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CELL CYCLE DEPENDENCY OF CISPLATIN CYTOTOXICITY ON OVARIAN CANCER CELLS

By

Seyed Farshad Hosseini Shirazi

A thesis submitted to the

Faculty of Graduate Studies and Research

in partial fulfilment of the requirements

for the degree of

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Faculty of Medicine,

Department of Cellular and Molecular Medicine

University of Ottawa

Ottawa, Ontario

26 January 1998

The undersigned recommend to the Faculty of Graduate Studies

and Research acceptance of this thesis

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Thesis Supervisor

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Chairman, Department of Cellular and Molecular Medicine

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ABSTRACT

The cytotoxicity of Cisplatin (CDDP) is traditionally believed to be cell cycle independent. This general belief is not in agree with CDDP-induced apoptosis. Therefore, cell cycle dependency of cisplatin was investigated using human ovarian carcinoma OV 2008 cell line synchronized at the different phases of the cell cycle (i.e. subpopulations). Clonogenic assay, flow cytometry, fluorescence microscopy, electron microscopy (EM), atomic absorption spectroscopy (AA), and various biochemical techniques were used in this project. OV 2008 cell subpopulations at the different phases of G1, G1/S, S and G2/M were exposed to 1 μg/ml of CDDP for one hour (n=3). CDDP resulted in 66$\pm$6(SE)%, 50$\pm$6%, 42$\pm$5%, and 41$\pm$5% survival for cells in G2/M, S, G1/S boundary and G1 of the cell cycle, respectively. Flow cytometry confirmed a general pattern of arrest in S and G2/M for all subpopulations, in spite of the different percentages of death. A cell cycle delay in arrest was noted when cells were exposed to CDDP in late S or G2/M, but not for the other two phases. EM revealed both apoptotic and necrotic cell death. For all subpopulations, the appearance of apoptotic and necrotic cells were 70% and 30% in

about 12 hours, with 25% and 75% in 30 hours, respectively. All subpopulations of cells had accumulated the same amount of intracellular elemental platinum after exposure to CDDP. However, different amounts of DNA adducts were formed for cells in each subpopulation. In general, the rank order of CDDP cytotoxicity on the different phases of OV 2008 cell cycle is as follows; G1>G1/S>S>G2/M. This rank order is found in good agreement with the rank order of CDDP-DNA binding, as well as intracellular acidity (pHi) of OV 2008 cells; being at the lowest pH in G1 and highest in G2/M. Control experiments have shown the conversion of CDDP to its active metabolite of aquated CDDP (AP)in acidic media. pH dependent metabolism of CDDP to AP, which will then bind to DNA was confirmed. We have concluded that OV 2008 cells have different sensitivity to CDDP at the different phases of the cell cycle, which is related to the variations in pHi, and consequently the intracellular metabolism of CDDP to AP.

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In the name of God

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This thesis is dedicated to my family; Anoosh, Sina and Nima, who have shown a great deal of patience, support and understanding during this program.

Finally, I would like to express my deepest appreciation to my parents for their advice, support and encouragement throughout my life. This thesis is a minor result of the invaluable guidance they have done to their children.

And in the memory of all who have made this happen; teachers throughout my education starting from grade one, my colleagues, friends, and the great people of my country. They all do have input in this work, and I owe to them.

May God bless them all.

TABLE OF CONTENTS

List of Figures

List of Figures

List of Figures

List of Tables

LIST OF ABBREVIATIONS:

AP	Aquated cisplatin
ATCC	American Type Culture Collection
Avg	Average
CDDP	Cisplatin
CP	Cisplatin
DNA	Deoxyribonucleic acid
EM	Electron microscopy
Fig	Figure
G	Guanine
pHi	Intracellular pH
Pt	platinum
RNA	Ribonucleic acid
SE	Standard Error
Tab	Table

* "Subpopulations", is the term used for OV 2008 cells synchronized at different phases of the cell cycle.

1

Cisplatin and its applications in cancer chemotherapy:

1.1 - History:

As has been the case in many other medical developments, the discovery of antitumour activity of platinum amine complexes was somewhat fortuitous (Cleare and Hydes, 1980). Professor Barnett Rosenberg of Michigan State University, was fascinated by the appearance of spindle cell formation during cell mitosis, as they appeared to him to resemble lines of magnetic force, such as those seen with iron filings around a magnet. Thus, a study was initiated to see if an electromagnetic field would influence cell division. The setting up of these experiments involved two pieces of good fortune. Firstly, AC current was passed through *Escherichia coli* bacteria in a growth chamber via a set of platinum electrodes, and secondly, the bacteria were supported in a nutrient medium containing ammonium chloride as the nitrogenous source. *E. coli,* and prokaryotic cells in general, did not show mitotic figures in division and were only being used to test the equipment; this was another piece of good fortune. Under the influence of the current, the bacteria underwent filamentous growth. Bacterial rods, normally some 2-5 μm in length, formed strands up to some 300 times that length. Thus, cell division was inhibited while cell growth was unaffected. Further tests showed that gram-negative rods were most sensitive, with gram positive rods much less so; spherical bacilli (cocci) were unaffected.

A long series of control experiments showed that the current was not causing the filamentous growth but was causing some 10 ppm of platinum to dissolve from the electrodes. The species formed was identified as $[PtCl_6]^{2-}$, which was present in part as the ammonium salt.

Fresh solutions of $(NH_4)_2[PtCl_6]$ were bacteriostatic and inhibited cell division at these concentrations (approximately 10 ppm); however, aged solutions (2-3 days) were very effective in producing filaments at low platinum concentrations. Further studies confirmed the photochemical reaction (Rosenberg *et al.*, 1967).

The inhibition of cell division, but not cell growth, suggested that these compounds might have antitumour properties. Initially, 4 platinum compounds, including cis-$[PtCl_4(NH_3)_2]$ and cis-$[PtCl_2(NH_3)_2]$, were tested against Sarcoma 180 in mice and were found to be effective in inhibiting tumour growth through their antimitotic effect (Rosenberg *et al.*,1969). Neither of the trans isomers showed any appreciable antitumour activity. The two cis isomers of the platinum compounds were submitted to the U. S. National Cancer Institute and screened against L1210 leukaemia in mice. These compounds showed potent antitumour activity with a high cure rate with a single injection at the therapeutic dose of 8 mg/kg of body weight. Rosenberg and Van Camp (1970) went on to show that cis-$[PtCl_2(NH_3)_2]$ was capable of regressing large solid Sarcoma-180 tumours (8 days old) in Swiss white mice. Cis-$[PtCl_2(NH_3)_2]$ (figure 1-1) appeared to be the most potent of the original compounds and has since been tested against many transplanted animal tumours, where it has proved to have a wide spectrum of activity. It has undergone extensive toxicological and clinical trials leading to governmental approval for certain

human tumours (see Sec. 1.5).

1.2 - Chemistry:

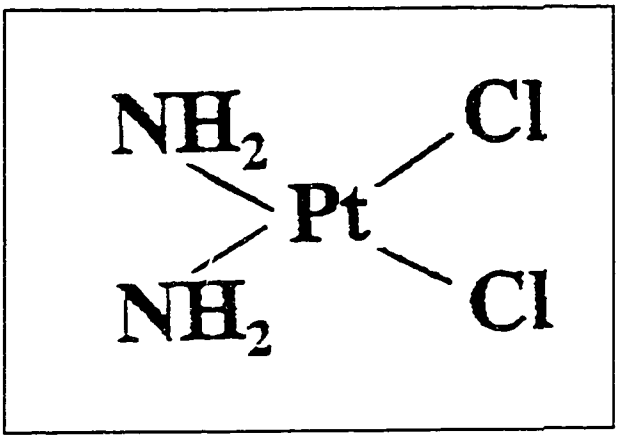


Figure 1-1: Chemical structure of cisplatin

Since the original discovery of anticancer effects of platinum complexes, numerous studies have been undertaken to determine the relationships that exist between chemical structure and antitumour activity, with a view to finding more active, less toxic drugs. This work has been discussed in several reviews and a summary is given here and in the following sections (Cleare, 1974).

An obvious similarity between Rosenberg's original active complexes is the cis arrangement of the chloride ligands (the definition of ligand is based on its meaning in organic chemistry; ligand is any molecule or ion that has at least one electron pair that can be so donated. Ligands may also be called Lewis bases; in the terms used in organic chemistry, they are nucleophiles [Cotton and Wilkinson, 1988].). The trans isomers have been found to be inactive (and usually non-toxic) in comparison to the corresponding active cis isomers. Thus, for platinum complexes, this had led to a concentration of effort on cis complexes of the general type cis-[PtA_2X_2]. Platinum complexes of the type [PtX_2A_2] (X_2 = two monodentate anionic ligands or one bidentate anionic ligand; A2 = two monodentate amine ligands or one bidentate amine ligand)

have attracted the most attention, while limited data on other systems have been reported. It has been established that antitumour activity is only found in neutral species and in cis rather than trans isomers. The X and A ligands have been systematically varied and the chemistry of active compounds has been investigated, particularly in kinetic terms. Trans isomers are consistently more reactive in chemical reactions than their cis analogues. Thus, trans-$[Pt(NH_3)_2Cl_2]$ aquates approximately four times faster than cis-$[Pt(NH_3)_2Cl_2]$, although the corresponding cis equilibrium constants for this reaction is larger. Thus, trans compounds are likely to react more quickly and with a wider variety of body constituents and should be rather less specific in their action than cis compounds (Hoeschele and Van Camp, 1971). Since only cis compounds have the potential to form chelates, this might imply that the anti-tumour activity is largely associated with a chelating interaction. Chemical studies on the $[Pt(NH_3)_2Cl_2]$ isomers have clearly established that the chlorides are the reactive ligands (See following sections). The Pt-NH_3 bond is very stable and ranks close to CN^- in affinity for PtII (Basolo and Pearson, 1967).

The chemistry of platinum (II) amine species of the type $[PtX_2A_2]$ is dominated by the high affinity of NH_3 for the Pt (II) centre. Affinities for common ligands vary (Basolo and Pearson, 1967):

$CN^- > NH3$ &/or $OH^- > I^- > SCN^- > Br^- > Cl^- > F^-$ &/or H_2O

The nature of the amine ligands (A) has a primary effect on the antitumour properties of the platinum compounds. Regarding this group (A), the most interesting results have come from complexes with alicyclic amines. The sequence from cyclopropylamine through

cyclooctylamine gives excellent results. These have the best therapeutic indexes ever reported for the ADJ/PC6 tumour system (Cleare, 1974).

The chemistry of cisplatin and its derivatives, which may lead to the discovery of new platinum compounds, has recently been reviewed extensively (Lippard, 1982).

1.3 - Chemical Reactions:

The pharmacologic behaviour of cisplatin is in part determined by its reactions in water. These are described below:

1.3.1 - Stability and Hydrolysis:

The platinum co-ordination complexes have square planar geometry. As mentioned before, they have two "inert" ligands (two NH_3) in two corners of the square and two "moderately labile" ligands (two Cl^-) in the other two corners of the square structure, with the platinum in the centre.

The stability of cisplatin in 0.9% sodium chloride solution and the effects of pH and light on its degradation were studied by Zieske and his co-workers (1991). Cisplatin undergoes hydrolysis in water (figure 1-2). These aqua complexes are weak acids and the related pH and pK_a values have been investigated by many authors (Theophanides, 1980). Reishus and Martin (1961) have investigated the acid hydrolysis of cisplatin at 25° and 35°C. For the first acid hydrolysis:

$$cis\text{-}[Pt(NH_3)_2Cl_2]+H_2O \rightarrow cis\text{-}[Pt(NH_3)_2(H_2O)(Cl)]^+ + Cl^-$$

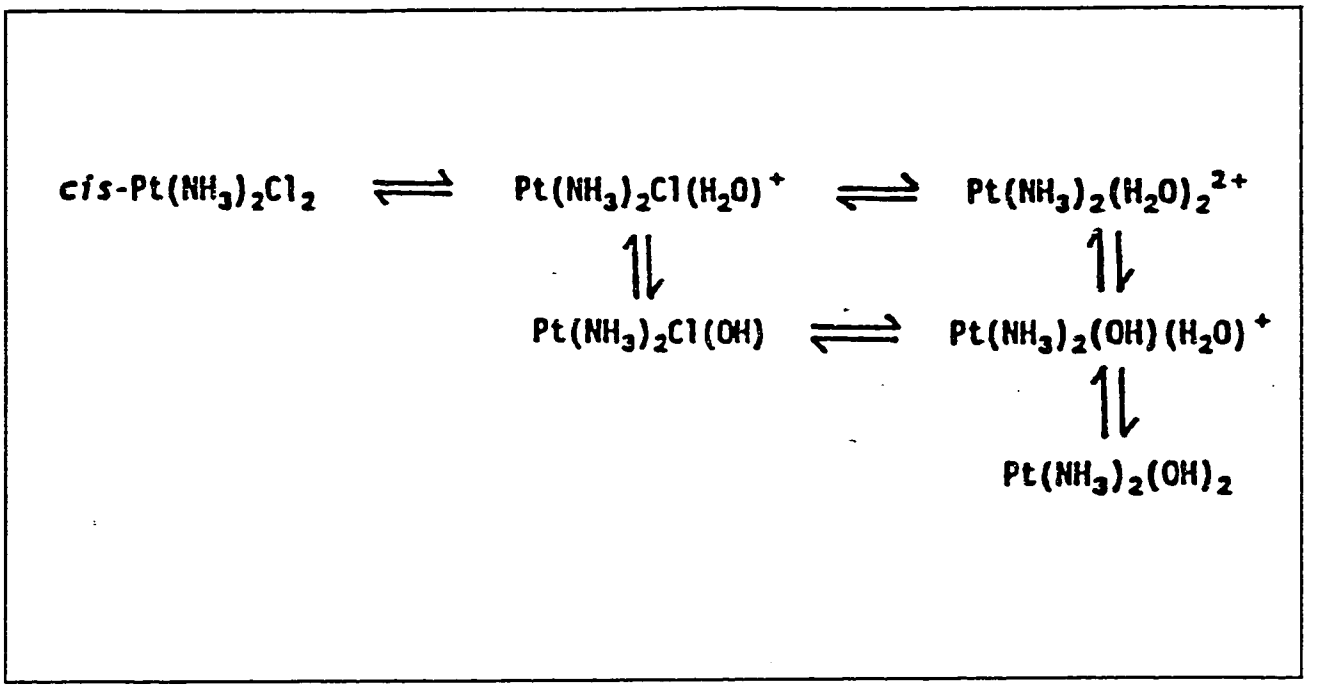


Figure 1-2: Hydrolysis of cisplatin in water.

the equilibrium constant, K_1, in their study was 3.3×10^{-3} mole/l and the rate constant was determined as 2.5×10^{-5} sec^{-1}. They did not find any significant direct exchange between the chloride ligands of cisplatin and Cl^-. For the second acid hydrolysis:

$cis\text{-}[Pt(NH_3)_2(H_2O)(Cl)]^+ + H_2O \rightarrow cis\text{-}[Pt(NH_3)_2(H_2O)_2]^{++} + Cl^-$

the equilibrium constant, K_2, was 4×10^{-5} mole/l, at 25° C. The exchange of chloride with mono-aquated cisplatin occurred at a rate which is chloride-independent and is characterized by a first order rate constant, $k_2 = 3.3 \times 10^{-5}$ sec^{-1} at 25° C. It was suggested that this exchange also occurs by only an acid hydrolysis mechanism.

Miller and House (1989) resulted in a model of the complete pH-rate profile for the

hydrolysis of cisplatin. This model is summarized in figure 1-3.

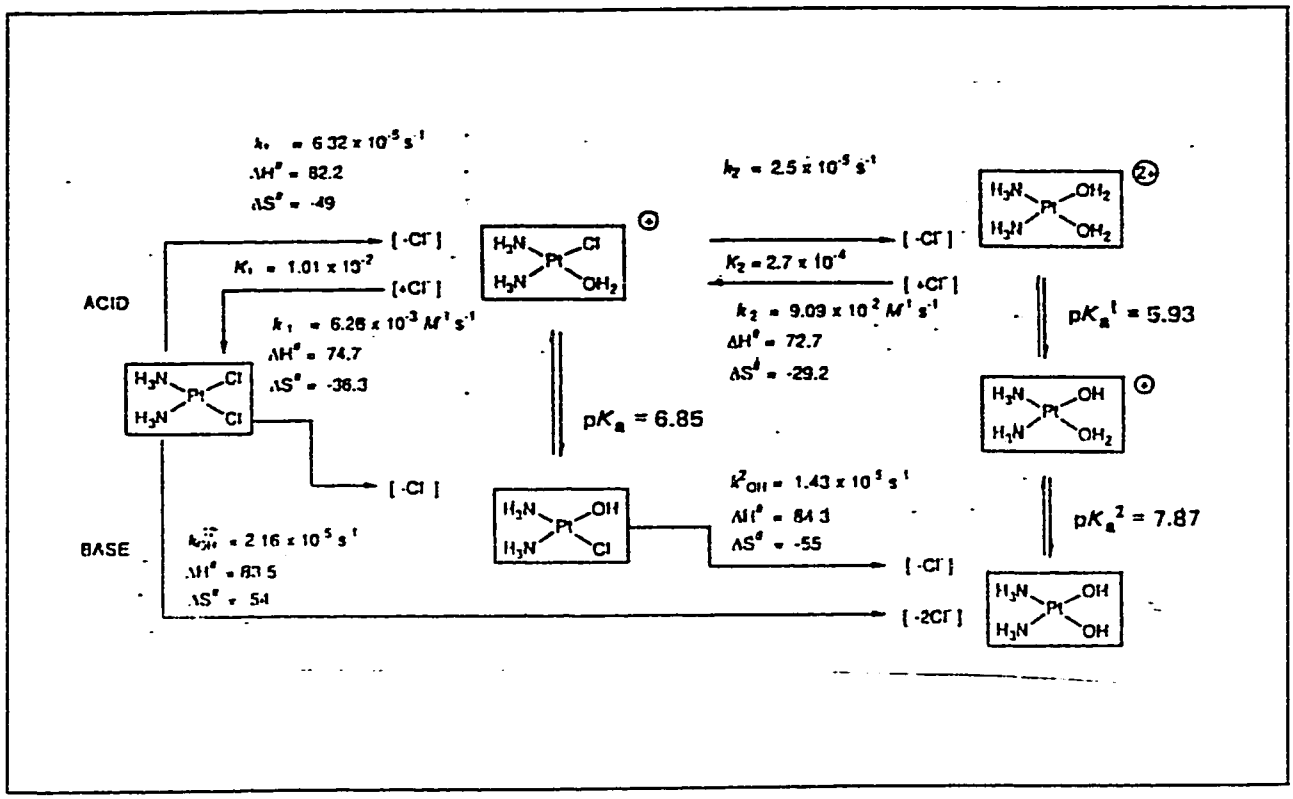


Figure 1-3: Rate and equilibrium constants for cisplatin and its hydrolysis products in acidic and basic conditions at 25° C.

Cisplatin undergoes acid hydrolysis in aqueous solution. This reaction is pH dependent. Berners-Price and co-workers (1992) have determined the pK_a values of mono- and di- aquated cisplatin to be 6.41 and 5.37, respectively, via the use of ^{1}H NMR spectroscopy on the ^{15}N analogue of cisplatin. They found that the reactions of these species are complicated by

further formation of hydroxo-bridged polymers (...Pt/^{O}\Pt...) at different pHs. These hydroxylated species have the following general formula:

cis-$[PtCl(OH)(NH_3)_2]$

cis-$[Pt(OH)_2(NH_3)_2]$

cis-$[PtCl(OH)(H_2O)(NH_3)_2]^+$

Figure 1-4 presents the plots of ^{1}H NMR chemical shifts vs. pH for the above mentioned species. Their results will therefore allow more accurate preparation of different species and detailed investigations of the pathways of reactions of cisplatin under biologically relevant conditions.

1.3.2 - Reactions with DNA:

Present knowledge is still not in a position to pinpoint the exact products responsible for anti-tumour activity of cisplatin in a biological system. However, a review of some work will help to predict the mechanism of action of cisplatin.

Blood plasma has a pH of about 7.4, and under these conditions, base hydrolysis becomes a real possibility. The equilibrium end product composition would then be about 50% cis-$Pt(OH)_2(NH_3)_2$ and 50% cis-$PtCl(OH)(NH_3)_2$ (Miller and House,1990) (See previous section).

The generally accepted mechanism for the interaction of cisplatin with DNA molecules is as follows (Rosenberg and Van Camp, 1970): The neutral cisplatin molecule passes through the cell membrane. Once inside the cell, hydrolysis will occur, and because of low

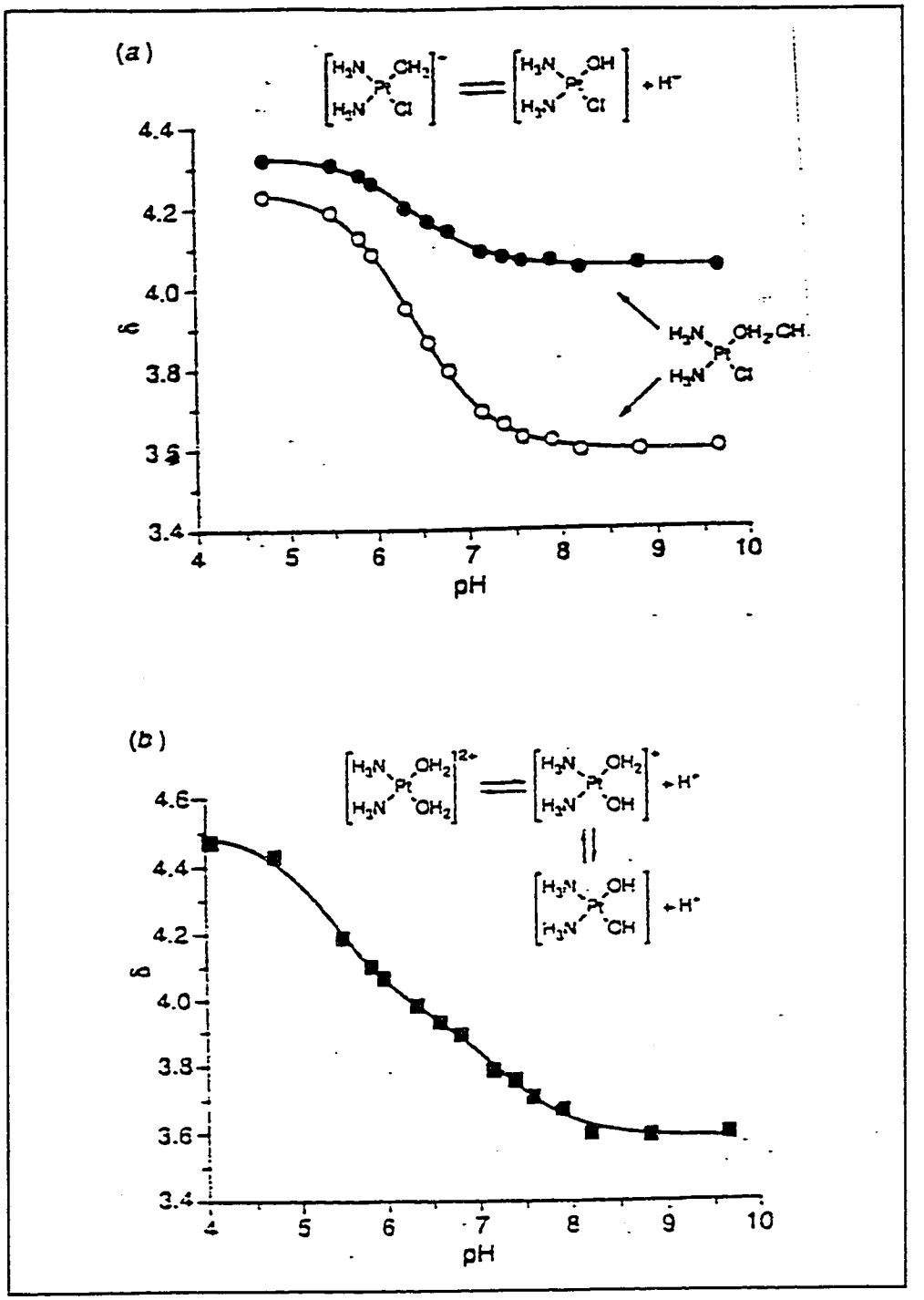


Figure1-4: Plots of ^{1}H NMR chemical shifts vs pH: a) cis-$[Pt(NH_3)_2(H_2O)(Cl)]^+$ and b) cis-$[Pt(NH_3)_2(H_2O)_2]^{++}$. From Berners-Price et al., (1992).

chloride concentration in the cell (about 4 mM), the positively charged (chloro)(aqua) product is the most likely labile complex for donor groups to the DNA target. On the other hand, if the base hydrolysis process were to operate at pH=7.4 (the pH of blood plasma), then an entirely different model could be proposed for the mechanism of cisplatin-DNA interaction. In this way, the final product would be a mixture of cis-$Pt(OH)(NH_3)_2(OH_2)^+$, cis-$PtCl(OH)(NH_3)_2$ and cis-$Pt(OH)_2(NH_3)_2$. The di(hydroxo) and (chloro)(hydroxo) forms are relatively inert to substitution, but capable of cell membrane penetration in the neutral form, whereas the (hydroxo)(aqua) of (chloro)(aqua) ions would be the active reagent for attack by polar donor groups in the DNA target molecule. The rapid protolytic equilibria between the (chloro)(hydroxo) and the

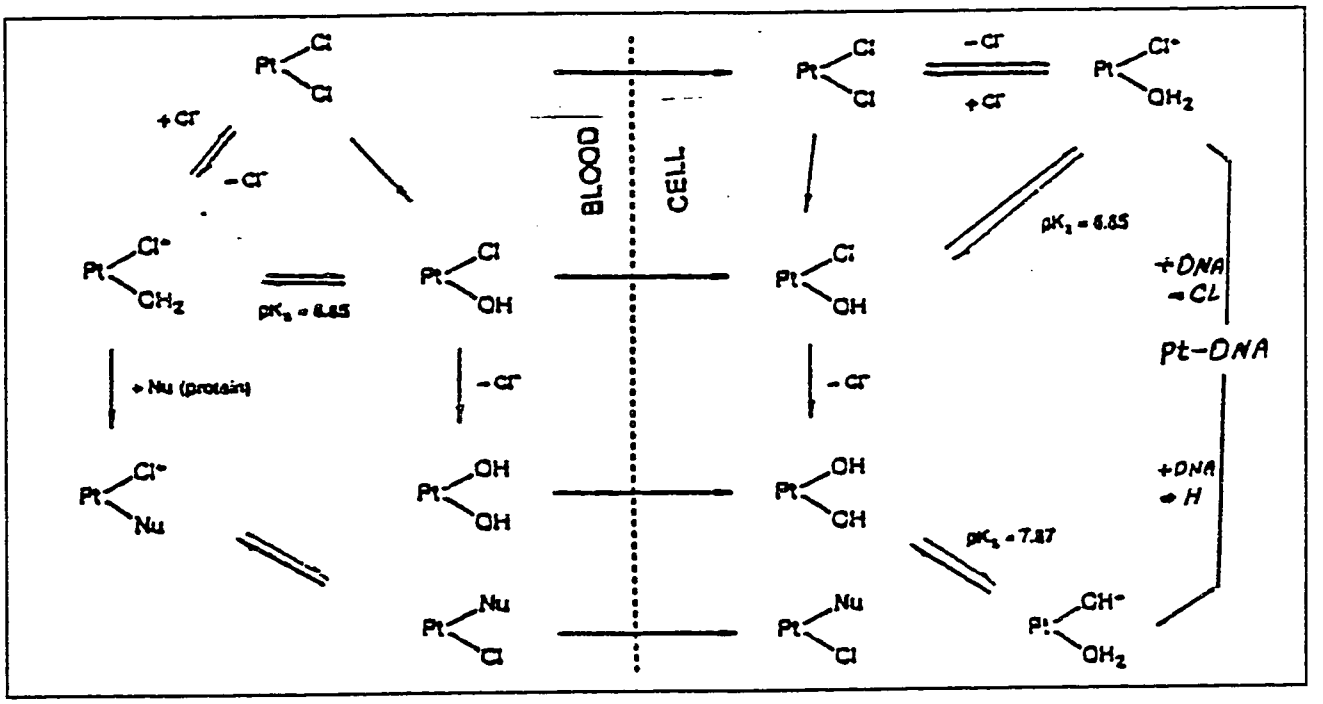


Figure 1-5: A model of cisplatin modifications at the different pHs from the blood to the DNA. (NH_3 groups have been omitted; Nu: nucleophile group).

(chloro)(aqua) species would allow facile transport of platinum species to suitable DNA sites. A speculative model is presented in figure 1-5.

A review of articles on the interaction of cisplatin with DNA is presented below. In the interaction of cisplatin with DNA, the two chloride ligands can (usually after aquation) react with two guanine sites so as to form crosslinks (Lippard, 1994). It has been established that platinum salts react preferentially with the N_7 site of guanine in nucleosides, nucleotides and DNA. Cisplatin reacts with guanine (G) first by substituting one of its two chloride atoms with the N_7 atom of guanine, thus making a covalent adduct on DNA the same as a carcinogen (See figure 1.6-A). The second chloride must react simultaneously or subsequently with a basic site nearby at a distance of 2.9 to 3.4 A. This second coordinating basic site may be another G molecule on the same strand of DNA just above or below the first G, again through a N_7 site and located at a distance of 3.4 A from the first N_7. This forms an intrastrand cross-linked complex (figures 1.6 and 1.7). The formation of an intrastrand cross-linked platinum connecting N_7 sites is repairable. The repair mechanism of excision, re-synthesis of DNA by a DNA polymerase and the welding repair by ligase is shown in figure 1.6.C. However, formation of a cross-link with the other base on the other strand of DNA is cytotoxic (Gellert and Bau, 1979).

1.4 - Pharmacology:

Chemistry and a part of the metabolism of cisplatin have been reviewed above.

