Chemoresistance to paclitaxel in human ovarian xenografts: the role of apoptosis-regulating proteins

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Abstract

Paclitaxel has established an evolving role in the management of ovarian cancer. However, the emergence of resistance to paclitaxel is likely to become an increasing problem, and thus a reason for failure of therapy of ovarian cancer. Understanding of the molecular pathways involved in the development of resistance to paclitaxel may provide suitable targets for novel therapies to help to circumvent resistance to paclitaxel. Three main mechanisms have been shown to be involved in paclitaxel resistance: (a) altered paclitaxel uptake, (b) altered paclitaxel-microtubules interaction, (c) alterations in apoptosis regulating proteins that control the induction of cell death.

The realization that most chemotherapeutic agents- including paclitaxel- induce cell death by a genetically programmed process termed apoptosis together with the demonstration that alteration of apoptosis regulating proteins such as bcl-2 family of proteins or p53 may induce chemoresistance to paclitaxel has led to intense research in this area. Almost all these studies were in vitro with an obvious lack of in vivo studies. Hence, these apoptosis regulating proteins were the focus of the present in vivo study as they may either represent oncogenes responsible for acquired resistance, or alternatively potential targets for molecular intervention to circumvent resistance to paclitaxel.

The first part of the study compared the alterations in apoptosis regulating proteins between CH1 paclitaxel sensitive xenografts (CH1/TS) and acquired paclitaxel resistant xenografts (CH1/TR). These xenografts were established from a CH1 paclitaxel sensitive cell line treated with paclitaxel which then acquired resistance in vitro. Following the confirmation of the sensitivity pattern of both tumours to paclitaxel in vivo, it was demonstrated that there was no paclitaxel uptake defect in CH1/TR tumours that may contribute to paclitaxel resistance. Flow cytometric and apoptosis studies provided an insight into the mechanism of action of paclitaxel. They confirmed the response pattern with paclitaxel inducing mitotic arrest, G2/M phase arrest, and apoptosis in CH1/TS tumours. In contrast, these parameters were unaltered in the CH1/TR tumours, consistent with its resistant phenotype. There were clear differences in the induction of apoptosis regulating proteins between the paclitaxel sensitive and resistant tumours. Following the treatment of CH1/TS tumours with paclitaxel, there was a significant

early induction of p53, and p21 with evidence of inactivation (phosphorylation) of survival promoting (anti-apoptotic) protein bcl-2, and down regulation of anti-apoptotic protein bcl-xl. These changes were not significantly altered in CH1/TR tumours.

Subsequently, these xenografts were treated with cisplatin to compare and contrast its effect to that of paclitaxel. The first difference demonstrated was that both CH1/TS and CH1/TR tumours displayed complete and partial response, respectively, to cisplatin. In contrast to paclitaxel, the main cell cycle alterations to cisplatin were accumulation of cells in S phase, and late G2 arrest. These cell cycle alterations were shown to be associated with induction of apoptosis. More importantly, it was observed that cisplatin was able to induce p53 and down regulate bcl-xl in CH1/TR tumours suggesting that these pathway are intact. This suggested that CH1/TR tumours acquired resistance to paclitaxel was not due to inherent defect in the expression of apoptosis regulating proteins, but instead due to an upstream defect possibly at the level of paclitaxel-microtubule interaction. This is consistent with previous in vitro studies that have shown that successful polymerization of microtubules is required for induction of cell death, induction of p53 and p21 and phosphorylation of bcl-2. Taking all these observations into consideration, it could postulated that resistance to paclitaxel in CH1/TR tumours that was reflected in the lack of significant alteration in apoptosis regulating protein was due to unsuccessful microtubule-paclitaxel interaction which is supported by the lack of G2/M arrest and induction of apoptosis. The paclitaxel-microtubule interaction will be the focus of a subsequent study.

The second part of this study demonstrated in vivo that the overexpression of a single apoptosis regulating protein resulted in the modulation of response to chemotherapy. Xenografts of CH1 paclitaxel sensitive cells overexpressing the anti-apoptotic protein bcl-xl were established and treated with paclitaxel and cisplatin. The overexpression of bcl-xl protein resulted in development of chemoresistance to paclitaxel and cisplatin. This observation was particularly important because it demonstrated that these proteins do control a common pathway controlling cell death, and further underline their importance as possible targets for molecular therapy.

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Chapter 1: Introduction

1.1 Development of chemotherapy treatment for ovarian cancer

1.1.1. Introduction

Epithelial ovarian cancer is the sixth most common neoplasm among women worldwide and the leading cause of death from gynaecological cancers, with an incidence rate of about 15 per 100,000 women in the western world. Despite the improvement in the management of ovarian cancer, it still has a poor prognosis. The fact that up to 64% of patients present with advanced disease (FIGO stage III and IV) and the development of chemoresistance are the two main contributing factors to the poor outcome (Trope C, 1997).

The present standard management of advanced ovarian cancer consists of aggressive surgical cytoreduction followed by chemotherapy. Surgery is the cornerstone of management and it is supported mainly by retrospective data that show a clear inverse correlation between volume of residual disease and both response to chemotherapy and survival (Hacker NF, 1983). Ovarian cancer is chemotherapy sensitive with 70% to 80% of patients responding to platinum-based chemotherapy. Furthermore, 20% of patients with suboptimally debulked disease (residual disease greater than or equal to 1 cm) and 50% with optimally debulked disease (residual disease less than 1 cm) will achieve complete pathological response as determined by second look lapratomy (Kristensen GB, 1997).

The evolution of chemotherapeutic regimens for management of ovarian has progressed through phases. Before 1978 the standard chemotherapy following surgery was single alkalyting agent (melphalan) or a combination of an alkalyting agent (cyclophosphamide) and doxorubicin (AC) (Tobias JS, 1976). Subsequently, two chemotherapeutic agents dominated the management of ovarian cancer, cisplatin and paclitaxel.

1.1.2 Clinical development of cisplatin-based chemotherapy

Following the demonstration of activity of cisplatin (P) in ovarian cancer, it was added to cyclophosphamide and doxorubicin (CAP). The Gynecologic Oncology Group (GOG) study 47 randomized patients with large volume advanced disease to receive either AC or CAP regimen (Omura G, 1986). The addition of cisplatin to the regimen resulted in improvement in complete and overall response rate, duration of response and survival in patients with measurable disease. Subsequently, the Netherlands joint study group confirmed the superiority of cisplatin containing regimen over non-platinum containing regimens with improvement in survival (Neijt JP, 1991). A large meta-analysis further substantiated the superiority of platinum compound containing regimens (Advanced Ovarian Cancer Trialists Group, 1991).

These studies established platinum-based chemotherapy as the treatment of choice and subsequent trials were aimed at improving the therapeutic index and reduction of toxicity. The first modification was to assess the role of doxorubicin. The GOG study 52 randomized patients with small volume stage III disease to receive either CAP or cyclophosphamide and cisplatin only (Omura GA, 1989). There was no difference between the two regimens in terms of response or survival with reduced toxicity for cisplatin combination. Four other trails reached the same conclusion and established the cisplatin and cyclophosphamide combination as the regimen of choice (Bertelsen K, 1987; Conte PF, 1986; Hernadi Z, 1988) ((Gruppo Interegionale Cooperativo Oncologico Ginecologia, 1987).

The second modification was to assess the role of escalating cisplatin dose intensity. Although not completely a close issue today, high dose cisplatin did not offer any significant outcome advantage for patients with advanced ovarian cancer (McGuire WP, 1998). The GOG study randomized 97 patients to receive either cisplatin 50 mg/m^2 or cyclophosphamide 500 mg/m^2 every 3 weeks for eight cycles or cisplatin 100 mg/m^2 and cyclophosphamide 1000 mg/m^2 every 3 weeks for four cycles. There was no statistically significant difference in terms of response or survival between the two regimens (McGuire WP, 1995). In contrast, a Scottish study randomized patients to receive cyclophosphamide 750 mg/m^2 and either 50 mg/m^2 or

100 mg/m^2 of cisplatin for 6 cycles. An improvement in response rate and overall survival was reported for higher dose (Kaye SB, 1992). However, the study design allowed the high dose arm to receive double dose intensity, but also double total dose. Thus, it was concluded that the total dose was a key issue in determining efficacy as opposed to total dose intensity and more mature data suggest overall survival curves are beginning to converge (Kaye SB, 1996b).

The third modification in platinum-based regimens was the introduction of less toxic platinum compound, carboplatinum. Four randomized trials have compared cisplatin-based combination and carboplatin-based combination (Alberts DS, 1992; Hannigan EV, 1993; Swenerton K, 1992; Ten Bokkel Huinink WW, 1988). There was no difference in response or overall survival, however carboplatinum had a more favourable toxicity profile. Recently the second international collaborative ovarian study (ICON2) randomized patients to receive cyclophosphamide, doxorubicin, and cisplatin combination or carboplatin alone. There was no difference in response or survival between the two arms (International Collaborative Ovarian Neoplasm Study, 1998).

Unfortunately, most patients with advanced ovarian cancer responding to platinum-based chemotherapy will ultimately relapse developing recurrent disease that is usually resistant to further chemotherapy (acquired resistance). In addition, 20-30% of patients with advanced tumours will not respond to chemotherapy (innate resistance) and these patients have poor prognosis with survival less than 6 to 12 months (McGuire WP, 1998). Hence, the need for development and incorporation of new drugs in the management of ovarian cancer has been an intense area of research over the last two decades. In the 1990s the taxanes, in particular paclitaxel, were developed and have now become incorporated in primary therapy. In fact, paclitaxel and platinum based regimen has already been shown to improve outcome in the management of advanced ovarian cancer in terms of response and survival (McGuire WP, 1996; Stuart G, 1998). The success of paclitaxel in management of ovarian cancer is probably due to its unique mechanism of action targeting the microtubules and cytotoxicity, which is different from DNA damaging agent such as platinum drugs.

1.1.3 Clinical development of paclitaxel-based chemotherapy

Following the demonstration of activity of paclitaxel in ovarian cancer in phase I studies, five phase II trials were conducted in total of 198 patients with progressive, relapsed ovarian cancer treated with cisplatin. There was an overall response rate of 29%, which was certainly encouraging in this group of patients with resistant disease (Einzig AI, 1992; Kohn EC, 1994; McGuire WP, 1989; Thigpen JT, 1994).

The next step was to explore the dosing and scheduling of paclitaxel and to develop paclitaxel-based regimens. A joint NCI-Canada/ European trial in patients who received up to 2 prior platinum based regimens, compared 2 doses (135 vs. 175 mg/m^2) and 2 infusion times (3 vs. 24hours) in a bifactorial randomized design (Eisenhauer EA, 1994). In 382 evaluable patients the overall response rate was 17.3% (12% in platinum-refractory patients). There was no statistical difference between those randomized to high versus low dose (response rate 20% vs. 15%) or long versus short infusion (19 vs. 16%). There was a statistically significant advantage to the higher dose and to shorter infusion in terms of time to progression, but no difference in survival or quality of life. This trial provided some important observation on toxicity. The frequency of hypersensitivity and significant cardiac toxicity were low (<1%) in all groups regardless of schedule. The myelosupression was significantly less common with 3 hour infusion. The incidence of peripheral neuropathy did not appear to be schedule-dependent, although there was a trend towards more neurotoxicity with higher paclitaxel dose.

The demonstration that paclitaxel is non-cross resistant with cisplatin gave the rational for the use of paclitaxel in combination with cisplatin in adjuvant setting in order to improve the outcome. So far three randomized trials have been reported, one reported in full and two in abstract form (table1-1). The GOG study 111 randomized 410 suboptimally debulked patients to receive either paclitaxel 135mg/m^2 over 24hours plus cisplatin 75mg/m^2 or cyclophosphamide 750mg/m^2 and the same dose of cisplatin. The paclitaxel arm was superior in terms of response rate (77% vs. 64%, $p<0.01$), median time to progression (18 vs. 13 months, $p=0.0001$) and median overall survival (37.5 vs. 24.4 months, $p=0.0001$)(McGuire WP, 1996). Recently, the results of an intergoup collaborative trial conducted in Europe and

Canda has become available. This study differed from the GOG study in that 35% of patients had low volume disease at entry, the paclitaxel was administered at 175 mg/m^2 over 3 hours, and second look surgery was not required to assess pathological response. The paclitaxel arm had improved response rate (77% vs. 66%, p=0.02), clinical CR rate (57% vs. 43%, p=0.01), progression free survival (16.6 vs. 12 months, p=0.0001), median survival (35 vs. 25 months) (Stuart G, 1998). Finally, the GOG study 132 also recently reported the results of a large study in 615 patients with bulky ovarian cancer randomized to receive cisplatin alone (100 mg/m^2), paclitaxel alone (200 mg/m over 24 hours), or paclitaxel (135mg/m^2 over 24 hours) and cisplatin (75 mg/m^2) combination (Muggia FM, 1997). As shown in table 1, the paclitaxel alone arm was inferior to the other two arms in terms of response and survival. The response rate and median survival were similar in cisplatin only arm and paclitaxel and cisplatin combination, however the toxicity was less in the combination regimen. However, in this trail there has been cross over between in large number of patients and at an early stage of treatment. Thus the interpretation of these results is difficult since most patients received both drugs.

More recently, a panel of oncologist treating ovarian cancer in United Kingdom published a consensus statement on standard management of ovarian cancer. It was recommended based on current evidence including the GOG 132 study that combination of paclitaxel and platinum compound is the regimen of choice for majority of patients with advanced ovarian cancer (Adams M, 1998).

Unfortunately, both the innate and acquired resistance to paclitaxel are now well recognized. Thus, the understanding of molecular pathways of mechanisms of action and resistance of paclitaxel has become an intense area of research over the last few years with the aim of developing therapies to defined molecular targets that will help to overcome resistance.

Resistance to paclitaxel can be broadly into proximal and distal mechanisms.
The proximal mechanisms include altered drug uptake and drug targets (microtubules). The distal mechanisms involve the alteration or modification of several proteins that regulate cell death or apoptosis. The concept of programmed cell death (apoptosis) has gained huge

interest over the two decades, particularly with demonstration that most chemotherapeutic agents induce cell death by apoptosis (Hannun YA, 1997). It now recognized that the alteration of apoptosis regulating proteins such as bcl-2 family of proteins or p53 may induce chemoresistance to paclitaxel (Haldar S, 1995; Wahl AF, 1996). If so, these apoptosis regulating proteins may provide molecular targets to help to circumvent resistance to paclitaxel.

Trial	Overall response(%)	Clinical CR (%)	Median time to progression (months)	Median survival (months)
GOG-111				
CP	66	31	13.0	24.4
TP	73	51	18.0	37.5
Intrgroup study				
CP	66	36	12.0	25
TP	77	50	16.6	38
GOG-132				
P alone.	67	NA	16.4	30.2
T alone.	46	NA	11.4	26
TP	67	NA	14.1	26.6

Table 1-1: Three randomized trials comparing paclitaxel and cisplatin based regimens. C= cyclophosphamide, P= cisplatin, and T= paclitaxel. For drug doses and delivery see text.

1.2 Paclitaxel

1.2.1 Discovery and development

The history of paclitaxel started around the turn of the century, when a British official in the Indian subcontinent noted that parts of the European yew, *Taxus baccata,* were used in a clarified butter preparation for the treatment of cancer (Song JI, 1991). Subsequent development of paclitaxel was conducted in the National Cancer Institute (NCI) in a program evaluating plants for anti-cancer activity. Initially, in 1963, crude bark extracts of the related pacific yew, *Taxus brevifolia,* were provided to NCI by Untied States forest service. The crude alcohol extract was shown to be cytotoxic against several murine tumours. The pure form of the active component (Paclitaxel) was isolated in 1966 and two years later; Wani et al described paclitaxel unique chemical structure (figure1-1) (Wani MC, 1971). However, further development was delayed because of modest activity of the drug in preclinical model systems, scarce supply, poor solubility, and apparent similarities in mechanism of action to vinca alkaloids. Interest was revived in 1979 when the unique mechanism of action of paclitaxel was discovered (Schiff PB, 1979). Unlike other microtubule targeting drugs, which inhibit the polymerization of microtubules, paclitaxel was shown to promote the assembly of tubulin and stabilize the resulting microtubules. Under the auspices of the NCI, paclitaxel underwent phase I and phase II studies in the early eighties. In 1989, paclitaxel was recognized as an important clinical advance when activity in platinum resistant ovarian cancer was demonstrated (McGuire WP, 1989). Paclitaxel has been commercially available since early 1993 and activity of the drug has been demonstrated now against ovarian cancer, breast cancer, lung cancer, malignant melanoma, head and neck cancer, and lymphoma.

1.2.2 Chemical structure and pharmacological properties

Paclitaxel (C4 H51 O14, M.W. 853.9) possesses a taxane nucleus built from a diterpene carbon skeleton (figure 1-2). The taxane nucleus consists of three rings (Kingston DG, 1994). Six membered A- and C-rings are fused at an almost perpendicular angle with an eight membered B-ring. The C13 side chain of paclitaxel, 2R- 3S N-benzoyl-3- phenyliserine methylester is esterified with C13-OH of taxane nucleus. This C-13 side chain seems to be mandatory for its anti-tumour activity because analogs of paclitaxel that do not possess it are not cytotoxic (Arbuck SG, 1993). Intramolecular hydrogen bonds between estercarbonyl, 2-OH and 3-NH stabilize the side chain into well-defined confirmation.

Paclitaxel is highly lipophilic and insoluble in water, but soluble in Cremophol EL, polyethylene glycols 300 and 400, chloroform, acetone, ethanol and methanol. Paclitaxel used in clinical practice is formulated in 50% Cremophor EL and 50% dehydrated alcohol (Verweij J, 1994). In early phase I studies the pharmacokinetic of paclitaxel showed linear biexponential disposition profile whereby the area under the plasma concentration versus time curve (AUC) is linearly related to dosage. Subsequent studies demonstrated non-linear pharmacokinetics whereby there was an unproportional increase in maximally reached plasma concentration and area under the curve, when increasing dosage of paclitaxel was given by 3-hr infusion suggesting triexponential disposition. There was an initial rapid decline representing significant elimination of drug from the circulation and tissue distribution and a prolonged terminal phase representing a slow reflux of paclitaxel into the circulation (Huizing MT, 1995).

Paclitaxel shows extensive protein binding (up 98%), and less than 6% of drug is recovered unchanged in urine. Thus the renal clearance is rather insignificant and the metabolism by biliary excretion, and extensive tissue binding is probably responsible for total body clearance of paclitaxel (Huizing MT, 1995).

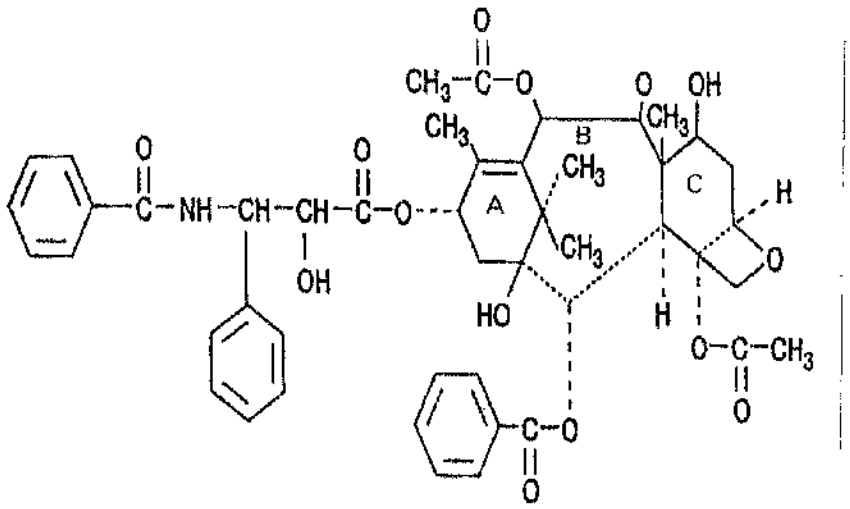


Figure 1-1: Chemical structure of paclitaxel (see text).

1.2.3 Mechanism of action

1.2.3.1 Microtubular structure

Paclitaxel induces its cytotoxic effect by targeting the microtubules (Schiff PB, 1979). In addition to the microtubule's important role during cell division as the major component of the mitotic spindle, they are required for maintenance of cell shape, motility and transport between organelles within the cell (Dustin P, 1980; Rowinsky EK, 1990). The Microtubules are composed of tubulin heterodimers, each one of which is itself a dimer composed of two very similar globular protein called α and β tubulin subunit bound tightly by non-covalent bonding (Correia JJ, 1991). The cylindrical structure of the microtubule is of made of 13 protofilaments composed of linear chain of tubulin subunits with α and β subunits alternating along its length and creating a structural polarity with the α subunit exposed at one end (termed minus end) and the β subunit at the other (plus end) (Derry WB, 1998). In the cell there is a dynamic equilibrium between the soluble tubulin dimers and microtubular polymers, which is vital for their function (figure 2) (Verweij J, 1994).

The study of mechanisms of action and resistance of paclitaxel is hindered by the fact that there are multiple tubulin isotypes that are encoded by a large multigene family consisting of both functioning and non-functioning genes. The greatest diversity between the β-tubulin isotypes occurs in the carboxy-terminal variable region sequence and to a lesser extent in the amino terminal variable region (Haber M, 1995). In humans, six β tubulin isotypes have been identified which display a distinct pattern of tissue expression. Their classification is as follows (Roman numerals represent the tubulin protein class, while Arabic numerals represent the gene: class I, HM40; class II, Hβ9; class III, Hβ4; class IV a, Hβ5; class IV b, Hβ2; class VI, Hβ1. In mammalian cells expression is as follows: class I and Ivb isotypes are constitutively expressed, class III and IV a are restricted to brain, class II is found predominantly in brain and at low levels in broad range of tissues, and class VI is restricted to haematopiotic tissue (Sullivan KF, 1986).

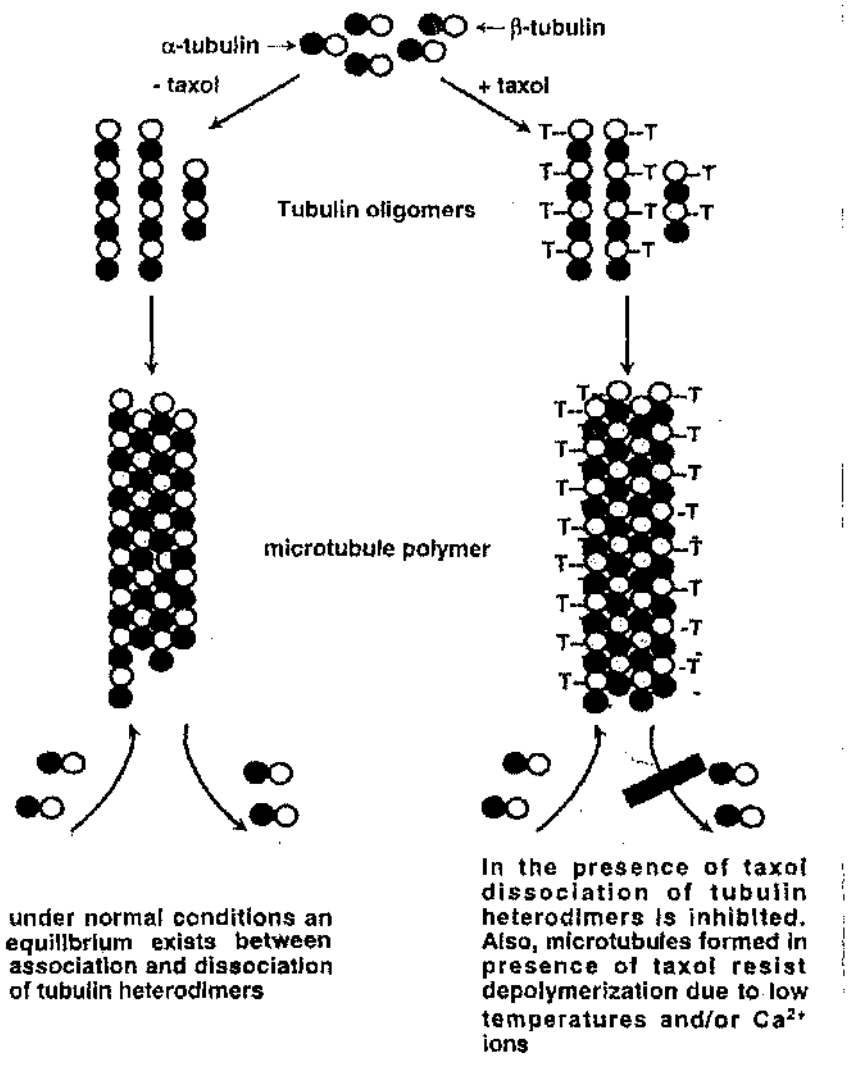


Figure 1-2: Microtubule's structure and interaction with paclitaxel (Parekh H, 1997a).

1.2.3.2 Paclitaxel interaction with microtubules

Unlike the vinca alkaloids, which binds tightly to the free tubulin and prevents its polymerization into microtubules, paclitaxel encourages polymerization of microtubules. It binds tightly to the microtubule polymers and prevents the loss of the tubulin subuits, at the same time allowing new subunits to be added. In doing so, paclitaxel stabilizes the inherently unstable assembly-disassembly process and alter the dynamic equilibrium between soluble tubulin dimers and microtubular polymer in favor of the latter, thereby reducing the critical concentration of tubulin required forming microtubules (Parekh H, 1997a). The polymerization occurred even in the absence of guanosine 5-triphosphate (GTP) and microtubule-associated proteins (MAPs), which are otherwise required for tubulin assembly (Horwitz SB, 1992; Horwitz SB, 1993). Microtubules assembled under influence of paclitaxel are refractory to depolymerization even at 4 °C and in the presence of calcium, conditions that otherwise induce rapid disassembly of microtubules (Horwitz SB, 1992; Horwitz SB, 1993).

Direct photoaffinity labeling has demonstrated that paclitaxel binds preferentially to the beta subunit of tubulin with stoichiometry approaching one mole of paclitaxel per one mole of polymerized tubulin dimer (Horwitz SB, 1992) (Manfredi JJ, 1984). The binding site of paclitaxel was localized to a 31-aminocid region at the N-terminal end of β tubulin subunit (Rao S, 1992a). Electron crystallography analysis revealed that the binding site of paclitaxel was present near the interprotofilament contact region in the microtubular polymer and not near tubulin monomer-monomer or dimer-dimer contact regions (Noagales E, 1995).

The effect of paclitaxel on the microtubules appears to be dose dependent. At high concentration (100-500 nM), paclitaxel enhances microtubule polymerization, increases mass of microtubules, causes extensive bundling of microtubule with production of spindle asters. These changes were observed in the interphase and mitotic phase of cell cycle (Jordan MA, 1993). However at low paclitaxel concentration (< 9 nM) paclitaxel blocks mitosis at the transition from metaphase to anaphase, with no increase in microtubular mass or bundling (Jordan MA, 1993). At this concentration, paclitaxel potently suppresses dynamics at the plus end of microtubules with no effect at the minus end permitting continued microtubule depolymerization at the spindle pole (Derry WB, 1998). Thus paclitaxel causes dysfunctional

spindles that can not properly polymerize and lead to abnormal chromosome segregation and aberrant cytokenesis (Derry WB, 1998). The latter results in cells with abnormal size and DNA content, eventually causing cell death. The molecular events that are thought to occur distal to binding of paclitaxel to microtubules such as phosphorylation of bcl-2 and bcl-xl and lead to cell death will be discussed in details in 1.4.2.

1.2.4 Paclitaxel and induction of mitotic arrest

The hallmark of Paclitaxel treatment on cells is the induction of mitotic arrest. Paclitaxel has been shown to interact with several important regulators of mitosis that include maturation-promoting factor (MPF), Raf-1, and p53. Several studies have demonstrated that the onset of mitosis is regulated by MPF complex (King KL, 1995; Nurse P, 1990). This complex consists of two subunits, a catalytic subunit, cdc2 kinase, and its regulatory subunit, cyclin B. The kinase activity of MPF complex is activated at G2/M transition and inactivated at the onset of anaphase. For the activation to occur, cyclin B should accumulate above certain threshold level followed by dephoshorylation of cdc2 kinase. At the end of mitosis, the activity of cdc2 kinase is abolished by proteolysis of cyclin B (King KL, 1995; Nurse P, 1990). Paclitaxel has been shown to produce a marked increase in cyclin B1 accumulation and activation of cyclin B1 dependent kinase (Ling YH, 1998a). The accumulation of these subunits was shown to parallel the mitotic arrest and precedes apoptosis (Ling YH, 1998a). More recently, the signal transduction molecule Raf-1 kinase activation has been shown to be important for the passage through mitosis (Torres K, 1998). Also, it has been observed that p53 participates in the spindle checkpoint process to ensure a correct DNA replication before mitosis (Cross SM, 1995). Paclitaxel activates both molecules, thus effecting the progress through mitosis as discussed in sections 1.4.1 and 1.4.2.

1.2.5 Non-microtubular paclitaxel effects

There is some evidence to suggest that paclitaxel induces other cellular changes, besides its paclitaxel microtubule-stablizing properties. Paclitaxel has been shown to stimulate the lipopolysacharide (LPS) signalling pathway in murine macrophages resulting in secretion of tumour necrosis factor α and down regulation of TNF-α receptor (Ding AH, 1990). This effect is independent of its ability to stabilize microtubules, because some derivatives of paclitaxel that retain the microtubular polymerizing activity but do not stimulate cytokine secretion. Burkhat et al demonstrated that induction of TNF α gene expression and secretion is only seen with paclitaxel and its 7-acetyl derivative and not with other taxanes including docetaxel (Burkhat AC, 1994). All the taxanes used in these studies were able to induce cell death suggesting that induction of TNF-α or LPS- like activity is separate from the cytotoxic effect of paclitaxel. In addition, the transcriptional upregulation of interleukin-1 and interleukin-8 following the treatment with paclitaxel has been reported and associated with ability of paclitaxel to activate nuclear factor kappa B (NF-kappa B)(Parekh H, 1997a).

