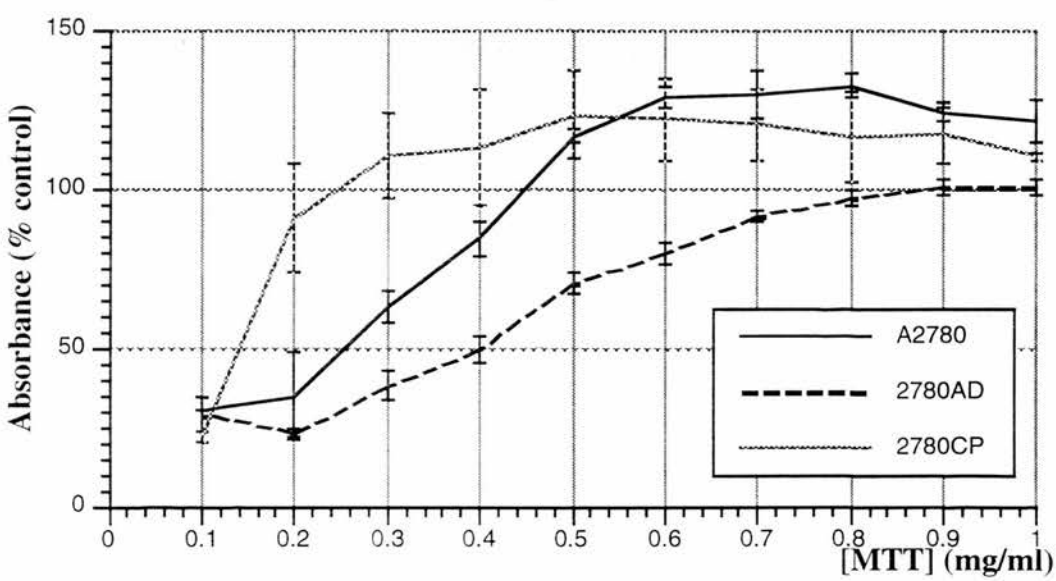


FIGURE 1: Sensitivity of ovarian cancer cell lines to MTT.

Cellular sensitivity to CDDP

Chemosensitivity profiles for all three cell lines after 24 hours exposure to CDDP are shown in Figures 2, 3, & 4, at both early and late passage number. For the latter, drug-resistant sublines were assayed after at least six months continuous culture in drug-free medium.

From these curves the mean ID_{50} value for CDDP against each cell line can be interpolated, as that concentration of drug which reduces cell survival by 50%. Table 3 summarises these findings. Both resistant sublines displayed a stable and moderate degree of CDDP resistance compared with A2780, the drug-sensitive parental cell line. 2780AD was 14.2-fold resistant, and 2780CP 26.8-fold resistant to CDDP using this assay. All experiments were conducted in triplicate and repeated at least twice.

Cell Line	Early	Late
A2780	0.19	0.19
2780AD	2.3	3.1
2780CP	5.1	5.1

TABLE 3: ID_{50} values (μM) for CDDP in ovarian cancer cell lines at early (≤ 12) and late (>12) passage number.

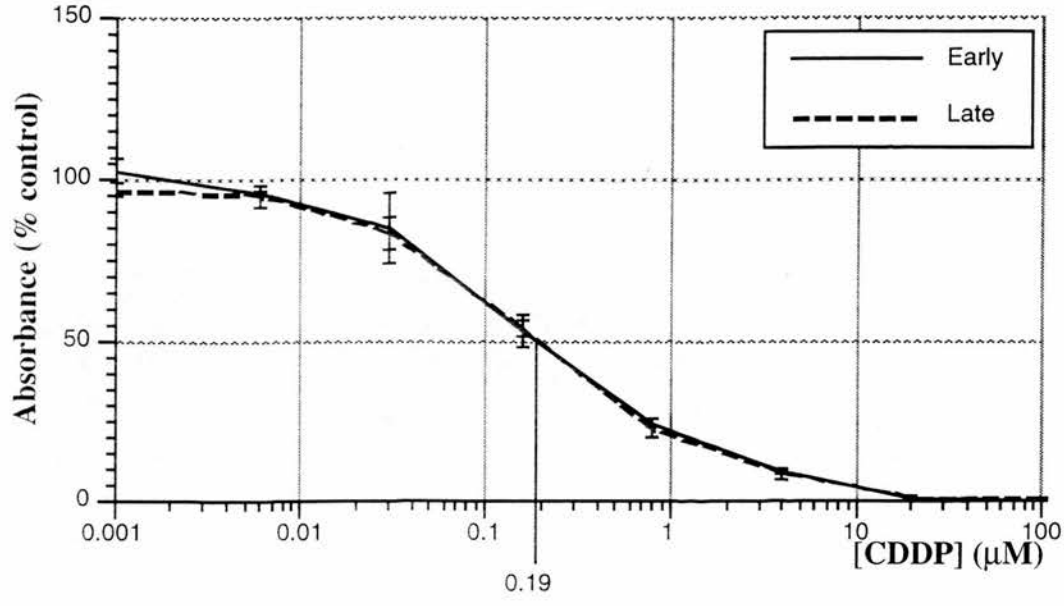
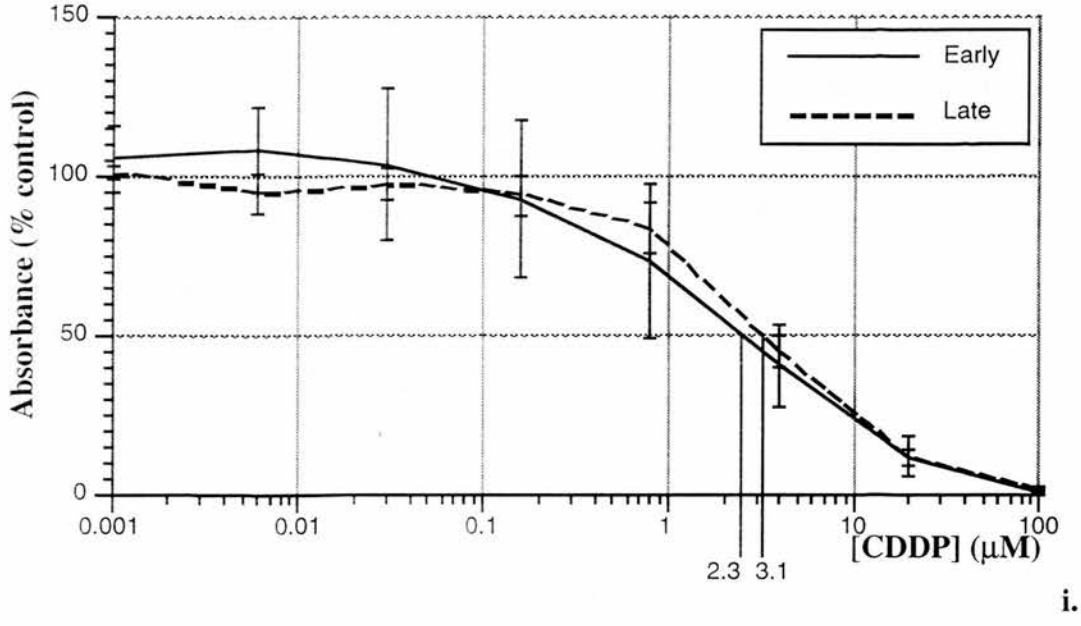


FIGURE 2: Sensitivity of A2780 to CDDP at early (≤ 12) and late (>12) passage number.



i.

FIGURE 3: Sensitivity of 2780AD to CDDP at early (≤12) and late (>12) passage number.

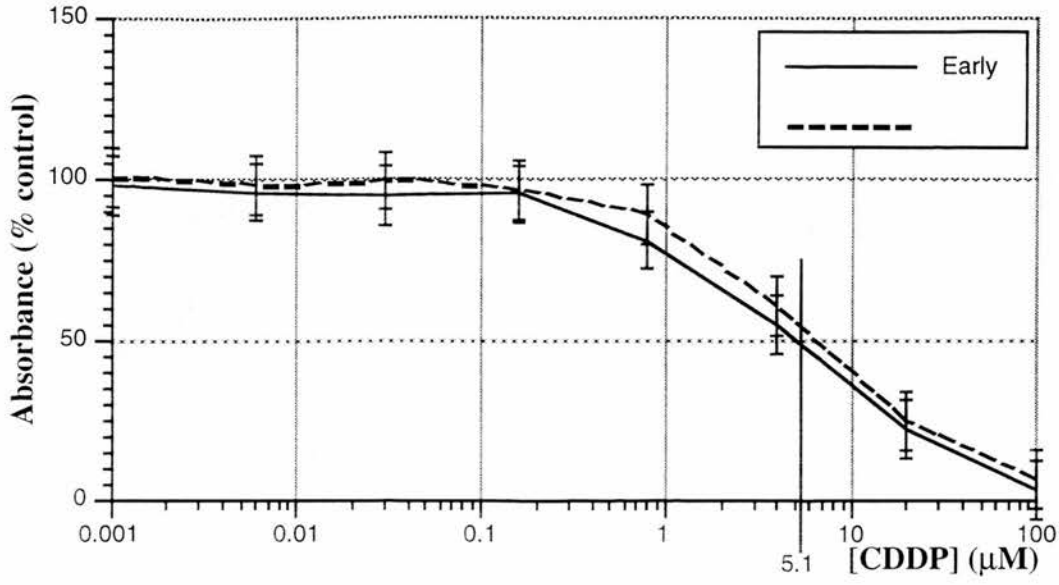


FIGURE 4: Sensitivity of 2780CP to CDDP at early (≤12) and late (>12) passage number.

CDDP sensitivity was also compared for 4-hour and 24-hour exposure in all three cell lines, within the same experiment (Figures 5, 6, & 7). Table 4 summarises these findings. For A2780 and 2780CP, 4 hour CDDP exposure reduced chemosensitivity by approximately 3-fold. For 2780AD, however, the reduction in chemosensitivity was less, approximately 2.3-fold. This experiment was conducted twice, in triplicate.

Cell Line	4h	24h
A2780	0.43	0.13
2780AD	7.1	3.05
2780CP	14	4.2

TABLE 4: ID_{50} values for CDDP in ovarian cancer cell lines after 4 and 24 hours exposure to CDDP.

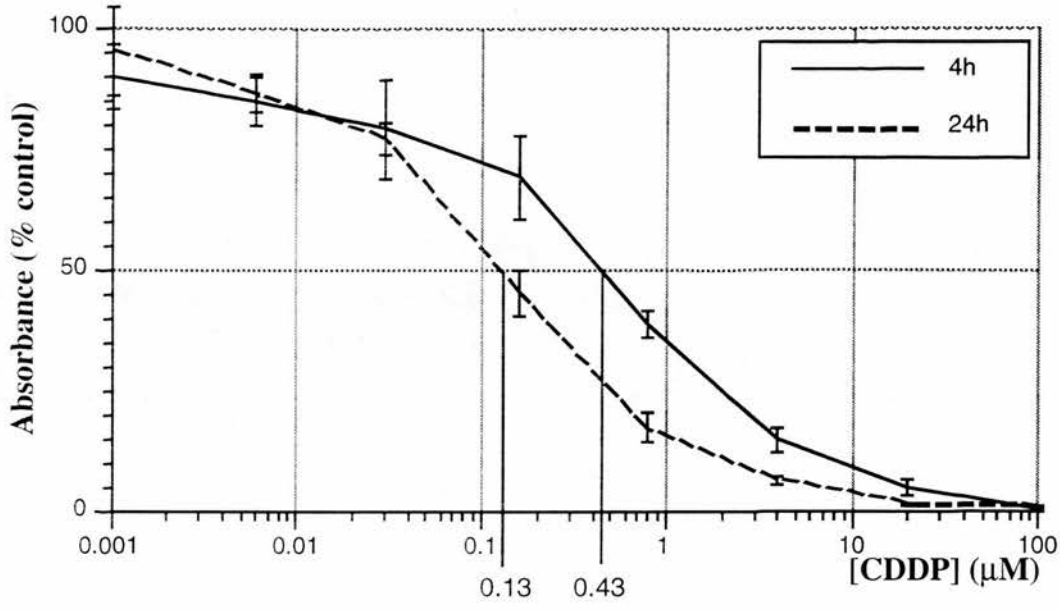


FIGURE 5: Sensitivity of A2780 cells to CDDP after 4 hours and 24 hours exposure.

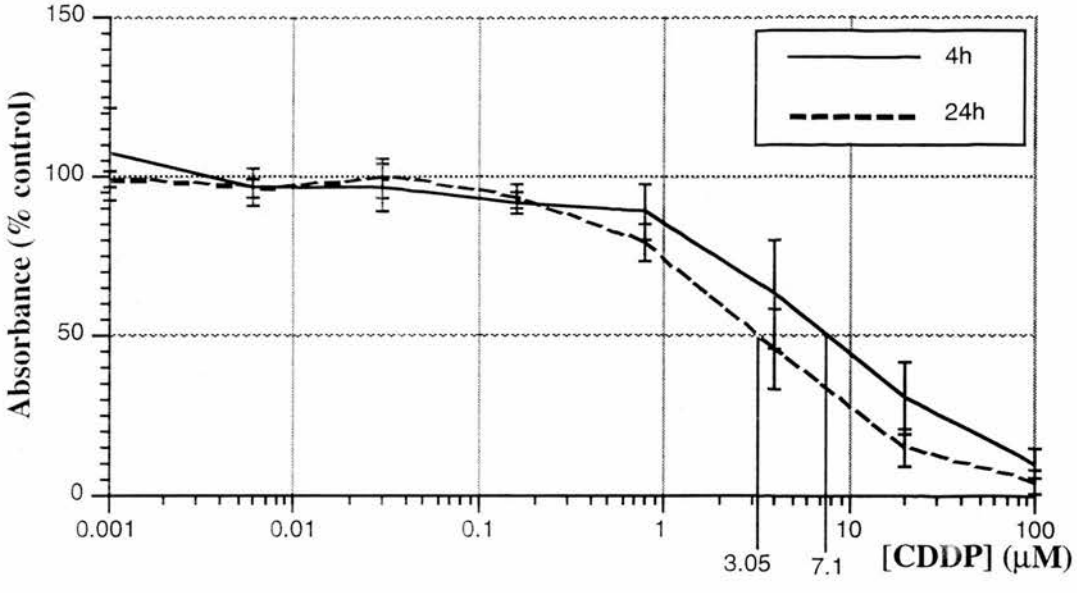


FIGURE 6: Sensitivity of 2780AD cells to CDDP after 4 hours and 24 hours exposure.

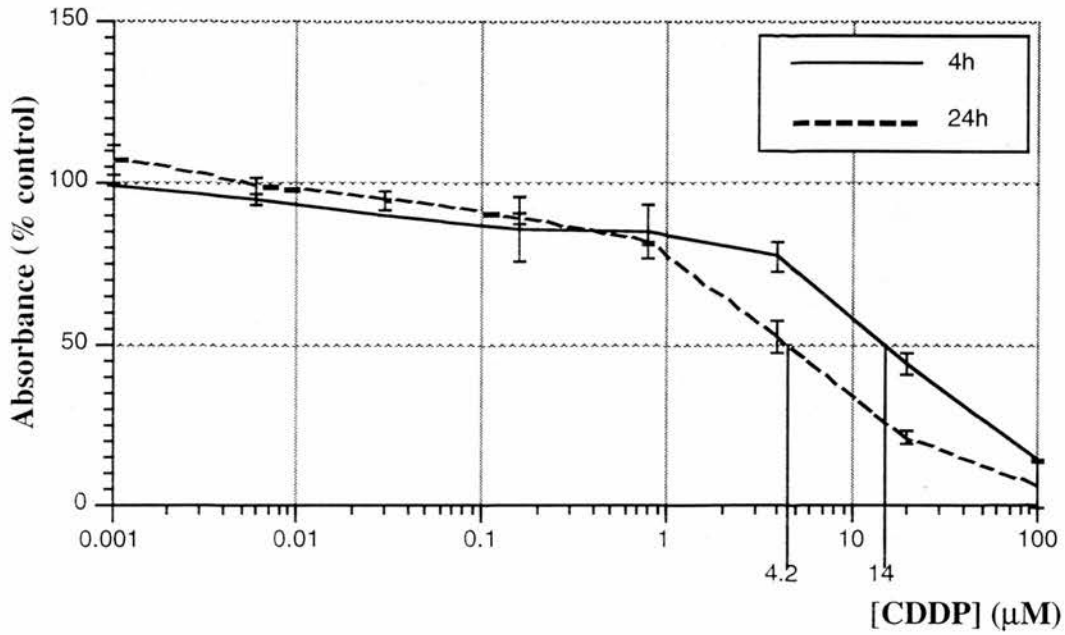


FIGURE 7: Sensitivity of 2780CP cells to CDDP after 4 hours and 24 hours exposure.

Immunohistochemistry for P-glycoprotein

Plate 5 shows typical immunostaining patterns for all three cell lines. 2780AD, the adriamycin-resistant subline,with moderate cross-resistance to CDDP, showed strong immunostaining, indicating the presence of significant P-glycoprotein. In contrast, A2780 and 2780CP showed no immunostaining, indicating the absence of P-glycoprotein in these cell lines. However, 2780AD cells which had been cultured for six months in drug-free medium appeared to have lost P-glycoprotein expression.

DISCUSSION

All three cell lines showed growth characteristics typical of epithelial cell lines. A range of karyotypic abnormalities was observed in the two resistant sublines. Chemosensitivity to CDDP was found to be stable over a six month period of continuous culture for all three cell lines,even when the resistant sublines were maintained in drug-free conditions. Both drug-resistant sublines exhibited a moderate degree of resistance to CDDP, with resistance ratios of 14.2 (2780AD) and 26.8 (2780CP) for 24 hours exposure. Exposure to CDDP for four hours produced similar resistance ratios for the lines, 16.5 for 2780AD and 32.6 for 2780CP. P-glycoprotein immunostaining demonstrated strong expression of this multidrug efflux pump in 2780AD only; this expression was lost after prolonged culture of this subline in adriamycin-free medium.

Complete characterisation of the ovarian carcinoma cell line A2780, and of its two established drug-resistant sublines, 2780AD and 2780CP, has not previously been published. These data provide further confirmation of their epithelial cell nature, as shown by their growth characteristics. Doubling times in optimal tissue culture conditions were similar for all three cell lines, with 2780CP growing slightly faster (doubling time 22.5 cf 26.4h) and 2780AD slightly slower (29.4 cf 26.4h) than the parental cell line. Karyotypic analysis revealed basic diploidy in A2780 and 2780CP, whilst 2780AD contained 47 chromosomes. Multiple chromosomal abnormalities were found, particularly deletions of chromosome 6, deletions of the long arm of chromosome 1, and multiple translocations. These findings are similar to those reported in other CDDP-resistant ovarian cancer cell lines (Wolf et al., 1987 and Briers et al., 1989).

The development of clinical resistance to CDDP is a well established phenomenon and has undoubtedly limited its therapeutic benefit. Solid tumours are known to be

heterogeneous in cellular content, and resistance could therefore be due to either the clonal expansion of a subpopulation of cells with inherent resistance (intrinsic) or or its development *de novo* in previously sensitive cells (acquired), or to a combination of these. Resistance mechanisms which may be operational at this level include: increased activity of a specific efflux pump, as in the multidrug resistance phenomenon; intracellular activation or deactivation; detoxification changes and alterations in DNA repair mechanisms.

Chemosensitivity assays of all three cell lines to CDDP, for 24 hours exposure, were performed over a prolonged period of continuous culture *in vitro* , and results compared for early and late passage number (Figures 2, 3, & 4). The modified MTT assay used throughout this work was found to give reproducible results.

The ID_{50} value for the parental EOC cell line, A2780, was shown to be unchanged over six months, at 0.19 μM CDDP. The ID_{50} value for CDDP against the adriamycin-resistant subline, 2780AD, was found to be 2.3 μM at early passage number: this moderate degree of cross-resistance is interesting and has not previously been reported. Resistance to CDDP was found to be stable over a period of six months continuous culture in adriamycin-free tissue culture medium (ID_{50} 3.1 μM at late passage number) For this subline, resistance to adriamycin is normally maintained *in vitro* by continuous culture in a low concentration of adriamycin (2 μM); continuous exposure to CDDP is therefore not necessary to maintain cross-resistance to CDDP. A greater degree of CDDP resistance was found in the CDDP-resistant subline 2780CP: the ID_{50} value being 5.1 μM, giving a resistance ratio of 26.8 compared with A2780, and 2.2 compared with 2780AD. Resistance to CDDP is normally maintained by intermittent exposure of cells to moderate concentrations of CDDP (1 μM); this work showed that CDDP resistance was stable over at least six months in drug-free culture medium (ID_{50} 5.1 μM also at late passage number, Table 3).

Chemosensitivity assays were also conducted over a 4-hour exposure period to CDDP, and compared within the same experiment to standard 24-hour ID_{50} values (Figures 5, 6, & 7). This shorter exposure time conferred a similar reduction in CDDP sensitivity for both A2780 and 2780CP (3.3-fold), perhaps indicating that cellular toxicity to CDDP is complete by four hours for these two cell lines. In contrast, there was a smaller reduction in sensitivity for 2780AD (2.3-fold), which may reflect differential rates of drug uptake in this subline (Table 4). Resistance ratios for the two resistant sublines after 4 hours exposure to CDDP were similar to those after 24 hours, 16.5 for 2780AD and 32.6 for 2780CP.

The multidrug-resistance efflux pump, P-glycoprotein, is known to be an important mechanism of cellular resistance for many unrelated anti-cancer drugs, including anthracyclines, vinca alkaloids and etoposide. Its expression in ovarian cancer patients has only convincingly been demonstrated following treatment with these agents: hence.it is not thought to be a major mechanism for primary CDDP resistance (Goldstein et al., 1989; Bourhis et al., 1989). 2780AD is known to express the gene for P glycoprotein, thought to be the main mechanism for its resistance to adriamycin (Sugawara et al., 1988). Cross-resistance of this cell line to CDDP has not previously been described. The observation that its sensitivity to adriamycin is not fully restored by calcium-channel blocking drugs (resistance modulators) implies additional mechanisms of resistance may exist (Rogan et al., 1984), some of which may also contribute to CDDP resistance.

Plate 5 shows typical P-glycoprotein immunostaining patterns for all three cell lines. A2780 and 2780CP did not show any evidence of P-glycoprotein expression, providing further evidence for the lack of involvement of this mechanism in CDDP resistance. Interestingly, whilst 2780AD showed strong expression at early passage number, this pattern was lost after prolonged culture in drug-free medium. Since CDDP sensitivity in 2780AD remained stable over this period, it can also be deduced

from this that the P-glycoprotein pump is not an important contributor to its cross-resistance to CDDP.

No single mechanism has been found to explain the development of CDDP resistance, which is thought to be a multifactorial phenomenon. Multidrug resistance (mdr) is not thought to be significant, although 2780CP has been shown to accumulate less CDDP for equivalent drug exposures, and efflux drug faster than its more sensitive parent (Parker et al., 1991). Increased DNA repair in the resistant subline, reversed by aphidicolin (a specific inhibitor of DNA polymerase α), has also been shown (Masuda et. al., 1990). Recent attention has focussed on the interaction between cellular DNA and platinum, in an attempt to elucidate the actual cytotoxic lesion and thus investigate further the mechanisms of platinum resistance.

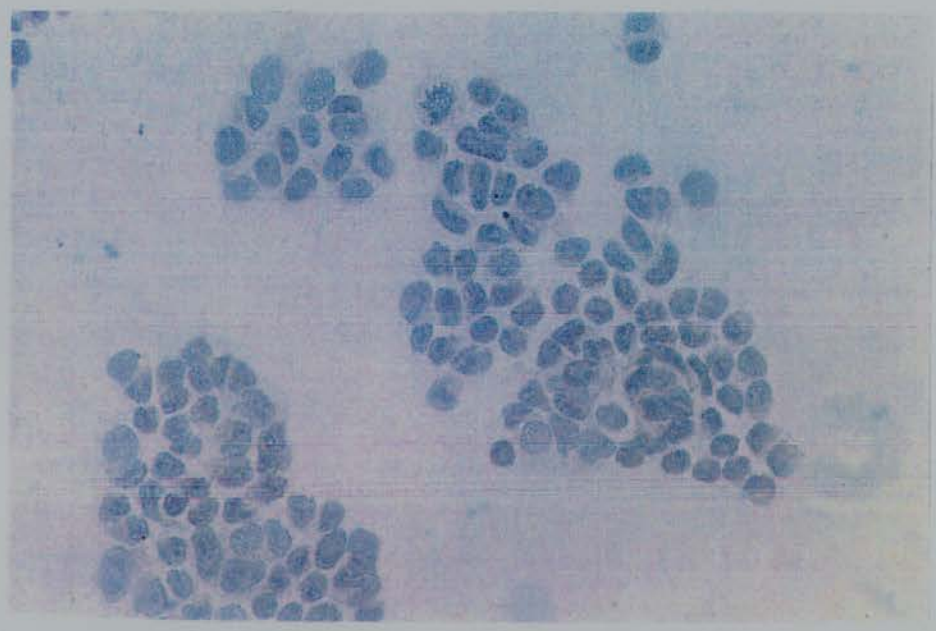


PLATE 1a : Morphological characteristics of A2780 at subconfluence.

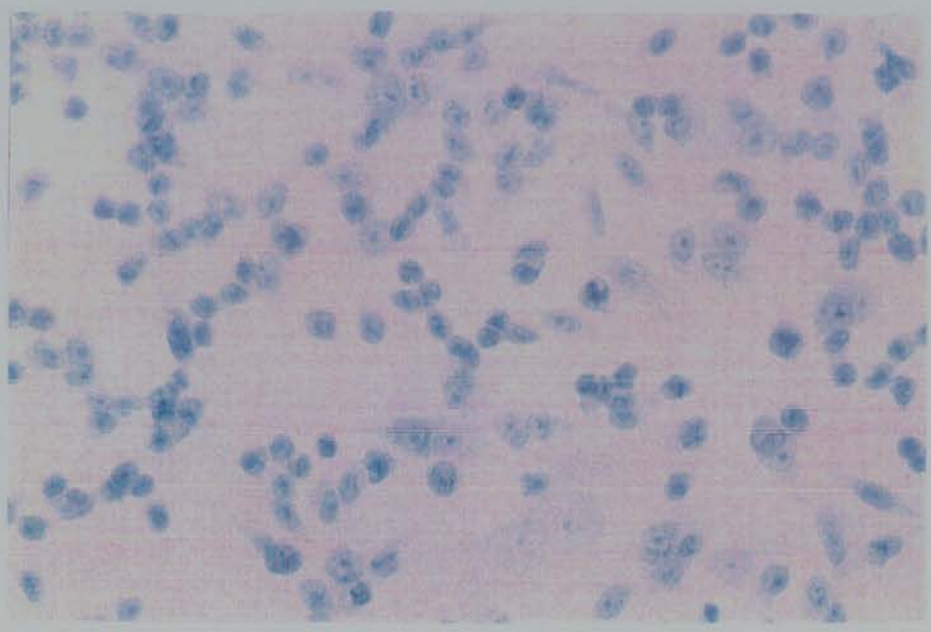


PLATE 1b: Morphological characteristics of 2780AD at subconfluence.

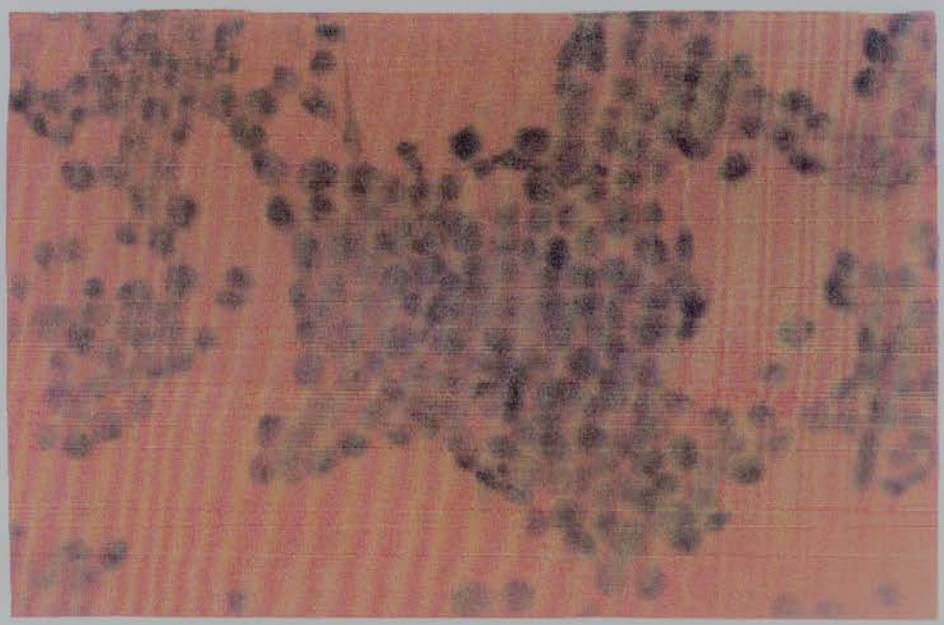


PLATE 1c : Morphological characteristics of 2780CP at subconfluence.

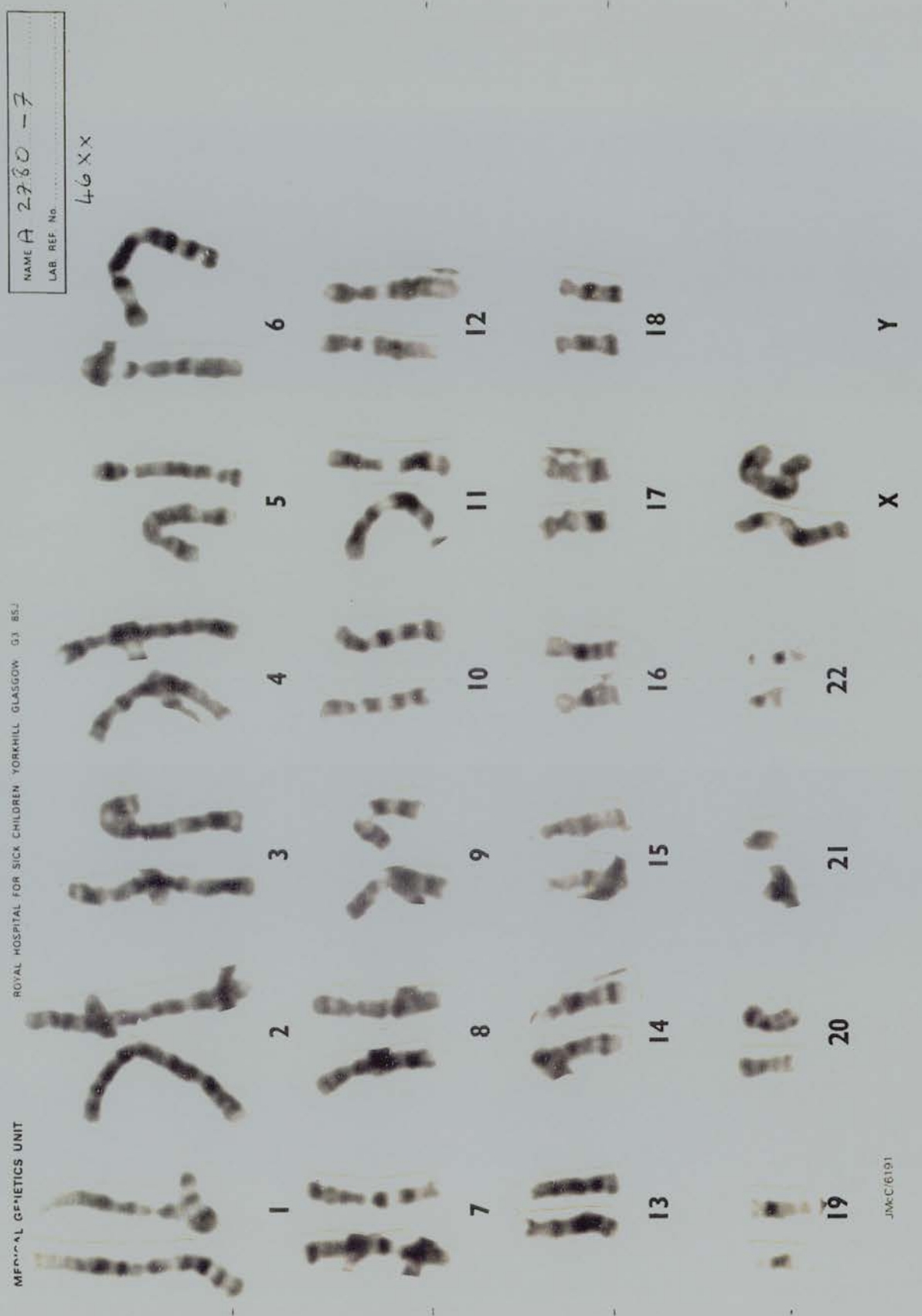


PLATE 2: Metaphase spread for A2780.

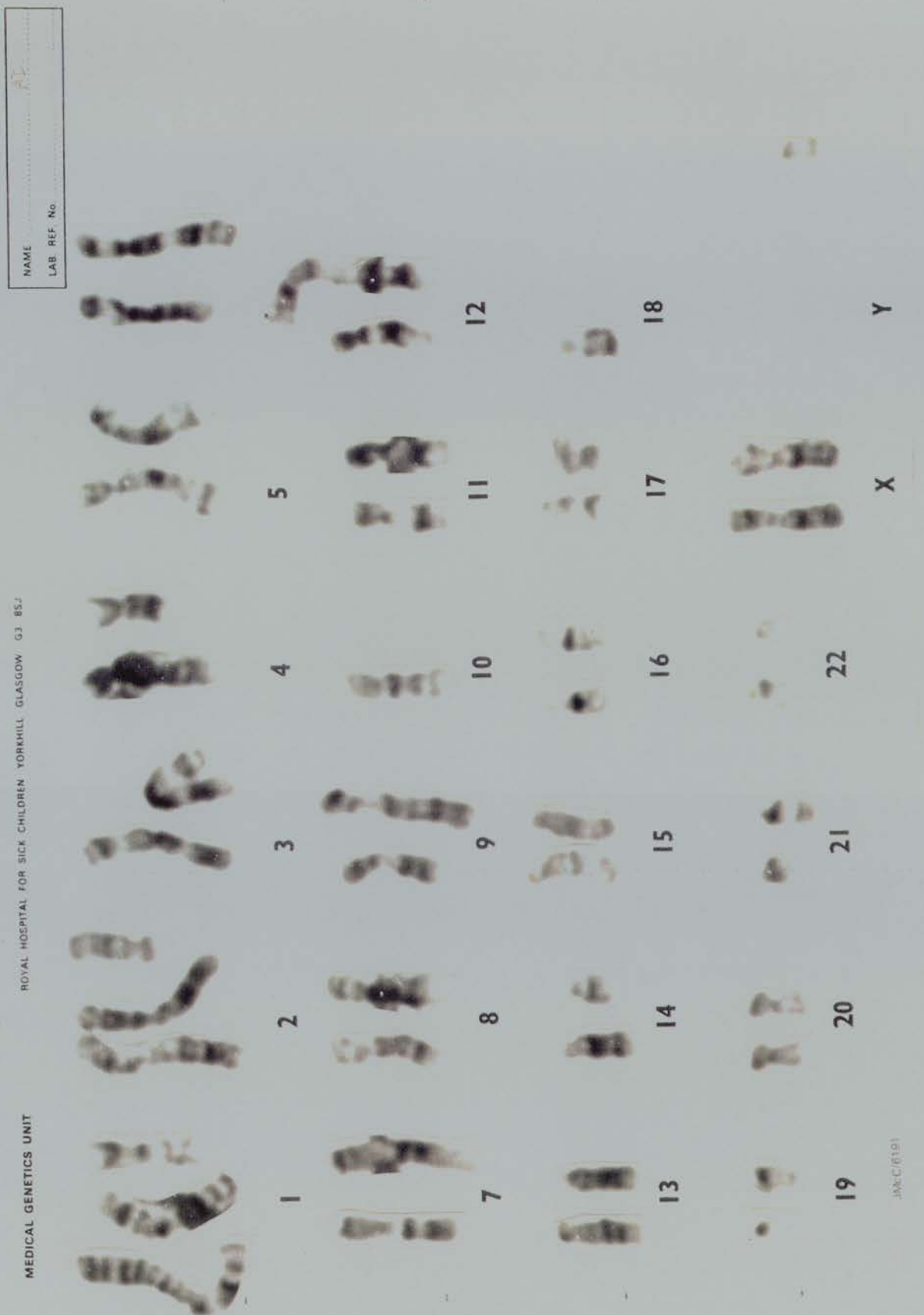


PLATE 3: Metaphase spread for 2780AD.