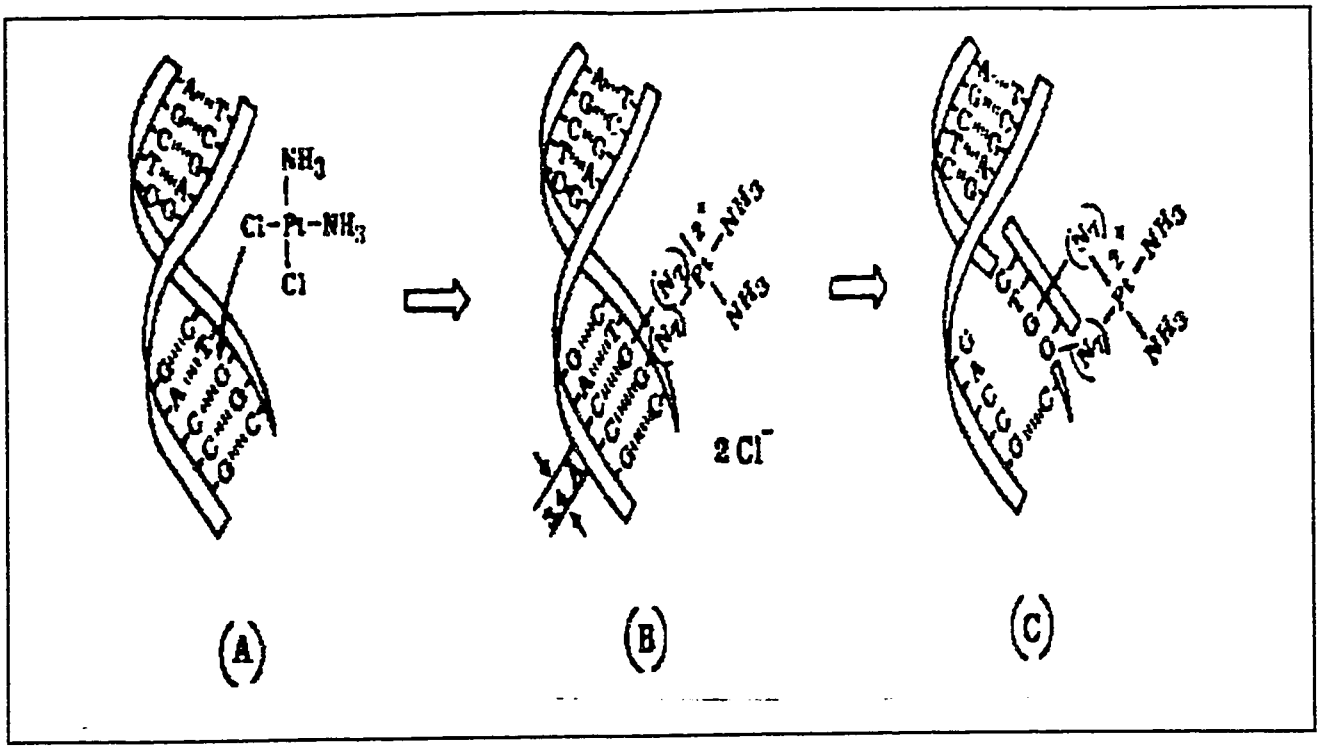


Figure 1-6: Interaction of cisplatin with DNA.

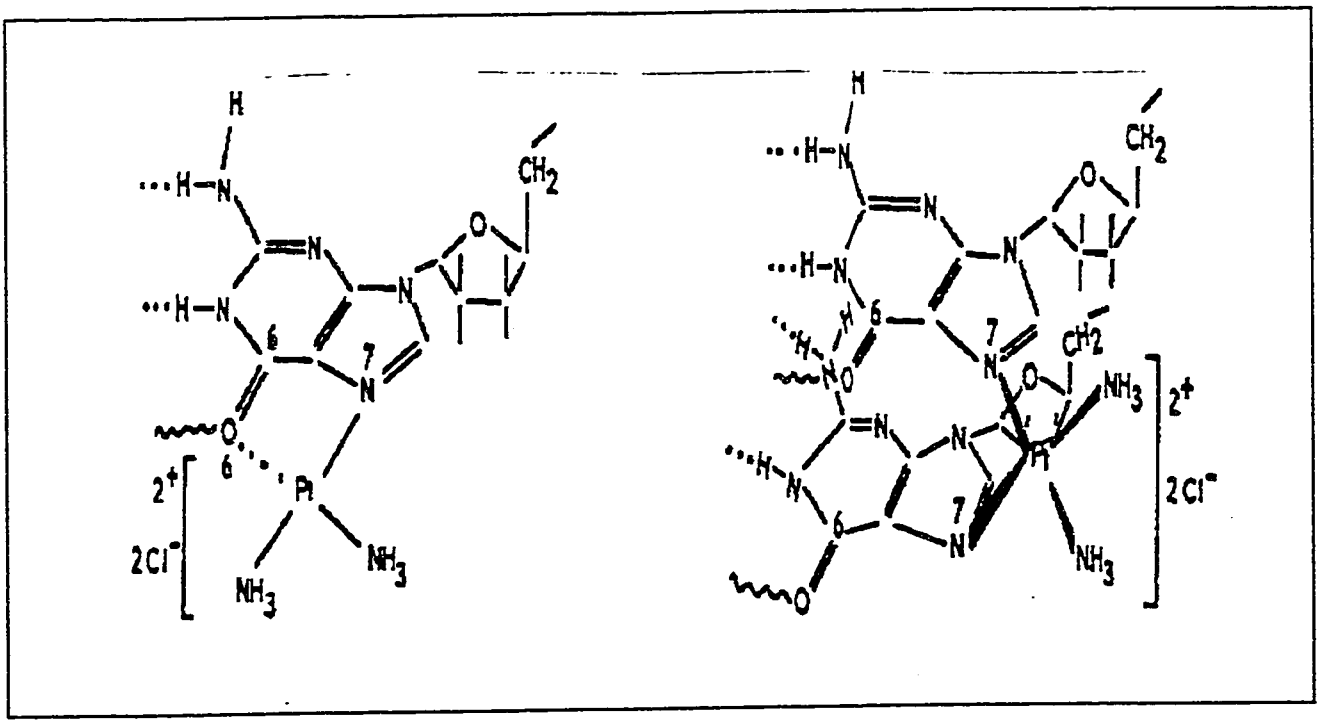


Figure 1-7: Proposed model of binding of cisplatin to guanine bases.

Some other important pharmacological aspects of this drug will be presented here.

1.4.1 - Clinical Applications:

Cisplatin is one of the most commonly used cancer chemotherapy drugs. Combination cisplatin chemotherapy with other chemotherapeutic drugs like etoposide is effective for 85% of patients with advanced testicular cancer (Xiao *et al.*, 1997). The drug is also potentially effective for advanced carcinoma of the ovary. Cisplatin causes significant responses in cancers of the bladder, head and neck (Douple, 1990), small cell and non-small cell carcinomas of the lung; and some neoplasms of childhood (Vokes *et al.*, 1993). Interestingly, the drug also sensitizes cells to the cytotoxic effects of radiation therapy (Stock *et al.*, 1997). Its usual intravenous dosage is 50-125 mg/m^2 with a frequency of once every four weeks, when given as a single agent (Kalant and Roschilau, 1989), 50-70 mg/m^2 as single dose every 3 weeks or 20 mg/m^2 for 5 days (Desoize *et al.*, 1996).

The usefulness of cisplatin as an anti-tumour drug is limited by drug toxicity and drug resistance. Some strategies that are being developed to overcome drug resistance include a) an increase in dose-intensity, b) modulation of drug resistance, and c) the development of analogues of cisplatin. The last strategy is also being used to decrease drug toxicity (Freed, 1993).

1.4.2 - Pharmacokinetics:

1.4.2.1 - General parameters:

Cisplatin is not effective when administered orally. After rapid intravenous administration of usual doses (Sec. 1.4.1), the drug has an initial half-life in plasma of 25 to 50 minutes; concentrations decline subsequently, with a half-life of 58 to 73 hours, based on the

administration program. The half-life of the drug is longer in patients who receive high doses of cisplatin.

More than 90% of the platinum in the blood is bound to plasma proteins. High concentrations of cisplatin are found in the kidney, liver, intestine, and testes, but there is poor penetration into the CNS. Only a small portion of the drug is excreted by the kidney during the first 6 hours; after 5 days up to 43% of the administered dose is recovered in the urine. When given by infusion instead of rapid injection, the plasma half-life is shorter and the amount of drug excreted is greater. Biliary or intestinal excretion of cisplatin appears to be minimal (Villanueva *et al.*, 1997).

1.4.2.2 - Cell Membrane Transport:

It has been generally accepted that cisplatin enters the cell largely through passive diffusion (Gately and Howell, 1993). However, there are several characteristics of intracellular cisplatin accumulation that seem incompatible with only simple passive uptake, since it is: 1) energy dependent, Na^+ dependent and ouabain inhibitable (Andrews *et al.*, 1988); 2) stimulated by reduction in osmotic strength and pH (Smith and Brock, 1989); 3) stimulated by elevation of cAMP level (Andrews and Howell, 1990); and 4) inhibited by aldehydes (Dornish and Petersen, 1989). Despite these factors, classical proof for a carrier system is lacking, in that accumulation is not saturable up to 3 mM and can not be inhibited with structural analogs (Mann *et al.*, 1990).

A double reciprocal plot of platinum accumulation vs drug exposure concentration yields a straight line through the origin, indicating that the rate limiting factor for platinum uptake is the concentration of drug (Gale *et al.*, 1973; Binks and Dobrota, 1990). These observations are

in agreement with a passive diffusion of cisplatin. A strong argument against the presence of active transport is that the accumulation is not inhibited by structural analogs of cisplatin (Andrews *et al.*, 1987). The only compound that inhibits cisplatin accumulation is cis-$PtCl_2(NH_3)$. However, even this analog produces only 5% inhibition, which is attributed to non-specific damage from the highly reactive drug. Uptake of cisplatin is not saturable. This observation was confirmed by measuring the cellular uptake of cisplatin using either atomic absorption spectroscopy or radioactive cisplatin (Hormas *et al.*, 1987).

On the other hand, there are data suggesting that some components of cisplatin uptake must be mediated by a form of transport mechanism. Byfield and Calabro-Jones, (1981) postulated that cisplatin may be entering the cell via a carrier mediated process. This hypothesis came from the observation that cisplatin showed differential toxicity in cycling and resting human T lymphocytes. This pattern is shared by alkylating agents with known carriers (eg, melphalan and nitrogen mustard), but not by agents that enter the cell through passive diffusion (eg, mitomycin C). However, this observation might be related to some other biological differences between cycling and resting cells, which will be discussed later.

The protein synthesis inhibitor benzaldehyde reduces the cytotoxicity of cisplatin, while another inhibitor of protein synthesis, cycloheximide, does not (Dornish *et al.*, 1986). Considering some control experiments, it has been postulated that benzaldehyde reacts with membrane proteins to inhibit the uptake of cisplatin (Dornish *et al.*, 1986). It has been determined that a large number of aldehyde compounds inhibit the uptake of cisplatin, presumably by forming Schiff bases with membrane proteins (Dornish *et al.*, 1989). Cisplatin accumulation is

potassium dependent, and therefore also appears to be dependent on the membrane potential. When the membrane is depolarized by incubation in high potassium media, the cells accumulate 5.4-fold more cisplatin than control cells (Andrews *et al.*, 1991). The uptake of cisplatin has also been shown to be inhibited by the over-expression of the c-Ha-ras oncogene by about 40% (Isonishi *et al.*, 1991). Cisplatin accumulation is also affected by a number of other intracellular signalling mechanisms, including protein kinase C (PKC), protein kinase A (PKA), and the Ca^{++} calmodulin pathway. Calmodulin antagonists have been shown to decrease cisplatin accumulation (Kikuchi *et al.*, 1990). AMP-induced activation of PKA increases the cellular accumulation and toxicity of cisplatin in human ovarian 2008 cells (Mann *et al.*, 1991). Howell *et al.*, (1987), showed that the nucleoside transport inhibitor dipyridamole increased cisplatin accumulation in OV2008 cells in a dose-dependent manner.

In conclusion, various studies present different mechanisms involved in the membrane transport of cisplatin, in favour of either active or passive uptake of the drug. These differences might be partially related to the different cell lines or experimental procedures that have been used by different investigators. On the other hand various cell culture media and solutions may change the chemical structure of cisplatin in a way that different transmembrane mechanisms be applied in any particular experiment. Low stability of cisplatin may make the comparison of different results very difficult.

1.4.3 - Mechanism of Action:

The cytotoxicity of cisplatin is believed to be due to the formation of DNA adducts (Munchausen and Rahn, 1975), which include DNA-protein cross-links, intermolecular cross-

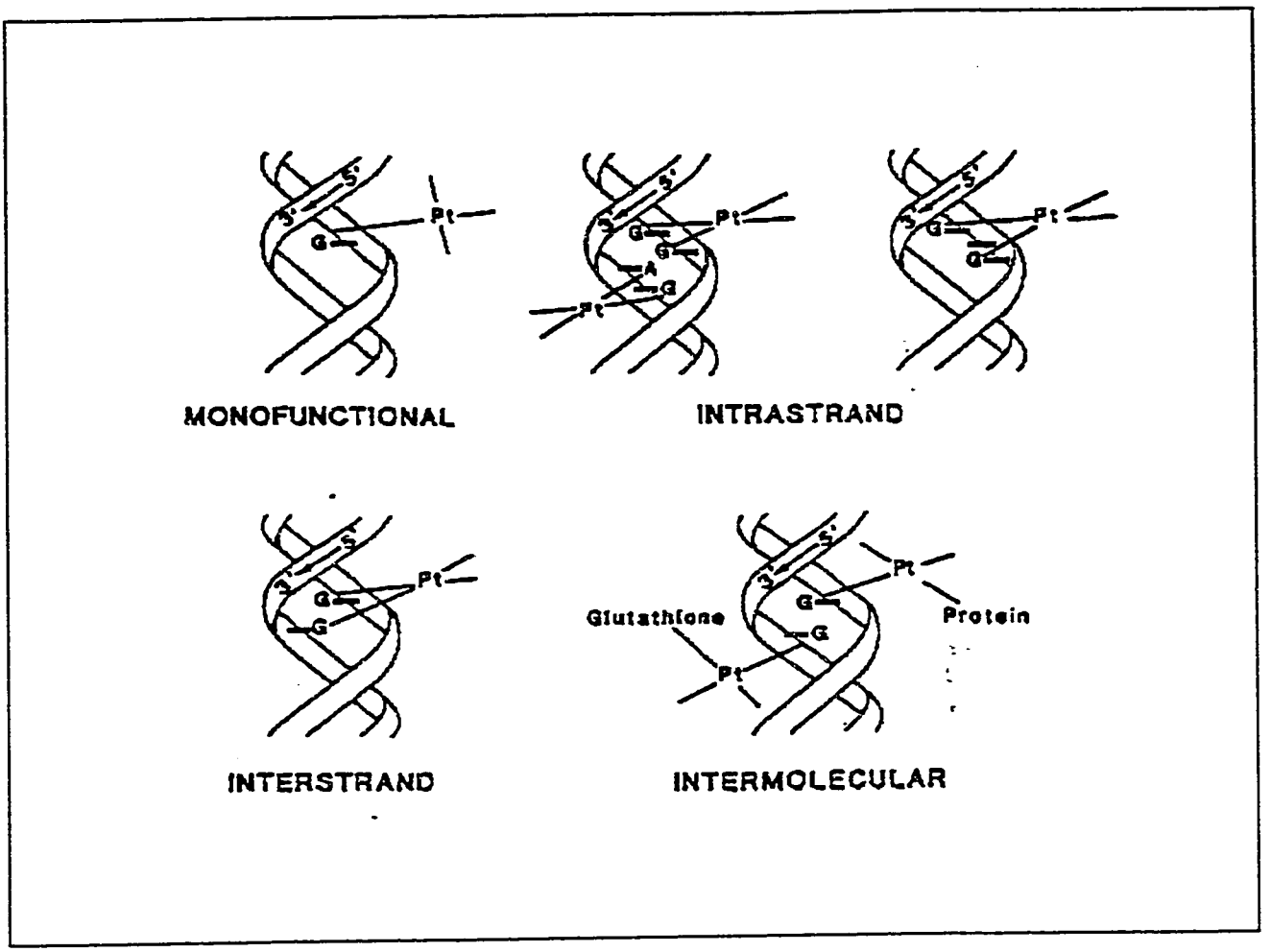


Figure 1-8: Structures of various adducts produced on DNA by cisplatin.

links, DNA monofunctional binding, and interstrand and intrastrand DNA cross-links (Fig. 1.8).

The interactions of cisplatin with DNA have been defined in detail; however, the interactions of this drug with other components of the cell are less well understood.

Quantitative studies show that 1,2-intrastrand d(GpG), and d(ApG) cross-links account for 65 and 25%, respectively, of the cisplatin adducts formed in vitro (G: Guanine; A: Adenine) (Eastman,1986). X-ray diffraction of the cross-linked dinucleotide cis-$Pt(NH_3)_2$(d(pGpG))

reveals that the two guanines are completely destacked. Thus, the intra-strand cisplatin cross-link produces a severe local distortion in the DNA double helix, leading to unwinding and kinking (Bellon *et al.*, 1991).

The extent of cisplatin-pGpG cross-link formation in leukocytes from different cancer patients may differ up to 4.5-fold (Vermorken *et al.*, 1986). Fichtinger-Schepman and co-workers, (1987) also reported a positive correlation between the extent of in vitro and in vivo formation of cisplatin-pGpG cross-links in the DNA of human leukocytes. Inter-individual variation in the level of cisplatin-DNA adducts has also been reported for rat tissues (Reed *et al.*, 1987).

The finding that DNA synthesis is inhibited in a variety of cells following platination has led to speculation that inhibition of DNA synthesis may be the critical step in cisplatin toxicity (Harder and Rosenberg, 1970). Studies of the relationship between cell killing and the binding of cisplatin to DNA in six different mammalian cell lines indicate that the initial level of cisplatin-DNA binding may be a reasonable predictor of sensitivity to this chemotherapeutic drug (Terheggen *et al.*, 1990). However, as I will describe later, this initial amount of binding changes over time; hence, correlations between DNA binding and cytotoxicity would depend on the timing of cisplatin-DNA measurement.

There are several DNA binding proteins that might confer sensitivity to cisplatin. Proteins may shield cisplatin adducts from repair, induce cell cycle arrest, or signal apoptosis (Donahue *et al.*, 1990). The binding of proteins to cisplatin adducts may also enhance blocks to replication or transcription, or may interfere with control systems that induce cell cycle arrest after

DNA damage (Chu, 1994). Clugston and co-workers (1992) have demonstrated the presence of at least two protein complexes in human cell extracts that bind selectively to DNA modified with cisplatin. Using monoclonal antibodies, they have shown that one complex, namely B1, contained human single-stranded DNA binding protein, a protein known to be involved in the in vitro repair synthesis assay of mammalian excision repair. Single-stranded DNA binding proteins have previously been suggested to have a role in the recognition of damaged DNA prior to DNA repair. Such proteins have a higher affinity for DNA modified by chemical adducts, including cisplatin, than for unmodified DNA (Toulme *et al.*, 1983). These proteins could potentially have an important role in cisplatin resistance.

1.4.4 - Cellular Resistance to Cisplatin:

There are questions with respect to how cisplatin gets into cells, how it is transformed and inactivated, how it effluxes from the cell, how the DNA damage is repaired, and how all of these processes can be modulated for therapeutic gain.

Decreased cisplatin accumulation has been a consistent finding in many cisplatin-resistant cell lines from a variety of species. The decreased drug accumulation found in most of these resistant cells could thus be attributed to decreased influx, decreased intracellular binding or sequestration, or increased efflux.

The biochemical change underlying this decreased drug influx is presently unclear. It is assumed that a change in the passive permeability of the plasma membrane is responsible (Popovic *et al.*, 1993) because cisplatin-resistant OV2008 cells do not have the same membrane fluidity as sensitive cells, and some changes in the cellular phospholipid composition were found

(Popovic,*et al.*, 1994). However, if this is true, this should affect both influx and efflux. Timmer-Bosscha *et al.*, (1989) have also found changes in the lipid composition of cisplatin-sensitive versus cisplatin-resistant small cell lung carcinoma cells, although accumulation of cisplatin is the same in these two cell lines. Polyunsaturated fatty acids can sensitize drug resistant cell lines to cytotoxic drugs. With respect to the interaction of cisplatin and γ-linolenic acid (a polyunsaturated fatty acid), the isobologram analyses showed additive toxicity for these agents (Plumb *et al.*, 1993).

Cisplatin accumulation is higher by treatment of cells at higher temperatures in cisplatin resistant as well as sensitive cells. Therefore hyperthermia may be a good resistance modulator when it is possible to reliably increase tumour temperature (Eichholtz-Wirth and Heitel, 1990).

A role for increased DNA repair capacity as a mechanism of cisplatin-resistance has been established. Donahue and co-workers (1990) provided direct evidence for increased DNA repair, showing that cisplatin-resistant cells remove the predominant lesion (the dGpdG intrastrand cross-link) more rapidly than do sensitive cells. These resistant cells can also reactivate a cisplatin-damaged plasmid more readily than can sensitive parent cells (Sheibani *et al.*, 1989).

Kraker and Moore (1988) reported that DNA polymerase beta activity was elevated in cisplatin-resistant P388 leukaemia cells and that these cells also exhibited increased unscheduled DNA synthesis. A DNA-binding protein that recognizes a cisplatin-damaged DNA fragment was described. Whether this protein is involved in DNA repair or altered in cisplatin-

resistant cells is unclear (Chu and Change, 1988).

1.5 - Cell cycle:

The basic mechanism of reproduction in eukaryotic cells is mitotic division. An understanding of the mechanisms that regulate cell division is very helpful in the treatment of diseases that are directly related to this event, e.g. cancer.

Until recently, very little was known about the biochemical or molecular events that precede and regulate mitosis. The cell cycle is defined as the interval between completion of mitosis in the parent cell and completion of the next mitosis in one or both daughter cells. Mitosis is divided into four phases: presynthetic interphase or postmitotic gap (G1), DNA synthesis phase (S), postsynthetic interphase or premitotic gap (G2) and mitosis (M). One of the first and classic papers on the cell cycle is the one by Quastler and Sherman (1959) which introduced these phases.

Scientists had long been aware that dividing cells have lengthy intervals of apparent rest between one mitosis and the next. Only in the past few years have they realized that this interval is not really a period of rest but is, on the contrary, a period of intense activity, during which the cell prepares for mitosis (Baserga, 1985). This finding eventually evolved to the concept of cell cycle checkpoints.

There are also nondividing cells, which can be of two types: G0 cells that are still capable of re-entering the cell cycle; and terminally differentiated cells that are destined to die without dividing. Many cells can not be arrested in G0. This includes Hela cells, most CHO cell

lines (Dethlefsen, 1979), and human ovarian cancer OV 2008 cells.

Completion of the cell cycle requires the co-ordination of a variety of macromolecular syntheses, assemblies, and movements. The chromosomes must be replicated, condensed, segregated, and decondensed. The spindle poles must duplicate, separate, and migrate to opposite ends of the nucleus. Co-ordination of these complex processes is thought to be achieved by a series of changes (phase transitions) in the cyclin-dependent kinases (CDKs). The passage of cells from one stage of the cell cycle to another is tightly regulated by a series of controls that act on the transcription of cyclin genes, the degradation of cyclin genes, the degradation of cyclin proteins, and the modification of the kinase subunits by phosphorylation (Hartwell and Kastan, 1994).

How do checkpoints control cell cycle progression? The simplest model is that the checkpoint prevents the next CDK phase transition until the delayed process (preparation) can be completed or, in case of any damage, until repair is effected. At least two checkpoints detect DNA damage: one at the G1-S transition and one at the G2-M transition. The first one is currently better understood than the other one. Cells with DNA damage rapidly increase p53 protein levels by a post transcriptional mechanism. Induction of p53 results in transcriptional activation of p53-dependent genes such as GADD45, WAF-1 and MDM2 which might cause either cellular arrest in G1 or apoptosis (Sherr, 1993).

Alterations of various components of the cell cycle regulatory machinery that controls the progression of cells from a quiescent to a growing state contribute to the development of many human cancers. Such alterations include the deregulated expression of G1 cyclins, the loss of function

of activities such as those of protein p16INK4a that control G1 cyclin-dependent kinase activity, and the loss of function of the retinoblastoma protein (Rb), which is normally regulated by the G1 cyclin-dependent kinases (Khleif *et al.*, 1996).

The retinoblastoma gene (RB) is the prototype of the tumour suppressor genes, which play critical roles in the genesis of cancer in humans. Rb sequesters the transcription factor of E2F to regulate entry of cells into the cell cycle but enhances the activities of another class of transcription factors, exemplified by NF-IL6, to initiate terminal cellular differentiation. Thus, the Rb protein can serve as a mediator for extracellular signals of growth or differentiation. The fundamental question of why only limited cell types are susceptible to tumour formation when Rb expression is lost remains unanswered at present. It is likely that D-type cyclins are important in the regulation of Rb in the cell cycle (Chen *et al.*, 1995). A role for the cyclin kinase D might be to phosphorylate Rb in order to release E2F to turn on genes required for S phase. The E2F family of transcription factors regulate genes, whose products are essential for progression through the mammalian cell cycle. The transcriptional activity of the E2Fs is inhibited through the specific binding of Rb and the Rb homologs p107 and p130 to their transactivation domains (Girling *et al.*, 1994).

There are some other non-molecular events happening along with the cell cycle progression which are also very important. A good example of these events is the fluctuation in intracellular content of different ions.

The internal pH (pHi) of animal cells is an important physiological parameter, as cells can function only in a narrow range of pHi values. The regulation of pHi is achieved through the action of the Na^+-H^+ ion exchanger (Seifter and Aronson, 1986). Cells have a large negative

membrane potential, which would cause the passive diffusion of H^+ ions across the plasma membrane. Without the extrusion of H^+ ions out of the cells through the action of the Na^+-H^+-exchanger, the cytoplasm of the cells would be too acidic for the cells to function. Through the action of the Na^+-K^+ ATPase, the concentration of Na^+ inside the cell is kept well below the concentration of Na^+ outside the cell. This provides the thermodynamic driving force for H^+ extrusion (Welsh and Al-Rubeai, 1996).

Intracellular alkalization has been linked to cell proliferation and synthetic activity. This has been extensively shown for cells as diverse as yeasts, slime moulds, ciliates and lymphocytes (Nuccitelli and Heiple, 1982). At higher pHi, it has been noted that cells are stimulated from quiescence to activity and propagation. As an example, the rate of protein synthesis would increase following intracellular alkalization, which is important for mitosis and also controls growth-factor induced ribosomal protein S6 phosphorylation and protein synthesis in the G0-G1 transition of fibroblasts (Chambard and Pouyssegur, 1986). On the other hand, Mosgrove *et al.* (1987) showed that acidification of the growth medium to a pH of 6.5 resulted in intracellular acidification and a marked increase in cells entering a quiescence state. The cells seemed to arrest at a particular point in the G1 phase that is pH sensitive. These findings highlight the point that the change in pHi can regulate the entrance into the S phase of the cell cycle. Apparently, the pHi has a crucial role in the regulation of both DNA and protein synthesis and, thereby, progression through the cell cycle (Ober and Pardee, 1987).

Cancer is a disease of the cell cycle.Cancer cells still progress through the four broadly defined phases of the cell cycle. However, some of the controls exerted on progression through

the cell cycle are lost when cells become transformed: in particular the controls at G1/S and G2/M checkpoints (Pines, 1995).

Cell cycle progression differences between cancer cells and normal cells offer a new set of potential targets for chemotherapeutic compounds. Tumours are composed of rapidly dividing cells and these cells have lost at least some of the normal growth controls that regulate progression through the cell division cycle. These concepts have a direct impact on the strategies adopted and methods employed in searching for anticancer therapies (Oliff and Friend, 1997). Agents that can preferentially kill dividing cells should be more effective cytotoxic agents in the treatment of cancer. This aspect of tumour cell physiology was most thoroughly explored by Skipper and co-workers (1964). Many anticancer drugs like alkylating agents (e.g. cyclophosphamide, busulfan) and topoisomerase inhibitors (e.g. etoposide and doxorubicin) take advantage of this fact. These agents target the nucleic acids and proteins which are necessary for cell replication and have more effects on rapidly dividing cells compared to normal cells. Antimetabolites like 6-mercaptopurine and 5-fluorouracil target the DNA synthesis procedure and apparently the S phase of the cell cycle for their antitumor actions. Tubulin-binding agents like vincristine and paclitaxel target the G2/M phase of the cell cycle, as they act on the mitotic spindle apparatus. There are some new drugs that take advantage of the molecular events occurring during the cell cycle. Some of these drugs such as tyrphostins and lavendustins may inhibit cyclin-dependent kinases (Oliff and Friend, 1997).

The concept of targeting dividing cells and cell cycle events as a mechanism for cancer therapy has clearly been validated by these successes. However, while a few molecular factors

in the cell cycle have been targeted, many other events during the cell cycle have been ignored by the majority of investigators, including pHi changes.

1.6 - Apoptosis:

Cell death has been divided into two categories: necrosis and apoptosis (Wyllie *et al.*, 1980). Most animal cells have the ability to self-destruct by activation of an intrinsic cell suicide program when they are no longer needed or have become seriously damaged. The execution of this death program is often associated with characteristic morphological and biochemical changes. This form of cell death has been termed apoptosis. This term, "apoptosis", was initially given by Kerr *et al.* (1972) to morphologic change culminating in cell death by a process clearly distinct from necrosis. The term "apoptosis" derives from a Greek word used for the dropping off of leaves from trees. Apoptosis, although originally thought of as programmed cell death, is now known to be induced by a number of xenobiotic compounds (Boobis *et al.*, 1990).

Apoptosis accounts for a range of biological processes, including differentiation, development, cell maturation, immunologic cell loss that accompanies atrophy of adult tissues following endocrine and other stimuli, and also for the most significant component of the well-established continuous cell loss of most tumors (Kyprianou *et al.*, 1991).

Apoptotic cells shrink in volume, lose contact with their neighbors and lose specialized surface elements such as microvilli and cell-cell junctions. The endoplasmic reticulum dilates and a series of crater-like cavities appear where the dilated cisternae fuse with the cell surface. Initially, and in contrast to cells dying by other means (e.g. necrosis), mitochondria are

normal in structure. The most outstanding internal structural changes, however, occur in the nucleus. Chromatin condenses into dense granular caps under the nuclear membrane (Earnshaw, 1995).

During the cell cycle, the content of p53 increases from G1 to S phase and further to G2 and M phase (Selter and Montenarh, 1994). It has been shown that p53 might be critical for transition from G0/G1 to S (Mercer *et al.*, 1984). The level of expression of wild-type p53 is increased by DNA damage (including that caused by many drugs), which causes G1 arrest in the cell cycle (Fritshe et al., 1993). DNA damage and/or increased levels of p53 cause increased expression of members of the gadd (growth arrest and DNA damage-inducible) and MyD gene families, which have been suggested to play a role in growth arrest and apoptosis (Zhan *et al.*, 1994). In any case, whether or not induction of p53 causes apoptosis or a G1 block (and subsequent DNA repair) seems to be cell type specific (Slichenmyer *et al.*, 1993).

1.7 - Cisplatin cytotoxicity, cell cycle and apoptosis:

In cells that eventually die from cisplatin, total transcription, polyadenylated RNA synthesis, and protein synthesis were found to be markedly inhibited only after 48 hours. Nicotinamide adenine dinucleotide (NAD) and adenosine triphosphate (ATP) levels decreased after 3 days. Cell membrane integrity was lost after 4 days. These results demonstrate that cells can be lethally damaged, yet continue to undergo apparently normal metabolic activities for several days. DNA double-strand breaks are detectable after 1 day, but breaks are visible as fragmentation in the nucleosome spacer region of chromatin after two days. This type of damage

is consistent with cell death occurring by the process of apoptosis (Sorenson *et al.*, 1990). Timing of DNA degradation varies between publications due to different experimental procedures and cell lines.

The analysis of cell death induced by cisplatin reveals DNA fragmentation into multimers of 180 base pairs, consistent with internucleosomal cleavage of chromatin by an endonuclease, followed by loss of membrane integrity and cell shrinkage. This process is inhibited by cycloheximide, suggesting that death requires new protein synthesis. These findings are some more proof of the induction of programmed cell death or apoptosis (Barry *et al.*, 1990).