1.3 Molecular aspects of response to paclitaxel

Chemotherapeutic agents including paclitaxel induce cell death by a process termed apoptosis. The term apoptosis is of Greek origin used to describe the falling of petal from a flower or the loss of leaves from a tree. In vertebrates, the term apoptosis is used to describe the characteristic the phenotypic and biochemical changes that accompany programmed cell death (PCD). The importance of programmed cell death as an essential process for the appropriate development and function of multicellular organisms is now well accepted. More importantly, It is realized now that failure to appropriately regulate or induce apoptosis may lead to suppression of cell death resulting in several pathological processes and chemoresistance.

There is accumulating evidence to suggest that prolonged mitotic arrest initiates apoptosis (Debernardis D, 1997; Donaldson KL, 1994; Ling YH, 1998a); however, little is known about

the signalling pathways involved. Several recent discoveries have shed more light on molecular events distal to binding of paclitaxel to microtubules involving apoptosis regulating proteins and leading to cell death. In fact, it is realized now that alterations in these apoptosis regulating proteins may contribute to paclitaxel resistance. In this section an overview of the important concept of apoptosis and its regulating proteins will be provided followed by discussion of paclitaxel induction cell death. This may provide insight how dysregulation of the apoptosis regulating proteins may lead to chemoresistance.

1.3.1 Apoptosis: General overview

The importance of this concept has gained a huge amount of interest following the description of the physiological form of cell death termed apoptosis (Kerr JF, 1972; Wyllie AH, 1980). The process of apoptosis can be subdivided into three different phases: initiation, effector and degradation. Whereas the latter two phases are common to all apoptotic processes, the initiation phase depends on the type of apoptosis-inducing stimulus. The morphological features described by Kerr et al occur during effector phase. The initial changes include cell shrinkage due to extrusion of water from the cell, condensation of cells chromatin giving rise to the formation of pyknotic nuclei, nuclear fragmentation followed by break up of cells into small sealed vesicles called apoptotic bodies. These apoptotic bodies are subsequently engulfed and destroyed by neighbouring cells or macrophages without inducing inflammatory response during the degradation phase. It is now recognized that the morphological changes are closely linked to biochemical changes. The biochemical hallmark of apoptosis during the effector phase is the fragmentation of the cells DNA into nucleosome size fragments, which can be seen in agrose gel as a DNA ladder (Arends MJ, 1990; Wyllie AH, 1980). The fragmentation occurs due to activation of one or more cation-dependent endogenous nucleases, first into large 30-50 kilobases, and subsequently into nucleosomal fragments of 180-200 base pairs.

1.3.2 Genetics and molecular basis of apoptosis

Most of the present knowledge of apoptosis in mammals comes from study of the nematode *Caenorhabditis elegans*. In this worm, 1090 cells are generated during development, of which 131 cells die by an intrinsic death program (Ellis HM, 1986). Three genes, *ced-3, ced-4 and ced-9,* play a crucial role in executing and regulating the death of these cells (Hengartner MO, 1992; Miura M, 1996; Yuan J, 1993). Both *ced-3 and ced-4* are essential for cell death to occur and, consequently, mutants lacking either of these genes have extra cells. The *ced-9* gene antagonizes the function of *ced-3 and ced-4* by protecting cells from death.

The first important clues indicating that the pathways regulating apoptosis are similar in *C. elegans* and mammals came from the discovery that the function of *ced-9* mutation can be restored by expression of the human *bcl-2* genes (Vaux DL, 1992). Indeed, Subsequent cloning of *ced-9* showed that its product is similar to *bcl-2* (Hengartner MO, 1994). The cloning of *ced-3* showed that this 503 amino acid protein is homologous to a mammalian interleukin- 1B-converting enzyme (ICE) (Yuan J, 1993). More recently, ced-4 has been shown to be homologous to a mammalian protein termed Apoptotic protease activating-1 factor (Apaf-1) (Li P, 1997).

1.3.3 ICE and caspases

ICE is a cysteine protease responsible for the proteolytic conversion of 31 kilodaton (KD) inactive cytokine precursor, pro-interleukin 1B (proIL-1B), to its 17.5 KD active form (Thornberry NA, 1992). ICE is synthesized as a dormant 45 kD proenzyme that resides in the cytoplasm, and is proteolytically activated by unknown mechanism. The active form is composed of two subunits, one 20 KD and one 10 kD, both which are derived from the 45 kDa proenzyme following removal of 11 kD N-terminal peptide and a 2 KD linker peptide (Thornberry NA, 1992). Cleavage of the proenzyme occurs at aspartic acid residues, suggesting that the enzymes involved in processing are ICE-like proteases.

Following the recognition of the similarity between *ced-3* and ICE in 1993, a further nine related highly conserved ICE-like proteases have been identified. Because all these proteins

are cysteine protease (c) that cleave after an aspartic acid residue (aspases), they are termed caspases (Alnemri ES, 1996). The caspases exist as inactive zymogen in the cytosol and become activated through proteolysis when the cell receives apoptotic signals.

Several lines of evidence suggest that caspases have a direct role in the regulation of apoptosis and indeed they are the effector death molecules. *Ced-3* is an absolute requirement for apoptosis to proceed in *c. elegans.* The overexpression of gene encoding caspase-1 in Rat-1 cells was shown to induce apoptosis (Miura M, 1993). Mutation in the catalytic Cys residue of ICE completely abolishes its apoptosis inducing activity (Miura M, 1993). Microinjection of ICE-c DNA expression vectors into chicken dorsal-root-ganglion also results in cell death while microinjection of CrmA, a viral inhibitor of ICE, prevents apoptosis induced by serum depletion (Gagliardini V, 1994) (Ray CA, 1992).

The exact sequence of caspase activation is still unknown. The fact that all caspases are cleaved at specific Asp residues, raise the possibility that some of the caspases might sequentially activate each other in a cascade (Nagata S, 1997). It has been suggested that caspases containing long NH-2 domain such as caspases 8 and 9 play a central role in this model and have been termed the initiator protease, which in turn activate an amplifier protease such as caspase1, which in turn activate a machinery protease such as caspase3. Enari et al demonstrated that caspase 3 activation is dependent on caspase1 activation in liver mice damaged by anti-fas antibody (Enari M, 1996). Further evidence for the cascade theory comes from the activation of caspase 8 and caspase 9 by anti-Fas antibody through two different mechanisms with subsequent activation of other caspases. Caspase 8 is recruited to the death induced signaling complex (DISC) on cell surface through interaction with FADD/MORTI and caspase 9 forms a complex with Apaf-1 and cytochrome c in presence of d ATP/ATP. The activated caspase 8 and 9 within these complexes inturn activate caspases 3, 6, and 7 which lack long NH2 terminal but constitute the main caspase activity in apoptotic cells. On the other hand, the observation that caspase1 null mice have normal phenotype (Li P, 1995) and caspase 3 null mice show profound neurological defects (Kuida K, 1996), suggest that some members of caspase family (e.g. caspase 1) are redundant cleaving the same substrate.

The activated caspases appear to target a discrete number of proteins for proteolytic cleavage. Some of these substrates that are cleaved during apoptosis include nuclear proteins such as poly-adenosine diphosphate, ribose polymerase, DNA dependent kinase, topoisomerase I, lamins and 70k D of U1sn RNP. Cytoskeletal and cytoplasmic components are also cleaved including D4-GDI, Fodrin, Pkc and other (Thornberry NA, 1992). The exact mechanism of cell death following cleavage of these substrates remains unknown.

1.3.4 Bcl-2 family

The Bcl-2 (B cell lymphoma / leukaemia-2) gene was initially cloned from a 14; 18 translocation break point associated with 85% of follicular lymphoma and 20% of diffuse B cell lymphoma. In this translocation the bcl-2 gene is moved from its normal chromosomal location at 18q21 into juxtaposition with heavy chain immunoglobulin promoter on chromosome 14 resulting in overproduction of bcl-2 m-RNA and their encoded proteins (Tsujimoto Y, 1986). In 1988, Vaux et al reported that overexpression of bcl-2 prolonged the survival of interleukin-3 (IL-3) dependent immature pre-B lymphocyte despite the absence of IL-3, but without concomitant cell proliferation (Vaux DL, 1988). Subsequently, it was shown that microinjection of bcl-2 expression plasmids into Nerve Growth Factor dependent sympathetic neurons delayed the rate of cell death upon withdrawal of growth factor (Garcia I, 1992). It was also shown that transfection of bcl-2 into haematopoietic cell lines prevented cell death upon growth factor withdrawal (Nunez G, 1990). Prompted by these observations bcl-2 has been found to protect variety of cells from a variety of apoptotic signals including glucocorticoids, γ-irradiation, phorbol estera, anti-CD-3antibody, viral and chemotherapy induced cell death (Miyashita T, 1993; Strasser, 1994; Yang E, 1996). Subsequently, Reed et al demonstrated that anti-sense mediated reduction in bcl-2 gene expression accelerated the rate of cell death in the setting of growth factor withdrawal (Reed JC, 1990).

All of the above observations suggested the critical role of bcl-2 in controlling cell death and that bcl-2 blocks a final common pathway leading to cell death. However, bcl2 has little effect

on apoptosis during negative selection or apoptosis in cytotoxic T cell activated killing (Reed JC, 1994). Also the observation that bcl-2 function is not required for embryonic development raised the possibility that other proteins modulate apoptosis in addition to bcl-2 (Kamada S, 1995; Reed JC, 1994). It is now recognized that bcl-2 belong to a growing family of apoptosis regulating gene products. At least fourteen cellular homologues have been described. Broadly, these fall into two categories: The anti-apoptotic death antagonist include bcl-2, bcl-xl, Mci-1, bcl-w, Brag-1and pro-apoptic cell death promoting proteins bax, bcl-xs, bak, bad, bik, bid, Hrk) (Reed JC, 1994).

1.3.4.1 Localization and the importance of carboxy terminus

Bcl-2 has been shown to localize mainly in the outer mitochondrial membrane, smooth endoplasmic reticulum and peri-nuclear membrane (Krajewski S, 1993; Monaghan P, 1992). Other proteins encoded by bcl-2 gene family are predominantly localized in the outer mitochondrial membrane such as bcl-xl and bax (Yang E, 1996). Most members of bcl-2 family possess highly conserved stretch of 19 hydrophobic amino acids near the carboxy terminus followed by two charged residues which serve to anchor the protein in membranes, thus exposing most of the polypeptide to the cytosol, where it is exposed to protease digestion (Kromer G, 1997). The deletion of this transmembrane carboxy terminus abrogates or diminishes bcl-2 the death inhibitory effect and prevents the targeting of bcl-2 interacting proteins (Zhu W, 1996). The deletion of the crboxy terminus from bax prevents targeting to mitochondria and completely abolishes its cytotoxicity in yeast (Kromer G, 1997). These observations suggest that, at least for some members, an integral membrane position is required for activity.

1.3.4.2 Bcl-2 homology domains

The bcl-2 family of proteins shares up to four highly conserved domains termed bcl-2 homology (BH) domains (Zha H, 1996). Most of Bcl-2 family members share BH1 (residues 136-155 of bcl-2) and BH2 (residues 187-202 of bcl-2). The substitution of Gly 145 in BH1 or Trp188 in BH2 abrogated the death suppressing activity of bcl-2 and prevented bcl-2

binding to bax without impairing homodimerization with endogenous wild type bcl-2 (Yin XM, 1994). In addition, it prevents the interaction targeting proteins such as Raf-1(Wang HG, 1996b). Similarly, some mutations in BH1 and BH2 domains of bcl-xl have been shown to abrogate and disrupt their ability to heterodimerize with bax and bak, but the mutant proteins retain most (70-80%) of their death repressor activity, suggesting that these molecules can also act independently of each other (Kromer G, 1997).

A third domain BH3 (residues 93-107 of bcl-2) was identified by studying the interaction between an anti-apoptotic adenovirus E1B 19K (homologue to bcl-2) and bax (Han J, 1996). In order for bax to interact with E1B protein, a minimal region of 28 aminoacids distinct from BH1 and BH2 are needed (Han J, 1996) (Wang K, 1998). This region is designated BH3 and is conserved among the various bcl-2 family members. Several observations suggest that this domain is uniquely important in the promotion of apoptosis. The two proapoptotic proteins Bik and bid have only BH3 domain (Boyd JM, 1995; Wang K, 1996). The deletion of BH3 in pro-apoptotic bax and bak disrupt their function (Zha H, 1996). In addition to that the swapping of a 23-aminoacid segment surrounding BH3 from bax to bcl-2, converted bcl-2 to a pro-apoptotic protein (Hunter JJ, 1996b). The sequence alignment studies have shown that BH3 domain sequence differs between the proapoptotic and antiapoptotic proteins (Rao L, 1997).

The fourth domain BH4 (residues 10-30 of bcl-2) is conserved among anti-apoptotic homologues, but not found in most pro-apoptotic bcl-2 family proteins except bcl-xs (Kromer G, 1997). This observation suggests that BH4 may play a unique role in the function of anti-apoptotic proteins. The deletion of BH4 abrogates the anti-apoptotic function of these proteins, but it is not required for dimerazation (Hunter JJ, 1996a). Also, BH4 may play role in the interaction of bcl-2 and bcl-xl with other non-structurally death-promoting proteins such Raf-1, ced-4 (Wang HG, 1996d) (Chinnaiyan AM, 1997b)

1.3.4.3 Tertiary structure

The determination of the three dimensional structure of bcl-xl by X-ray and MRI analysis provided an insight to bcl-2 family protein function. It consists of two central hydrophobic alpha helices penetrating the lipid bilayer surrounded by five amphipathic helices (Muchmore SW, 1996). The alpha helices are connected by a variable region consisting of 60-residue flexible loop located between BH-3 and BH-4 domain. An elongated hydrophobic pocket is created by close spatial proximity between BH1, BH2, and BH3 domains. Structurally, bcl-xl is analogous to bacterial toxins such as diphtheria toxin and colicins, having particularly striking similarity in their membrane insertion domain (Kromer G, 1997). Also, the addition of recombinant bax to synthetic membrane results in formation of ion channels (Kromer G, 1997). These observations suggest that many bcl-2 family proteins share the ability to form pores in cytoplasmic and nuclear membranes and function by regulating signals dependent on pH, voltage, or ionic strength.

1.3.4.4 Function of bcl-2 and related proteins

The exact role of bcl-2 and related proteins in the regulation of cell death or survival remains unknown. It is thought that these proteins function as regulators of cell death pathways proximal to caspases. At the molecular level, it has been suggested that bcl-2 may function as an anti-oxidant, a regulator of endoplasmic reticulum-associated calcium fluxes or an inducer of cytosolic acidification (Hockenbery DM, 1993; Lam M, 1994). However, these theories were invalidated by demonstration that bcl-2 inhibited apoptosis in cells that lacked mitochondria and under nearly anaerobic condition, the induction of nuclear apoptosis in calcium free media or by calcium depletion, and induction of apoptosis in some models by cytosolic acidification (Liu X, 1996; Tsao N, 1996; Zamzami N, 1996). Recent discoveries suggest that bcl-2 family proteins may modulate the process of apoptosis by two main mechanisms, the alteration of mitochondrial permeability transition and interaction with related and non-related proteins.

1.3.4.5 Mitochondrial permeability transition and pore formation

This model is based on the critical role of the mitochondria during apoptosis and the sub-cellular localization of bcl-2 and some of related proteins to the outer mitochondrial membrane. The biochemical and structural changes of mitochondria during apoptosis include mitochondrial swelling, disruption of mitochondrial outer membrane; disruption of the mitochondrial transmembrane potential and release of protease activators including cytochrome c and apoptosis inducing factor (AIF). It is postulated that the opening of large pores located at contact sites in mitochondria where the inner and outer membranes abut and where various transport processes involving ions and proteins occur lead to generation of mitchondrial permeability transition This is followed by dissipation of H+ gradient, osmotic swelling due to the high solute concentration of mitochondrial matrix, rupture of the outer mitochondrial membrane and release of cytochrome c (Kromer G, 1997; Reed JC, 1997a).

It has been demonstrated that Bcl-2 and bcl-xl can inhibit both the loss of mitochondrial potential and the release of factors from mitochondria into the cytosol that can activate the caspases (Susin SA, 1996). In addition to that it has been shown that bcl-2 and bcl-xl prevented cytochrome c release upon induction of programmed cell death in intact cells and cell-free systems (Kluck RM, 1997; Yang J, 1997). Bcl-2 and bcl-xl could not inhibit apoptotic changes induced by cytochrome c after it was released or added exogenously suggesting that bcl-2 and bcl-xl exert their function on mitochondria rather than cytosol (Kluck RM, 1997). The mechanism of regulation of cytochrome c by bcl-2 and bcl-xl is unclear. It is postulated that bcl-xl may function as an ion channel that regulates the permeability of mitochondria and could minimize the osmotic stress and in doing so prevent cytochrome c release due to mitochondrial matrix swelling and outer membrane rupture (Kromer G, 1997; Muchmore SW, 1996).

1.3.4.6Interaction with related proteins and post-transitional modification

The fate of a cell to undergo apoptosis or not may be determined in part by a balance between pro-apoptotic and anti-apoptotic proteins. These proteins are able to form homodimers with

self and heterodimers with other proteins by interacting through BH domains discussed above (Farrow SN, 1996; Rao L, 1997; Reed JC, 1997a). Post-translational modification or phosphorylation alters the balance between these proteins. For example, Bcl-2 can be phosphorylated on serine residues within a flexible loop region by various stimuli (Chang BS, 1997). The deletion of this loop domain enhances the anti-apoptotic activity of bcl-2 and phoshorylation of this loop lead to inactivation of bcl-2 impairing its ability to heterodimerize with bax inducing apoptotic cell death (Haldar S, 1995). The phosphorylation site of Bcl-2 appears to be one or more of 17 serine residue(s) included in bcl-2 variable region. Haldar et al mutated several serine residue(s) to homologus alanine residue(s) using overlaping PCR method. These mutants were transfected into DU 145 prostate cancer cells (lack expression of bcl-2) to test the ability of these mutants to undergo phosphorylation (Haldar S, 1998). It was shown that mutation of Ser 70 of variable region abolishes the ability of paclitaxel to induce bcl-2 phosphorylation of that site. It is postulated that this site may act as a major phosphorylation site or binding site for putitive kinase that induces phosphorylation (Haldar S, 1998).

In addition to that, Bad has been shown to be a target of serine phosphorylation. Bad is a pro-apoptotic protein which heterodimerizes with membrane bound bcl-xl or bcl-2 neutralizing their activity and promoting death. In the presence of the survival factor IL-3, bad become phosphorylated in two serine residues (Zha J, 1996). It is thought that the targeting of Raf-1 to the mitochondria by bcl-2 initiates local signaling that leads to bad phsphorylation (Wang HG, 1996a). The phosphorylated bad forms a complex with cytosolic protein 14-3-3 preventing its interaction with bcl-xl located in mitochondria. Upon growth factor withdrawal and dephosphorylation, bad is able to dimerize with membrane associated bcl-xl promoting cell death (Zha J, 1996).

Another form of post-transitional modification is inactivation of bcl-2 family proteins by protease. The variable region of bcl-2 is highly susceptible to digestion by protease such as trypsin and chymotrypsin. In particular, HIV protease has been shown to cleave bcl-2, which may explain cell death during infection (Strack PR, 1996).

1.3.4.7 Interaction with unrelated proteins

Bcl-2 family proteins interacts with up to eleven proteins that have been identified including Raf-1, R-Ras and H-Ras, and ced-4, which interact with bcl-2 and bcl-xl but not bax. Although the importance of such interactions is not entirely clear, recent discoveries have been made.

Ced-9 (nematode equivalent of mammalian bcl-2) has been shown to physically interact with ced-4, and that ced-4 can independently bind to ced-3/caspases (Chinnaiyan AM, 1997a; Chinnaiyan AM, 1997b). These observations suggest that bcl-2 regulates the activation of caspases via ced-4 which acts as an adaptor (Jacobson MD, 1997). Interestingly, the ced-4, which normally found in the cytosol, when co-expressed with ced-9 or bcl-xl, is sequestered to intracellular membranes where ced-9 and bcl-xl are located (Chang BS, 1997). Conversely, when pro-apoptotic members of bcl-2 family such as bax are co-expressed with bcl-xl, they dimerize with bcl-xl, there by displacing ced-4 into cytosol and probably activating the caspases (Jacobson MD, 1997).

The signal transduction molecule Raf-1 kinase was shown to bind via its catalytic domain (Cat) to bcl-2 in BH-4 dependent manner (Shaham S, 1996; Wang HG, 1996b). Bcl-2 was found to target Raf-1 to mitochondria allowing this kinase to contribute to survival by phosphorylating the pro-apoptotic protein bad as discussed above. Also, the bcl-2 binding protein (BAG-1) was shown not only to bind raf-1 but also increases the activity of kinase through protein-protein interaction (Wang HG, 1996c). Thus, it is postulated that dimers of bcl-2 could form a docking site that allow Raf-1 and BAG-1 to interact and result in transient reversible activation of the Raf-1 kinase locally in the vicinity of bcl-2 on the surface of mitochondria, ER, or nuclear envelope membrane (Reed JC, 1997a).

1.3.5 P53

The loss and mutation of tumor suppressor gene is the commonest genetic lesion in human cancers occurring in approximately 50% of mainly late stage ovarian cancer (Shelling AN, 1995). P53 is a sequence specific DNA transcription factor that activates the expression of a

number of well-defined target genes. Wild type p53 can transcriptonaly transactivate genes involved in cell cycle arrest e.g. p21, interact with DNA repair machinery GADD45, or protein modulating apoptosis e.g. Bax and Fas (Harris CC, 1996). Thus following DNA damage, p53 may induce cell cycle arrest allowing DNA repair before proceeding through cell cycle or induce apoptosis if the damage is severe (figure 1-3). These two functions appear to be separable; in tumor cell lines lacking p53 in which inducible restoration of p53 results in cell cycle arrest and apoptosis, bcl-2 gene transfer blocks apoptosis but not cell cycle arrest (Ryan JJ, 1994; Wang Y, 1993).

1.3.5.1 P53 and cell cycle arrest

Following sublethal damage, p53 protein rises rapidly followed by concomitant activation of a number of p53 dependent genes including Gadd45that inhibits the progression of cells into S phase, and p21 that acts as a potent inhibitor of cyclin dependent kinase cdk2 that halt the cell cycle at G1/S boundary by binding to proliferating cell number antigen (PCNA) preventing its interaction with DNA ploymerase (Harris CC, 1996). Furthermore, p53 regulates the cell cycle by interacting with Retinoblastoma (Rb) gene. In the active hypophosphorylated form, Rb inhibits cell cycle progression and entry into S phase by forming complexes with and inhibiting the activity of the E2F family of transcription factors, which are thought to regulate expression of genes necessary for S phase entry (Sellers WR, 1997). Advancement through the cell cycle is thought to be mediated by sequential phsphorylation of Rb by G1 cyclin-dependent kinases, resulting in release of active E2F, which then proceeds to transcriptionally activate genes necessary for S-phase progression. The interaction between p53 and Rb gene is based on the action of two genes, mdm2 and p21, which are regulated by p53. It has been shown that mdm2 proteins bind to p53 and act as a negative regulator inhibiting wild type p53 transcriptionaly regulatory activity and creating autoregulatory feedback loop (Harris CC, 1996). Rb binds to carboxyl terminal domain of mdm2 restarting its function by altering the confirmation of the pocket region. An alternative pathway of p53-Rb interaction is mediated by p21, which could inactivate cyclin cdk complexes that target Rb phosphorylation. In addition to the well described action of p53 on G1/S part of cell cycle, there is evidence to

suggest that induction of wild type p53 at high enough levels may arrest cells in G2/M (Cross SM, 1995).

1.3.5.2 P53 and apoptosis

Although the exact role of p53 in induction of apoptosis has not been defined, its central role has been implied by a series of observations. Expression of wild type p53 has been shown to initiate apoptosis in a variety of cell lines in which p53 is either absent or mutated. For example expression of p53 in myeloid 32-cell line was shown to rapidly induce apoptosis after growth factor withdrawal (Yonish-Rouach E, 1991). In addition when temperature sensitive wild type p53 was transfected in CML cell line K562, the cells underwent apoptosis when cells were switched to the permissive temperature (Yonish-Rouach E, 1991). Studies of p53 transgenic mice have also demonstrated that p53 is required for induction of apoptosis by radiation and DNA damaging agents in thymocytes (Lowe SW, 1993). However, p53 genotype had no effect on glucocorticoid- induced apoptosis in these cells, suggesting both p53 dependent and p53 independent mechanism of apoptosis (Macfarlane M, 1996). The existence of a p53 independent mechanism is supported by normal development of p53 knock out mice (Lowe SW, 1993). These results may suggest that p53 status and role in apoptosis may be dependent on tissue type and stimuli inducing apoptosis.

There is some evidence to suggest that p53 modulates apoptosis regulating proteins. It has been shown that restoration of p53 in a murine leukemia, M1, cell line was associated with increases in Bax m-RNA and protein, accompanied by a decrease in bcl-2 levels (Miyashita T, 1996; Miyashita T, 1994). Similar elevation of bax m-RNA occurred after exposure to ionizing irradiation of several cell types that express wild type p53. On the other hand, it appears that bax induction is cell dependent. A lack of change in bax protein level was demonstrated in immature rat thymocyte in p53 wild type and p53 null thymocyte. The Fas gene, an apoptosis-intiating cell surface receptor of the tumour necrosis factor receptor superfamily, has been recently shown to be upregulated in response to increases in wild type p53 activity. However; it has not yet been determined whether this apoptosis-promoting gene is product of direct transcriptional target of p53 (Owen-Schaub LB, 1995).

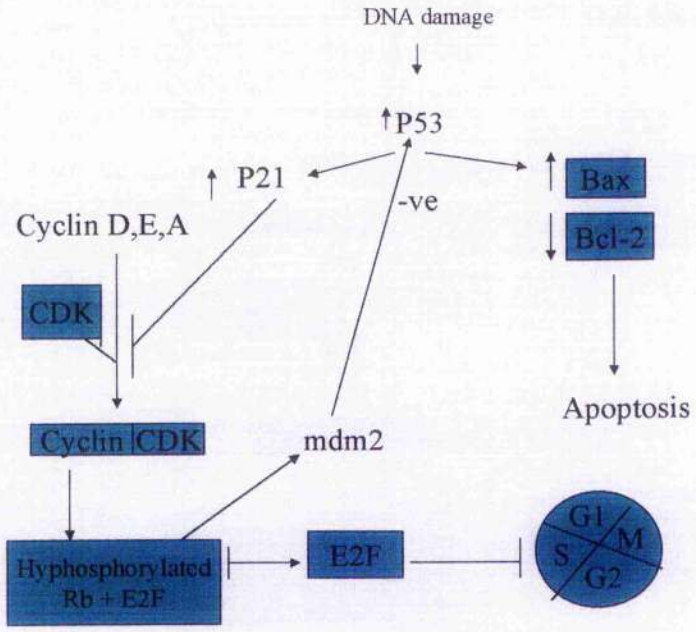


Figure 1-3: the role of p53 in regulating cell cycle arrest and apoptosis. (See text)

1.4 Paclitaxel and interaction with apoptosis regulating proteins

1.4.1 P53 and Paclitaxel

The role of P53 in induction of apoptosis following DNA damaging chemotherapeutic agents is well described (Debernardis D, 1997; Pestell KE, 1998). Although paclitaxel does not directly damage the DNA, p53 and p21 induction has been demonstrated in some cell lines (Blagosklonny MV, 1995; Tishler RB, 1995). P21 induction by paclitaxel in some cell lines (PC3M) appears to be p53 independent because it occurred in cells lacking p53 expression (Blagosklonny MV, 1995). The rise in p53 protein appears to be mediated in large part by increase stabilization of protein itself. The increase in p53 and p21 protein levels has been associated with paclitaxel's ability to induce a c-raf-1 cascade (Blagosklonny MV, 1995). Recent evidence suggests that p53 and p21 induction at low paclitaxel concentration may be raf-1 kinase independent suggesting that a different pathway is activated at low paclitaxel concentration (<9 nM) (Torres K, 1998).

1.4.2 Anti-apoptotic proteins and paclitaxel

Several studies have suggested that paclitaxel induces cell death by post-translational hyperphosphorylation of anti-apoptotic proteins (figure 1-4). Initially, Haldar et al showed that the treatment of human leukaemic cells with paclitaxel led to bcl-2 phosphorylation (Haldar S, 1996; Haldar S, 1995). Subsequently these observations were extended to lymphoma cell lines (RS 11846), prostate cell lines (PC3 and LNCap), breast cancer cell lines (MCF-7) and leukemia cell line (HL60) (Blagosklonny MV, 1997; Haldar S, 1996). The phosphorylated form of bcl-2 is incapable of forming heterodimers with bax, and a 50% reduction in formation of bcl-2 results in apoptotic cell death. Recently, it has been shown that paclitaxel induces hyperphosphorylation of bcl-xl abrogating its anti-apoptotic function in a manner similar to bcl-2 (Poruchynsky MS, 1998).