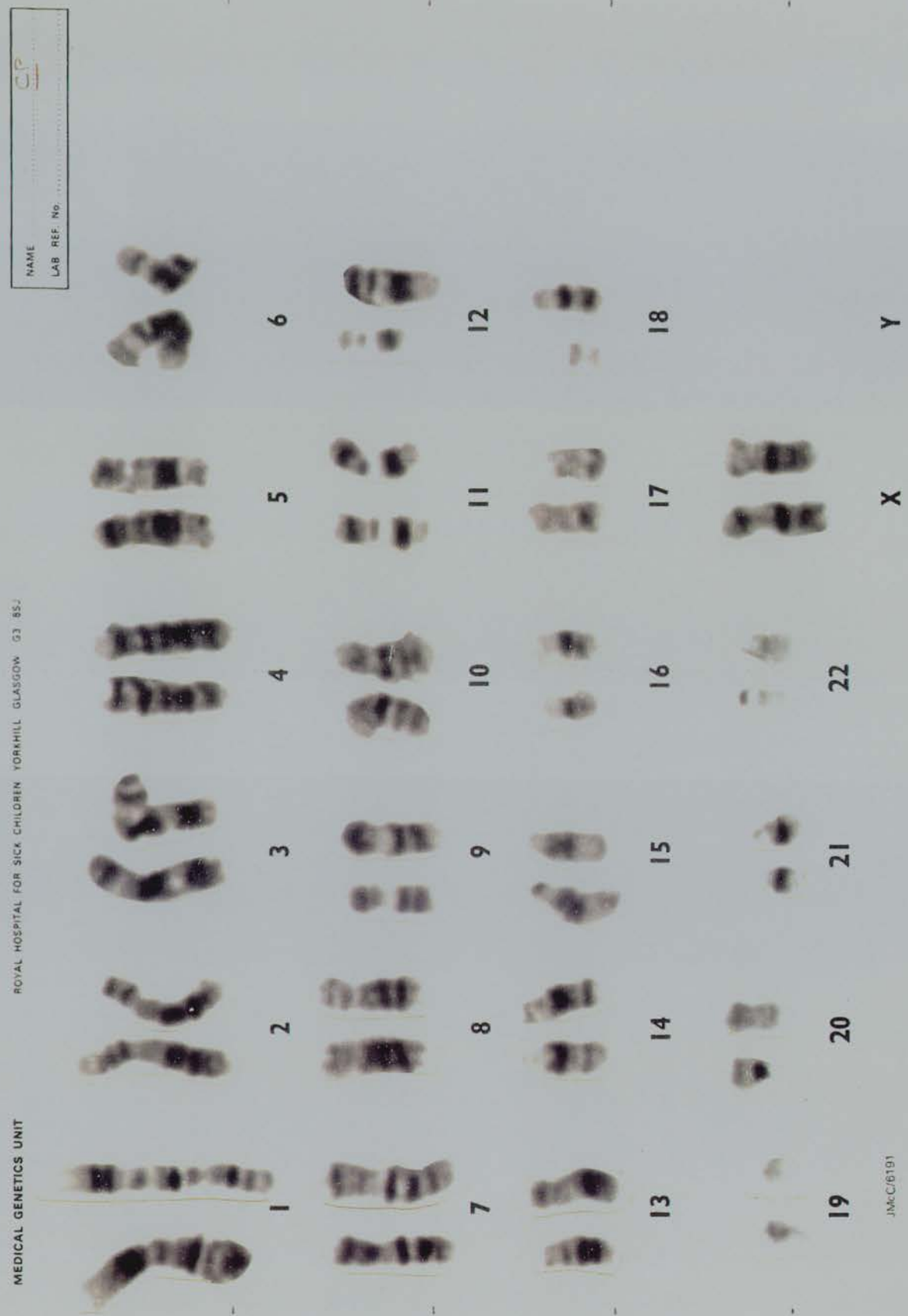


PLATE 4: Metaphase spread for 2780CP.

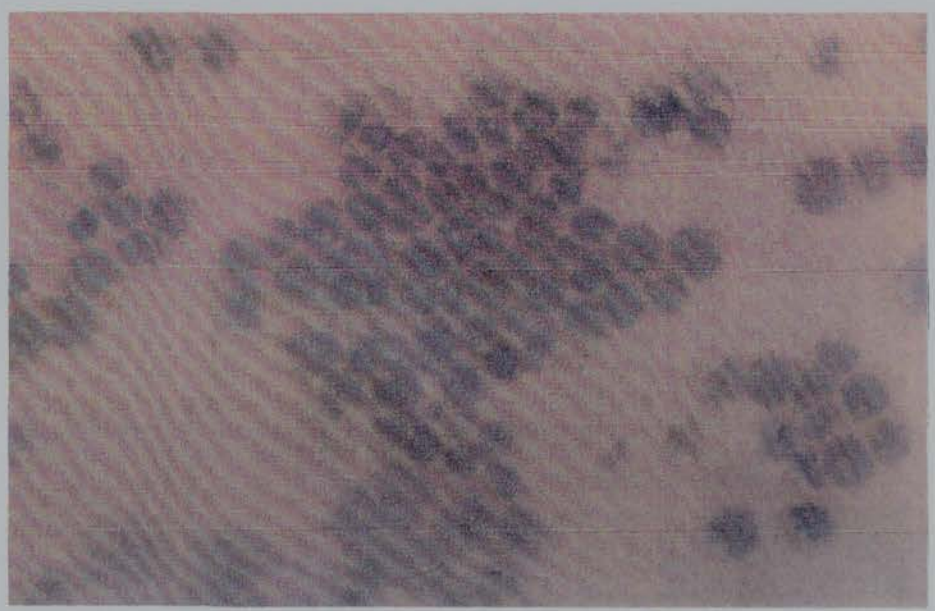


PLATE 5a: P-glycoprotein immunostaining for A2780

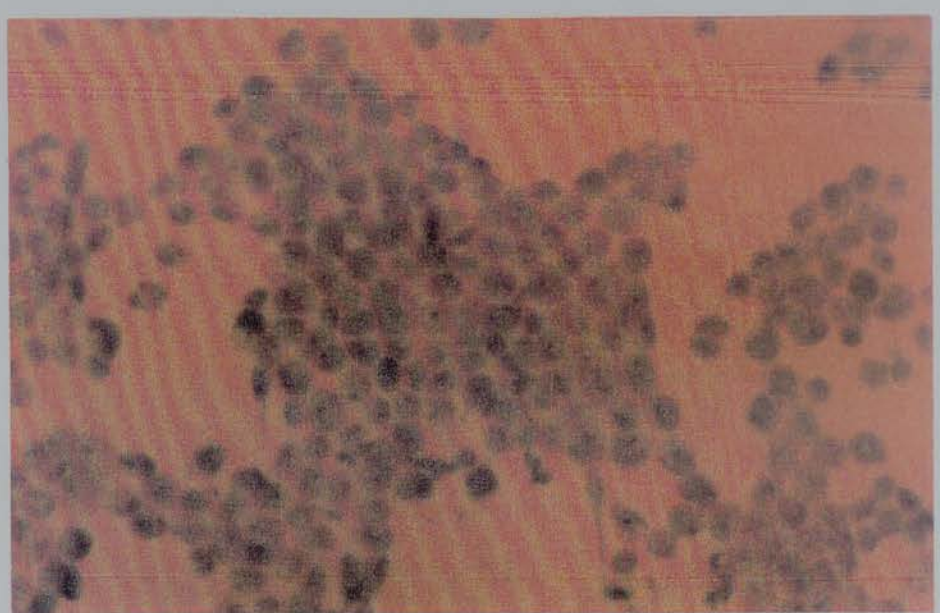


PLATE 5b: P-glycoprotein immunostaining for 2780CP

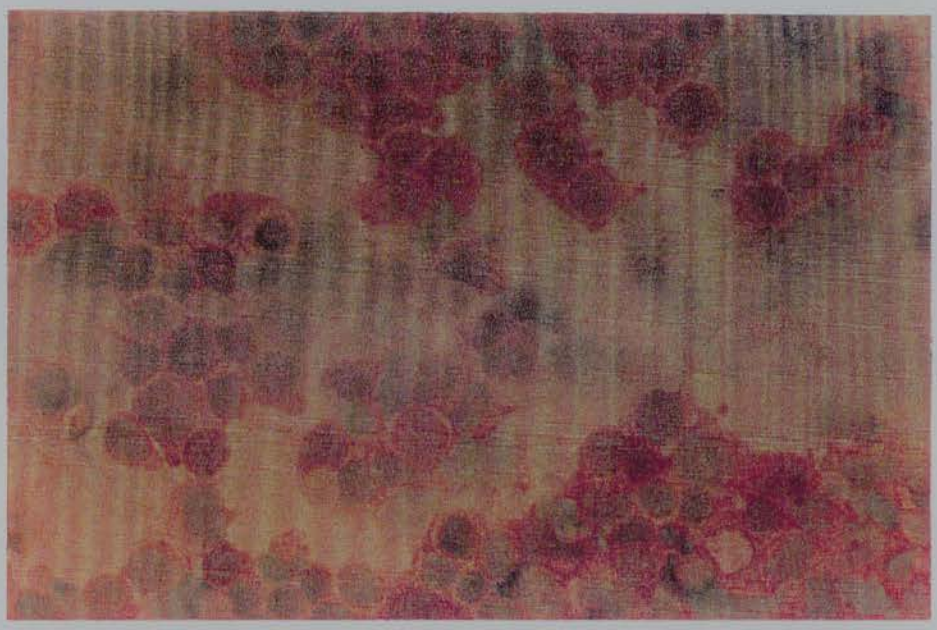


PLATE 5c: P-glycoprotein immunostaining for 2780AD, after recent culture in adriamycin-containing medium.

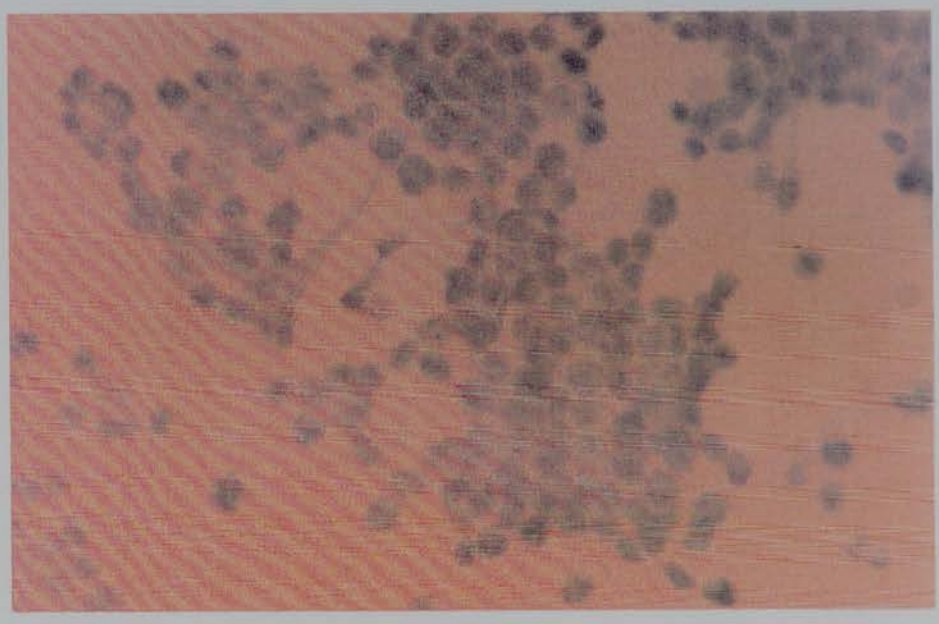


PLATE 5d: P-glycoprotein immunostaining for 2780AD, after 6 months culture in drug free medium.

CHAPTER 3

DEVELOPMENT OF A DIRECT ASSAY FOR THE MEASUREMENT OF INTRASTRAND PLATINUM-DNA ADDUCTS

INTRODUCTION

It is now widely accepted that the antitumour activity of CDDP (and analogues) is due to its interaction with DNA, around 1% of platinum entering cells binding to genomic DNA (Andrews & Howell, 1990).

The nature of the interaction between CDDP and cellular DNA

Classical monofunctional analogues of CDDP, e.g. cis-[(dien)Pt Cl^-]Cl , show no anti-tumour activity (Pinto & Lippard, 1985). Hence it is believed that the bifunctional interaction of CDDP with DNA is crucial to its therapeutic activity. CDDP forms a variety of bidentate adducts between platinum and DNA, including interstrand adducts, intrastrand adducts, DNA-protein adducts and DNA-glutathione adducts (Figure 1).

Interstrand platinum-DNA (Pt-DNA) adducts have been widely studied, mainly by the technique of alkaline elution, and attempts made to correlate interstrand crosslinking with CDDP cytotoxicity. Since it is estimated that these adducts account for around only 0.1% of total Pt-DNA adducts (Plooy et al., 1984), any such correlation is likely to be masked by the massive number of other adducts present. Studies with CDDP-sensitive and -resistant pairs of L1210 cells have shown that interstrand adducts alone are not sufficient to account for the differential sensitivity to CDDP. In this work, three sublines of L1210 cells were used, exhibiting different

degrees of induced CDDP resistance. At equitoxic doses of CDDP, interstrand adduct formation varied considerably in the resistant sublines and thus did not correlate with either cytotoxicity or resistance. (Strandberg et al., 1982).

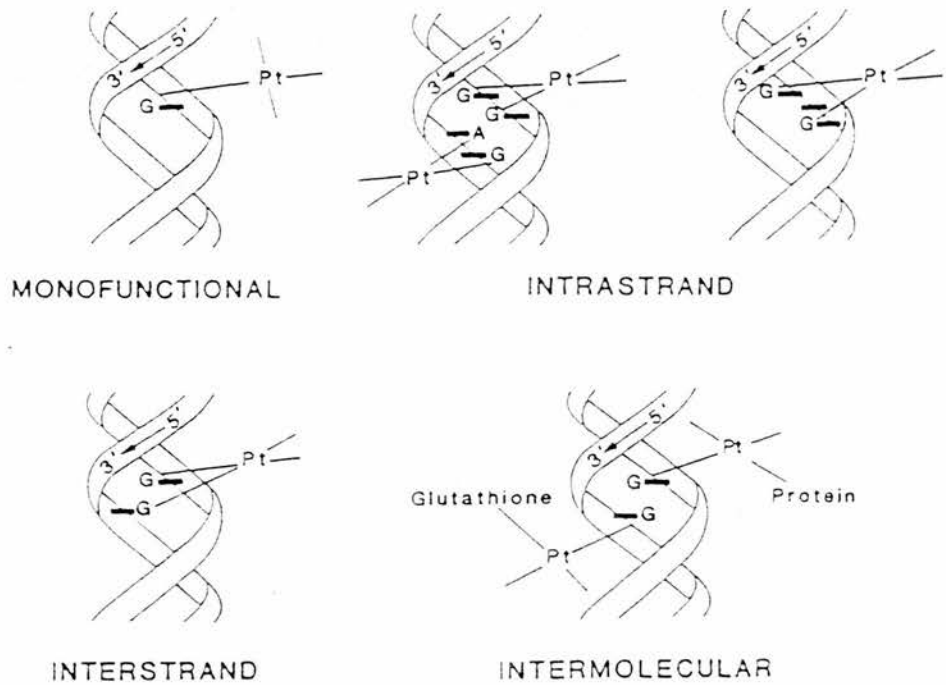


FIGURE 1: Structures of platinum-DNA adducts

DNA-protein adducts can also be formed with platinum compounds: although persistent, they account for only a very small fraction (0.15%) of total Pt-DNA adducts (Plooy et al., 1984).Experiments with both *cis* and *trans* platinum compounds in a variety of continuous cell lines have failed to show any consistent relationship between platinum-DNA-protein adducts and cytotoxicity (Zwelling et al.,1979).

Evidence for the importance of intrastrand platinum-DNA adducts to cytotoxicity of CDDP

The therapeutically inactive trans isomer of CDDP, TDDP, also reacts bifunctionally with DNA, but only exhibits cytotoxic activity at much higher doses than CDDP (Pinto & Lippard, 1985). It will, however, block DNA replication at equitoxic doses

to CDDP (Donohue et al., 1990). TDDP can also form monofunctional, interstrand, intrastrand and DNA-protein crosslinks but the observation that it cannot form intrastrand adducts between adjacent nucleotides has led to the suggestion that these adducts are responsible for the anti-tumour activity of CDDP.

Early biochemical studies (Lippard, 1982) predicted that CDDP binds preferentially to guanine-rich DNA, especially at the N7 position which allows stabilisation of platinum by hydrogen bonding. The observed unwinding of the DNA double helix following platinum binding (Cohen et al., 1979) is probably caused by the consequent weakening of hydrogen bonding of the guanine-cytidine (G-C) base pair. That platinum binds preferentially to guanine-rich sites in DNA was further confirmed by the demonstration that the large increase in buoyant density of CDDP-treated DNA was directly proportional both to its G-C content and drug-nucleotide ratio (Stone et al., 1974). Spectrophotometric studies of CDDP binding to the four nucleoside monophosphates (guanine-, adenine-, cytidine- and thymidine-,) established an order of preferential reactivity of GMP > AMP > CMP >> TMP (Pinto & Lippard, 1985); at physiological pH values the N7 site was again demonstrated to be the principal reaction site. It is thought that the high electron density of the N7 atom of guanine increases its susceptibility to attack by electrophilic metal ions. Its position in the major groove of the DNA helix also renders it more accessible to attack by reagents. Further confirmation of the formation of intrastrand adducts between platinum and two neighbouring guanine bases (Pt-GG) is provided by studies on inhibition of the restriction endonuclease *Bam*H1 (which makes a unique cut between two neighbouring guanine bases) at increasing drug:nucleotide ratios (Ushay et al., 1981) and on the inhibition of exonuclease cleavage at sites identified by DNA sequencing gel electrophoresis to be guanine-rich (Royer-Pakora et al., 1981). The stereochemical inability of the therapeutically inactive TDDP molecule to chelate to the N7 atoms of

adjacent guanines (Pinto & Lippard, 1985) lends further support to the relevance of the intrastrand Pt-GG adduct to cytotoxicity.

These intrastrand Pt-GG adducts represent around 65% of all such adducts formed between CDDP and cellular DNA (Fichtinger-Schepman et al., 1985b); other intrastrand adducts thus formed include Pt-AG, between adjacent adenine and guanine (25%), and a third minor adduct between two guanines separated by a single nucleobase (Pt-GXG).

Techniques for the analysis of intrastrand platinum-DNA adducts

Enzymatic digestion of platinated DNA, followed by chromatographic separation of the resulting products, has allowed direct characterisation of these intrastrand crosslinks. Eastman (Eastman, 1983) used a radiolabelled CDDP analogue, [^{3}H]-cis-dichloro(ethylenediamine) platinum II ([^{3}H]-cis-DEP), known to react with DNA in an identical fashion to CDDP, followed by digestion and separation of products by reverse phase chromatography. Fichtinger-Schepman and colleagues (Fichtinger-Schepman et al., 1985b) used non-radioactive CDDP, followed by enzyme digestion and separation of resultant products by anion exchange chromatography. Nuclear magnetic resonance (NMR) spectroscopy was used for further characterisation of the adducts thus produced. Both groups have confirmed that the major adduct in platinated DNA is the intrastrand Pt-GG crosslink, with Pt-AG and Pt-GXG occurring less frequently. The use of ammonium bicarbonate ($NH_4C O_3$) to inactivate monofunctionally bound CDDP (Fichtinger-Schepman et al., 1984), proved such adducts to be numerically insignificant.

Direct quantitation of intrastrand platinum-DNA adducts is difficult owing to their extremely low concentration within cellular DNA treated with therapeutically relevant doses of CDDP. Atomic absorption spectroscopy (AAS) has a detection limit too high (1 - 10 ng/ml) at realistic CDDP exposure levels (Fichtinger-Schepman et al.,

1985a), whilst radiolabelled platinum compounds are difficult to synthesise and do not permit human experimentation. A sensitive enzyme-linked immunosorbent assay (ELISA) method for the detection of CDDP-modified DNA was first developed by Poirier and colleagues (Poirier et al., 1982), based on a similar method for the detection of adducts formed between chemical carcinogens and DNA. Antibodies, specific for a particular three-dimensional adduct structure, are used in immunoassays (both radioimmunoassay, RIA, and ELISA) to a detection limit of around one adduct per 10^7 nucleotides of biological DNA. An alternative approach (Fichtinger-Schepman et al., 1985a) entailed the synthesis of polyclonal antibodies against haptens mimicking the platinum-containing digestion products of enzymatically-treated Pt-DNA. Polyclonal antisera thus produced now allow the sensitive detection (to femtomolar levels) of intrastrand crosslinks in cells exposed to biologically relevant concentrations of CDDP. The accurate quantitation in biological samples of all three identifiable Pt-DNA adducts (Pt-GG, Pt-AG and G-Pt-G) and the monofunctional adduct Pt-G is now possible (Fichtinger-Schepman et al., 1987a). Experiments using these antisera have proved that antigenic determinants found on DNA modified by CDDP both *in vitro* and *in vivo* are stereochemically similar (Poirier et al., 1982; Fichtinger-Schepman et al., 1987a).

Improvements in elemental analysis of platinum

Over the last decade, great improvements have been made in elemental analysis by mass spectrometry, such that the direct detection of femtomolar levels of platinum in biological samples is now within the realms of possibility. A variety of analytical techniques for the estimation of platinum in biological tissues, following treatment with CDDP or its analogues, was reviewed recently (Riley, 1988). These mainly entail non-selective determination of platinum (rather than CDDP) and include: X-ray fluorescence (XRF), proton-induced X-ray emission (PIXE), flameless atomic absorption spectroscopy (FAA), and inductively-coupled plasma atomic emission

spectrometry (ICP-AES). Selective methods for CDDP detection usually involve normal or reverse-phase high performance liquid chromatography (HPLC) with pre-column derivatisation using diethyl dithiocarbamate (DDTC).

XRF and PIXE are moderately sensitive techniques for platinum analysis (detection limits around 200 ng/ml) which require complex procedures for the pre-treatment of samples. The poor availability of these techniques, in addition, limits their use for routine analysis. FAA is more widely available and requires relatively little preparation of biological samples; it is an order of magnitude more sensitive (detection limits 1 - 10 ng/ml).

ICP-AES appears to show some promise as a more accurate and sensitive technique for platinum analysis. Measurement precision, calculated as the relative standard deviation (RSD) -between two independent series of data, was found to be 0.95 - 2.5% in biological fluids with platinum levels of 0.25 - 5 ng/ml (Dominici et al.,1986). The much wider linear dynamic concentration range (four orders of magnitude compared with two for AAS), combined with its higher precision and accuracy, make ICP-AES a powerful tool in the biomedical investigation of platinum compounds. Matrix interference, (the suppression of analyte signal by moderate to high concentrations of other matrix constituents), can affect the analytical sensitivity of both AAS and ICP-AES, however.

Specific methods for CDDP analysis (e.g. direct UV absorption and reductive electrochemistry) have been mainly used in pharmacokinetic studies, and generally are not sufficiently sensitive for the accurate detection of Pt-DNA adducts.

Inductively coupled plasma mass spectrometry (ICP-MS) is a relatively recent technique with considerably lower detection limits than ICP-AES for elemental analysis (Houk, 1988).The development of ICP as a superior excitation source for the analysis of solutions by AES, together with the demonstration that useful mass

spectra could be obtained from elemental constituents in solution by the application of a DC plasma, led to the first commercial ICP-MS instrument being developed in 1983.The technique is capable, in general, of analysing elements, including platinum, to detection limits of 0.01 - 0.05 ng/ml (Houk & Thompson, 1988) in solution. Its sensitivity is thus far superior to any other technique currently available, introducing for the first time the possibility of direct analysis of DNA-Pt adducts.

ICP-MS in the direct analysis of platinum-DNA adducts

The scarcity of [^{3}H]-*cis*-DEP and the lack of commercial availability of polyclonal antibodies to the identified intrastrand Pt-DNA adducts are major obstacles to the further study of the role of these adducts in CDDP resistance. The availability of ICP-MS provided the opportunity to investigate the suitability of this extremely sensitive, but technically very complex, technique in the analysis of Pt-DNA adducts in an experimental model for CDDP resistance in ovarian cancer.

This chapter describes the development of a direct assay for the analysis of DNA-platinum interaction by CDDP, using highly platinated calf thymus DNA. At this high level of platination, it was possible to compare AAS and ICP-MS as techniques for platinum analysis.

MATERIALS AND METHODS

Platination of calf thymus DNA

Calf thymus DNA (Sigma, Poole, England) was first dissolved in 10mM sodium phosphate buffer (pH 7) to a final concentration of 0.5 mg/ml. A fresh solution of CDDP (Sigma, Poole, England) dissolved in saline (NaCl, 0.9g/100 ml) to a stock concentration of 0.25 mg/ml was prepared. Prewarmed 10 ml aliquots of DNA solution were incubated with CDDP (25 µg/ml) at 50° C for five hours in the dark. 2 ml 0.5M NH_4HCO_3 was then added to inactivate any monofunctionally DNA-bound CDDP (Fichtinger-Schepman et al., 1984) and the solution was then dialysed at room temperature to stop the reaction, first against NH_4HCO_3 (0.1M) for 18 hours and then against distilled water for 8 hours.

CDDP-DNA was recovered from aqueous solution by precipitation in two volumes of a mixture of cold absolute alcohol and sodium acetate (NaAc, 3M) containing EDTA (1mM) at 9:1 v/v, followed by centrifugation at 600g at 14° C for 30 minutes.

Enzymatic Degradation

The resultant pellet of CDDP-treated calf thymus DNA was redissolved in 5 ml Buffer A (Tris HCl, 10mM, pH 7.2, containing Na_2EDTA, 0.1mM, and $MgCl_2$, 4mM). A portion (350 µl) of this solution was taken, to which was added 7.2 µl $ZnSO_4$, 10mM, 6 µl DNase I (Sigma, Poole, England) and 30 µl P_1 Nuclease (Sigma, Poole, England), to form an enzymatic digestion mixture containing DNA at a final concentration of 1 mg/ml. This was incubated overnight at 37° C to allow degradation of DNA to nucleotides and (platinum-containing) oligonucleotides. The resulting digest was then incubated with Proteinase K (Sigma, Poole, England) at approximately 1 mg / ml for 2 hours at 37° C followed by heat inactivation of the

enzyme, and clarification by centrifugation (microfuge, 13K for 5 minutes). The final nucleotide concentration was determined from the absorbance at 260 nm.

High Performance Liquid Chromatography (HPLC)

Analytical separation of the four major intrastrand Pt-DNA adducts and unmodified deoxyribonucleotides was performed on an HPLC system (Waters, Millipore, U.K.) with a Mono-Q anion exchange column (Pharmacia, U.K.). The mobile phase used was Tris 12.5 mM, pH 8.8. The column was first equilibrated with mobile phase containing NaCl (50mM). Following injection of digested CDDP-DNA (200µg in 200µl), adducts were eluted off the column using a salt gradient, increasing from 50mM to 25mM over 14 minutes. Eluting nucleotides were detected by UV absorbance at 260 nm and peak spectral data was collected over the range 200 - 320 nm by a photo-diode array detector (Waters, Millipore, U.K.). The identity of the individual nucleotides and the Pt-DNA adducts was confirmed by co-chromatography with authentic standards, previously characterised by NMR and kindly donated by Dr Fichtinger-Schepman (TNO Medical Biological Laboratories, Rijswijk, Netherland). Eluting fractions were collected at half minute intervals for platinum determination.

Calibration of the Mono-Q HPLC Chromatogram

The correlation between nucleotide formation and DNA content, measured by spectrophotometric absorbance at 260 nm, was compared for all four nucleotides. Unplatinated calf thymus DNA (20 µg) was enzymatically disaggregated, fivefold dilutions made, and samples separated by HPLC as described. Nucleotide content of the resulting spectral peaks were calculated by integration, and compared with initial DNA concentration.

Platinum Determination by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Figure 2 shows a typical layout for an ICP used in conjunction with a mass spectrometer. An inductively coupled plasma is formed by the application of high voltage to argon gas at atmospheric pressure. The solution to be analysed is introduced, usually by nebulisation, into the axial channel and carried to the normal analytical zone (A in Figure 2) by the laminar flow of argon. Here the high gas kinetic temperature (5000 - 7000K) permits efficient elemental ionisation within two milliseconds. Relatively few doubly charged ions are formed; singly charged analyte ions are numerically surpassed by neutral argon atoms (about 10^{18} per cm^3) and by atoms formed by the ionisation of solvent material. Such matrix elements, if ionised, may contribute to the spectral profile, usually by less than 20%. Nitric acid is the preferred solvent for ICP-MS because of the simplicity of its background spectrum when nebulised.

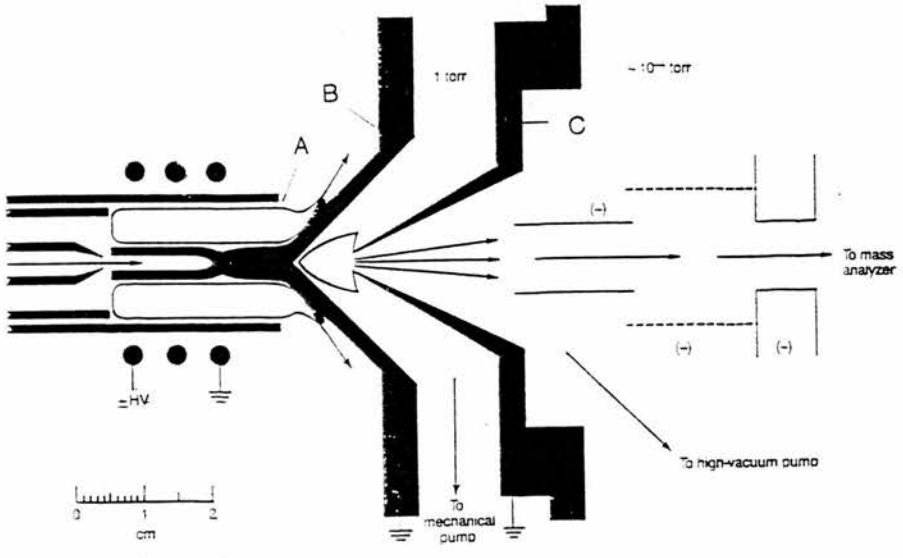


FIGURE 2: ICP apparatus (from Houk, 1986).

An ion sampling interface for extraction of ions into a vacuum (B in Figure 2) is interposed between the ICP and the mass spectrometer, as a large negative operating pressure is required for mass spectrometry. A cooled nickel zone, with a 0.5 - 1 mm circular orifice is inserted directly into the ICP. Gas from the ICP, containing ions for analysis, enters this orifice from the axial channel. This relatively large sampling orifice allows a greater flow of ions into the vacuum system, and provides resistance to blockage by solids condensed from the injected solution. A second conical skimmer (C in Figure 3) is positioned behind the sampler, to permit maximal ion transmission to the mass analyser.

For this work, a VG Plasmaquad PQ1 ICP-MS machine (VG Elemental Ltd, Winsford, U.K.) was used, incorporating a Fassel-type torch, a Gilson 222 automatic sampler and an IBM PC-AT data processing system. A sample volume of 3 - 4 ml in HCl, 0.1%, was used, at a flow rate of 0.7 ml / minute; total analysis time was about three minutes per sample.A solution of K_2PtCl_6 (kindly supplied by Dr Fichtinger-Schepman) was used for calibration at a final concentration of 4 µM. Spectral peak "jumping" on In 115, Pt 194, Pt 195 and Pt 196 was used for maximal analytical efficiency; three measurements were made of each.

Half-minute elution fractions (0.5 ml) from HPLC separation of enzymatically digested DNA were analysed for platinum content, diluted to 3 or 4 ml in HCl, 0.1%, and introduced to the ICP-MS by nebulisation. As internal standard, a solution of indium (J & M Specpure, U.K.) was made in HNO_3, 5%, and 25 µl added to each sample.

Platinum standards, prepared from a stock solution of platinum, 1 mg/ml, (J & M Specpure, U.K.) in HCl, 1%, were made up at 100 ng/ml and 10ng/ml; 1% HCl was used as a blank.Since linearity of dose-response for platinum standards was known to be excellent, it was possible to perform retrospective regression analysis of results

from the blanks and standards to produce a calibration equation. With simultaneous correction for indium standards, this equation could be rapidly used for the calculation of platinum concentration for each sample. To evaluate internal consistency, a platinum standard (10 ng / ml) was interpolated approximately every ten samples. Total solute content of HPLC fractions did not exceed 0.2%, therefore processing of solutions to reduce matrix contamination was not necessary.

Platinum Determination by Atomic Absorption Spectrometry (AAS)

To confirm the accuracy of ICP-MS for platinum analysis in these experiments, parallel analysis was also performed, on one set of samples only, by AAS by Dr Fichtinger-Schepman in Rijswijk, Netherland. For this, a Perkin Elmer model 4000 atomic absorption spectrophotometer was used, with a HGA-500 graphite furnace and an AS-400 autosampling system. K_2PtCl_6 solution, diluted to 4 µM, was again used for calibration.

Platinum Binding to DNA

Previous work has demonstrated a linear response between the concentration of CDDP used in the reaction with DNA, and the number of platinum atoms bound per nucleotide (r_b value) to a maximum of 25 µg CDDP/ml (Fichtinger-Schepman A-M. J. et al., 1982). For a CDDP concentration of 25 µg / ml, a r_b value of around 0.088 would be expected (Figure 3).

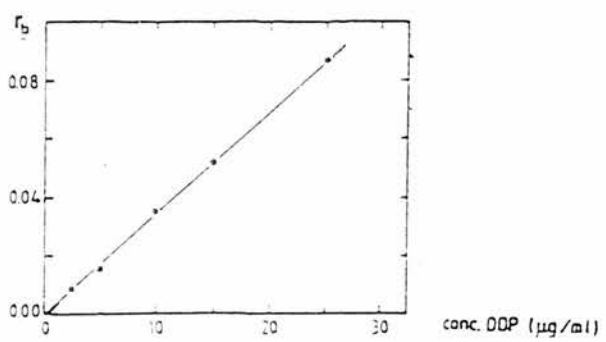


FIGURE 3: Relationship between the number of platinum atoms bound per nucleotide (r_b) in CDDP treated DNA and the amount of DNA in the incubation mixture (from Fichtinger-Schepman at al, 1982).

RESULTS

Separation of nucleotides

A typical HPLC elution profile (at 260 nm) for unmodified DNA is shown in Figure 4, showing excellent nucleotide separation over a run time of around 14 minutes. Four major peaks are seen and were identified as follows:

Peak 1 (4.8 min)	dCMP
Peak 2 (6.5 min):	dAMP
Peak 3 (8.5 min):	dTMP
Peak 4 (11.8 min):	dGMP

3D spectral analysis (Figure 5) confirmed the identity and purity of each of these peaks.

Identification and calibration of the HPLC Mono-Q Chromatogram

Elution times for each major intrastrand Pt-DNA adduct were established for this HPLC system and compared with the reference compounds kindly supplied by Dr Fichtinger-Schepman (Figure 6 and Table 1). Good agreement was also found between the HPLC separation method described here and the FPLC system used by Dr Fichtinger-Schepman.