There is nothing unique about cisplatin that facilitates the activation of apoptosis. However, it is of particular interest that under conditions that kill 90% of the cells, the appearance of DNA degradation is almost always delayed for several days after drug treatment. With cisplatin, this delay is associated with arrest in G_2 phase of the cell cycle. In contrast, following heat treatment, DNA digestion occurs within 30 min. At high concentrations of cisplatin, DNA digestion can be observed within 6 hours. Hence, passage to G_2 is not essential for cell death but may facilitate the process. Indeed, cell death induced by toxic agents often appears to occur during the G_1 phase of the cell cycle (Eastman, 1990). A human ovarian carcinoma cell line showed apoptotic changes in 60% of cells by 72 hours after treatment with 10 uM cisplatin and in 70% of cells after treatment with 25 uM cisplatin. However, this apoptotic cell death was not associated with degradation of DNA into nucleosomal-sized fragments (Ormerod *et al.*, 1994 a & b). On the other hand, indirect immunofluorescence staining of L929 cells confirmed that these cells accumulated nuclear p53 protein 16 hours after exposure to cisplatin (Fritsche *et al.*, 1993).

Electron microscopy of kidney tissue taken from male BDF_1 mice 4 days after i.p. cisplatin showed many lesions characteristic of apoptosis (Montpetit *et al.*, 1994). Variability in results in these different studies may have resulted from either different experimental conditions or differences in methods used to detect apoptosis. Moreover, apoptosis is not the only mechanism of cisplatin cytotoxicity. Necrosis is also involved.

In regard to the timing of toxicity and cell cycle events, the binding of cisplatin to DNA is not in itself sufficient to cause cell death. Cisplatin is much more toxic to dividing cells than to resting cells, suggesting that some cell-cycle associated event is required for its toxicity (Frava and Roberts,1979). Although traditionally it is believed that cisplatin cytotoxicity is not dependent on a specific phase of the cell cycle (Chu , 1994), cisplatin can be up to 10 times more toxic to cells that are about to enter into S phase (Dornish *et al.*, 1987). Cytotoxicity of cisplatin is a function of the amount of DNA damage remaining at the time cells enter S phase (Pera *et al.*, 1981). However, DNA repair-deficient Chinese hamster ovary cells that were highly sensitive to cisplatin were found to progress through S phase at a normal rate following exposure to the drug. The cells then arrested in G_2 and subsequently died. There was no apparent inhibition of DNA synthesis in these cells during S phase (Sorenson and Eastman, 1988). Thus cell death does not correlate with inhibition of DNA synthesis. In contrast, Eastman (1990) has reported that DNA repair-competent cells could survive incubation with higher concentrations of cisplatin that inhibited DNA synthesis and slowed passage through the S phase. These cells also arrested in G_2 following exposure to the drug. It should be emphasized that although G_2 arrest appears to be

a prerequisite for cell death (except at very high drug concentrations), all such arrested cells do not die. At minimally toxic concentrations of cisplatin, cells may eventually bypass the block and return to normal cycling. Hence, there are two fates for a G_2-arrested cell: survival or death. While few publications have addressed the cell cycle dependency of cisplatin cytotoxicity, some very recent reports on this subject have resulted from the study of cisplatin-induced apoptosis.

2

Background and Hypothesis for this Project:

2.1 - Background:

Generally, it is believed that cisplatin is not a cell cycle specific agent; however, it is more effective in the treatment of solid tumours than in haematological malignancies. Solid tumours are believed to have a higher proportion of cells in G1 or G0 phases of the cell cycle than do haematological cancers (Kastan, 1997). Some early reports have claimed that cisplatin is more toxic to cells in G1 than in any other phase of the cell cycle. However, this has not been the finding of all investigators. It has also been reported that cisplatin has a different degree of toxicity to cells in plateau phase compared to exponentially growing cells (Frava and Roberts, 1979). Although the cell cycle specificity of cisplatin cytotoxicity has not been well investigated, some recent papers have claimed that cells have different sensitivity to cisplatin-induced apoptosis in different phases of the cell cycle. These few reports are controversial, and different investigators have indicated that different phases of the cell cycle (G1 [Roberts and Fraval, 1980], S [Demarcq *et al.*, 1994] and/or at the boundary of G1/S [Dornish *et al.*, 1987]) are the most sensitive phase to cisplatin-induced apoptosis. I am not aware of any publications offering a

detailed explanation for cell cycle specificity of susceptibility to cisplatin-induced apoptosis. This question is examined in this thesis, using the OV 2008 human ovarian cancer cell line. This cell line has been selected for these studies since cisplatin is one of the most important drugs in the treatment of human ovarian carcinoma and since the OV 2008 cell line was readily available to us. It is my feeling that the information gained might suggest specific new cytokinetically guided approaches to cisplatin-based combination chemotherapy.

2.2 - Hypothesis:

The sensitivity of the human ovarian carcinoma OV 2008 cell line to cisplatin varies as a function of the phase of the cell cycle. These differences are related to the DNA-binding of cisplatin, which in turn is related to the conversion of cisplatin to a more potent DNA-binding metabolite. Intracellular transformation of cisplatin varies with intracellular biochemical changes in different phases of the cell cycle.

From the chemistry point of view, the reactions that would be expected to take place during cisplatin cytotoxicity would be as follows (Lippard, 1994):

1) cisplatin (Cp) + $H^+ \rightarrow$ Aquated cisplatin (Ap)

2) Ap + DNA $\rightarrow$ DNA-Pt

3) DNA-Pt $\rightarrow$ cell cycle arrest and death

Step 1 would be expected to be limited by the availability of hydrogen ions, but would be expected to be very rapid (Reishus and Martin, 1961). Since DNA is a rich pool of nucleophilic targets for the electrophile, aquated cisplatin (Ap), it would be expected that step 2

above would also be quite rapid (Rosenberg and Van Camp, 1970). Therefore, for a cell with a given intracellular hydrogen ion concentration, I would expect that the rate-limiting step for cytotoxicity would be cisplatin penetration into the cell.

In the comparison of sensitive vs resistant cell lines, there are many publications that indicate that the resistant line has reduced net cisplatin accumulation (Popovic, *et al.*, 1994; Timmer-Bosscha *et al.*, 1989; Plumb *et al.*, 1993; Eichholtz-Wirth and Heitel, 1990). It remains uncertain whether this reduced accumulation is due to reduced cisplatin influx uptake or to increased efflux.

However, the mechanism of this slowest step is unknown. In spite of some reports on the active transport of cisplatin (Andrews *et al.*, 1988; Smith and Brock, 1989; Andrews and Howell, 1990) the general belief is that cisplatin enters the cell through passive diffusion (Gately and Howell, 1993). A double reciprocal plot of platinum accumulation vs drug exposure concentration yields a straight line through the origin, indicating that the rate limiting factor for platinum uptake in cisplatin sensitive cell lines is the concentration of drug and therefore is likely passive diffusion (Gale *et al.*, 1973; Binks and Dobrota, 1990).

In the comparison of different cell lines to distinguish active from passive transport, in which the intracellular hydrogen ion content might be different, low intracellular pH would favour active diffusion of a charged aquated species, while high pH would favour passive diffusion of a neutral hydroxyl species. Hence, in some resistant cell lines, high intracellular pH might lead to a reduction in both cellular platinum accumulation and cisplatin DNA binding by reducing the formation of charged aquated species. Since cell membrane transport is likely to be

the same for a given cell line at the different phases of the cell cycle, I would assume that the intracellular conversion of cisplatin to its more active aquated form is more important in its level of cytotoxicity at the different phases of the cell cycle.

2.3 - Experimental design and controls:

To elaborate on my idea, I would like to take advantage of the chemistry of cisplatin as was described in chapter one. Cisplatin needs to be converted to its aquated form to bind to the DNA as its main mechanism of cytotoxicity. Acidic pH favours the formation of aquated cisplatin. Therefore, lower intracellular pH should be able to convert cisplatin to its more active form of aquated cisplatin. In case of the different phases of the same cell line (human OV 2008 cells), any possible changes in pH during the cell cycle will result in alterations of formation of the highly active species of aquated cisplatin, and this will cause more cisplatin DNA adduct formation and cytotoxicity for cells in G1 versus S and or G2/M. Therefore, I need to show that DNA binding of cisplatin will increase as pH drops. The question is then whether or not a minor variation in pH between G1 cells and G2/M cells would be enough to account for the significant differences in platinum DNA binding? The following chemical equations and stoichiometric calculations are designed to address this question:

cisplatin (Cp) + $H^+ \rightarrow$ Aquated cisplatin (Ap)

For each 0.1 unit difference in the mean intracellular pH of cells from the most acidic state to the least acidic form at the different phases of the cell cycle, the pH is equal to $10^{-0.1}$ M. With the assumption of a constant rate for the above reaction (k), the following equation will

represent the ratio of aquated cisplatin (Ap) to the parent drug (Cp) at any given pH:

$k = [Ap] / ([Cp] [H^+]) \Longrightarrow [Ap]/[Cp] = k [H^+]$

For the different pH levels, one may calculate different ratios of [Ap]/[Cp]. Taking "A" as the ratio in an acidic environment and "B" for a basic environment, "A/B" will express the relative production of aquated cisplatin at any two given pH levels. Therefore, a correlation between this ratio and the ratio of accumulation or cytotoxicity of cisplatin at the different phases of the cell cycle is necessary to address my idea.

To test the predictions of this hypothesis, it is first necessary to correlate cisplatin cytotoxicity, rate of cisplatin interaction with DNA and cell cycle phase of OV 2008 cells. Then the cytotoxicity and cisplatin DNA binding during different phases of the cell cycle must be determined. Next, the intracellular pH must be measured as a function of cell cycle phase, since the conversion of parental cisplatin to its highly reactive aquated form should substantially increase its DNA binding. On the other hand, since this conversion is pH dependent the changes in pH with progression through the cell cycle must be followed to determined if any observed alterations in cisplatin DNA binding and cytotoxicity were cell cycle phase specific. These studies were intended to clarify the cell cycle dependency of cisplatin cytotoxicity and to provide a possible mechanism for such a cell cycle dependency.

To undertake all these control conditions, the following experiments were set up:

1) As a preliminary step in examining whether cisplatin cytotoxicity is cell cycle dependent, the cisplatin cytotoxicity in plateau needs to be compared to exponentially growing cells, since cell cycle phase distribution would be expected to be different in these two populations.

2) Next, the cytotoxicity of cisplatin needs to be observed in different phases of the cell cycle, to see any differences in the comparison of cisplatin cytotoxicity in the plateau phase vs exponentially growing cells.

3) The mechanism of cell death (apoptosis vs necrosis) must be established and its variation as a function of cell cycle phase of cisplatin exposure is required. The idea is as follows: OV 2008 cells die either by apoptosis or necrosis. Presumably, different mechanisms are responsible for each of these two types of cell death. Looking at the differences in the percentages of apoptosis vs necrosis induced by cisplatin in each subpopulation could provide clues as to whether or not the mechanism of cytotoxicity is different for different subpopulations.

4) Cisplatin cellular accumulation as a function of cell cycle phase is also required since higher intracellular concentrations might cause greater cytotoxicity. Since cisplatin accumulation at the site of its action might be expected to be more important than total intracellular cisplatin accumulation, DNA cisplatin content as a function of cell cycle phase must also be measured.

5) It is also necessary to observe whether cisplatin DNA binding could correlate with cisplatin cytotoxicity in different phases of the cell cycle despite the fact that different cell cycle phases could have comparable total intracellular platinum content. This will show whether cell cycle specificity of cisplatin correlates to the variability in cisplatin DNA binding from one phase to the next of the cell cycle, and in turn to cell cycle specific variations in intracellular pH.

3

Experimental Procedures and Results:

3.1 Experimental Procedures:

A variety of methods have been used in different parts of this project to answer different questions. The following methods have been used in this project: standard cell culture practices, clonogenic assays, flow cytometry, UV/visible spectrophotometry, atomic absorption spectroscopy, HPLC, light, electron and fluorescence microscopy, BioRad protein assay, cell fractionation techniques, and others. Throughout the project, the following points apply, unless specified otherwise:

a) All experiments were done in triplicate. Results are reported as mean ± standard error (N=3), unless specified otherwise.

b) OV 2008 cells were grown in RPMI 1640 media supplemented with 10% fetal calf serum (FCS) and incubated in 5% CO_2 at 37° C.

c) In all experiments, drug exposure has been with the one hour exposure of cells in RPMI media to 1 μg/ml of cisplatin at 37° C.

d) Statistical analysis of the results has been done using paired t-tests and/or ANOVA.

e) The term "subpopulation" throughout this thesis means either cells in exponential *vs* plateau phase of growth, or cells synchronised in different cell cycle phases (G1, S, G2/M or at the boundary of G1/S).

3.1.1 - Materials:

3.1.1.1 - Chemicals: Clinical formulation of cisplatin 1 mg/ml in 0.9% saline was obtained from Horner Laboratories (Montreal, Canada). Different concentrations of cisplatin were formed by diluting the clinical cisplatin formulation with 0.9% saline. RPMI media with glutamine, fetal calf serum and trypsin were purchased from Gibco BRL Laboratories (Burlington, Canada). Fetal calf serum was inactivated with heat (56° C for 30 min.) to inactivate the possible active serum proteins involved in proteolytic cleavage reactions. This serum was added to the media in 1:10 ratio prior to experiments. RPMI media containing 10% FCS was kept in the fridge as storage, then heated to 37° C before each experiment.

Phosphate buffered saline was purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada), as 10X solution and was diluted to be used for different experiments with double distilled water.

Standard crystal violet stain for visualization of cell colonies was supplied by Sigma-Aldrich Canada Ltd. and was dissolved as 0.2% w/v in 70% ethanol.

Ethanol, phenol, chloroform, glacial acetic acid, Absolute ethanol (Minimum assay with GLC equals to 99.7%), HPLC grade methanol, and HPLC grade acetonitrile were supplied from BDH Inc. (Toronto, Ontario, Canada) for the different experiments.

The following chemicals for various experiments were supplied by Sigma-Aldrich Canada Ltd.: propidium iodide, trypan blue, thymidine, glutaraldehyde, spurr kit and spurr resin, uranyl acetate, acridine orange, NaOH, $MgCl_2$, NaCl, Nonidet p-40, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Tris-HCl, $CaCl_2$, KCl, sucrose, glycerol, ethylenediamine tetra acetic acid (EDTA), KH_2PO_4, lauryl sulfate sodium (SDS) and dimethyl sulfoxide (DMSO).

RNAse and DNAse were purchased from Promega corporation (Madison, WI, USA). Bovine serum albumin (BSA) was purchased from Gibco BRL Laboratories (Burlington, Canada).

Bio-Rad kit for the measurement of protein was supplied from Bio-Rad Laboratories (Bio-Rad Laboratories, Life Science Group, CA, USA). The dye was diluted 10 times and filtered before experiments.

Carboxy-SNARF-1-AM and nigricin were supplied from Molecular Probes, Inc. (Eugene, OR, USA).

ET buffer was prepared with 10 mM Tris-HCl, pH=8 and 1 mM EDTA and was stored in a cold room.

The following formulations were used for the different buffers throughout this thesis:

a) Cell lysis buffer: 10 mM Tris-HCl, pH 7.5 + 1.5 mM $MgCl_2$ + 10 mM NaCl + 1% Nonidet P-40 + 1 mM PMSF + 1 mM DTT and double distilled water up to the volume (10 ml)

b) TKCM buffer: 50 mM Tris-HCl, pH 7.5 + 25 mM KCl + 2 mM $CaCl_2$ + 5 mM $MgCl_2$ + 1 mM PMSF + 1 mM DTT and double distilled water up to volume (10 ml).

c) TKM buffer: 50 mM Tris-HCl, pH 7.5 + 25 mM KCl + 5 mM $MgCl_2$ + 1 mM PMSF + 1 mM DTT and double distilled water up to the volume (10 ml).

d) TKM + sucrose: TKM buffer + 0.25M sucrose.

e) TKCM+ sucrose: TKCM buffer + 0.6 M sucrose.

f) Nuclear lysis buffer (NLB): 80 mM Tris-HCl, pH 7.5 + 2 mM EDTA + 0.53 M NaCl + 20% glycerol + 1 mM PMSF + 1 mM DTT.

3.1.1.2 - Cell line: The human ovarian cancer OV 2008 cell line was a gift from Dr. Stephen B. Howell, San Diego, USA. This cell line was originally derived from an untreated patient (Disaia *et al.*, 1972). The cells were grown on tissue culture dishes in an incubator at 37° C with a humidified atmosphere of 5% CO_2 and 95% air.

3.1.1.3- Instruments: The following instruments were routinely used for the cell culture and other experiments: Forma Scientific CO_2 incubator from Fisher Scientific Ltd, (Ottawa, Ontario, Canada), H.E.P.A. filter Clean Air System hood from Canadian Cabinet Company Limited (Ottawa, Ontario, Canada), a Mega-Pure, MP-3A Corning distillator system from Leigh Ryckman Company, (Ottawa, Ontario, Canada), WILD Heerbrugg M40-580 light microscope (Ottawa, Ontario, Canada), Beckman CS-16 R centrifuge, from Beckman Instruments, Inc. (Palo Alto, California, USA), Mettler HK 60 balance made in Greifensee, Zurich, and an Olympus SZ-STS light microscope for colony counting.

For the purpose of fluorescence microscopy, an Olympus CK2 microscope was

equipped with a green fluorescence light source. A Hitachi 7100 Transmission electron microscope (Hitachi Inc., USA) has been used for electron microscopy.

A Cary-1E, Varian UV-Visible spectrophotometer (Varian Canada Inc., St-Laurent, QU, Canada) and a Philips PYE UNICAM PU 8610 UV/VIS kinetics spectrophotometer were used for experiments.

An Epics XL Coulter Counter flow cytometer was used for the flow cytometry experiments (Coulter Electronics of Canada, Ltd., Burlington, ON, Canada). 488 nm excitation and 520 nm emission have been used for propidium iodide (PI) staining of cells. For the purpose of intracellular pH measurement, the excitation was set at 488 nm, while two emissions of 575 nm and 620 nm were used simultaneously and the ratio of the absorbances at these two emissions were calculated. Flow cytometry data were processed using MultiCycle AV software supplied with the instrument.

Atomic absorption spectroscopy was performed using a Varian Techtrom AA-1475 spectrophotometer coupled with a GTA-95 pyrolitically coated graphite tube atomizer. The spectrophotometer was operated at an excitation wavelength of 265.9 nm. The three-stage temperature program consisted of drying at 95° C for 50 seconds, ashing at 1400° C for 20 seconds, and atomizing at 2700° C for 3 seconds.

For high performance liquid chromatography, Waters HPLC equipment was used. This system was equipped with a model 510 pump and a model 6000 pump for the gradient flow of mobile phases, a U6K manual injector, an 8x10 RCM module with a C_{18} cartridge column, a Guard-PAK precolumn module with a C_{18} precolumn and a Retriever II fraction collector.

3.1.2- Methods:

3.1.2.1- Cell culture: Human ovarian OV 2008 cell line has been used for these experiments. The cells were grown on tissue culture dishes in an incubator at 37° C with a humidified atmosphere of 5% CO_2 and 95% air. The cells were maintained in logarithmic growth in complete medium consisting of RPMI 1640 containing 10% heat-inactivated fetal calf serum.

For the assay of OV 2008 growth curves (Baserga, 1995), 10^4 cells were seeded in 25 cm^2 cell culture flasks with 5 ml RPMI 1460 media. Three flasks were trypsinized each day and the number of cells were counted using a haemocytometer and trypan blue staining. In two different sets of experiments, cells for which the media was changed to fresh media at 6 days ("fed cells") were compared to cells which did not undergo a change in media ("non-fed cells"). Doubling time was calculated from the fitted line through the exponential phase of growth.

3.1.2.2- Cytotoxicity assay: Of the several different methods available for the assessment of cytotoxicity, I selected the clonogenic assay. Most other cytotoxicity assays (such as procedures like 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue (MTT), 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) and/or trypan blue staining) are based on particular aspects of cellular physiology. As an example XTT and MTT look at the functioning of cellular reducing enzymes (Lamontagne *et al.*, 1994) and trypan blue measures the health of cell membranes (Cole, 1986). Cells might still be alive or in a repair period in spite of some problems with their enzymatic systems, and/or they might be dead in spite of still functioning enzymes in the extracellular matrix. The clonogenic assay, on the other hand, assesses cellular health in general, and particularly their propagation capability. The percentage

of live colonies is determined about two weeks after drug exposure.

Clonogenic assay has been used for this purpose based on the method of Dornish *et al.*, 1987. In this experiment, cultured cells were exposed to 50 µl of different concentrations of cisplatin for one hour at 37°C. These dilutions of cisplatin were prepared from the clinical formulation of cisplatin in saline. Medium was removed and cells were washed with PBS three times before trypsinization. 300 to 800 cells from each flask (depending on the concentration of cisplatin to which the cells had been exposed) were seeded in each of three other cell culture petri dishes with 5 ml of fresh media. These flasks were incubated for 10 to 14 days and colonies were counted following crystal violet staining for a better visibility of colonies. Three control flasks followed the same procedure with the addition of 50 µl saline instead of cisplatin, for each set of experiments.

In the comparison of cisplatin cytotoxicity in plateau versus exponentially growing cells, flasks of cells on days 2 (as exponential population) or 10 (as plateau population) were used.

In the comparison of cisplatin cytotoxicity in the different phases of OV 2008 cell cycle, subpopulations of cells in each phase were used.

3.1.2.3- Flow cytometry: To study the DNA content and cell cycle kinetic status of OV 2008 cells using PI staining, about $2x10^6$ cells were suspended in 2 ml PBS. Cell suspensions were added into 5 ml ice cold 70% ethanol, drop-wise. To reduce clumping of cells, ethanol was vortexed while adding the cells. Fixed cells in ethanol were kept in -20° C freezer until the day of flow cytometry assay. On the day of assay, cells were centrifuged and ethanol was aspirated.

Cells were resuspended in 1 ml of PBS containing 100 µl of RNAase (100 µg/ml) and incubated for one hour. 5 µg/ml of propidium iodide (PI) was added and the cell suspension was transferred to a flow cytometry machine after about 30 min (Ormerod *et al.*, 1994-b). The resulting histograms were analysed using the Multicycle AV program.

In the study of cisplatin effects on OV 2008 cells using flow cytometry, different subpopulations of cells were exposed to 1 µg/ml cisplatin for one hour, then washed and replated in fresh medium. Cells were collected at 0 hours (ie, right after termination of cisplatin exposure and addition of fresh media), and at 3, 6, 12, 18, 24, 36, 48, 72 and 96 hours. Both attached and floating cells were collected at each time point. Trypsinization was used in the collection of attached cells.

3.1.2.4- Synchronization: Determination of the duration of cell cycle phases of OV 2008 cells was necessary for synchronization. To design any method of cell synchronization for any given cell line, an understanding of the duration of each phase of that particular cell line is mandatory. The most common method in the determination of the duration of the various phases of the cell cycle is to use of [^{3}H]thymidine and fluorescence microscope (Baserga and Malamud, 1969). The method is based on the addition of [^{3}H]thymidine to exponentially growing cells on a cover slip, usually about 48 h after plating. After 30 min, media is exchanged for normal, non-radioactive growth media. At various intervals after the removal of [^{3}H]thymidine, groups of cover slips are washed and fixed for autoradiography. As the time interval between removal of [^{3}H]thymidine and fixation of cells increases, the percentage of labelled mitoses increases rapidly to 100%. The percentage of labelled mitoses remains near 100% for a time that is roughly

equivalent to the duration of the DNA synthesis phase of the cell cycle (S phase). It then drops and the percentage reaches a low point in G1 phase. The determination of the various phases of the cell cycle is based conventionally on the 50% points between these increase and decrease points. This method was not possible in our lab because of our limitation in the use of radioactive materials. Besides, a very simple flow cytometric method is also available. Hence, the flow cytometric method of Baserga (1995) was used for this purpose.

In summary, three groups of flasks containing either 10^4, $5x10^4$ or 10^5 cells were seeded in 5 ml RPMI. Every day for up to three days, three flasks of each group were trypsinized and cells were collected. Cells were washed with PBS and fixed in 2 ml of 70% alcohol. Cells were then exposed to propidium iodide for flow cytometry. Percentages of cells in G1, G2/M and S were calculated for each group of cells on each day. Duration of each phase of the cell cycle was calculated using the percentage of cells in each phase (for each group on each day) and the doubling time of OV 2008 cells (from the growth curve, as is described before), in a way that the doubling time was divided into various periods of time based on the percentages of each corresponding cell cycle phase.

Using these data, synchronization was performed in exponentially growing OV 2008 cells (day 2 after seeding in a fresh media). Media was replaced with RPMI containing 0.1% FCS for 17 hours, then the media was replaced with RPMI containing 20% FCS for another 12 hours. At this point, cells were exposed to 2 mM thymidine in RPMI media containing 0.5% FCS for 16 hours. The rational of these steps in the synchronization of OV 2008 cells is described in the results section.

OV 2008 cells were collected every 15 minutes after relase from thymidine block, and the following times were selected for the collection of different subpopulations: time 0 for G1/S, time 3.5 h for S, time 10 h for G2/M and time 20 h for G1 cells.

3.1.2.5- Electron microscopy: General electron microscopy practice has been used for this experiment (Falcieri *et al.*, 1994) Subpopulations of cells were either selected as controls, or exposed to 1 μg/ml of cisplatin for one hour. Cells were then incubated in fresh media at 37° C. 48 hours after exposure, attached or floating cells were collected, washed in PBS and fixed in 1.6% glutaraldehyde in 0.1 M cacodylate buffer at 4° C for 45 min, and centrifuged to obtain cell pellets. Cell pellets were post-fixed in 1% sodium tetroxide for one hour, dehydrated through a graded ethanol series and then infiltrated as well as embedded in spurr resin. 60 nm sections were stained with uranyl acetate and lead citrate (Falcieri *et al.*, 1994), and examined by a Hitachi 7100 Transmission electron microscope.

3.1.2.6- Double dye staining: To distinguish between apoptotic and necrotic cells a double dye staining procedure was developed based on the previously published methods (Mpoke and Wolfe, 1997). Subpopulations of cells were exposed to 1 μg/ml cisplatin for one hour. Drug was washed out, and after the addition of fresh media, cells were returned to the incubator. At different intervals up to 96 hours, attached and floating cells were then collected separately. These cells were counted with the help of trypan blue. A drop of cell suspension was placed on a microscope slide and mixed with a drop of PI solution (50 μg/ml) and a drop of acridine orange (AO; 5 μg/ml). Cells were mixed well and observed under a fluorescence microscope. Under the microscope, living cells were bright yellow (the fluorescence appearance of AO bound on DNA),

while necrotic cells were red (the fluorescence colour of PI bound to DNA) and apoptotic cells had a granular green appearance. Both the colour and the granularity of apoptotic cells helped differentiate them from healthy cells under the microscope. Cells of each type were counted and the percentage of each of apoptotic, alive and necrotic cells were calculated.

3.1.2.7- Cellular platinum accumulation: Subpopulations of OV 2008 cells were exposed to 1 μg/ml cisplatin for one hour. Cells were washed with PBS and fresh media was added at the end of the one hour cisplatin exposure. Setting the time of cisplatin addition to the media as time zero, flasks of cells from each group of synchronized cells were washed rapidly three times with cold PBS. Control experiments showed that no measurable cisplatin remained in the extracellular environment of the flask following this method of washing. Cells were trypsinized and collected in 1 ml of RPMI at the following times: 0, 10, 20, 30, 40, 50, 60 (end of cisplatin exposure), 65, 75, 90, 105, 120, 150, 180, 210, 240 and 300 minutes. 500 μl of each sample was collected for protein assay, and the rest of the sample was collected for the measurement of elemental platinum by atomic absorption (AA) spectroscopy (Preisner *et al.*, 1981). A standard curve of platinum concentration versus absorbance was generated with the injection of 20 μl of known concentrations of cisplatin. Each determination of elemental platinum in unknown samples was based on the absorbance average of triplicate injections of 20 μl of any given sample into the AA spectroscope and comparison with a standard curve.

The protein assay was performed using the BioRad protein assay kit (Macart *et al.*, 1994). In summary, 500 μl of cell suspension was exposed to the same volume of molar NaOH for 8 hours. 50 μl of the resulting suspension was added to 5 ml of the BioRad diluted dye and

the absorbance was measured at 595 nm. Total amount of protein was calculated compared to a known amount of bovine serum albumin (BSA) solution in water. In a set of control experiments, OV 2008 cells synchronized in different phases of the cell cycle were examined for the total amount of protein per 10^6 cells.

Since cisplatin exposure ended at 60 minutes, samples from the first 60 minutes represented the uptake phase, the sample at 60 minutes gave the cisplatin accumulation right after exposure, and the rest represented the efflux curve of cisplatin from cells. The platinum concentrations were calculated as nanograms of platinum per μg protein for cells in each phase of the cell cycle, after correction based on the relative amount of protein in each phase of the cell cycle.

3.1.2.8- Platinum assay in cell fractions: Subpopulations were exposed to 10 μg/ml of cisplatin for one hour at 37° C. A higher concentration of cisplatin was used for this assay due to the sensitivity limits of atomic absorption spectroscopy in the measurement of elemental platinum in each fraction of cells. After one hour exposure, cell fractions were isolated using a modification of the method of Duguet *et al.* (1983).

Cells were trypsinized and collected after washing 3 times with ice cold saline. A sample was taken, cells were counted and the total protein amount was measured, as is described above. Cells were lysed in cell lysis buffer (composition of this buffer is mentioned in section 3.1.1.1) by gently resuspending them, leaving them for 2 min on ice, then homogenizing with 10 strokes with a dounce homogenizer. The suspension was centrifuged at 1000 RPM (120 xg) for 10 minutes, and the supernatant was collected. The nuclei (contained in the precipitate) were

resuspended in 2 ml TKCM buffer and layered over 0.6 ml of 0.25 M sucrose with an added cushion of the same buffer. They were then centrifuged at 800 RPM (75 xg) for 15 minutes and the supernatant was collected. The precipitate was washed, centrifuged, and supernatant was again collected. All of the supernatant fractions from the above steps were added together as cell cytoplasm. The nuclei (in the final precipitate) were resuspended in TKCM buffer, and 20 µl of 0.2 M EDTA and 0.44 ml nuclear lysis buffer (composition of this buffer is mentioned in section 3.1.1.1) were added while stirring gently for 60 min on ice. This suspension was centrifuged for 10 min at 800 RPM (75 xg) and the supernatant was collected. Precipitate was resuspended in phenol/chloroform (1:1 v:v), vortexed and centrifuged (1000 RPM [120 xg] for 10 min). The upper viscose layer was collected and the lower phenol/chloroform layer was added to the previous supernatant as cell nucleoplasm. DNA in the upper viscose layer was precipitated with the help of two volumes of absolute ethanol on ice for an hour, spun down with a microcentrifuge and precipitated. DNA precipitate was dissolved in 500 µl of Tris-EDTA buffer before storage. About 100 µl of this solution was taken for the measurement of DNA purity using a UV-Visible spectrophotometer, based on the Warburg-Christian Concentration Assay (Sambrook *et al.*, 1989).