The signalling pathways linking the microtubule-paclitaxel interaction and prolonged mitotic arrest to bcl-2 phosphorylation and apoptosis remain obscure. Initially, Blagosklonny suggested that paclitaxel induced apoptosis seems to have some dependency on Raf-1 kinase

activity by demonstrating that paclitaxel stimulated the hyperphosphorylation of Raf-1 kinase in different cell line (Blagosklonny MV, 1996). The pharmacological depletion of Raf-1 by geldanamycin resulted in lack of bcl-2 phosphorylation and cell death, suggesting that Raf-1 induction is pre-requisite step in bcl-2 phosphorylation. Subsequently, Blagosklonny et al showed that in order for paclitaxel to induce Raf-1 kinase, it required the induction of tubulin polymerization (Blagosklonny MV, 1997). Raf-1 activation was diminished markedly in paclitaxel resistant sublines in which tubulin polymerization did not occur following paclitaxel, although other microtubule agents such as vincristine were able to induce Raf-1 phosphorylation.

Thus, a model has been suggested by this series of experiments. Following microtubule polymerization in response to paclitaxel, Raf-1 kinase is activated with subsequent phosphorylation of bcl-2 and cell death. Similarly, bcl-xl phosphorylation may be dependent on Raf-1 phosphorylation (Poruchynsky MS, 1998). Recently, it has been demonstrated that Raf-1 activation is paclitaxel concentration dependent (Torres K, 1998). Disruption of normal microtubule occurs at lower paclitaxel concentration (1-9nM) in absence of Raf-1 activation. Raf-1 activation correlates with induction of G2-M block, and depletion of Raf-1 resulted in accumulation of cells in G2/M phase suggesting that Raf-1 may play a role in the passage through mitosis. These observations suggest that paclitaxel mediated cell death may occur through two different mechanisms. At low concentration cell death occurs after an aberrant mitosis by Raf-1 independent pathway, whereas at high paclitaxel concentration cell death may be the result of terminal mitotic arrest occurring by a Raf-1 dependent pathway.

The central role of activation of Raf-1 kinase activity in induction of apoptosis following paclitaxel treatment has been challenged –at least in cervical carcinoma- by a recent study. Rasouli-Nia demonstrated in twelve unperturbed cervical tumor cell lines exhibiting a range of sensitivities to paclitaxel that cells with low levels of endogenous Raf-1 kinase exhibited increased sensitivity to paclitaxel (Rasouli-Nia A, 1998). The genetic down regulation of Raf-1 kinase activity by two folds resulted in four-fold enhancement in paclitaxel cytotoxity in these cell lines. However, it is unclear whether these results could be extrapolated into ovarian

tumours as cervical tumours are generally infected by human papilloma virus, which may alter their genetics and response to chemotherapy.

On the other hand, bcl-2 phosphorylation was demonstrated to occur in cells expressing a mutant raf-1 kinase that was not phosphorylated. Thus, the involvement of other kinases in the signaling pathway between the mitotic arrest stage and bcl-2 phosphorylation has been suggested. Scatena et al demonstrated that bcl-2 undergoes cell cycle-dependent phosphorylation during mitosis when there is elevated Cdc2 kinase activity followed by cell death. In this study the exact nature of the phosphorylating kinase was not identified (Scatena CD, 1998). The immunoprecipitation experiments revealed an Bcl-2-associated kinase capable of phosphorylating histone H1 in vitro but the kinase was likely not cyclin B1/Cdc2 because cyclin B1/Cdc2 was not detectable in Bcl-2 immunoprecipitates, nor was recombinant Bcl-2 phosphorylated in vitro by cyclin B1/Cdc2. Thus, it was suggested that paclitaxel may increase cellular susceptibility to apoptosis by amplifying the normal downstream events associated with mitotic kinase activation. In fact, Ling et al suggested that bcl-2 phosphorylation by paclitaxel in cervical and ovarian cell line (SKOV3) is associated with mitotic arrest and is not a determinant of progression into apoptosis (Ling YH, 1998b). In this study, the time course of bcl-2 phosphorylation, mitotic arrest, and apoptosis was correlated. It was demonstrated that Bcl-2 phosphorylation was closely associated in time with M phase arrest, accumulation of cyclin B1, and activation of Cdc2/cyclin B1 kinase, but not with apoptosis.

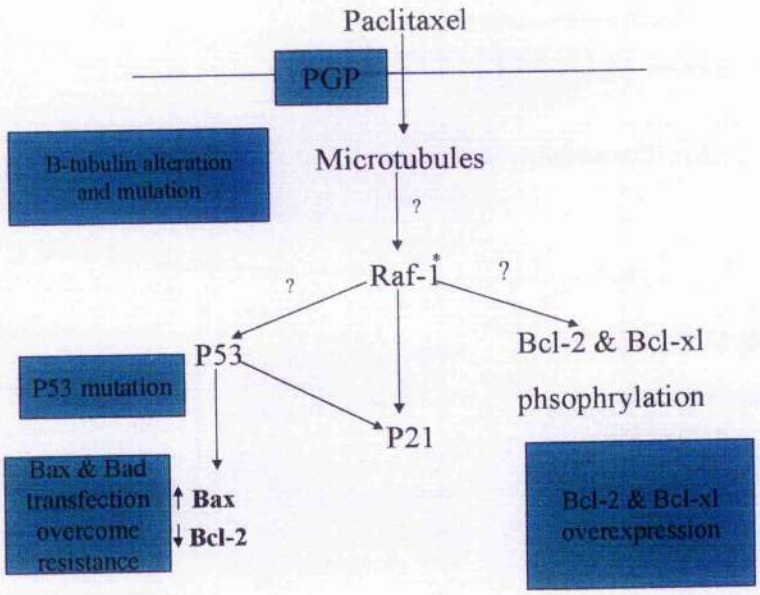


Figure 1-4: Schematic representation of steps of mechanism of action of paclitaxel. Following passive diffusion of paclitaxel into the cell, it acts on the microtubules.Following induction of polymerization of microtubules, signal transduction molecules are activated and lead to alterations in apoptotsis regulating proteins and cell death. (See text). (?) other signal transduction molecules may be involved. (*) Activation when paclitaxel concentration greater than 9 nM. At each level of action,a mechanism of resistance has been descrided and it is shown in the green boxes.

1.5 Paclitaxel resistance

Chemotherapy resistance in general can be caused by multiple mechanisms. These mechanisms include altered drug uptake, defective metabolic drug deactivation, structural changes in drug target, changes in cellular components that interact with target, or changes in response of pro-apoptotic or anti-apoptotic proteins that modulate cell death. The resistance to paclitaxel can be broadly divided into proximal mechanisms of resistance including altered drug uptake, changes in drug targets, or distal mechanisms related to apoptosis regulating proteins such as bcl-2 family of proteins and p53.

1.5.1 Altered drug uptake

Because of the hydrophobic nature of paclitaxel, chemoresistance has been associated with induction of multidrug resistance phenotype (MDR) and overproduction of P-glycoprotein (PGP). PGP is an integral membrane protein that passes through the membrane 12 times and has both its amino and carboxy ends within the cell. A linker region separates the two halves of PGP, each of which contains a nucleotide binding site. PGP is thought to function as an energy dependent drug-efflux pump that maintains the drug within the cell below toxic level.

Initially expression of the MDR phenotype was detected in Chinese hamster (CHO) selected for resistance to paclitaxel in a single step selection (Gupta RS, 1985). Horwitz et al demonstrated that highly paclitaxel resistant murine macrophages cell line J7.T1 (900 fold resistant) selected by stepwise increasing concentration of paclitaxel expressed MDR1 phenotype with PGP overproduction (Horwitz SB, 1993). Cell lines that have been selected for resistance to other anticancer agents and display multi-drug resistance were shown to be crossresistant to paclitaxel. For example, colon carcinoma HCT116 (VM) 46, selected for resistance to VM-26 and expressing the MDR phenotype was shown to be crossresistant to paclitaxel (Long B, 1991). Recently, Bhalla et al reported the isolation of paclitaxel –resistant human myeloid HL-60 cells (100 and 1000 resistant to paclitaxel that express MDR-1 gene, and were cross-resistant to the other drugs known to be pumped out by PGP (Bhalla K, 1994).

Similarly, Parekh et al demonstrated that paclitaxel resistance in ovarian cell lines was due to overexpression of PGP (Parekh H, 1997b).

Recent studies suggested that MDR phenotype might also be mediated by other proteins. Overexpression of a novel 135 kDa membrane glycoprotein distinct from the 170-180 kDa PGP has been reported to be responsible for reduced intracellular accumulation of paclitaxel in a paclitaxel resistant murine macrophage cell lines (Roy SN, 1985). In some cases, multi-drug resistance can be mediated by increased transcription of membrane- associate protein MRP. To date, there is little evidence to suggest that MRP plays any role in paclitaxel resistance (Breuninger LM, 1995; Cole SP, 1994).

1.5.2 Drug target alterations

Alterations in the microtubules causing reduced drug-binding affinities and decreased intracellular levels of polymerized tubulin have been associated with paclitaxel resistance. The study of tubulin alterations can be divided into two phases. The initial studies identified alterations in tubulin by detection of changes in electophoretic mobility of tubulin molecules or in total amount of tubulin m-RNA or protein present in these cells. Schibler et al reported that resistance in paclitaxel dependent Chinese hamster ovary (CHO) cells was due to altered forms of α and β tubulin detected by two-dimensional gel analysis (Schibler MJ, 1986). Also, Minotti et al using CHO cells demonstrated that paclitaxel resistance correlated to decreased polymerized tubulin (Minotti AM, 1991). Ohta et al reported an increased acetylation of α-tubulin subunit in paclitaxel resistant human small cell lung cancer (Ohta S, 1994). Although α-tubulin is not known to be a target for paclitaxel, alterations in this subunit may alter the topology of the tubulin polymer in such a way that paclitaxel affinity for the microtubules is reduced, thus resulting in the development of paclitaxel resistance.

Subsequent studies used PCR-based methodology to analyze the expression of β tubulin isotypes and sequence specific target regions without the need for cloning of individual genes. However, these studies were hindered by the presence of functional and non-functional genes encoding for different α and β isotypes discussed in section 1.2.3.1. Despite that several

studies have correlated altered expression of tubulin isotypes – in particular β tubulin isotype- to paclitaxel resistance. Increased expression of Mβ2, the class II β-tubulin isotype has been reported in a murine macrophage cell line with high level of paclitaxel resistance (Rao S, 1992b). Similarly, paclitaxel resistant human sarcoma mutants displayed reduced expression of the β4 and the β5 forms of the β-tubulin isotypes as compared with parental cells (Dumontet C, 1996). Kavallaris et al demonstrated differential expression of β-tubulin isotype in paclitaxel resistant ovarian tumours (Kavallaris M, 1997). Using specific oligonucleotides and polymerase chain reaction, the expression of six β- tubulin genes were compared in seven untreated primary ovarian tumours and four paclitaxel resistant ovarian tumour bearing ascites. Prior to that, specific β tubulin changes were studied in non-small cell lung cancer with low level of paclitaxel resistance. These cells displayed increased expression of β-tubulin genes: HM40 which encodes the class I isotype, Hβ9 which encodes for class II isotype, Hβ4 which encodes for class III isotype, and Hβ5 which encodes for class IVa isotype. In ovarian tumours with de novo resistance, HM40, Hβ4, and Hβ5gene expression was significantly increased compared to untreated tumours. The increased expression of the same specific tubulin isotypes in both paclitaxel selected tumour cell lines and clinically derived paclitaxel resistant cells suggest that these alterations may be involved in the resistant phenotype. Similarly, Ranganathan et al demonstrated increased in Hβ4 (class III) and Hβ5 (class IV) isotypes in human prostate carinoma cell lines resistant to paclitaxel. (Ranganathan S, 1998).

Further evidence that alterations in β tubulin isotype composition may modulate paclitaxel resistance came from a recent study using purified tubulin isotypes. Microtubules composed of class III and class IV β tubulin isotypes were found to be 7.4 and 7.7 less sensitive, respectively, to the effects of bound paclitaxel than microtubules assembled from unfractionated tubulin (Derry WB, 1997). It is postulated that altered expression of distinct isotypes could modify tubulin/microtubules dynamics or stability of microtubules in such a way that the action of paclitaxel is diminished. The selection of less stable tubulin isotypes could offer cells a survival advantage when exposed to microtubule stabilizing drug such as paclitaxel.

More recently, point mutations in β isotubulin have been demonstrated in 1A9 ovarian cell line (Giannakakou P, 1997) .In this study the tubulin content and polymerization was equal in sensitive and resistant cell lines. There was small increase (1.5 fold) in M40 gene expression (class 1isotype). These genes were found to be mutated in residue β 270 with substitution of phenylalanine to valine and amino acid β 364 with substitution from alanine to threonine. It is postulated that these point mutations may alter the paclitaxel binding site such that paclitaxel binding produces less stable microtubules.

1.5.3 Distal mechanism of resistance

1.5.3.1 P53 alterations and resistance to paclitaxel

Several in vitro studies attempted to address the issue of p53 status and resistance to paclitaxel yielding contradictory results. Whal et al reported that in different cell lines (HeLa and fibroblast-derived cell lines), the disruption of wild type p53 function resulted in seven to nine fold increase sensitivity of cells to paclitaxel compared with cells expressing wild type p53 (Wahl AF, 1996). Similarly, Vikhanskaya et al demonstrated that disruption of wild type p53 by transfection with E6 protein of human papilloma virus (HPV) type 16 led to 50 fold increase sensitivity to paclitaxel in A2780 ovarian cancer cells (Vikhanskaya F, 1998). In contrast, Vasey et al demonstrated that transfection of A 2780 ovarian cancer cells expressing wild type p53 with a dominant negative p53 mutant gene acquired resistance to cisplatin and doxorubicin, but not to paclitaxel (Vasey PA, 1996). Moreover, the introduction of wild type p53 in ovarian cell lines not expressing p53 did not change their sensitivity to paclitaxel (Graniela Sire EA, 1995). Subsequent study showed that the disruption of wild type p53 by transfection with HPV E6 resulted in decrease of apoptosis in ovarian cell line following treatment with paclitaxel (Wu GS, 1996). In addition to that, it was demonstrated that paclitaxel treatment of nine ovarian cell lines exhibiting different p53 status showed difference cytotoxicity (Debernardis D, 1997). Overall most of the above results suggest that p53 does not influence response to paclitaxel.

Several prognostic studies in patients treated with platinum based chemotherapy have shown that p53 mutations confer poor prognosis with shortening of progression free survival and reduction of overall survival rates (Herod JJ, 1996; Marx D, 1998). Recently, Sorensen et al correlated p53 status analyzed at DNA level with response rate and disease free survival in patients treated with paclitaxel. In total 45 patients were randomized to be treated with paclitaxel and cisplatin combination or cisplatin and cyclophosphamide combination (Smith-Sorensen B, 1998). When relapse free survival was estimated in the 33 patients (73%) who had detectable p53 gene alterations, there was a significantly better outcome for patients treated with paclitaxel based combination compared to the cyclophosphamide arm. Moreover, there was an association between p53 status and prognosis for patients who received platinum-based combination but not for the paclitaxel arm.

Taken together, these observations suggest that paclitaxel mediated cytotoxicity in ovarian cancer is not influenced by p53. Since p53 mutations occur in about 50% of patients presenting with ovarian cancer and these mutations influence the outcome cisplatin treatment, the use of first line paclitaxel and cisplatin treatment may help to improve the outcome as has been shown by the GOG 111 trial and intergroup study discussed in section 1.1.3.

1.5.3.2 Bcl-2 family

Several experimental models have shown that bcl-2 family proteins can modulate the sensitivity to paclitaxel in different cancer cell lines. Liu et al investigated the role of Bcl-xL in resistance to chemotherapy-induced apoptosis in ovarian carcinoma. Two human ovarian cell lines A2780 and SKOV3 and tumour lysates from human ascitic samples were analyzed for bcl-xl expression by Western blotting. The A2780 cell lines expressed low endogenous levels and displayed sensitivity to cisplatin and paclitaxel while SKOV3 cell lines expressed high endogenous levels of bcl-xl and resistance to chemotherapy (Liu JR, 1998). All the tumour lysates from ascitic fluid expressed high levels of bcl-xl. When the chemotherapy

sensitive A2780 cell line were transfected with bcl-xl, it became resistant to paclitaxel and cisplatin (Liu JR, 1998). Similarly, the overexpression of bcl-xl or bcl-2 by transfection of human leukaemic cells (HL60) inhibited paclitaxel induced apoptosis without affecting its antimicrotubule or cell cycle effects (Ibrado AM, 1997) and high levels of bcl-xl were shown to reduce free bax levels by hetrerodimerization.

In contrast, the pro-apoptotic proteins appear to enhance paclitaxel cytotoxicity. The transfection of MCF7 breast cancer cells that express high levels of bcl-xl with pro-apoptotic protein bcl-x_s induced a marked increase in chemosensitivity to paclitaxel (Sumantran VN, 1995). Similarly, Strobel et al suggested that bax may play an important role in paclitaxel induced apoptosis. Ovarian cancer cell (SW626) with p53 deficiency were transfected with c DNA encoding for murine bax resulting in significant enhancement of cytotoxicity to paclitaxel, vincristine, and doxorubicin, but not carboplatin. The presence of high levels of bax itself was not sufficient to trigger apoptosis without additional stimulus (Strobel T, 1996). The exact mechanism of how bax transfection augment paclitaxel induced apoptosis remain unclear, however several paclitaxel mediated events were enhanced including G2/M phase arrest, tubulin polymerization and phosphorylation. More recently it has been demonstrated that there is an increased accumulation of paclitaxel in bax-overexpressing cells, an effect due to diminished drug efflux (Strobel T, 1998a). Similarly, the enhancement of apoptotic pathway function through stable expression of the pro-apoptotic protein bad is in order to sensitize human epithelial ovarian cancer cells to chemotherapy has been studied. Expression of HA-bad in six separate clonal transfectants from two different ovarian cancer cell lines was found to significantly enhance the cytotoxic effects of paclitaxel, vincristine, and, to a lesser extent, etoposide. Importantly, this effect was associated with binding of HA-bad to bcl-xl and concomitant disruption of bax: bcl-xl interaction.

Taken together, these data suggest two main options to circumvent resistance to paclitaxel other than MDR reversal. The first the development of small molecules which mimic the effects of pro-apoptotic proteins such as bax and bad that may represent a new class of drugs capable of preventing or reversing resistance to chemotherapy agents such as paclitaxel (Strobel T, 1998b). The second is the development of antisense bcl-2 or bcl-xl oligonucleotide

to reduce their protective effects. In fact, the antisense bcl-2 oligonucleotide is already in clinical studies (Webb A, 1997) (Reed JC, 1997b)

On the other hand, the prognostic significance of bcl-2 family in the prediction of response to chemotherapy and survival in solid malignancy has been controversial and mostly inconsistent with in vitro studies. Most prognostic studies in ovarian cancer treated with cisplatinum suggested that high bcl-2 expression correlated with better prognosis. Herod et demonstrated that high bcl-2 expression detected by immunohistochemical staining in 45 patients with advanced ovarian cancers treated with platinum based chemotherapy was associated with improved survival (Herod JJ, 1996). Similarly, Diebold et al and Henriksen showed that high bcl-2 expression in patients with advanced ovarian cancer was predictive of better outcome (Diebold J, 1996; Henriksen R, 1995). All these studies concentrated on bcl-2 expression alone without correlating bcl-2 expression to other pro-apoptotic proteins such as bax that is clearly important in determining the fate of the cell. Marx et al examined the issue of differential expression of bcl-2 and bax in 215 ovarian cancers using immunohistochemical staining. Although bcl-2 expressing tumours had better prognosis, bax-expressing tumour had worse outcome in terms of overall survival. The overall survival in patients with bcl-2 positive/ bax negative tumours was significantly longer than bcl-2 and bax negative tumours (Marx D, 1997). On the other hand, Tai et al analyzed the expression of pro-apoptotic protein bax expression by immunohistochemistry and m-RNA transcript levels in 45 newly diagnosed ovarian cancer patients. Ovarian tumours expressing high levels of bax had higher response rates and had a clear disease free survival advantage independent of other prognostic factors (Tai YT, 1998).

Several explanation has been put forward to explain the inverse relationship between bcl-2 expression and prognosis. The first is that the interaction between the pro-apoptotic and anti-apoptotic are complex and dynamic, hence, the attempt to assess a single protein will be misleading. For example, bcl-2 predominates in well differentiated colorectal tumours and declines, as the tumour becomes undifferentiated. In contrast, bcl-xl tends to predominate in undifferentiated tumours. Similarly, for these studies to be meaningful, the anti-apoptotic proteins should be correlated to pro-apoptotic proteins. It has been demonstrated that

aggressive metastatic breast cancer had reduction in bax with reduced bcl-2 levels. Thus, low levels of bcl-2 were offset by reduction in bax and probably resulted in a survival advantage. The second is that immunohistochemical staining of histological biopsies provide a snap picture, which is technique and operator dependent.

1.6 Aims of the study

The first aim of this thesis was to test in vivo the hypothesis that significant alterations in the expression of the apoptosis regulating proteins occur to account for the development of resistance to paclitaxel. To achieve this aim, paclitaxel sensitive and resistant xenografts were established form CH1 and CH1 resistant cell lines respectively. The CH1 cell lines were chosen because of lack of expression of MDR phenotype, which would help to eliminate one mechanism of resistance to paclitaxel. In addition, both CH1 paclitaxel sensitive (CH1/TS) tumours and CH1 paclitaxel resistant tumours (CH1/TR) express wild type p53 allowing the assessment of its central role in the regulation of other death inducing proteins described in vitro. Following the establishment of these xenografts, the sensitivity and resistance to paclitaxel was examined in vivo. Subsequently, intra-tumoural paclitaxel concentrations were measured following paclitaxel administration to rule out any uptake defects. Then, the effect of paclitaxel on the cell cycle and proliferation, and induction of cell death was compared in both types of tumours. Finally, the changes in levels of apoptosis regulating proteins following paclitaxel treatment were assessed by western blotting. It was essential to take into consideration the dynamic nature of all these parameters, so they were assessed at 24, 48, and 72 hours following paclitaxel administration.

Because of the importance of cisplatin in the management of ovarian cancer, it was important to assess its effect on these tumours. Hence, the response of both tumours, changes in cell cycle and proliferation, induction of cell death, and changes in apoptosis regulating proteins were studied following cisplatin treatment. Thus, we were able to compare and contrast the effect of paclitaxel and cisplatin on all these parameters.

The importance of studying these proteins stems from their role in controlling the most distal steps of induction cell death following chemotherapy treatment, therefore, providing a suitable targets for molecular therapy. We have chosen to conduct this study in vivo because of two main reasons. Firstly, in vivo studies have the advantages of taking into consideration the tissue-tissue interactions, and drug delivery which are major determinants of tumour growth. Secondly, there is a lack of in vivo studies addressing these issues despite the abundance of in vitro studies. Consequently, the present study will help to determine if the changes in apoptosis regulating proteins occur in vivo.

The second aim of the thesis was to test the hypothesis that the overexperssion of a given anti-apoptotic protein can modulate the response to paclitaxel and cisplatin in vivo. To that effect, xenografts of CH1 ovarian cancer cells transfected with anti-apoptotic protein bcl-xl were established and subsequently treated with cisplatin and paclitaxel. Similarly, the intra-tumour paclitaxel concentration was determined in these tumours and compared to the parent CH1 tumour to rule any uptake defects. The importance of this experiment would be to examine whether the alteration in expression of a single effector distal protein could modulate the response to chemotherapy, which would further strengthen their possible role as target for molecular therapy.

With these experiments, we aimed to:

1. Demonstrate in vivo the effect of paclitaxel on cell cycle and proliferation, and induction of apoptosis.
2. Compare and contrast the mechanisms of action, apoptosis induction, and the effect on apoptosis regulating protein of paclitaxel and cisplatin in resistant and sensitive tumours.
3. Determine whether in acquired resistance, there is any alteration in the expression or induction of apoptosis regulating proteins.
4. Determine whether overexpression in vivo of a given anti-apototic gene, bcl-xl, could result in development of resistance to paclitaxel.

Chapter 2: Materials & Methods

2.1 Cell lines and establishment of xenografts

2.1.1 Solution & Buffers

(1) Dulbecco's Modified Eagle Medium (DMEM) without sodium pyruvate and with 4500mg/l glucose. (Gibco catalogue number (cat no.) 41965-039). Stored at 4°C.

(2) *Foetal Bovine Serum (FBS) (Imperial Laboratories, Andover, UK). Stored at –20 to –40 °C.*

(3) *L-glutamine (200 MM 100X Gibco cat. no.11140-035). Stored at –20 °C.*

(4) Modified Eagle Medium non-essential amino acids (100X Gibco cat. No.11140-0335). Stored at 4 °C.

(5) *Hydrocortisone 25 mg of hydrocortisone dissolved in 5 ml 95% ethanol and stored at 4 °C. When Required 0.5ml of stock is added to 12 ml Earle' s balanced salt solution, Filtered and 1.1 ml per 500 ml bottle.*

(6) *Trypsin (Life Technologies, U.K.).*

(7) Ethyelenediaminatetraacetic acid (EDTA) (Sigma).

2.1.2 Cell lines and culture conditions

The CH1 ovarian carcinoma cell line was derived from a moderately differentiated human ovarian carcinoma (Hills CA, 1989), and has been shown previously to be relatively sensitive to cisplatin, and paclitaxel. The paclitaxel resistant subline of CH1 (CH1/TR) was provided courtesy of Dr Kelland, and was generated by continuous exposure to paclitaxel at escalating concentrations up to 20 nM. The 96 hrs IC_{50} of CH1 and CH1/TR to paclitaxel were 0.66nM, and 7.93nM respectively, making the CH1/TR cell line 12 fold resistant to paclitaxel compared to parent cell line (Rogers P, 1996). Both cell lines were maintained in DMEM supplemented with 10% fetal calf serum, 2mM of L-glutamine, 5 ml of MEM non-essential amino acids and 0.5 mg/ml of hydrocortisone. Both cell lines expressed wild type p53 sequence, with no overexpression of PGP (Pestell KE, 1998; Rogers P, 1996).

2.1.3 Preparation of cell line for implantation

The cells were harvested by trypsinisation for 5 minutes at 37 °C using 0.02% EDTA/0.05% trypsin and centrifuged at 500 g for 15 minutes. Cells were washed once in phosphate buffered saline (PBS), and then resuspended in 2ml of DMEM containing 10% foetal calf serum. Cells were then injected in 0.2ml volume containing approximately $5x10^6$ cells.

2.1.4 Establishment of CH1 and CH1 paclitaxel resistant xenograft

2.1.4.1 Experimental animals

Specific pathogen-free 4-6 week old athymic nude (nu/nu) mice were used. The mice were housed in sterilized filter topped cages kept in laminar flow isolators and were fed sterilized food and water. All procedures involving the animals were performed under sterile conditions in a laminar flow hood. All studies were performed according to the approval of the Institute of cancer research Animal Ethics Committee and according to UKCCCR guidelines.

2.1.4.2 Establishment of Xenografts

In total ten nude mice were injected subcutaneously in the right inguinal region with $5x10^6$cells of either CH1/TS or CH1/TR cells. When the tumours reached 10-15mm in diameter they were serially passaged by subcutaneous (s.c.) implantation of explants of xenografted tissue. The passage was done by implanting a small non-necrotic fragment of harvested tumours subcutaneously in right inguinal and the wound was sutured by a metal clip. The tumours were passaged for at least four times before conducting the growth experiments. All these procedures were done with mice fully anaesthetized by halothane.

2.2 Biological growth experiments

The aim of this experiment was to confirm the sensitivity and resistance pattern of CH1/TR and CH1/TS cells in vivo.

2.2.1 Drugs

A) Paclitaxel (*Bristol Myers Squibb, Wallingford, Connecticut, and U.S.A.*) was dissolved in a vehicle containing 50% polyoxyethylated castor oil (Cremophor EL) and 50% ethanol, and further diluted in 5% glucose in water.

B) Cisplatin (*Johnson Matthey Technology Centre, Reading, Berks, UK*) was dissolved in 0.9% NaCl.

2.2.2 Methodology

50 nude mice were implanted with CH1/TS tumours and 50 nude mice were implanted with CH1/TR tumours. The take rate which is defined as the number of tumours that grew progressively after implantation and could be serially passaged were 75% and 50% for CH1/TS and CH1/TR respectively.

The tumours were measured once per week in two dimensions (length and width) using calipers. A baseline weight of each animal was taken and subsequently weighed once per week. When the tumor diameter size reached 8±2 mm, the mice were randomized into three groups of five for each line:

A) Group 1 controls (no treatment)

B) Group2 treated with paclitaxel 25mg/kg in 0.2ml IP on days 0,4,and 8.

C) Group 3 treated with cisplatin 4mg/kg in 0.2ml IP on days 0,4, and 8.

The end point was reached when tumour is 20 mm in diameter, or 60 days post treatment. The tumour volume was calculated using formula $Tv = a \times b^2 \times \pi/6$, where a is the length measurement of longest axis, and b is width perpendicular to the length. The biological growth curves were generated by plotting the relative tumour volume against time.

The specific growth delay of CH1/TS and CH1/TR tumours treated with cisplatin or paclitaxel was calculated by formula:

$$\text{Specific growth delay} = \frac{\text{TD treated} - \text{TD control}}{\text{TD control}}$$

TD= tumour doubling time.

2.3 Paclitaxel uptake and pharmacokinetic studies

The aim of this experiment was to assess the paclitaxel concentration levels and pharmacokintetic parameters of CH1/TS, and CH1/TR following intraperitoneal (i.p.) administration of 25mg/kg of paclitaxel using High Performance Liquid Chromatography (HPLC).