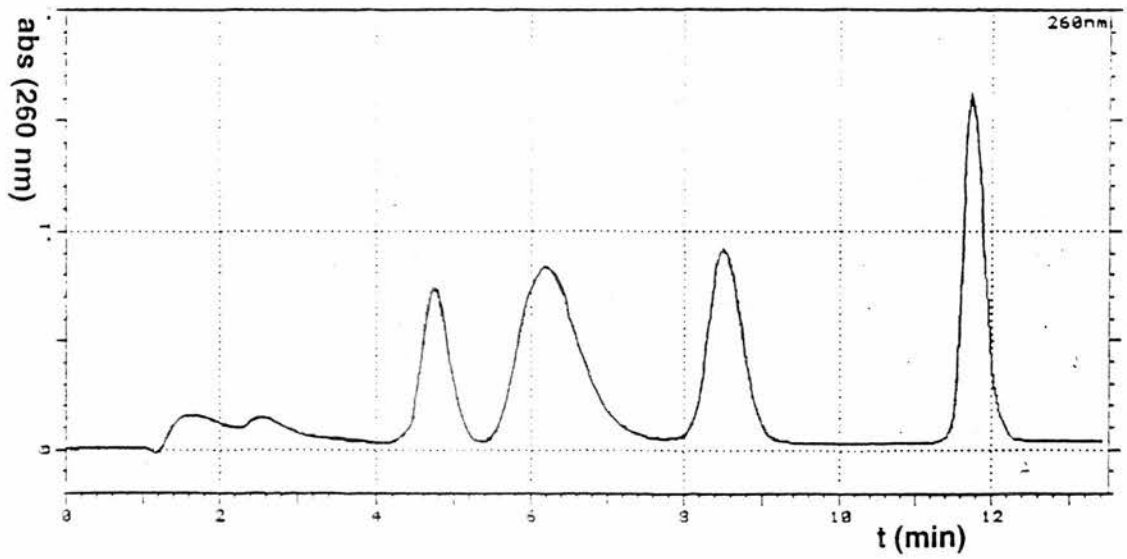


FIGURE 4: Separation of unplatinated calf thymus nucleotides by HPLC.

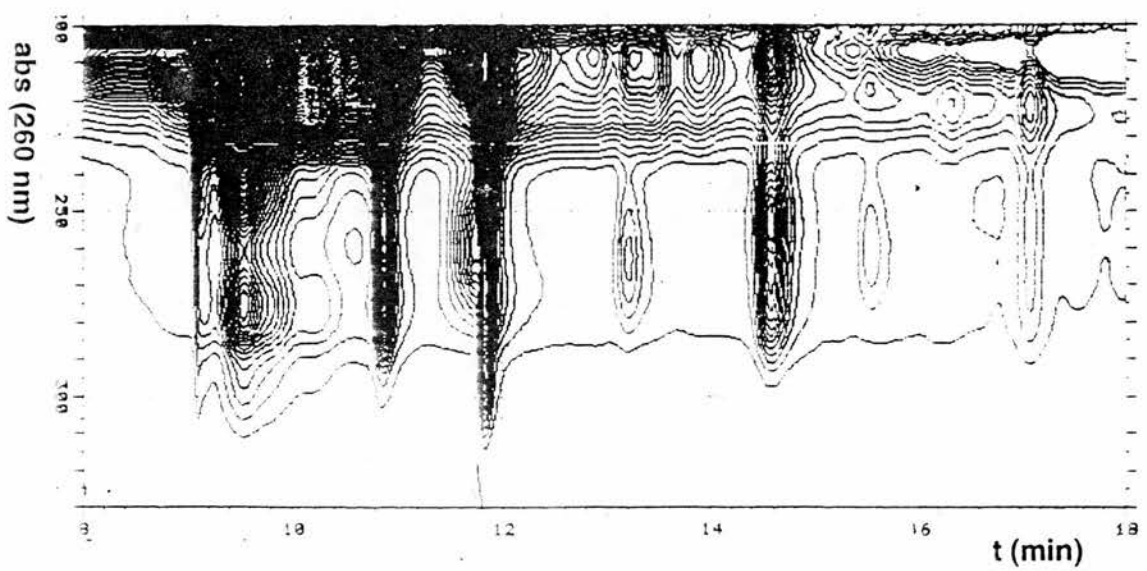


FIGURE 5: Spectral analysis of HPLC chromatogram, confirming the identity and purity of the peaks.

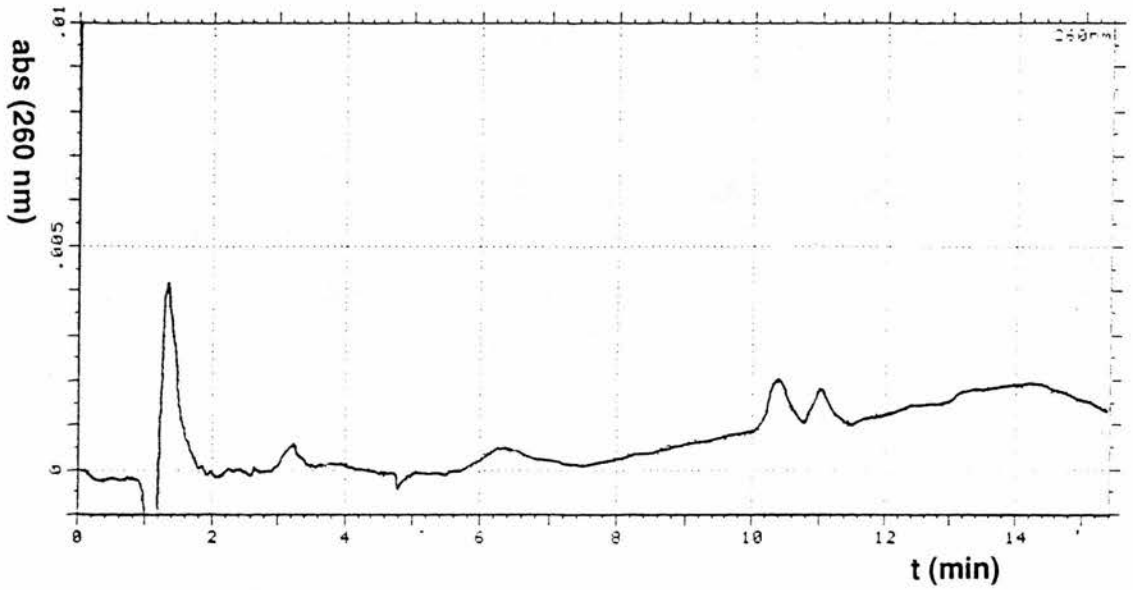


FIGURE 6: Elution times for purified intrastrand Pt-DNA adducts (donated by Dr Fichtinger-Schepman).

	Pt-AG (min)	Pt-GG (min)	G-Pt-G (min)
calf thymus DNA-Pt (HPLC)	2.9	10.1	10.9
reference compounds[1] (HPLC)	3.1	10.4	11
reference compounds (FPLC)[2]	3.5	7.8	9.7

TABLE 1: Elution times for the major intrastrand platinum-DNA adducts using the Mono-Q HPLC chromatogram for their separation. ([1]: kindly donated by Dr Fichtinger-Schepman; [2]: Fichtinger-Schepman et al., 1985b)

For calibration of the column, chromatograms were obtained of serial dilutions of a known amount of nucleotide solution. Excellent correlation (r= 0.99) was obtained between DNA content and integrated peak area on chromatogram (Figure 7).

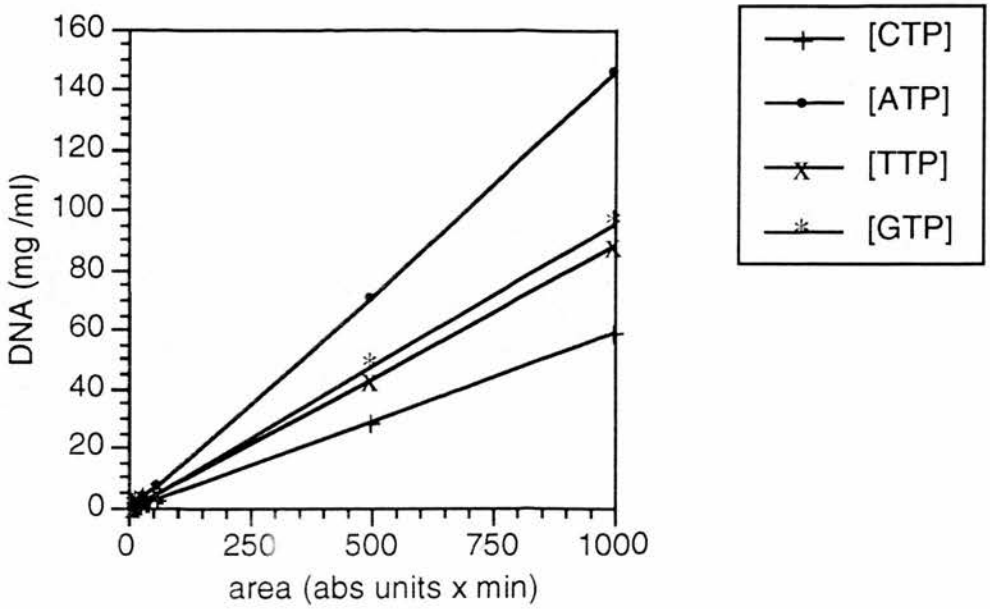


FIGURE 7: Calibration of HPLC Chromatogram.

Separation of platinated DNA

A typical HPLC chromatogram for highly platinated calf thymus DNA, showing excellent resolution of peaks for both unmodified nucleotides and the three main bifunctional intrastrand Pt-DNA adducts, is shown in Figure 8. Seven major peaks are seen and were identified as follows:

Peak 1 (2.9 min)	*cis*-Pt(NH_3)$_2$d(pApG) - Pt-AG
Peak 2 (4.8 min)	dCMP
Peak 3 (6.4 min)	dAMP
Peak 4 (8.5 min)	dTMP
Peak 5 (10.1 min)	*cis*-Pt(NH_3)$_2$d(pGpG) - Pt-GG
Peak 6 (10.8 min)	*cis*-Pt(NH_3)$_2$d($GMP)_2$ - G-Pt-G
Peak 7 (11.8 min)	dGMP

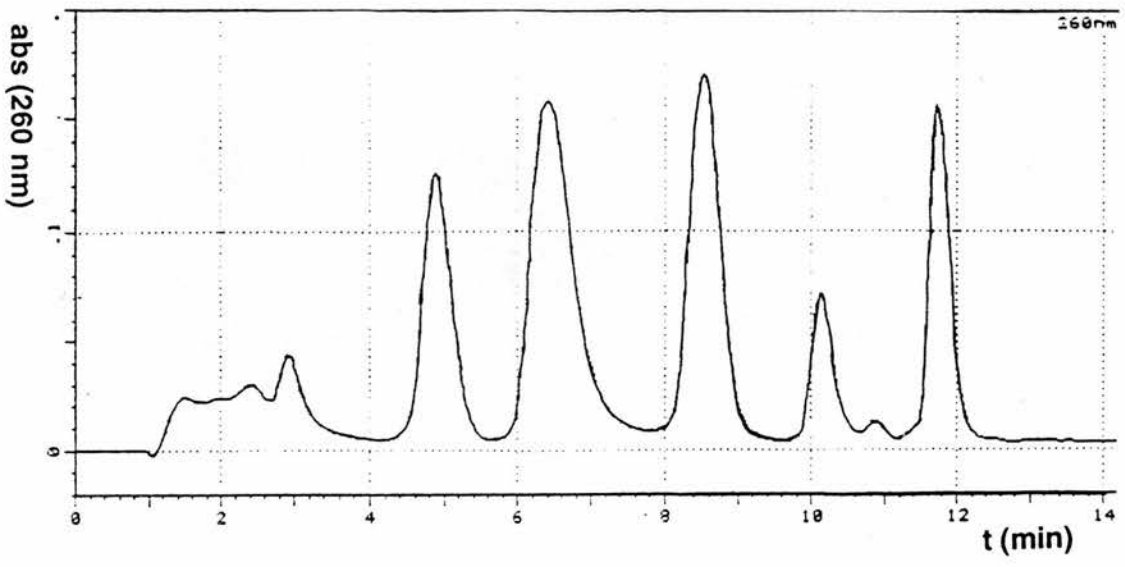


FIGURE 8: Separation of highly platinated calf thymus nucleotides and oligonucleotides by HPLC.

Platinum analysis of HPLC fractions

When elution samples were analysed for platinum content, by ICP-MS or AAS, localisation of platinum to the expected elution positions of the two major (quantitatively) intrastrand Pt-DNA adducts, Pt-GG and Pt-AG, was found (Figure 9). In addition, a smaller, intermediate peak occurred (elution time 7 min), which did not correlate with any previously identified Pt-DNA adduct. Excellent agreement was achieved between the two analytical techniques at this level of platination.

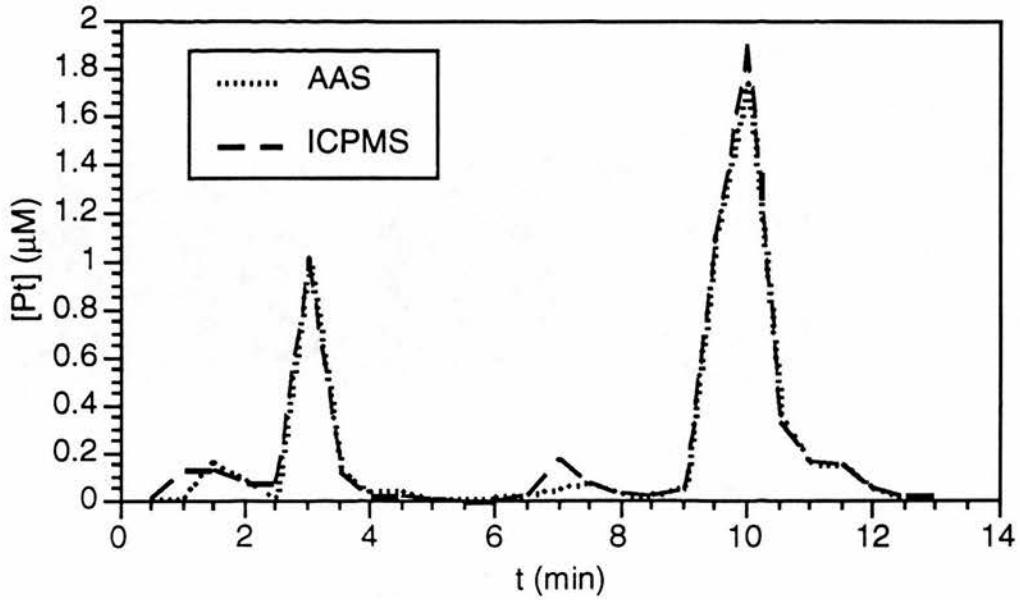


FIGURE 9: Platinum analysis of eluate fractions by AAS and ICP-MS.

DISCUSSION

The cytotoxic lesion responsible for the therapeutic effect of CDDP remains to be clearly established. Much evidence exists to implicate the intrastrand Pt-DNA adducts, Pt-GG and PT-AG, in this role, although an assay sensitive to femtomolar levels is necessary for their detection in biological systems when therapeutically relevant doses of CDDP are used.

Highly sensitive immunochemical (ELISA) techniques are available (Poirier et al., 1982; Fichtinger-Schepman et al., 1985a) but have some disadvantages. In particular, they are indirect methods, requiring the use of polyclonal antisera to predicted DNA modifications by CDDP, and therefore would be unable to detect any unanticipated lesions. Large discrepancies exist between Pt-DNA adduct values obtained with these two immunochemical methods (Fichtinger-Schepman et al., 1989).The competitive ELISA devised by Poirier and colleagues uses a polyclonal rabbit antiserum to highly platinated calf thymus DNA (r_b value 0.088), which recognises both Pt-GG and Pt-AG together. This may cause underestimation of adduct levels. The indirect ELISA developed by Fichtinger-Schepman and colleagues uses polyclonal antibodies raised against CDDP-containing nucleotides coupled to haptens. Three different antisera allow the sensitive detection of the Pt-GG/G-Pt-G, Pt-AG and Pt-GMP adducts separately. A one to one correlation with AAS values for total Pt-DNA binding has been demonstrated for this method (Reed et al., 1990). Several recent studies, however, have confirmed that significant underestimation of Pt-DNA adducts occurs with the anti-DNA ELISA method developed by Poirier and colleagues (Poirier et al., 1988; Mustonen et al., 1989; Fichtinger- Schepman et al., 1989).

Until recently, no method for platinum analysis has been sensitive or reliable enough to consider the direct analysis of intrastrand Pt-DNA adducts. Advances in the field of elemental analysis by ICP-MS have now engendered this possibility. The work

described in this chapter used calf thymus DNA, reacted with CDDP and disaggregated enzymatically in a similar way to that used previously with salmon sperm DNA (Fichtinger-Schepman et al.,1985). Using HPLC rather than fast protein liquid chromatography (FPLC) excellent separation of deoxyribonucleotides was obtained. The three major intrastrand Pt-DNA adducts, Pt-GG, Pt-AG and G-Pt-G, were clearly identified. Retention times matched those of the reference compounds kindly donated by Dr Fichtinger-Schepman, and were very similar to those obtained by this group using FPLC (Table 1). The linear relationship demonstrated between disaggregated DNA dose and the integrated peak area of a representative eluted deoxyribonucleotide on chromatogram, allowed calculation of the total DNA content of each sample analysed. The wider availability of HPLC, compared with FPLC, as an analytical technique necessitated the use of this method for anion exchange chromatography. Comparable results were obtained, over a similar elution time, with no obvious loss of sensitivity.

Platinum analysis of individual elution fractions obtained by HPLC separation of a highly platinated sample of calf thymus DNA was performed by ICP-MS. This confirmed localisation of platinum to the expected elution positions of the two major Pt-DNA adducts (Pt-GG and Pt-AG). Comparison of this analytical technique with the better established method of AAS yielded excellent correlation (Figure 9). An additional, much smaller intermediate peak was also observed which may represent a previously unidentified Pt-DNA adduct. The sensitivity of ICP-MS for platinum analysis (detection limits 0.01 - 0.05 ng/ml) is far superior to any other technique currently available. At higher atomic numbers, such as platinum (195), practically no elemental interference is encountered. The simplicity of mass spectra obtained is such that any overlap interference can be readily predicted, allowing an alternative isotope to be sought. Sampling time, for the three major platinum isotopes, usually takes around three minutes per sample, including rinses. Short-term precision and accuracy

are comparable with those found in both ICP-AES and AAS. Intra-experimental error is usually within 10% (Houk, 1986), but some drift of sensitivity can be observed with time This effect can be minimised by maintaining low solute levels (less than 0.2%) for nebulised solutions. The use of an internal standard with a distinct spectral peak, such as indium, corrects any residual sensitivity problems.

Matrix interference, however, remains a problem with ICP-MS. This effect manifests as suppression of analyte signal by other matrix constituents present at a moderate to high level, and stems from the inherent efficiency of the ICP as a universal ion source for virtually every element within a sample (Houk, 1986). In particular, the total electron density within the ICP is dependent on the sodium concentration within the sample solution. Intraexperimental variation in sodium concentration will alter the analyte ion to atom ratio within the ICP, and cause signal suppression. The isocratic elution process used in this work during anion-exchange chromatography could cause significant matrix effect with increasing fraction number analysed, owing to the increasing sodium concentration. However, comparison of Pt-GG peak levels, obtained by platinum analysis by both ICP-MS and AAS, show no significant difference (Figure 9). This could be explained by equivalent matrix interference occurring during AAS analysis. Alternatively, the dilution of each fraction by 6 - 8 fold, to enable sufficient volume for nebulisation, could have minimised this effect. The use of indium as an internal standard was intended to compensate for mild ionisation suppression effects.

In this work, existing methods for Pt-DNA adduct identification in highly platinated DNA have been modified, and a novel technique for platinum analysis of resultant adducts investigated. In biological systems DNA is much less highly modified and adducts are found at femtomolar levels. The sensitivity of ICP-MS in platinum analysis, however, raised the possibility of the direct measurement of DNA modifications by CDDP in biological systems.

CHAPTER 4

MEASUREMENT OF INTRASTRAND ADDUCT LEVELS IN CONTINUOUS OVARIAN CARCINOMA CELL LINES

INTRODUCTION

The relationship between intrastrand Pt-DNA adducts and CDDP resistance

Indirect immunochemical techniques have been widely used in the study of intrastrand Pt-DNA adducts and their relationship with CDDP resistance in a number of experimental model systems. The induction and repair of four intrastrand Pt-DNA adducts in two cell lines derived from germ cell tumours of testis (inherently sensitive to CDDP) and one cell line derived from a transitional cell carcinoma of bladder (inherently resistant to CDDP) were compared (Bedford et al., 1988). Proficient repair of all four adducts was found in the bladder carcinoma cell line. The two testicular carcinoma cell lines expressed similar CDDP sensitivities, but one was found to be deficient in adduct repair, of Pt-GG and Pt-AG. It was therefore suggested that inability to repair platinated DNA might account for the sensitivity of this cell line to CDDP. Nuclear immunostaining, using a polyclonal antiserum which recognises G-linked CDDP in DNA, also correlated adduct-specific nuclear staining density with induced CDDP resistance (Terheggen et al, 1990). These observations could all be explained by the importance of increased Pt-DNA repair capacity as a resistance mechanism in CDDP resistance.

In another study, increased Pt-GG and Pt-AG adduct levels were found in a CDDP-sensitive human small cell lung carcinoma cell line, compared with its resistant subline. Following incubation in drug-free medium, however, deficient removal of Pt-

GG was noted in the resistant subline (Hospers et al., 1990). In a comparison of intrastrand Pt-DNA adduct formation in rats bearing either a CDDP-sensitive or a CDDP-resistant immunocytoma, no significant difference was found between adduct levels in normal or tumour tissues (Fichtinger-Schepman et al., 1989). A recent study comparing the formation and removal of intrastrand Pt-DNA adducts in two human ovarian cancer cell lines with an intrinsic CDDP resistance ratio of more than 23 noted similar levels of adduct formation but an apparent inability to remove the Pt-GG and G-Pt-G adducts in the resistant cell line.

ICP-MS-ETV for the direct analysis of platinum-DNA adducts

A direct method for the analysis of Pt-DNA adducts would circumvent most of the criticisms applied to the indirect immunochemical techniques and provide further, independent, insight into the nature of these lesions in biological systems. The previous chapter described the development of a direct assay to measure intrastrand Pt-DNA adducts in highly platinated calf thymus DNA, using ICP-MS. The natural extension of this work was to refine this assay to enable the study of these adducts in whole cells, following treatment with CDDP at therapeutically relevant doses.

In standard usage, however, ICP-MS has a lower limit of sensitivity for platinum of around 50 picomolar (pM), which is around three orders of magnitude too high for the direct analysis of intrastrand Pt-DNA adducts at the cellular level. The use of electrothermal vapourisation (ETV) for sample introduction improves the detection power of ICP-MS by up to 1000-fold (Hulmston & Hutton, 1991). This is achieved by the direct volatilisation of the sample into the gas stream using a controlled electric current to ash the sample (Figure 1). Thus the inefficient nebulisation step is eliminated, and thermal pre-treatment of the solvent minimises matrix effects. The small sample volume used (10 - 100 µl) allows further improvements in analytical sensitivity. A 50% increase in sensitivity for platinum analysis by ICP-MS-ETV was found when this method of sample introduction was compared with standard

nebulisation (Gregoire, 1988) for the analysis of platinum group elements. It thus seemed possible that the increased sensitivity afforded by the use of ICP-MS-ETV would allow the direct measurement of Pt-DNA adducts in biological systems.

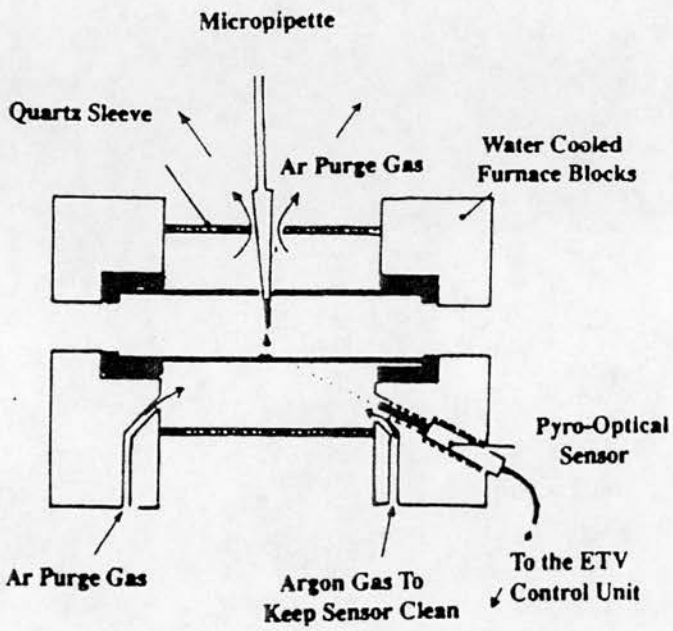


FIGURE 1: Electrothermal vapourisation (ETV) apparatus for ICP-MS.

This chapter describes refinement of the direct assay for adduct measurement to allow determination of intrastrand Pt-GG and Pt-AG adduct levels in whole cells, and thus explore the relationship between CDDP sensitivity and the kinetics of adduct formation and removal . The three ovarian carcinoma cell lines characterised in Chapter 2 (A2780, 2780AD and 2780CP) were used throughout, and exposed to CDDP at approximately equitoxic doses (10 times their ID_{50} value) for 4 hours and 24 hours initially. The kinetics of adduct formation over 24 hours was investigated; adduct repair was then assessed by measuring levels after a similar time-course of CDDP exposure, followed by a 24 hour recovery period in drug-free medium. Equitoxic doses of CDDP were chosen to try and standardise the extent of adduct formation throughout all experiments.

MATERIALS AND METHODS

Cell culture

The three ovarian carcinoma continuous cell lines (A2780, 2780AD and 2780CP) described in Chapter 2, with a stable range of induced CDDP resistance of up to 27-fold, were used Tissue culture conditions were as described in Chapter 2.

Treatment of cell lines with CDDP

Cells were plated out at an initial density of 10^4 cells / cm^2 in 175 cm^2 flasks, allowed to attach, and then grown for 6 days under optimal tissue culture conditions until subconfluent. They were then fed with fresh RPMI medium and left for one hour to equilibrate with 2% CO_2 at 37° C prior to treatment with CDDP at a final concentration of about 10 times their ID_{50} value. Cells were washed and NH_4CO_3 (final concentration 0.1M) was added to inactivate monofunctionally bound CDDP. They were harvested either immediately following drug exposure (for the investigation of adduct formation) or after a further 24 hours incubation in drug-free medium (for the investigation of adduct recovery). Cell pellets were frozen at -20° C until DNA was extracted.

Extraction of DNA

Cell pellets, consisting of about 10^7 -10^8 cells, were washed with PBS and resuspended in 2.5 ml Tris buffer, 10mM, containing EDTA (1mM), pH 7.8, to which NH_4CO_3 (0.1M) had been added just before use. Cell lysis was performed by overnight incubation at 37° C with SDS (1% w/v); proteinase K, 250 µg/ml, was also added to digest proteins and DNA-protein adducts.The following day, an equal volume of phenol, (previously saturated with Tris buffer, 10mM), pH 7.8, containing EDTA (1mM) and NaCl, (0.1M) was added and the lysates extracted for 15 minutes at 37° C. DNA was then precipitated from the aqueous phase by the addition of two

volumes cold absolute ethanol with sodium acetate buffer (0.3 mM), pH 5.5, containing EDTA (0.1mM). DNA was collected by spooling onto glass rods, rinsed briefly in 70% ethanol, and redissolved in 1 ml Tris / EDTA buffer. Coprecipitated RNA was digested by incubating with RNase A (Sigma, Poole, England), 75 µg/ml, (heated at 80° C for 5 minutes to destroy any DNase activity) and RNase T_1,(Boehringer, Bracknell, England), 75 IU/ml, at 37° C for two hours. A second DNA extraction was then performed with an equal volume of chloroform / isoamyl alcohol (24:1) and DNA reprecipitated with absolute ethanol.

Verification of DNA purity

In order to confirm the purity of DNA thus extracted, representative samples of DNA, dissolved in a small volume of Buffer A, were run in a 1% agarose gel electrophoresis system, and compared with a lambda bacteriophage DNA standard.

Enzymatic degradation of DNA

DNA was redissolved in 300 µl Buffer A (as described in Chapter 3), with 7.2 µl $ZnSO_4$, 10 mM, 6 µl DNase I (at 1 mg / ml) and 30 µl P_1 Nuclease, to form a digestion mixture of approximately 1 µg DNA / ml. Enzymatic degradation was then performed as described in Chapter 3.

Identification of Pt-DNA adducts

The various DNA digestion products (nucleotides and platinum-containing oligonucleotides) obtained after enzymatic degradation were separated by anion exchange chromatography using a mono-Q column and a HPLC system as described in Chapter 3. The column was first calibrated with unplatinated digested calf thymus DNA, to identify the elution positions of the four unmodified nucleotides.

Fractions (0.5 ml) were collected throughout the separation process for analysis of elemental platinum content by ICP-MS-ETV. For this, 100 µl of concentrated HNO_3

and 100 µl of concentrated HCl were added to each fraction, and heated to dryness at 120° C. On the day prior to platinum analysis this residue was dissolved in 100 µl HCl, 1%. 50 µl samples were introduced to the ETV furnace and elemental platinum analysed by single ion monitoring. Platinum solutions of 1 ppb (0.05 ng/ml) were used as internal standards and checked between every 6 samples to reduce sensitivity drift with time.

Adduct levels were calculated from the platinum concentration of the sample as analysed by ICP-MS-ETV, and expressed as a function of the DNA content of the sample (calculated by integration from the HPLC chromatogram) and the CDDP dose used (fmol Pt / µg DNA / µmole CDDP).

Kinetics of adduct formation and recovery

For the investigation of adduct kinetics, cells were treated with CDDP, as described, for either 4, 8, 12, or 24 hours. Cells were then either trypsinised and stored at -20° C prior to DNA extraction (adduct formation -F) or incubated for 24 hours in drug-free medium prior to trypsinisation and DNA extraction (adduct recovery -R).

Enzymatic disaggregation and HPLC separation of extracted DNA was then performed as before. Fractions eluting over the predicted time for the two major intrastrand adducts, Pt-AG (retention time around 3.1 minutes) and Pt-GG (retention time around 10.4 minutes) were pooled to produce samples with a total volume of 2-3 ml. In view of the known high salt content of later fractions, Pt-GG samples were desalted using solid phase extraction cartridges (Bondelut C18, Varian, Jones Chromatography, Glamorgan). After the addition of both concentrated HCl and concentrated HNO_3 (100 µl), samples were evaporated to dryness and analysed for platinum content by ICP-MS-ETV as described above. Platinum content for each sample was calculated with a one-point calibration using the integral counts obtained from the 1 ppb platinum standards.

Correction for dilution by DNA synthesis

A separate experiment was performed to investigate the significance of any dilutional effect due to DNA synthesis during the 24 hour recovery period in drug-free medium. Cells from all three cell lines were grown to subconfluence in 24 well plates and exposed to CDDP at the same concentrations as were used above (see Table 1) for 4, 8, 12, and 24 hours. Control wells were fed with medium alone. After each exposure time, medium was removed and all cells washed with PBS. Cells were then either trypsinised and stored at -20° C prior to DNA estimation (adduct formation -F) or incubated for 24 hours in drug-free medium prior to trypsinisation and DNA estimation (adduct recovery -R).

DNA content was estimated essentially as described by Labarca & Paigen (1980). Briefly, 2 ml of sodium phosphate buffer containing NaCl, 2M and EDTA, 0.002M, pH 7.4, and Hoechst dye 33258 (Hoechst, Hounslow, England), 1 μg/ml, was added per well. After brief sonication, emission was measured at 458 nm in a spectrofluorimeter. Calf thymus DNA was used as standard.

Cell line	[CDDP] used (μM)	ID_{50} - CDDP (μM)
A2780	2.0	0.19
2780AD	20	2.3
2780CP	50	5.3

TABLE 1: Concentrations of CDDP used to treat each cell line, approximately ten times the ID_{50} value.

RESULTS

Verification of DNA purity

Figure 2 shows a typical example of an agarose 2D electrophoresis gel. Lanes 1 - 13 contain aliquots of extracted DNA (approximately 5 μg) from all three cell lines. The low mobility of DNA in rows 1 - 13 confirms the high purity of extracted cellular DNA.

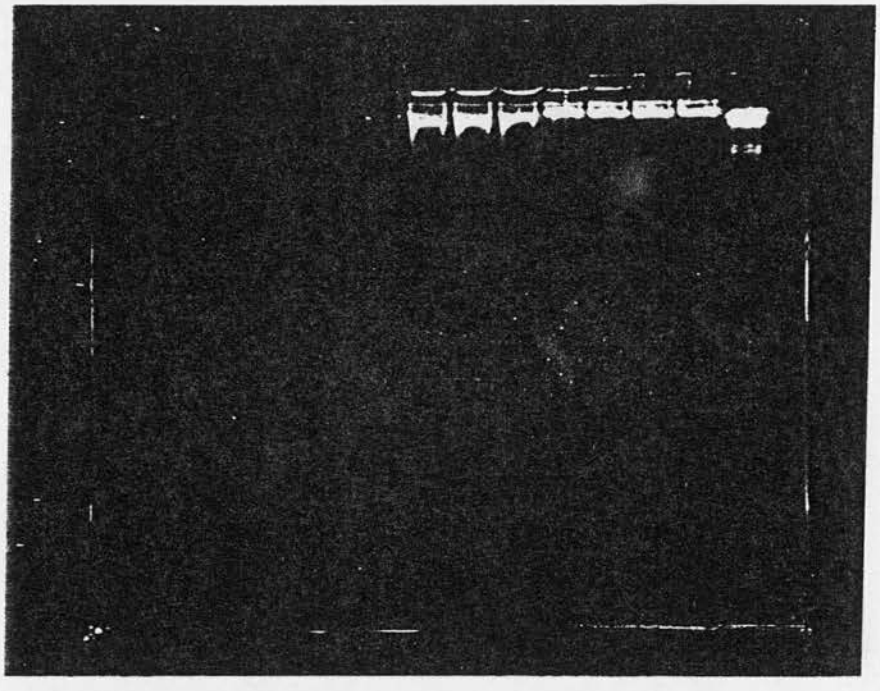


FIGURE 2: Electrophoresis gel confirming the purity of DNA extracted from ovarian carcinoma cell lines. Rows 1 to 13 contain DNA extracted from A2780, 2780AD and 2780CP. Row 14 contains bacteriophage λ DNA as standard.

Identification of Pt-DNA adducts for cell lines treated with CDDP *in vitro*

Initial studies were designed to assess the sensitivity of ICP-MS-ETV for the determination of Pt-DNA oligonucleotides following HPLC separation. For this, DNA was extracted from all three cell lines after treatment with approximately equitoxic doses of CDDP for either 4 hours or 24 hours, and disaggregated

enzymatically as described. Table 1 lists the concentrations of CDDP used for each cell line.

Typical HPLC chromatograms for digested DNA extracted from unplatinated (Figure 3) and platinated (Figure 4) 2780CP cells are shown. Although individual oligonucleotides can be seen clearly, it is not possible to identify the position of Pt-containing oligonucleotides at this scale. Elution positions of Pt-GG and Pt-AG adducts are inferred from those of purified standard samples, as described in Chapter 3.

The platinum content of each fraction was then determined and the profile for each of the three cell lines is shown for two independent experiments in Figures 5 and 6. Two major platinum peaks can be identified for all three cell lines, the first occurring early (3 - 6 minutes), the second late (12 - 14 minutes) in the elution process. In addition, there appears to be a third, much smaller, peak present between these two.

The first major peak is larger than the second in most of the platinum profiles displayed in Figures 5 and 6. For the two resistant lines, Pt-DNA content was greater after 24 hours exposure to CDDP than after 4 hours exposure. DNA platination, after both CDDP-treatment times, appears greatest for the most sensitive cell line, A2780 and least for the most resistant cell line, 2780CP, with intermediate platinum levels achieved for 2780AD.

Kinetics of adduct formation and recovery

For this work, a more accurate representation of Pt-DNA adduct levels was attempted by collecting eluate fractions over the expected elution period of the two major Pt-DNA adducts. The total platinum content of pooled HPLC fractions was then analysed by ICP-MS-ETV.

Figures 7 - 9 show Pt-DNA levels corresponding to the elution position of the intrastrand adduct Pt-AG in three independent experiments for the cell lines A2780, 2780AD and 2780CP, following exposure to CDDP for periods up to 24 hours. Platinum levels for DNA extracted from cells immediately following CDDP exposure (formation -F) and after a 24 hour recovery period in drug-free medium (recovery -R) are shown.

There is a general trend for increasing platinum content with time, seen in all three cell lines for both adduct formation and recovery. Platinum levels, in general, seem highest in the most sensitive cell line, A2780, and for both adduct formation and recovery are higher than in the two CDDP-resistant sub-lines. Pt-AG adduct formation, however, appears higher in 2780CP than 2780AD. Platinum levels show a trend to be marginally higher in A2780 following recovery in drug-free medium, seem little changed in 2780AD, and (in Experiments 1 and 3) appear lower in 2780CP.