Those samples with purity of 1.6-2 were accepted for the measurement of platinum on DNA. The platinum levels of cytoplasm, nucleoplasm and DNA fractions of cells were measured by atomic absorption spectroscopy, as described above. Total cellular protein was used as a correction factor for the measurement of elemental platinum in cytoplasm or nucleoplasm fractions. Concentration of DNA from each sample was used as a correction factor for the

measurement of pg of platinum per μg of DNA.

3.1.2.9- Intracellular pH: To directly measure intracellular pH of OV 2008 cells synchronized in different phases of the cell cycle, I have used a modified method of Welsh and Al-Rubeai (1996).

In summary, subpopulations of cells in G1, G1/S, S and G2/M were spun down and resuspended in 2.5 ml of RPMI media. 50 μg of a PH-sensitive probe consisting of carboxy-SNARF-1-AM (seminaphtorhodafluor-1-acetoxymethylester) was diluted in 150 μl of DMSO, of which 20 μl was added to the cell suspension. Cells were spun down after a 30 minute incubation, and the pink pellet was washed with RPMI media and then PBS. The final precipitate of cells was resuspended in PBS for the measurement of intracellular pH. In a parallel experiment, the final precipitate of cells was resuspended in a range of different buffers at different pHs (from 6 to 8) and 2 μg/ml of nigricin to allow the equilibration of internal and external pH values for construction of a standard curve. Suspensions were transferred to flow cytometry tubes and the emission of SNARF-1 at two different wavelengths of 620 and 575 nm was measured, with excitation at 488 nm. Basically, SNARF-1 enters the cell by passive diffusion as a nonpolar ester. Once in the cytoplasm, it is hydrolysed by esterases into a polar compound that cannot pass through membranes easily, and so it accumulates inside the cell. SNARF-1 shows large changes in pH-dependent fluorescence. When excited at 488 nm by an argon laser so that the fluorescence ratio of basic and acidic forms of SNARF-1 can be measured, the ratio of its emissions at two different wavelengths will change with pH.

This experiment was done six times and the result is as mean ± standard deviation of

these six numbers.

3.1.2.10- HPLC fractionation: A high performance liquid chromatographic (HPLC) method in conjunction with atomic absorption spectroscopy has been used for the measurement of cisplatin transformations in different pHs. HPLC studies were based on a published method for the separation of platinum complexes (Mauldin *et al.*, 1989).

The pH of the clinical formulation of cisplatin was adjusted to different points in a range from 4 to 9 using Tris buffer. The pH values of 4 and 9 were selected as controls for the transformation of cisplatin in acidic and basic environments, respectively. 100 µl of each solution was injected into the HPLC system and 40 fractions of waste (from the column) were collected at the rate of 1 ml/min. 20 µl of each fraction was assayed by atomic absorption spectrometry for elemental platinum content.

A gradient system consisting of two mobile phases was used for HPLC separation of cisplatin species at different pHs. Mobile phase A was a 1 mM solution of 1-heptanesulfonate, pH 3.4 (with the pH being adjusted with glacial acetic acid) and mobile phase B was 90% methanol in double distilled water. The elution profile on the gradient HPLC system was 10 min of buffer A, a 30 min linear gradient from A to B, and 10 min of B at a flow rate of 1 ml/min. The column was reequilibrated for 60 min with buffer A at 1 ml/min prior to the next injection.

3.1.2.11- Cisplatin binding on genomic DNA: Genomic DNA was extracted from OV 2008 cells using lysis buffer made up of 10mM Tris-HCl at pH 8, 10 mM EDTA, 75 mM NaCl and 0.5% SDS (Ormerod *et al.*, 1996). 1.25 ml of lysis buffer was added to 100 µl of cell suspension, mixed gently and allowed to incubate at room temperature for 15 min. Cells were spun down in

an Eppendorf tube with a microcentrifuge at 13,000 g for 15 min at room temperature. The cell pellet was discarded and 100 μg/ml of RNAse was added and incubated at 37°C for 30 min. After this step, cells were incubated for another 30 min with the addition of 100 μg/ml of proteinase K at 50°C. DNA was then precipitated with 50% ethanol and 0.5 M NaCl overnight at -20°C. Eppendorf tubes were microcentrifuged the next day at 4°C for 15 min, ethanol was discarded and the pellet of genomic DNA was allowed to dry. After the measurement of the purity and amount of DNA with a UV-visible spectrophotometer as was described before, the DNA was incubated with cisplatin solutions at various pHs from 7 to 7.5 for one hour at 37°C. DNA was precipitated and washed with water 3 times. The DNA was dissolved in ET buffer and prepared for injection into the atomic absorption spectroscope. 20 μl of each sample was assayed for platinum by atomic absorption spectrometry.

3.1.2.12- Statistics: Statistical analysis of the results has been done using the GraphPad Instat TM V2.03 software (GraphPad Software, San Dieago, CA, USA).

3.2- Results:

3.2.1- Growth and cytotoxicity: Cells in culture typically show a sigmoidal pattern of proliferative activity that reflects adaptation to culture, conditioning of environment, and the availability of physical factors (e.g. space) and nutrient supply necessary to support a healthy life and the production of new cells (McAtter and Davis, 1994). It was necessary to make sure of this point before starting any other experiment. As with many other healthy, growing cells, the growth curve of OV 2008 cells presents two distinguishable parts: an exponential growth phase, in which cells are actively propagating, and a plateau phase in which the number of cells per unit of flask surface does not change significantly.

Figure 3.1 represents the growth curve of OV 2008 cells in RPMI media. Each point is the mean $\pm$ standard error of three sets of experiments, each done in triplicate. Fed cells continued their growth and reached a plateau level after about 9 days, while non-fed cells died around day 12. Using this graph, the doubling time of exponentially growing OV 2008 cells was calculated to be 24 hours. This pattern of growth confirms normal physiological status of OV 2008 cells in my experimental conditions. The rate of proliferation of non fed OV 2008 cells slows down and levels off as the cell population attains confluency. However, fed cells still show some degree of mitotic activity at confluency, which would balance cell death and keep the number of live cells almost constant. These cells will continue to divide and pack more tightly until about day 12, when daughter cells start to detach and release into the overlying medium.

Plateau cells accumulate mainly in G1 (and even G0 in some other cell lines) of the

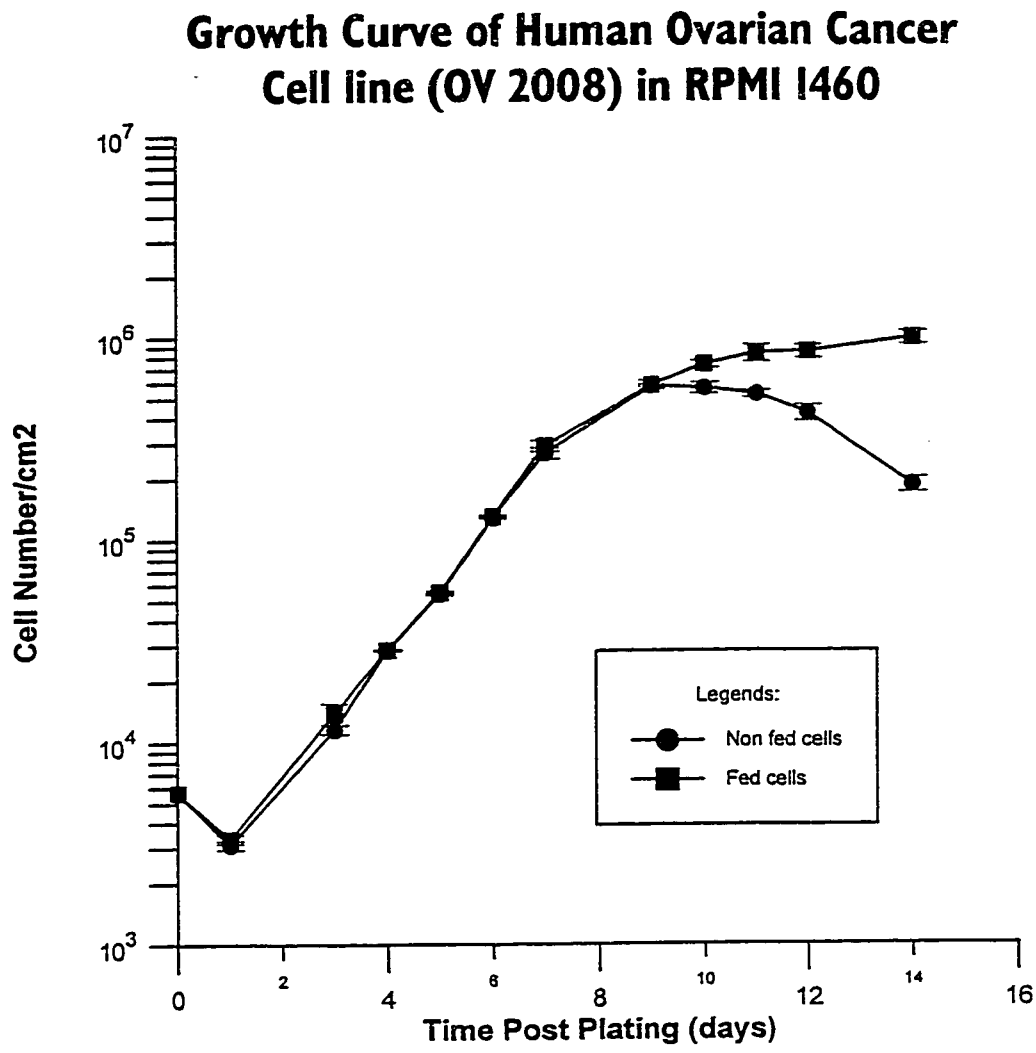


Fig. 3.1: Growth curve of human ovarian OV 2008 cell line. 10^4 cells were seeded in 25 cm^2 cell culture flasks and RPMI 1640 medium. Number of cells were counted each day for up to two weeks. A comparison of fed and non fed cells was made with the change of medium on day 6, for the first group. OV 2008 cell line follows the general pattern of exponential and plateau phases of growth which confirm a suitable experimental conditions for its growth.

cell cycle. The higher proliferation rate in exponentially growing cells produces a greater percentage of cells in S and G2/M phases of the cell cycle. I used this differential cell cycle distribution pattern to perform a preliminary assessment of the relative cytotoxicity of cisplatin in different phases of the cell cycle by assessing cytotoxicity in exponential vs plateau phase cells.

Figure 3.2 shows the percentage of colonies formed after exposure of OV 2008 cells to different concentrations of cisplatin compared to their matched controls for either plateau or exponentially growing cells. Each point is a mean ± standard error of 3 experiments performed in triplicate. As is shown in this figure, plateau phase OV 2008 cells were more sensitive (IC_{50} = 0.45 μg/ml) to cisplatin than were exponentially growing cells (IC_{50} = 0.9 μg/ml). A t-test on the comparison of percentages of survival of plateau vs exponentially growing cells at each corresponding concentration shows a significant difference at 1 μg/ml cisplatin, with a p value of 0.0482.

Since cell cycle phase distribution at the different phases of growth is variable (as is described above), I then investigated the relative cytotoxicity of cisplatin in different subpopulations of OV 2008 cells accumulated in different phases of the cell cycle. To do so, preparation of synchronized subpopulations was necessary.

3.2.2- Synchronization of OV 2008 cells:

Table 3.1 summarizes the results for the calculation of the cell cycle phase duration of the human ovarian OV 2008 cell line. As is shown in this table, OV 2008 cells have a G1 of about 13 hours, an S phase of about 8 hours and a G2/M of 3 hours. In the exponential phase of

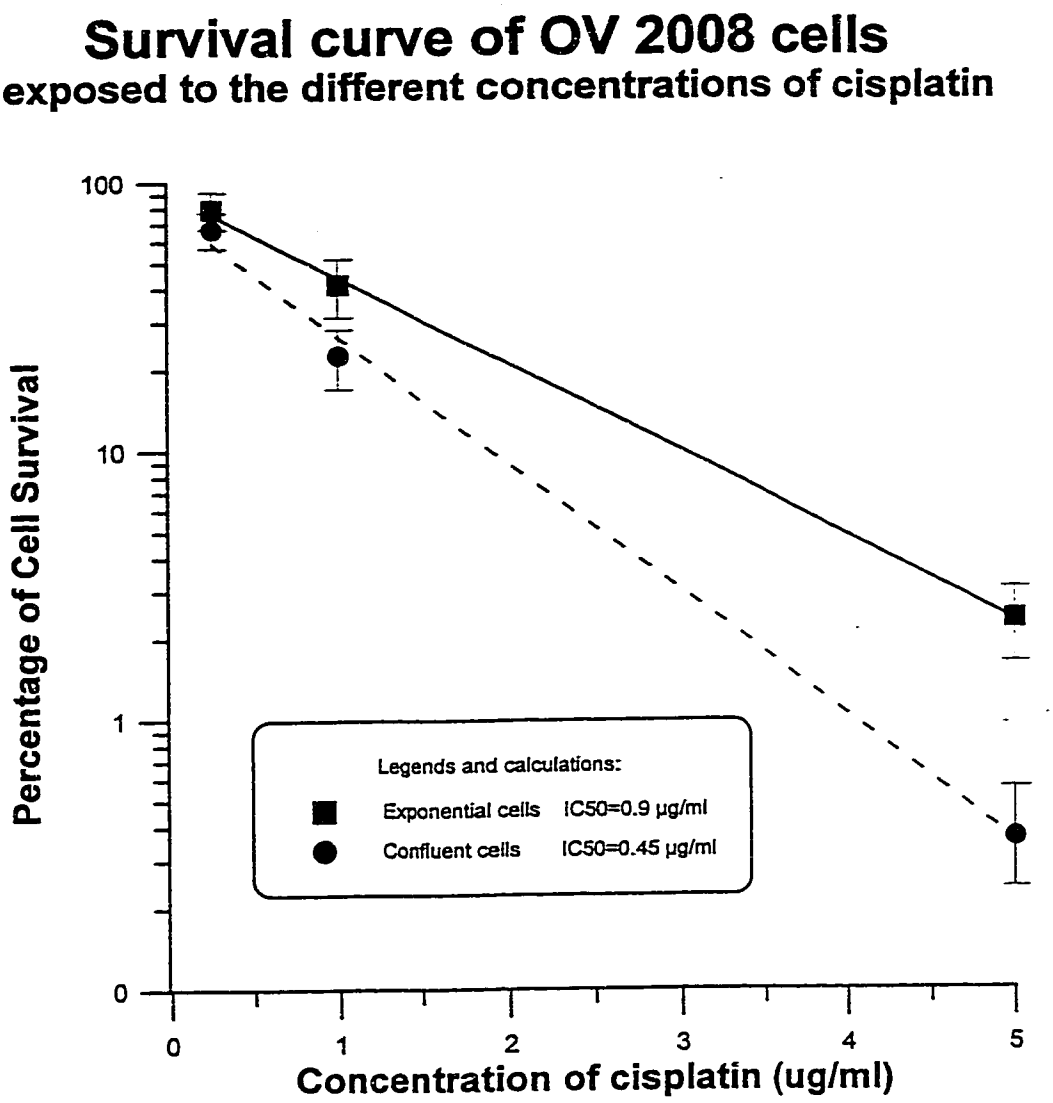


Fig. 3.2: Survival curves of plateau phase vs. exponentially growing OV 2008 cells. Each group of cells was exposed to different concentrations of cisplatin for one hour and colony count was performed about 14 days later. The percentages of survived colonies are plotted on the Y axis and the concentrations of cisplatin are plotted on the X axis. Data points are mean of percentage of colonies formed compared to the control ± SE (n=3). Percentages of survival for cells exposed to 1 µg/ml of cisplatin for one hour show that the plateau cells have higher sensitivity to cisplatin compared to the exponentially growing cells (Two-tailed p=0.0482, calculated by student's t-test).

growth, 56±4 percent of cells are in G1 phase of cell cycle. This changes to 76±5 percent in the plateau phase of growth. During exponential growth, the percentage of cells in S phase is 32±4, which indicates a large population of cells in the process of DNA synthesis. The duration of cell cycle phases for OV 2008 cells was used for the synchronization of these cells in different phases of the cell cycle.

I combined various synchronization methods to establish a new method utilizing protein deficiency followed by thymidine block. I confirmed that this method was suitable for the synchronization of OV 2008 cells at the boundary of G1/S. As explained in the Methods section, a serum deficiency condition was established for 17 hours to decrease the metabolic activity of cells and to accumulate cells in G1. Cells would then enter cell cycle progression in the next step with the addition of 20% FCS. Thymidine would then accumulate these propagating cells at the border of G1 and S in the third step. The rationale is that in inhibiting ribonucleotide reductase activity, thymidine, at high concentrations, inhibits DNA synthesis in S-phase cells by depleting the nucleotide precursor pools of dCTP.

94±3% of the cell population was accumulated at the border of G1/S by this method. Plating efficiency experiments on synchronized cells revealed that only about a 5.5% decrease in plating was caused by this method. Figure 3.3 shows an example flow cytometry histogram of the synchronized cells after thymidine was washed out and cells were stained with propidium iodide. Figure 3.4 represents the percentage of cells in each phase of the cell cycle after release from thymidine block (time 0) and after the addition of fresh RPMI with 10% FCS for up to 24

Table 3.1: Percentages of exponentially growing OV 2008 cells in each phase of the cell cycle.			
	G1	G2/M	S
Day 1 (% of cells)	52±1	13±0.7	33±0.7
Day 2(% of cells)	56±1	11±0.4	35±0.7
Day 3 (% of cells)	59±3	12±0.5	29±3
Average of 3 days (% of cells)	56±4	12±1	32±4
Duration of each cell cycle phase (h)	13.4	2.9	7.7

Tab. 3.1: Percentages of OV 2008 cells in different phases of the cell cycle. Flasks with different numbers of cell were seeded in RPMI media. Every day, for up to 3 days, samples of each group were stained with propidium iodide and the percentages of cells in different phases of the cell cycle were measured by flow cytometry. Data are mean ± SE of percentage of cells in each phase from the three groups of cells, as is mentioned above, in each day. Calculation of the duration of each phase is based on the doubling time of OV 2008 cell (24 h) and the average percentage of cells in each phase of cell cycle as is presented in method section. The result of this calculation, i.e. duration of each phase, is presented above in a separate row under the table.

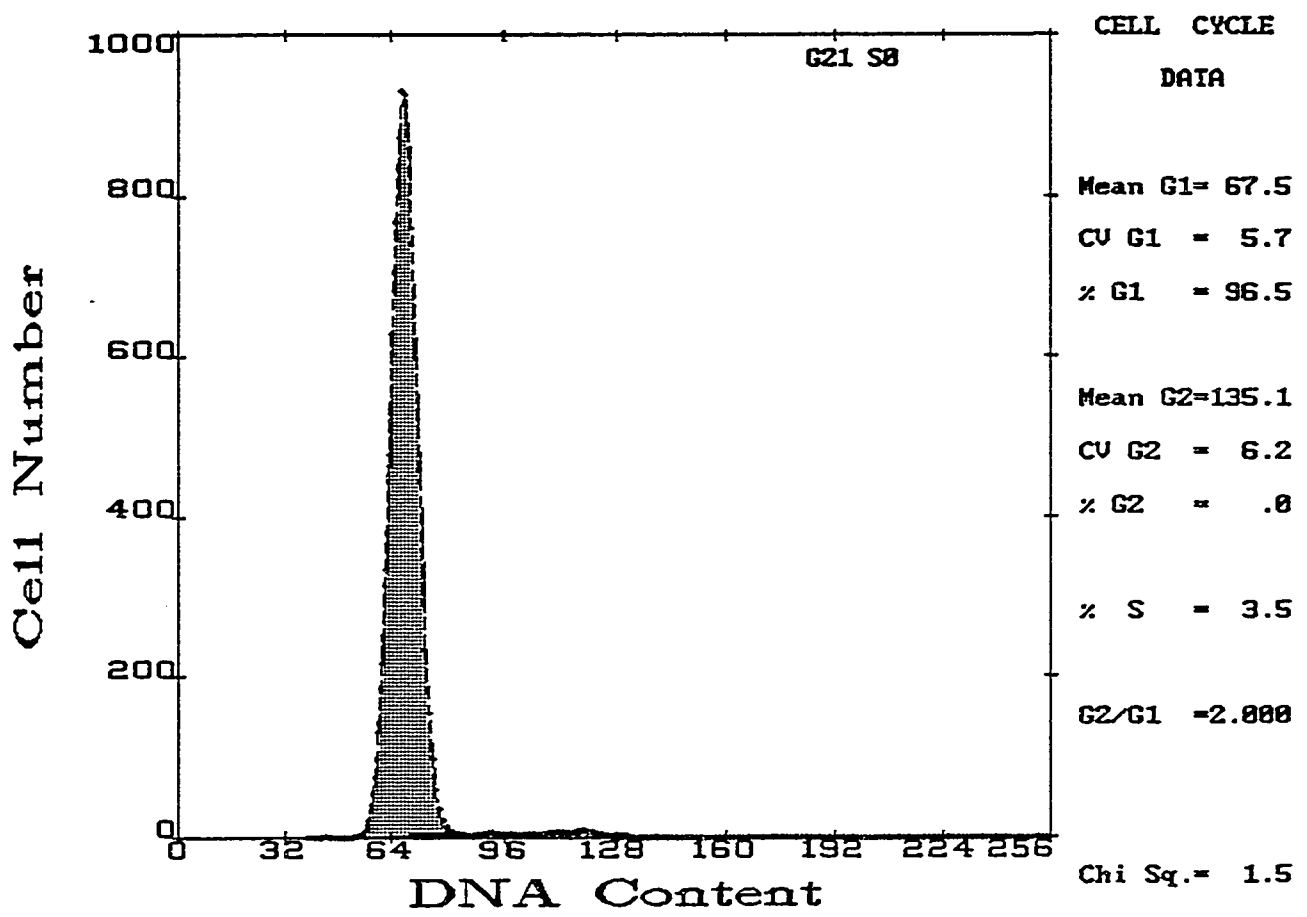


Fig. 3.3: DNA histogram of synchronized OV 2008 cells. At the exponentially growing phase, a serum deficiency condition of 0.1% FCS was applied for 17 hours. Medium was then replaced with fresh media containing 20% FCS for 12 hours. At this point, cells were exposed to 2 mM thymidine in RPMI medium containing 0.5% FCS for 16 hours. Cells were released from arrest at the boundary of G1/S with the change of medium to fresh RPMI containing 10% FCS. This figure shows flow cytometry of OV 2008 cells using propidium iodide soon after release from thymidine block which confirms a 96.5% accumulation of cells at this boundary in this example.

Cell Cycle Progression

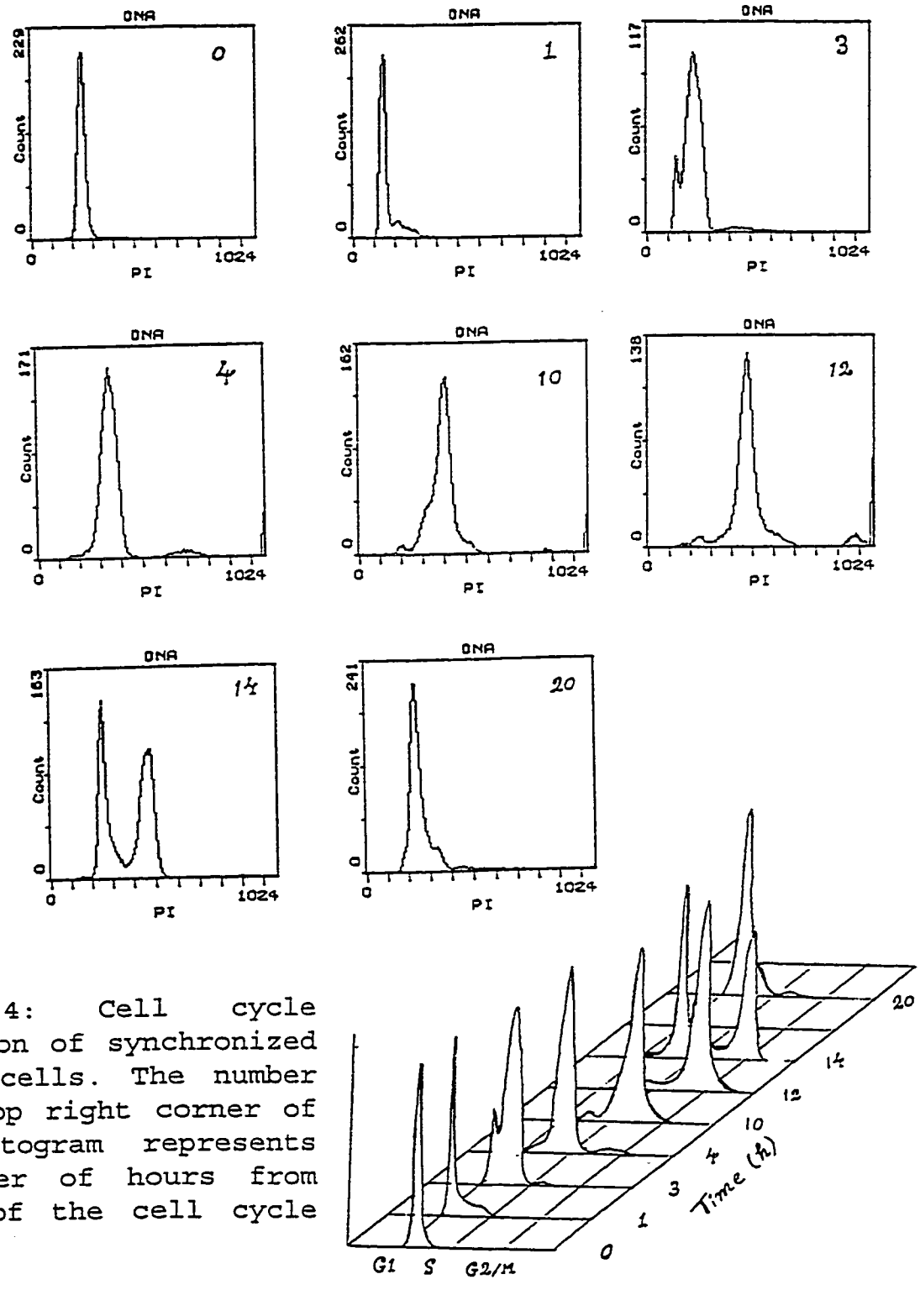


Fig. 3.4: Cell cycle progression of synchronized OV 2008 cells. The number in the top right corner of each histogram represents the number of hours from release of the cell cycle block.

hours.

This experiment confirmed that OV 2008 cells can be synchronized using a double block technique with the help of serum deficiency and thymidine. These cells will synchronously pass through the phases of the cell cycle at least for another 30 hours, which is enough to follow their cell cycle phases during one doubling time.

3.2.3- Cisplatin cytotoxicity in different cell cycle phases:

Now that I had subpopulations of cells in different cell cycle phases, I could repeat my clonogenic cytotoxicity assay to see if there are any differences in the cytotoxicity of cisplatin in different phases of the cell cycle.

As is shown in figure 3.5, there is a difference in cisplatin sensitivity of OV 2008 cells in different phases of the cell cycle exposed to the same concentration of cisplatin for the same duration of time. The rank order of cisplatin cytotoxicity in cells in different phases of the cell cycle is as follow: G1>G1/S>S>G2/M. The major difference in the cell survival curves for G1, G1/S, and S cell cycle phases is in the average slope of the curves for cisplatin concentrations up to 0.5 μg/ml, while the slope of the cell survival curve for G2/M cells may be less than that for other phases of the cell cycle over the entire length of the curve.

The following table summarized the application of ANOVA testing to the comparison of cisplatin cytotoxicity for the different subpopulations of OV 2008 cells presented in figure 3.5. As is shown in this table, significant differences exist between each pair of subpopulations for at least some cisplatin concentrations (NS= not significant).

Cisplatin → *Cell cycle phases* ↓	**0.025 (μg/ml)**	**0.125 (μg/ml)**	**0.25 (μg/ml)**	**0.5 (μg/ml)**	**0.75 (μg/ml)**
G1 vs G1/S	NS	NS	NS	$p<0.01$	NS
G1 vs S	NS	NS	$p<0.001$	$p<0.001$	$p<0.01$
G1 vs G2/M	NS	NS	$p<0.001$	$p<0.001$	$p<0.001$
G1/S vs S	NS	NS	NS	NS	$p<0.05$
G1/S vs G2/M	NS	NS	$p<0.05$	$P<0.001$	$p<0.001$
S vs G2/M	NS	NS	NS	$p<0.01$	$p<0.001$

It has been postulated that the observed change in curve slope might arise from "passive resistance" mechanisms, where passive resistance (analogous to non-competitive inhibition of drug effect) is due to a deficiency or mutation of a factor that is essential for cell killing (Stewart *et al.*, 1996). "Active resistance", on the other hand, would give a shoulder on the dose-response curve. Active resistance is analogous to competitive inhibition of drug effect, and is due to over-expression of a protective factor. If dose-response curve shape does, in fact, reflect resistance mechanisms, my curves would indicate that G1, G1/S, and S cells differ mainly from one another with respect to passive resistance mechanisms that influence cell survival most over the 0-0.5 μg/ml cisplatin dose range. G2/M cells have increased passive resistance at least

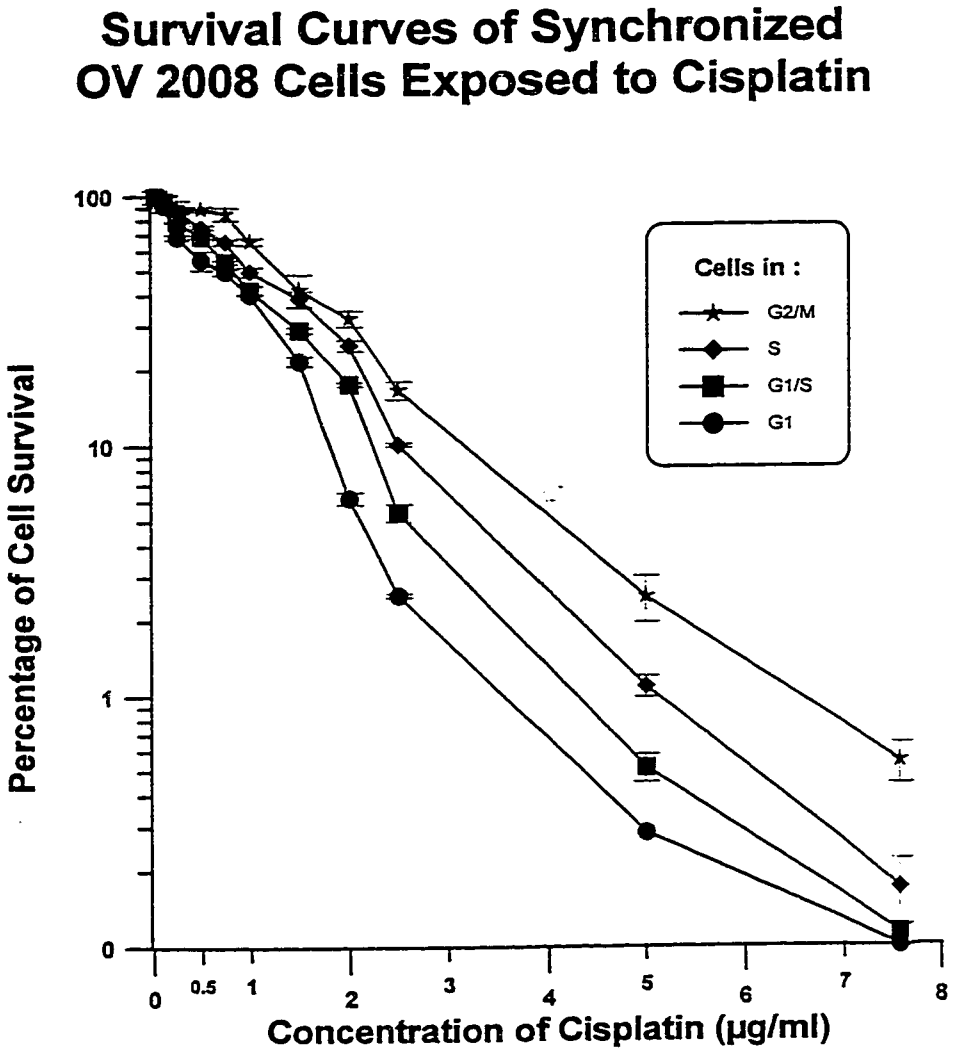


Fig. 3.5: Survival curves of synchronized OV 2008 cells exposed to cisplatin for one hour while in different phases of the cell cycle. Percentage of cell survival is based on the clonogenic assay as is described in the method section (data are mean ± SE of treated samples compared to control). Survival of subpopulations show a significant difference from each other by the concentration of 0.5 μg/ml of cisplatin, which would cause a general differences in the slope of curves for the rest of cisplatin concentrations ($p<0.001$, calculated by ANOVA).

up to a cisplatin concentration of 5 μg/ml, as well as possibly having increased active resistance at cisplatin concentrations ≤ 1 μg/ml.