2.3.1 Methodology

Three tumours per time point were harvested from athymic nude mice bearing CH1/TS, and CH1/TR xenografts at 1, 3, 6, and 24 hours following administration of 25mg/kg i.p. paclitaxel. All tumours were snap frozen with liquid nitrogen for subsequent use.
At the time of analysis, the tumours were disrupted by Polymix homogenizer with a tight-fitting ground glass pestle after the addition of 2ml of PBS per gram of tissue. Paclitaxel was extracted from tumours by adding 2.5 ml of acetonitrile: then the samples were vortexed for 30 seconds and centrifuged at 400 g for 10 minutes. The supernatants were collected and dried under nitrogen flow at 37 °C. The dry residues were reconstituted in 200µl of acetonitrile, methanol, and de-ionized water solution (4:1:5) by vortexing for 30 seconds. They were then centrifuged at 400g for 5 minutes, and 100 µl of supernatants was injected into the HPLC column.

The standards were prepared from untreated control tumours that were homogenized as above. Paclitaxel was added to tumour tissue and serial dilutions from 5000 ng/ml to 50 ng/ml were made.

2.3.1.1HPLC measurement

Paclitaxel concentration in tumour tissue was measured by means of the reversed-phase HPLC method. The HPLC system consisted of a Waters 240 UNICAM module equipped with a UV detector and a Supelcosil (Supelco) 1.5 cm x 4.6mm 5 μm (C18) column. The UV detection was done at 227 nm, with a limit of sensitivity of 0.125 μg/ml. The mobile phase consisted of 10 ml of ammonium acetate, 490 ml of de-ionized water, 100 ml of ethanol, and 400 ml of acteonitrile per liter. The tissue extracts were chromatographed at flow of 1 ml/min. The peak heights in microvolts of paclitaxel standards and tissue extracts were measured. A Standard curve of concentration ranging from 50 to 5000 ng/ml versus peak values was generated using paclitaxel standard. The correlation coefficient was used to evaluate the linearity of the standard curve. The tissue paclitaxel concentration was derived from the standard curve by linear regression using Prism2 of Graph Pad software.

2.3.1.2 Pharmacokinetic analysis

Concentration-time curves were plotted using mean concentration of paclitaxel per time point for each tumour type. These curves were then used to calculate the following tissue pharmacokinetic parameters $\mathbf{C_{max}}$, $\mathbf{t_{1/2}}$ Lambda z, **AUC**last, and **AUC**INF. The calculation was done using Win non lin professional software (non-compartemental analysis). **AUC** from time 0 to the last sampling point was computed for tissue concentration data by using trapezoidal method.

2.4 Chemotherapy induced pharmacodynamics: Study design

The aim of this experiment was to design a xenograft model from CH1/TS and CH1/TR tumours to study the cell cycle changes, induction of apoptosis, and dynamic changes of bcl-2 family of proteins, p53, and p21, Raf-1 following paclitaxel and cisplatin treatment. All these parameters were assessed at 24, 48, and 72 hrs following treatment because of their dynamic nature.

2.4.1 Buffers &Solution

(1) *Bromodeoxyuridine (Sigma, B-9285)*

(2) *Tris-HCL (50 mM) and NaCl buffer*

0.6057g Tris-HCL + 0.8766g NaCl/100 de-ionized water (dd. H2O) adjusted to pH 7.6

(3) *Sodium Orthovanadate (100 mM) (Na VO4)(Sigma)*

18.4 mg of Na VO4/ 1ml ddH2O

(4) *Phenylmethylsulfonyl fluoride PMSF (20m M) (Sigma, P-7626)*

(5) *Sodium Dodecyl Sulphate (SDS) (20%) (Sigma)*

10mg SDS/ 50 ml ddH20

(6) *Aprotinin (10mg/ml) (Sigma, A-6279)*

(7) *Leupeptin (10mg/ml) (Sigma, L-9783)*

(8) *Nonidet P-40 (NP40) (Sigma, N-6507)*

(9) *Lysis buffer*

10 ml 50 mM Tris-HCL, 150 mM NaCl, 100µl 100% NP40, 100µl 20%SDS, 500µL NaVO4 (100 mM), 2µL Aprotinin (10mg/ ml), 2µlLeupeptin (10mg/ ml) was made in place immediately prior to use.

2.4.2 *Methodology*

Forty two athymic nude bearing CH1/TS tumours and forty two athymic nude mice bearing CH1/TR were randomized into three groups when the tumour size was 8±2 mm in diameter. The groups and the treatment for each cell line were as follows:

1) 18 mice received paclitaxel 25mg/kg i.p. and they were sacrificed in groups of six mice at 24h, 48h, 72h after treatment.
2) 18 mice received cisplatin 4mg/kg i.p. and they were sacrificed in groups of six mice at 24h, 48h, 72h after treatment.
3) 6 mice received no treatment and they were sacrificed at 24h after start of experiment.

All mice were injected with 100mg/kg of bromodeoxyuridine (Brd Urd) made up in 0.9% NaCl saline (10mg/ml) five hours before harvesting of tumours to facilitate the cell cycle kinetics by flow cytometry. Subsequently, the tumours were harvested and divided in to three sections:

1) First section was section was immersed in ice-cold 70% ethanol to be used for flow cytometry.
2) The second section was immersed in 10% formalin before making of paraffin section.
3) The third lysed with ice-cold lysis buffer and used for protein extraction.

2.5 Measurement of cell kinetics by bromodeoxyuridine/anti-bromodeoxyuridine method

There are several methods that can be used to assess proliferation and cell kinetic measurement, each having advantages and disadvantages. Most methods such as mitotic index, S phase fraction from the DNA histogram, or Ki-67 and PCNA staining provide a snapshot assessment of the cells in a particular phase of cell cycle or cells actively proliferating. Techniques which include the use of DNA precursors such as bromodeoxyuridine (Brd Urd) are recognized to be better, in cell kinetic terms, because they measure the functional aspects of the cell proliferation. The assessment of proliferation by Brd Urd technique using flow cytometry is based on incorporation of Brd Urd (detected by monoclonal antibody) relative to DNA content measured by propidium iodide (PI). Thus allowing measurement of cell kinetics and more importantly the movement of cohort cells in any phase of cycle in labelled and unlabelled cells using appropriate computer generated regions.

The flow cytometric analysis involved two steps: obtaining the nuclei from ethanol fixed tumours and denaturation procedure of DNA for Brd Urd detection.

2.5.1 Obtaining nuclei from ethanol fixed tumours

The tumour samples were removed from the 70% ethanol and minced into small ($1mm^3$) fragments, then dissociated by addition of 5 mls of pepsin solution (0.4 mg/ml/in 0.1M HCL) to each sample and incubated at shaking water (37 °C) water bath for 30 minutes. The samples were then drawn into 1ml pipette and continued disassociation until only connective tissue remained. The suspension was filtered through 35 μm nylon mesh into conical-bottomed 10ml tube. The nuclei were pelleted by centrifugation at 500g for 5 minutes and then washed and resuspended in PBS.

2.5.2 Denaturation of DNA and Brd Urd detection

The freshly prepared nuclie were pelleted by centrifugation at 500 g for 5 minutes and then resuspended in 1 ml of 2 M HCL. The suspension was incubated at room temperature for 30 minutes to denature the DNA after which 5 ml of PBS was added and the nuclei centrifuged at 500 g for 5 minutes. The pelleted nuclei were washed twice with PBS, then resuspended in labelling solution containing 1ml of 0.5% Tween 20/1% BSA/PBS and 20 µl of Anti-Brd Urd FITC-conjugated monoclonal antibody (Becton-Dickinson, cat. no.347583) and incubated for 30 minutes. The suspension was then washed twice in 1ml 0.5% tween 20/ 1% BSA/PBS and resuspended in 1 ml of PBS containing 20µg/ml propidium iodode (PI) (Sigma) and 100 µg/ml RNAse (Sigma).

2.5.3 Flow cytometry

Flow cytometric measurements were made on a Coulter EPICS Elite ESP (Beckman coulter, High Wycombe, UK) using a Spectra-Physics argon-ion laser tuned to produce 200 mW at 488 nm. The DNA content was measured as red fluorescence (>630 nm) from PI. A display of the peak versus the area of the signal red fluorescence was used to gate out any debris or any clumped nuclei (Ormerod, 1990).

A display of green representing cells incorporating Brd Urd during DNA synthesis (fluorescein, 520 nm) versus red (DNA) was recorded in bivaraite histogram. A separate histogram of DNA fluorescence was also recorded. The percentage of tumour cells in each phase of the cell cycle was analyzed on multicycle-cell analysis program (Phoenix flow systems, CA, USA) that deconvolves a DNA histogram into G1, S, and G2/M phases. A region was set on the histogram to exclude the host cells from the analysis.

Several regions were created in the bivaraite histogram; a) region containing all cells, b) region containing all cells with G1 DNA content, c) region containing the Brd Urd labelled G1 phase cells, d) region containing labelled in S phase, e) region containing cells with G2

DNA content. The labelling index was expressed as the percentage of tumour cells that had taken up Brd Urd (Ormerod MG, 1994). Because some cells would have divided to produce two daughter cells, labelled cells which appear in G1 phase were halved and subtracted from the total number of labelled cells and from the total cell number. The relative movement was calculated by subtracting the mean DNA of G1 population from that of the labelled cells and dividing it by G1 population from that of the labelled cells and dividing it by G1 subtracted from G2 (Ormerod MG, 1994).

2.5.4 Detection of apoptotic cells by flow cytometry

Cells dying of apoptosis from G1 give a sub-G1 (hypoploid) peak in the DNA histogram that correlate with DNA degradation. The sub-G1 debris in all 84 samples was measured from the DNA histogram to give crude estimation of apoptosis and subsequently correlated with values obtained from TUNEL assay.

2.6 TdT - mediated d UTP-biotin nick end labelling (TUNEL) assay of apoptosis in paraffin-wax embedded sections

The detection of apoptosis by TUNEL assay is based on specific binding of terminal deoxynucleotidyl transferase (TdT) to 3-OH ends of DNA produced during DNA fragmentation. Following the exposure of nuclear DNA on histological sections by proteolytic treatment, TdT is used to incorporate biotinylated deoxyuridine at sites of DNA breaks. The signal is amplified by Avidin-biotin peroxidase conjugate enabling conventional histochemical identification by light microscopy.

2.6.1 Solution & BUFFERS

1) *Phosphate Buffered Saline, pH 7.3 (PBS)*

   (Tablets- 1o tablets per 1000 ml de-ionized water, each tablet contain 7.33 g sodium chloride (NaCL), 2.36g di-sodium hydrogen phosphate, and 1.32g sodium di-hydrogen orthophosphate.

2) *Tris buffered saline*

   0.606 g Tris base + 0.88g NaCl/ 100 mls di-water adjusted to pH 7.6

3) *Cobalt chloride (50m M)*

   0.119-g cobalt chloride in 10 mls di-water. 200 aliquots stored at –20°C.

4) *Reaction buffer (X5)*

   1 M sodium cacodylate (3.2g) + 125 mM Tris HCl (3.15g) + 0.025mg/ml BSA in di-water.

5) *Reaction mixture (RM)*

   Each 100µl= 0.75µl TdT, *0.50µl* Biotin16dutp, 10.00µl cobalt chloride, 20.00µl [X5] reaction buffer.

6) *pepsin solution*

   0.5g pepsin (molecular biology grade: Sigma P-7012) was dissolved in 100 ml of de-ionized water, and diluted in 0.1 M HCL to give a final pH OF 2.0. The enzyme solution was stored in 500 µl aliquotos at –20 °C in eppendorf tubes.

2.6.2 Paraffin sections

The harvested tumours were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sequential adjacent sections (3μm) were cut from the paraffin-tumours onto slides coated with 3-aminopropyltriethoxysilane (Sigma), dried at 37 °C overnight, and stored at room temperature in standard slide shelving until use.

2.6.3 Labelling of paraffin sections

Three micron paraffin sections were dewaxed in xylene twice and then immersed in increasing dilutions of Industrial Methylated Spirits (IMS) (100% x3, 95% x1, 80% x1, and 70% x1) and hydrated in de-ionized water x2. The endogenous peroxidase was blocked by immersing in 1% H2O2 for 15 minutes and then washed in de-ionized water x3. Each section was incubated with 100μl of 0.5% pepsin (pH 2) for 30 minutes in humidified chamber at 37 °C. The digestion with pepsin was terminated by rinsing for 1 minute, then washing for 5 times in de-ionized water and Tris buffered saline (pH 7.6) for 5 minutes x2. Each section was covered with 100μl of reaction mixture (RM) and incubated in humidified oven for 1 hour at 37 °C. After incubation, the sections were washed with de-ionized water x2 and PBS X3, then, incubated for 30 minutes with Streptavidin Biotin Complex conjugated to horseradish peroxidase (Strep ABC/HPR Dako) diluted 1:400 in PBS, 1% BSA, and 0.5% tween 20. After washing sections in PBS/Tween 20 for three times, the peroxidase activity were demonstrated with 0.5% diaminobenzidine (DAB; Sigma) and 0.07% imidazole for 30 seconds, then 100 μl of H_2O_2 was added and sections were incubated for a further 10 minutes. The sections were then washed in running tap water for 5 minutes and then immersed in 0.5% copper sulphate and 0.9% sodium chloride in de-ionized water for 1 minute. After washing in running tap for 5 minutes, the sections were counterstained with Mayers haematoxylin for 60 seconds, washed in tap water, dehydrated through increasing concentration of IMS (as above) followed by xylene, cleared and mounted in DPX.

2.6.4 *Determination of apoptotic index (AI)*

Every slide was examined carefully a using a standard light microscope (x40 objecive) (Nikon Optiphot II, Japan) with 10x10 eyepiece graticule. A total of 3000 cells were counted randomly and percentage of positively labelled cells was determined to obtain AI.

2.7 Western Blot of total protein extracts of tumours

2.7.1 *Protein recovery for Western blotting*

The tumors were harvested and homogenized in 2ml of ice-cold lysis buffer by an electrical homogenizer and left on ice for 15 minutes with occasional mixing. The mixture was centrifuged at 12,000 g at 4°C for 20 minutes. The supernatant was carefully removed and an aliquot of 50µl placed into a labeled tube (for protein concentration measurement by BCA assay) and the rest was placed in another appropriately labelled tube. Then samples were stored at –70 °C.

2.7.2 *Protein concentration estimation by Pierce protein BCA assay*

Protein concentration estimation was done using Pierce protein BCA assay. In this assay, proteins react with Cu^{+2} to yield Cu^{+}. Cu+ reacts selectively with bioinchoninic acid of BCA to yield an H2O Soluble purple reaction product that absorbs light strongly at 562 nm. This assay was used because of its compatibility with detergents present in lysis buffer.

Initially Bovine Serum Albumin (BSA) (Pierce, cat no. 23209) standards were prepared. Then, BCA reagents were prepared by mixing 50 vol. Reagent A (Pierce cat no. 23223) with 1 vol. Reagent B (Pierce cat no. 23224) – stable for day at room temperature. The samples were diluted between 1/20 to 1/50 for cellular total protein samples. Duplicates of 50µl sample or standard were pipetted into a small test tube, and 1 ml of BCA reagent was added. The samples were vortex mixed. The samples and standards were incubated at 37 °C for 30

minutes, and then allowed to cool for 10 minutes. The absorbance of standard and samples were measured at 562 nm. The absorbance of the standards at 562 nm was plotted against protein concentration and a standard curve was generated. The sample protein concentration was determined from the standard curve by linear regression using Prism 2 program of Graph pad software.

2.7.3 Western blotting of cellular total protein extracts

2.7.3.1 Gel electrophoresis

Precast gels were obtained from Novex (cat no. EC60485, Germany) with 8-16% gradient separating gel. The gel specification

Tris-glycine Gel matrix, 1.5 mm gel thickness, 8 cm x 8 cm gel
Size, cassettes size 10 cm x 10 cm, and 15 well configuration.

2.7.3.2 Buffers & Solutions

1) *Laemmli sample buffer (x2)*
   20 ml glycerol (20%), 10 ml mercaptoethanol (10%), 23ml of 20% SDS (4.6%), 6.25 ml of 1 M Tris (pH 6.8), 5 ml bromophenol blue (0.1%) diluted to 100 ml with de-ionized water. Aliquot into 250µl and freeze at –20°C until required.
2) *Running buffer (10x, Novex, cat no. LC 2675)*
   29 g Tris (0.2 M), 144 g Glycine (1.9 M), 10 g SDS (1%).
   80 ml of running buffer was added to 720 ml of de-ionized water.
3) *Transfer buffer (25x, Novex, cat no. LC 3675)*
   12 mM Tris, 96 mM Glycine
   20ml of Transfer buffer is added 430 of de-ionized water and 50 ml of methanol.
4) *Casein blocking buffer (pH 7.6)*
   0.5% Casein (2.5g) (Biochemical, cat no 4402034), 0.02% thimerosal (0.1g) (Sigma).
   The mixture is dissolved in 500ml of PBS and heated to 60°C until Casein dissolves, then allowed to cool and adjusted to pH 7.6.

2.7.3.3 Antibodies

The primary antibodies used were as follows:

(1) Bcl-2

Source: Santa Cruz Biotechnology

Antibody Type: polyclonal rabbit N-19 (cat no. Sc-492)

Epitope: Amino acids 4-21 of bcl-2

Dilution: 1:1000

Duration of incubation: Overnight incubation.

(2) Bax

Source: Santa Cruz Biotechnology

Antibody Type: Polyclonal rabbit (cat no. Sc-493)

Epitope: Amino acids 11-30 of bax

Dilution: 1:500

Duration of incubation: Overnight incubation

(3) Bcl-$xl_{S/L}$

Source: Santa Cruz Biotechnology

Antibody Type: Polyclonal rabbit (cat. no. Sc-634)

Epitope: Aminoacid sequence mapping at amino terminus of Bcl-x of human origin.

Dilution: 1:500

Duration of incubation: Overnight incubation

(4) P53

Source: Santa Cruz Biotechnology

Antibody Type: monoclonal mouse (DO-1) (cat. no. Sc126)

Epitope: Amino acid residues 11-25 of human origin

Dilution: 1:500

Duration of incubation: Overnight incubation

(4) P21

Source: Santa Cruz Biotechnology

Antibody Type: Polyclonal rabbit C-19 (cat no sc-397)

Epitope: Amino acid mapping at the carboxy terminus of human p21.

Dilution: 1:500

Duration of incubation: Overnight incubation

(5) Raf-1

Source: Santa Cruz Biotechnology

Antibody Type: Polyclonal rabbit C-12 (cat no sc-133)

Epitope: Amino acid mapping at the carboxy terminus.

Dilution: 1:500

Duration of incubation: Overnight incubation

The secondary antibody were as follows:

(1) Anti-rabbit

Source: Santa Cruz Biotechnology

Antibody Type: Anti-rabbit IgG-HPR labelled (cat.no. sc-2004)

Dilution: 1:1000

Duration of incubation: 45 minutes

(2) Anti-mouse

Source: Amersham

Antibody Type: HPR-labelled anti-mouse antibody

Dilution: 1:1000

Duration of incubation: One hour

2.7.3.4 Methodology

The protein extracts were diluted with ddH2O so that all the samples contained the same protein concentration (40-60μg). The samples were diluted 1:1 with laemmli buffer, and heated at 95°C for 3 minutes. 5 μg of rainbow marker was diluted with 1:1 laemmli buffer and heated at 95°C for 1 minute. Both samples and rainbow marker were allowed to cool and kept on ice until use.

Equal amount of proteins 40 μg were loaded into 15 wells of a 8-16% polyacrylamide gel. The gels were run under constant current of 30 mA per gel using running buffer. Following completion of separation of proteins, they were transferred onto nitrocellulose filter. The transfer step was carried in cold room (4°C), under constant current of 300 MA and using running buffer. Subsequently, the electroblotting efficiency and equal protein content per lane were verified by staining of membranes with 0.1% Ponceau S. The membranes were blocked using blocking buffer for 60 minutes at room temperature. Following this step the membranes are ready for immunodetection.

2.7.3.5 Immunodetection of specific proteins on nitrocellulose filters

Initially the nitrocellulose filters were washed with PBS containing 0.1% Tween 20 (PBST), then the filters were exposed to the appropriate antibody diluted in blocking buffer as above. All antibodies were incubated overnight to enhance detection of proteins. Following incubation with primary antibody, the nitrocellulose filters were washed with PBST for 15 minutes once and 5 minutes three times. Then the filters were exposed to the appropriate secondary antibody for one hour. Then the filters were washed with PBST as above.
The proteins were detected using Enhanced Chemiluminescent (ECL) and autoradiography films in dark room. The filter was exposed to mixture of 5 mls of reagent A and 5 mls of reagent B for 60 seconds. The reaction was terminated by the removal of the filter from the mixture, and quickly exposed to autoradiography film for 1,2,5 minutes.

2.7.3.6 *Imagequantification*

The expression of different apoptotic proteins were assessed in two ways:

(1) The effect of paclitaxel treatment on bcl-2 family of proteins, p53, p21, and Raf-1 in CH1/TS and CH1/TR tumours was compared at 24, 48, 72 hours. The pre-cast gels were loaded with CH1/TS control, CH1/TR control, protein samples of the six CH1/TR xenografts treated with paclitaxel, and protein samples of the six CH1/TS xenografts treated with paclitaxel to perform this comparison.

(2) The effect of cisplatin and paclitaxel on expression of the above proteins in CH1/TR xenograft was compared because of different response of these xenografts to the two drugs. The pre-cast gels were loaded with CH1/TR control, protein samples of the six CH1/TR xenografts treated with cisplatin, and protein samples of the six CH1/TR xenografts treated with paclitaxel.

The imagequantification of protein bands was done using Imagequant Molecular Dynamics software. The imagequntification provided a value reflecting the band density of the protein probed for each individual sample. The band density values were expressed as ratio folds increase or decrease from the control proteins. Prior to that, the controls for CH1/TR and CH1/TS xenografts were blotted and probed for different proteins above. The expression of the probed proteins in the control samples was found to be almost equal. Hence, one control was chosen for each group and used throughout the experiments.

2.7.3.7 *Characterization of bcl-2-related proteins*

In addition to the 26 kilodalton (KD) bcl-2 band, two other bands with molecular weight of 27 KD and 30 KD bcl-2 related bands. To further characterize these bands, 50 μg of tumour lysate of three paclitaxel treated CH1/TS tumours lysates were either incubated with or without 2 units of alkaline phosphotase at 37 °C for 4 hours. Then, 40 μg were loaded into pre-cast gels and immunoblotted with anti-bcl-2 antibody. The tumour lysates were prepared with same lysis buffer described in section 2.4.1, but Sodium Orthovanadate was omitted because it inhibits the action of alkaline phosphates.

2.8 Growth studies of xenografts of bcl-xl transfected CH1 paclitaxel sensitive cells

The aim of this experiment was to assess the effect of the transfection of the anti-apoptotic bcl-xl on the responsiveness of CH1/TS tumours to cisplatin and paclitaxel.

2.8.1 methodology

Cell line of CH1 tumour cells transfected with bcl-xl was provided courtesy of Dr. Kelland. The bicistronic plasmid vector pIRES-P (code number 250, EMBL accession number Z75185) was used to express bcl-xl in CH1 cells (figure 2-1) (Sharp SY, 1998). This vector expresses a single bicistronic mRNA driven from the human cytomegalovirus immediate early enhancer/promoter and uses the internal ribosome entry site from encephalomyocarditis virus to direct translation of the downstream pac gene encoding for puromycin acetyl transferase. Xenografts of CH1 cells transfected with bcl-xl (CH1/bcl-xl) and xenografts CH1 cells transfected with vector only (CH1/F 280) were established using the same procedures in section 2.1.4 were used to establish these xenografts. These xenografts were passaged at least for three times before conducting the growth experiments. The level of expression of bcl-xl protein CH1/bcl-xl xenografts was assessed by western blotting and expressed as fold increase from CH1/TS tumours.

Subsequently, 105 athymic nude mice were divided into three equal groups and implanted with either CH1/TS, CH1/bcl-xl, or CH1/F 280 tumours. The take rates were 75%, 60%, and 65% for CH1/TS, CH1/bcl-xl, and CH1/F 280 tumours respectively. The tumours were measured once per week in two dimensions (length and width) using calipers. A baseline weight of each animal was taken and subsequently weighed once per week. When the tumor size reached 8 ±2 mm, the mice were randomized into three groups of six for each line:

D) Group 1 controls (no treatment)
E) Group2 treated with paclitaxel 25mg/kg in 0.2ml IP on days 0,4,and 8.
F) Group 3 treated with cisplatin 4mg/kg in 0.2ml IP on days 0,4, and 8.

The end point reached when tumour is 20 mm in diameter, or 60 days post treatment. The tumour volume and specific growth delay was calculated as described previously.

2.9 Paclitaxel uptake and pharmacokinetic studies of CH1/bcl-xl

It was important to rule out any uptake defect that contributes to the pattern of response of CH1/bcl-xl tumours to paclitxel. Hence, Three tumours per time point were harvested from athymic nude mice bearing either CH1/bcl-xl or CH1/TS tumours at 1, 3, 6, and 24 hours following administration of 25mg/kg i.p. Then intra-tumoural concentration of paclitxel in CH1/bcl-xl tumours was measured and compared to parent tumour CH1/TS tumours using the method described in section 2.3.1.

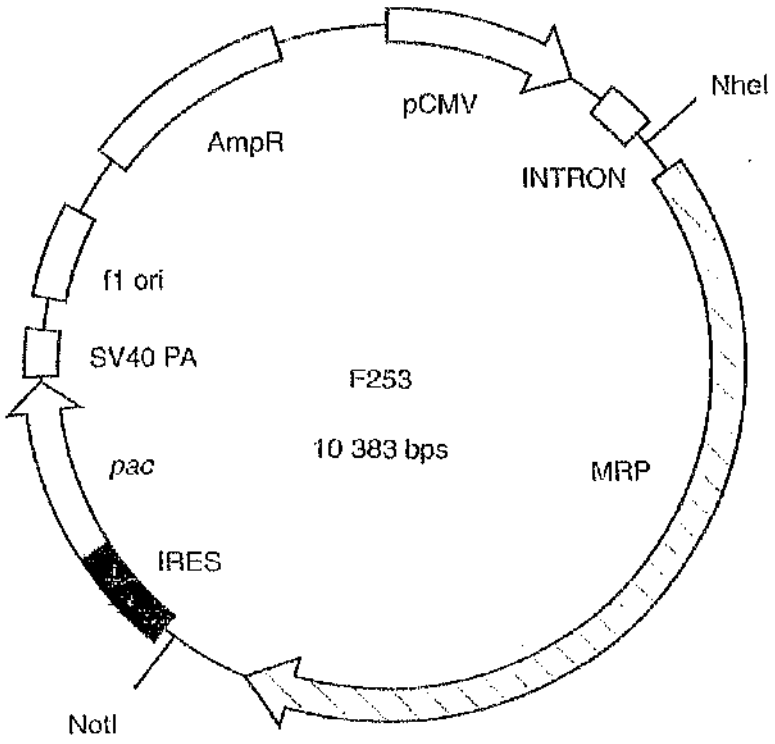


Figure 2-1: The puromycin-resistant bicistronic IRES vector containing the complete coding sequence for MRP1 (F250 vector).

2.10 Statistical analysis

2.10.1 Biological growth studies

Following the calculation of the mean relative tumour volume and standard error of the mean (SEM), it was blotted against time (days), and no further statistical analysis was carried out.

2.10.2 Flow cytometeric studies and proliferation

The mean percentage values and SEM of cells in sub G1, G1, S, and G2/M phases were calculated in control untreated tumours and CH1/TS tumours following cisplatin and paclitaxel treatment at 24, 48, and 72 hours. In addition, the mean percentage values and SEM of labeling index and mean relative movement values were calculated in control tumors, and in CH1/TS and CH1/TR tumors following capsulation and paclitaxel treatment at 24, 48, 72 hours. The Mann-Whitney test was used to detect significant statistical difference ($p < 0.05$) for each group of values compared to the control. The Mann-Whitney test, also called the rank sum test, is a nonparametric test compares two unpaired groups. It assumes that the data is representative of a population with each subject was selected independently, but the populations do not need to follow any particular distribution.

2.10.3 Apoptotic studies

The mean values and SEM of apoptotic indices were calculated for control tumours, and CH1/TS and CH1/TR tumours following paclitaxel and cisplatin treatment at 24, 48, and 72 hours. The Mann-Whitney test was used to detect significant statistical difference ($p < 0.05$) for each group of values compared to the control.

2.10.4 Apoptosis regulating proteins

Following the estimation of each protein level per tumour by densitometry, the levels were, then, expressed as ratio increase or decrease to control. The mean values and SEM of these ratios were calculated for each protein. The statistical differences between levels of protein expression were compared in CH1/TS and CH1/TR tumours at 24, 48, and 72 hours.

Chapter 3: Results

3.1 Biological growth studies

3.1.1 CH1 paclitaxel sensitive (CH1/TS) xenografts

The average growth rates of CH1/TS and CH1/TR tumours following paclitaxel (25mg/kg) and cisplatin (4mg/kg) treatment on days 0,4,and 8 are shown in figure 3-1. CH1/TS tumours demonstrated sensitivity to paclitaxel with complete disappearance of all the tumours 4 days post last dose of treatment. Three mice were still tumour free 52 days post treatment, in one mouse tumour began to grow slowly 24 days post treatment, and the fifth mice was culled at day 43 post treatment because of toxicity with no evidence of growing tumour. The doubling time of untreated control CH1/TS tumours was 2.9 days and a growth delay of paclitaxel treated CH1/TS tumours was more than 60.1 days. Similarly, cisplatin induced complete disappearance of CH1/TS tumors 4 days post last dose of treatment. Four mice were still tumour free 52 days post treatment, and in one mouse the tumour began to grow slowly 24 days post treatment. The growth delay for these tumours was, also, more than 60.1 days.