Figures 10 - 12 show Pt-DNA levels corresponding to the elution position of the intrastrand adduct Pt-GG for A2780, 2780AD and 2780CP in three independent experiments. Results for later exposure times to CDDP were not always available, owing to problems encountered either in calculating DNA content from the HPLC chromatogram or in cell yield.

There is again a general trend for increasing platinum content with time in all three cell lines, although for adduct formation there may be a peak level after 8 or 12 hours exposure to CDDP, following which platinum level often falls. Adduct formation appears highest again in the most sensitive cell line, A2780, although differences are less marked. Pt-GG adduct formation seems, in general, higher in the most resistant cell line, 2780CP than in the less resistant subline 2780AD, although this pattern is not always consistent. For Pt-GG, in contrast to Pt-AG, levels following recovery in

drug-free medium are consistently lower than at formation in A2780. For the two resistant sublines, levels for this adduct following recovery are either higher or very similar.

Correction for dilution by DNA synthesis

DNA content of cells from a parallel experiment to assess the impact of potential adduct dilution (due to fresh DNA synthesis) is shown in Table 2. For all three cell lines, after exposure to CDDP for up to 24 hours, DNA content following a 24 hour recovery period in drug-free medium is consistently less (17 - 93%) than immediately after drug exposure.

A2780 - CDDP exposure (h)	**[DNA] - F (μg/ml)**	**[DNA] - R (μg/ml)**	**[DNA] -R/F (%)**
4	9.29 ± 0.8	3.75 ± 0.52	40.4
8	7.49 ± 0.64	2.20 ± 0.99	29.4
12	8.44 ± 1.03	3.89 ± 1.55	46.1
24	5.43 ± 0.81	2.41 ± 0.76	44.4
2780AD - CDDP exposure (h)			
4	17.8 ± 0.67	16.6 ± 0.52	93.1
8	17.3 ± 0.75	13.4 ± 1.37	77.4
12	18.2 ± 0.57	13.9 ± 1.88	76.4
24	15.7 ± 0.97	9.6 ± 0.63	61.1
2780CP - CDDP exposure (h)			
4	5.7 ± 0.61	1.1 ± 0.11	20.0
8	4.4 ± 0.77	1.5 ± 0.19	33.0
12	4.3 ± 0.46	0.73 ± 0.15	17.0
24	1.7 ± 0.25	1.4 ± 0.20	82.4

TABLE 2: DNA content of cells exposed to CDDP before (F) and after (R) 24 hour recovery in drug-free medium

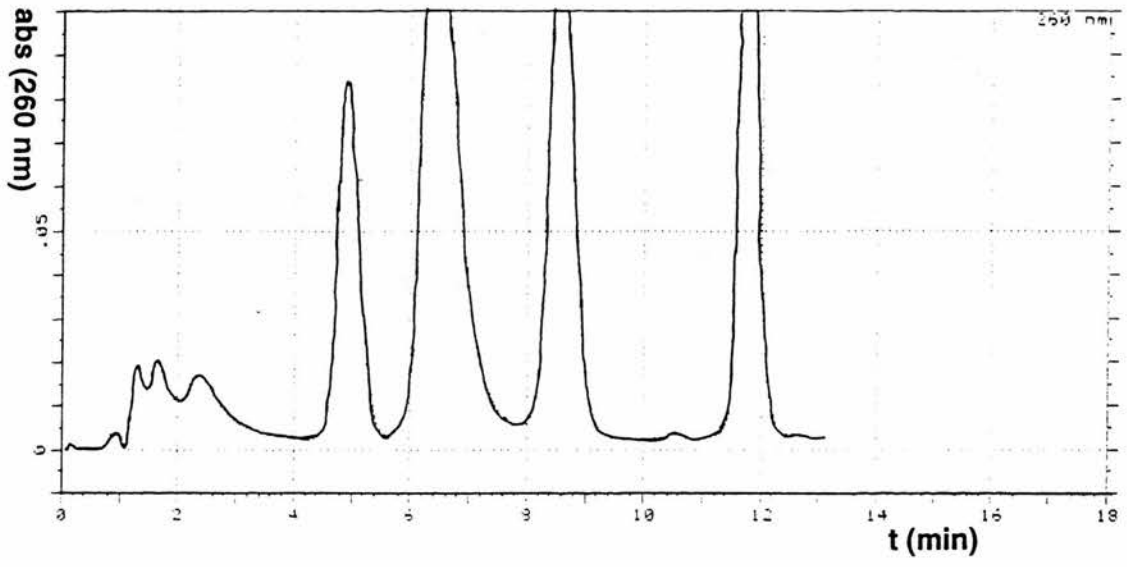


FIGURE 3: Separation of unplatinated digested DNA (2780CP) into nucleotides.

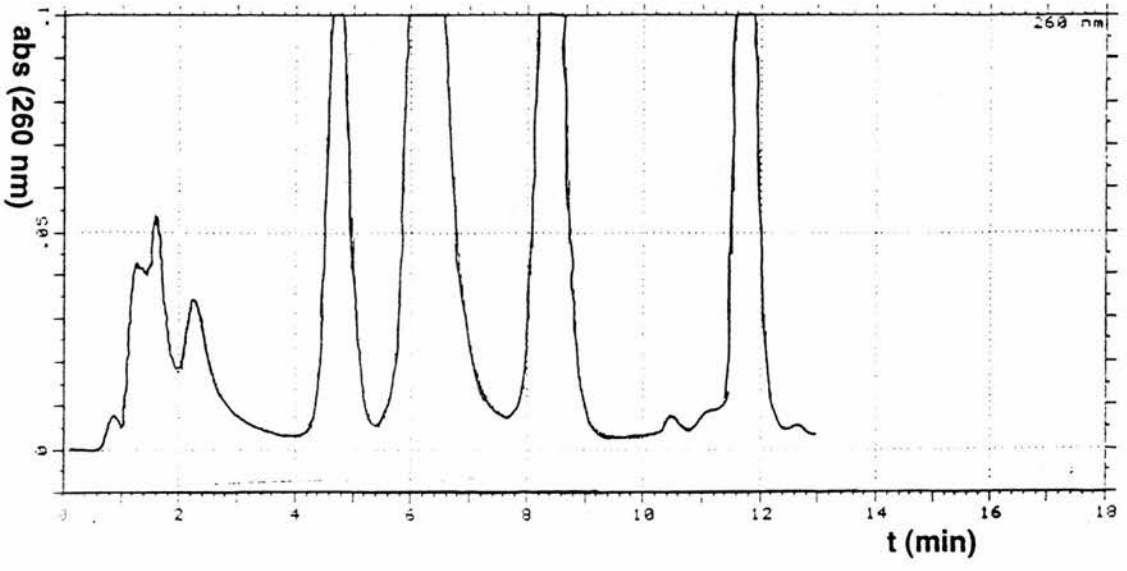


FIGURE 4: Separation of platinated digested DNA (2780CP) into nucleotides and platinum - oligonucleotides by HPLC. At this level of platination, platinum-DNA adducts cannot be identified.

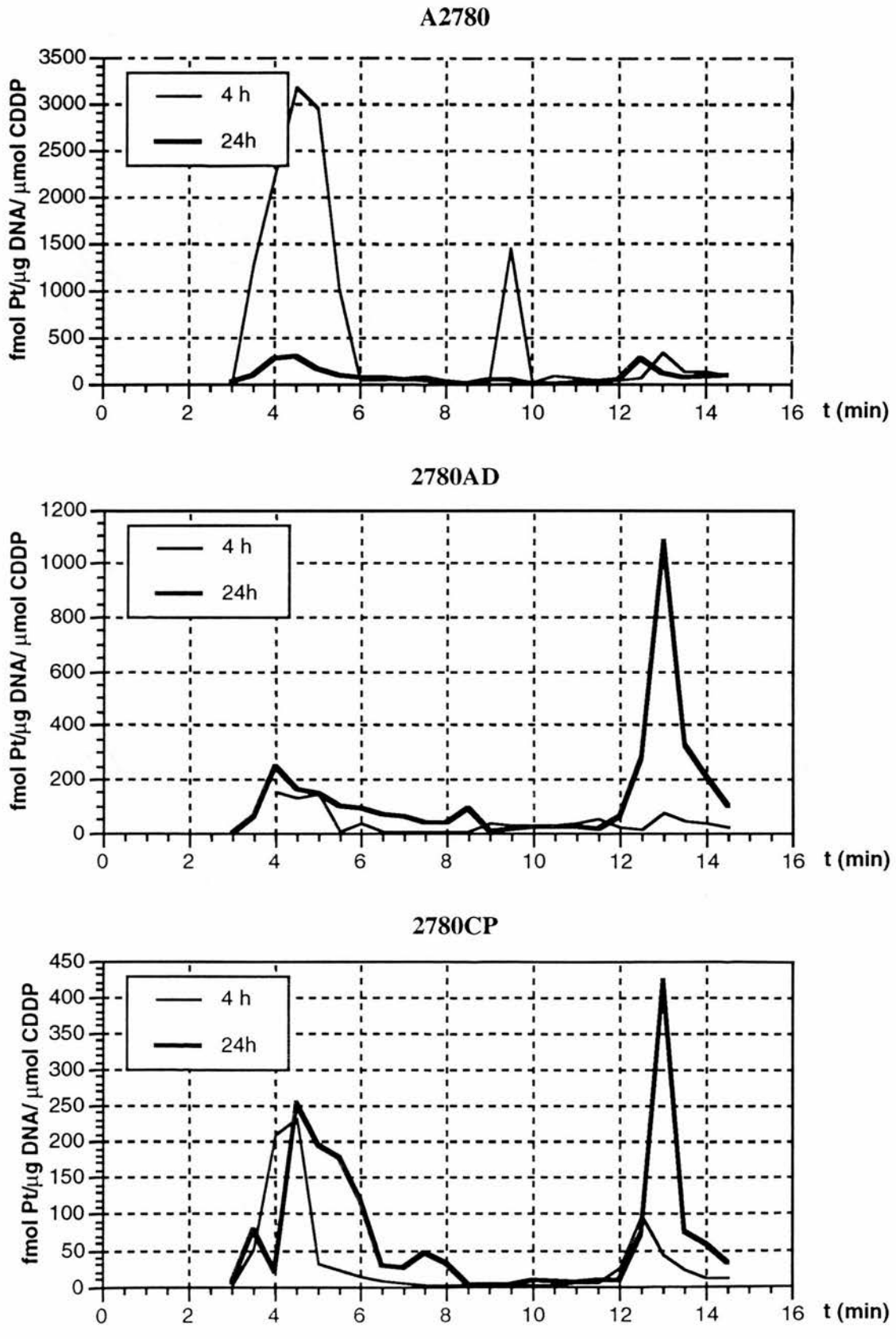


FIGURE 5: Platinum distribution for HPLC chromatogram - Experiment 1

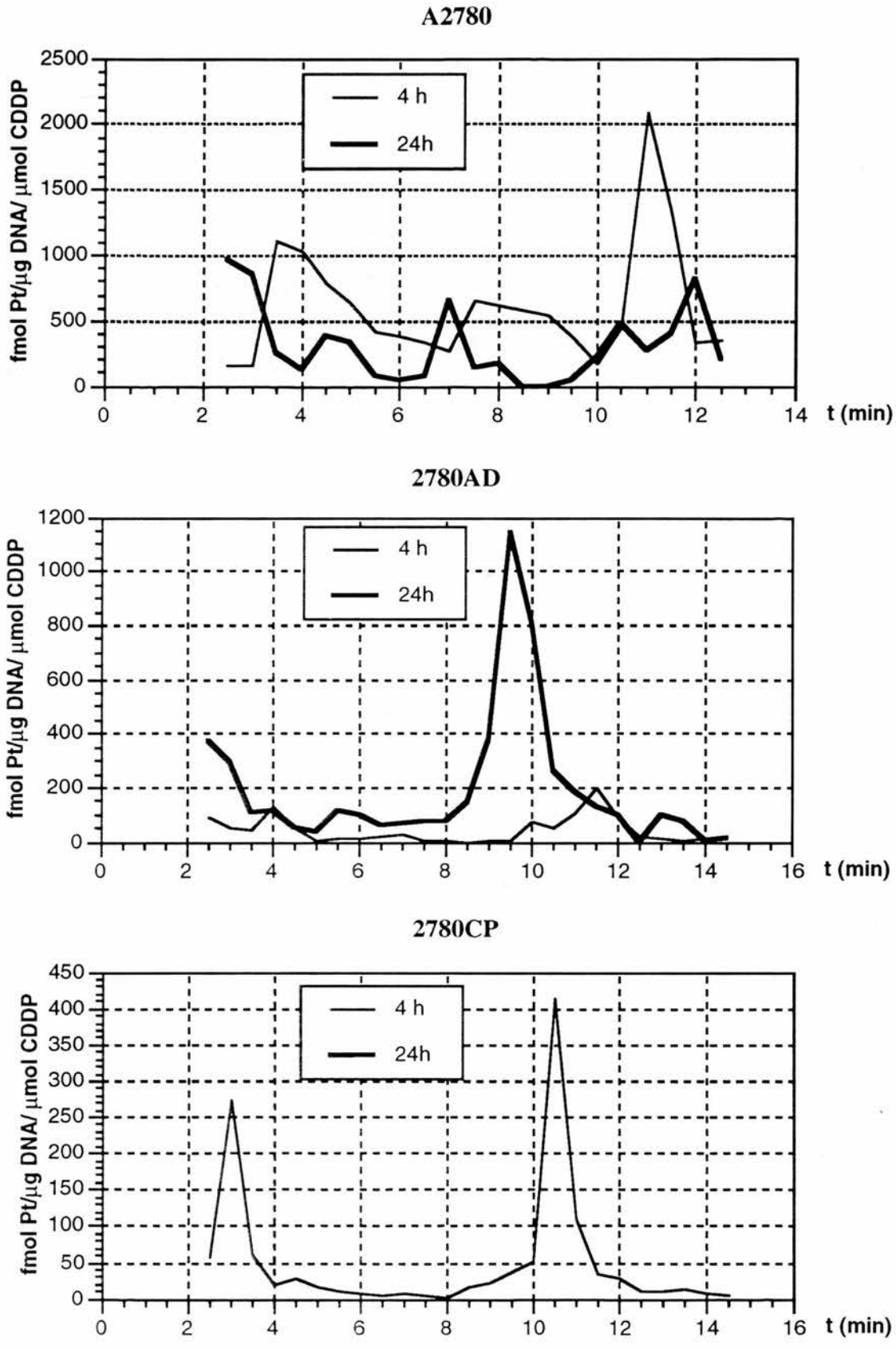


FIGURE 6: Platinum distribution for HPLC chromatogram - Experiment 2

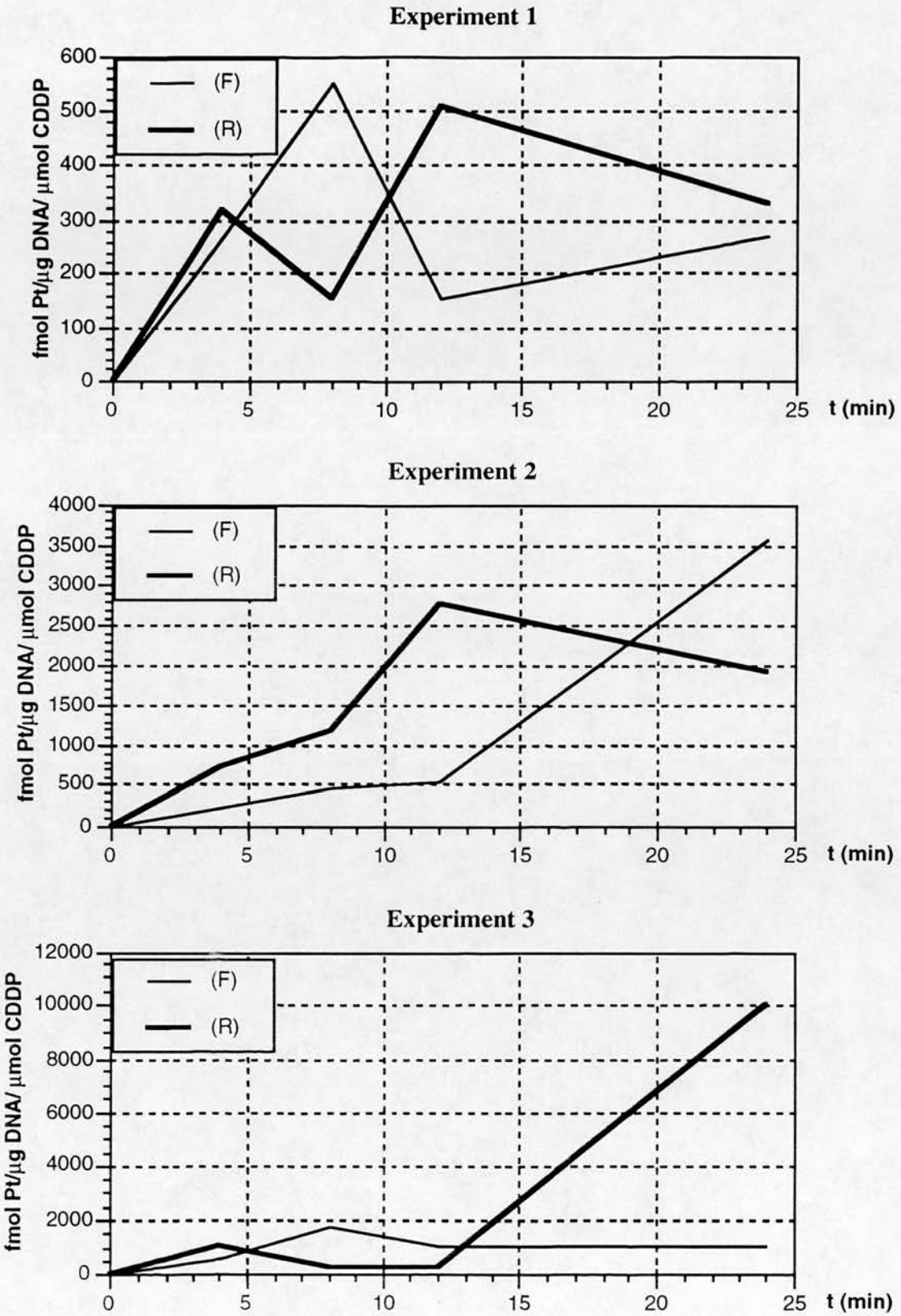


FIGURE 7: Pt-AG adduct levels for A2780 -F: formation; -R: recovery

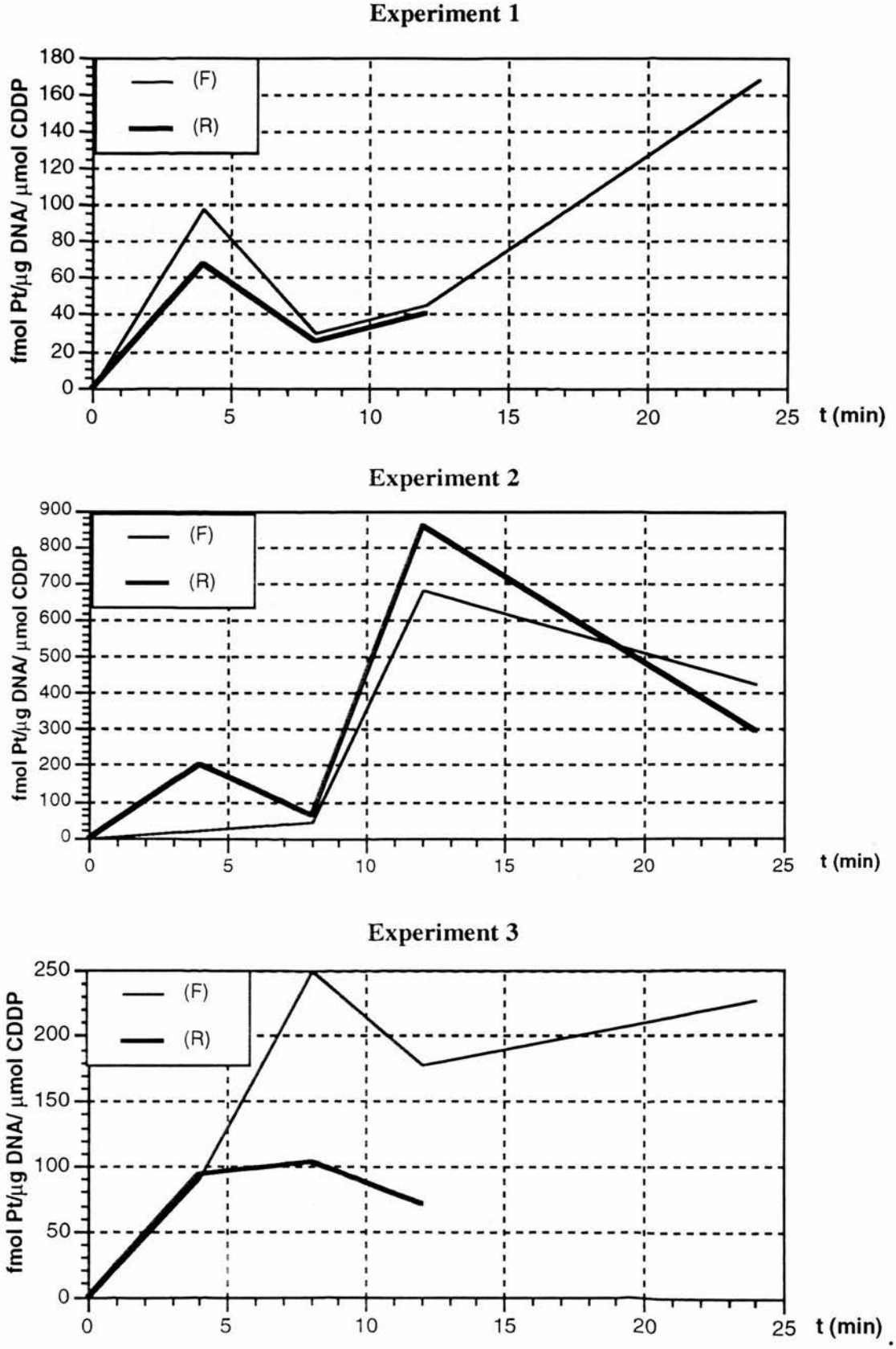


FIGURE 8: Pt-AG adduct levels for 2780AD; F- formation; R- recovery

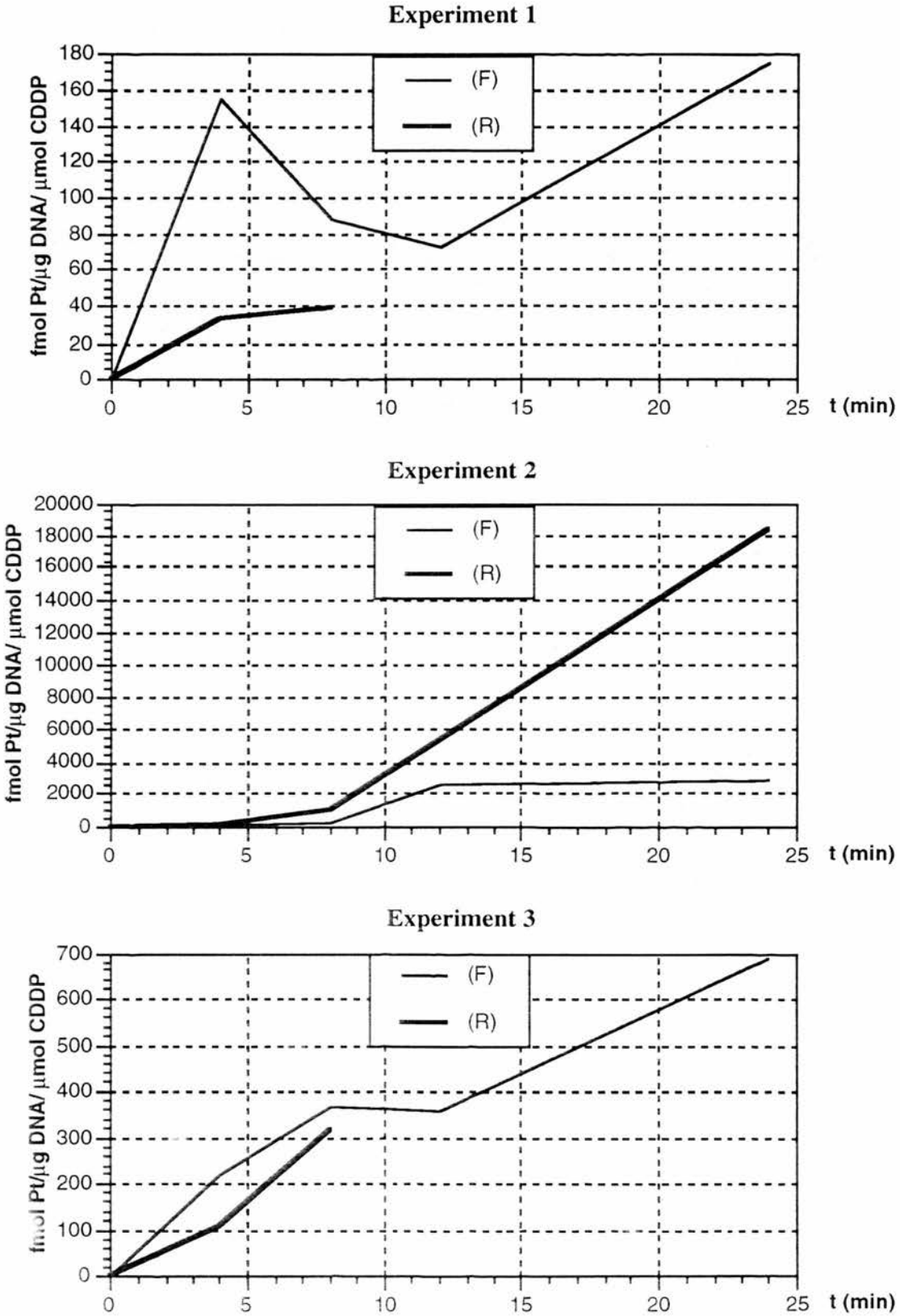


FIGURE 9: Pt-AG adduct levels for 2780CP - F: formation; R: recovery

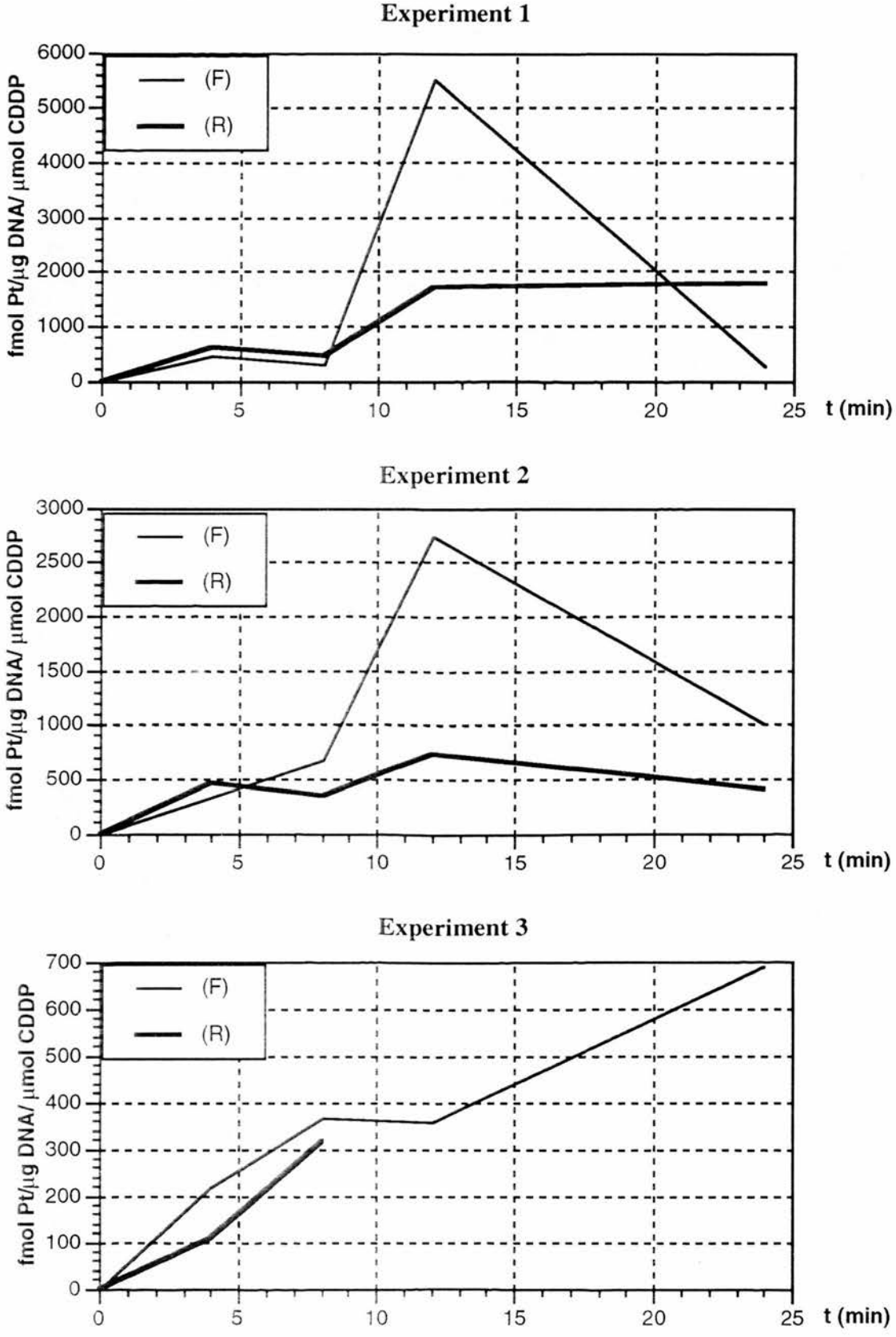


FIGURE 10: Pt-GG adduct levels for A2780-F: formation; R: recovery

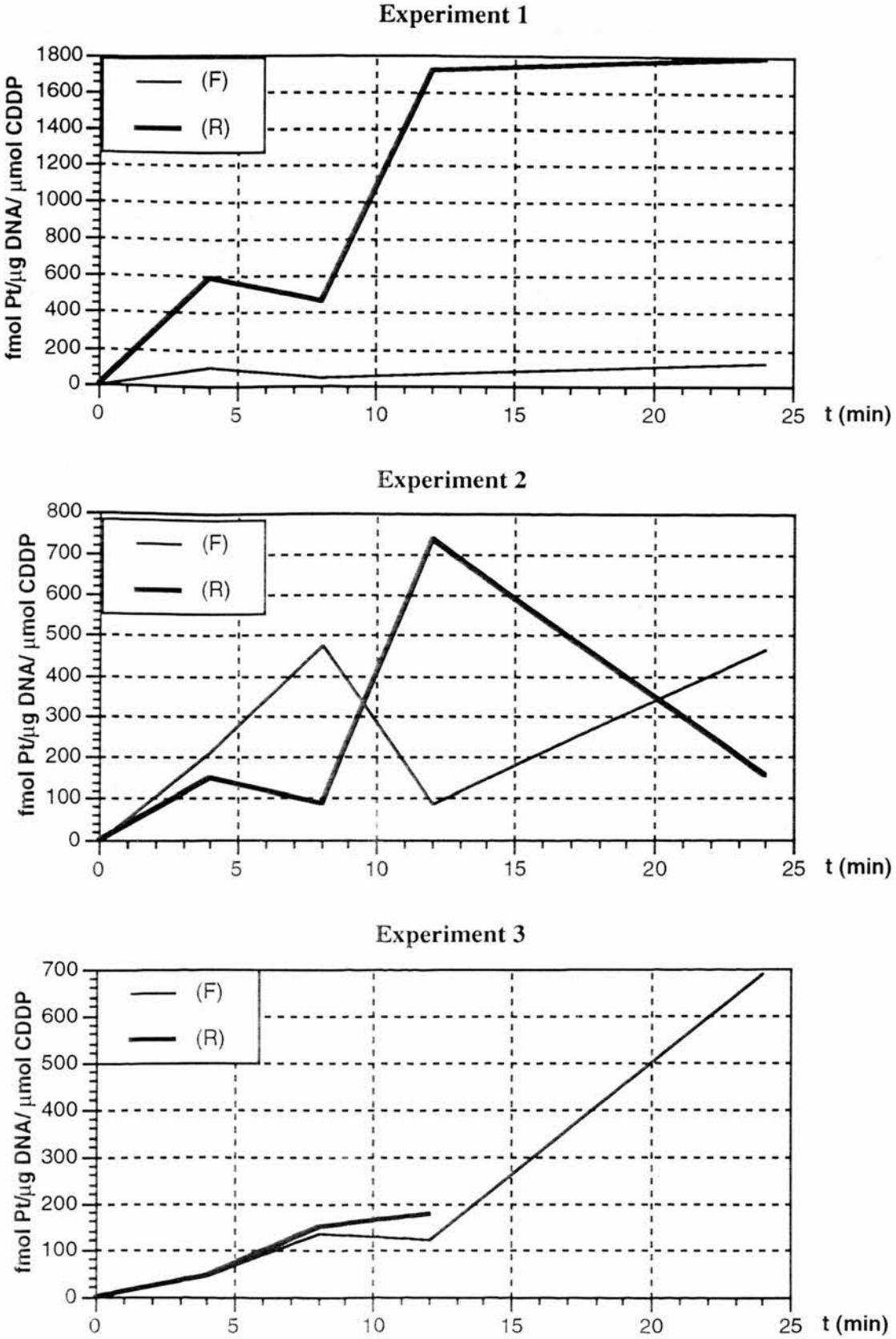


FIGURE 11: Pt-GG adduct levels for 2780AD - F: formation; R: recovery

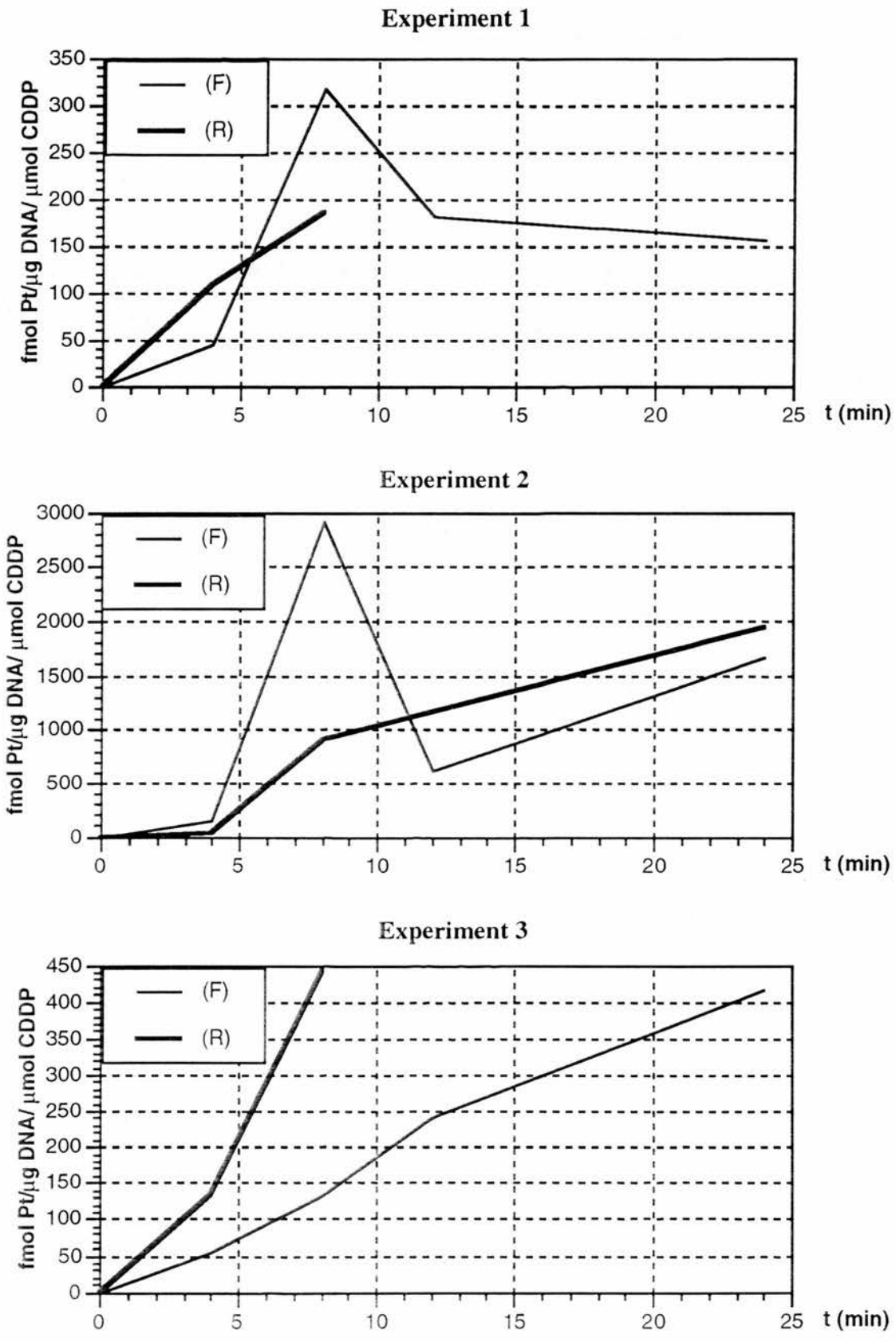


FIGURE 12: Pt-GG adduct levels for 2780CP - F:formation; R:recovery

DISCUSSION

Earlier work described in this thesis (Chapter 3) validated the techniques of anion exchange chromatography by HPLC and platinum analysis by ICP-MS for direct detection of the two major intrastrand Pt-DNA adducts, Pt-AG and Pt-GG, in enzymatic digests of highly platinated calf thymus DNA. These methods have now been extended to the study of these adducts in the ovarian carcinoma cell lines A2780, 2780AD and 2780CP. Initial experiments confirmed that, following treatment with approximately equitoxic (tenfold ID_{50} value) doses of CDDP *in vitro*, and HPLC separation of the resulting DNA digests, platinum peaks were identifiable by ICP-MS-ETV at elution times corresponding roughly to those expected for Pt-AG and Pt-GG. The kinetics of adduct formation and recovery was then studied over a time-course for CDDP exposure of 24 hours, followed by a 24 hour recovery period in drug-free medium for each time point. Significant interassay variability prevented detailed analysis of adduct formation and recovery, but some trends were evident.