3.2.4- OV 2008 cells mode of death by cisplatin: OV 2008 cells exposed to lethal cisplatin concentrations would die by either apoptosis or necrosis. These cells, however, have different sensitivities to cisplatin in different phases of the cell cycle. For further clarification, I investigated the mode of OV 2008 cell death for cells exposed to cisplatin in different phases of the cell cycle.

There are many different ways to investigate the mode of death caused by a toxic agent on a particular cell line, including electron microscopy, DNA gel electrophoresis, flow cytometry and histochemical and cytochemical techniques such as the TUNEL (TdT-mediated dUTP-X nick end labeling) method (Gaverieli *et al.*, 1992). I have used a variety of these methods (electron microscopy, flow cytometry and double dye staining) to answer this question and also to accumulate clues as to the mechanism by which cisplatin cytotoxicity is different in the different phases of the cell cycle. Electron microscopy is claimed to be the most reliable method to show both apoptotic and necrotic cell death in asynchronous and/or synchronized cells in different phases of the cell cycle (Kerr *et al.*, 1994). I have therefore confirmed these two modes of cell death in OV 2008 cells caused by cisplatin.

Figure 3.6 shows the electron photograph of control OV 2008 cells that had not been exposed to cisplatin. As is shown, normal OV 2008 cells show numerous plasma cell membrane extensions with many microvilli, an indented euchromatin nucleus, and homogeneously

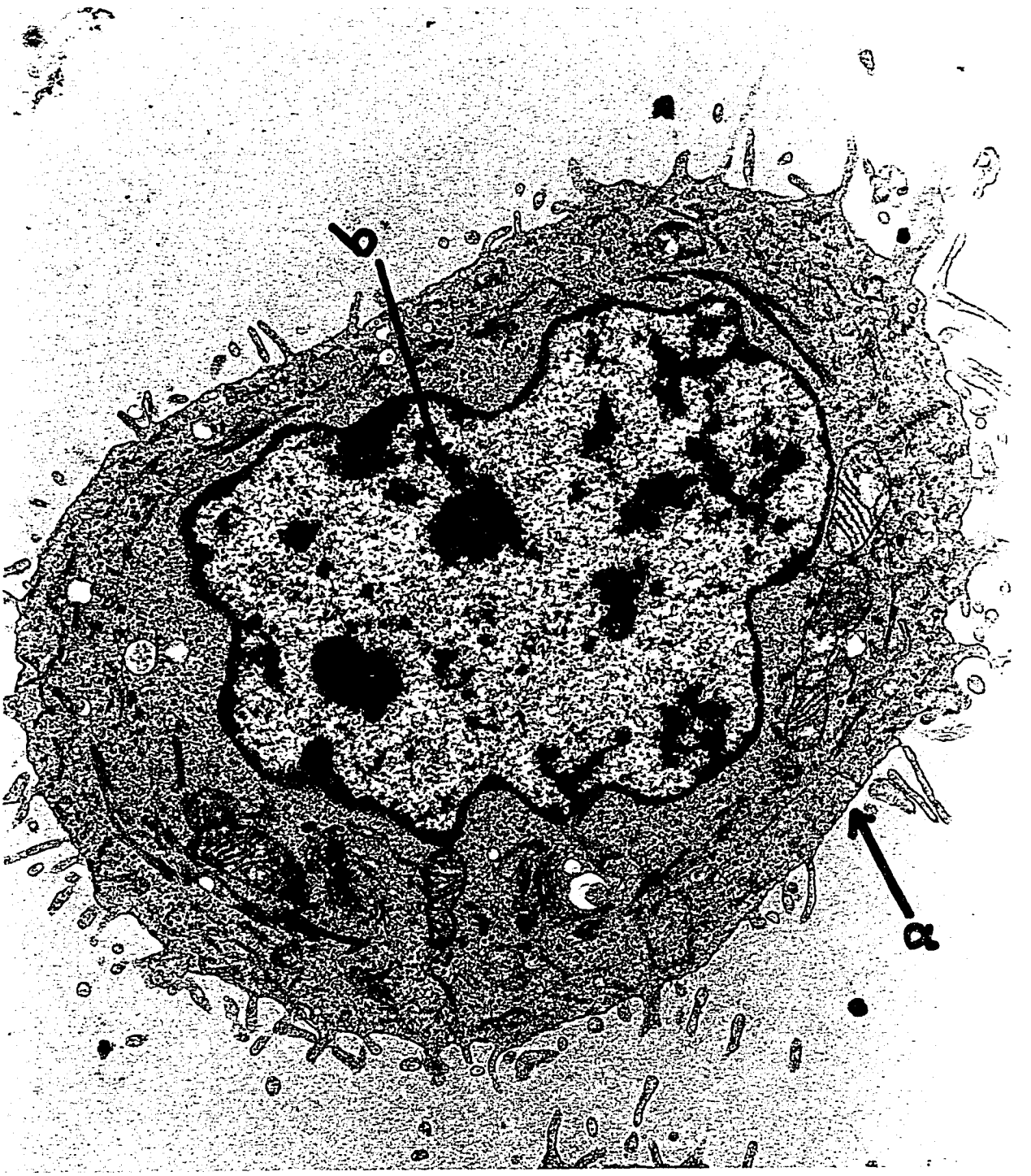


Fig. 3.6: Electron microscopy of a normal OV 2008 cell (magnification is x6000). Electron microscopy revealed that normal OV 2008 cells are associated with numerous plasma cell membrane extensions with many microvilli (a), normal euchromatin nucleus (b) and also cellular organelles homogenously distributed in the cytoplasm.

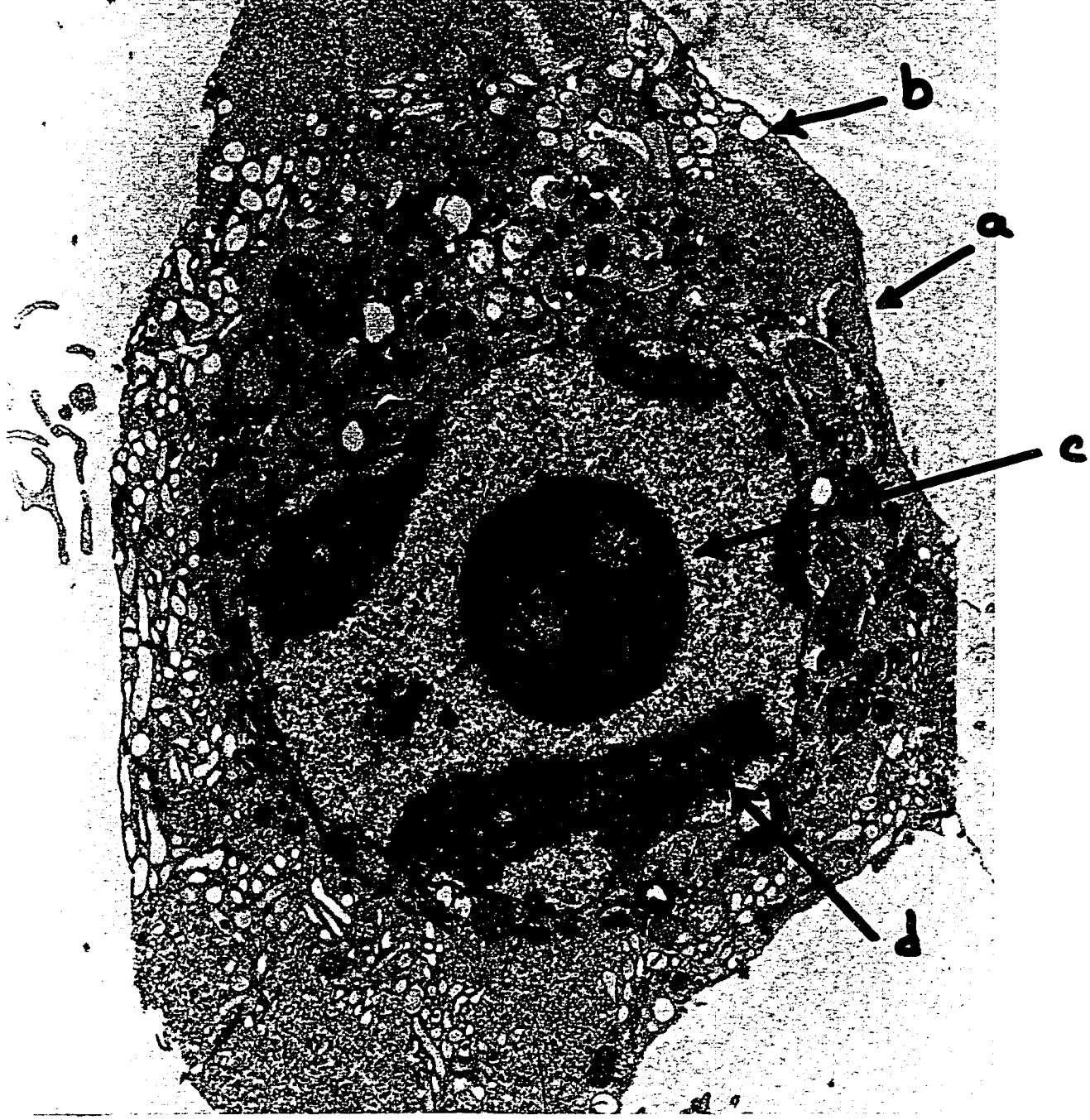


Fig. 3.7: Electron microscopy of OV 2008 cell after one hour exposure to 1 μg/ml cisplatin; early apoptosis stage (magnification is x6000). The early morphological changes in apoptotic OV 2008 cells are characterized by: compaction and condensation of cell, smoothing of plasma membrane as well as elimination of microvilli (a), appearance of intra cytoplasmic vacuoles (b), rounding of nucleus (c), and chromatin condensation (d).

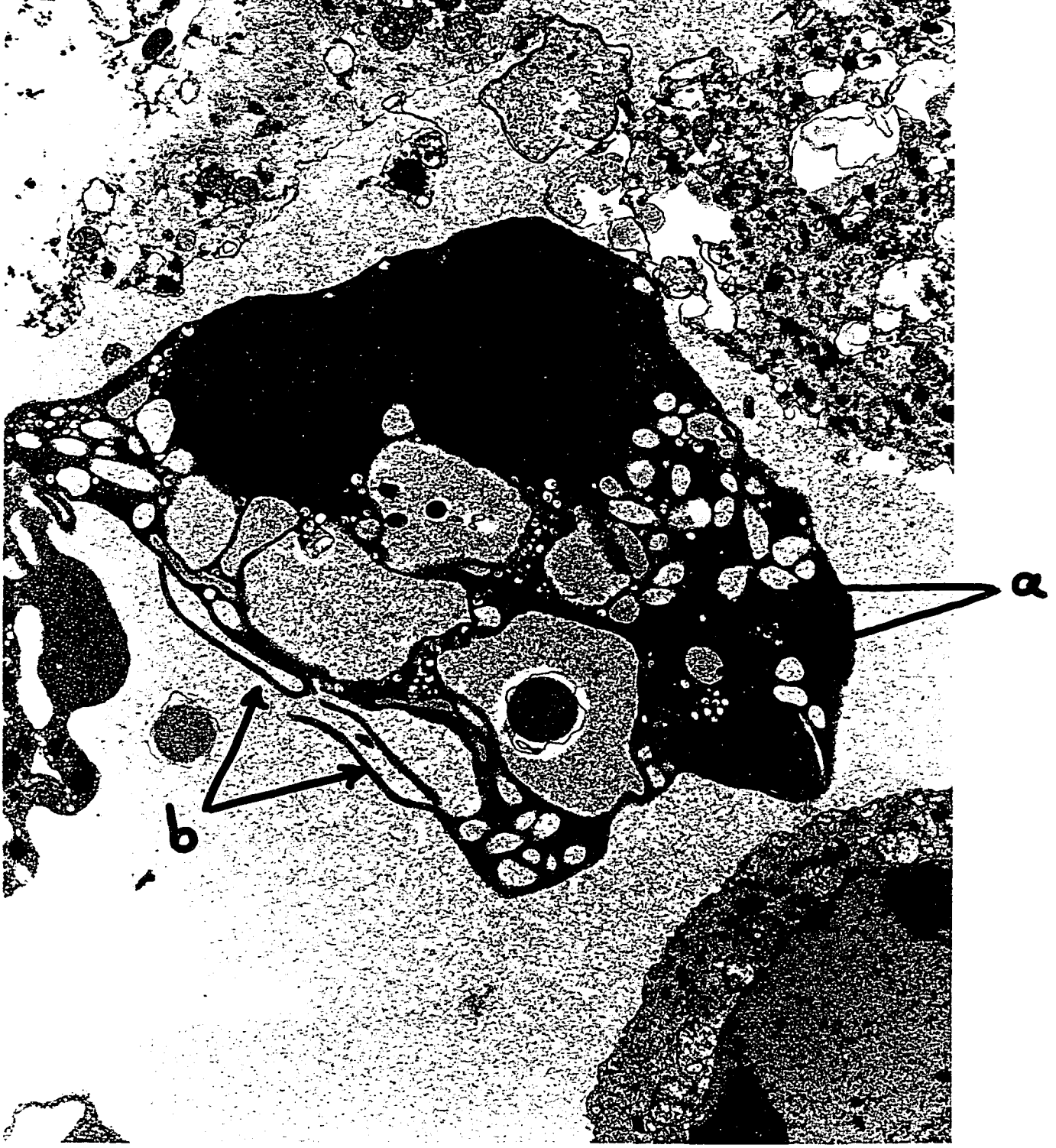


Fig. 3.8: Electron microscopy of OV 2008 cells after one hour exposure to 1 μg/ml cisplatin; late apoptosis (magnification is x6000). Late changes are associated with increasing intra cytoplasmic vacuoles (a), attachment of vacuoles to plasma membrane (b), organelles entrapping in the membrane structure (apoptotic body) (c), and apoptotic bodies separation from apoptotic cells (which is not shown in this picture).

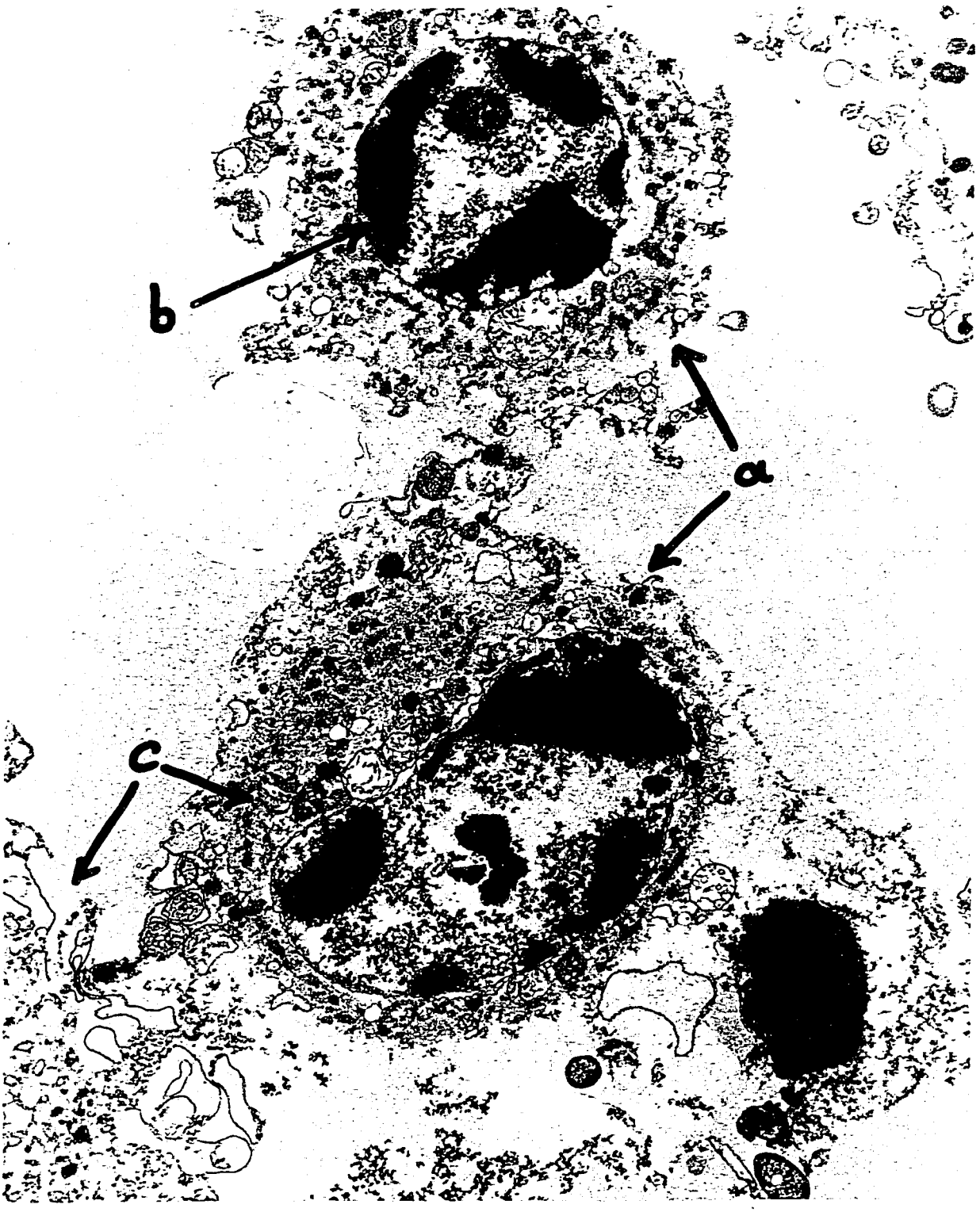


Fig. 3.9: Electron microscopy of OV 2008 cells after one hour exposure to 1 μg/ml cisplatin; necrotic cells (magnification x6000). Necrotic cells are observed to have damaged cell membrane (a) with condensed nuclei (b), and/or completely lysed (c).

distributed cellular organelles within the cytoplasm. Electron microscopy showed apoptosis in floating cells but not in attached cells. As is shown in figure 3.7, early morphological changes in apoptotic OV 2008 cells are characterized by compaction and condensation of cells, smoothing of plasma membranes, as well as by elimination of microvilli, formation of intracytoplasmic vacuoles, rounding of nuclei and chromatin condensation. Figure 3.8 shows a later stage of OV 2008 apoptotic cells, with increasing intracytoplasmic vacuoles, vacuole attachment to plasma cell membranes, organelle entrapment in the membrane structure (apoptotic body), and eventually, separation of apoptotic bodies from apoptotic cells. Figure 3.9 shows completely lysed cells as the main indication of necrotic (as opposed to apoptotic) cell death.

Electron microscopy confirmed apoptotic and/or necrotic cell death in OV 2008 cells growing exponentially or in plateau phase, and/or cells accumulated in the different phases of the cell cycle. However, this technique did not show any differences in the appearance of apoptotic and/or necrotic cells in any subpopulation of synchronized cells. On the other hand, electron microscopy is a qualitative way of exploring the mode of cell death rather than quantitative. Therefore, some other methods are needed to get a better understanding of the mode of death caused by cisplatin on OV 2008 cells and the possible differences in different phases of the cell cycle.

3.2.5- Cell cycle kinetics and arrest: Flow cytometry has been used to further investigate the process of death in the OV 2008 synchronized subpopulations. This technique is able to present the kinetics of cellular events following exposure to cisplatin.

My results showed that all subpopulations of OV 2008 cells were arrested in S and G2/M phases of the cell cycle after exposure to cisplatin. Arrested cells then died by apoptosis or continued the cell cycle progression, as is shown with the percentage of arrested or released cells. Figure 3.10 compares the general shape of flow cytometry histograms from three samples of normal and arrested cells and cells that had undergone apoptotic death (represented by a component of the SubG1 peak). Arrested cells could be found in both attached and floating cells, but subG1 (apoptotic cells) could only be seen in the floating cells. The percentage of arrested cells in the floating cell population was much higher than the percentage in attached cells. Floating cells have been used to study the kinetics of affected cells following cisplatin exposure.

For each of the subpopulations exposed to cisplatin, figures 3.11 to 3.14 present means ± standard errors of percent of cells in different phases of the cell cycle at various times after cisplatin exposure. Figure 3.15 summarizes the observations from the results of these studies.

As is clear from these figures, there was a delay in cisplatin-induced arrest and apoptosis for the subpopulations of S and G2/M cells. In the study of synchronized subpopulations of G1 or G1/S cells, subsequent arrest in S and G2/M phases was seen in the same cell cycle during which cisplatin exposure occurred. However, cells would go through one additional cell cycle before arrest if the cisplatin exposure occurred in S or G2/M phases. This pattern emphasizes the importance of passage through G1 or early S phase of the cell cycle for the cytotoxicity of cisplatin.

Although these experiments were not able to show the percent of dead vs live cells,

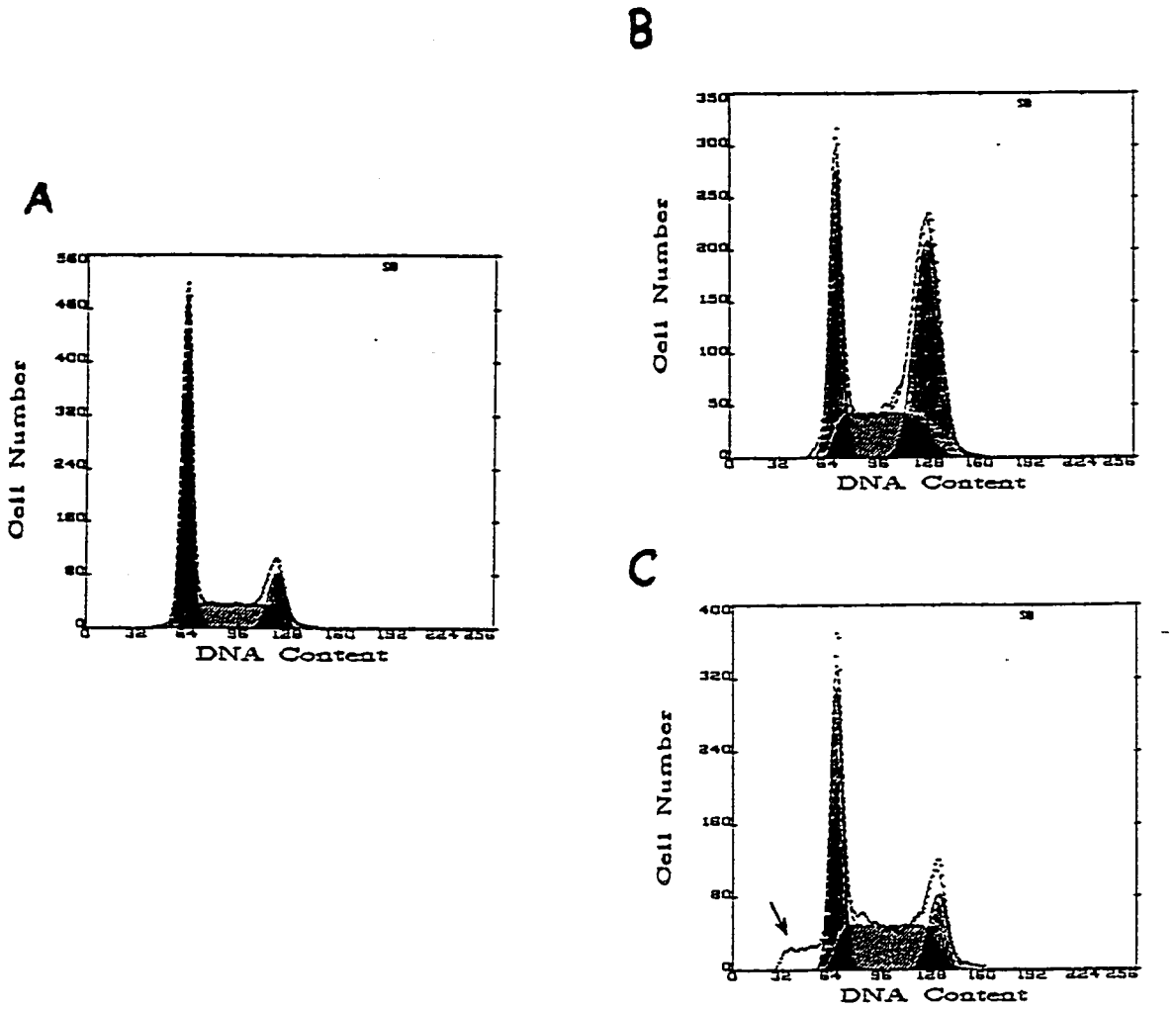
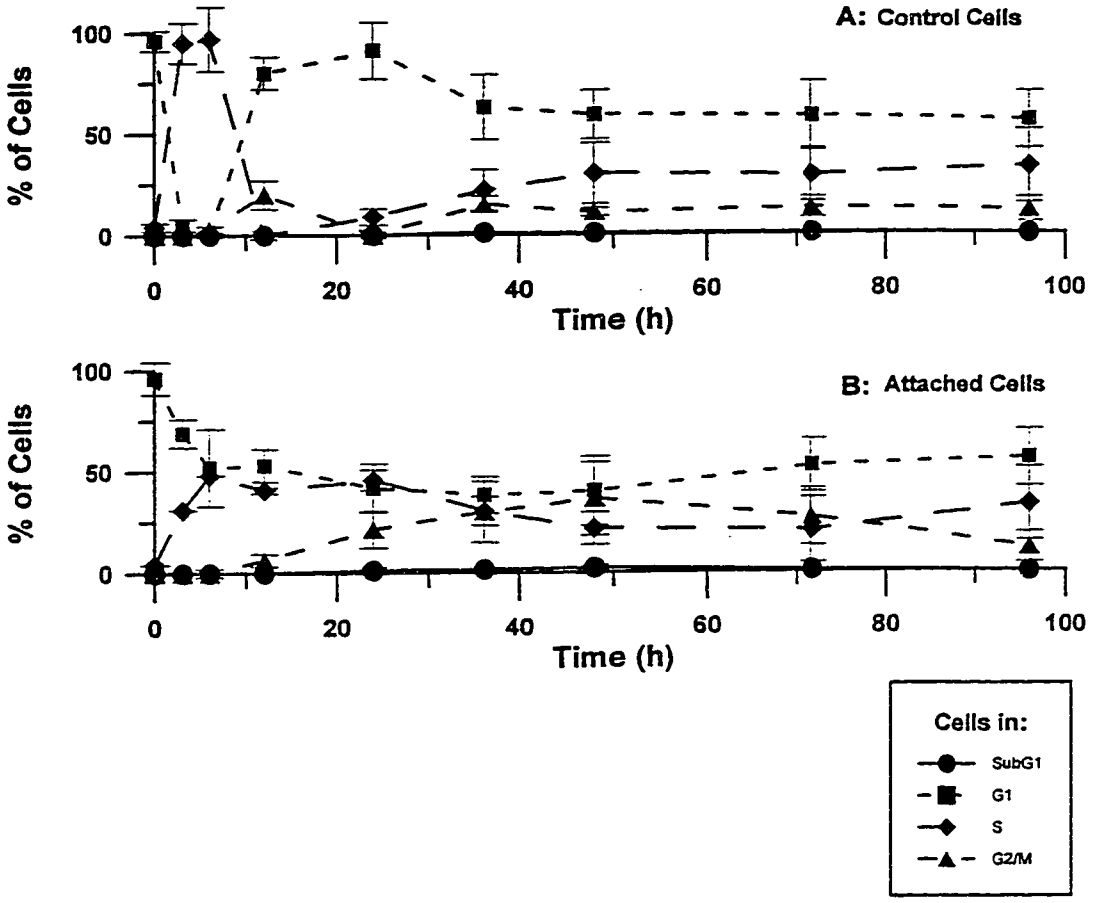


Fig. 3.10: Flow cytometry histograms of OV 2008 cells. A) Histogram of normal OV 2008 cells show a high population in G1 (at 67.5±5.7 on X axis), small population of cells in G2/M (at 135.1±6.2 on X axis) and S cells in between these two points. B) Arrested cells in S and G2/M clearly show a much higher percentages of cells in S and G2/M peaks. C) A subpopulation of cells appear as a peak before the G1 population (an indication of the apoptotic fraction) and represent the cells with lower amount of DNA due to DNA cuts in the apoptotic cells (which is represented as laddering phenomenon in gel electrophoresis).