3.1.2 CH1 Paclitaxel resistant (CH1/TR) xenografts

The average growth rates of CH1/TR tumours following paclitaxel and cisplatin treatment are shown in figure 3-2. CH1/TR tumours displayed no response to paclitaxel with tumours reaching the end-point size and weight 16 days post last dose of treatment in all five mice. The doubling time of control untreated CH1/TR tumours were 3 days and the a growth delay of paclitaxel treated CH1/TR tumours were 8.7 days. Following cisplatin treatment there was partial response of CH1/TR tumours in all mice that was maintained on average for 21 days post treatment indicating partial resistance to cisplatin with a growth delay of 32.4 days.

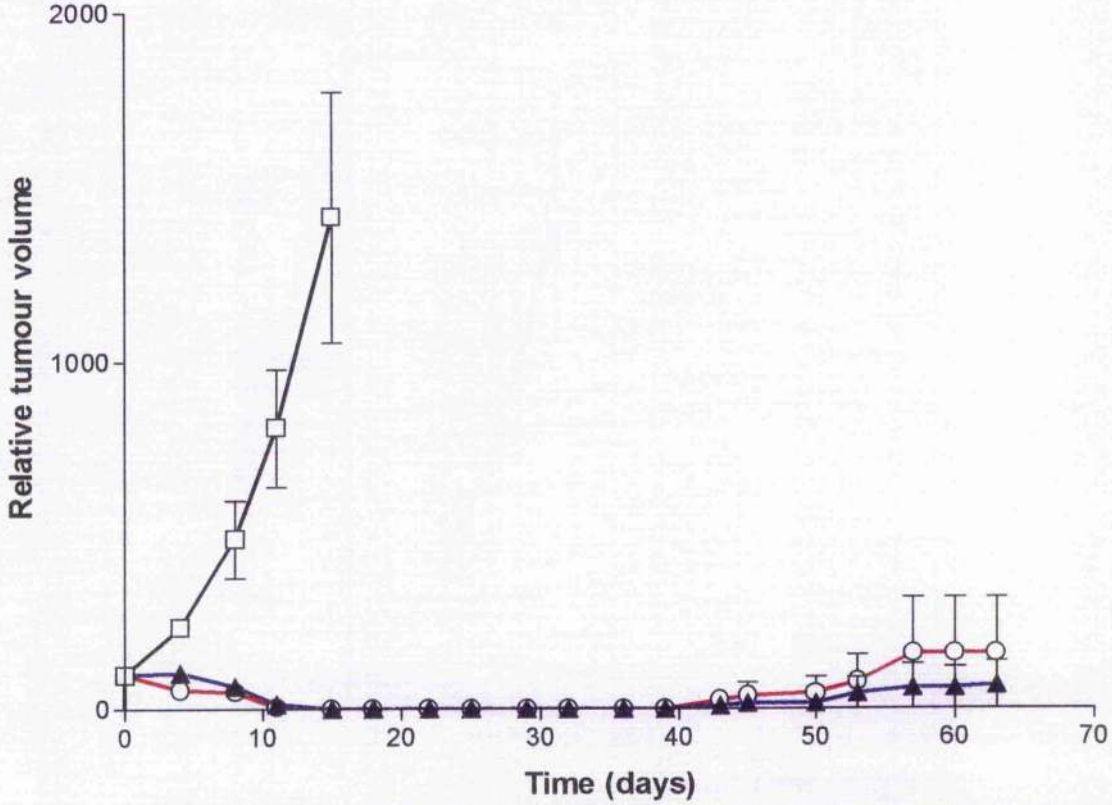


Figure 3-1:Averge growth curves of CH1/TS tumours following paclitaxel (25mg/kg) and cisplatin (4mg/kg) given on days 0,4, and 8.

—□— CH1/TS CONTROL —▲— CH1/TS PACLITAXEL

—○— CH1/TS CISPLATIN

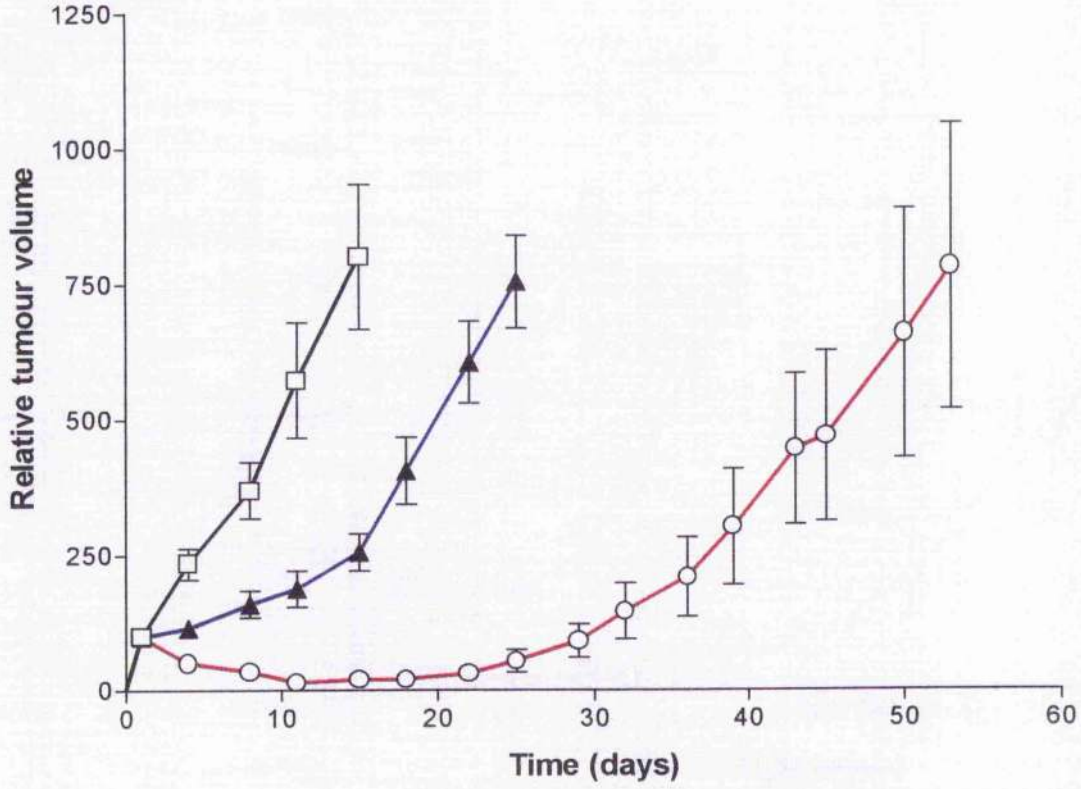


Figure 3-2: Average growth curves of CH1/TR tumours following paclitaxel (25 mg/kg) and cisplatin (4 mg/kg) on days 0,4,8.

—□— CH1/TR CONTROL —▲— CH1/TR/PACLITAXEL

—○— CH1/TR CISPLTIN

3.2 Paclitaxel uptake studies and pharmacokinetic analysis

The chormatographic analysis of samples showed that paclitaxel eluted with a mean retention time of 9.5 minutes. The mean paclitaxel concentrations and concentration versus time curves of CH1/TS, and CH1/TR tumours are shown in table 3-1 and figure 3-3 respectively. There was no significant difference in mean intra-tumoural paclitaxel concentration of CH1/TS, CH1/TR 1, 3, 6, or 24 hours post treatment. In particular, the difference of paclitaxel concentration noted at 1 hour post treatment with values of 0.22μg/ml, 0.067μg/ml, respectively, was not significant (p=0.4) .The mean paclitaxel concentrations 6 hours post treatment were almost equal and represented the mean peak values for both tumours.
The pharmacokinetic parameters measured are summarized in table 3-2.The intratumour paclitaxel C_{max} occurred at 6 hours post treatment with similar values of 3.18 and 3.46μg/ml in CH1/TS, CH1/TR tumours respectively. The calculated $AUC_{0\rightarrow24}$ value for CH1/TS was 100.8 hr x μg/ml, however, there was an increase of 8% in $AUC_{0\rightarrow24}$ values for CH1/TR as shown in table 3-2. The terminal $T_{1/2}$ values of CH1/TS, and CH1/TR were 15.2, and 28.5 respectively.

The above results suggest that there are no major differences in either intratumoural paclitaxel concentrations or pharmacokinetics to account for the resistant pattern observed in CH1/TR tumours as demonstrated in biological growth studies previously. Thus, these results confirm that there are no alterations in paclitaxel uptake and suggest that the observed resistant phenotype is due to more distal mechanisms.

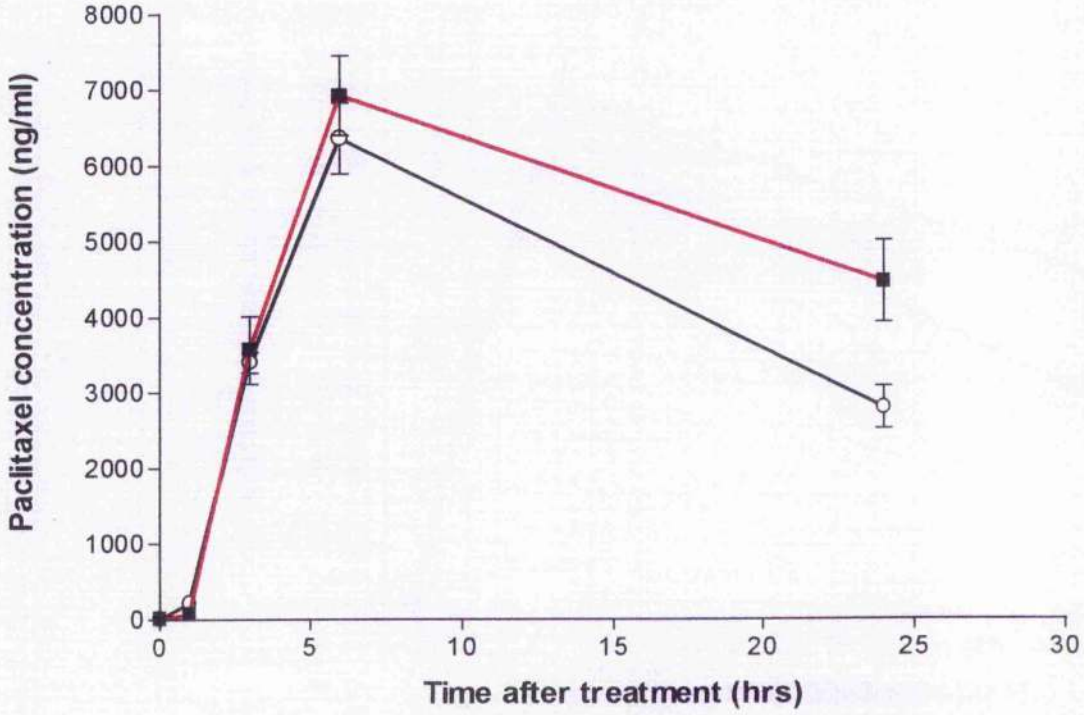


Figure 3-3: Intratumoural paxlitaxel concentration versus time of —○— CH1/TS and —■— CH1/TR following administration of 25 mg/kg of paclitaxel i.p.

Time (hrs)	CH1/TR (μg/ml)	CH1/TS (μg/ml)
1	0.067±0.021	0.22±0.09
3	3.56±0.45	3.42±0.13
6	6.93±0.52	6.36±0.47
24	4.47±0.54	2.8±0.28

Table 3-1: the intra-tumoural concentration of paclitaxel in CH1/TS, and CH1/TR tumours following treatment with 25 mg/kg of paclitaxel i.p.

Parameters	CH1/TR	CH1/TS
C_{max} (ng/ml)	3462	3181
AUClast (ng/ml x hr)	121997.8	100798
$T_{1/2}$ Lambda z (hr)	28.51	15.16
AUCINF (ng/ml x hr)	152931.07	80957.95

Table 3-2: the pharmacokinetics parameters measured in CH1/TS and CH1/TR tumours following treatment with 25 mg/kg of paclitaxel i.p.

3.3 Flow cytometry

3.3.1 CH1/paclitaxel sensitive

3.3.1.1 Post paclitaxel treatment

There were 18 athymic nude mice bearing CH1/TS tumours that were treated by single dose of paclitaxel 25mg/kg i.p. and sacrificed in groups of six mice at 24hr, 48hr, 72hr following treatment. There were six control untreated mice. Table 3-3 shows the mean percentage (%) values of cells in S phase and G2/M phase, labelling index (LI), and calculated relative movement through S phase (RM) of treated and controls xenografts. The characteristic feature of the DNA histograms (figure 3-4) is accumulation of cells in G2/M phase of cell cycle. This effect was apparent at 24 hours post treatment with mean percentage of cells in G2/M of 17.54 (p=0.11), but became more marked at 48 hrs and 72 hrs post treatment with mean % of cells in G2/M of 24.23% (p=0.0159) and 32.37% (p=0.0095) respectively. Interestingly, there was an increase in % of cells in S phase with mean % of cells of 40.2 (p=0.0317), 36.02 (p=0.0303), and 36.98% (p=0.0087) at 24, 48, and 72 hours respectively. These observations are consistent with previous in vitro studies.

The green (Brd Urd uptake by cells actively synthesizing DNA at time of injection) versus red (DNA content) histograms demonstrated that movement of cells through S phase is unhindered following paclitaxel treatment (figure 3-5). This is reflected in relative movement values (table 3-3) with control, 24h, 48h,and 72h values of 0.68, 0.70, 0.69, and 0.69 respectively. The percentage of tumour cells labelled with BrdU (LI) was largely unaltered at 24 hours and 48 hours post treatment with control, 24 hr, 48 hr mean % values of 26.9, 29.43 (p=0.4206), 24.15 (p=0.9048) (table 3-3). However, 72 hr post paclitaxel treatment there was inhibition of S phase proliferation with decreased in mean % of LI value to 19.9 (p=0.0303).

3.3.1.2 Post cisplatin treatment

Eighteen athymic nude mice bearing CH1/TS tumours were treated by single dose of cisplatin 4mg/kg i.p. and sacrificed in groups of six mice at 24hr, 48hr, 72hr post treatment. There were six control untreated mice. Table 3-4 shows the mean percentage values of cell in S phase and G2/M, labelling index, and relative movement through S phase following cisplatin treatment. The characteristic feature of DNA histograms (figure 3-6) is accumulation of cells in S phase. This effect was apparent at 24 hours (p=0.03) post-cisplatin treatment and became more marked at 48 and 72 hours with mean percentage of cells in S phase 54.37% (p=0.0173) and 51.98% (p=0.0087) respectively. There was a statistically significant increase in mean percentage of cells in G2/M (18.22%) at 72 hours post cisplatin treatment. These observations were previously demonstrated in ovarian cancer cell lines including CH1 and other cell lines following cisplatin treatment (Ormerod MG, 1996).

The green versus red histogram (figure 3-7) demonstrated slowing down in the movement of cells through S phase with little corresponding hindrance at G1-S phase transition. The control untreated tumours incorporated sufficient amount Brd Urd and showed steady cell cycle distribution pattern. Three out of six tumours 24 hours post treatment and five out of six tumours 48 hours post treatment had no or poor incorporation of Brd Urd. This part of the experiment was repeated twice to exclude any methodological errors, however the same results were obtained. The S phase proliferation is markedly inhibited in the tumours that incorporated Brd Urd with mean percentage values of LI of 14.8% and 5.8% for three tumours at 24 hours and one tumour at 48 hours post treatment respectively (table 3-4). In addition the RM values of these tumours reflected the marked retardation of movement through S phase with mean values of 0.41 and 0.47 at 24 hrs and 48 hrs post treatment respectively. These observations suggest that the lack of Brd Urd uptake in the other tumours may be due to complete inhibition of DNA synthesis. In contrast at 72 hours post cisplatin treatment there was sufficient incorporation of BrdUrd with an increase in LI (46.34%) suggesting an escape from temporary effect of cisplatin on DNA synthesis. However, the RM through S phase at 72 hours post cisplatin treatment was still retarded with value of 0.58 (p=0.002).

3.3.2 CH1/TR paclitaxel resistant xenografts

The DNA histogram of most of these xenografts showed multiple G1 peaks suggesting the presence of multiple subclones within the tumours. Because of that specific values of G1, S phase, and G2/M values were difficult to obtain. However, in descriptive terms the DNA histograms and red versus green histogram reflected the response of these tumours to paclitaxel and cisplatin. In addition, an estimate of percentage of G1 and G2/M cell cycle phases were obtained by generating region on the DNA histograms using the WinMIDI program. This enabled the calculation of G2 to G1 ratio was to obtain an estimate of the changes in G2/M phase of cell cycle in CH1/TR tumours following paclitaxel treatment.

3.3.2.1 Post-paclitaxel treatment

The same number of mice and design was used as in section 3.3.1.1. The LI, RM, G2/G1 values of CH1/TR tumours following paclitaxel treatment are summarized in table 3-5. The DNA histograms (figure 3-8) demonstrated no cell cycle alterations in these tumours following paclitaxel treatment, in particular there was no G2/Mblock. The G2/G1 ratio of the CH1/TR control xenografts and at 24, 48, and 72 hours post paclitaxel treatment were 0.3, 0.35, 0.37, and 0.35 respectively. In contrast the G2/G1 ratio of the CH1/TS controls and at 24, 48, 72 hours post paclitaxel treatment were 0.3, 0.39, 0.56, 1.19 respectively. Moreover, the green versus red histograms (figure 3-9) shows no change in the LI and RM between untreated control and treated tumours as shown in table 3-5. These observations confirm the resistance of these tumours to paclitaxel.

3.3.2.2 Post-cisplatin treatment

The same number of mice and design was used as in section 3.3.1.2. The LI, RM values of CH1/TR tumours following cisplatin are summarized in table 3-6. The DNA histograms of CH1/TR treated with cisplatin displayed the S phase slow down previously demonstrated in CH1/TS tumours (figure 3-10). Similarly, the green versus red histogram demonstrate the pile up of cells in early S phase with RM values of control and treated tumours at 24h, 48h, and

72h of 0.69, 0.42(p=0.0022), 0.52 (p=0.0043), and 0.68 (p=0.93) respectively (figure 3-11). In contrast to CH1/TS tumours there was no inhibition of proliferation with mean percentage LI of control, and treated tumours at 24, 48, and 72h values of 30.55,40.68, 49.48, and 30.88. These observations are consistent with partial resistance of CH1/TR xenografts demonstrated previously in the biological growth studies.

	Control	24h	48h	72h
G2 %	11.88 ±1.03	17.54 ±2.61	24.23±3.51*	32.37 ±5.8**
S phase (%)	27.4 ±0.92	40.2 ±3.62*	36.02±2.42*	36.98±2.25*
LI (%)	26.09 ±1.72	29.43 ±4.55	24.15 ±3.05	19.19±2.48*
RM	0.68 ±0.007	0.71 ±0.021	0.70 ±0.01	0.69 ±0.017

Table 3-3: the mean percentage values and standard error of the mean (SEM) of cell cycle G2/M and S phases, labelling index (LI), and mean values of relative movement through S phase (RM) of CH1/TS tumours following treatment with paclitaxel. (*)=P <0.05, (**)= P <0.01, (***)= P <0.0001.

	Control	24h	48h	72h
G2 %	11.88 ±1.03	11.3 ±0.72	7.83 ±0.98	18.22±2.14*
S phase %	27.4 ±0.92	40.7 ±2.89*	54.37±4.08**	51.98±4.66**
LI %	26.09 ±1.72	14.28±6.45*	5.18 ±5.18^{+}	46.34 ±2.45
RM	0.68 ±0.007	0.41 ±0.012	0.47±0.47^{+}	0.58 ±0.013**

Table 3-4: The mean percentage values and standard error of the mean (SEM) of cell G2/M and S phases, labelling index (LI), and mean values of relative movement through S phase (RM) of CH1/TS tumours following treatment with cisplatin. (*)=P <0.05, (**)= P <0.01, (***)= P <0.0001. (+) Represent value from single tumour (see text).

	Control	24 hr	48 hr	72 hr
LI %	30.55 ±0.69	32.02 ±2.07	32.7 ±1.9	29.26 ±1.27
RM %	0.69 ±0.04	0.63 ±0.25	0.65 ±0.015	0.67 ±0.007
G2/G1	0.3	0.35	0.37	0.35

Table 3-5: the mean percentage values and standard error of the mean (SEM) labelling index (LI), relative movement through S phase (RM) of CH1/TR tumours following treatment with paclitaxel, and the mean values of G2/G1 ratios. (*)=P <0.05, (**)= P <0.01, (***)= P <0.0001.

	Control	24 hr.	48 hr.	72 hr.
LI %	30.55 ±0.69	40.68 ±3.68	49.48 ±5.22	30.88 ±2.53
RM %	0.69 ±0.04	0.42 ±0.027**	0.52 ±0.028**	0.68 ±0.04

Table 3-6: the mean percentage values and standard error of the mean (SEM) labelling index (LI), and relative movement through S phase (RM) of CH1/TR tumours following treatment with cisplatin. (*)=P <0.05, (**)= P <0.01, (***)= P <0.0001.

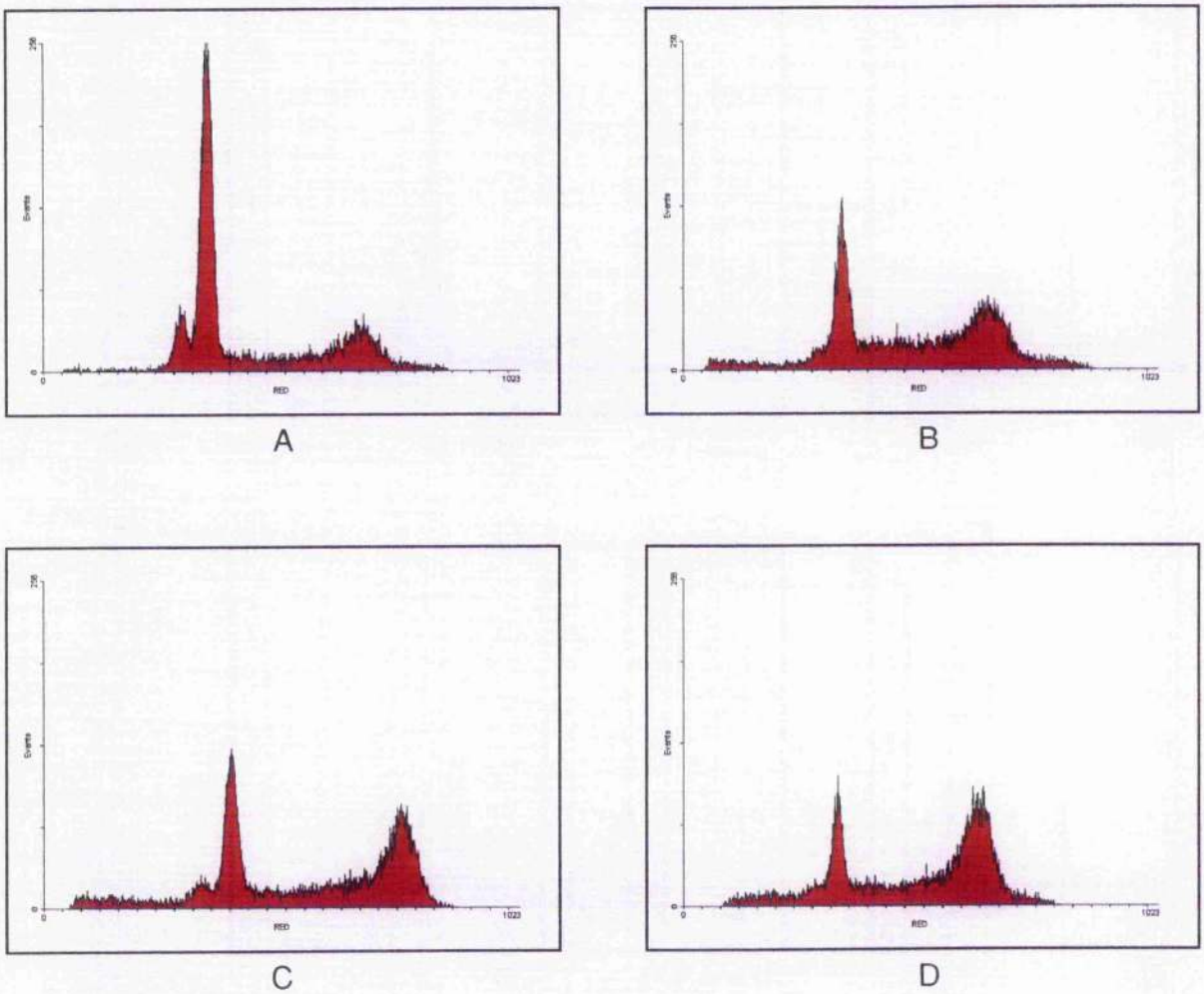


Figure 3-4: DNA histograms of CH1/TS tumours following paclitaxel treatment showing accumulation of cells in G2/M phase of cell cycle. (A) Control, (B) 24 hrs post-treatment, (C) 48 hrs post-treatment, (D) 72 hours post treatment.

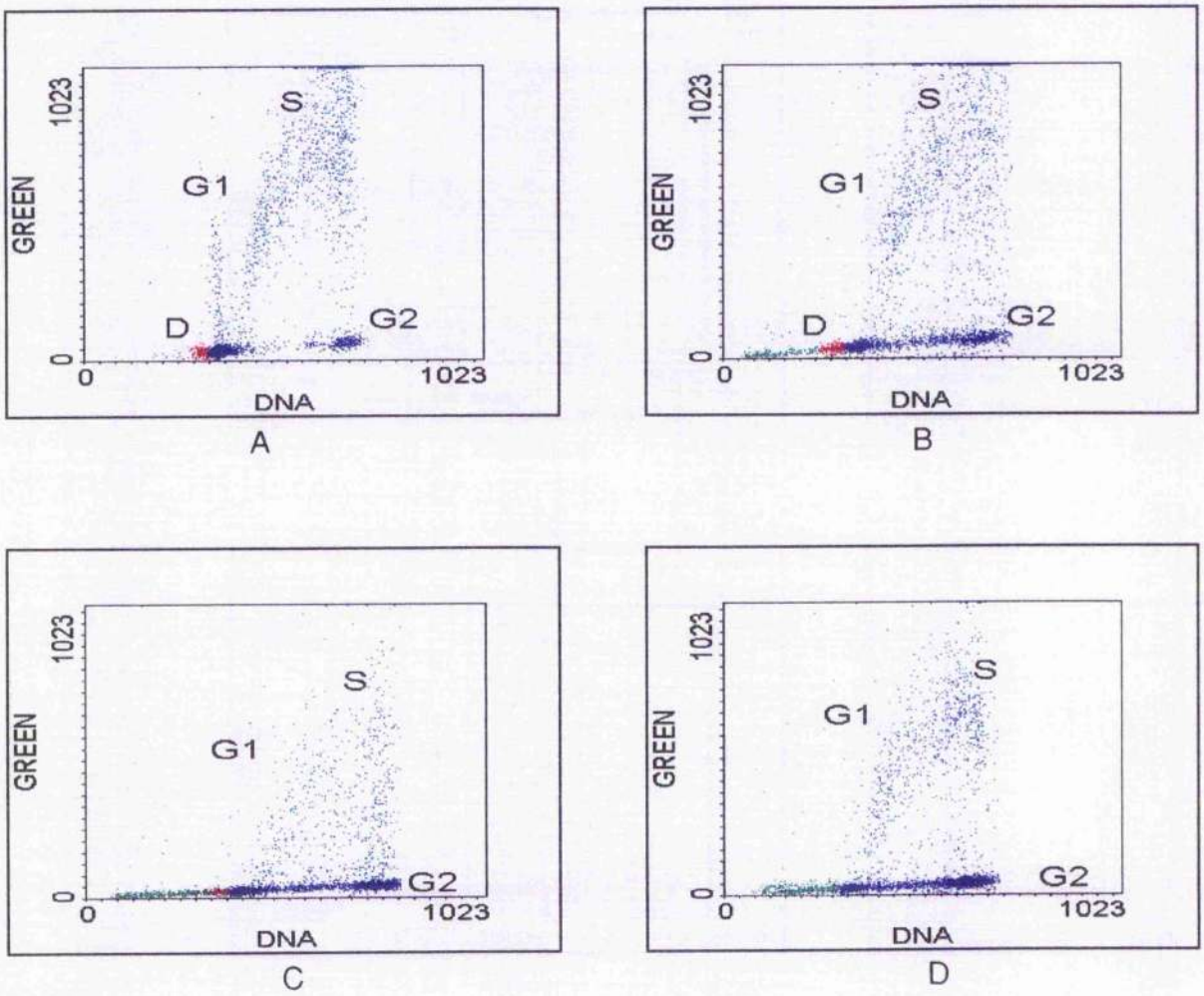


Figure 3-5: Red (DNA) vs. green (Brd Urd uptake) histograms of CH1/TS tumours following paclitaxel treatment showing unhindred movement of through S phase, -D-on the histograms refer to cells that have undergone division. (A) Control, (B) 24-hrs post-treatment, (C) 48 hrs post-treatment, (D) 72 hours post treatment.