ICP-MS-ETV analysis of individual eluate fractions for all three cell lines showed good separation of platinum content, with two major peaks identifiable (Figures 5 & 6) at elution times approximating to those of the two main intrastrand Pt-DNA adducts. Minor discrepancies in the exact positions of these peaks were probably due to differences in HPLC separation between the two experiments. Fractions eluting before 2.5 minutes were not analysed for platinum content since the earliest expected bifunctional Pt-DNA adduct, Pt-AG, was known under these conditions to have an elution time of 3.1 minutes.

For A2780, three platinum peaks appeared: the earliest, between 3 and 6 minutes presumably representing Pt-AG, the latest, between 12 and 14 minutes presumably representing Pt-GG, and an intermediate peak between 7 and 10 minutes which may represent an unrecognised Pt-oligonucleotide lesion. Platinum levels, in general, for

this cell line were higher after 4 hours than after 24 hours exposure to CDDP. This could be explained either by repair of platinated DNA occurring by 24 hours, or by cell death occurring during this time period. Whilst the DNA content of cells exposed to CDDP was reduced after 24 hours recovery in drug-free medium (Table 2), this effect was similar for the 4 hour treatment period (40.4 %) and 24 hour treatment period (44.4 %).

For 2780AD and 2780CP, the two major platinum peaks corresponding to the Pt-AG and Pt-GG adducts were clearly identified. The intermediate peak, however, was much less obvious, suggesting that the putative new Pt-DNA lesion (present in the chromatogram of A2780 DNA) was either much less relevant, or more readily repaired, in the two CDDP-resistant sublines. In contrast to A2780, platinum levels for both 2780AD and 2780CP were higher after 24 hours than after 4 hours exposure to CDDP. This may reflect either faster immmediate intracellular and / or intranuclear accumulation of platinum, or more rapid removal mechanisms for Pt-DNA lesions in the more sensitive parental cell line. Maximum platinum levels for Pt-GG were higher than for Pt-AG for both 2780AD and 2780CP, in keeping with other studies using the indirect ELISA for measurement of Pt-DNA adducts (Bedford et al., 1988 and Dempke et al., 1991). For A2780, however, this pattern was only found in one of the experiments. Although this initial work should only be regarded as semiquantitative, it also appeared that peak platinum levels achieved were directly related to the sensitivity of the cell line, in that highest levels (up to 3200 fmoles Pt / µg DNA / µmole CDDP) were achieved for A2780, intermediate levels (up to 1100 fmoles Pt / µg DNA / µmole CDDP) for 2780AD, and lowest levels (up to 430 fmoles Pt / µg DNA / µmole CDDP) for 2780CP. This may also reflect differences in either platinum accumulation or the rate of repair of Pt-DNA lesions between these cell lines.

The next experiment attempted to achieve more accurate analysis of the kinetics of formation and recovery (over 24 hours) for the two major adducts, Pt-AG and Pt-GG, by collecting and pooling HPLC fractions at around their expected elution times, as calculated from the individual chromatograms. The consequent reduction in samples for platinum analysis allowed this work to be performed three times in total. Unfortunately, however, significant interassay variability (of two- to three-fold) prevented combination of this data. Each experiment was therefore plotted separately (Figures 7 - 12), and results can only be considered as semiquantitative.

Levels of the Pt-AG adduct increased with time of CDDP exposure for all three cell lines, and appeared higher for A2780 than for either of the resistant sublines, 2780AD and 2780CP. For Pt-GG, a similar "dose-response" relationship was less clear. Levels of this adduct in A2780 DNA increased up to 12 hours CDDP exposure, but then fell in two out of three experiments. For 2780CP, a similar initial pattern was seen, followed by a later, slow rise again in platinum levels. For 2780AD, no consistent pattern emerged. In general, the peak Pt-GG level reached was highest for A2780 than for 2780AD or 2780CP, in keeping with observations for Pt-AG. This general trend confirmed that seen in the first experiment, and again may reflect faster platinum accumulation or slower Pt-DNA repair mechanisms for A2780, compared with 2780AD and 2780CP.

Following a 24 hour recovery period in drug-free medium, Pt-DNA adducts measured for the three cell lines showed some interesting differences (Figures 7 - 12). For Pt-AG, levels in A2780 were generally higher than those measured immediately following CDDP exposure, whereas in 2780AD and 2780CP levels were found to be lower. For Pt-GG, the converse was found: levels were lower in A2780, but higher in 2780AD and 2780CP following the recovery period. This implies repair of Pt-GG adducts, but not Pt-AG, for the CDDP-sensitive cell line, A2780. The two CDDP-resistant sublines appear to have repair mechanisms for Pt-AG, but not for Pt-GG,

when compared with A2780. CDDP resistance in 2780AD and 2780CP, therefore, appears to correlate with improved repair capacity for the Pt-AG adduct, or tolerance for the presence of the Pt-GG adduct.

Other studies of Pt-DNA adduct formation in CDDP resistance have used a constant dose throughout experiments, despite a range of sensitivity to CDDP in the cell lines used. Thus CDDP has been used from 1.1 to 29 times the ID_{50} value (Bedford et al., 1988; Shellard et al., 1991 & Dempke et al., 1991), for analysis of adduct levels by an indirect ELISA (Fichtinger-Schepman et al., 1985). Since the detection power of this assay is affected by the degree of platinum modification of cellular DNA analysed, it would seem more sensible to minimise this variable by standardising CDDP dose. In this work, ten times the ID_{50} values of CDDP for each cell line were used throughout. In some experiments, DNA extracted following the recovery period was insufficient to allow HPLC separation, presumably reflecting the long-lasting cytotoxicity of CDDP even after its removal from tissue culture medium. This impression was confirmed by an experiment conducted to assess the (potentially dilutant) contribution of fresh DNA synthesis to adduct levels in DNA from cells after the recovery period (Table 2). No such effect occurred in any cell line: in fact significant cytotoxicity of up to 83% was noted for 2780CP. This reflects the difficulty of achieving true equitoxic treatments with CDDP for these cell lines with such a wide range of CDDP resistance. Ideally, adduct formation in all three cell lines should be assessed over a wide range of concentrations of CDDP, to validate the existence of a dose-response relationship. Such an experiment would require analysis of large numbers of samples, which would be difficult given the current limitations of ICP-MS-ETV for this work.

The experiments described here confirm, however, the potential usefulness of ICP-MS-ETV in platinum analysis for the direct detection of intrastrand Pt-DNA adducts. However, at present it is necessary to operate at the extreme lowest levels of sensitivity for this technique, allowing only semiquantitative evaluation of results.

Interassay variability in these experiments was greater than is normally cited for ICP-MS-ETV (5 - 10%, Gregoire, 1988). This may have been due to a combination of factors, particularly the high matrix suppression effect noted in the pooled Pt-GG fractions, despite desalting of these. Other technical problems include its capital expense, and the very high degree of operator precision and technical understanding required for this. Sample preparation is time-consuming, and when ETV is used for sample introduction,a maximum of only 30 samples per day can be processed. At present, therefore, it is an expensive and cumbersome technique. However, direct analyis of Pt-DNA adducts has been achieved; technical advances and greater automation may improve its suitability for their future analysis.

CHAPTER 5

PLATINUM UPTAKE IN OVARIAN CARCINOMA CONTINUOUS CELL LINES

INTRODUCTION

Whilst it is now generally accepted that intrastrand Pt-DNA adducts account for the cytotoxicity of platinum agents, only a tiny proportion of total cellular platinum (<1%) actually binds to DNA (Andrews & Howell, 1990). A large intracellular pool of platinum also exists, around half of which is associated with diffusible small molecules such as histidine, methionine and glutathione (Sharma & Edwards, 1983). It is possible, therefore, that cytosolic platinum interactions, and their reversibility, may influence the cytotoxic action of CDDP. Mechanisms of platinum transport across both the cell and nuclear membrane may also have significance, particularly in the development of platinum resistance. The phenomenon of multidrug resistance, whereby cross-resistance to a number of unrelated but naturally occurring cytotoxic drugs develops (see Chapter 1), has been shown to be due to increased drug efflux via the P-glycoprotein membrane pump. This is an important mechanism for the development of resistance to some drugs, but is not considered relevant for platinum resistance in ovarian cancer (Bourhis et al., 1989; Goldstein et al., 1989 & van der Zee et al., 1991).

Mechanisms for intracellular platinum transport

A direct relationship between platinum accumulation and CDDP cytotoxicity in cancer cells has, however, been observed (Eicholtz-Wirth & Hietel, 1986). The actual mechanism by which CDDP enters cells remains unclear. Initially it was assumed that this occurred by passive diffusion, on the basis of a lack of saturation kinetics

observed for CDDP (Hromas et al., 1987) and also for its less potent analogue *cis* - PPC-^{3}H (Gale et al., 1973). However, this latter model does not provide good criteria for categorising uptake, particularly as the pyridine ligands contained within this CDDP analogue may significantly alter its uptake (Andrews et al., 1988). Further support for the role of passive diffusion in CDDP uptake is provided by the ability of some membrane-disrupting agents to enhance cellular platinum accumulation (Timmer - Bosscha et al., 1989) and by the inability of structural analogues of CDDP to saturate or to inhibit competitively platinum accumulation (Andrews et al., 1991). Membrane fluidity studies in paired ovarian carcinoma cell lines (with a 2-fold range of CDDP resistance) did not reveal any significant differences in bulk membrane lipid composition or fluidity, despite clear and early differences observed in platinum accumulation (Mann et al., 1988).

A role for carrier-mediated platinum transport has been suggested in the development of CDDP resistance. CDDP was found to cause selective inhibition of sodium-dependent amino acid uptake into L1210 murine leukaemia cells (Scanlon et al., 1983), suggesting an interaction between CDDP and the cell membrane. Indirect evidence for the role of amino acid carrier proteins in CDDP uptake was suggested by the discovery that its cytotoxicity can be inhibited in a competitive manner by coincident exposure to certain amino acids, such as leucine (Byfield & Calabro-Jones, 1982). This phenomenon could contribute to the nephrotoxicity of CDDP. Various aldehydes have been shown to protect cells from the cytotoxic action of CDDP, and have been linked, via binding to cell membrane and consequent Schiff base formation, with its reduced accumulation at sites which may be involved in carrier-mediated CDDP transport (Dornish et al., 1986). An energy dependent component in CDDP accumulation has also been proposed (Andrews et al., 1988) on the basis that (despite lack of saturability of CDDP accumulation) platinum transport was inhibited by a combination of dinitrophenol (an inhibitor of oxidative phosphorylation) and the

glycolysis inhibitors NaF and iodoacetate. Ouabain, a specific inhibitor of Na^+, K^+-ATPase, was also shown to inhibit CDDP accumulation, indicating a possible transport role for this ionic pump. The stimulation of platinum uptake by reduced osmolarity (Smith & Brock, 1989), reduced membrane potential and elevation of cAMP levels (Andrews & Howell, 1990) also tend to support a role for some sort of carrier system.

Differential platinum accumulation as a mechanism for CDDP resistance

Elucidation of the mechanism of platinum accumulation, and its relationship to the development of platinum resistance, is clearly important and offers potential for therapeutic manipulation of CDDP. Continuous cell lines of various origins, with differing degrees of induced CDDP resistance, have been used to investigate the relationship between platinum accumulation and cellular platinum sensitivity. In general, platinum accumulation was found to be reduced in resistant sublines over a range of time schedules and CDDP doses.

In paired ovarian cancer cell lines with moderate degrees of induced CDDP resistance (up to 10-fold), cellular platinum levels were measured as a function of CDDP concentration and exposure time (up to 24 hours). Accumulation was found to be reduced by 25 - 50% in the resistant sublines (Andrews et al., 1988; Kuppen et al., 1988). In other (non-ovarian) cell lines with induced CDDP resistance, platinum accumulation in resistant cells was found to be reduced in some studies (Shionoya et al., 1986; Foka et al., 1988; Kraker & Moore, 1988) but not in others (Sekiya et al., 1989).

Short-term platinum accumulation may, however, best reflect reductions in influx. No difference in accumulation was found when platinum was measured immediately after CDDP exposure in paired A2780 cells (Parker et al., 1987) and human bladder cancer

cells (Bedford et al., 1987). In murine leukaemia cells, with a CDDP resistance ratio of 20, platinum accumulation was unchanged over 12 minutes (Hromas et al., 1987).

Platinum efflux was also measured in some of these paired cell lines: this was unchanged in one study (Parker et al., 1991), and reduced in resistant sublines, by a similar degree to accumulation, in others (Andrews et al., 1988; Waud, 1987). The absence of a consistent association between either platinum accumulation or efflux and CDDP resistance provides further (indirect) evidence for the lack of importance of passive transport in platinum accumulation.

All the previous studies described have used either AAS or ^{195m}Pt to measure intracellular platinum. Development of an assay for the direct measurement of Pt-DNA adducts, described in Chapters 3 & 4, using ICP-MS for platinum analysis, allowed access to a highly sensitive technique for the investigation of intracellular platinum accumulation. The three ovarian carcinoma cell lines described in Chapter 2, with a moderate range of CDDP sensitivity, were used to study the role of differential platinum accumulation in induced CDDP resistance, as a function of both time and CDDP concentration.

MATERIALS AND METHODS

Cell culture

The three ovarian carcinoma continuous cell lines (A2780, 2780AD and 2780CP) described in Chapter 2, with a stable range of induced CDDP resistance of up to 27-fold, were used Tissue culture conditions were as described in Chapter 2.

Measurement of intracellular platinum accumulation

For studies of total intracellular platinum accumulation, cells were seeded into 6-well tissue culture plates at a density of 5 x 10^4 cells per well, and grown to confluence over 4 - 6 days. Tissue culture medium was then removed and replaced with medium containing CDDP at a final concentration of 20 μM, previously equilibrated with 2% CO_2 in air. At various times, plates were placed on ice, the medium removed and cells washed twice with ice-cold PBS. After trypsinisation, the cells were resuspended in 3 ml ice-cold PBS and pelleted by centrifugation at 200g for 5 minutes at 4° C. Triplicate wells were harvested for each cell line. Cell pellets were stored at -20°C until platinum analysis was performed. Total cell numbers in each well were counted from triplicate wells of a control (untreated) plate for all three cell lines.

In a second experiment to compare platinum accumulation with CDDP concentration, cells were incubated with CDDP at a range of concentrations from 10^{-4} M to 1.3 x 10^{-9} M for 4 hours. Cell pellets were prepared and stored as described above, prior to platinum analysis.

Measurement of DNA-bound platinum

For the measurement of platinum bound to DNA, cells were seeded into 75 cm^2 flasks at a density of 5 x 10^4 per ml, and grown to confluence over 6 - 8 days. They were then fed with 25 ml of tissue culture medium containing CDDP (20 μM) as described in the previous experiments. At various time-points, medium was removed

from triplicate flasks and the cells were washed twice with ice-cold PBS. DNA was then extracted as follows. Tissue culture medium was removed, 10 ml phenol equilibrated with NaAc (0.3 mM) was added to each flask and incubated for 10 - 15 minutes at 37° C in an agitator, then poured into 50 ml polypropylene tubes (Falcon, Becton Dickinson, London). Equal volumes of lysis buffer (containing NaAc, 0.3M, SDS, 0.5%, and EDTA, 5 mM) and chloroform (containing isoamyl alcohol in the proportions 24:1) were then added, and mixed continuously for 15 minutes at room temperature. The tubes were then centrifuged at 600g for 20 minutes at 14° C, or until a compact interphase layer had formed. The aqueous phase from each tube was then drawn off carefully into 25 ml polypropylene tubes (Falcon, London), and 2 volumes cold absolute ethanol added. Precipitated nucleic acids were later recovered by centrifugation at 600g, 14° C, for 20 minutes. The resulting pellet was washed with 70% ethanol and centrifugation repeated as before, prior to resuspension in 200 µl TNM buffer (NaCl, 0.14M, Tris buffer 0.01M , pH 7.4, $MgCl_2$, 1.5 mM) with NaAc, 0.3M. RNA was removed by incubation with 2 µl RNase A (Sigma, Poole, U.K.) at 37° C for 20 minutes. The phenol and chloroform extraction steps described above were then repeated, and resulting DNA resuspended in TNM buffer, 0.5 ml, prior to platinum analysis.

Energy dependence of platinum uptake

To investigate the energy dependence of platinum transport, cells were grown in the absence of glucose and in the presence of sodium azide to inhibit energy production via oxidative phosphorylation within the cell. For this, A2780, 2780AD and 2780CP cells were seeded into 75 cm^2 tissue culture flasks at a density of 5 x 10^4 per ml and grown to confluence as described. Glucose-free medium (Hanks Balanced Salt Solution, ICN Biomedicals, Rickmansworth, Hertfordshire), equilibrated with $NaHCO_3$, was used thereafter for tissue culture . The cells were fed with either glucose-free medium alone, or containing sodium azide, 10 mM, and equilibrated

with 2 % CO_2 in air at 37°C for 10 minutes. CDDP at a final concentration of 2 μM was then added to all flasks and cells incubated under standard conditions.for one hour. Medium was then removed, the cells washed twice with ice-cold PBS, trypsinised as described previously and resuspended in 1 ml PBS. A small aliquot, 100 μl, of the suspension was used to count cell number in an electronic counter as described in Chapter 2. Remaining cells were pelleted as described above and stored at -20°C prior to platinum analysis.

Platinum analysis by ICP-MS

For all samples, the cell pellet was resuspended in concentrated HNO_3, 50 μl, and concentrated HCl, 50 μl. Lysed cells were heated to 120° C until dry and resuspended in 3 ml 1% HCl immediately prior to platinum analysis by ICP-MS. Liquid nebulisation was used for sample introduction; solutions of indium and platinum were used as internal standards and for calibration as described in Chapter 3.

RESULTS

Measurement of intracellular platinum accumulation

Cells from all three cell lines were exposed to CDDP, 20 μM, over a six hour time course, and total cell platinum content measured by ICP-MS (Figure 1). For all three cell lines, there was an increase in platinum content over time. Platinum accumulation was more rapid for A2780, and reached a level of 106.5 ± 9.3 pmoles / 10^6 cells after six hours exposure. For the two resistant sublines the rates of platinum accumulation were similar, but were less than for A2780. After six hours 2780AD had accumulated 56.2 ± 14.6 pmoles / 10^6 cells and 2780CP 49.2 ± 21.4 pmoles / 10^6 cells. Platinum accumulation compared with A2780 was 49.6 ± 16.2% at 4 hours and 52.7 ± 13.3% at 6 hours for 2780AD, and 50.7 ± 19.6% at 4 hours and 45.5 ± 16.9% at 6 hours for 2780CP.

Figure 2 shows the results of a similar experiment measuring total cell platinum content as a function of CDDP concentration. Cells were incubated with CDDP for four hours over the same range of concentrations used in the MTT chemosensitivity assay (see Chapter 2), in order to allow comparison of platinum accumulation with cytotoxicity. Platinum accumulation was linear over the range 0.0013 - 100 μM CDDP for all three cell lines, and was again greatest for A2780. For the two resistant sublines, however, 2780CP consistently accumulated more platinum than 2780AD, despite its greater resistance to CDDP at this exposure time.(resistance ratio 32.6 for 2780CP, 16.5 for 2780AD - see Table 4, Chapter 2). Regression analysis for these lines gave equations of:

$y = 1.36x + 0.55$ ($r = 0.9995$) for A2780

$y = 1.09x + 0.78$ ($r = 0.9896$) for 2780CP

$y = 0.455x + 0.34$ ($r = 0.9830$) for 2780AD

where r is the correlation coefficient. Thus, for 2780CP the rate of total cell platinum accumulation over this range of CDDP concentration, compared with that found in the CDDP-sensitive parental cell line, A2780, was 80.1%, and for 2780AD 33.5%.

Since it was only possible to perform this experiment once, in duplicate, results given are the mean of two values.

Table 1 shows the data for platinum content at 20 μM CDDP in this experiment: mean accumulation : cytotoxicity was 31.2 % for A2780, 15.2 % for 2780AD and 49.6 % for 2780CP.

	pmoles Pt / 10^6 cells†	**% cell kill**
A2780	24.8 35.4	96.4
2780AD	11.6 9.7	69.6
2780CP	23.7 28.5	52.6

TABLE 1: Intracellular platinum accumulation and cytotoxicity of CDDP when 20 μM CDDP was used for cell treatment. (†: duplicate values for the same experiment)

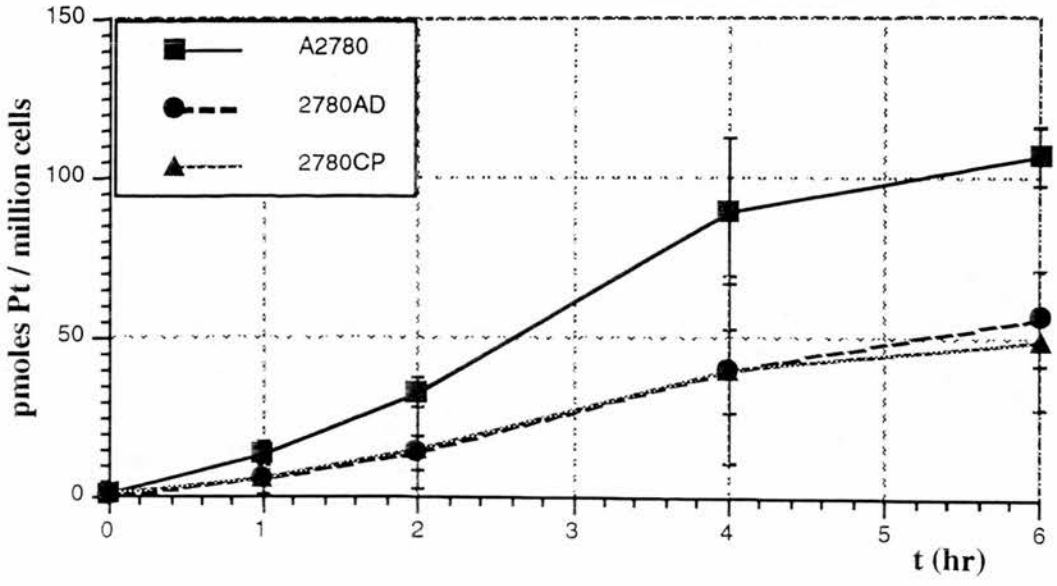


FIGURE 1: Intracellular platinum accumulation as a function of time. Results given are the mean ± S.E. of three separate experiments.

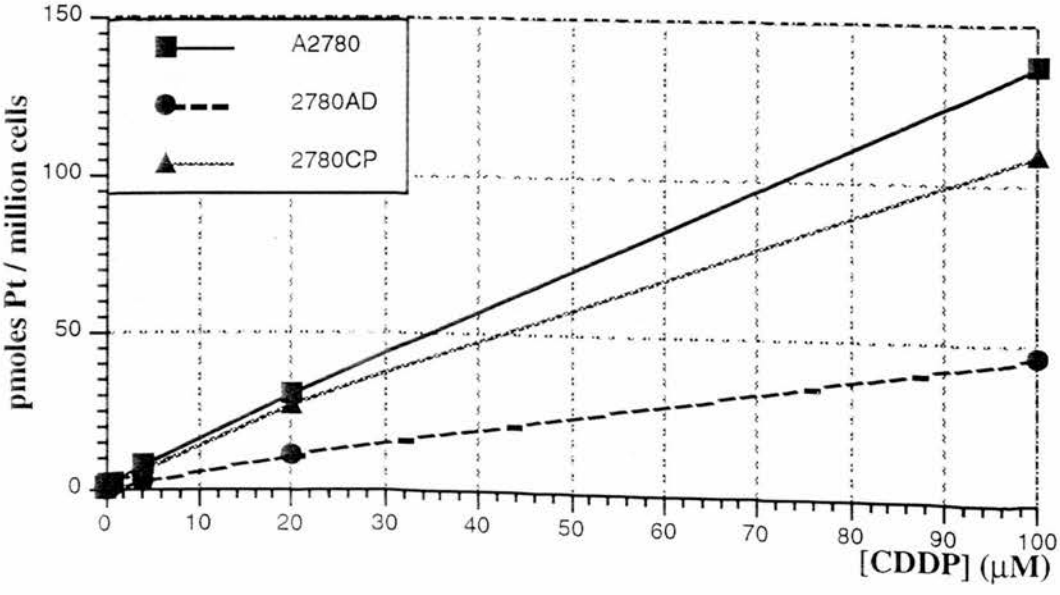


FIGURE 2: Intracellular platinum accumulation as a function of CDDP concentration used to treat cells.

Measurement of DNA-bound platinum

Estimations of total cellular platinum accumulation and DNA-bound platinum were performed in parallel, using CDDP at 20 μM over a six hour time-course. Analytical constraints again meant only one experiment, in duplicate, could be performed: results are therefore given as the mean of two values (Figures 3a & 3b).

Total cell platinum was again found to increase with time; results were similar to those for the earlier experiment (Figure 1) with platinum accumulating faster, and reaching a higher peak level, for A2780 than for the two resistant sublines. For these, rates of accumulation and peak platinum levels were very similar, despite their twofold difference in resistance ratio to CDDP over four hours (Table 4, Chapter 2).

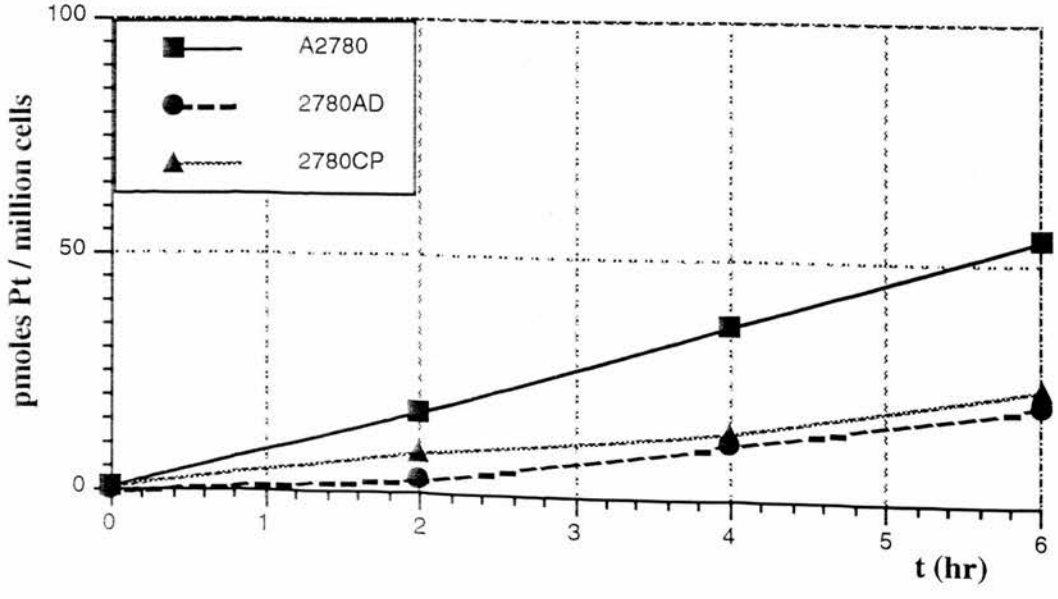


FIGURE 3a: Total cellular platinum accumulation after treatment of cells with 20 μM CDDP.

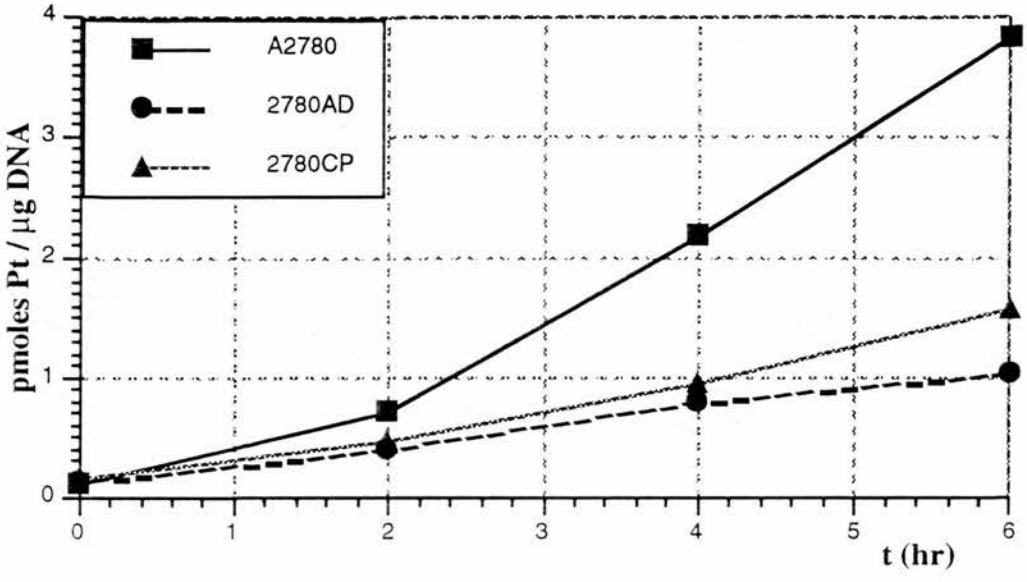


FIGURE 3b: DNA-bound platinum levels after treatment of cells with 20 µM CDDP. Results given for Figures 3a and 3b are the mean of two values obtained for one experiment performed in parallel.

Similar results were found for DNA-bound platinum over this time-course (Figure 3b). All three cell lines showed an increase in Pt-DNA with time, this being greatest for A2780; for the two resistant sublines levels of DNA-platination were slightly higher for 2780CP than for 2780AD. Rate of accumulation was greatest between 2 and 6 hours: regression analysis for these lines between these time points gave equations of:

$y = 0.78x - 0.87$ ($r = 0.9989$) for A2780

$y = 0.16x + 0.1$ ($r = 0.9743$) for 2780AD

$y = 0.13x - 0.13$ ($r = 0.9962$) for 2780CP

where r is the correlation coefficient. Thus, the rate of DNA-platination between 4 and 6 hours, compared with A2780, was 20.5% for 2780CP and 16.7% for 2780AD.

Table 2 shows the proportion of total cell platinum that was bound to DNA for each time point in this experiment, for all three cell lines. There is a clear trend for this to be highest immediately after CDDP exposure, although this may be an artefact owing

to the very low platinum values obtained immediately after addition of CDDP to cells. For all other time-points proportions were much lower and no significant differences were found.

t (hr)	A2780 (%)	2780AD (%)	2780CP (%)
0	31	400	79
2	4.3	13.9	5.7
4	6.1	7.1	7.0
6	6.9	5.0	6.5

TABLE 2: The proportion of total cellular platinum bound to DNA at each time point.

Relationship between cellular platinum accumulation and sensitivity to CDDP

Figure 4 shows the cytotoxicity of CDDP after 4 hours exposure as a function of its intracellular accumulation. For this, the percentage cytotoxicity noted at each concentration of CDDP (4 hours exposure) was calculated for each cell line from the MTT chemosensitivity data described in Chapter 2. These values were then compared with total cellular platinum levels over the same range of CDDP concentration. The curve for A2780 is furthest to the left, indicating the increased sensitivity of this cell line to CDDP compared with its resistant sublines 2780AD and 2780CP, at all levels of platinum accumulation. The most resistant subline, 2780CP, appeared able to tolerate higher levels of intracellular platinum: for 50% cell kill, total cell platinum accumulation was 2 pmoles / 10^6 cells for A2780, 6 pmoles / 10^6 cells for 2780AD and 25 pmoles / 10^6 cells for 2780CP.

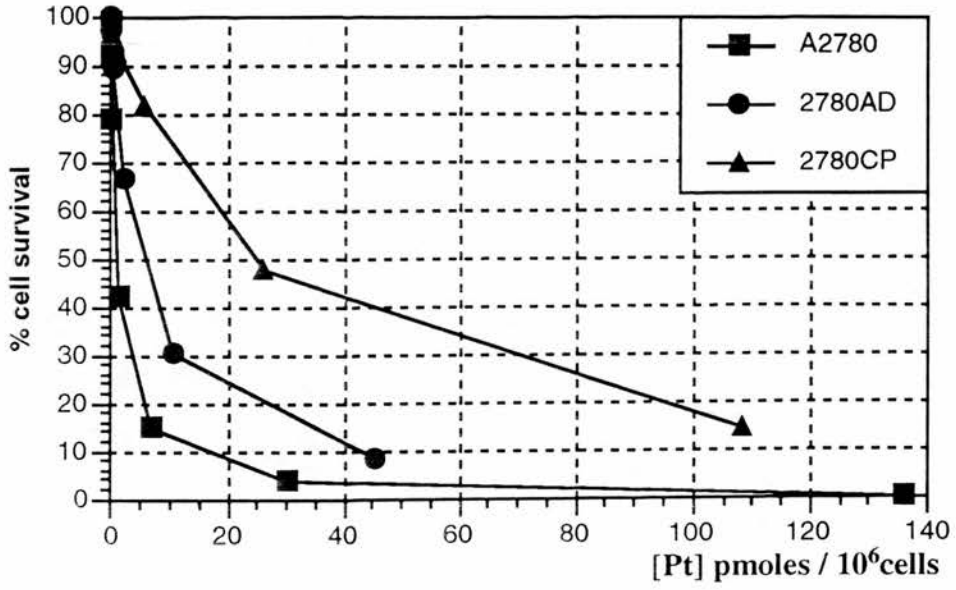


FIGURE 4: Cytotoxicity of CDDP as a function of concentration of CDDP used.

Energy dependence of platinum uptake

Table 3 shows platinum accumulation when sodium azide was used to inhibit energy formation within the cell, and thus prevent any actively-mediated platinum transport. Cells were exposed to CDDP, 2 μM, for one hour: this low dose-schedule was chosen to minimise the effect of CDDP cytotoxicity on cell transport mechanisms. No significant change in total cell platinum accumulation was found for the most resistant subline, 2780CP, whilst a moderate decrease (23.6%) in platinum accumulation was noted for 2780AD. Sodium azide at the concentration used, 10 mM, was noted to be significantly cytotoxic to A2780.

	pmoles Pt / 10^6 cells	
Cell line	**- sodium azide**	**+ sodium azide**
A2780	15.58	63.98†
2780AD	12.46	9.52
2780CP	14.88	14.82

† significant cytotoxicity noted for A2780 only

TABLE 3: Total cellular platinum accumulation (glucose free medium) in the presence and absence of sodium azide to inhibit intracellular oxidative phosphorylation.

DISCUSSION

This work aimed to investigate the relationship between sensitivity to CDDP and platinum accumulation, intracellular and nuclear, in the three ovarian cancer continuous cell lines described in Chapter 2. These displayed a range of resistance after 4 hours exposure to CDDP, with resistance ratios of 16.5-fold for 2780AD and 32.6-fold for 2780CP (see Chapter 2, Table 4).