Kinetics of Cell Cycle Progression
for G1/S Cells after Exposure to Cisplatin



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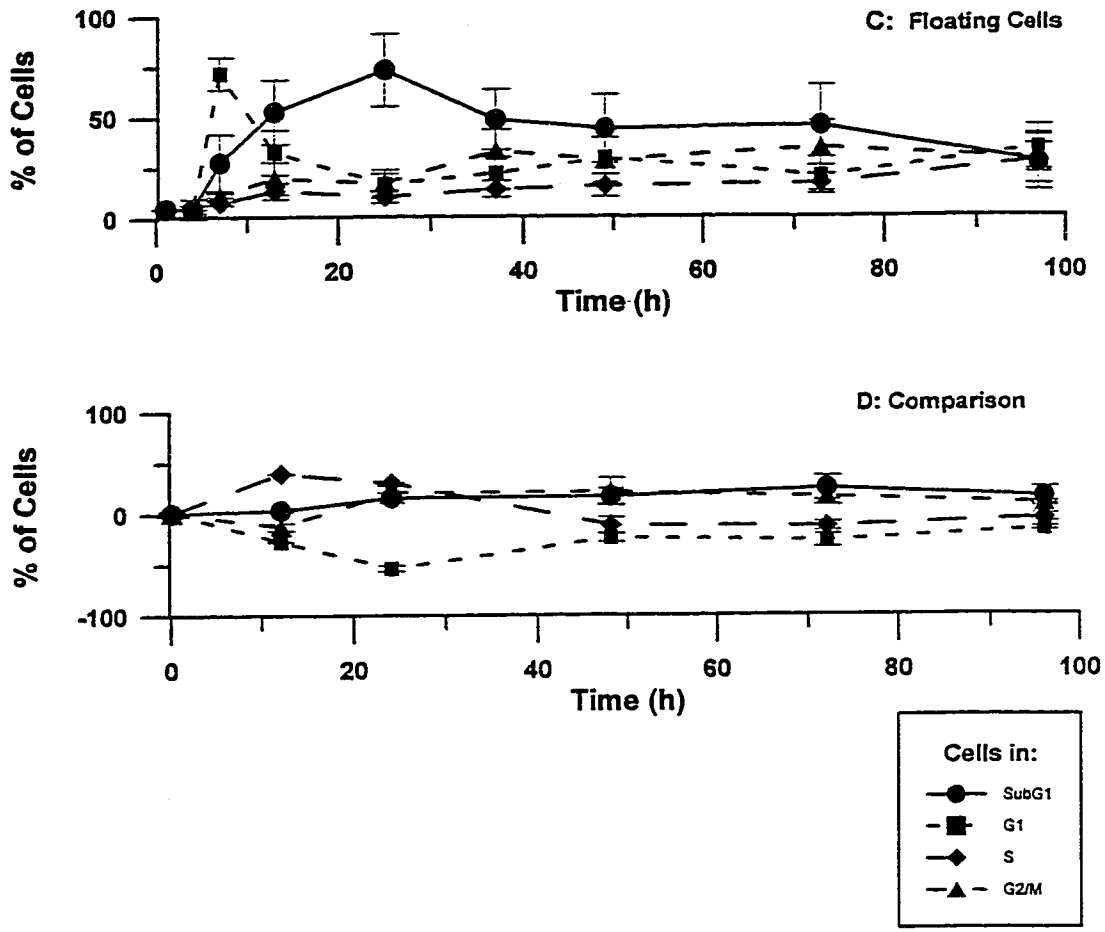
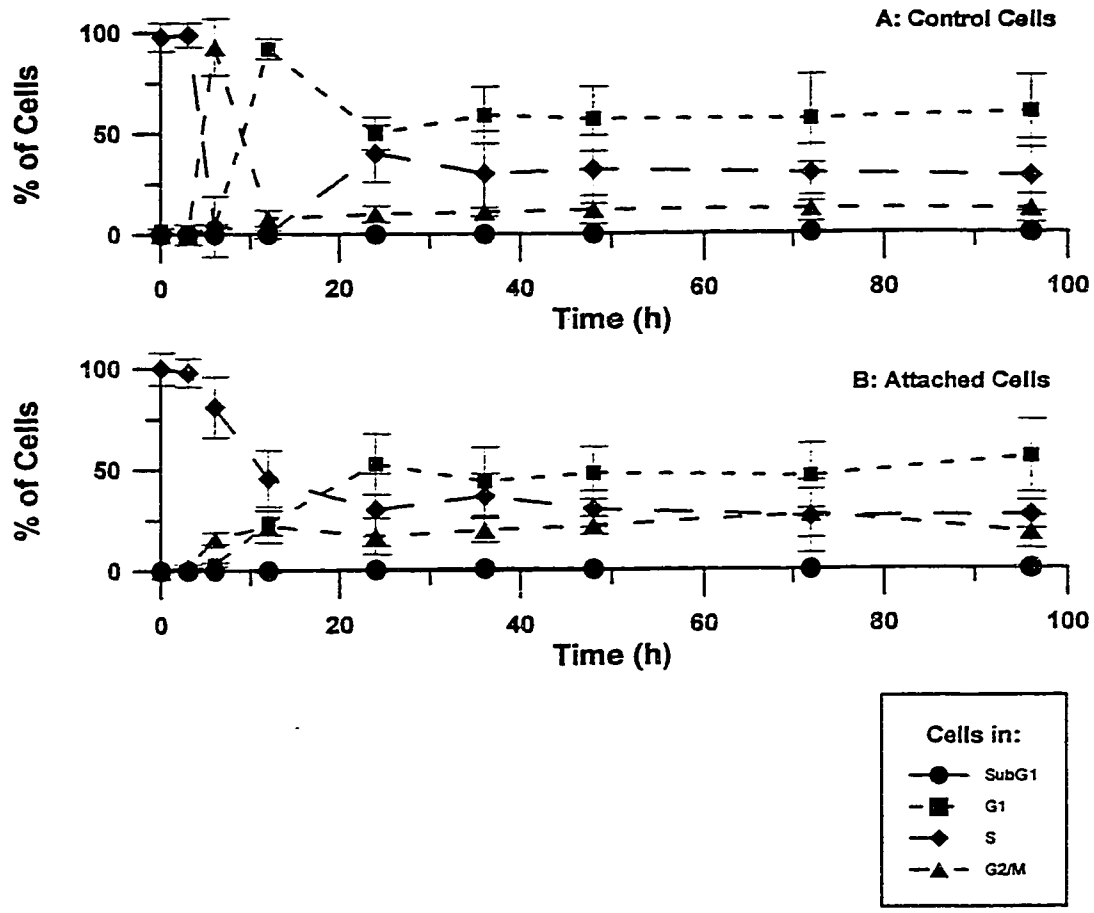


Fig. 3.11: Cell cycle kinetics after one hour exposure of G1/S cells to 1 μg/ml cisplatin. Attached and detached cells were collected and their cell cycle status were compared to the control cells at different intervals using flow cytometry (data are as mean ± SE of percentage of cells in each phase of the cell cycle); A) control cells, B) attached cells after exposure to cisplatin, C) detached cells after exposure to cisplatin, and D) variation in precent of cells at different phases of the cell cycle after exposure to cisplatin compared to the control cells (A and B are presented on page 72). In graph D, a positive number means increase and negative number means decrease in the percentage of cells compare to control. Each point on this graph resulted from the following calculation:
((% in B × % of attached at that time*) + (% in C × % of detached at that time*)) − % in A
* % of attached or detached cells at any time point is presented in figure 3.17.

Kinetics of Cell Cycle Progression for S Cells after Exposure to Cisplatin



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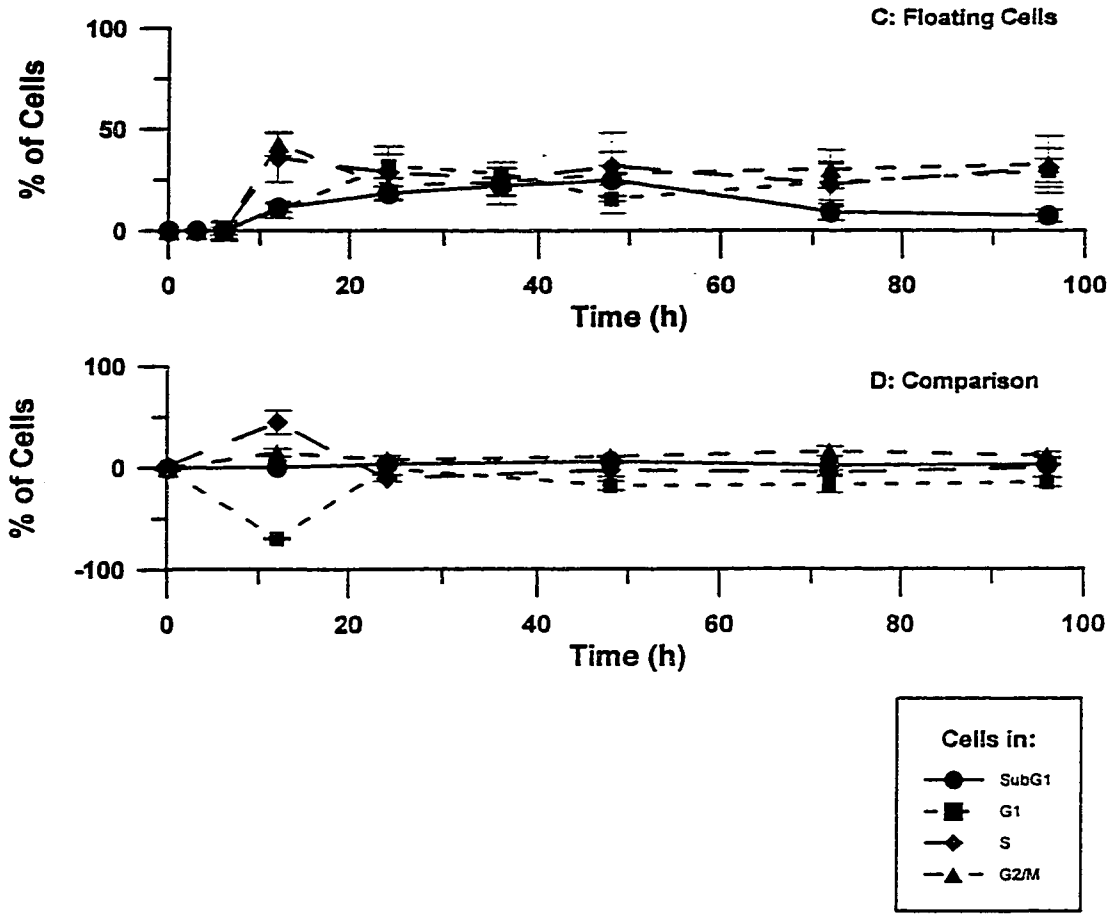
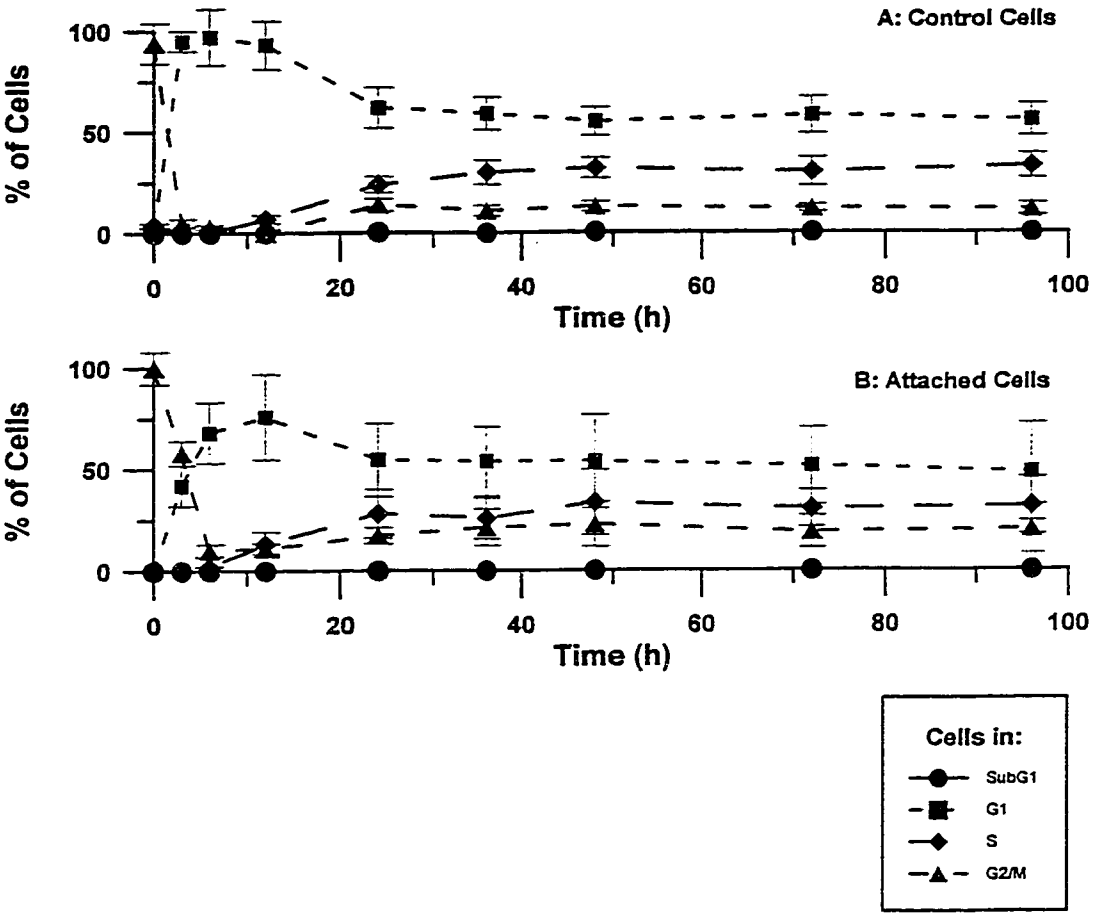


Fig. 3.12: Cell cycle kinetics after one hour exposure of S cells to 1 μg/ml cisplatin. Attached and detached cells were collected and their cell cycle status were compared to the control cells at different intervals using flow cytometry (data are as mean ± SE of percentage of cells in each phase of the cell cycle); A) control cells, B) attached cells after exposure to cisplatin, C) detached cells after exposure to cisplatin, and D) variation in precent of cells at different phases of the cell cycle after exposure to cisplatin compared to the control cells (A and B are presented on page 74). In graph D, a positive number means increase and negative number means decrease in the percentage of cells compare to control. Each point on this graph resulted from the following calculation:
((% in B × % of attached at that time*) + (% in C × % of detached at that time*)) - % in A
* % of attached or detached cells at any time point is presented in figure 3.17.

Kinetics of Cell Cycle Progression for G2/M Cells after Exposure to Cisplatin



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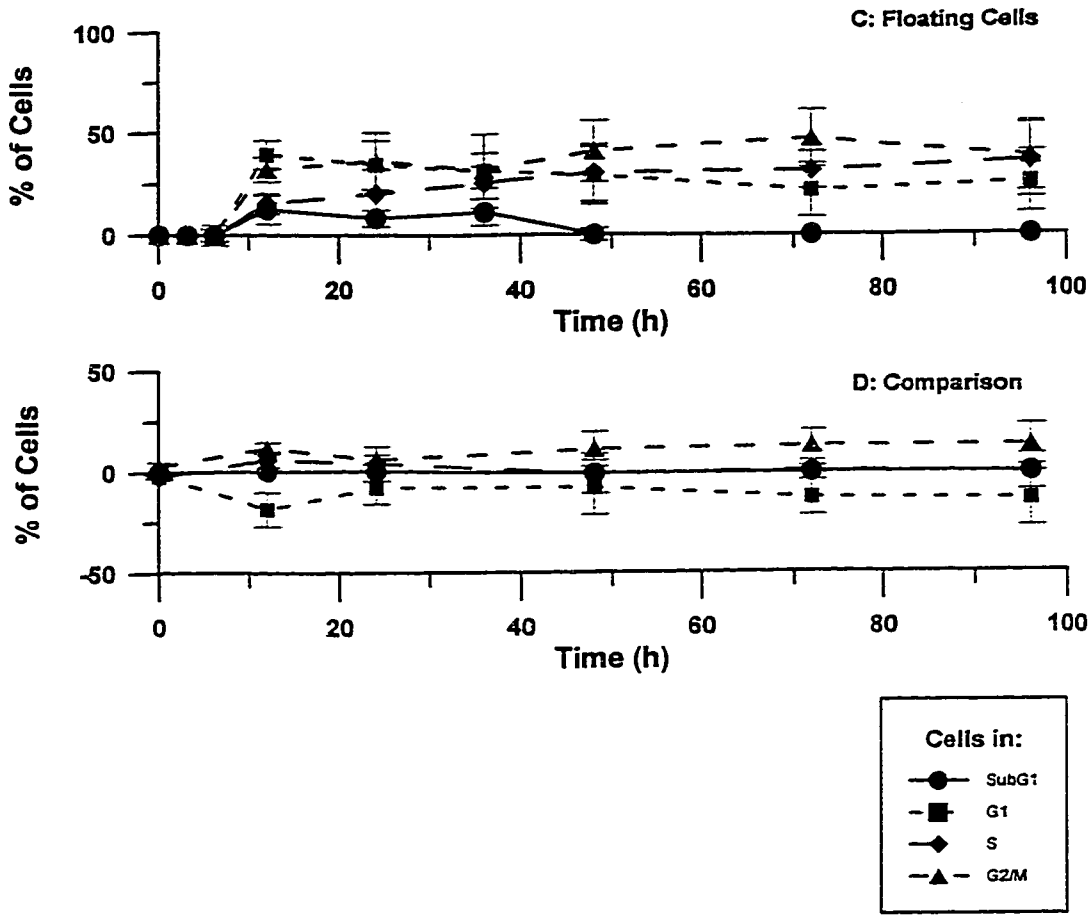
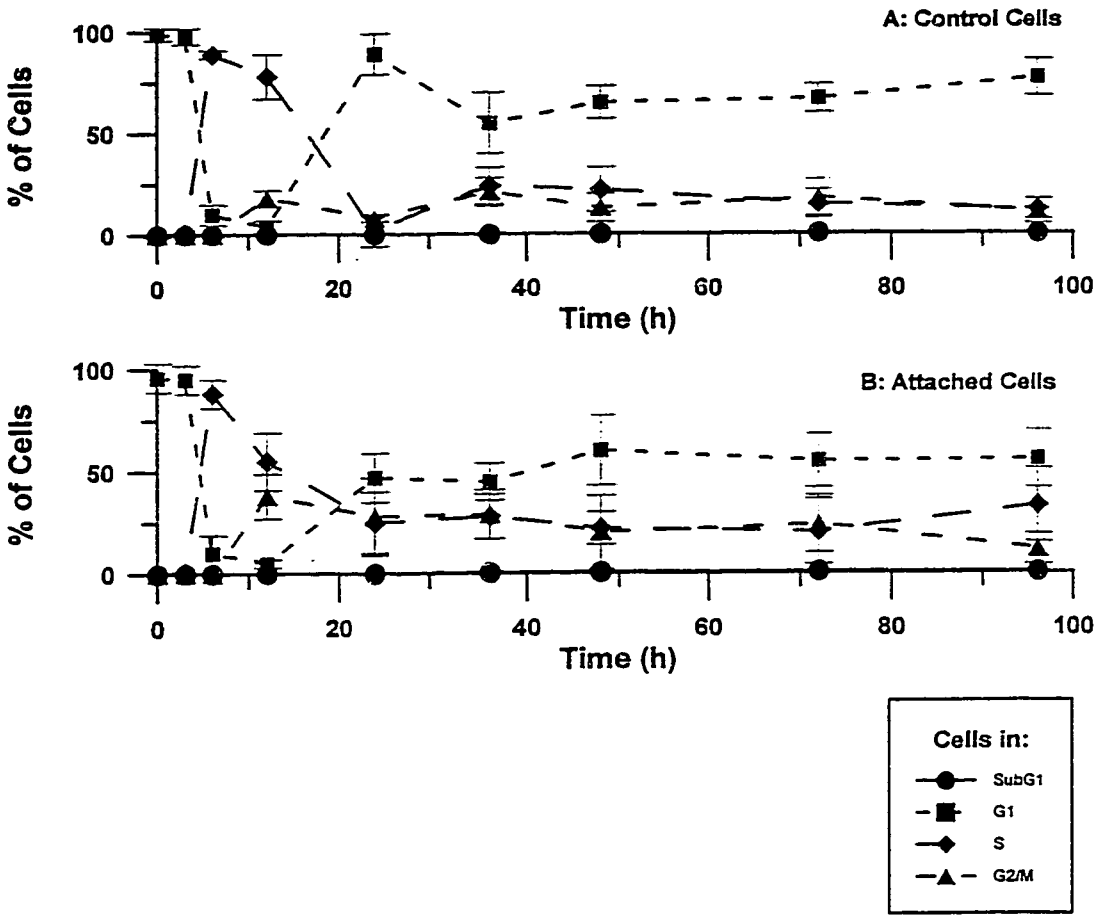


Fig. 3.13: Cell cycle kinetics after one hour exposure of G2/M cells to 1 μg/ml cisplatin. Attached and detached cells were collected and their cell cycle status were compared to the control cells at different intervals using flow cytometry (data are as mean ± SE of percentage of cells in each phase of the cell cycle); A) control cells, B) attached cells after exposure to cisplatin, C) detached cells after exposure to cisplatin, and D) variation in precent of cells at different phases of the cell cycle after exposure to cisplatin compare to the control cells (A and B are presented on page 76). In graph D, a positive number means increase and negative number means decrease in the percentage of cells compare to control. Each point on this graph resulted from the following calculation:
((% in B × % of attached at that time*) + (% in C × % of detached at that time*)) - % in A
* % of attached or detached cells at any time point is presented in figure 3.17.

Kinetics of Cell Cycle Progression for G1 Cells after Exposure to Cisplatin



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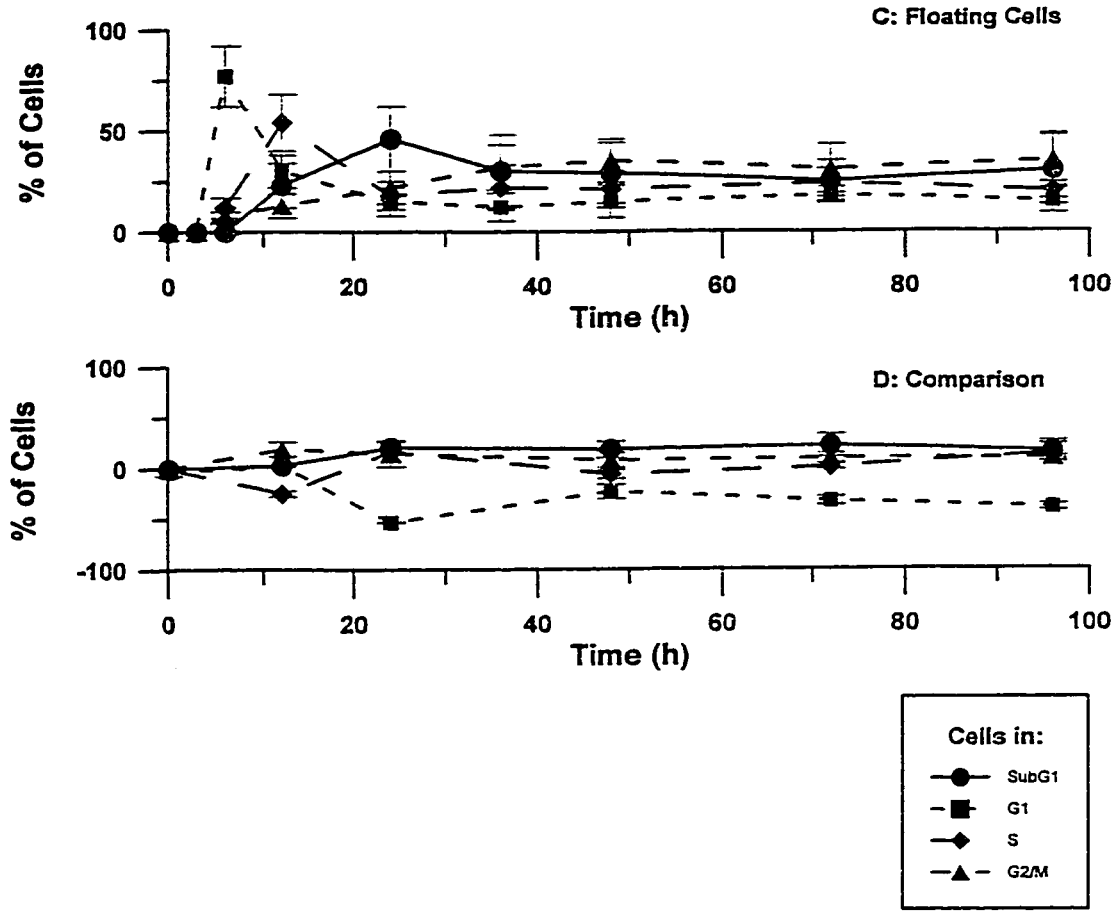
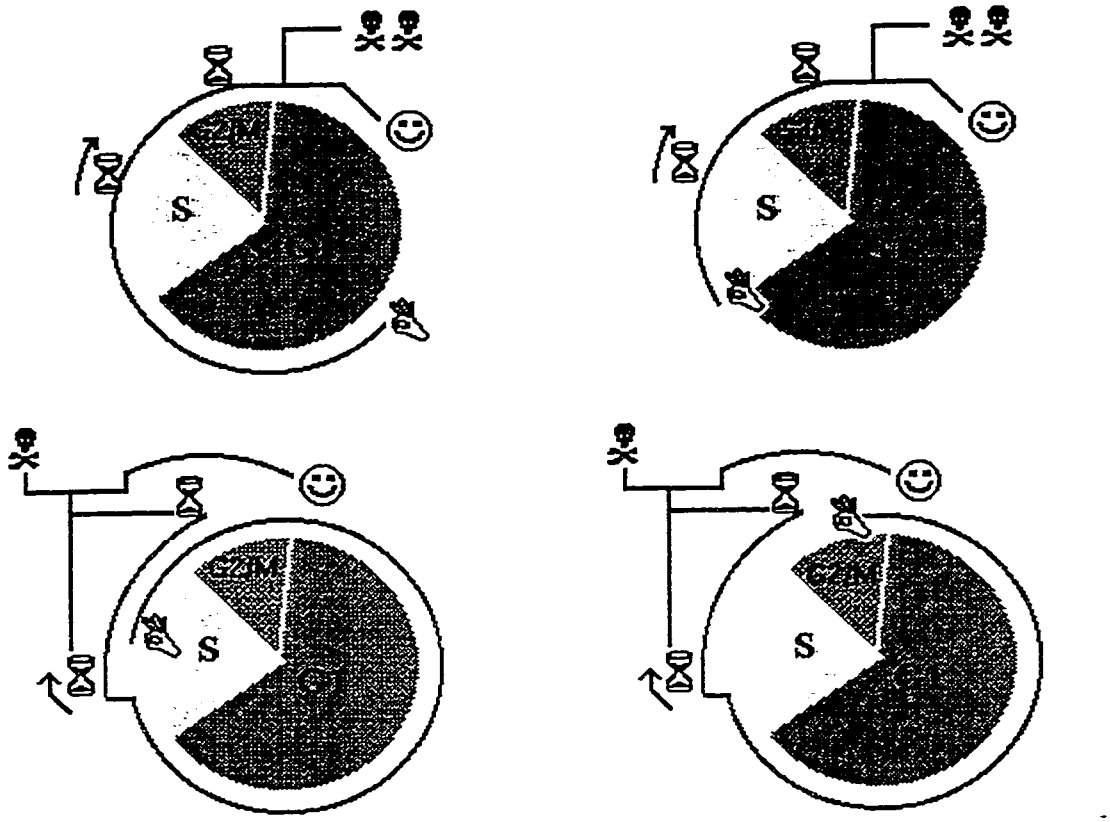


Fig. 3.14: Cell cycle kinetics after one hour exposure of G1 cells to 1 μg/ml cisplatin. Attached and detached cells were collected and their cell cycle status were compared to the control cells at different intervals using flow cytometry (data are as mean ± SE of percentage of cells in each phase of the cell cycle); A) control cells, B) attached cells after exposure to cisplatin, C) detached cells after exposure to cisplatin, and D) variation in precent of cells at different phases of the cell cycle after exposure to cisplatin compare to the control cells (A and B are presented on page 78). In graph D, a positive number means increase and negative number means decrease in the percentage of cells compare to control. Each point on this graph resulted from the following calculation:
((% in B × % of attached at that time*) + (% in C × % of detached at that time*)) − % in A
* % of attached or detached cells at any time point is presented in figure 3.17.



Legends:

Exposure to cisplatin

Cell survival

Cell cycle arrest

Cell death

Fig. 3.15: A schematic presentation to summarize the cell cycle arrest and apoptosis of OV 2008 cell subpopulations after exposure to cisplatin. As is presented in results, subpopulations of G1 and G1/S would arrest in S or G2/M of the same cycle after exposure to cisplatin, while subpopulations of S and G2/M would pass through another cell cycle to arrest in S or G2/M.

there appeared to be far more floating dead cells following exposure to cisplatin in G1 or G1/S, compared to exposure to cisplatin in S or G2/M (Quantitative results will come later).

3.2.6- Apoptosis versus necrosis: Electron microscopy confirmed that OV 2008 cells undergo both apoptosis and necrotic cell death as a result of exposure to cisplatin. Flow cytometry characteristics of necrosis have not yet been well defined, but my flow cytometry studies did demonstrate that cisplatin induced both cell cycle arrest and apoptotic cell death. So far, these experiments confirmed a difference in the kinetics of cytotoxicity when cells are exposed to cisplatin in different phases of the cell cycle. However, to answer the question of any differences in the mode of death through the comparison of apoptotic vs necrotic death, a more quantitative method was needed.

To my knowledge, there is no precise method to distinguish between apoptosis and necrosis in a quantitative way. Different fluorescence microscopic procedures for the detection and quantification of apoptosis are often used and provide an easy method to detect apoptosis. Fluorochrome dyes such as Hoechst and acridine orange have been used to study apoptosis (Blanco-Radriguez and Martinez-Garcia, 1996). Hoechst and propidium iodide dyes have also been used for the determination of necrosis (Stapper *et al.*, 1995). However, there is no established quantitative method for the measurement of apoptosis and necrosis in a single assay. A new method using Annexin V has recently been claimed to be able to distinguish apoptosis *vs* necrosis (Gorczyca *et al.*, 1993). This method is based on the loss of plasma membrane asymmetry during the process of apoptosis. In practice, Annexin V is only able to distinguish

apoptotic cells. Necrotic cells are determined as the remaining non-apoptotic and non-live population. Therefore, there is not a well defined and preferred method for a quantitative measurement of apoptotic and necrotic cells in the same cell population. After several control experiments, a method involving a double staining procedure for the measurement of apoptosis *vs* necrosis in a single experiment was developed for this thesis.

The double dye staining method, which is described in the Methods section, was confirmed in control experiments to be a reliable, simple and rapid technique for quantitative measurement of apoptosis and necrosis in a single experiment. In a control experiment using DNAse I, it was shown that living cells are bright yellow (the fluorescence appearance of AO bound on DNA), while necrotic cells are red (the fluorescence colour of PI bound to DNA). Propidium iodide (PI) won't penetrate apoptotic cells due to their healthy cell membrane. Acridine orange (AO) is able to penetrate these cells but will appear as green dots in the cell under a fluorescence microscope due to the granular condensed chromatin of apoptotic cells.

Figure 3.16 shows photographs of the appearance of live, apoptotic and necrotic cells with this method of staining under the fluorescence microscope. Figure 3.17 presents the percentages of floating (dead) vs attached OV 2008 cells at different intervals after one hour exposure to 1 μg/ml cisplatin and figure 3.18 compares these results at 48 hours after one hour exposure to 1 μg/ml cisplatin. As is shown in this graph, the rank order of the number of dead cells after exposure to cisplatin for cells exposed during different cell cycle phases is as follows: G1>G1/S>S>G2/M. This confirms that more cells will die when cisplatin is added in G1 or at the boundary of G1/S of the cell cycle than in S or G2/M. Figure 3.19 shows the percentage of

A)

B)

Fig. 3.16 Pictures of OV 2008 cells under the fluorescence microscope after staining with AO and PI. Apoptotic cells are green and necrotic cells appeared red (picture A), while viable cells were yellow (picture B).