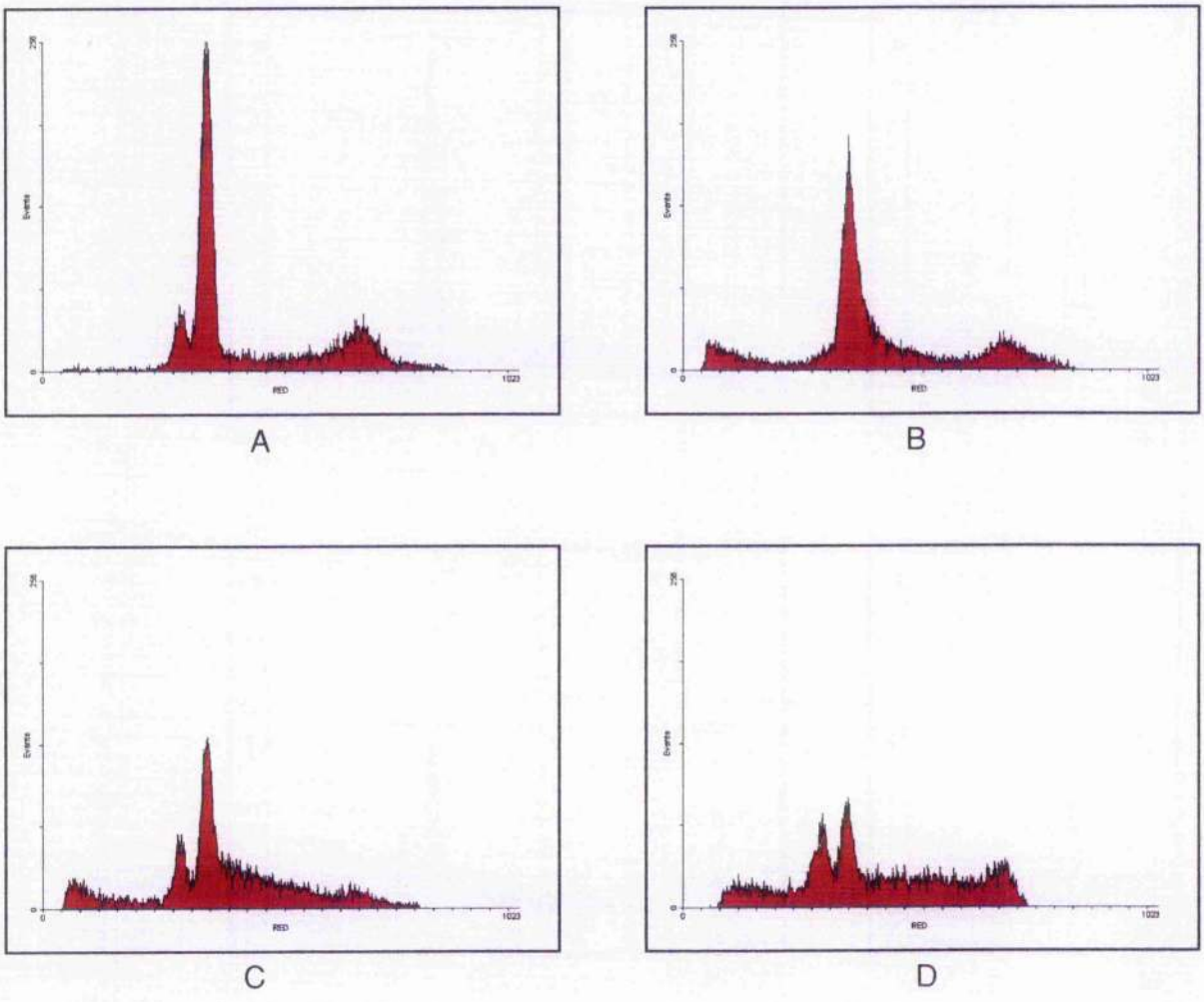


Figure 3-6: DNA histograms of CH1/TS tumours following cisplatin treatment showing accumulation of cells in S phase. (A) control, (B) 24 hrs post-treatment, (C) 48 hrs post-treatment, (D) 72 hours post treatment.

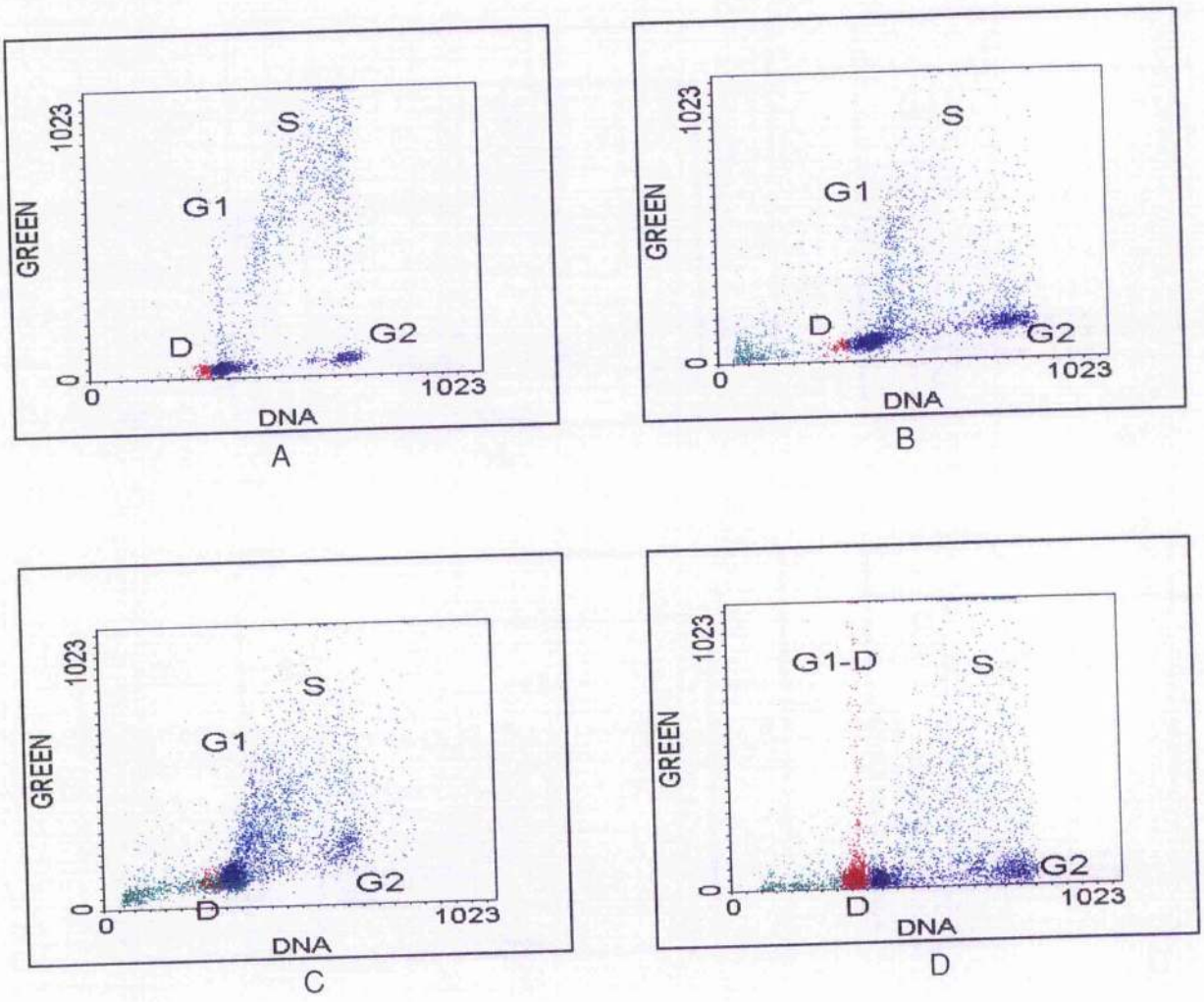


Figure 3-7: Red (DNA) vs. green (Brd Urd) histograms of CH1/TS tumours following cisplatin treatment showing pile up cells in early S phase. G1-D refers to cells that took up Brd Urd and divided, then entered G1 phase of cell cycle. (A) Control, (B) 24 hrs post-treatment, (C) 48 hrs post-treatment, (D) 72 hours post treatment.

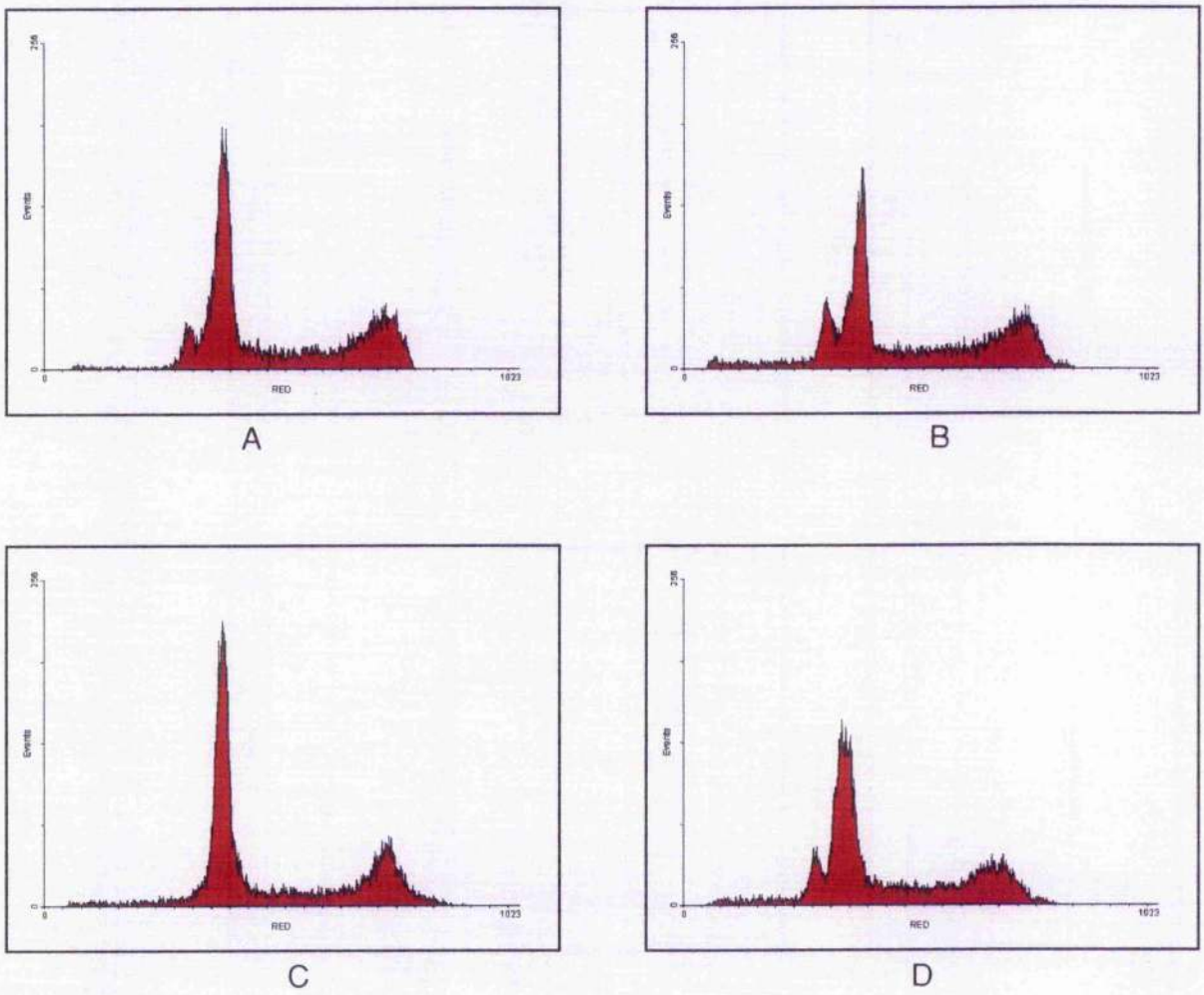


Figure 3-8: DNA histograms of CH1/TR tumours following paclitaxel treatment showing no alterations in the cell cycle. (A) Control, (B) 24 hrs post-treatment, (C) 48 hrs post-treatment, (D) 72 hours post treatment.

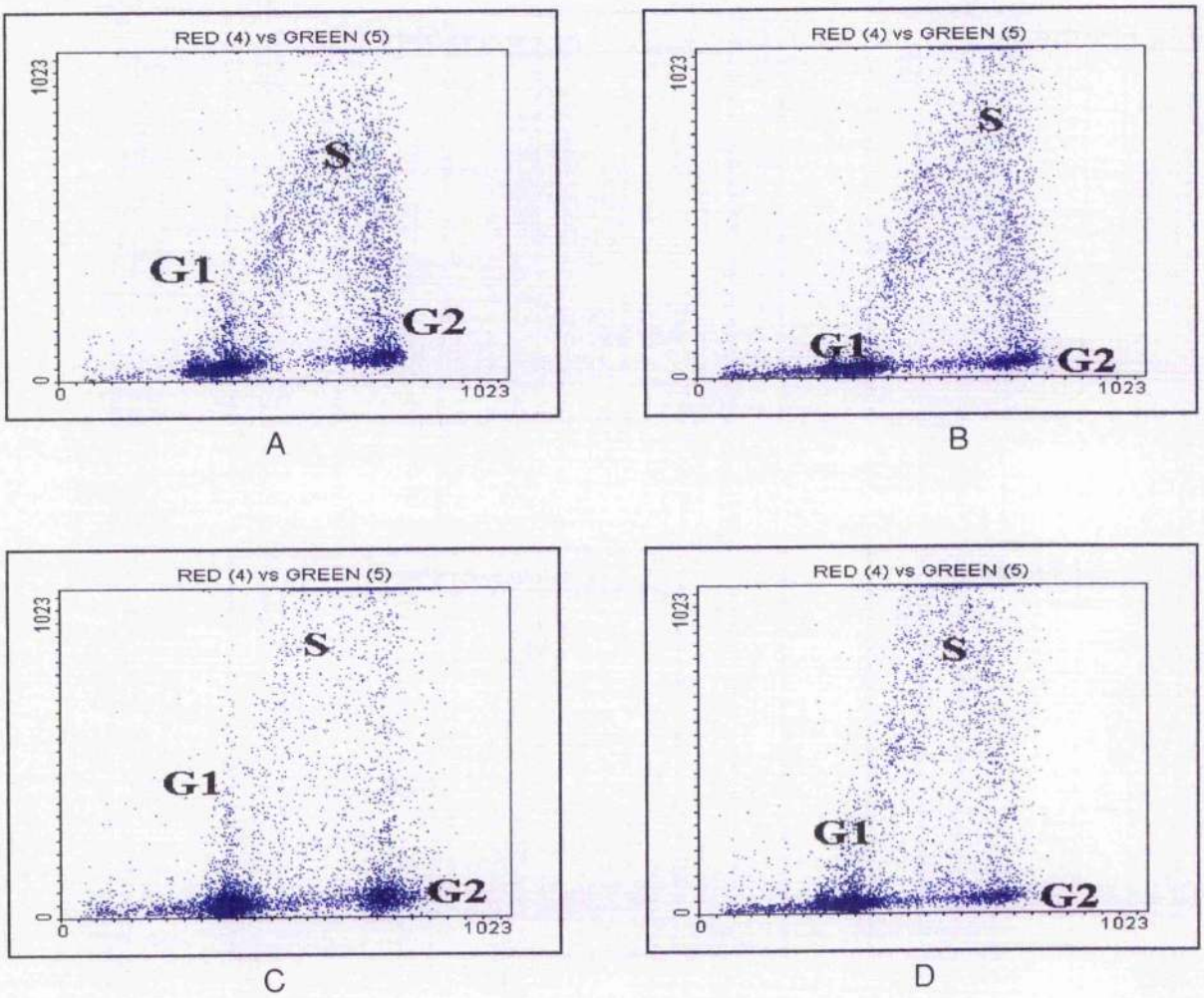


Figure 3-9: Red (DNA) vs. green (Brd Urd) histograms of CH1/TR tumours following paclitaxel treatment showing no alteration in cell cycle. (A) control, (B) 24 hrs post-treatment, (C) 48 hrs post-treatment, (D) 72 hours post treatment.

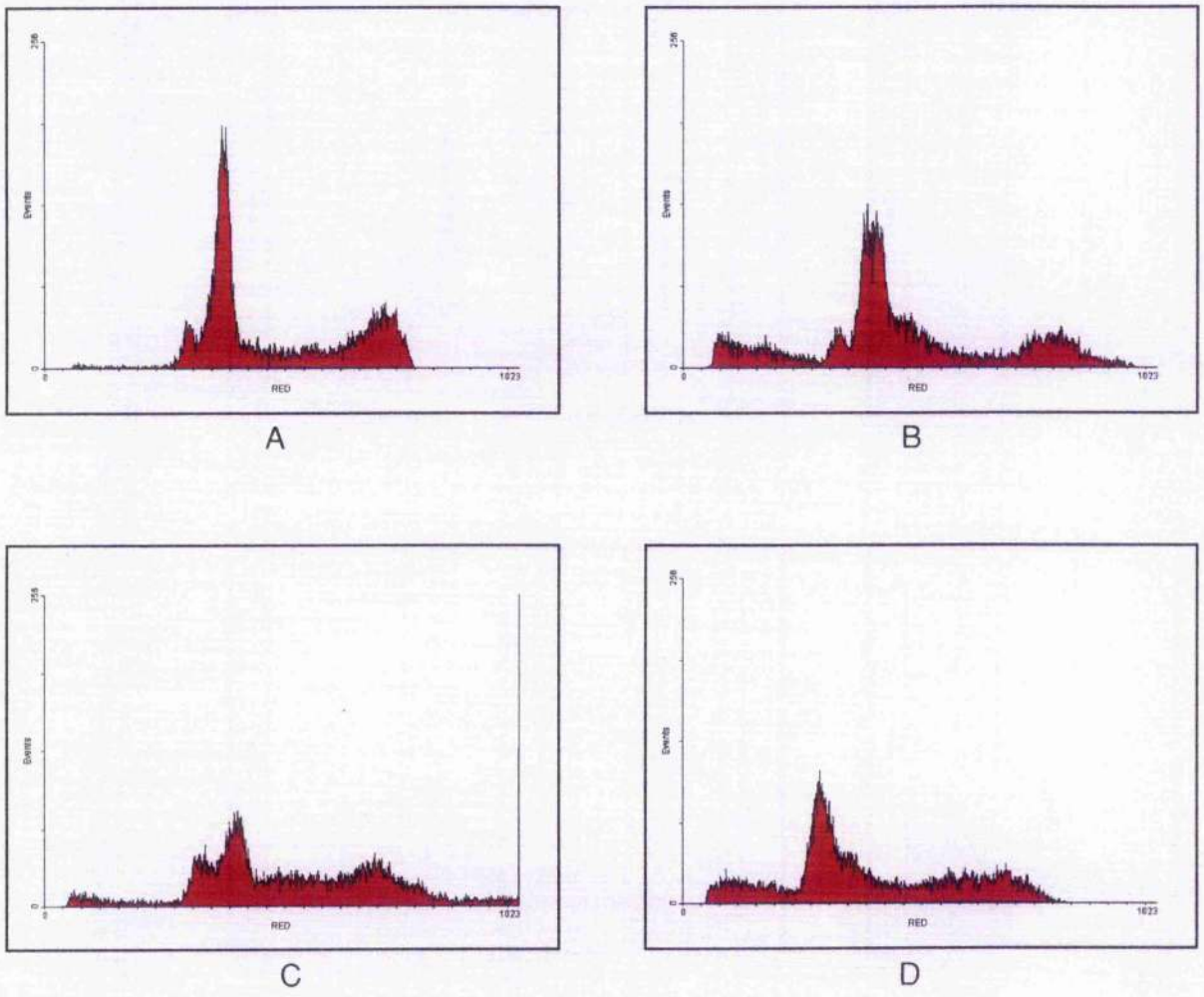


Figure 3-10: DNA histograms of CH1/TR tumours following cisplatin treatment showing accumulation of cells in early S phase. (A) Control, (B) 24 hrs post-treatment, (C) 48 hrs post-treatment, (D) 72 hours post treatment.

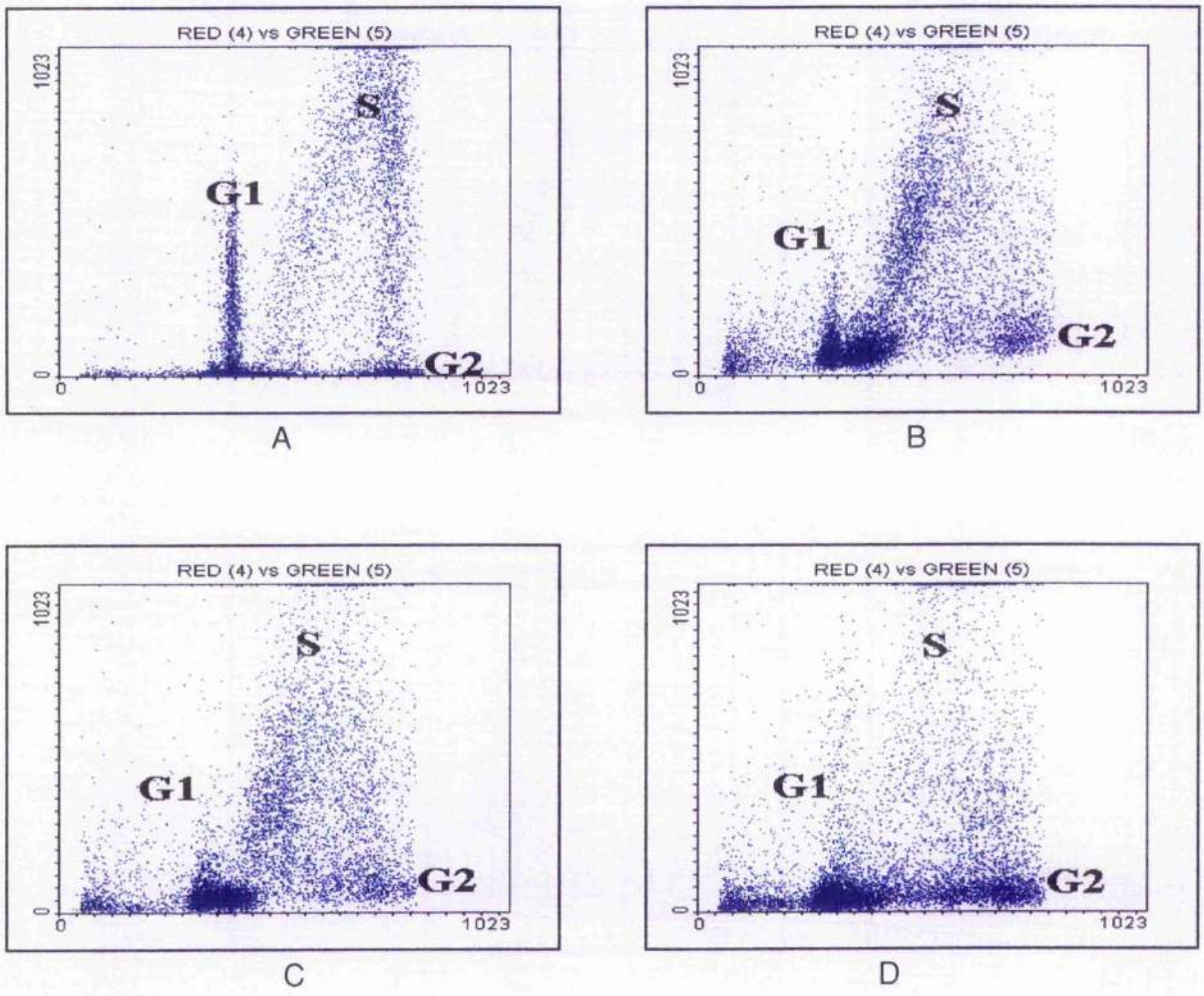


Figure 3-11: Red (DNA) vs. green (Brd Urd) histograms of CH1/TR tumours following cisplatin treatment showing pile up of cells in early S phase. (A) control, (B) 24 hrs post-treatment, (C) 48 hrs post-treatment, (D) 72 hours post treatment.

3.4 Apoptosis

The main method for detection of apoptosis in this study was labelling of paraffin embedded sections by TUNEL assay. There were no discrete sub-G1 peaks that are frequently demonstrated in vitro and correlated with DNA degradation. However, the careful examination of DNA histograms showed that there were sub-G1 debris that exhibited a pattern in both treated and untreated sensitive and resistant tumours. These sub-G1 debris were estimated from the DNA histograms using the WinMIDI program and correlated with apoptotic index (AI) obtained by TUNEL assay. Despite the usefulness estimating of sub-G1 debris as supporting evidence for apoptosis, it remains a crude method for detection of apoptosis because it may consist of apoptotic and necrotic cells. Thus the results here are interpreted with caution and in correlation with results from the TUNEL assay.

3.4.1 Detection of apoptosis by TUNEL assay

3.4.1.1 CH1/TS Tumours

The mean percentage apoptotic indexes of CH1/TS tumours are summarized in table 3-7. Figures 3-12, 3-13, and 3-14 provide representative CH1/TS tumour slides labelled by TUNEL assay of control tumours, and paclitaxel and cisplatin treated tumours respectively. The sensitivity of CH1/TS tumours to cisplatin and paclitaxel is demonstrated by a statistically significant increase in AI following treatment. Cisplatin induced an increase in AI with mean percentage values for control, and treated tumours at 24, 48, and 72 hours of 0.83, 13.7 ($p=0.002$), 20.4($p=0.002$), 19.4($p=0.002$) respectively. Similarly, there was an increase in mean percentage AI following paclitaxel treatment with values at 24, 48, and 72 hours of 8.6 ($p=0.002$), 9.3 ($p=0.002$), 6.5($p=0.002$) respectively. The difference in increase of AI between cisplatin and paclitaxel could be explained in part by presence of large number of cells in mitotic arrest that may subsequently undergo apoptosis as shown in figure 3-13. The mean percentages of cells in mitotic arrest for control tumours and at 24, 48, and 72 hours following paclitaxel treatment were 1, 9.6, 6.4, and 4.2 respectively.

3.4.1.2 CH1/TR tumours

The mean apoptotic indexes of CH1/TR tumours are summarized in table 3-7. Figures 3-15, 3-16, and 3-17 provide representative CH1/TR tumour slides labelled by TUNEL assay of control tumours and paclitaxel and cisplatin treated tumours respectively. Following paclitaxel treatment of CH1/TR tumours, there was statistically non-significant increase in AI with mean % values of control, and treated tumours at 24, 48, 72 hours of 0.99, 1.79 (p=0.064), 1.91(p=0.093), and 1.93(p=0.093) respectively. Furthermore, cells displaying mitotic arrest were seen very occasionally in small number of slides (less than 1%). These observations confirm the resistance of these tumours to paclitaxel and the lack of induction of apoptosis. On the other hand, cisplatin induced statistically significant increase in apoptosis in CH1/TR tumours with mean percentage of AI at 24, 48, and 72 hours of 10.4(p=0.002), 7.9(p=0.002), and 7.4(p=0.002). In comparison to CH1/TS tumours, cisplatin induced less apoptosis in CH1/TR tumours consistent with inherent partial resistance to cisplatin that has been previously demonstrated in the biological growth studies.

3.4.2 Measurement of sub-G1 debris

The results of sub-G1 debris measurement are summarized in table 3-8. There was a small sub-G1 debris peak for CH1/TS and CH1/TR controls with mean values of 3.27 and 2.3 respectively. There was marked statistically significant increase in sub-G1 debris in CH1/TS following paclitaxel and cisplatin treatment. The mean % of sub-G1 debris following cisplatin treatment was 13.6, 29.42, and 15.12 (p=0.002) at 24, 48, and 72 hours respectively. The mean % of sub-G1 debris following paclitaxel treatment was 8.45, 17.62, and 16.42 (p=0.002) at 24, 48, and 72 hours respectively. Interestingly, The rise in sub-G1 values was more marked following cisplatin treatment and in keeping with apototic indexes measured by TUNEL assay.