For all three cell lines, platinum accumulation, both total and DNA-bound, increased with time but was greatest for the most sensitive cell line, A2780. Little difference was found between the two resistant sublines for either parameter. Cytotoxicity was found to be directly related to intracellular platinum levels, after 4 hours exposure to CDDP, with A2780 again the most sensitive, 2780CP the most resistant, and 2780AD intermediate in sensitivity. Inhibition of active transport, with sodium azide, did not alter platinum accumulation for 2780CP, but caused a moderate decrease for 2780AD without obvious cytotoxicity being noted.

For A2780, the rate of cellular platinum accumulation increased with time at a greater rate, and reached a higher level, than for 2780AD or 2780CP (Figure 1). After 4 hours, total cell platinum content when compared with A2780, was 49.6 ± 16.2% for 2780AD (resistance ratio 16.5), and 50.7 ± 19.6% for 2780CP (resistance ratio 32.6). After 6 hours, 2780AD had reached a total cell platinum level of 52.7 ± 13.3% that of A2780; for 2780CP accumulation was 45.5 ± 16.9% that of A2780. Thus the two resistant sublines displayed very similar reductions in total cell platinum accumulation compared with A2780, despite a twofold difference in their resistance ratios under these experimental conditions. This suggests that differential platinum accumulation, whilst contributing, is not the sole mechanism in CDDP resistance for these two resistant sublines.

When cellular platinum content was compared with CDDP concentration in the incubation medium (after 4 hours exposure), a linear increase was observed for all three cell lines, again greatest for A2780. However, in this experiment 2780CP was found to accumulate more platinum for a given CDDP concentration than the more sensitive subline 2780AD (rate of accumulation compared with A2780: 80.1% for 2780CP and 33.5% for 2780AD). This supports the theory that, particularly for 2780AD, there may be a mechanism operating to reduce intracellular platinum accumulation which may account for some of the observed CDDP resistance. At a CDDP concentration of 20 μM, cytotoxicity for 2780AD was 72.2% and for 2780CP 54.6%, compared with that found for A2780 at this concentration. Platinum accumulation for 2780AD was 35.2% and for 2780CP was 86.7% that found for A2780 at this concentration of CDDP (Table 1). These figures support the above theory that, for 2780AD, CDDP resistance may reflect reduced platinum accumulation. However, for 2780CP, relative platinum accumulation was higher with increasing concentration of CDDP, despite its greater resistance to CDDP: this may reflect an increased tolerance to platinum.

For DNA-bound platinum, similar observations were made: for all three cell lines, DNA-Pt levels increased with time, particularly between 2 and 6 hours (Figure 3b). Between these times the rate of DNA-platination, compared with that for A2780, was 16.7% for 2780AD and 20.5% for 2780CP. Thus both the rate of platinum-DNA binding, and the peak level achieved, was again greatest for A2780. In this experiment, platinum values were similar for the two resistant sublines, but the relative reductions in platinum-DNA binding were greater than those found for total cell platinum. This could be explained by a reduction in platinum transport (or increased efflux) across the nuclear membrane in the resistant sublines, or alternatively by increased intranuclear platinum transport for A2780 to account for its increased sensitivity to CDDP. Alternatively, there could be fewer available binding

sites on DNA, or more rapid removal of Pt-DNA adducts in the resistant sublines. The proportions of DNA-Pt compared with total cell platinum are shown in Table 2. Values immediately after CDDP exposure are certainly higher than thereafter, but this could be explained by the very small values observed at this time-point, particularly for total cell platinum. For most of the other time-points, however, the proportion of total cell platinum that is DNA-bound is remarkably similar, between 5 and 7%. This finding does not support increased intranuclear platinum transport as a mechanism for the increased CDDP sensitivity of this cell line.

When cellular platinum accumulation was related to CDDP sensitivity a direct relationship was observed, with A2780 displaying the greatest cytotoxicity for a given platinum level, 2780AD intermediate cytotoxicity and 2780CP the most resistance (Figure 4). From these results extrapolations of cellular platinum content at 50% cytotoxicity could be made. For 2780AD, compared with A2780, platinum levels were increased 3-fold, and for 2780CP were increased 12.5-fold. Thus, again, 2780CP seemed able to tolerate higher levels of CDDP, which may explain some of its observed resistance.

These results demonstrate that reduction in platinum accumulation, both cellular and nuclear, contributed to CDDP resistance in the two resistant sublines, 2780AD and 2780CP. However, the degree to which this contributed to the observed resistance ratio appeared to differ. For 2780AD reduced accumulation appeared to be relevant. This cell line was known to express strongly the P-glycoprotein efflux pump that is responsible for the multidrug resistance phenomenon; however expression of this membrane pump was shown to disappear in 2780AD after prolonged culture in drug-free medium (Chapter 2, Plate 5), whilst the degree of resistance observed was unchanged. It was not, therefore, thought likely that this mechanism explained the reduced accumulation. The effect of energy depletion on platinum accumulation for this cell line (Table 3) did not suggest a role for an energy-dependent efflux pump.

The most resistant subline, 2780CP, whilst showing reduced accumulation, seemed able to tolerate higher levels of platinum, when compared with the two more sensitive cell lines for cytotoxicity. No difference in platinum accumulation was noted when energy was depleted with sodium azide. For 2780CP, therefore, increased DNA repair mechanisms may be relevant to its observed high level of CDDP resistance.

These findings are in agreement with those reported by other workers, using either AAS or radiolabelled CDDP or its analogues. Reduced platinum accumulation was a consistent finding in many CDDP-resistant cell lines (Andrews & Howell,1990). In pairs of ovarian carcinoma cell lines, platinum accumulation was reduced by 50 - 75% in the resistant subline (Kuppen et al., 1988; Andrews et al., 1989). In other models no such difference was found between sensitive and resistant cell lines (Hospers et al., 1988; Sekiya et al., 1989). This discrepancy could, however, be explained by differences in experimental design: the relative reduction in platinum accumulation was least (or non-existent) when measured within minutes of exposure to CDDP (Hromas et al., 1987; Bedford et al., 1987; Shionoya et al., 1986). Over longer time periods of exposure to CDDP other mechanisms, such as decreased influx or increased efflux could have contributed to any reduction in platinum accumulation in resistant cell lines. In this work platinum levels measured immediately after addition of CDDP were too low (for all three cell lines) for meaningful comparisons to be made. There was an apparent trend for the proportion of total cell platinum that was DNA-bound to be highest immediately after CDDP administration (Table 2), but this may simply be an artefact due to the very low values recorded.

ICP-MS was used throughout these experiments for analysis of platinum levels, using liquid nebulisation as a method for sample introduction (see Chapter 3). Typical measurements were between 0 and 150 ng platinum, well within the limits of sensitivity for this technique. As described in Chapter 3, an internal platinum standard was measured after every ten samples, to reduce interassay drift in sensitivity.

Interassay variation in platinum analysis was between 5 and 10%. This method for platinum analysis is, therefore, sufficiently sensitive to be used in the study of differential cellular and nuclear platinum uptake. However, it is expensive, slow and time-consuming: thus limiting its usefulness as a technique for routine experimental platinum analysis. The high degree of technical complexity currently associated with this method, requiring dedicated, consistent and experienced operators, is also a drawback.

CHAPTER 6

INTRACELLULAR INACTIVATION OF PLATINUM BY GLUTATHIONE

INTRODUCTION

Glutathione (GSH)

Glutathione (GSH), a naturally occurring low molecular weight thiol tripeptide, is a major constituent of the intracellular non-protein sulphydryl pool. Its chemical structure was first described in 1935 (Meister, 1981), since which time the physiological functions of this important molecule, and its oxidised form glutathione disulphide (GSSG), have gradually been elucidated. It participates directly or indirectly in many important biological processes, including DNA and protein synthesis, transport mechanisms, enzymic activity and cellular metabolism. In addition, it also has an important function in the protection of cells against reactive oxygen compounds and free radicals (Meister & Anderson, 1983). This latter detoxification property has stimulated interest in the role of GSH in the protection of cells, both normal and cancerous, against xenobiotic and cytotoxic compounds such as alkylating agents. The electrophilic nature of CDDP, particularly towards sulphur-containing compounds, also predicted a role for the modulation of both its cytotoxicity and nephrotoxicity by GSH.

The selective manipulation of GSH became possible with the advent of a specific substrate inhibitor of its synthesis, buthionine-S-R-sulfoxamine (BSO), and of an intracellular cysteine delivery system, 2-oxothiazolidine-4-carboxylate (OTZ). Intracellular GSH levels can be reduced by treatment with BSO, and can be increased by exposure to OTZ.

GSH and resistance to cytotoxic agents

Many studies have since investigated the relationship between GSH levels, their manipulation, and sensitivity to various cytotoxic agents in a variety of experimental systems. In human ovarian cancer cell lines a correlation between GSH levels and melphalan resistance was initially noted; GSH depletion (using BSO) increased cellular response to melphalan (Green et al., 1984). This observation was further supported by a study of melphalan, adriamycin and cisplatin sensitivity in paired drug-sensitive and drug-resistant human ovarian cancer cell lines (Hamilton et al., 1985). Elevated GSH levels were found in the resistant cell lines, and depletion of GSH was associated with increased sensitivity to CDDP. A model for human ovarian cancer *in vivo* has been developed in which disseminated intra-abdominal carcinomatosis is produced in athymic mice (Ozols et al., 1987). The administration of BSO in drinking water significantly lowered GSH levels in tumour cells, and produced a 72% increase in median survival time following melphalan therapy, compared with mice given melphalan alone. Elevation of GSH levels with OTZ produced a small increase in CDDP resistance in normal human lung fibroblasts but not in a lung adenocarcinoma cell line (Russo et al., 1986) or in Chinese hamster ovary (CHO) cells (Freeman et al., 1990).

GSH and CDDP resistance

Many authors have since investigated the relationship between GSH level, and its manipulation, in a number of CDDP-sensitive and -resistant paired cell lines of various origins. In general, an association between CDDP resistance and elevation of GSH levels was only noted when the resistant cell line had been selected by continuous exposure to CDDP. Prolonged depletion of GSH was then required to produce potentiation of cytotoxicity (Andrews & Howell, 1990).

Results from experiments performed *in vivo* are also rather conflicting. A recent study investigating CDDP biodistribution in tumour-bearing rats found that depletion of GSH caused elevation of platinum levels in all subcellular tumour fractions, but to an extent not directly proportional to the GSH depletion (Parti & Wolf, 1991). Human primary ovarian cancer biopsies, assessed by flow cytometry, have shown pronounced heterogeneity in intratumour GSH content (Lee et al., 1989) Other workers (Britten et al., 1989) have demonstrated an association between GSH levels in fresh human primary biopsies and earlier chemotherapy with alkylating agents. Manipulation of GSH levels *in vivo* has been hampered by the additional toxic effect of BSO at doses effective in their reduction (Kelley & Rosenoweig, 1989), although this problem has been successfully circumvented by others (Ozols et al., 1988).

GSH may reduce the cytotoxicity of CDDP towards target tissues in a number of ways: the exact mechanism has not yet been elucidated (de Graeff et al., 1988). Suggestions include: alterations in transport of CDDP at the cell membrane level, cytoplasmic inactivation by the formation of an inactive complex between CDDP and GSH, decreased CDDP-DNA binding, quenching of CDDP-DNA monoadducts by GSH (thus preventing their conversion to the more toxic bifunctional form) and changes in DNA repair mechanisms. Despite a multitude of studies investigating the association between intracellular GSH levels, CDDP resistance, and their manipulation via BSO or OTZ, little conclusive evidence exists to prove the hypothesis that raised intracellular GSH levels are causally linked with CDDP cytotoxicity..

In this work, the three ovarian carcinoma continuous cell lines originally described in Chapter 2, with a broad (26-fold) range of CDDP resistance, were used as a model to investigate further the relationship between intracellular GSH levels, their manipulation with BSO, and CDDP resistance induced *in vitro* . The effect of reduced

GSH levels on cellular platinum uptake was also measured, using ICP-MS for platinum analysis.

MATERIALS AND METHODS

Cell culture

The three ovarian carcinoma continuous cell lines A2780, 2780AD and 2780CP, described in Chapter 2, with a stable range of induced CDDP resistance of up to 27-fold were used. Tissue culture conditions were as described in Chapter 2.

Cellular glutathione analysis

Cells were seeded into 6-well tissue culture plates at a density of 5 x 10^4 per well, and grown to confluence over 8 - 10 days. In order to minimise the contribution of tissue culture medium (RPMI 1640, containing GSH 1 mg / ml), to cellular GSH content, medium was not replaced during the growth period of these cells.

Total glutathione (GSH + GSSG, TGSH) was measured according to the enzymic recycling assay of Tietze (Tietze, 1969), with some modifications. This method utilises the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by glutathione reductase to form 2-nitro-5-thiobenzoic acid, the rate of formation of which can be measured spectrophotometrically. The linearity of this reaction was confirmed at the start of each experiment, when a standard curve was performed using glutathione (Sigma, Poole, England), made up in 125 mM sodium phosphate buffer containing EDTA, 6.3 mM, pH 7.5, in a range of concentrations from 0 - 500 ng / ml. 200 µl of sample was then placed in a cuvette with 700 µl NADPH, 0.3 mM, and 100 µl DTNB, 6 mM, and equilibrated to 30^0C. 10 µl glutathione reductase, 50 U / ml (Sigma, Poole, England) was then added immediately prior to monitoring the absorbance at 412 nm over 3 minutes with a spectrophotometer. For assaying cellular GSH, cells in the stationary phase of growth were trypsinised and a small aliquot removed to allow estimation of the total number of cells present in each sample by Coulter counter analysis, as described in Chapter 2. The remaining cell suspension

was washed three times with PBS. The cell pellet was then resuspended in 1 ml perchloric acid, 1M, containing EDTA, 2 mM, and spun for 1 minute at 6.5K in an Eppendorf microfuge. A portion of supernatant was then titrated to pH 7.4 against a small volume of KOH, 2M, with MOPS, 0.3 M, and centrifuged again for 1 minute at 6.5K in a microfuge. The resulting supernatant was then used, appropriately diluted, to measure TGSH by the spectrophotometric assay described above. GSSG alone was also measured, by its initial derivatisation with 2 vinyl pyridine (2VP). 400 µl of supernatant was incubated at room temperature with 8 µl 2VP for around one hour prior to analysis as described. Reduced glutathione (GSH alone) could then be calculated.

Cytotoxicity of BSO

The cytotoxicity of D,L-BSO (Sigma, Poole, England), which was used to inhibit the synthesis of cellular GSH in later experiments, was investigated in the three ovarian cancer cell lines using the modified MTT chemosensitivity assay described in Chapter 2. Cells were seeded into 96-well microtitre plates at low density (between 5×10^2 and 1×10^3 per well). After 3 days growth in standard conditions, the central 8 columns of cells were fed with tissue culture medium containing D,L-BSO at a final concentration between 10^{-3} M and 1.3×10^{-8} M; medium alone was added to the remaining cells, which acted as controls. After 24 or 48 hours exposure, D,L-BSO was removed and cells were fed daily with medium alone, until day 7, when medium containing HEPES buffer (10 mM) and MTT (50 µl / well) was used. Plates were incubated for four hours in darkness and absorbance of the formazan crystals thus produced, redissolved in DMSO with Sorenson's glycine buffer, was measured in an ELISA plate reader as described in Chapter 2. The concentration of BSO which produced 50% absorbance of the control (untreated) cells.at 570 nm, was calculated and expressed as the ID_{50} value.

Effect of BSO on cellular glutathione content

The ability of D,L-BSO, at concentrations of 25 μM and 50 μM, to reduce GSH formation in all three cell lines was assessed over 24 and 48 hours. Cells were grown to confluence in 6 well plates over 6 - 8 days as previously described. Wells were then fed with either 25 μM D,L-BSO (in tissue culture medium), 50 μM D,L-BSO (in tissue culture medium) or medium alone as control. Cells were incubated at 37° C in 2% CO_2 for 24 hours, at which point either GSH content was assayed, or a further 24 hours of D,L-BSO treatment was performed prior to GSH assay.

Modulation of CDDP toxicity by d-L-BSO

The modulating effect of D,L-BSO on the cytotoxicity of CDDP in ovarian cancer cell lines was assessed using the modified MTT assay, described in Chapter 2. In addition to the use of CDDP as cytotoxic agent (in five-fold dilutions from 10^{-4} M) to all wells, D,L-BSO in tissue culture medium (at 25 μM or 50 μM, for either 24 or 48 hours) was added to the first four wells of each column. Medium was removed after 24 or 48 hours, and cells fed daily until day 7, when plates were analysed spectrophotometrically as described earlier.

To investigate the necessity of having already lowered cellular GSH levels at the time of addition of CDDP, an alternative experimental protocol of 24 hours pre-incubation with D,L-BSO (25 μM), prior to 4 hours exposure to CDDP, was also used.

The modulating factor (MF) for D,L-BSO was calculated by dividing the ID_{50} value of CDDP alone by that obtained in the presence of D,L-BSO.

Effect of D,L-BSO on platinum uptake

To assess the effect of D,L-BSO on platinum accumulation *in vitro*, by the reduction of intracellular GSH levels, ovarian carcinoma cells were grown to confluence in 75 cm^2 tissue culture flasks over 6 -8 days, as described above. The cells were then fed

with either D,L-BSO, 25 μM, in tissue culture medium, or with medium alone. After 24 hours incubation, CDDP was added at a final concentration of 2 μM in all cell lines. After incubation with CDDP for either 1 hour or 6 hours, cells were washed twice with PBS, trypsinised and a small aliquot removed for estimation of total cell number in each flask by Coulter counter. Cell pellets were then stored at -20° C prior to analysis of total intracellular platinum content or DNA-bound platinum by ICPMS, as described in Chapter 3.

RESULTS

Cellular glutathione analysis

At the start of each assay the rate of formation of DTNB from a range of concentrations of commercial GSH solution, measured spectrophotometrically at 412 nm, was measured to allow interpolation of cellular GSH and GSSG content. This relationship was found to be linear (Figure 1).

Total GSH and GSSG levels for all three cell lines in the stationary phase of growth were calculated; GSSG content was consistently negligible and results for total GSH therefore reflected those for GSH alone (Table 1).

TGSH content appeared to be directly related to CDDP resistance, being lowest in A2780, intermediate in 2780AD and highest in 2780CP ($r = 0.93$, Figure 2).

[TGSH} (ng / 10^6 cells)		
A2780	**2780AD**	**2780CP**
63.19 ± 21.5	150.8 ± 10.7	304.8 ± 78.9

TABLE 1: Glutathione content of ovarian cancer cell lines in the stationary phase of growth, calculated from the rate of formation of DTNB. Values given are the mean ± S.E. for three separate experiments.

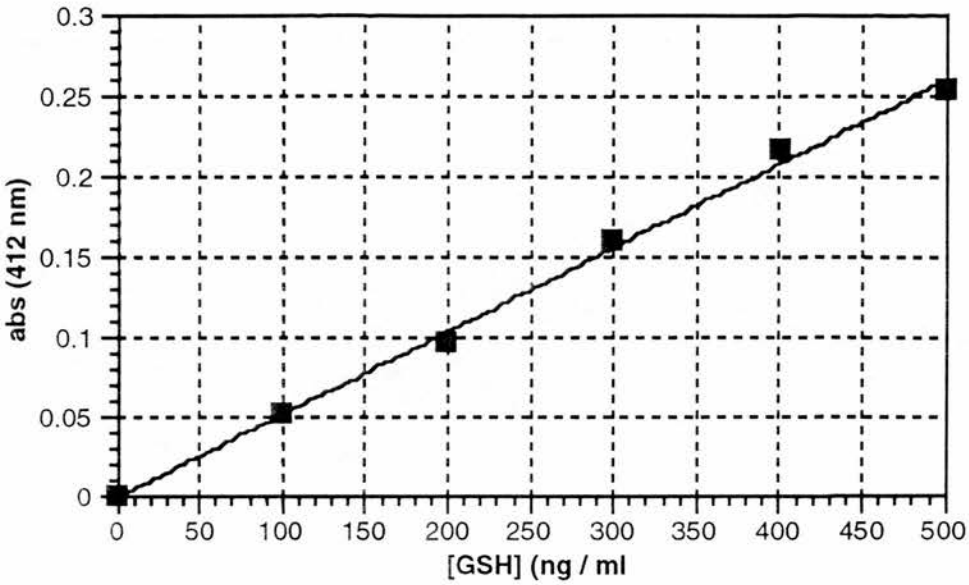


FIGURE 1: Typical standard curve showing the rate of formation of DTNB from GSH.

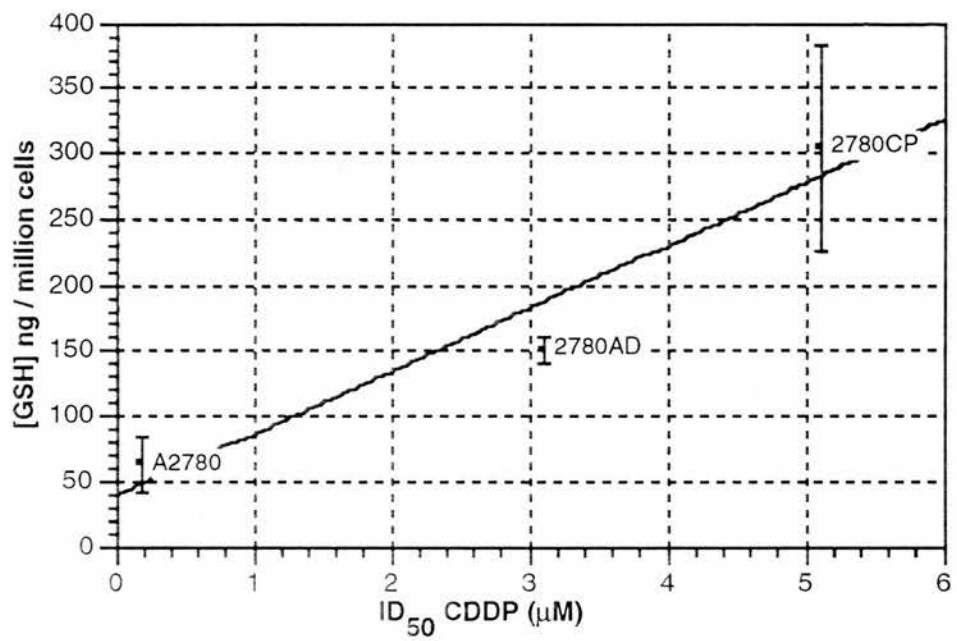


FIGURE 2: Correlation between intracellular GSH content and sensitivity to CDDP for ovarian cancer cell lines (r = 0.93).

Cytotoxicity of D,L-BSO

The toxicity of D,L-BSO towards the ovarian cancer cell lines A2780, 2780AD and 2780CP was measured using the modified MTT chemosensitivity described fully in Chapter 2. ID_{50} values for D,L-BSO, after 24 and 48 hours exposure, are shown in Table 2. Over 24 hours, significant cytotoxicity for D,L-BSO was not found for any of the cell lines. 2780CP was the most resistant to any cytotoxicity of D,L-BSO, whereas A2780 was the most sensitive. Only after 48 hours exposure, for A2780, and to a lesser extent for 2780AD, was the intrinsic cellular cytotoxicity of D,L-BSO itself likely to interfere with the interpretation of results for the later experiments on platinum uptake and modulation of chemosensitivity to CDDP.

ID_{50} (μM) for D,L-BSO			
Exposure time (hr)	**A2780**	**2780AD**	**2780CP**
24	643.3± 277.3	> 10^{-3} M	> 10^{-3} M
48	46.3 ± 11.6	102.0 ± 29.0	> 10^{-3} M

TABLE 2: Cytotoxicity of D,L-BSO towards ovarian cancer cell lines. Values given are the mean ± S.E. for three separate experiments.

Effect of D,L-BSO on cellular glutathione content

Cellular GSH levels, following incubation with D,L-BSO, at concentrations of 25 μM and 50 μM, over 24 and 48 hours, are shown in Table 3. Once again, GSSG levels were negligible. Table 4 shows the mean percentage reduction in TGSH content following each incubation period with D,L-BSO, for each cell line.

Cell line	Incubation time (hr)	[TGSH] (ng / 10^6 cells) : A2780		
		C (no D,L-BSO)	+ 25 μM D,L-BSO	+ 50 μM D,L-BSO
A2780	24	66.7 ± 25.9	4.1 ± 5.2	0
	48	33.0 ± 19.5	0	0
2780AD	24	111.4 ± 16.2	5.5 ± 6.5	0
	48	40.9 ± 24.4	0	0
2780CP	24	91.7 ± 12.2	3.2 ± 5.2	5.0 ± 6.2
	48	95.2 ± 60.9	4.6 ± 4.9	7.6 ± 9.0

TABLE 3: Glutathione content of ovarian cancer cell lines following incubation with D,L-BSO. Values given are the mean ± S.E. for three separate experiments.

	A2780 (%)		2780AD (%)		2780CP (%)	
t (hr)	25 μM D,L-BSO	50 μM D,L-BSO	25 μM D,L-BSO	50 μM D,L-BSO	25 μM D,L-BSO	50 μM D,L-BSO
24	95.4 ± 5.9	100 ± 0	94.6 ± 6.4	100 ± 0	95.8 ± 7.1	94.4 ± 6.6
48	100 ± 0	100 ± 0	94.7 ± 10.6	100 ± 0	92.5 ± 8.9	94.4 ± 6.0

TABLE 4: Percentage reduction in cellular glutathione content in ovarian cancer cell lines following incubation with D,L-BSO. Values given are the mean ± S.E. for three separate experiments.

Reductions of over 92% in cellular glutathione content were seen for all three cell lines under all experimental conditions used. A reduction in TGSH levels in control cells, after 48 hours incubation without D,L-BSO, was also observed. The most CDDP-resistant cell line, 2780CP, was the only one to show recordable TGSH values, albeit much reduced, following all four combinations of D,L-BSO addition to culture medium. It was thus found possible to achieve near complete reduction in cellular TGSH using the regime for D,L-BSO administration that was least toxic to the cell lines (25 μM over 24 hours).

Modulation of CDDP toxicity by D,L-BSO

The potential for D,L-BSO to act as a modulator of the cytotoxicity of CDDP towards ovarian cancer cells through its reduction in cellular GSH levels, was assessed using the modified MTT assay described. Cells were exposed to D,L-BSO, either 25 μM or 50 μM, for 24 or 48 hours, followed by treatment with CDDP for 24 hours. To assess the importance of lowering cellular GSH levels prior to the addition of CDDP, in another experiment cells were also pre-incubated with d- L-BSO, 25 μM, for 24 hours prior to treatment with CDDP for 4 hours.

Figure 3 shows typical cell survival curves following preincubation with D,L-BSO, 25 μM. No significant difference was found in 4-hour ID_{50} values for CDDP for this regime (Table 5), indicating that the cytotoxicity of CDDP was not affected by the reduction in GSH levels in any of the cell lines studied. Figures 4 - 7 show similar results for cell lines incubated with D,L-BSO at both 25 μM and 50 μM for 24 and 48 hours. For A2780, following 48 hour exposure, the cytotoxicity of D,L-BSO was such that cell survival curves could not be plotted for this cell line. The depletion of cellular GSH by D,L-BSO, for any of the regimes used, appeared to have only modest enhancing effect on the cytotoxic effect of CDDP in the three cell lines studied (Tables 5 and 6).

Effect of D,L-BSO on platinum uptake

No effect on total cellular platinum uptake (over 6 hours) was observed in any of the cell lines studied following reduction of intracellular GSH by D,L-BSO (Figure 8). Platinum uptake into the nucleus, and its binding to DNA, was similarly unaffected, indicating that there is no obvious redistribution of intracellular platinum across the range of CDDP sensitivities observed in these cell lines (Figure 9).

D,L-BSO	ID_{50} (CDDP) -A2780 (μM)		ID_{50} (CDDP) - 2780AD (μM)		ID_{50} (CDDP) - 2780CP (μM)	
	+ BSO	- BSO	+ BSO	- BSO	+ BSO	- BSO
25 μM[1] - preincubated	1.4 (0.55-2.8)	2.0 (0.96-3.6)	17 (1.3-36)	15 (3.1-56)	38 (10-90)	46 (7.2->100)
25 μM[2] - 24 hours	0.1 (0.04-0.25)	0.15 (0.05-0.36)	1.2 (0.49-2.3)	1.9 (0.82-3.4)	2.9 (1.5-4.8)	4.2 (2.0-7.6)
25 μM[2] - 48 hours	-	-	3.9 (2.2-9.1)	6.0 (4.8-7.2)	6.0 (3.6-10)	10.0 (7.2-18)
50 μM[2] - 24 hours	0.28 (0.16-0.4)	0.4 (0.32-0.54)	2.3 (2.0-2.7)	2.7 (2.6-2.8	6.0 (4.6-8.2)	8.0 (7.2-9.0)
50 μM[2] - 48 hours	-	-	2.2 (1.9-2.6)	5.5 (4.3-6.7)	5.1 (2.5-8.8)	7.2 (4.5-11.0)

TABLE 5: Effect of d-L-BSO on the cytotoxicity of CDDP towards ovarian cancer cell lines. Values given are the ID_{50} values for CDDP after either 4 hours ([1]) or 24 hours ([2]) exposure, and the range over two or three separate experiments. Where no value is quoted, significant cytotoxicity of D,L-BSO was noted.

BSO regime	A2780	2780AD	2780CP
25 μM- preincubated	0.70	1.13	0.83
25 μM- 24 hours	0.67	0.63	0.69
25 μM- 48 hours	-	0.65	0.60
50 μM- 24 hours	0.70	0.85	0.75
50 μM- 48 hours	-	0.40	0.71

TABLE 6: Modifying factors for d-L-BSO on the cytotoxicity of CDDP towards ovarian cancer cell lines

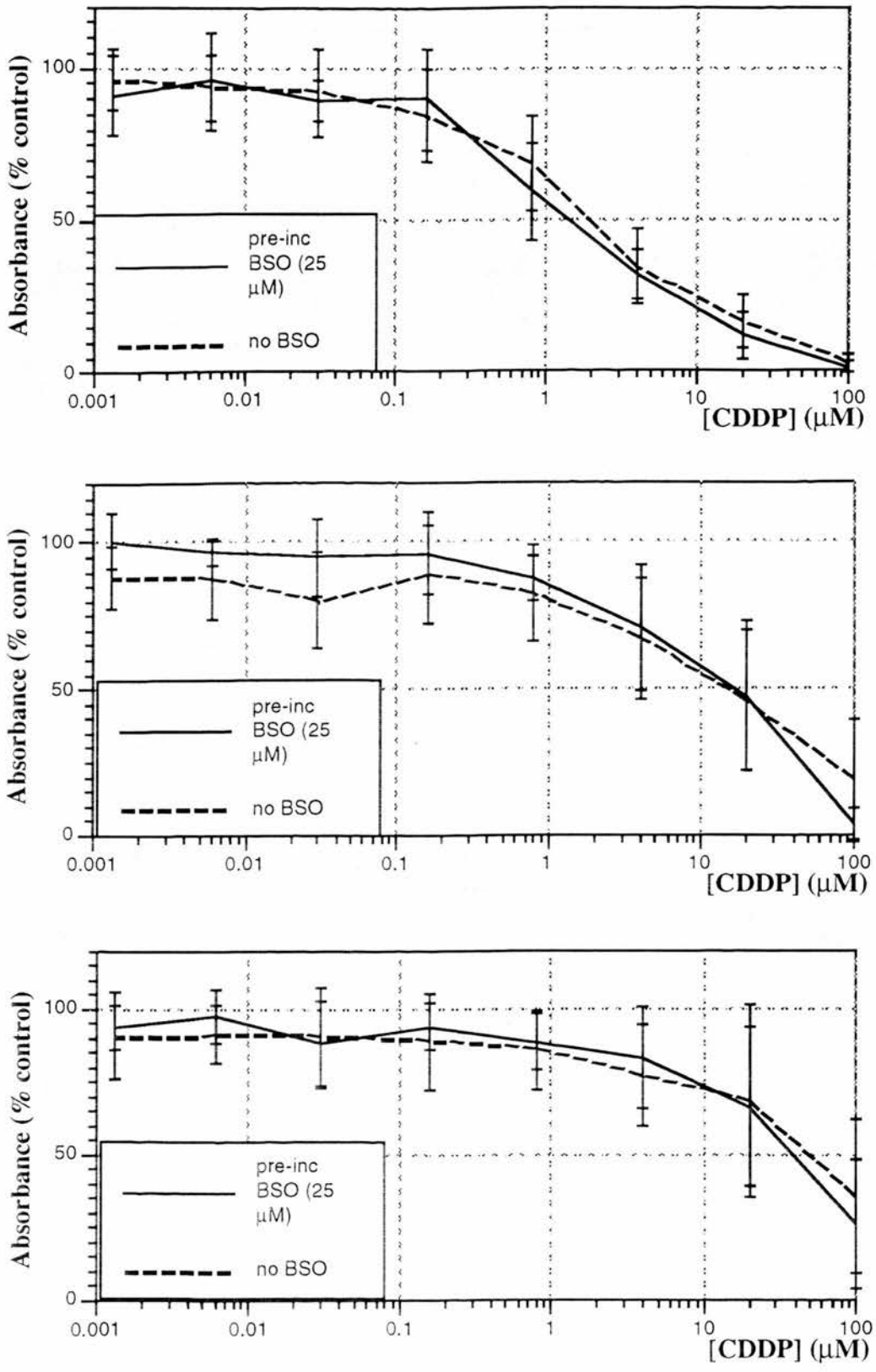


FIGURE 3: Cytotoxicity of ovarian cancer cells to CDDP (4 hours) following pre-incubation with d-L-BSO, 25μM for 24 hours. Values given are the mean ± S.E. for three separate experiments.

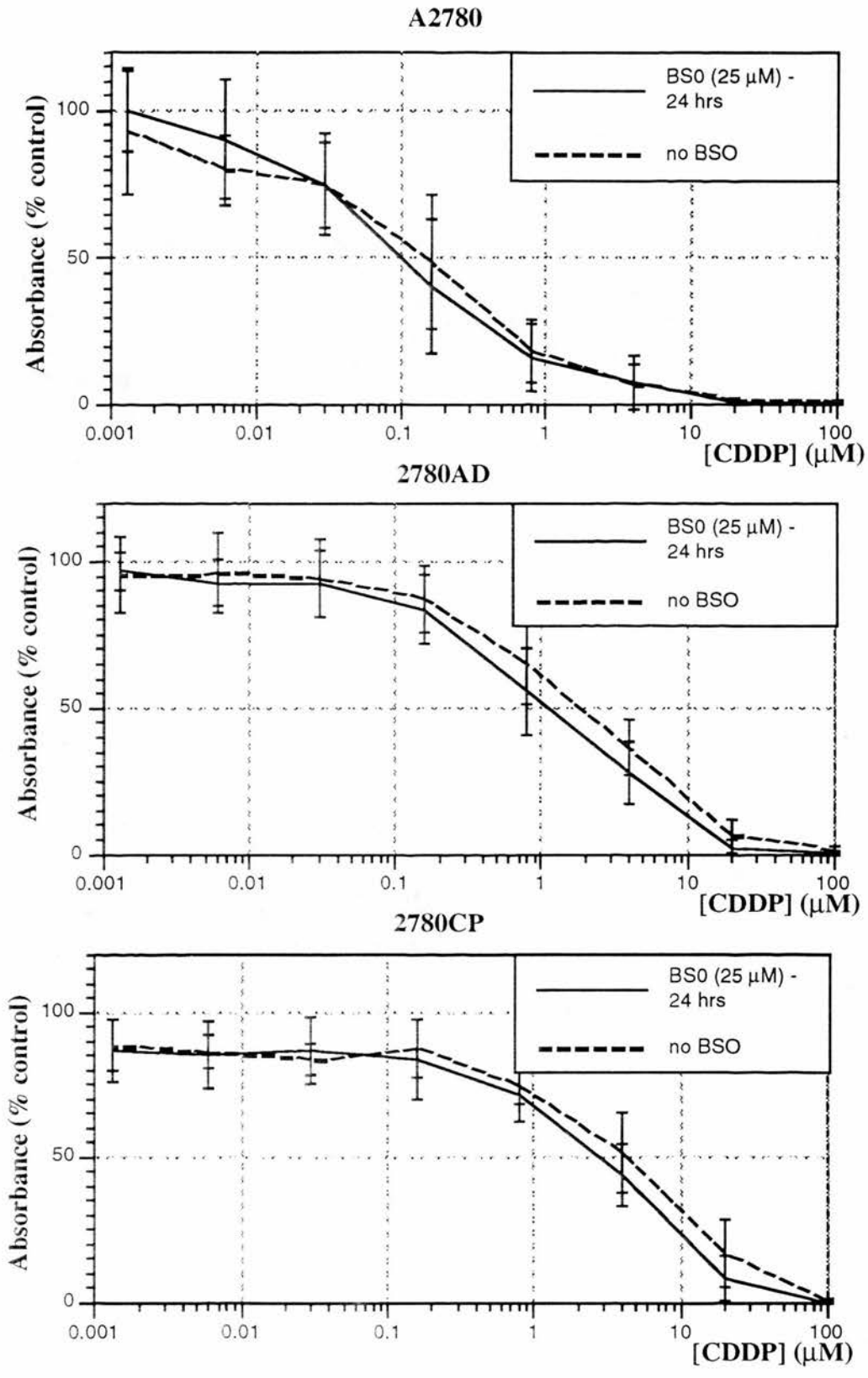


FIGURE 4: Cytotoxicity of CDDP (24 hours) towards ovarian cancer cells following incubation with d-L-BSO, 25 µM for 24 hours (no pre-incubation). Values given are the mean ± S.E. for three separate experiments.