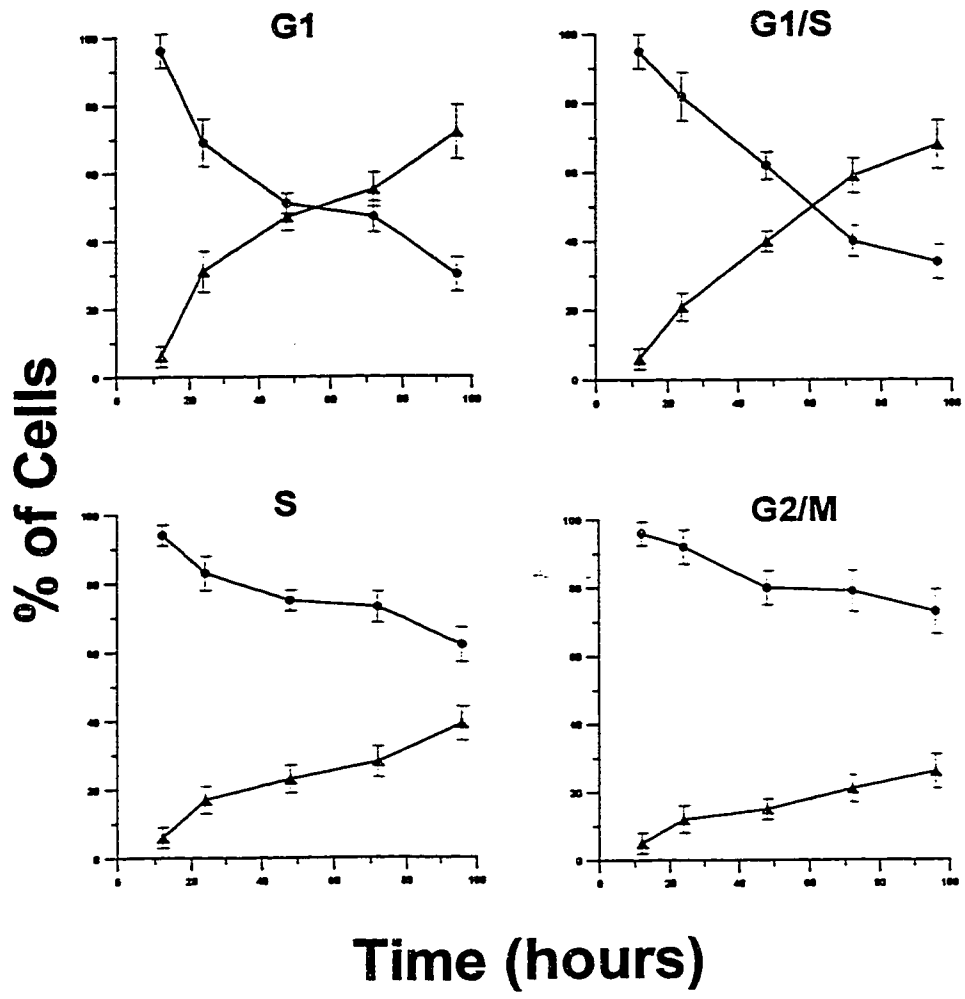
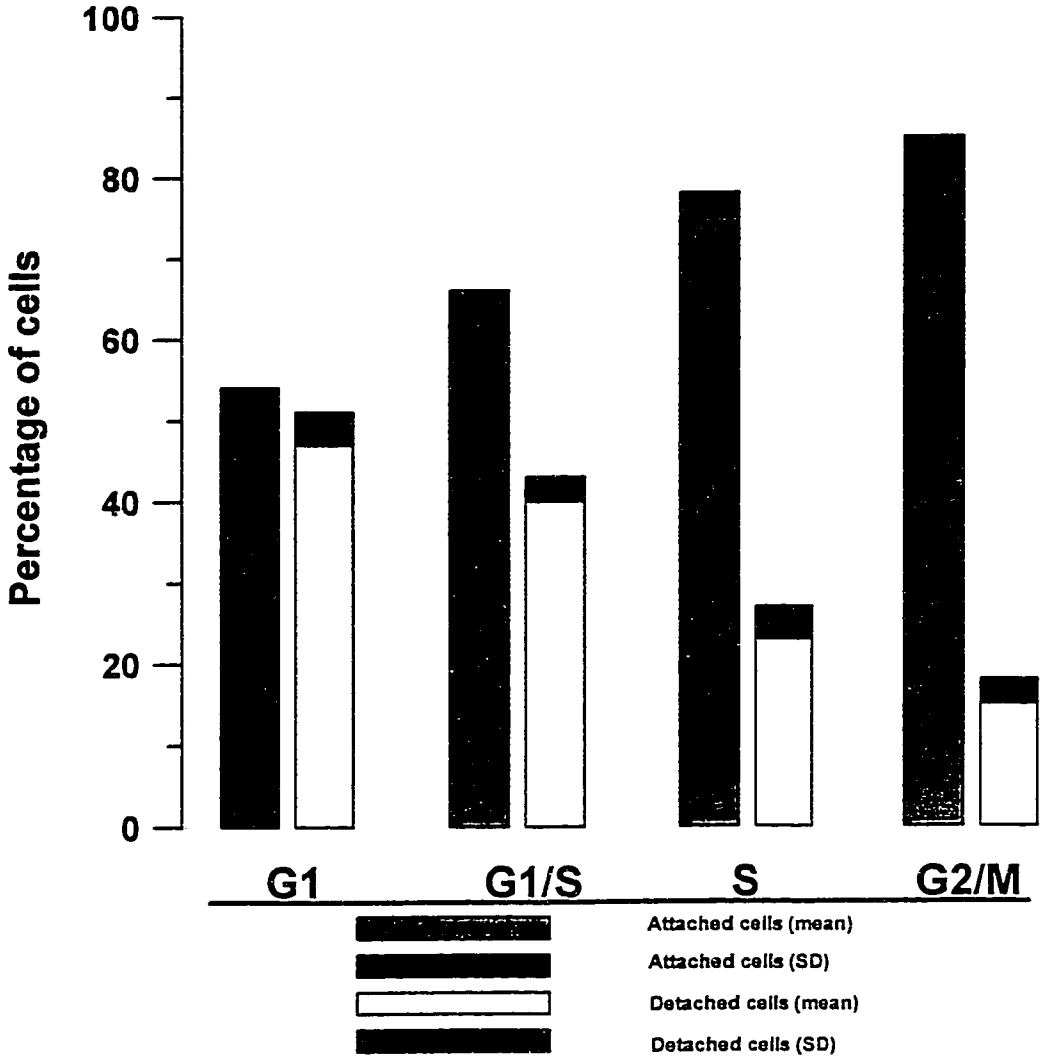


Fig. 3.17: Percentages of attached and detached OV 2008 cells at different times after one hour exposure of the different subpopulations to 1 μg/ml cisplatin. Attached cells were trypsinized at different time points and counted after staining with crystal violet. Detached cells were collected from floating cells at different time points and counted after staining with crystal violet. Data are mean ± SE. Statistical analysis for percentages of detached cells, as a measurement of cisplatin cytotoxicity (described in method section), is presented in Fig. 3.18, on page 85.



Statistical Analysis of above results using ANOVA test;

comparison	*p value*	*Comparison*	*p value*
G1 vs G1/S	N.S.	G1/S vs S	p<0.01
G1 vs S	p<0.001	G1/S vs G2/M	p<0.001
G1 vs G2/M	p<0.001	S vs G2/M	N.S.

(Above results are from Tukey-Kramer Multiple Comparison ANOVA post test.)

N.S.; not significant

Fig. 3.18: Percentages of detached OV 2008 cells, 48 hours after a one hour exposure of different subpopulations to cisplatin (mean ± SD). The proportion of nonviable detached cells is highest for G1 cells and lowest for G2/M cells. Statistical analysis on the comparison of detached cells is also presented.

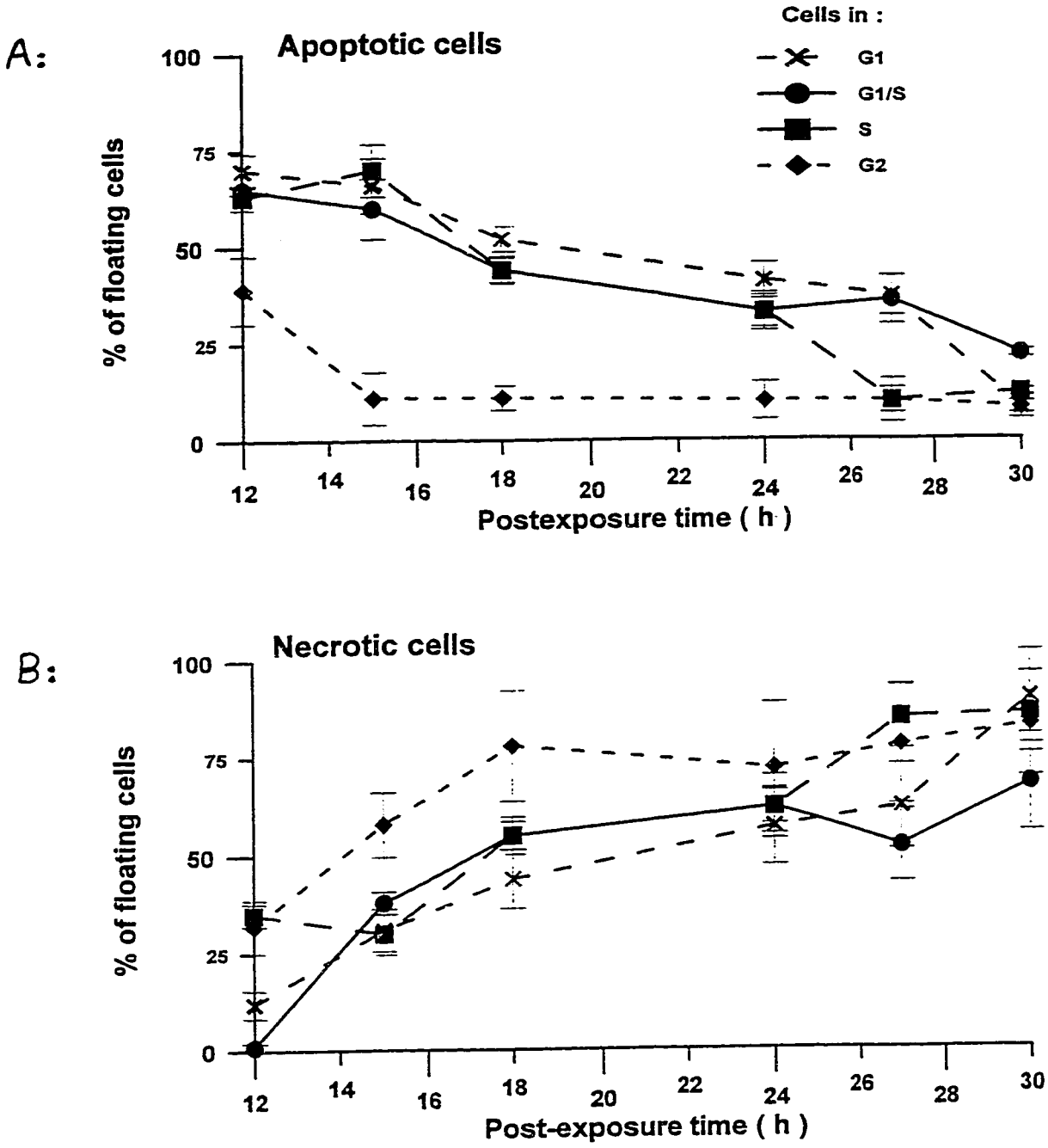


Fig. 3.19: Percentages of floating (detached) cells (mean ± SE, n=3) that are apoptotic (graph A), or necrotic (graph B) as a function of time, after one hour exposure of different subpopulations of OV 2008 cells to 1 μg/ml of cisplatin. ANOVA did not show any significant differences in the general pattern of increase of apoptotic cell and/or decrease of necrotic cell populations, except for the subpopulation in G2/M phase ($p<0.001$).

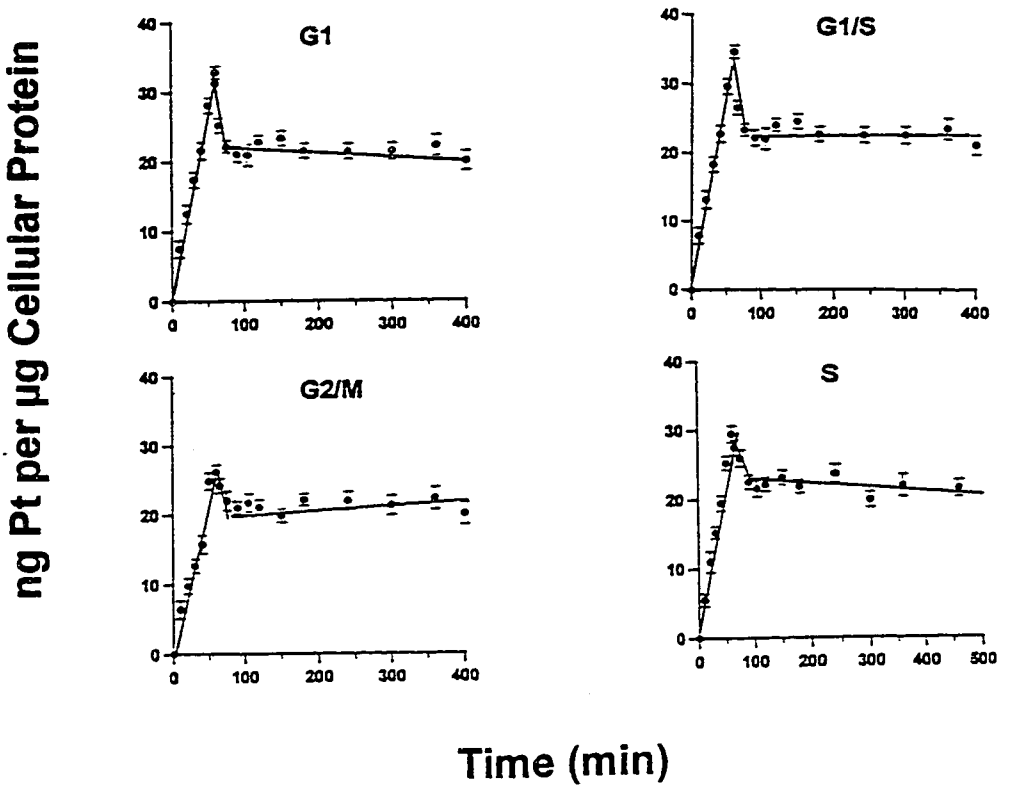
apoptotic and necrotic cells among floating cells 48 hours after exposure to cisplatin. These graphs reveal the same general pattern for different subpopulations, with the early appearance of apoptotic cells after cisplatin exposure, and a later shift to a higher proportion of necrotic cells. Hence, although the proportion of cells killed by cisplatin varies between subpopulations, the mode of cell death appears to be similar for different subpopulations.

The only disagreement in this general pattern of decreasing apoptosis and increasing necrosis is for the G2/M subpopulation, in which the percentage of apoptotic cells fell more rapidly than in other subpopulations. To speculate on this difference and to emphasize that it does not affect the acceptability of similar kinetics of cell death following the exposure to cisplatin, the concept of "secondary necrosis" might help. Secondary necrosis was first described in animal tissues (Shah *et al.*, 1997), but was then shown to occur in a variety of cell lines and in vitro experiments (Wiger *et al.*, 1997). According to this concept, cells affected by a toxic agent may enter the apoptotic pathway, but will then change to necrosis without the completion of apoptosis. Cells initially appear to be dying by apoptosis, while later observations show necrosis. The mechanism of this switch from apoptosis to necrosis remains uncertain. The general pattern I observed in OV 2008 cells is compatible with apoptosis followed by secondary necrosis. This was true for all OV 2008 subpopulations following cisplatin exposure. Necrosis is directly related to cell lysis; hence, cell membrane health and/or susceptibility to disruption may be an important factor in the onset of necrosis. One might speculate that this would explain why my G2/M cells appear to more readily go from apoptosis to necrosis than do other subpopulations. Their cell membranes are already preparing to "disrupt" as part of cell division. Overall, however, the mode

of cisplatin induced cell death appears to be comparable in the different subpopulations, and this would suggest that the basic mechanisms behind cell death are also the same.

3.2.7- Cellular accumulation of cisplatin: The general look at the different cellular toxicity pattern based on the comparison of apoptosis *vs* necrosis failed to reveal any significant differences in the different subpopulations of OV 2008 cells exposed to cisplatin. However, as noted earlier, the major difference in the shape of the dose-response curves for different subpopulations was a change in slope, compatible with passive resistance. Theoretically, a number of factors could give rise to passive resistance, including either decreased active or passive influx of drug, decreased drug binding, or increased passive efflux of drug (Stewart *et al.*, 1996).

Figure 3.20 represents the cellular platinum accumulation of OV 2008 cells as a function of phase of the cell cycle during cisplatin exposure. As is shown in this figure, cellular accumulation of platinum is linear with exposure time. On the other hand, after the termination of exposure, two different phases of efflux are distinguishable: an initial rapid phase of efflux, which lasts for about 20 minutes, and a slow phase of efflux or a plateau, during which platinum concentrations do not change significantly for up to the end of the sampling time at 5 hours. Statistical evaluation of the mathematical parameters of this graph is also presented. There are basically no significant differences in the total cellular accumulation of cisplatin in OV 2008 cells between any phases of the cell cycle.



Cells in:	Accumulation of Cellular platinum (ng/µg protein) at t=60 min	Accumulation of Cellular platinum (ng/µg protein) at plateau phase
G1/S	33 ± 2	21 ± 4
S	30 ± 3	21 ± 3
G2/M	26 ± 4	20 ± 4
G1	32 ± 3	22 ± 3

Fig. 3.20:Cellular accumulation of cispaltin, as is measured by the ng of elemental platinum per µg of cellular protein in the different subpopulations of OV 2008 cells after one hour exposure to cisplatin (description in method section). Data points are presented as mean ± SE (n=3). ANOVA test on the accumulation results at t=60 min (end of exposure) did not show any significant differences. The same test for the comparison of final platinum accumulation (determined from the extrapolation of the fitted curve) in the plateau phase has also failed to show any significant differences between subpopulations. Protein content of cells at the beginning of S, mid S and/or G2/M phases of the cell cycle are 1.1±0.05, 1.5±0.1 and 1.8±0.1 times more than G1 cells, respectively.

3.2.8- Cisplatin localization on DNA: The total cellular cisplatin accumulation studies showed no statistically significant differences between cells in different phases of the cell cycle. It is generally believed that DNA is the main target of cisplatin and other platinum compounds. I wondered if the accumulation of cisplatin on DNA would change in spite of comparable cellular cisplatin uptake for the different phases of the cell cycle. Cytoplasm, nucleoplasm and DNA of OV 2008 cell subpopulations were separated after one hour exposures to 10 μg/ml cisplatin and the platinum content was measured as described in the Methods section.

Table 3.2 summarizes the amount of elemental platinum in the different fractions of OV 2008 cells exposed to cisplatin during different phases of the cell cycle. As is shown in this table, the rank order of the accumulation of platinum on DNA is as follows for different phases of the cell cycle: G1 > G1/S > S > G2/M. This rank order is in agreement with the rank order of cisplatin cytotoxicity in OV 2008 cells in different phases of the cell cycle. Statistical evaluation on these results (ANOVA) has shown a significant difference for the accumulation of cisplatin on DNA ($p=0.0024$) and also in cytoplasm ($p=0.0136$). However, differences in nucleoplasm were not statistically significant ($p=0.755$). Therefore, it is concluded that in spite of the same total cellular accumulation of cisplatin (as was shown before), the intracellular distribution of platinum differs, depending on cell cycle phase during which cisplatin exposure occurred. Platinum would most likely bind to the DNA soon after passage through the nuclear membrane, since the amount of cisplatin in nucleoplasm was much lower than on DNA. I would also conclude that the interaction of cisplatin with DNA is more important for its cytotoxicity than its interaction with the other components of nucleus. This conclusion was made since the rank order

a) Platinum accumulation on DNA (pg / μg DNA)	
Phase	Pt on DNA
G1	43.2 ± 4
G1/S	38.5 ± 4
S	26.9 ± 3
G2/M	15.8 ± 3

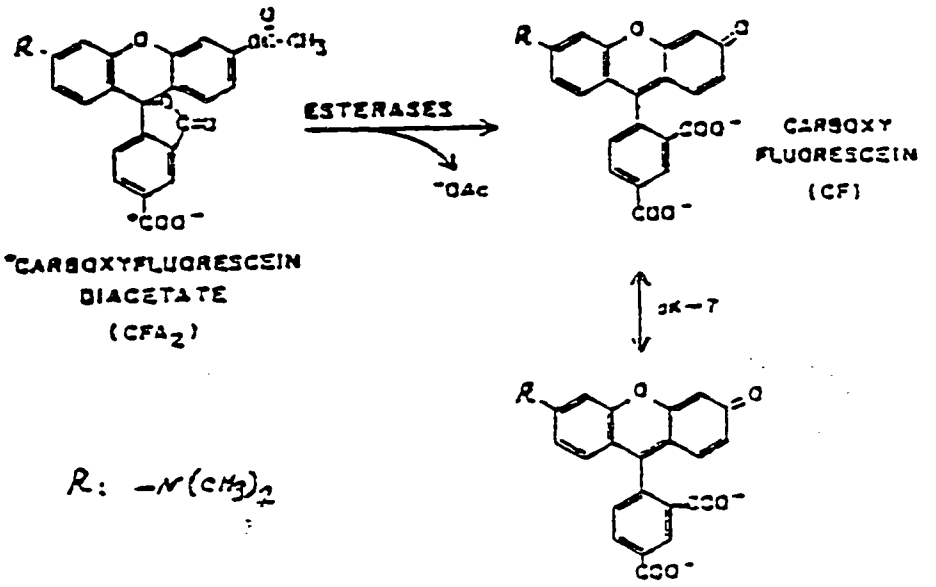
Tab 3.2: Platinum accumulation in the different fractions of different subpopulations of OV 2008 cells following one hour exposure to cisplatin. a) platinum accumulation on DNA as the pg of platinum per μg of DNA, and b) platinum accumulation in nucleoplasm and cytoplasm as the ng of platinum per pmol of protein. Data are presented as mean ± SE. Statistical evaluation of these results using an ANOVA test has confirmed significant differences in the amount of platinum on DNA and in the cytoplasm in the different subpopulations (p values 0.0024 and 0.0136, respectively). No significant difference was found in the amount of pt in the nucleoplasm in the different subpopulations.

b) Platinum accumulation in other fractions (ng pt/ pmol of protein)		
Phase	Nucleoplasm	Cytoplasm
G1	0.3 ± 0.1	14 ± 1
G1/S	1.2 ± 0.5	16 ± 2
S	2 ± 2	28 ± 5
G2/M	1.6 ± 1	32 ± 4

of accumulation on DNA was in agreement with the rank order of cytotoxicity but nucleoplasm content did not vary between subpopulations. Since the rank order of cisplatin content in cytoplasm is also significantly different between different subpopulations and is opposite to the rank order of cisplatin cytotoxicity, it is concluded that a cell cycle related factor facilitated either the passage of cisplatin through the nuclear membrane or its interaction with DNA. Again, since the amount of cisplatin is almost the same in the nucleoplasm of different subpopulations, it would be most likely that the latter hypothesis is correct, i.e. some cell cycle related factor facilitates cisplatin interaction with DNA, thereby increasing its cytotoxicity. Either a cell cycle factor may behave as a carrier that transports cisplatin into the nucleus, or else cisplatin may change to a more potent DNA-reacting agent due to cell cycle specific intracellular changes. Molecular studies done in the past by a variety of groups (Andrews and Howell, 1990; Gately and Howell, 1993) have failed to show a carrier for cisplatin, but changes in intracellular pH could potentially lead to important changes in the chemical structure of cisplatin.

3.2.9- Intracellular pH: Using SNARF-1 and flow cytometry, the intracellular pH of OV 2008 subpopulations was measured as described in the Methods section. Figure 3.21 represents a sample histogram of cells stained with SNARF-1. Figure 3.22-a shows the standard curve of the changes of SNARF-1 emissions ratio by changes in the intracellular pH. A linear fitted curve on this graph has an R-squared value of 0.988, which indicates a linear correlation in the pH range of interest. Using this calibration curve, figure 3.22-b represents the intracellular pH changes of synchronized OV 2008 cells in different phases of the cell cycle. As is seen in this graph, cells

I:



*CARBOXYFLUORESCEIN DIACETATE (CFA2)

II:

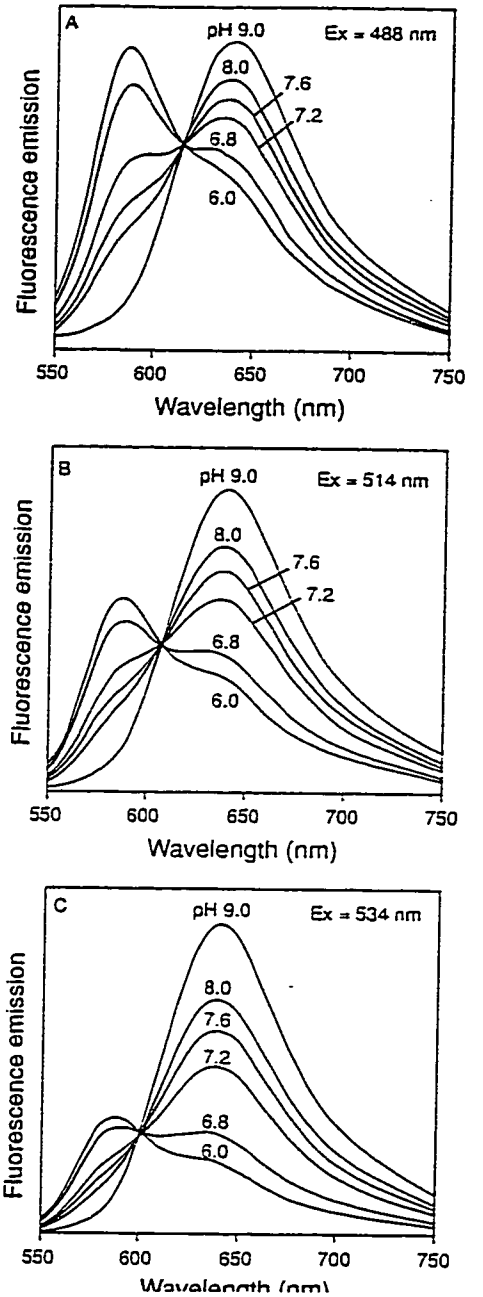
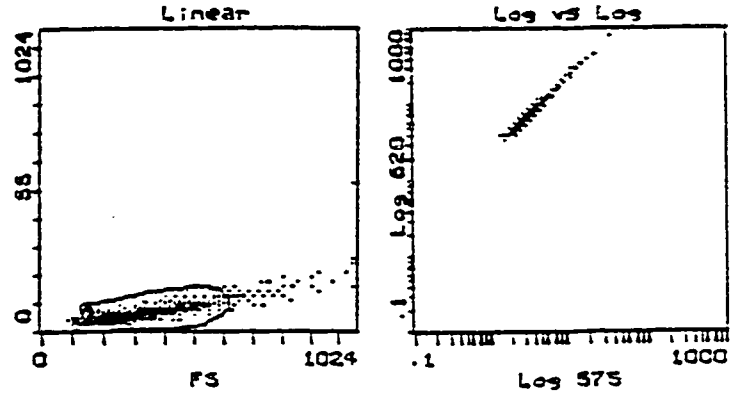
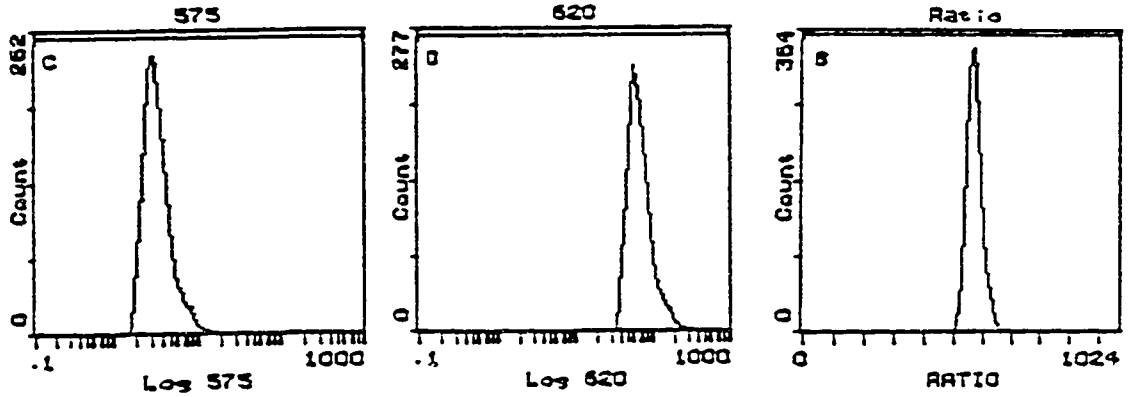


Fig. 3.21: Flow cytometric histogram of OV 2008 cells after incubation with SNARF-1. I: Chemical bases of SNARF-1 action; SNARF-1 enters the cell by passive diffusion as a nonpolar agent. While in the cell, it will convert to its ionic form with the help of cellular esterases. The ionic form of SNARF-1 inhibits its leakage from the cell. This form of SNARF-1 may change to its isomer as a result of intracellular pH changes. Two different intracellular isomers of SNARF-1 have different emissions for the excitation at 488 nm. II: These graphs confirm the linearity of SNARF-1 excitation and/or emissions at two different wavelengths and in different pHs. III: OV 2008 cell distribution counted on Epics XL Coulter flow cytometer. Live cells on the forward vs side scatter histogram have been selected for the measurement at two different emissions of 575 nm and 620 nm. IV: Emission of SNARF-1 in OV 2008 cells at two different pHs at 575 nm and 620 nm. A third graph represents the ratio of these two emissions, which varies with the changes in intracellular pH.