There was less marked increase in sub-G1 debri in CH1/TR tumours following cisplatin and paclitaxel treatment. The mean % of sub-G1 debris values following paclitaxel treatment of CH1/TR tumours were 6.83, 6.25, and 7.01 (p=0.002). Despite the statistical significance of

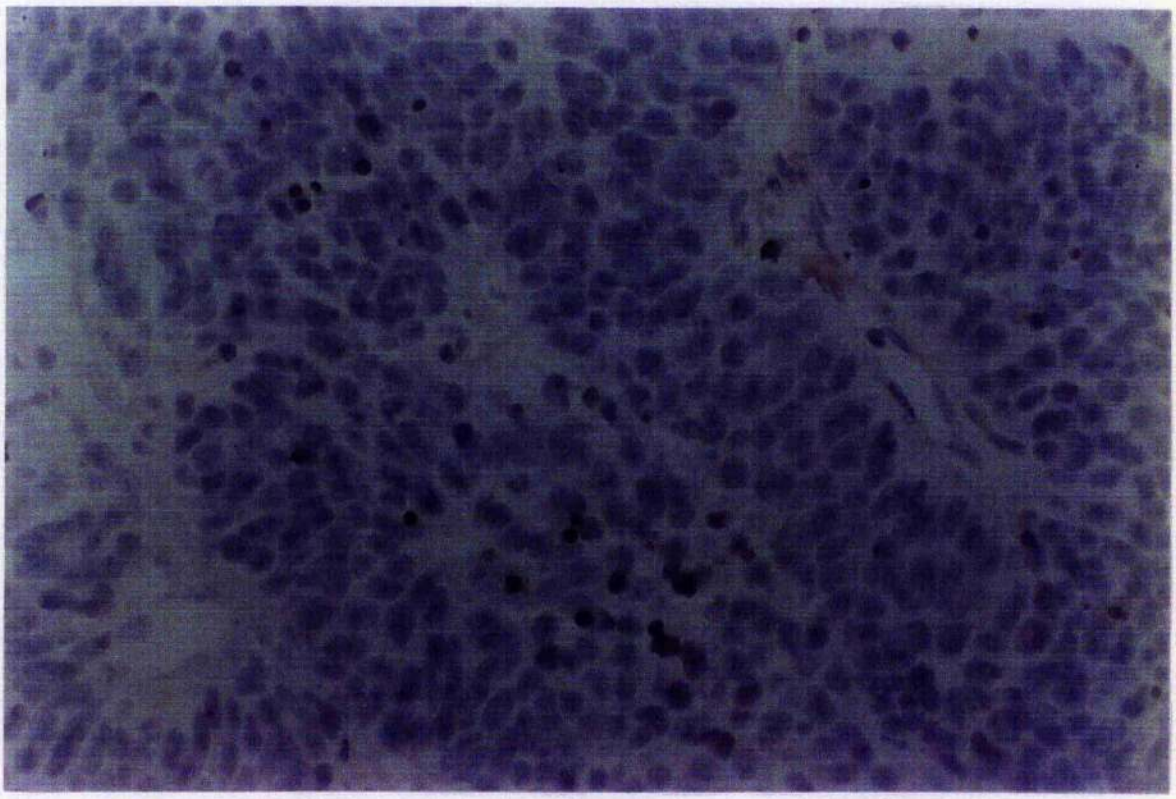
these values, the increase in sub-G1 debris in CH1/TR tumours following paclitaxel treatment is much less than the values obtained following treatment of CH1/TS tumours with paclitaxel. These observations are in keeping with results of A.I estimation by TUNEL assay. There was a statistically significant increase in mean % of sub-G1 with values of 12.5, 9.45, and 9.28 at 24, 48, 72 hours post treatment respectively. Similarly this increase in sub-G1 debris in CH1/TR tumours is less than values obtained following the cisplatin treatment of CH1/TS tumours in keeping with biological growth studies and estimation of A.I results that suggest a degree of partial resistance

	Control	24 hr	48 hr	72 hr
CH1/TS/Tx	0.83 ±0.15	8.6 ±1.08**	9.3 ±0.98**	6.5 ±1.31**
CH1/TR/Tx	0.99 ±0.16	1.79 ±0.77	1.9 12 ±0.39	1.93 ±0.28
CH1/TS/Cis	0.83 ±0.15	13.17 ±0.58**	20.4 ±3.21**	19.4 ±4.54**
CH1/TR/Cis	0.99 ±0.16	10.4 ±1.1**	7.9 ±0.81**	7.4 ±0.84**

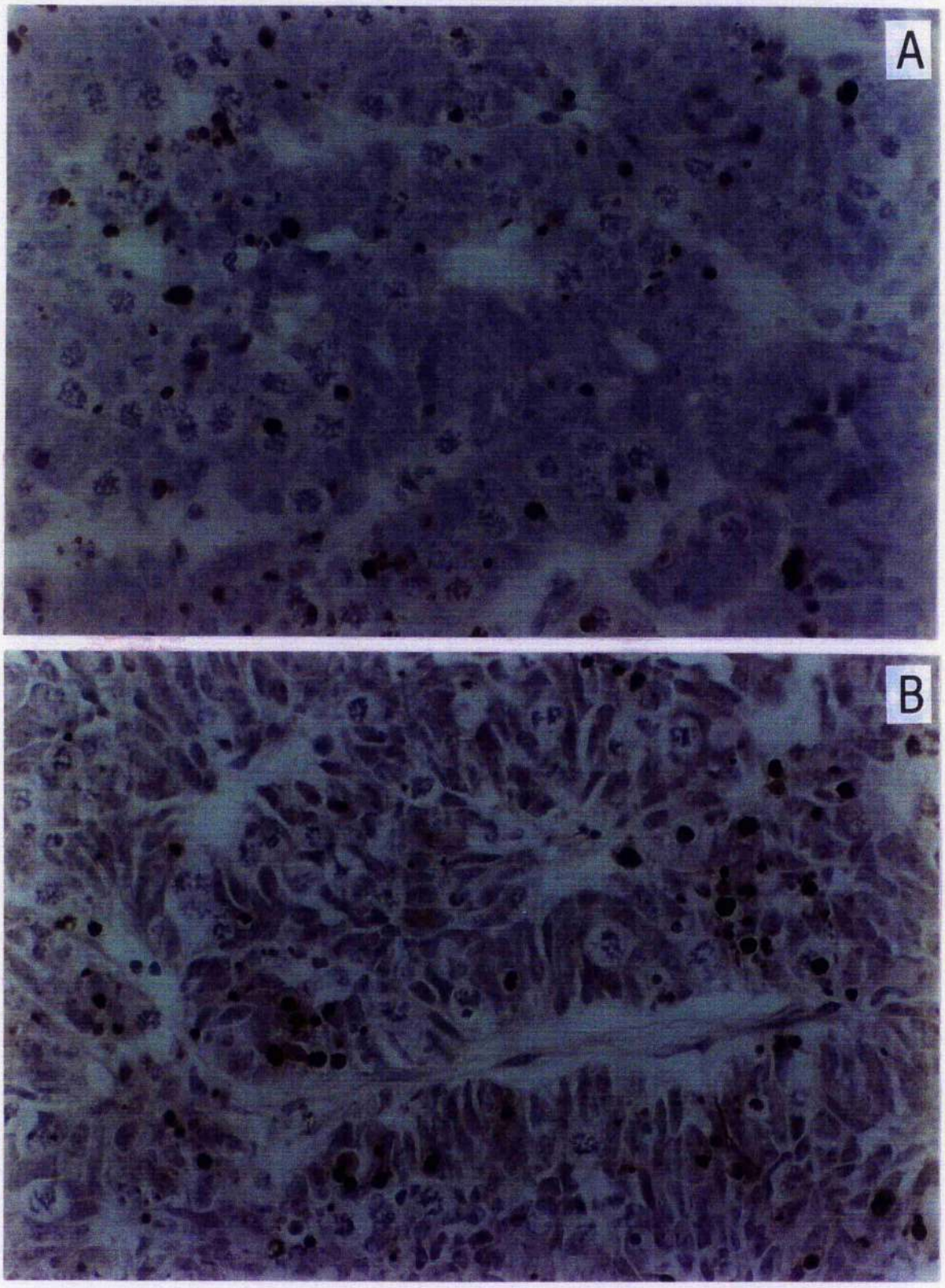
Table 3-7: Mean % of apoptotic index and standard error of mean (SEM) of CH1/TS and CH1/TR tumours treated with both paclitaxel (Tx) and cisplatin (Cis). (*)=P <0.05, (**)= P <0.01, (***)= P <0.0001.

	Control	24 hr	48 hr	72 hr
CH1/TS/Tx	3.27 ±0.85	8.45 ±0.54**	17.62 ±3.37**	16.42 ±5.28**
CH1/TR/Tx	2.3 ±0.56	6.83 ±1.014**	6.25 ±0.58**	7.017 ±0.94**
CH1/TS/Cis	3.27 ±0.85	13.6 ±1.7**	29.42 ±6.33**	15.12 ±5.73**
CH1/TR/Cis	2.3 ±0.56	12.5 ±2.54**	9.45 ±1.27**	9.28 ±1.93**

Table 3-8: Mean % of sub-G1 values and SEM of CH1/TS and CH1/TR tumours treated with both paclitaxel (Tx) and cisplatin (Cis). (*)=P <0.05, (**)= P <0.01, (***)= P <0.0001.



3-12: Representative photomicrograph (x40) of control (untreated) CH1/TS tumour showing few darkly stained apoptotic bodies.



3-13: Representative photomicrographs (x40) of CH1/TS tumours treated with paclitxel showing darkly stained apoptotic bodies and numerous cells in mitotic arrest. (A) 24 hrs following treatment, (B) 48 hrs following treatment, (C) 72 hrs following treatment (overleaf).

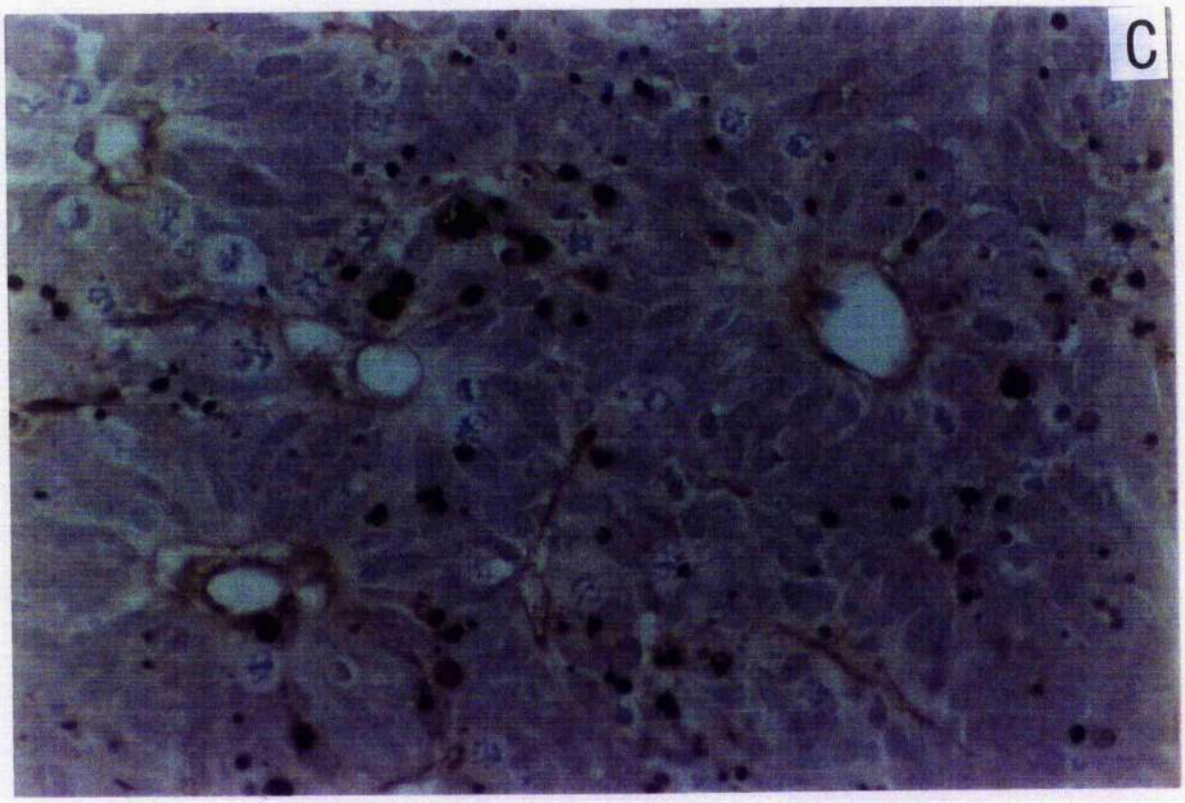
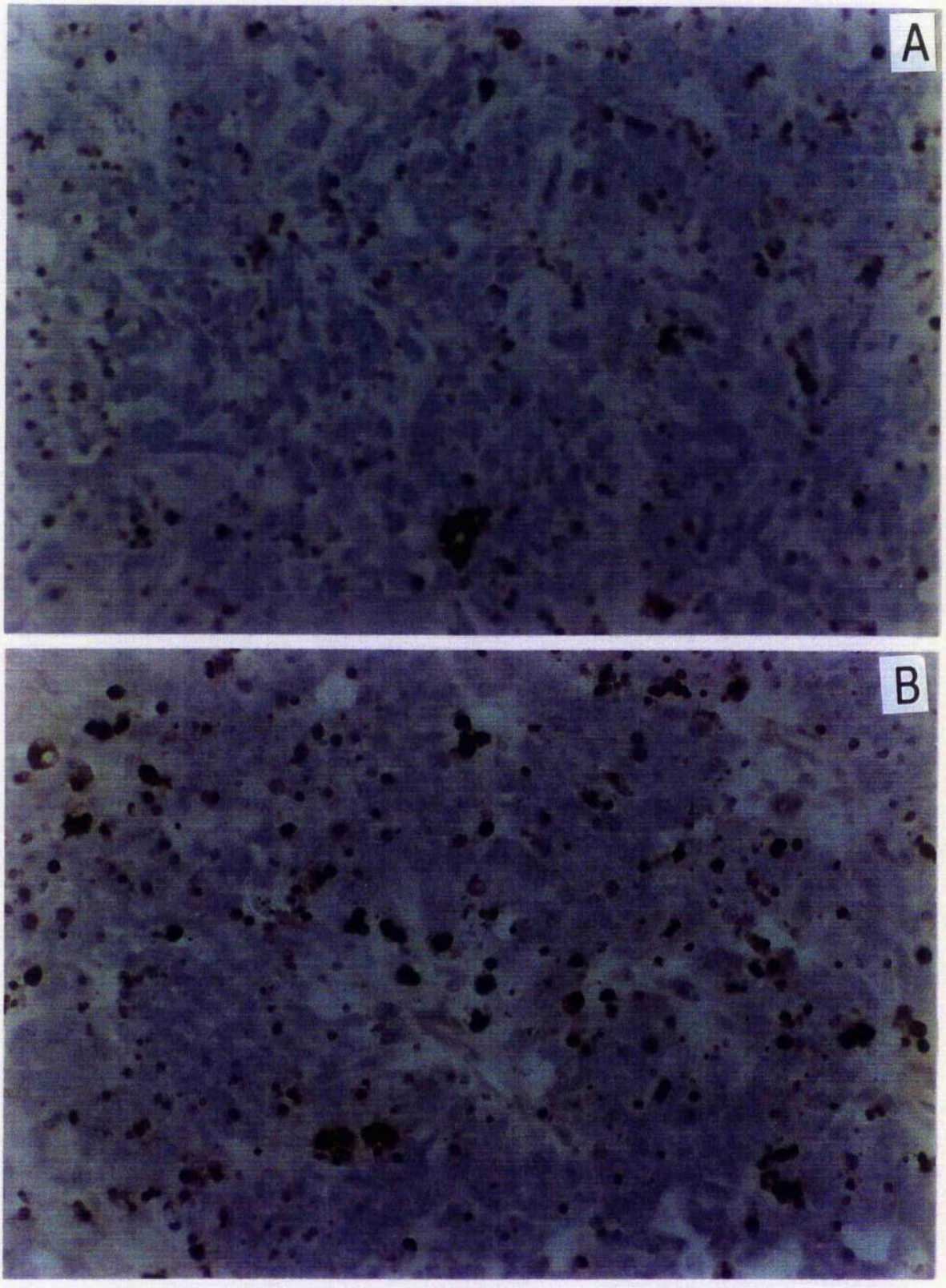


Figure 3-13(cont.) - (C) CH1/TS tumour 72 hrs following paclitaxel treatment.



3-14: Representative of photomicrographs (x40) of CH1/TS tumours treated with cisplatin showing numerous darkly stained apoptotic bodies. (A) 24 hrs following treatment, (B) 48hrs following treatment, (C) 72 hrs following treatment (overleaf).

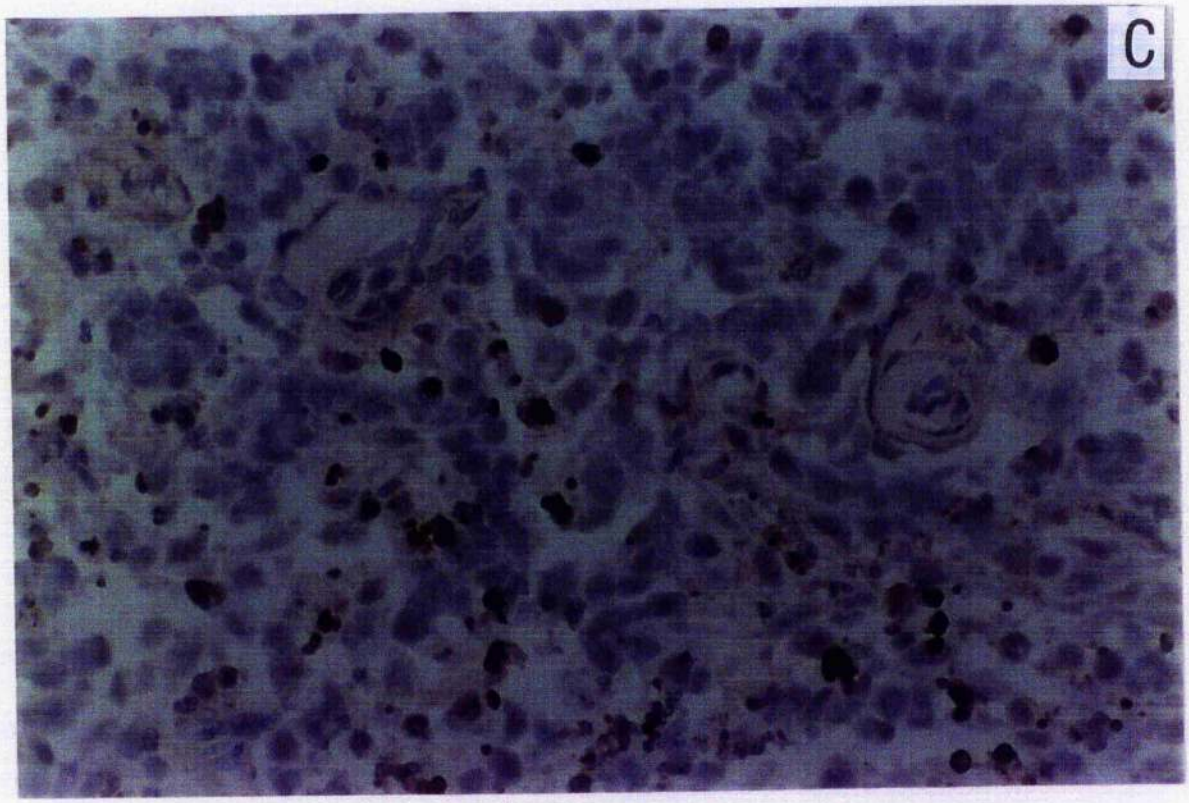
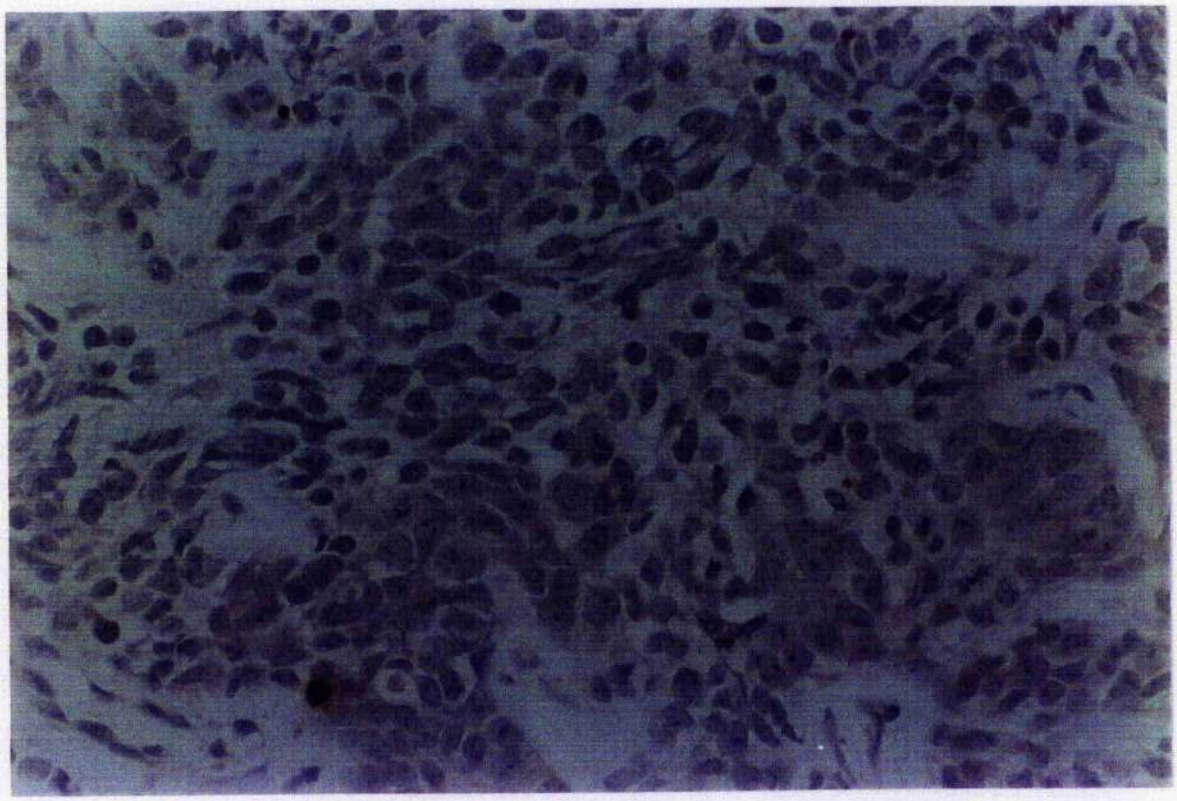
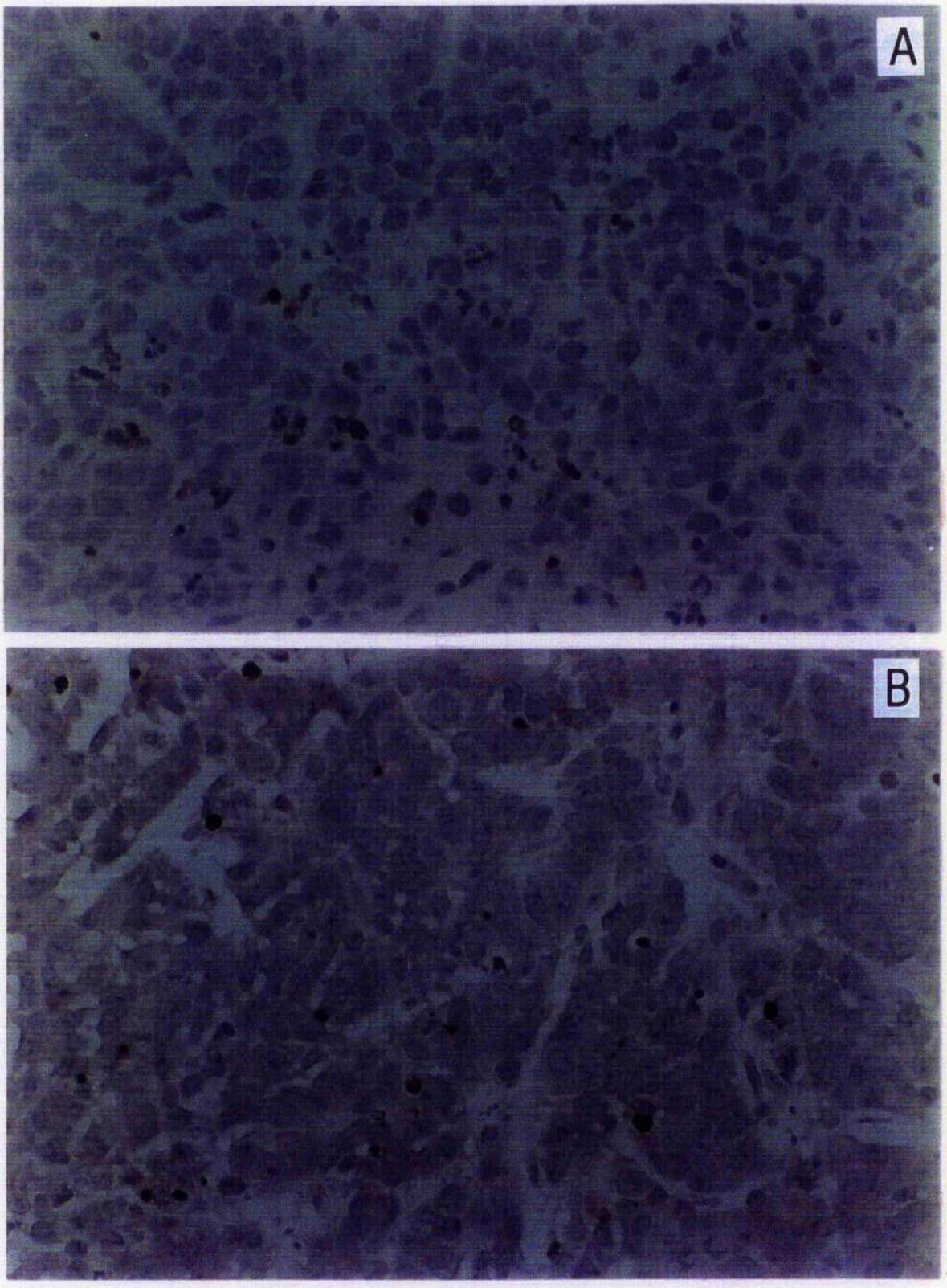


Figure 3-14 (cont.) - (C) CH1/TS tumours 72 hrs following cisplatin treatment.



3-15: Representative of photomicrographs (x40) of control (untreated) CH1/TR tumour showing f two darkly stained apoptotic bodies.



3-16: Representative photomicrographs (x40) of CH1/TR tumours treated with paclitaxel showing few darkly stained apoptotic bodies.(A) 24 hrs following treatment, (B) 48 hrs following treatment, (C) 72 hrs following treatment (overleaf).

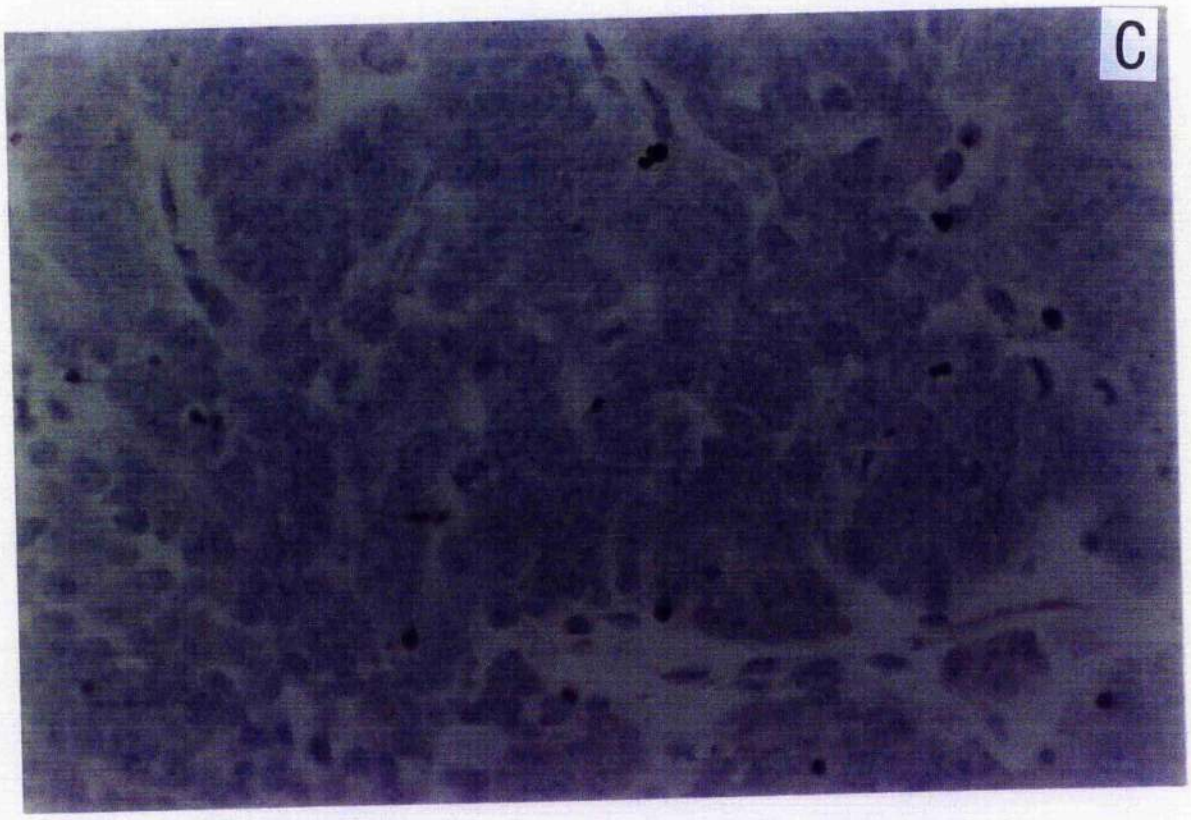
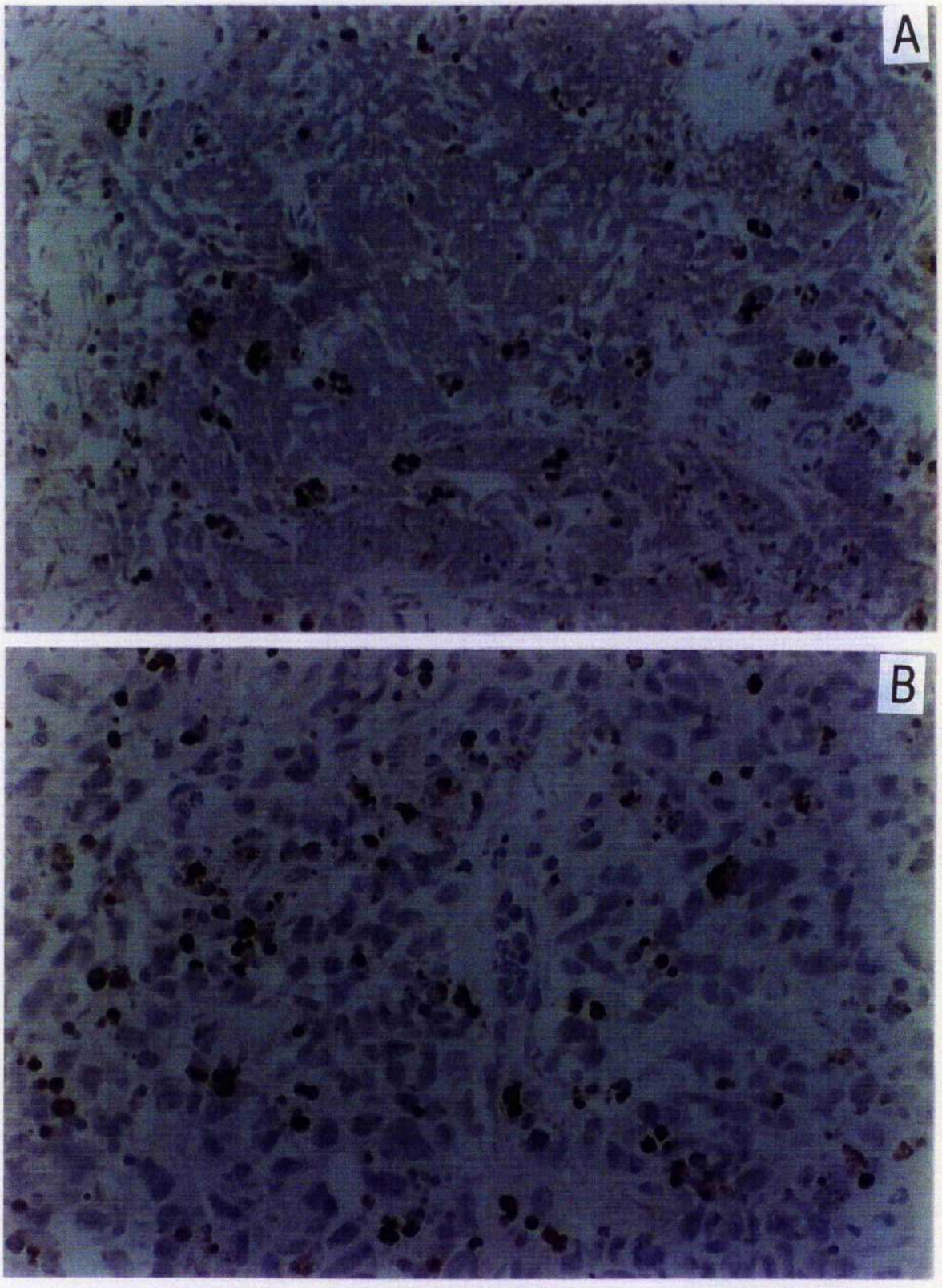


Figure 3-16(cont.) - (C) CH1/TR tumours 72 hrs following paclitaxel treatment.