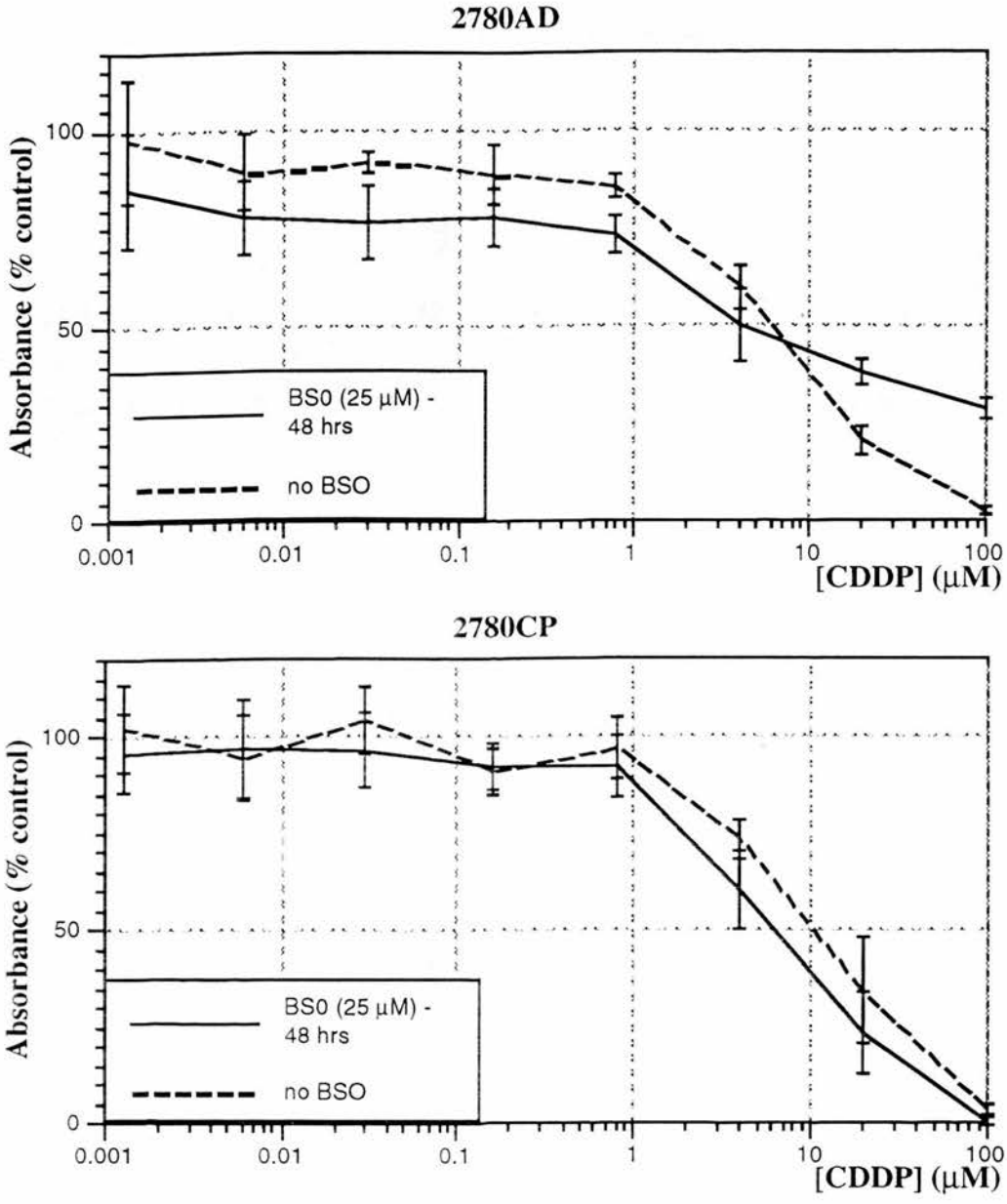


FIGURE 5: Cytotoxicity of CDDP (24 hours) towards ovarian cancer cells following incubation with d-L-BSO, 25 µM for 48 hours. Values given are the mean ± S.E. for three separate experiments.

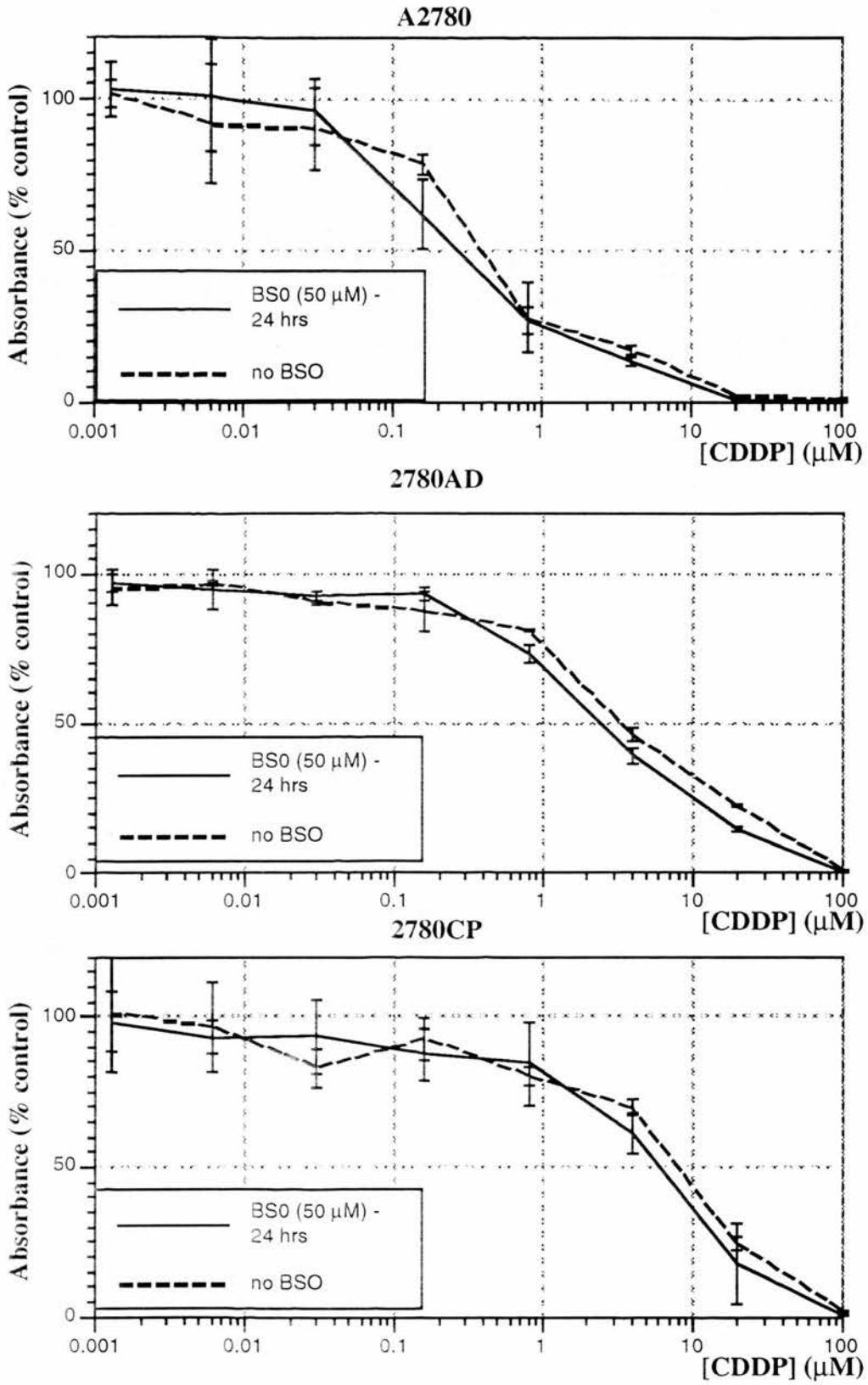


FIGURE 6: Cytotoxicity of CDDP (24 hours) towards ovarian cancer cells following incubation with d-L-BSO, 50 μM for 24 hours. Values given are the mean ± S.E. for three separate experiments.

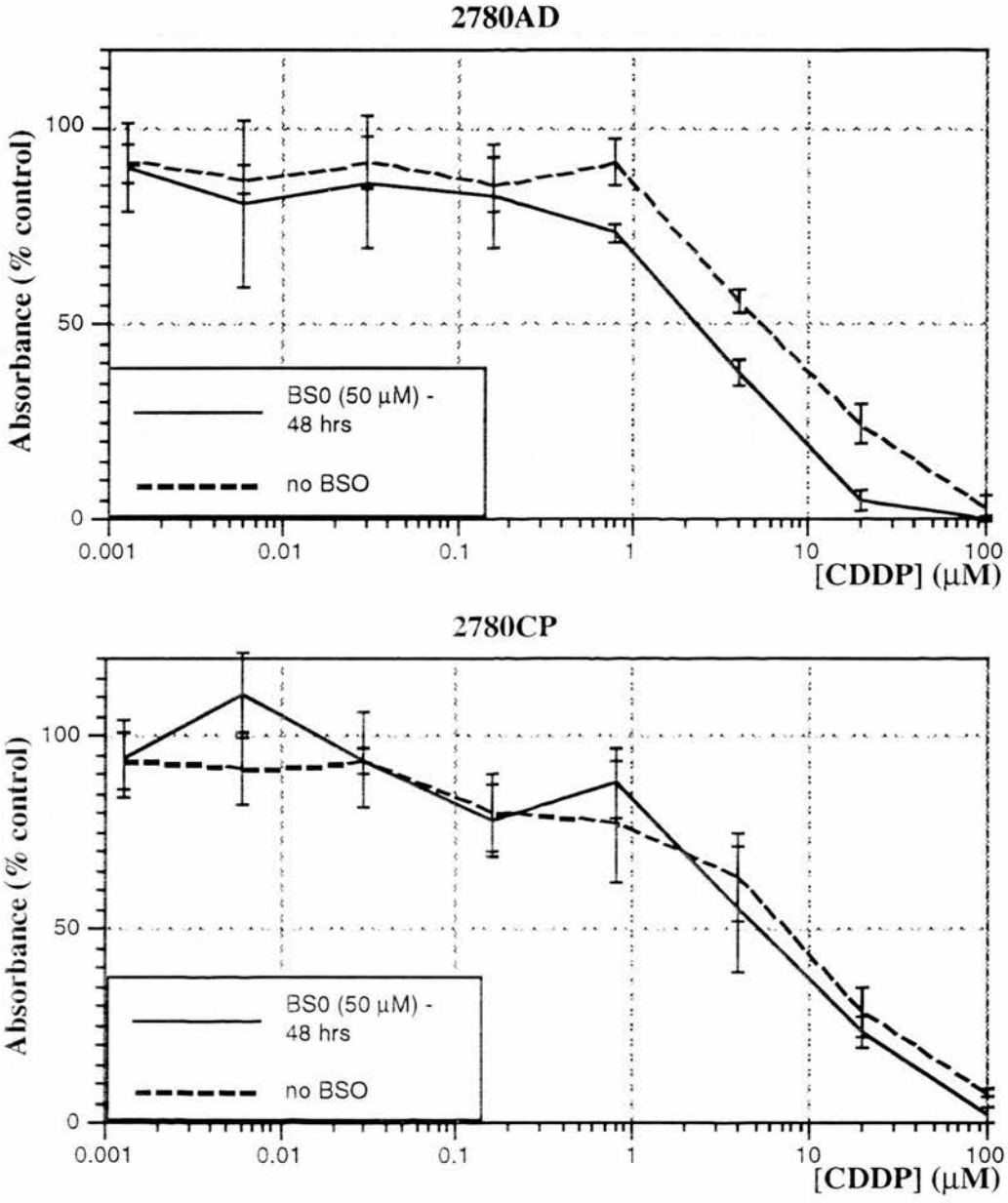


FIGURE 7: Cytotoxicity of CDDP (24 hours) towards ovarian cancer cells following incubation with d-L-BSO, 50 µM for 48 hours. Values given are the mean ± S.E. for three separate experiments.

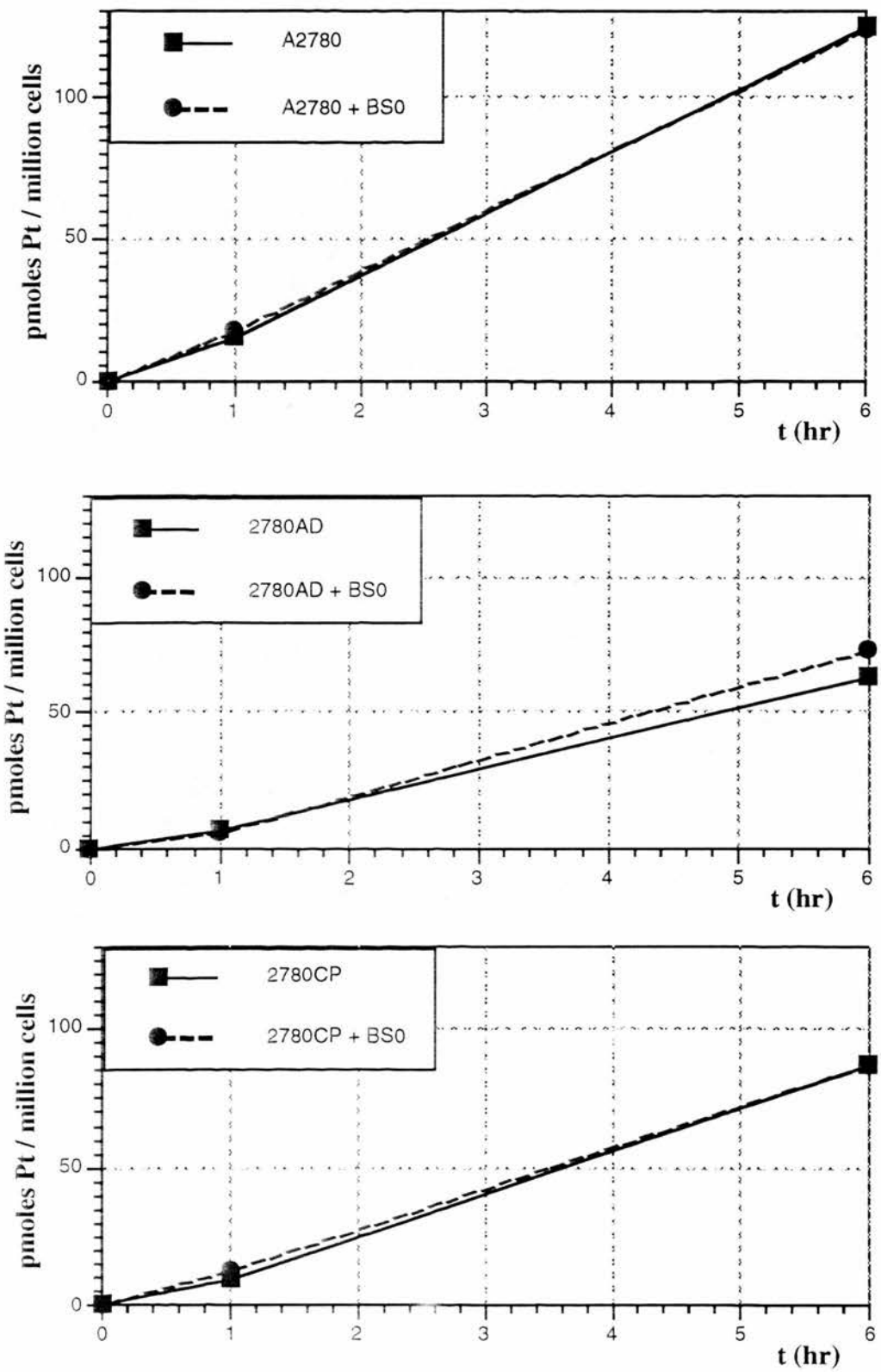


FIGURE 8: Total cellular platinum uptake in ovarian cancer cells treated with CDDP (2 μM) following incubation with d-L-BSO, 25 μM for 24 hours.

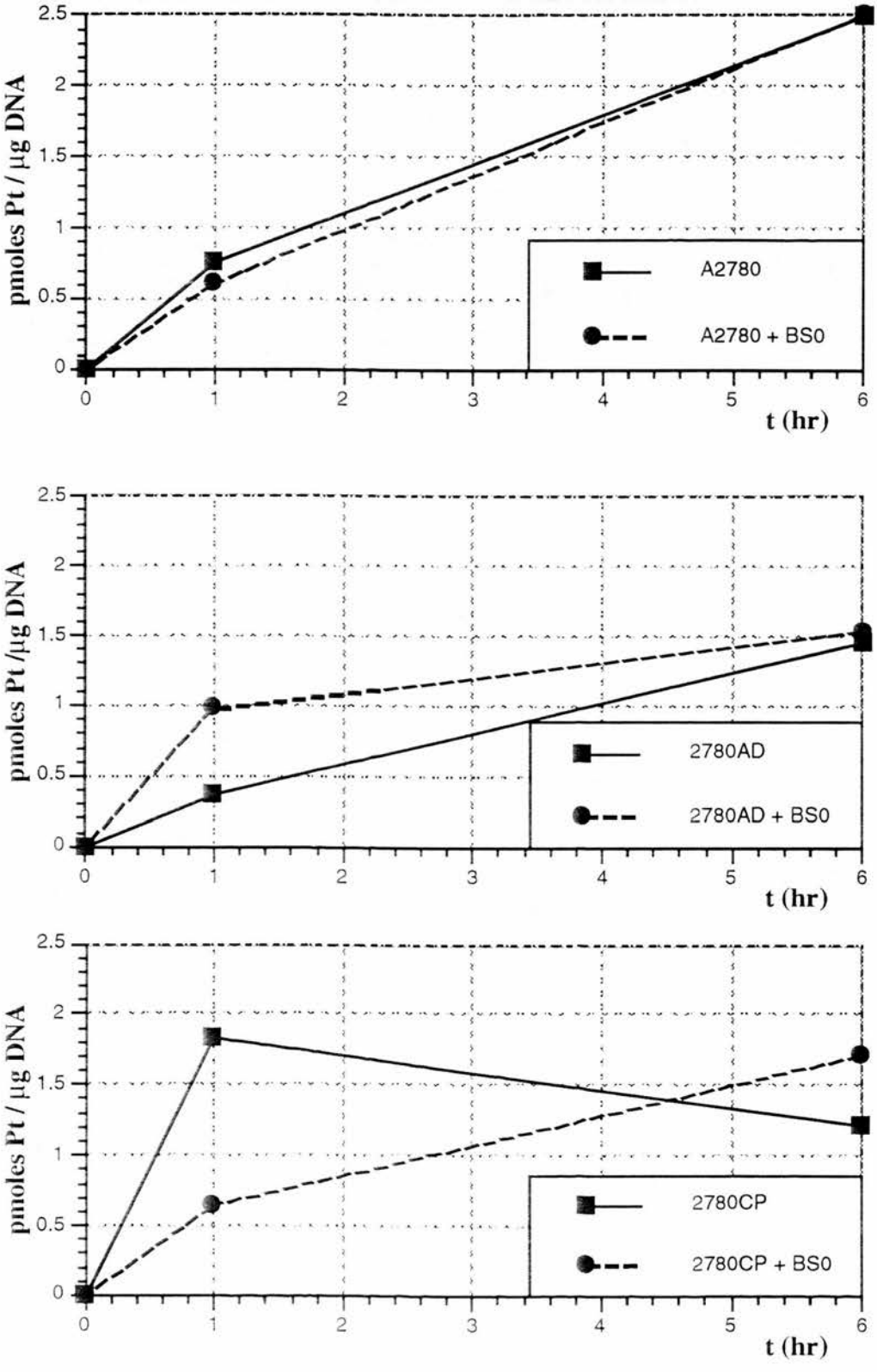


FIGURE 9: Platinum-DNA binding in ovarian cancer cells treated with CDDP (2 µM) following incubation with d-L-BSO, 25 µM for 24 hours.

DISCUSSION

These experiments aimed to investigate further the relationship between absolute cellular GSH levels in ovarian cancer cells, their manipulation with D,L-BSO, and their relative sensitivities to CDDP *in vitro*. The two resistant sublines, 2780AD and 2780CP, were believed to represent two differing mechanisms for CDDP resistance. For 2780CP, resistance to CDDP had been induced *in vitro* by stepwise increases in CDDP exposure, whereas for 2780AD, resistance had been induced primarily to adriamycin: this cell line was shown to display cross-resistance to CDDP (Chapter 2), which was thought to represent intrinsic CDDP-resistance, since this cell line had never previously been exposed to CDDP.

Total GSH levels were found to be directly related to CDDP resistance in the three ovarian cancer cell lines studied (Table 1); profound reductions in GSH levels (> 92 %) were achieved using different regimes for D,L-BSO administration (Tables 3 & 4). The cytotoxicity of D,L-BSO itself was studied; the most CDDP-resistant subline, 2780CP, was found to be resistant to D,L-BSO for any of the regimes used in this work. Cytotoxicity of this compound to A2780 and (to a lesser extent) 2780AD, however, after 48 hours incubation, was found to be sufficient to interfere with its use as a potential modulator for the cytotoxicity of CDDP over this time period (Table 2). This interesting finding, which may influence the apparent modulating activity of D,L-BSO has not previously been reported. When the modulating activity of D,L-BSO towards the cytotoxic action of CDDP was studied, a modest effect was observed for all cell lines. No advantage for this modulating effect was found by lowering GSH levels prior to the addition of CDDP (Tables 5 & 6).

Cellular platinum uptake, and DNA-Pt binding, were not significantly affected by incubation with D,L-BSO, at a dose sufficient to cause significant reduction in total GSH levels, prior to exposure to CDDP for up to 6 hours (Figures 8 & 9).

Total GSH levels for these three cell lines were found to be directly related to CDDP resistance. The most sensitive cell line, A2780, displayed the lowest level (63.19 ± 21.5 ng / 10^6 cells), the most resistant cell line, 2780CP, the highest level (304.8 ± 78.9 ng / 10^6 cells) and the cell line with intermediate resistance, 2780AD, an intermediate level (150.8 ± 10.7 ng / 10^6 cells) (Figure 2). This significant correlation (r= 0.93) reflects that found in another recent study using a wide panel of ovarian cancer cell lines (Mistry et al., 1991), but has generally only been noted elsewhere for resistant sublines selected by continuous exposure to CDDP. In contrast, the two resistant sublines used in this work, 2780AD and 2780CP, were produced by continuous exposure to adriamycin (2780AD) and by intermittent exposure to CDDP (2780CP). This implies a wider role for GSH in intrinsic CDDP resistance, since the induced resistance in these cell lines was not produced by continuous exposure to CDDP. GSH with its oxidised form GSSG forms a redox pair; GSSG levels for these experiments were consistently negligible. The high ratio of GSH to GSSG thus found reflects the general reductive status of the intracellular milieu (Russo et al., 1986) and may mean that absolute levels of GSH are not the most relevant measurements for its detoxification action, and hence its role in promoting drug resistance.

D,L-BSO, the specific inhibitor of GSH synthesis, has been used in animal studies to reduce tumour GSH levels and thereby promote sensitivity of tumour cells to cytotoxic agents such as melphalan (Ozols et al., 1987). This has raised the possibility of its clinical usefulness as a potential modulator for the cytotoxicity of CDDP in human ovarian cancer.

In this work, D,L-BSO was used, at concentrations of 25 μM and 50 μM, over 24 and 48 hours, to achieve reductions in total GSH levels of between 92 and 100% for all three cell lines. Prior to assessing a role for D,L-BSO in modulating the cytotoxicity of CDDP, the intrinsic cytotoxicity of this compound towards ovarian cancer cells was first investigated. For all three cell lines, after 24 hours exposure to D,L-BSO,

and for 2780CP after 48 hours exposure, no significant toxicity was observed. However for A2780 and 2780AD, after 48 hours exposure, ID_{50} values were such (46.3 ± 11.6 μM and 102.0 ± 29.0 μM respectively) that D,L-BSO could cause additional cytotoxicity when used over this time period in chemosensitivity assays with CDDP.

All four combinations of D,L-BSO dosage were assessed in a modified MTT chemosensitivity assay against CDDP, to evaluate any modulating effect caused by a reduction in total GSH levels on CDDP cytotoxicity. A modest modulation (modification factors 0.4 - 0.85) was seen for all cell lines, unrelated to their intrinsic CDDP sensitivity. This finding contrasts with other work (Hamilton et al., 1985; Ozols et al., 1988) which found an indirect association between modulating effect and baseline GSH level. Thus, in this work, despite a direct relationship between baseline cellular GSH levels and CDDP resistance, no association was noted between total GSH levels, their reduction by D,L-BSO, and CDDP sensitivity. Similar findings have been reported in human ovarian cancer cell lines (Andrews et al., 1985) and human squamous cancer cell lines (Teicher et al., 1987).

When intracellular GSH levels were reduced by 95%, prior to CDDP exposure, no consistent increase in modulating activity was found. It therefore did not seem to matter, in this experimental model, whether cellular GSH levels had already been reduced prior to CDDP administration, compared with the addition of CDDP and d-L-BSO concurrently. The maintenance of GSH depletion after drug treatment may, however, be more important (Andrews et al., 1988). The possibility that BSO may actively interfere with the action of CDDP, particularly in resistant cells, is indirectly supported by the association between increased CDDP-induced nephrotoxicity and administration of BSO (Mayer et al., 1987).

The effect of D,L-BSO on platinum uptake, both cellular and nuclear, over 6 hours, was assessed using ICP-MS as a highly accurate method for platinum analysis. Total cellular platinum uptake was found to be unaffected by the reduction in total GSH levels achieved, for all three cell lines (Figure 8). GSH levels, and their manipulation, do not therefore seem to influence platinum uptake across the cell membrane. For DNA-Pt binding a similar picture was found at 6 hours (Figure 9); however, at 1 hour, for 2780AD an increase, and for 2780CP a small decrease was observed. This may imply a consequent alteration in nuclear platinum uptake, caused by GSH reduction, for these two resistant sublines, but the effect is not very large and had disappeared by 6 hours exposure to CDDP. No significant effect on platinum transport across the nuclear membrane was thus found in the reduction of cellular GSH content by D,L-BSO. Unfortunately, analytical constraints with ICP-MS meant it was only possible to perform this experiment once, in duplicate. This finding, however, contrasts with a similar study on human small cell lung cancer cell lines with induced resistance to CDDP, in which an increase in both total DNA-Pt binding and levels of the major intrastrand Pt-DNA adduct, Pt-GG, was noted following GSH depletion in the resistant subline (Meijer et al., 1990).

Despite a clear association between total cellular GSH levels and CDDP resistance, the findings described here do not appear to support a major role for intracellular GSH as a mechanism for CDDP resistance in this experimental model. Other models, particularly animal studies, may, however, produce different results. The analysis of more dynamic aspects of GSH metabolism, such as the enzymes glutathione reductase, glutathione peroxidase or the various iso-enzymes of glutathione-S-transferase, could ultimately be more informative. Recent studies on newly-developed platinum (IV) drugs, such as iproplatin and tetraplatin, suggest that GSH may be more relevant in the development of platinum resistance in these compounds than in platinum (II) agents such as CDDP (Mistry et al., 1991).

CHAPTER 7

GENERAL DISCUSSION

The work described in this thesis aimed to explore further the nature of platinum resistance in ovarian cancer. Improved understanding of the cytotoxic action of CDDP (and analogues), and the mechanisms underlying platinum resistance, may ultimately help in the circumvention of this major clinical problem. Many different mechanisms have been suggested to explain the development of platinum resistance, including differential platinum accumulation, inactivation of CDDP by the nucleophilic non-protein thiol compound reduced glutathione (GSH), and differences in the formation and / or repair of Pt-DNA adducts. In this work, Inductively Coupled Plasma Mass Spectrometry (ICP-MS), a novel and highly sensitive analytical technique, was used in the measurement of intracellular and DNA-platinum levels in ovarian cancer cells. an innovative new assay for the direct analysis of Pt-DNA lesions was developed, and applied to a model for CDDP resistance in ovarian cancer cells *in vitro*. The same technique for platinum analysis was also used to investigate the relationship between platinum accumulation, both intracellular and intranuclear, and CDDP resistance in this experimental model. Finally, the role of intracellular inactivation of CDDP by GSH was studied using buthionine-S-R-sulfoxamine (D,L-BSO), a specific inhibitor of GSH synthesis, and a potential clinical modulator for CDDP resistance *in vivo*. The results of this work provided further confirmation of the multifactorial nature of CDDP resistance.

Intrastrand Pt-DNA adducts, the putative cytotoxic lesions after CDDP treatment, are only formed in biological systems at extremely low levels (10^{-15} to 10^{-18} M). Until

now the only techniques sufficiently sensitive for their analysis were indirect ELISA assays using polyclonal antibodies to stereochemically predicted Pt-DNA adducts. Such assays have been used to assess the relationship between adduct formation and repair in a variety of experimental models both *in vitro* and *in vivo*. In general, formation and / or repair capacity of the two major intrastrand adducts, Pt-AG and Pt-GG, have been found to be related to CDDP resistance in testicular cancer cell lines (Bedford et al., 1988), ovarian cancer cell lines (Terheggen et al., 1990), and small cell lung adenocarcinoma cell lines (Hospers et al., 1990). No difference was found in the induction of these adducts, or the kinetics of their excision in a resistance pair of human colonic cancer cell lines (Fram et al,. 1990).

The same indirect immunochemical assay has also been used to compare levels of the major intrastrand adducts formed in peripheral cells of patients receiving CDDP therapy with clinical responsiveness. A prospective study comparing Pt-GG and Pt-AG adduct levels with treatment response was performed in 55 ovarian cancer patients receiving platinum-based chemotherapy (Reed et al., 1987). A significant correlation was noted, particularly in those patients receiving single-agent platinum therapy. Polyclonal antisera were also used to study the formation of four intrastrand Pt-DNA adducts, Pt-GG, Pt-AG, G-Pt-G and Pt-GMP, in digested DNA from peripheral leucocytes of a small number of cancer patients receiving their first dose of CDDP (Fichtinger-Schepman et al., 1987a). This confirmed that these same four adducts are formed in human cells following platinum therapy, and that their relative proportions were similar to those found *in vitro*. A clear interindividual variation was noted for the induction of the major adduct, Pt-GG, thought to be due to differences in repair capacity for damaged DNA. This hypothesis was investigated further by a comparison of adduct formation in cultured peripheral blood leucocytes *in vitro* with adduct formation *in vivo* during subsequent CDDP therapy (Fichtinger-Schepman et al., 1987b). A good correlation (r= 0.91) was found for Pt-GG, providing some

prospect for the development of a predictive assay *in vitro* for individual clinical responsiveness to CDDP treatment.

The large discrepancies which have been noted between values obtained for Pt-DNA adduct levels by the two different quantitative immunochemical techniques used at present (Fichtinger-Schepman et al., 1989) provided an incentive for developing a more direct method for adduct analysis, by ICP-MS. This method, with its potential for use in biological systems, would also allow scrutiny for previously unrecognised Pt-DNA lesions of potential therapeutic significance. Ultimately, a predictive assay for CDDP responsiveness in ovarian cancer specimens could be feasible.

No concensus of opinion exists on the significance of differential platinum accumulation in clinical CDDP resistance. Reduced accumulation has been found in some CDDP-resistant cell lines (Andrews et al., 1988; Kuppen et al., 1988; Shionoya et al., 1986; Foka et al., 1988; Kraker and Moore, 1988) but not in others (Sekiya et al., 1989; Shellard et al., 1991). Increased drug efflux, through the P-glycoprotein transmembrane pump, has been largely excluded as an important mechanism for CDDP resistance in ovarian cancer (Goldstein et al., 1989).

Similarly, evidence for a link between intracellular GSH levels and CDDP resistance in ovarian cancer is conflicting. Whilst, in general, GSH levels are frequently elevated in CDDP-resistant cell lines, this may be due to a general stress reaction following treatment with CDDP. The method of selection for drug resistance also seems relevant, with continuous exposure to CDDP apparently important. The use of BSO to deplete intracellular GSH levels in an *in vivo* model, and thereby improve clinical response to melphalan (Ozols et al., 1987), introduced the possibility for the development of this compound as a clinical modulator for CDDP resistance in humans.

The work described in this thesis used three related ovarian cancer continuous cell lines with a wide range of resistance to CDDP *in vitro*. The two resistant sublines used displayed theoretically different mechanisms for the development of CDDP resistance. 2780AD had been initially developed from A2780, a drug-sensitive cell line, as a model for induced adriamycin resistance. It was subsequently found to be moderately cross-resistant to CDDP and was thought to represent an example of intrinsic platinum resistance, since it had never been previously exposed to CDDP. 2780CP had been developed from A2780 primarily as a model for a high degree of induced CDDP resistance. The further characterisation of these three cell lines is described in Chapter 2, confirming their typical epithelial cell characteristics. 2780AD was found to be moderately resistant to CDDP, with a resistance ratio of 14.2 after 24 hours treatment compared with A2780. 2780CP was highly resistant to CDDP, with a resistance ratio of 26.8 after 24 hours CDDP treatment. Resistance ratios after only 4 hours CDDP treatment were smaller. Immunostaining for P-glycoprotein, performed on all three cell lines, was only found for 2780AD. This expression was lost after 6 months culture in drug-free medium, despite maintenance of CDDP resistance over this time. These findings further support the lack of involvement of this transmembrane multidrug efflux pump in CDDP resistance.

The availability of ICP-MS, as an extremely sensitive elemental analytical tool, combined with the lack of commercial availability of the polyclonal antisera to Pt-DNA adducts used in the indirect immunochemical techniques, led to the investigation of the potential for this method in the direct detection of Pt-DNA adducts (Chapter 3). Initially highly platinated commercial DNA was used to verify the sensitivity of ICP-MS in the detection of the two major intrastrand adducts, Pt-AG and Pt-GG, at relatively high concentrations. Calibration of the anion-exchange chromatography column used in their separation was performed with purified samples of these adducts, kindly donated by Dr Fichtinger-Schepman. Excellent agreement

was obtained between ICP-MS and the more conventional AAS in platinum analysis of elution fractions, confirming the sensitivity of ICP-MS for adduct detection at μM levels.

Extrapolation of this new assay to adduct measurement in biological systems was the next logical step. The lower level of DNA modification achieved following treatment of cells with CDDP at therapeutically relevant concentrations required a method for direct analysis of Pt-DNA adducts to be at least 3 orders of magnitude more sensitive than standard ICP-MS. In Chapter 4 the incorporation of electrothermal vaporisation (ETV) as a method for sample introduction is described. Theoretically, the combination of smaller sample volume and increased ionisation efficiency provided by this refinement should have provided sufficient sensitivity for the analysis of these adducts in the experimental model for ovarian cancer described in Chapter 2. In practice, adduct detection was at the extreme lowest levels of sensitivity for the apparatus, and results described are thus subject to wide interexperimental variation. Nevertheless, it was possible to identify Pt-DNA lesions eluting from the separation column at times corresponding to those for Pt-AG and Pt-GG in all three ovarian cancer cell lines. Trends in induction of adducts, and the kinetics of their formation and recovery, were also evident. In addition, a platinum-containing peak, eluting between Pt-AG and Pt-GG was noted. This peak was most obvious for the sensitive parental cell line, A2780, and was less evident in the two resistant sublines. It may constitute an additional, previously unrecognised, Pt-DNA lesion; it was not, however, possible to isolate and identify this product following ICP-MS-ETV analysis.