III :


Linear
1024
85
0
0
1024
FS
Log vs Log
1000
Log 620
.1
.1
1000
Log 575

IV :


575
262
C
Count
0
.1
1000
Log 575
620
277
D
Count
0
.1
1000
Log 620
Ratio
364
E
Count
0
0
1024
RATIO

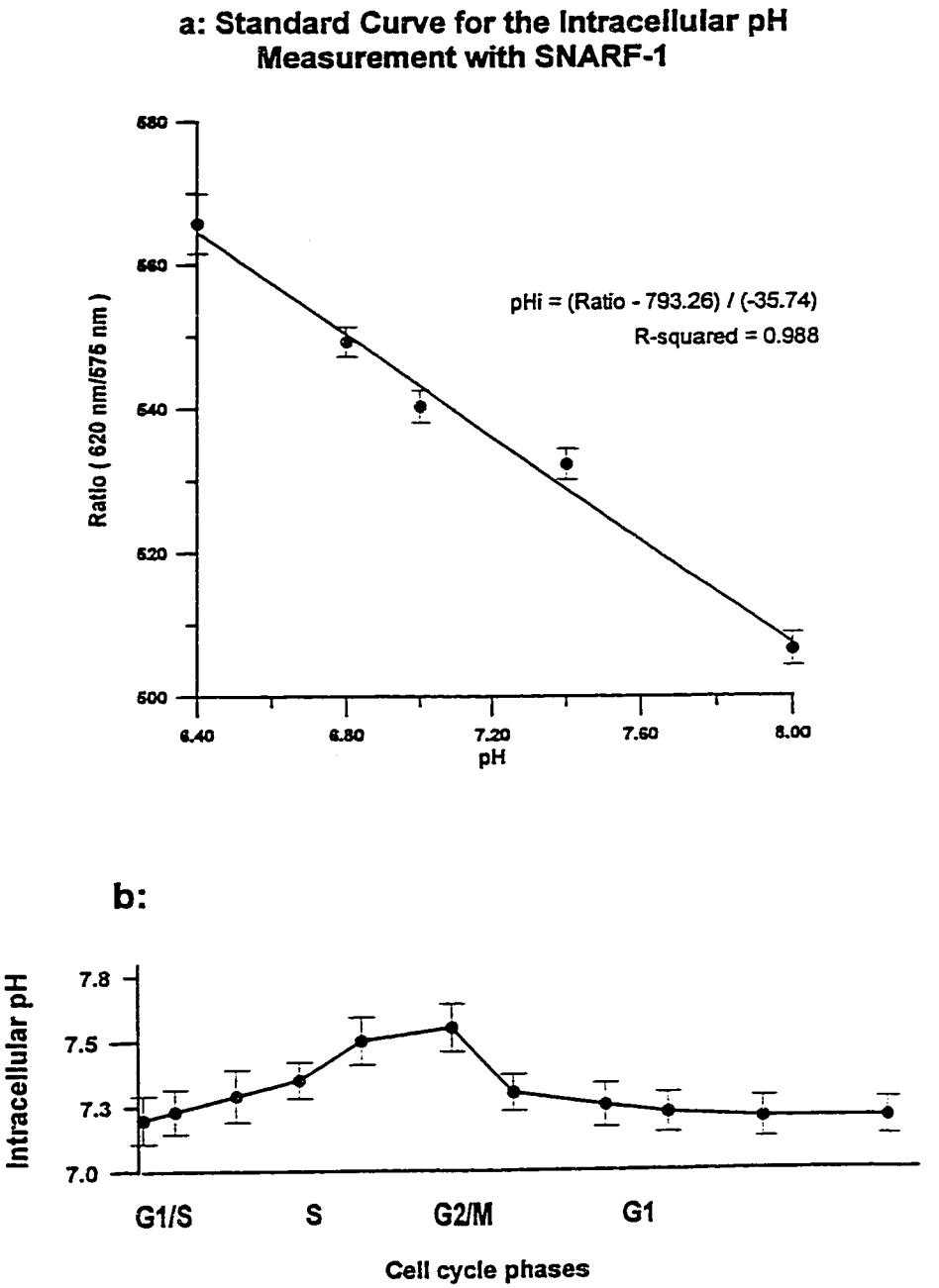


Fig. 3.22: Intracellular pH (pHi) measurement of OV 2008 cells: a) standard curve for the measurement of OV 2008 pHi using SNARF-1, as described in method, and b) pHi of OV 2008 cells during different phases of the cell cycle. Data are as mean ± SE. There is a significant difference between the pHi of cells in G1 and G2/M ($p=0.0186$, calculated by ANOVA).

are in their most acidic state in G1. Intracellular pH will increase from the entrance of cells to the S phase and will reach its peak in G2/M. Analysis of variance (ANOVA) showed a significant difference between the intracellular pH of cells in G1 and cells in G2/M, with a p value of 0.0186.

3.2.10- Cisplatin transformation as a function of pH: This experiment (as described in the Methods section) was designed to use HPLC to confirm the transformation of cisplatin into different species as a function of pH.

As is shown in figure 3.23, four distinguishable forms of platinum-containing substances were found using HPLC separation and atomic absorption spectroscopy quantitation. Two different pHs of 4 and 9 were selected as controls for the transformation of cisplatin in acidic and basic environments, respectively (as described in method). Polar compounds would be expected to pass through the C_{18} columns soonest in this reverse phase HPLC method. Therefore, the first peak (Retention time [Rt]= 5 min) shown in figures A, B and very small in C of figure 3.19 is presumed to be related to polar diaquated cisplatin, which is formed in an acidic environment. As is shown in graphs A, B and C, the presumed diaquated cisplatin is the major species at pH=4, but decreases as the pH rises, particularly from pH 6 to 7. In pH=7, presumed monoaquated cisplatin (Rt=9 min) is at a much higher concentration than is diaquated cisplatin. Hydroxylated cisplatin species (formed in a basic environment) are presumed to be represented by the peaks after 12 minutes. This is supported by the results of studies done at pH=9 (graph D). The last peak (Rt=22) is thought to be due to parental cisplatin. Graph C, which resulted

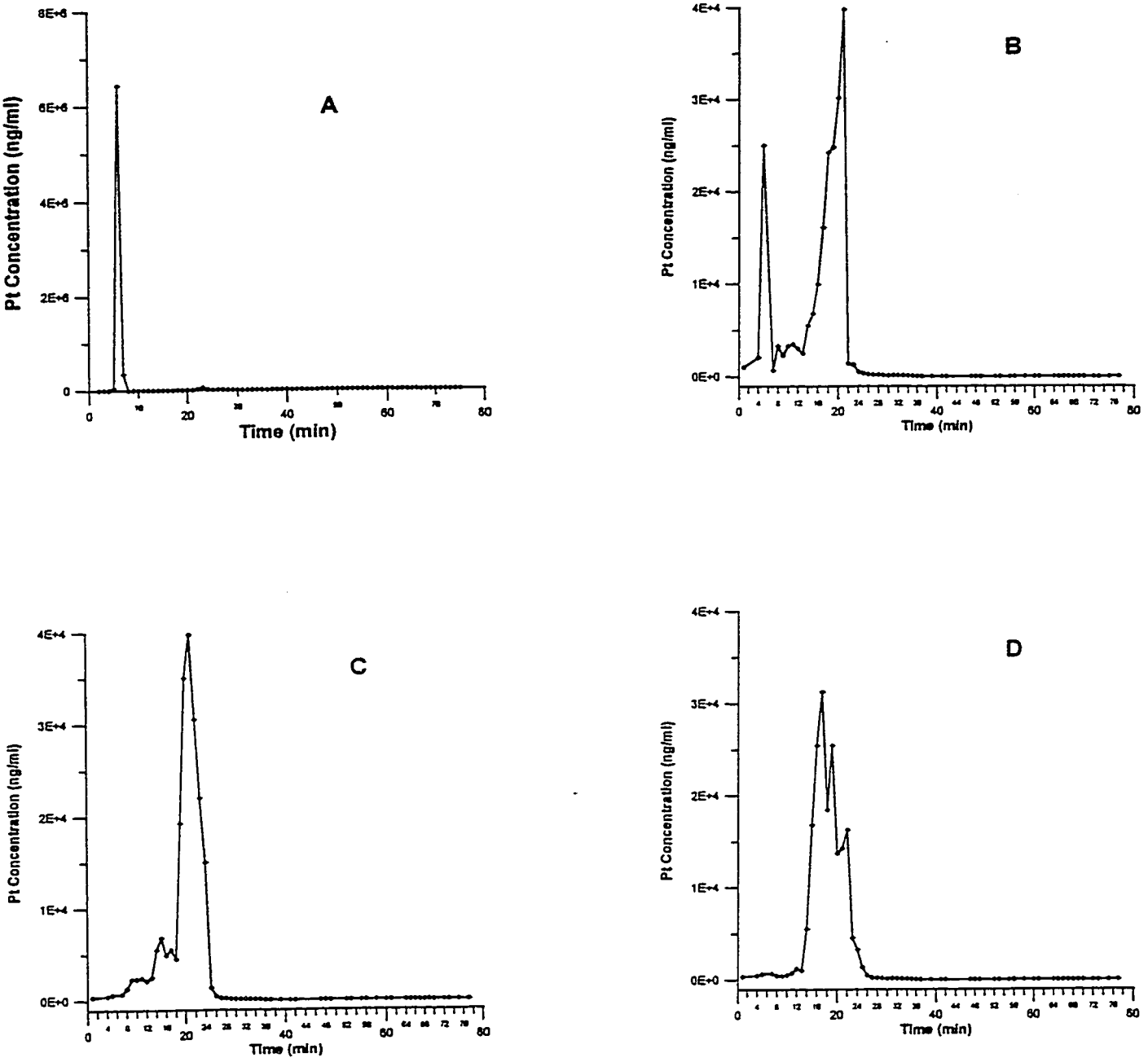


Fig. 3.23: HPLC analysis of platinum species produced in vitro by changing the environmental pH of cisplatin to: A) pH=4, B) pH=6, C) pH=7, and D) pH=9. A 100 µl of 1 mg/ml cisplatin solution at the different pHs were injected into a C18 column on a gradient HPLC system. 40 fractions of extract from column after the injection was collected in different tubes, at the rate of 1 ml/min and the platinum content of each fraction was measured using atomic absorption spectroscopy. Due to unavailability of controls for the peaks at different retention times, cisplatin dissolved at two different pHs of 4 and 9 have been selected as positive control for peaks generated in acidic and basic environments.

from cisplatin in Tris buffer at pH 7, supports this assumption. As is shown from these graphs, more aquated cisplatin is probably formed at lower pH values and a more hydroxylated cisplatin is formed at higher pH values. Retention time of different peaks in this experiment are also affected by the Tris buffer which has been used to control pH.

3.2.11- DNA binding of cisplatin at different pH levels: It has been shown that the aquated form of cisplatin (and not the parent drug) is the best candidate to interact with DNA (chapter 1). Hence, the DNA binding of cisplatin at different pH levels was measured, to assess whether it would vary over the pH range I found in different cell cycle phases.

Figure 3.24 represents the amount of platinum (in ng platinum per μg DNA) covalently bound to OV 2008 DNA after a one hour exposure to cisplatin at different pH levels. As shown in this graph, there was a higher degree of DNA adduct formation with greater acidity (R-squared = 0.95). DNA accumulated about 21 percent more platinum adducts at pH=7 than pH=7.5 ($p<0.05$ by ANOVA). Therefore, cisplatin binds more avidly to DNA in an acidic pH milieu than in a more basic pH milieu. Considering the fact that the intracellular pH of OV 2008 cells changes with the cell cycle phases, it may explain why the cytotoxicity of cisplatin is different for different subpopulations of OV 2008 cells.

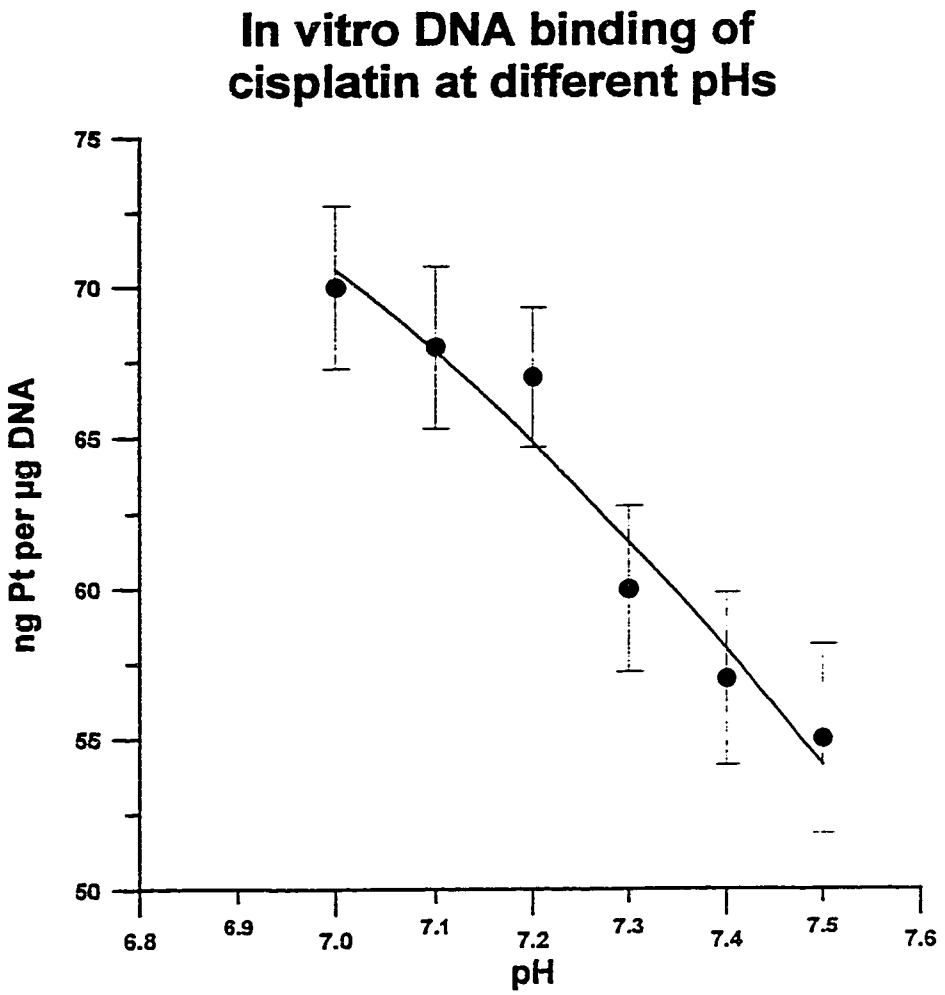


Fig. 3.24: DNA binding of cisplatin at different pHs. Genomic DNA was extracted from OV 2008 cells and was exposed to cisplatin at different pHs, for one hour. Elemental platinum was measured with atomic absorption spectroscopy in each sample and is expressed per amount of DNA. Points are presented as mean ± SD. Platinum DNA binding increases as the environmental pH decreases (R-squared=0.95). Statistical analysis showed a significant differences in the DNA binding of cisplatin at pH=7 compared to pH=7.5 ($p<0.05$, calculated by ANOVA).

4

Discussion:

The ability of a cell to produce exact replicates of itself is an essential component of cellular life. It is a process that must be done with great precision for organisms and species to propagate. Any error in the cellular reproduction process creates a situation of genetic instability, which appears to be a significant contributing factor to the development of malignant cells in higher eukaryotes (Calabresi and Parks, 1980). Thus, it is not surprising that cancer is a disease characterized by abnormal regulation of cellular growth and reproduction.

In a dividing cell population, the replication and division of a cell into genetically identical daughter cells depends on two functional phases and two preparatory phases. The functional phases of the cell cycle are the precise copying of the DNA, known as S phase or DNA replication, and the accurate segregation of duplicated sets of chromosomes between daughter cells, the M phase or mitosis. The cell prepares itself biochemically for S phase in a preparatory phase known as G1 (for gap 1) and prepares for mitosis in a poorly understood preparatory phase known as G2 (gap 2)(Baserga, 1985).

It has been suggested that one could take advantage of altered cell cycle checkpoints in tumor cells to make chemotherapy or radiotherapy given in a particular sequence more

specifically toxic for tumor cells (Hartwell and Kastan, 1994). For example, recent data suggest that tumor cells, particularly tumor cells that have lost the G1 checkpoint because of mutations in p53, may be particularly sensitive to inhibition of the G2 checkpoint (Russell *et al.*, 1995). As a second example, a number of agents that abrogate the G2 checkpoint and induce premature mitosis, such as caffeine, rapamycin, and staurosporine have been shown to enhance the cytotoxicity of cisplatin (Shi *et al.*, 1995). Checkpoints have been found to be very important in at least one of the major mechanisms of cell death, apoptosis (Oltvai and Korsmeyer, 1994).

Apoptosis is a mode of cellular death that appears to be an energy-dependent, programmed event that occurs in response to certain stimuli. A variety of stimuli (e.g., irradiation and chemotherapy, viral infection, growth factor or hormone withdrawal, and cytotoxic lymphocyte killing) can initiate this death program (Wyllie, 1987). Some of the gene products that control the cell cycle also appear to influence apoptosis (e.g. c-myc, p53, and pRb)(Thompson, 1995). Wyllie (1993) has suggested that apoptotic death is the natural default outcome for cycling cells unless a survival factor (hormone or growth factor) is present to keep the cell alive as it progresses through the cycle. Because responses to current antineoplastic therapies are also likely to be affected by the apoptosis tendencies of cells, this process has therapeutic implications.

Cisplatin has been proven to induce apoptosis as one of its mechanisms of cytotoxicity (Ormerod *et al.*, 1994-a). It has been proposed that resistance to cisplatin in human ovarian cell lines may be due to inactivation of a cisplatin-dependent p53-accumulation pathway (Vikhanskaya *et al.*, 1997). However, mutations in the p53 gene do not have any effect on the induction of apoptosis in human breast and ovarian cancer cells (Hunt *et al.*, 1997). Although the

relationship between p53 gene status and cisplatin-induced apoptosis and/or cisplatin resistance has been analyzed in a variety of human ovarian carcinoma cell lines (Fajac *et al.*, 1996; Perego *et al.*, 1996; Skilling *et al.*, 1996), I am unaware of any data on p53 status in the OV 2008 cell line.

Many intracellular factors of potential importance to chemotherapy efficacy will change along with the cell cycle phases. Intracellular concentration of different ions and minerals are among these factors. Changes in intracellular concentration of hydrogen ion (pH) have been found to accompany a variety of cellular functions, and in some of these, the intracellular pH change is an essential part of the control mechanism. Intracellular pH was first postulated to change during the cell cycle in 1960 (Frydenberg and Zeuthen, 1960). However, demonstration of this phenomenon was not forthcoming until 1976, when Gerson and Burton (1976), showed fluctuations of intracellular pH during the cell cycle of Physarum. In this system, the intracellular pH maximum occurred just prior to mitosis, and the minimum occurred during S phase. The amplitude of the fluctuations in this system was approximately 0.4 pH units. However, since Physarum does not express a G1 period, it remained unknown whether the alkalinization of intracellular pH correlated with mitosis or with the initiation of DNA synthesis. Intracellular pH oscillations accompany progression through the cell cycle in a number of different systems. In general, these oscillations appear to bear some fixed relation to nuclear events, specifically, mitosis and/or DNA synthesis. The general impression is that increased proliferation correlates with increased intracellular pH (Nuccitelli and Heiple, 1982).

In recent years, the development of new fluorochromes and related instrumentation

has enabled the measurement and imaging of intracellular pH within single cells (Tsien and Poenie, 1986). Measurements are usually not based on absolute determinations of fluorescence, but on the ratio of excitation or emission at two different wavelengths. This approach is generally believed to normalize for variation in path length, photobleaching and dye concentration (Bright *et al.*, 1987). The pH-sensitive fluoroprobe SNARF-1 has been extensively used for the measurement of intracellular pH in a variety of cell lines (Buckler and Vaughan-Jones, 1990).

Cisplatin is a neutral, square planar molecule containing two chlorine leaving groups oriented in a cis configuration. This structure is stable and relatively nonreactive in aqueous environments such as plasma, in which the chloride concentration is greater than 100 mM. However, at lower chloride concentrations (e.g., intracellularly), cisplatin loses its chloride atoms and is converted to an aquated, reactive electrophile that can bind covalently to a variety of macromolecules, including DNA (Pinto and Lippard, 1985). The precise mechanism by which platinum-DNA damage results in cell death is unknown.

Traditionally it was believed that cisplatin is not a cell cycle specific agent. However, using human ovarian OV 2008 carcinoma cells, I found that cisplatin is more toxic to plateau phase cells than to actively propagating cells. This result was in agreement with some previous publications (Raaphorst *et al.*, 1995), but disagreed with others (Evans *et al.*, 1994). This disagreement might be partially due to the different cell lines used in different experiments.

Considering the fact that cells in plateau phase *vs* exponential phase differ with respect to percentages of cells in different phases of the cell cycle, I hypothesised that human ovarian cancer OV 2008 cells have different sensitivities to cisplatin in different phases of their

cell cycle. This project confirmed this hypothesis using cells synchronized in different phases of the cell cycle. G1 cells were the most sensitive to cisplatin, followed by cells at the boundary of G1/S, S and eventually G2/M cells.

As discussed previously, cisplatin cytotoxicity is mediated either through necrosis or apoptosis. I thought that the percentage of apoptotic *vs* necrotic dead cells might change if different mechanisms of cell death were involved in the cytotoxicity of cisplatin, or that the kinetics of apoptosis or necrosis would change. My apoptosis experiments showed the same pattern of arrest and death for OV 2008 cells as has been published for other cell lines exposed to cisplatin-ie, arrest in S and G2/M (Fujikane *et al.*, 1989; Demarcq *et al.*, 1992).

In a comparison of apoptosis and necrosis resulting from the exposure to cisplatin of OV 2008 cells, the kinetics of appearance or disappearance of apoptotic and/or necrotic dead cells was found to be the same for OV2008 cells exposed to cisplatin during different phases of the cell cycle. This pattern of apoptosis and necrosis appearance and disappearance is in agreement with the theory of apoptosis evolving into secondary necrosis, as suggested by some investigators (Shah *et al.*, 1997). The results of this experiment not only indicated dual mechanisms of necrosis and apoptosis for cisplatin cytotoxicity, but also supported the argument that changes in cisplatin cytotoxicity between cell cycle phases were due to changes in cisplatin potency, rather than being due to changes in the mechanism by which cisplatin kills cells.

One possible explanation for differences in cisplatin cytotoxicity during different phases of the cell cycle would be due to variations in drug accumulation in cells. Many (but not all) cisplatin-resistant cell lines have been found to have reduced cisplatin accumulation

compared to their sensitive variants (Andrews *et al.*, 1988). However, cellular accumulation of platinum over time was found to be similar for OV 2008 cells in different phases of the cell cycle. Demarcq and co-workers (1994) have shown the presence of a cell cycle dependent protein which appears at the boundary of G1/S and binds strongly with cisplatin. This protein, however, is not as important in the other phases of the cell cycle and, hence, would not be able to explain the pattern of different cisplatin cytotoxicity from G1 to G2/M. Furthermore, this protein might not be of importance in OV 2008 cells, which have shown a higher sensitivity to cisplatin in G1 than at the boundary of G1/S.

In this project, and for OV 2008 cells, an increasing accumulation of cisplatin on its target site (DNA) was found to be in good agreement with the rank order of cisplatin cytotoxicity in different phases of the cell cycle. This raised the question of why cells in different cell cycle phases had different amounts of cisplatin DNA binding despite having comparable cellular accumulation of platinum.

Different scenarios might explain this phenomenon. Is the nucleomembrane transport of cisplatin different for cells in different phases of the cell cycle? Does the different configuration of DNA make it more or less attractive for cisplatin to form a covalent adduct? Or does intracellular conversion of cisplatin to its metabolites vary with cell cycle phases? To investigate this, I concentrated on factors that might both change with the cell cycle and that might also affect cisplatin affinity for DNA.

A negligible amount of platinum was found in the nucleoplasm, and cisplatin content in the nucleoplasm did not vary significantly in different cellular subpopulations. If an

intracellular carrier was responsible for the variable transport of cisplatin from cytoplasm to the nucleus, the same rank order of platinum content should have been found in the nucleoplasm as the rank order of cisplatin cytotoxicity, but this was not the case. Therefore, I suspected that the cisplatin molecule is being converted to a stronger and/or weaker "DNA binding agent" in the different phases of the cell cycle.

As mentioned in chapter 1, in aqueous solution, cisplatin undergoes reversible substitution of its chloride ligands with water to form aquated cisplatin. This substitution mechanism involves equilibria that depend on the chloride ion concentration and pH (Zieske *et al.*, 1991). Since intracellular chloride ion concentrations are quite stable in different phases of the cell cycle (Jennerwein and Andrews, 1994), I concentrated my attention instead on intracellular pH.

My experiments confirmed intracellular changes of pH that are in good agreement with the cytotoxicity of cisplatin. OV 2008 cells in G1 phase are at the lowest pH, followed by an increasing pH from S, to a peak in pH during G2/M. Interestingly, recent data on the comparison of intracellular pH in the cisplatin sensitive vs resistant HTB56 human lung adenocarcinoma cell line indicated a higher intracellular pH in the resistant cell line compared to the sensitive one (Chau Q., Department of Pharmacology, University of Ottawa, unpublished data, 1998).

As is shown in my results, the intracellular pH levels in G1 and G2/M, are 7.1 and 7.45, respectively. Therefore, it will cause a 2.24 times differences in the ratio of aquated cisplatin to the parent drug in G1 versus G2/M phases of the cell cycle. i.e. $10^{-7.1 - (-7.45)} = 2.24$

The result is very close to the 3 fold accumulation of cisplatin on DNA in G1 vs G2/M in OV 2008 cells. The question then arises whether this would be enough to lead to a 10 fold increase in cytotoxicity. The answer to this question is affirmative. Many pharmacological processes are driven by first order kinetics, and chemotherapy cytotoxicity is one such process. Hence, with most chemotherapy drugs, a doubling of the dose leads to a one log (i.e., 10 fold) increase in cell kill. In this case, a doubling of DNA cisplatin binding would be equivalent to a doubling of cisplatin dose. In a similar vein, note that for any one phase of the cell cycle, figure 3-5 indicates that a doubling of cisplatin dose translates into roughly a 10 fold reduction in cell survival, as would be expected with killing by first order kinetics.

In summary, cisplatin cytotoxicity in human ovarian OV 2008 carcinoma cells is cell cycle dependent. The rank order of cytotoxicity is as follows: G1>G1/S>S>G2/M. Both necrosis and apoptosis have been found as modes of cell death caused by cisplatin cytotoxicity. However, there are no differences in the percentages and kinetics of the different modes of cell death, when cells were exposed to cisplatin in any phase of the cell cycle. The rank order of intracellular acidity of OV 2008 cells is exactly the same as its cytotoxicity for the different phases of the cell cycle. Cisplatin was shown to make increasingly more DNA adducts with a decrease in pH. This is a rational explanation for why more cisplatin accumulated on its target (DNA), in spite of the same intracellular platinum content for cells in different phases of the cell cycle. Therefore, the intracellular metabolism of cisplatin probably varies with cell cycle phase in OV 2008 cells, and it is mainly under the control of the intracellular pH. Alterations in intracellular pH would be expected to result in the following rank order for formation of the active aquated species of

cisplatin: G1>G1/S>S>G2/M. To my knowledge, this study is the first to indicate that the cell cycle dependency of cisplatin cytotoxicity may be due to changes in DNA binding of cisplatin resulting from variations in intracellular pH.

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IMAGE EVALUATION
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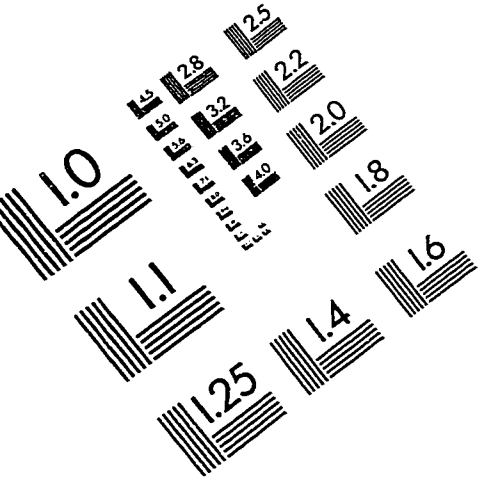

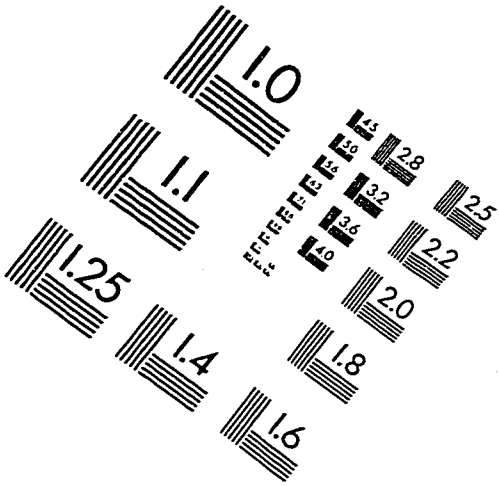

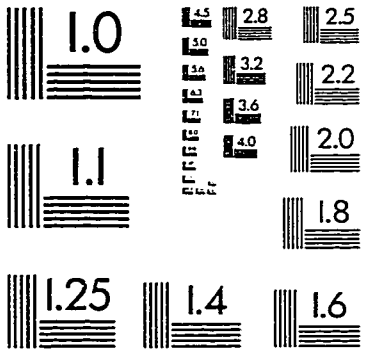

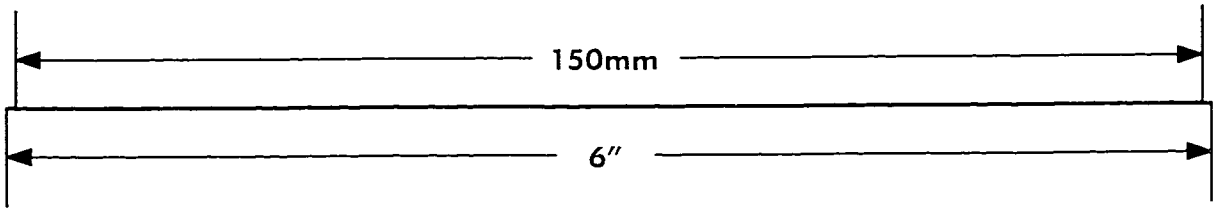

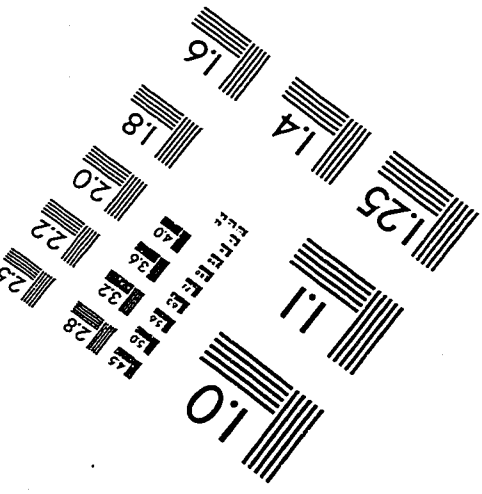

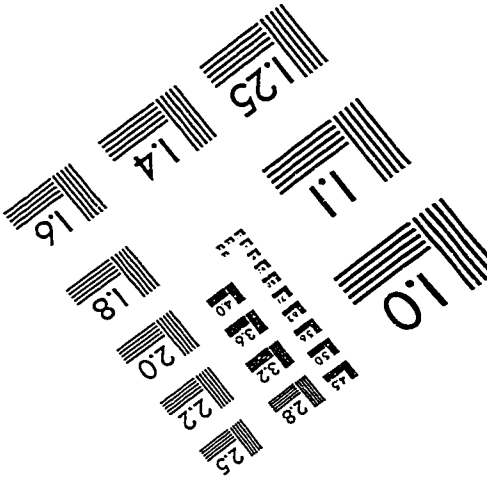

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