In general, the level of DNA-platination found for these cell lines was directly related to CDDP sensitivity, with highest levels found for A2780 and lowest levels for 2780CP. For A2780, following a 24 hour recovery period in drug-free medium, Pt-AG levels were higher and Pt-GG levels lower than immediately after CDDP

exposure. For 2780AD and 2780CP, the converse was true. These findings could all be explained by a lack of repair capacity for Pt-AG, but not Pt-GG for A2780, with the reverse being true for 2780AD and 2780CP. A2780 could thus be regarded as having increased sensitivity to CDDP as a result of its inability to repair the Pt-AG adduct. Alternatively, resistance in 2780AD and 2780CP could correlate with ability to repair Pt-AG, but not Pt-GG. Tolerance, of the Pt-GG adduct in particular, seemed to confer resistance in the two CDDP-resistant sublines. These findings have recently been corroborated in another CDDP-resistant human ovarian cancer cell line, using one of the indirect immunochemical assays for adduct analysis (Shellard et al., 1991).

These results show some promise for ICP-MS-ETV as a potentially useful method for the direct detection and measurement of intrastrand Pt-DNA adducts, despite obvious initial technical difficulties. Much of the experimental variability can probably be explained by matrix suppression effects, despite efforts to reduce solute concentration prior to ICP-MS-ETV analysis. Isotope dilution can be used as a technique to circumvent this problem: for this, a known amount of a pure, stable isotope of the element to be analysed is added to part of the sample. A more precise measurement can thus be obtained by comparing the isotope ratio of treated and untreated samples, taking into consideration the amount of pure isotope added. For platinum, pure isotope samples are scarce and very expensive at present; hence it was not possible to use this modification in this work.

In Chapter 5 ICP-MS was used to investigate the contribution of differential platinum accumulation in this model for CDDP resistance in ovarian cancer. Both total cellular platinum uptake and Pt-DNA binding increased in a linear fashion with time, with accumulation occurring faster in A2780. When compared with A2780, CDDP accumulation for both resistant sublines was reduced by similar amounts, 49.6 - 50.7% (intracellular) and 16.7 - 20.5% (DNA-platinum), despite the twofold difference in CDDP sensitivity between these two sublines. When the rate of

intracellular platinum accumulation was compared with CDDP concentration over a fixed time period of 4 hours, a wider difference emerged between the two resistant sublines: 80.1% for 2780CP and 33.5% for 2780AD. Thus it appeared that, for 2780CP in particular, there was an increased tolerance to platinum accumulation. When cytotoxicity of CDDP, after 4 hours exposure, was compared with intracellular accumulation over this time period, once again the resistant sublines appeared able to tolerate higher platinum levels. At 50% cell kill, 2780AD had accumulated 3-fold more platinum (resistance ratio after 4 hours 16.5), and 2780CP 12.5-fold (resistance ratio 32.6), indicating perhaps a greater contribution for an increased tolerance to intracellular platinum for 2780CP, as a resistance mechanism. Energy deprivation within the cell, using sodium azide, had a mild reducing effect on intracellular platinum accumulation for 2780AD only, which may indicate the residual contribution of an energy-requiring transport mechanism for this subline.

The role of GSH in mediating CDDP resistance is at present unclear; it has been suggested that increased GSH levels are found only in resistant sublines following their selection by continuous exposure to CDDP. This work allows the comparison of two different resistant sublines, only one of which (2780CP) was produced in this way. Chapter 6 gives values for intracellular GSH content for all three ovarian cancer cell lines. Levels are raised for both resistant sublines: 2.4-fold for 2780AD and 4.8 fold for 2780CP, neatly reflecting the twofold difference in CDDP sensitivity noted between these two sublines. Thus GSH may still be relevant to CDDP resistance even when selection has not been by continuous exposure *in vitro*. However, elevated GSH levels may merely reflect a general stress response to CDDP treatment, so it was important to assess the effect of intracellular GSH depletion on CDDP response. D,L-BSO, the specific inhibitor of GSH synthesis used in these experiments, has recently entered clinical trials as a potential modulator for CDDP resistance. Using D,L-BSO it was possible to deplete intracellular GSH levels by over 90% with no significant

cytotoxic effect on the ovarian cancer cells. In this work, depletion of GSH had only minimal modulating effect on the cytotoxicity of CDDP towards these cells *in vitro* ; however, this may still be sufficient to exert some clinical effect on disease response to CDDP. No effect on intracellular platinum uptake was noted in any of the cell lines when GSH levels were depleted with D,L-BSO, thus apparently excluding any role for GSH in platinum transport.

There is some evidence that GSH depletion is associated with inhibition of DNA repair, either through inhibition of DNA polymerase α or more directly, since it is essential for the synthesis of DNA precursors (Lai et al., 1989). DNA polymerase α is an important enzyme in DNA excision repair, and may thus participate in the repair of platinated cellular DNA following CDDP therapy. This enzyme can be competitively inhibited by aphidicolin glycinate (APG), an antibiotic compound currently undergoing early clinical trials as a potential modulator for clinical CDDP resistance. In theory, therefore, resistant tumour cells which have become proficient in the repair of platinum-DNA lesions could become more sensitive to CDDP in the presence of APG. However, a recent study in two repair-proficient ovarian carcinoma cell lines failed to show any evidence for increased repair of the major identified intrastrand Pt-DNA adducts. Cytotoxicity of CDDP did not seem altered, either (Dempke W.C.M. et al., 1991). Further investigation of the activity of this potential modulator in the experimental model described in this thesis would be interesting.

In summary, this work describes the use of three continuous ovarian cancer cell lines to provide a model for intrinsic and induced CDDP resistance. The majority of published work on CDDP resistance has used pairs of CDDP-sensitive and -resistant cell lines, and is therefore unable to test the consistency of proposed resistance mechanisms across a panel of related cell lines such as those described herein. A novel new technique using ICP-MS for the direct analysis of intrastrand Pt-DNA adducts was devised and extended to the model for CDDP resistance in ovarian

cancer cells described. Despite technical limitations, not insurmountable, a tendency for higher adduct levels in the sensitive cell line, compared with its resistant sublines was noted. CDDP resistance, in the two sublines, could be explained either by an increased capacity for repair of the Pt-AG adduct or by increased tolerance of the Pt-GG adduct.

The use of ICP-MS for platinum analysis confirmed the association, noted by other groups, between reduced platinum accumulation and resistance to CDDP, at both intracellular and intranuclear levels. GSH levels were also found to be directly associated with phenotypic CDDP resistance, although their depletion with D,L-BSO had no more than minimal modulating effect on CDDP resistance assayed *in vitro*.

Platinum analysis in biological samples by ICP-MS represents some improvement over AAS particularly with respect to its improved sensitivity and wider dynamic range. Residual technical difficulties, such as its expense and the length of time taken to analyse and process samples, remain to be overcome if the technique is to gain wider credibility as a practical method for platinum analysis in biological systems. Advances in apparatus design, improvements in sample preparation and thereby a reduction in matrix effect, should help to reduce the interexperimental variability found in this work.

REFERENCES

Advanced Ovarian Trialists Group. Chemotherapy in advanced ovarian cancer: An overview of randomised clinical trials. *Br. Med. J.*, **303**, 884 - 893.

Ajani J.A., Baker F.L., Spitzer G. & 6 others. (1987). Comparison between clinical response and *in vitro* drug sensitivity of primary human tumors in the adhesive tumor cell culture system. *J. Clin. Oncol.*, **5**: 1912-1921.

Andolf E., Svalenius E. & Astedt B. (1986). Ultrasonography for early detection of early ovarian carcinoma. *Br. J. Obstet. Gynaecol.*, **93**: 1286 - 1289.

Andrews P.A., Mann S.C., Huynh H.H. & Albright K.D. (1991). Role of Na^+, K^+-adenosine triphosphatase in the accumulation of *cis*-Diamminedichloroplatinum(II) in human ovarian carcinoma cells. *Cancer Res.*, **51**: 3677 - 3681.

Andrews P.A. & Howell S.B. (1990). Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. *Cancer Cells*, **2**: 35-43.

Andrews P.A., Murphy M.P. & Howell S.B. (1985). Differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion. *Cancer Res.*, **45**: 6250 - 6253.

Andrews P.A., Murphy M.P. & Howell S.B. (1989). Characterization of cisplatin-resistant COLO 316 human ovarian carcinoma cells. *Eur. J. Cancer Clin. Oncol.*, **25**: 619 - 625.

Andrews P.A., Velury S., Mann S.C. & Howell S.B. (1988). *cis*-Diamminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian cancer cells. *Cancer Res.*, **48**: 68 - 73.

Baak J.P.A., Schipper N.W., Wisse-Brekelmans E.C.M. & 4 others. (1988). The prognostic value of morphimetric features and cellular DNA content in cisplatin treated late ovarian cancer patients. *Br. J. Cancer*, **57**: 503 - 508.

Balconi G., Broggini M., Erba E. & 4 others. (1988). Human ovarian tumors in primary culture: growth, characterization and initial evaluation of the response to cisplatinum treatment in vitro. *Int. J. Cancer*, **41**: 809-818.

Balkwill F.R., Malik S.T.A., & Griffin G.B. (1990). Animal models of ovarian cancer. In: Ovarian cancer - biologic and therapeutic challenges. (ed F.Sharp, W.P. Mason & R.E. Leake), pp 37-44. Chapman & Hall, London.

Barber H.R.K. (1989).In: Manual of Gynecologic Oncology (2nd ed). p 248. Lippincott & Co, Philadelphia.

Bedford P., Fichtinger-Schepman A-M.J., Shellard S.A. & 2 others. (1988). Differential repair of platinum-DNA adducts in human bladder and testicular tumor continuous cell lines. *Cancer Res.*, **48**: 3019 - 3024.

Bedford P., Walker M.C., Sharma H.L. & 4 others. (1987). Factors influencing the sensitivity of two human bladder carcinoma cell lines to *cis*-Diamminedichloroplatinum (II). *Chem.-Biol. Interact.*, **61**: 1 - 15.

Bell D.R., Gerlach J.H., Kartner N. & 2 others. (1985). Detection of P-glycoprotein in ovarian cancer: a molecular marker associated with drug resistance. *J. Clin. Oncol.*, **3**: 311 - 314.

Bourhis J., Goldstein L.J., Riou G. & 3 others. (1989). Expression of a human multidrug resistance gene in ovarian carcinomas. *Cancer Res.*, **49**: 5062 - 5065.

Briers T.W., Stroobants P., Vandeputte T.M. & 5 others. (1989). Establishment and characterisation of a human ovarian neoplastic cell line, DO-5. *Cancer Res.*, **49**: 5153-5161.

Britten R.A., Green J.A. & Warenius H.M. (1989). Cellular glutathione (GSH) and glutathione-S-transferase (GST) activity in human ovarian tumor-biopsies following exposure to alkylating agents. *Int. J. Rad. Oncol. Biol. Phys.*, **24**: 527 - 531.

Byfield J.E. & Calabro-Jones P. (1982) Further evidence for carrier-mediated cell uptake of CDDP. *Proc AACR*, **23**,167.

Cannistra S.A. (1993) Cancer of the ovary. *N. Engl. J. Med.*, **329**, 1550 - 1559.

Cohen G.L., Bauer W.R., Barton J.K. & Lippard S.J. (1979). Binding of *cis*- and *trans*-Dichlorodiammineplatinum(II) to DNA: evidence for unwinding and shortening of the double helix. *Science*, **203**: 1014 - 1016.

Courtenay V.D., Selby P.J., Smith I.E. & 2 others. (1988). Growth of human tumour cell colonies from biopsies using two soft-agar techniques. *Br. J. Cancer*, **38**: 77-81.

de Graeff A., Slebos R.J.C. & Rodenhuis S. (1988). Resistance to cisplatin and analogues : mechanisms and potential clinical implications. *Cancer Chemother. Pharmacol.*, **22**: 325 - 332.

Dempke W.C.M., Shellard S.A., Fichtinger-Schepman A-M.J. & Hill B.T. (1991). Lack of significant modulation of the formation and removal of platinum-DNA adducts by aphidicolin glycinate in two logarithmically-growing ovarian tumour cell lines in vitro. *Carcinogenesis*, **12**: 525 - 528.

Dominici C., Alimonti A., Caroli S. & 2 others. (1986). Chemotherapeutic agent CDDP monitoring in biological fluids by means of ICP-AES. *Clin. Chim. Acta*, **158**: 207 - 215.

Donahue B.A., Augot M., Bellon S.F., & 4 others. (1990). Characterization of a DNA damage-recognition protein from mammalian cells that binds specifically to intrastrand d(GpG) and d(ApG) DNA adducts of the anticancer drug cisplatin. *Biochemistry*, **29**: 5872 - 5880.

Dornish J.M., Melvik J.E. & Pettersen E.O. (1986). Reduced cellular uptake of *cis*-Dichlorodiammineplatinum by benzaldehyde. *Anticancer Res.*, **6**: 583 - 588.

Eastman A. (1983). Characterization of the adducts produced in DNA by *cis*-diamminedichloroplatinum(II) and *cis*-dichloro(ethylenediamine)platinum(II). *Biochemistry*, **22**: 3927 - 3933.

Eastman A. (1987). The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol. Ther.*, **34**: 155 - 166.

Eicholtz- Wirth H. & Hietel B. (1986). The relationship between cisplatin sensitivity and drug uptake into mammalian cells *in vitro*. *Br. J. Cancer*, **54**: 239 - 243.

Eva A., Robbins K.C., Anderson P.R. & 11 others. (1982). Cellular genes analogous to retroviral onc gene are transcribed in human tumor cells. *Nature*, **295**: 116 - 119.

Fichtinger-Schepman A-M.J., Baan R.A. & Berends F. (1989). Influence of the degree of DNA modification on the immunochemical determination of cisplatin-DNA adduct levels. *Carcinogenesis*, **10**: 2367 - 2369.

Fichtinger-Schepman A-M.J., Baan R.A., Luiten-Schuite A. & 2 others. (1985a). Immunochemical quantitation of adducts induced in DNA by CDDP and analysis of adduct-related DNA unwinding. *Chem.-Biol. Interact.*, **55**: 275 - 288.

Fichtinger-Schepman A-M.J., Lohman P.H.M. & Reedijk J. (1982). Detection and quantification of adducts formed upon interaction of diamminedichloroplatinum (II) with DNA, by anion-exchange chromatography after enzymatic degradation. *Nucl. Acids Res.*, **10**: 5345 - 5356.

Fichtinger-Schepman A-M.J., van der Veer J.L., den Hartog J.H.J. & 2 others. (1985b). Adducts of the anti-tumour drug *cis*-Diamminedichloroplatinum (II) with DNA: formation, identification and quantification. *Biochemistry*, **24**: 707 - 713.

Fichtinger-Schepman A-M.J., van der Veer J.L., Lohman P.H.M. & Reedijk J. (1984). A simple method for the inactivation of monofunctionally DNA-bound CDDP. *J. Inorg. Biochem.*, **21**: 103 - 112.

Fichtinger-Schepman A-M.J., van Oosterom A.T., Lohman P.H.M. & Berends F. (1987a). CDDP-induced DNA adducts in peripheral leucocytes from seven cancer patients: quantitative immunochemical detection of the adduct induction and removal after a single dose of CDDP. *Cancer Res.*, **47**: 3000 - 3004.

Fichtinger-Schepman A-M.J., van Oosterom A.T., Lohman P.H.M. & Berends F. (1987b). Interindividual human variation in cisplatinum sensitivity, predictable in an in vitro assay? *Mutat. Res.*, **190**: 59 - 62.

Fichtinger-Schepman A-M.J., Vendrik C.P.J., van Dijk-Knijnenburg W.C.M. & 9 others. (1989). Platinum concentrations and DNA adduct levels in tumors and organs of cisplatin-treated LOU/M rats inoculated with cisplatin-sensitive or -resistant Immunoglobulin M immunocytoma. *Cancer Res.*, **49**: 2862 - 2867.

Foka M., Belehradek J. & Paoletti J. (1988). Interaction of *cis*-Diamminedichloroplatinum(II) with sensitive and resistant cell lines. Drug binding to nuclei and DNA. *Biochem. Pharmacol.*, **37**: 3467 - 3472.

Fox H. (1985). Pathology of ovarian carcinoma. In: Clinical Gynaecological Oncology, ed. J.H. Shepherd & J.M. Monaghan. pp. 167 - 186. Blackwell, Oxford.

Fram R.J., Woda B.A., Wilson J.M. & Robichaud N. (1990). Characterization of acquired resistance to *cis*-Diamminedichloroplatinum(II) in BE human colon carcinoma cells. *Cancer Res.*, **50**: 72 - 77.

Freeman M.L., Meredith M.J. & Eisert D.R. (1990). Failure of chronic glutathione elevation to reduce cytotoxicity produced by exposure to *cis*-Diamminedichloroplatinum(II), ionizing radiation, or hyperthermia. *Cancer Res.*, **50**: 5296 - 5300.

Freshney R.I., & Dendy P.P. (1983). Culture methods for assays of intermediate duration. In: Human Tumour Drug Sensitivity Testing *In Vitro* . (ed P.P. Dendy & B.T. Hill), pp 69 - 89. Academic, London.

Gale G.R., Morris C.R. Atkins L.M. & Smith A.B. (1973). Binding of an antitumour platinum compound to cells as influenced by physical factors and pharmacologically active agents. *Cancer Res.*, **33**: 813 - 818.

Goldstein L.J., Galski H., Fojo A., & 11 others. (1989). Expression of a multidrug resistance gene in human cancers. *J. Natl. Cancer Inst.*, **81**: 116 - 124.

Green J.A., Vistica D.T., Young R.C. & 3 others. (1984). Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. *Cancer Res.*, **44**: 5427 -

Gregoire D.C. (1988). Determination of platinum, palladium, ruthenium and iridium geological materials by Inductively Coupled Plasma Mass Spectrometry with sample introduction by Electrothermal Vaporisation. *J. Anal. Atom. Spectrom.*, **3**: 309 - 314.

Griffiths C.T. (1975). *Surgical Resection of Tumor Bulk in the Primary Treatment of Ovarian Carcinoma.* National Cancer Institute Monographs, **42**, pp 101 - 104.

Hacker N.F. (1989). Controversial aspects of cytoreductive surgery in epithelial ovarian cancer. In *Bailliere's Clin. Obstet. Gynecol.*, **3**, 49 - 57.

Hacker N.F. & van der Burg M.E.L. (1993). Debulking and intervention surgery. *Ann. Oncol.*, **4**, S41 - S48.

Hamburger A.W. & Salmon S.E. (1977). Primary bioassay of human tumor stem cells. *Science*, **197**:, 461 - 463.

Hamilton T.C., Lai G., Rothenburg M. & 3 others. (1989). Mechanisms of resistance to cisplatin and alkylating agents. In: Drug resistance in cancer therapy (ed. R.F.Ozols), pp 151 - 169. Kluwer, Boston.

Hamilton T.C., Winker M.A., Louie K.G. & 7 others. (1985). Augmentation of adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem. Pharmacol.*, **34**: 2583 - 2586.

Harder H.C., Smith R.G. & Leroy A.F. (1976). Template primer inactivation by *cis*- and *trans*-Dichlorodiammineplatinum for human DNA polymerase α, β and Raucher murine leukaemia virus reverse transcriptase, as a mechanism of cytotoxicity. *Cancer Res.*, **36**: 3821 - 3829.

Hill B.T. (1983). Use of continuous human tumour cell lines to evaluate drugs by clonogenic assays. In: Human Tumour Drug Sensitivity Testing In Vitro (ed. P.P. Dendy & B.T. Hill), pp 129 - 146. Academic, London.

Hospers G.A.P., de Vries E.G.E. & Mulder N.H. (1990). The formation and removal of cisplatin (CDDP) induced DNA adducts in a CDDP sensitive and resistant human small cell lung carcinoma (HSCLC) cell line. *Br. J. Cancer,* **61**: 79 - 82.

Houk R.S. (1986). Mass spectrometry of inductively coupled plasmas. *Analyt. Chem.*, **58**: 97 - 105.

Houk R.S. & Thompson (1988). Inductively coupled plasma mass spectrometry. *Mass Spectrom. Rev.*, **7**: 425 - 461.

Hromas R.A., Andrews P.A., Murphy M.P. & Burns C.P. (1987). Glutathione depletion reverses cisplatin resistance in murine L1210 leukaemia cells. *Cancer Lett.*, **34**: 9 - 13.

Hulmston P. & Hutton R.C. (1991). Analytical capabilities of Electrothermal Vaporization-Inductively Coupled Plasma-Mass Spectrometry. *Spectroscopy International*, **6**: 35 - 38.

Kaye S.B., Lewis C.R., Paul J. & 16 others. (1992). Randomised study of two doses of cisplatin with cyclophosphamide in epithelial ovarian cancer. *Lancet* , **340**: 329 - 333.

Kelley S.L. & Rosencweig M. (1989).Resistance to platinum compounds: mechanisms and beyond. *Eur. J. Cancer Clin. Oncol.*, **25**: 1135 - 1140.

Kuppen P.J.K., Schuitemaker H., van't Veer L.J. & 3 others. (1988). *cis*-Diamminedichloroplatinum(II)-resistant sublines derived from two human ovarian tumor cell lines. *Cancer Res.*, **48**: 3355 - 3359.

Kraker A.J. & Moore C.W. (1988). Accumulation of *cis*-Diamminedichloroplatinum (II) and platinum analogues by platinum-resistant murine leukaemia cells *in vitro*. *Cancer Res.*, **48**: 9 - 13.

Labarca C. & Paigen K. (1980). A simple, rapid and sensitive DNA assay procedure. *Analyt. Biochem,*. **102**: 344 - 352.

Lai G-M., Ozols R.F., Young R.C. & Hamilton T.C. (1989). Effect of glutathione on DNA repair in cisplatin-resistant human ovarian cancer cell lines. *J. Natl. Cancer Inst.*, **81**: 535 - 539.

Langdon S.P., Lawrie S.S., Hay F.G. & 7 others. (1988). Characterization and properties of nine human ovarian adenocarcinoma cell lines. *Cancer Res.*, **48**: 6166-6172.

Lazo J.S. & Bahnson R.R. (1989). Pharmacological modulators of DNA-interactive antitumour drugs. *TIPS*, **10**: 369 - 373.

Lee F.Y.F., Vessey A., Rofstad E. & 2 others. (1989). Heterogeneity of glutathione content in human ovarian cancer. *Cancer Res.*, **49**: 5244 - 5248.

Levin L & Hryniuk W.M. (1987). Dose intensity analysis of chemotherapy regimes in ovarian carcinoma. *J. Clin. Oncol.*, **5**: 756 - 767.

Lippard S.J. (1982). New chemistry of an old molecule: *cis*-$[Pt(NH_3)_2Cl_2]$.*Science*, **218**: 1075-1082.

Lynch H.T., Conway T. & Lynch J. (1990). Hereditary ovarian cancer. In: Ovarian cancer- biologic and therapeutic challenges ed. F. Sharp, W.P. Mason & R.E. Leake. pp 7 - 19. Chapman & Hall, London.

Mann S.C., Andrews P.A. & Howell S.B. (1988). Comparison of lipid content, surface membrane fluidity, and temperature dependence of *cis*-

Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian cancer cells. *Anticancer Res.*, **8**: 1211 - 1216.

Mann S.C., Andrews P.A. & Howell S.B. (1991). Modulation of *cis*-Diamminedichloroplatinum(II) accumulation and sensitivity by forskolin and 3-isobutyl-1-methylxanthine in sensitive and resistant human ovarian carcinoma cells. *Int. J. Cancer*, **48**: 866 - 872.

Massaza G., Tomasoni A., Lucchini V. & 7 others. (1989). Intraperitoneal and subcutaneous xenografts of human ovarian carcinoma in nude mice and their potential in experimental therapy. *Int . J. Cancer*, **44**: 494-500.

Masuda H., Tanaka T., Matsuda H. & Kusaba I. (1990). Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to CDDP. *Cancer Res.*, **50**: 1863 - 1866.

Mayer R.D., Lee K. & Cockett T.K. (1987). Inhibition of cisplatin-induced nephrotoxicity in rats by buthionine sulfoximine, a glutathione synthesis inhibitor. *Cancer Chemother. Pharmacol.*, **20**: 207 - 210.

Meijer C., Mulder N.H., Hospers G.A.P. & 2 others. (1990). The role of glutathione in resistance to cisplatin in a human small cell lung cancer cell line. *Br. J. Cancer*, **62**: 72 - 77.

Meister A. (1981). Metabolism and functions of glutathione. *TIBS*, **6**: 231 - 234.

Meister A. & Anderson M.E. (1983). Glutathione. *Ann. Rev. Biochem.*, **52**: 711 - 760.

Mistry P., Kelland L.R. Abel G. & 2 others. (1991). The relationship between glutathione, glutathione-S-transferase and cytotoxicity of platinum drugs and melphalan in 8 human ovarian carcinoma cell lines. *Br. J. Cancer*, **64**: 215 - 220.

Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**: 56 - 63.

MRC Gynaecological Working Party. (1990). An overview in the treatment of advanced ovarian cancer. *Br. J. Cancer*, **61**: 495 - 496.

Mustonen R., Takala M., Leppala S. & Hemminki K. (1989). Dose-dependence and stability of cisplatin binding to tissue DNA and blood proteins in rats. *Carcinogenesis*, **10**: 365 - 368.

Nio Y., Zighelboim J., Berek J. & Bonavida B. (1989). Cytotoxic and cytostatic effects of the streptococcal preparation OK-432 and its subcellular fractions on human ovarian tumor cells. *Cancer*, **64**: 434-441.

Onishi Y., Handel L.M., Hall L. & 4 others. (1989). Multidrug resistance (*mdr* - 1) gene amplification and overexpression in human ovarian cancer cell lines from untreated patients and patients refractory to combination chemotherapy. *Proc AACR*, **30**: 573.

Ozols R.F. (1989). Cisplatin dose intensity. *Sem. Oncol.*, **16(S)**: 22 -30.

Ozols R.F., Louie K.G., Plowman J. & 4 others. (1987). Enhanced melphalan cytotoxicity in human ovarian cancer in vitro and in tumor-bearing nude mice by buthionine sulfoximine depletion of glutathione. *Biochem. Pharmacol.*, **36**: 147 - 153.

Ozols R.F., Masuda H. & Hamilton T.C. (1988). Keynote address: Mechanisms of cross-resistance between radiation and antineoplastic drugs. *NCI Monographs*, **6**: 159 -165.

Parker R.J., Eastman A., Bostick-Bruton F. & Reed E. (1991). Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. *J. Clin. Invest.*, **87**: 772-777.

Parti R. & Wolf W. (1991). Effect of d-L-Buthionine sulfoxamine on the uptake and subcellular biodistribution of cisplatin in tumor-bearing rats. *Proc AACR.*, **32**: 119.

Pera M.F., Friedlos F., Mills J. & Roberts J.J. (1987). Inherent sensitivity of cultured human embryonal carcinoma cells to adducts of *cis*-Diamminedichloroplatinum(II) on DNA. *Cancer Res.*, **47**: 6810 - 6813.

Pinto A.L. & Lippard S.J. (1985). Binding of the antitumor drug *cis*-diamminedichloroplatinum(II) (Cisplatin) to DNA. *Biochim. Biophys. Acta*, **780**: 167 - 180.

Plooy A.C.M., Van Dijk M. & Lohman P.H.M. (1984). Induction and repair of DNA-crosslinks in Chinese Hamster Ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity and antitumour activity. *Cancer Res.*, **44**: 2043-2051.

Plumb J.A., Milroy R. & Kaye S.B. (1989). Effects of the pH dependence of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res.*, **49**: 4435-4440.

Poirier M.C., Egorin M.J., Fichtinger-Schepman A-M.J. & 2 others. (1988). DNA adducts of cisplatin and carboplatin in tissues of cancer patients. In: Damaging Agents in Humans: applications in cancer epidemiology and prevention. (ed H. Bartsch, K. Hemminki & J.K. O'Neill), IARC Scientific Publication No 89, 313 - 340. IARC.

Poirier M.C., Lippard S.J., Zwelling L.A. & 6 others. (1982). Antibodies elicited against CDDP-modified DNA are specific for CDDP-DNA adducts formed *in vivo* and *in vitro. Proc. Natl. Acad. Sci. USA*, **79**: 6443 - 6447.

Reed E., Gupta-Burt S., Litterst C.L. & Poirier M.C. (1990). Characterization of the DNA-damage recognised by an antiserum elicited against CDDP-modified DNA. *Carcinogenesis*, **11**: 2117 - 2121.

Reed E., Ozols R.F., Tarone R. & 2 others. (1987). Platinum-DNA adducts in leukocyte DNA correlate with disease response in ovarian cancer patients receiving platinum-based chemotherapy. *Proc. Natl. Acad. Sci .USA*, **84**: 5024 - 5028.

Riley C.M. (1988). Bioanalysis of cisplatin analogues - a selective review. *J. Pharm. Biomed. Anal.*, **6**: 669 - 676.

Rogan A.M., Hamilton T.C., Young R.C. & 2 others. (1984). Reversal of adriamycin resistance by verapamil in human ovarian cancer. *Science*, **224**: 994 - 996.

Rosenberg B., Van Camp L., & Krigas T. (1965). Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. *Nature* , **205**: 698 - 699.

Rotman B., Teplitz C., Dickinson K. & Cozzolino J.P. (1988). Individual human tumors in short-term micro-organ cultures: chemosensitivity testing by fluorescent cytoprinting. *In Vitro Cell. Devptl. Biol.*, **24**: 1137-1146.

Royer-Pakora B., Gordon L.K. & Haseltine W.A. (1981). Use of exonuclease III to determine the site of stable lesions in defined sequences of DNA: the cyclobutane pyrimidine dimer and *cis* and *trans* DDP examples. *Nucl. Ac. Res.*, **9**: 4595 - 4608.

Russo A., Carmichael J., Friedman N. & 4 others. (1986). The roles of intracellular glutathione in antineoplastic chemotherapy. *Int. J. Rad. Oncol. Biol. Phys.*, **12**: 1347 - 1354.

Scanlon K.J., Safirstein R.L., Thies H. & 3 others. (1983). Inhibition of amino acid transport by *cis*-Diamminedichloroplatinum (II) derivatives in L1210 murine leukaemia cells. *Cancer Res.*, **43**: 4211 - 4215.

Sekiya S., Oosaki T., Andoh S. & 3 others. (1989). Mechanisms of resistance to *cis*-diamminedichloroplatinum (II) in a rat ovarian carcinoma cell line. *Eur. J. Cancer Clin. Oncol,*. **25**: 429 - 437.

Sharma R.P. & Edwards I.R. (1983). *cis* -Platinum: subcellular distribution and binding to cytosolic ligands. *Biochem. Pharmacol.*, **32**: 2665 - 2669.

Sharp F., Mason W.P. & Leake R.E. (1989). Preface: Ovarian cancer- biologic and therapeutic challenges. p. xiv. Chapman & Hall, London

Shellard S.A., Hosking L.K. & Hill B.T. (1991). Anomalous relationship between cisplatin sensitivity and the formation and removal of platinum-DNA adducts in two human ovarian carcinoma cell lines *in vitro*. *Cancer Res.*, **51**: 4557 - 4564.

Shionoya S., Lu Y. & Scanlon K.J. (1986). Properties of aminoacid transport systems in K562 cells sensitive and resistant to *cis* -Diamminedichloroplatinum(II). *Cancer Res.*, **46**: 3445 - 3448.

Smith E. & Brock A.P. (1989). The effect of reduced osmolarity on platinum drug toxicity. *Br. J.Cancer*, **59**: 873 - 875.

Sorenson C.M. & Eastman A. (1988a). Mechanism of *cis*-Diamminedichloroplatinum(II)-induced cytotoxicity: role of G2 arrest and DNA double strand breaks. *Cancer Res.*, **48**: 4484 - 4488.

Sorenson C.M. & Eastman A. (1988b). Influence of *cis*-Diamminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese Hamster Ovary cells. *Cancer Res.*, **48**: 6703 - 6707.

Stampfer M., Hallowes R.C. & Hackett A.J. (1980). Growth of normal human mammary cells in culture. *In Vitro Cell. Devptl. Biol.*, **24**: 1137 - 1146.

Stone P.J., Kelman A.D. & Sinex F.M. (1974). Specific binding of antitumour drug cis-$Pt(NH_3)_2Cl_2$ to DNA rich in guanine and cytosine. *Nature*, **251**: 736 - 737.

Strandberg M.C., Bresnick E. & Eastman A. (1982). The significance of DNA cross-limking to *cis* -diamminedichloroplatinum(II)-induced cytotoxicity in sensitive and resistant lines of murine leukaemia L1210 cells. *Chem.-Biol. Interact.*, **39**: 169 - 180.

Sugawara I., Kataoka I., Morishita Y. & 4 others. (1988). Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. *Cancer Res.*, **48**: 1926 - 1929.

Sutton G.P., Stehman F.B., Einhorn L.H. & 3 others. (1989). Ten-year follow-up of patients receiving cisplatin, doxorubicin and cyclophosphamide therapy for advanced epithelial ovarian cancer. *J. Clin. Oncol.*, **7**: 223 - 229.

Teicher B.A., Holden S.A., Kelley M.J. & 5 others. (1987). Characterization of a human squamous carcinoma cell line resistant to *cis* -Diamminedichloroplatinum(II). *Cancer Res.*, **47**: 388 - 393.

Terheggen P.M.A.B., Emondt J.Y., Floot B.J.G. & 4 others. (1990). Correlation between cell killing by *cis* -Diamminedichloroplatinum (II) in six mammalian cell lines and binding of a *cis* -Diamminedichloroplatinum (II)-DNA antiserum. *Cancer Res.*, **50**: 3556 - 3561.

Tietze F. (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidised glutathione: applications to mammalian blood and other tissues. *Analyt. Biochem.*, **27**: 502 - 522.

Timmer-Bosscha H., Hospers G.A.P., Meijer C. & 5 others. (1989). Influence of docosahexaenoic acid on cisplatin resistance in a human small cell lung carcinoma cell line. *J. Natl. Cancer Inst.*, **81**: 1069 - 1075.

Ushay H.M., Tullius T.D. & Lippard S.J. (1981). Inhibition of the Bam H1 cleavage and unwinding of pBR322 DNA by the antitumour drug *cis*-Diamminedichloroplatinum(II). *Biochemistry*, **20**: 3744 - 3748.

van der Burg M.E.L., van Lent M., Kobierska A. & 7 others (1993). Interventional debulking surgery (IDS) does improve survival in advanced ovarian cancer: an EORTC Gynaecological Cancer Cooperative Group Study. *ASCO Abstract.*

Vermorken J.B., ten Bokkel Huinink W.W., Eisenhauer E.A., & 4 others (1993). Carboplatin versus cisplatin. *Ann. Oncol.*, **4**, S41 - S48.

von Hoff D.D. (1983). "Send this patient's tumor for culture and sensitivity". *N. Engl. J. Med.*, **308**: 154-155.

Waud W.R. (1987). Differential uptake of *cis* -Diamminedichloroplatinum(II) by sensitive and resistant murine L1210 leukaemia cells. *Cancer Res.*, **47**: 6549 - 6555.

Wiltshaw E. (1985). Chemotherapy of ovarian carcinoma and other gynaecological malignancies. In: Clinical Gynaecological Oncology ed. J.H. Shepherd & J.M. Monaghan, pp. 215 - 238. Blackwell, Oxford.

Wiltshaw E. & Kroner T. (1976). Phase II study of *cis* -diaminedichloroplatinum(II) (NSC-119875) in advanced adenocarcinoma of the ovary. *Cancer Treat. Rep.*, **60**: 55 - 60.

Wolf C.R., Hayward I.P., Lawrie S.S., & 6 others. (1987). Cellular heterogeneity and drug resistance in two ovarian adenocarcinoma cell lines derived from a single patient. *Int. J. Cancer*, **39**: 695-702.

Young R.C. (1987). Initial therapy for early ovarian carcinoma. *Cancer*, **60(S)**: 2042 - 2049 .

Zwelling L.A., Anderson T. & Kohn K.W. (1979). DNA-protein and DNA interstrand crosslinking by *cis* - and *trans* -Pt(II)diamminodichloride in L1210 mouse leukaemia cells and relation to cytotoxicity. *Cancer Res.*, **39**: 365-369.