3-17: Representative photomicrographs (x40) of CH1/TR tumours treated with cisplatin.(A) 24 hrs following treatment, (B) 48 hrs following treatment, (C) 72 hrs following treatment (overleaf).

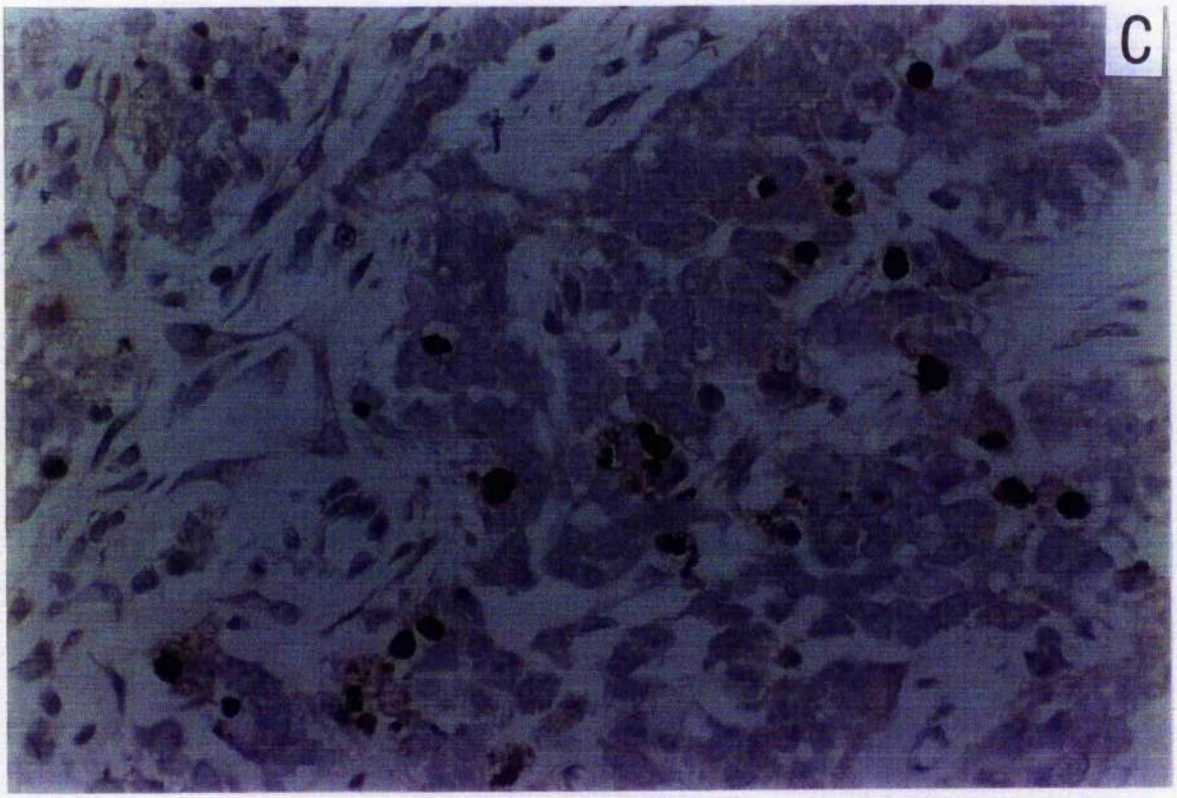


Figure 3-17 (cont.) - (C) CH1/TR tumour 72 hours following cisplatin treatment.

3.5 Assessment of apoptosis regulating proteins by Western blotting

The changes in apoptosis regulating protein p53, p21, bax, bcl-2, bcl-xl, and Raf-1 were studied CH1/TS and CH1/TR tumours following treatment with paclitaxel (25 mg/kg i.p) and cisplatin (4mg/kg i.p.). The established xenograft model allowed the dynamic assessment of the relative expression of these proteins at 24, 48, and 72 hours post treatment using western blotting. The levels of expression of these proteins were compared in two ways as discuss in section 2.7.3.6. Briefly, Because of the different response to paclitaxel, the effect of paclitaxel on the expression of these proteins was compared in CH1/TS and CH1/TR tumours. The CH1/TR tumours displayed a moderate sensitivity to cisplatin; hence the effect of cisplatin and paclitaxel on apoptosis regulating proteins in CH1/TR tumours was compared.

Here, the changes in the levels of each protein will be discussed separately, but taking into account the different interactions between these proteins. The mean fold increase or decrease in levels of proteins above in CH1/TS and CH1/TR tumours are summarized in tables 3-9 and 3-10. The mean fold increase or decreases in levels of proteins above in CH1/TR tumours following cisplatin treatment are summarized in table 3-11.

3.5.1 P53

CH1/TS and CH1/TR cell lines are known to express wild type p53 (Pestell KE, 1998). Paclitaxel induced significant increase in p53 levels in CH1/TS tumours (figure 3-18 and table 3-9) with mean fold increases from the control of 4.7, 3.4, and 4.3 at 24, 48, and 72 respectively. In contrast, paclitaxel induced slight increase in p53 in CH1/TR tumours with p53 levels of 1.3, 1.5, and 1.4 at 24, 48, and 72 hours respectively. The difference in induction of p53 between CH1/TS and CH1/TR was statistically significant ($p=0.002$) at 24 hrs, 48 hrs, and 72 hrs. Interestingly, cisplatin induced significant increase in p53 levels (figure 3-19) in CH1/TR with mean fold increases of 2.2, 2.55, and 3.9 ($p=0.002$) at 24, 48, and 72 hours respectively.

3.5.2 P21

P21 is downstream protein that is transactivated by p53 protein as discussed in section 1.3.5.1. The induction of p53 by paclitaxel in CH1/TS tumours resulted in similar rise in p21 levels (figure 3-20 and table 3-9) with fold increases from control of 1.46, 3.84, 3.6 at 24, 48, and 72 hours respectively. Paclitaxel resulted in slight changes in p21 levels in CH1/TR tumours of 1.37, 0.82, at 24, 48 hours respectively, however, at 72 hours there was more significant increase with increase from control of 2.2 fold. Although there was marked in induction of p53 protein in CH1/TR tumours by cisplatin, the level of induction of p21 was less (figure 3-21 and table 3-11) with fold increases from control of 1.5, 1.1, and 1.9 at 24, 48, and 72 hours respectively.

3.5.3 Bax

Bax protein (21 KD) transactivation by p53 protein has been demonstrated in ovarian cancer cell lines in vitro following paclitaxel treatment as discussed in sections 1.3.5.2 and 1.4.1(Jones NA, 1998). In this study, despite the marked induction of p53 in paclitaxel treated CH1/TS tumours, there was only slight induction of bax at 48 hours following paclitaxel treatment (figure 3-22and table 3-10) with fold increases from control of 0.86, 1.21, 1.17 at 24, 48, and 72 hours respectively. There was no induction of bax protein in CH1/TR tumours following paclitaxel treatment levels of 0.99, 0.84, and 0.85 at 24, 48, and 72 hours respectively. Similarly, there was no induction of bax protein in CH1/TR tumours following cisplatin treatment with levels of 0.9, 1.02, and 1.0 at 24, 48, and 72 hours respectively (figure 3-23 and table 3-11).

3.5.4 BCL-2

The anti-apoptotic protein bcl-2 has been shown to be phosphorylated following paclitaxel treatment in vitro and that phosphorylation leads to inactivation of bcl-2 and cell death as discussed in 1.4.2. The bcl-2 protein normally runs at molecular weight of 26 KD on SDS-PAGE under reducing conditions (Blagosklonny MV, 1997). In most cell line such as breast cancer cell line MCF-7, prostate cancer cell line PC3, and HL-60 leukemia, bcl-2 phosphorylation results in retardation of electrophoretical mobility of the original band with appearance of 27 KD bcl-2 band (Blagosklonny MV, 1996).

Figure 3-24 shows the immunoblotting of bcl-2 in CH1/TR and CH1/TS tumours following paclitaxel treatment. The 26 KD band is faintly expressed in CH1/TR control untreated tumours and more prominent in CH1/TS control tumours. In addition to the regular 26 KD bcl-2 band, two other bands at 27 KD and 30 KD were demonstrated. This pattern has been described previously. Tang et al detected the presence of 26, 27, and 30 KD bands in prostate cancer cell lines PC3 and DU145 using the same anti-bcl-2 antibody used in the present study (sc-492, Santa Cruz) (Tang DG, 1998). Tang et al demonstrated that 27 KD band is the phosphorylated isoform of the 26 KD band, but the significance of 30 KD band was not known. Guan et al detected in colonic cell line the presence of 30 KD band, in addition to the 26 KD band and demonstrated that the 30 KD band is the phosphorylated form of the original band (Guan RJ, 1996). Of note that in the latter two studies no chemotherapy was administered suggesting that these phosphorylated forms are endogenously expressed.

In this study, both 27KD and 30 KD bcl-2 bands were detected in CH1/TS control tumours suggesting that these proteins are endogenously expressed, although 30 KD bcl-2 band is more prominent (figure 3-24). The densitometery of the 30 KD band following paclitaxel treatment showed no significant alteration in its levels in CH1/TS and CH1/TR tumours. The ratio level to control in CH1/TS tumours were 1.2, 1.8, and 1.14 and in CH1/TR tumours were 1.1, 0.87, and 0.85 at 24, 48, and 72 hours respectively (table 3-10). The quantification of 27 KD band by densitometery was difficult to perform because of its position close to the 26 KD band. However, two main observations could be made. The first is that the 27 KD band is

present almost solely in the CH1/TS treated with paclitaxel. The second is that the 26 KD band became faint with increased prominence of 27 KD band following paclitaxel treatment. These observations suggested that the 27 KD band may represent phosphorylated form of the original 26 KD band, the pattern of which is affected by paclitaxel treatment. The 27 KD was further characterized by incubation of paclitaxel treated CH1/TS tumour lysate with alkaline phosphostase. The lysis buffer used to prepare these tumour lysates had no Sodium Orthovanadate that inhibit alkaline phosphatase action. The alkaline phosphatse treatment resulted in marked increase in 26 KD band with decrease in intensity of 27 KD band suggesting that the latter band is phosphorylated form of the 26 KD band (figure 2-26).

Following the treatment of CH1/TR tumours with paclitaxel, The 26 KD band is expressed faintly in CH1/TR tumours at 24, and 48hours and becoming more prominent at 72 hours with very faint expression of 27 KD band (figure 3-24). Following the treatment of CH1/TR tumours with cisplatin (figure 3-25), the 26 KD band could be seen faintly and the 27 KD band was not detected. The densitometery the 30 KD band showed no significant alteration at 24, 48, and 72 hours with ratio to control values of 0.71, 1.02, and 1.27 at 24, 48, and 72 hours.

Taking together these observations suggest that 27 KD bcl-2 band represented the phosphorylated form of 26 KD bcl-2 band which is present endogenously and further altered by paclitaxel treatment. It appears that paclitaxel treatment decrease the 26 KD band and increase the phosphorylated (inactive form) 27 KD band.

3.5.5 BCL-XL

The anti-apoptotic protein bcl-xl has been shown to be phosphorylated following paclitaxel treatment in vitro, which lead to bcl-xl inactivation and lead to apoptosis (Poruchynsky MS, 1998). The immunoblting pattern of bcl-xl in CH1/TR and CH1/TS follows paclitaxel treatment as shown in figure 3-27. In the present study there was no evidence of phosphorylation in either cell line following paclitaxel treatment. However, there was a decline of the level of the anti-apoptotic protein bcl-xl in CH1/TS but not CH1/TR tumours

treated with paclitaxel with the ratio bcl-xl levels to control in CH1/TS tumours of 0.63 (P=0.09), 0.54 (P=0.026), and 0.35 (P=0.026) at 24, 48, and 72 hours, in contrast, the ratio to of bcl-xl levels to control in CH1/TR were 0.83, 0.98, and 0.83 at 24, 48, 72 hours respectively (table 3-10). Figure 3-28 shows the immunoblotting pattern following treatment of CH1/TR with paclitaxel and cisplatin. Cisplain induced similar decrease in bcl-xl levels, With ratio to control levels of 1.0 (P=0.4), 0.73 (P=0.13), and 0.46 (P=0.015) at 24, 48, 72 hours respectively.

3.5.6 Raf-1

Following treatment of different cell lines with paclitaxel, it was demonstrated that c-Raf-1 phosphorylation occurs (Blagosklonny MV, 1997). More recently, it has been demonstrated that low concentration paclitaxel can induce mitotic arrest and cell death without phosphorylation of c-Raf-1. Figure 3-29 shows immunoblotting for Raf-1 of CH1/TS and CH1/TR tumours following the treatment with paclitaxel. There is no evidence of phosphorylation of 74,000 Raf-1 protein.

Protein	24 hrs	48 hrs	72 hrs
P53/CH1-TR	1.27±0.19	1.12±0.14	1.42±0.17
P53/CH1-TS	4.73±0.64	3.42±0.29	4.33±0.39
P21/CH1-TR	1.37±0.13	0.82±0.1	2.2±0.18
P21/CH1-TS	1.46±0.45	3.84±0.65	3.6±1.2

Table 3-9: The ratios of p53 and p21 levels to control in CH1/TS (paclitaxel sensitive) and CH1/TR (paclitaxel resistant) at 24, 48, 72 hrs following the treatment with paclitxel.

Protein	24 hrs	48 hrs	72 hrs
Bax/CH1-TR	0.99±0.06	0.84±0.12	0.85±0.12
Bax/CH1-TS	0.87±0.09	1.21±0.06	1.17±0.075
Bcl-2/CH1-TR	1.1±0.08	0.87±0.28	0.85±0.07
Bcl-2/CH1-TS	1.21±0.18	1.8±0.24	1.14±0.12
Bcl-xl/CH1-TR	0.83±0.046	0.98±0.13	0.83±0.19
Bcl-xl/CH1-TS	0.63±0.077	0.54±0.09	0.35±0.09

Table 3-10: The ratios of apoptotic proteins levels to control in CH1/TS (paclitaxel sensitive) and CH1/TR (paclitaxel resistant) at 24, 48, 72 hrs following the treatment.

Protein	24 hrs	48 hrs	72 hrs
P53	2.23±0.15	2.55±0.23	3.3±0.07
Bcl-xl	1.022±0.12	0.73±0.14	0.46±0.013
Bax	0.91±0.05	1.02±0.12	1.0±0.04
Bcl-2	0.71±0.07	1.02±0.25	1.27±0.3
P21	1.51±0.11	0.95±0.17	1.9±0.49

Table 3-11: The ratios of apoptotic protein levels to control in CH1/TR tumours at 24, 48, and 72 hrs following cisplatin treatment.

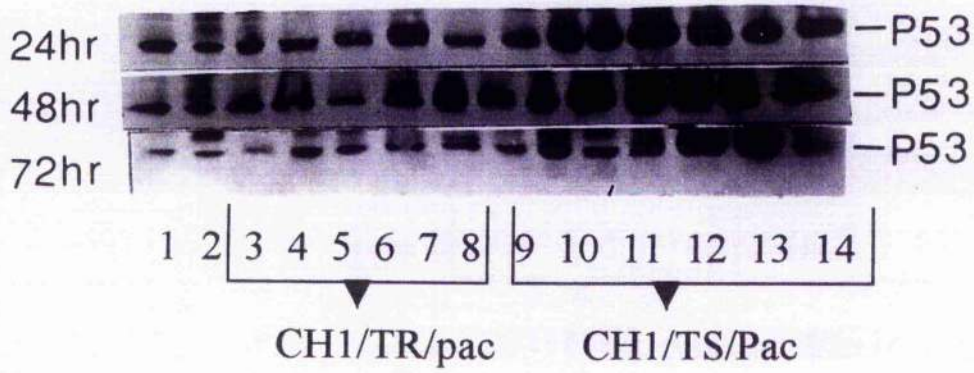


Figure 3-18: Immunoblotting of p53 (53 KD) in CH1/TS and CH1/TR tumours following paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2: CH1/TS control

Lanes 3-8: CH1/TR tumours treated by paclitaxel.

Lanes 9-14: CH1/TS tumours treated by paclitaxel.

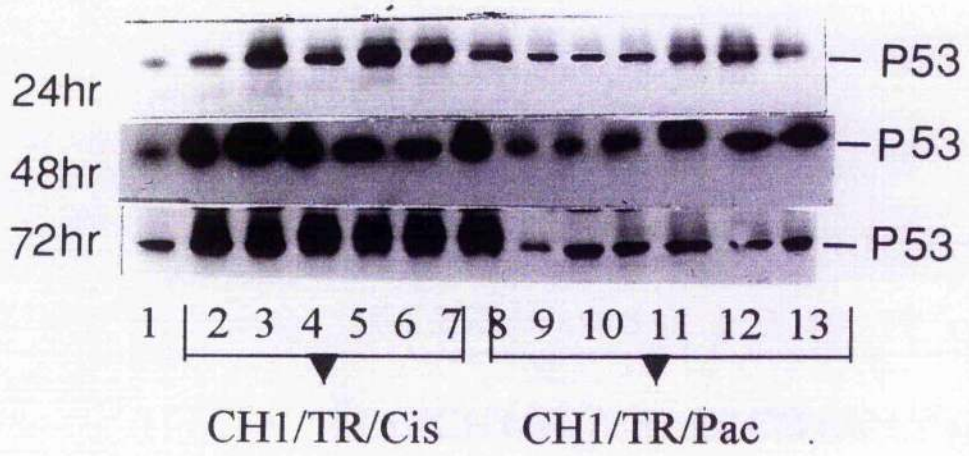


Figure 3-19: immunoblotting of p53 (53 KD) in CH1/TR tumours following cisplatin and paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2-7: CH1/TR tumours treated with cisplatin

Lane 8-13: CH1/TR tumours treated with paclitaxel.

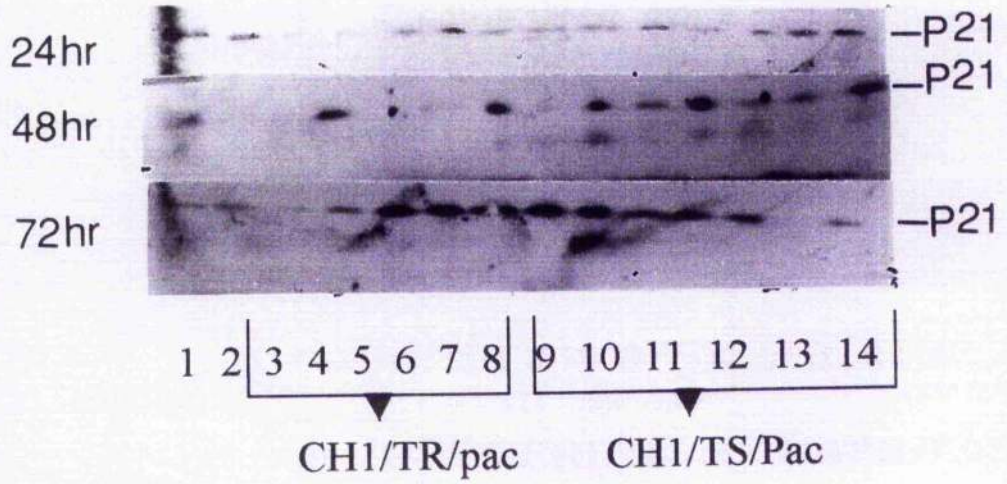


Figure 3-20: Immunoblotting of p21 (21 KD) in CH1/TS and CH1/TR tumours following paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2: CH1/TS control

Lanes 3-8: CH1/TR tumours treated by paclitaxel.

Lanes 9-14: CH1/TS tumours treated by paclitaxel.

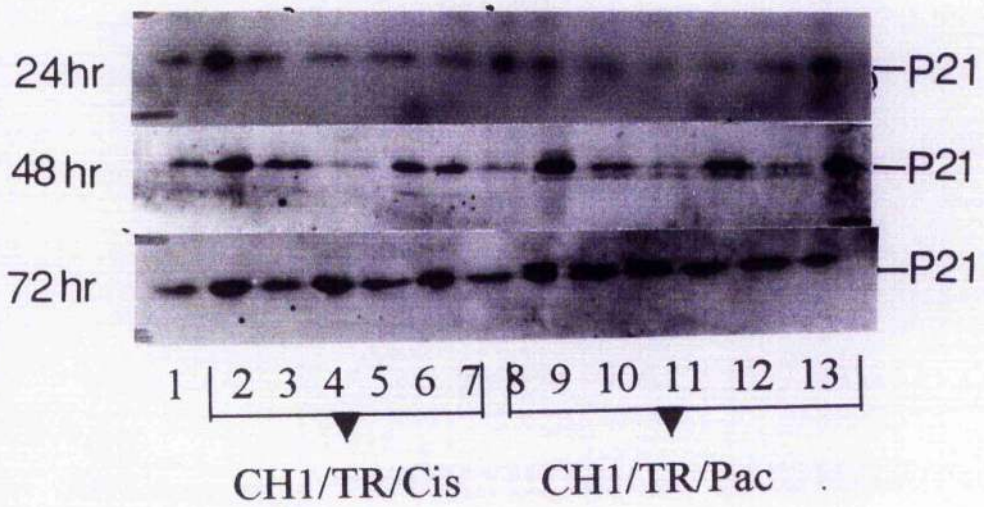


Figure 3-21: immunoblotting of p21 (21 KD) in CH1/TR tumours following cisplatin and paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2-7: CH1/TR tumours treated with cisplatin

Lane 8-13: CH1/TR tumours treated with paclitaxel.

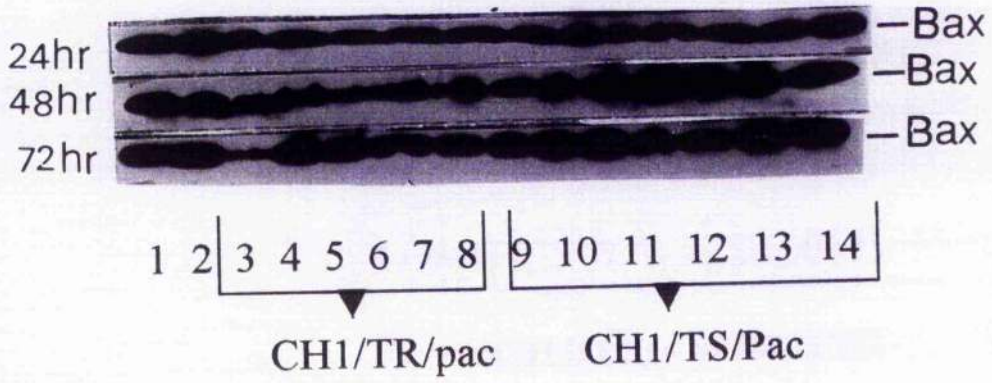


Figure 3-22: Immunoblotting of bax (21 KD) in CH1/TS and CH1/TR tumours following paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2: CH1/TS control

Lanes 3-8: CH1/TR tumours treated by paclitaxel.

Lanes 9-14: CH1/TS tumours treated by paclitaxel.

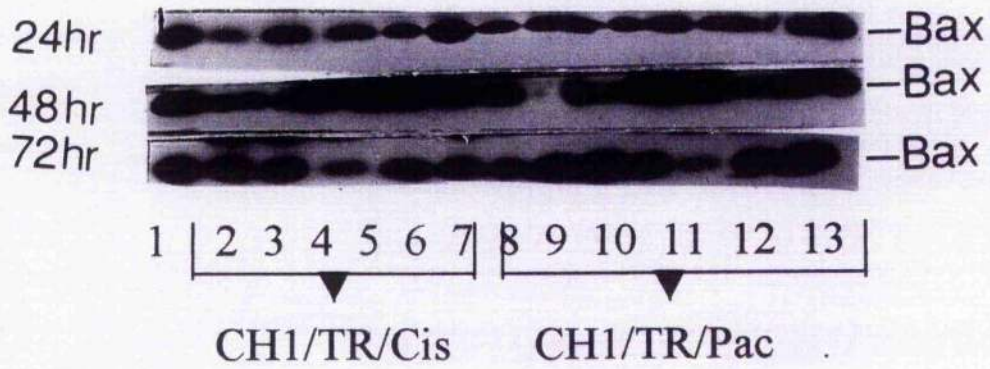


Figure 3-23: immunoblotting of bax (21 KD) in CH1/TR tumours following cisplatin and paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2-7: CH1/TR tumours treated with cisplatin

Lane 8-13: CH1/TR tumours treated with paclitaxel.

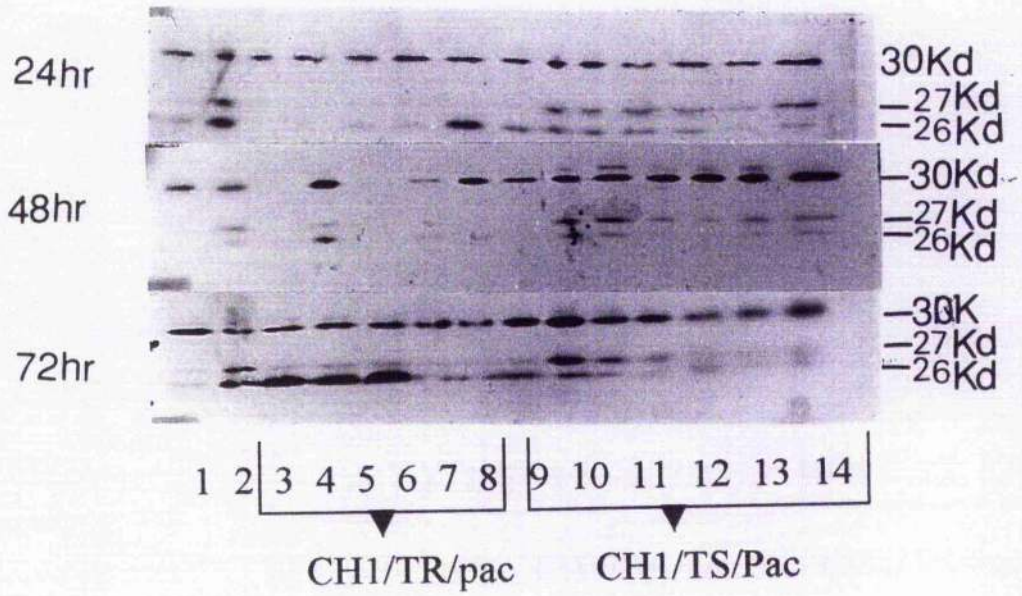


Figure 3-24: Bcl-2 immunoblotting in CH1/TS and CH1/TR tumours following paclitaxel treatment at 24, 48, and 72 hrs showing the 26 KD bcl-2 band and the related 27 and 30 KD bands.

Lane 1: CH1/TR control

Lane 2: CH1/TS control

Lanes 3-8: CH1/TR tumours treated by paclitaxel.

Lanes 9-14: CH1/TS tumours treated by paclitaxel.

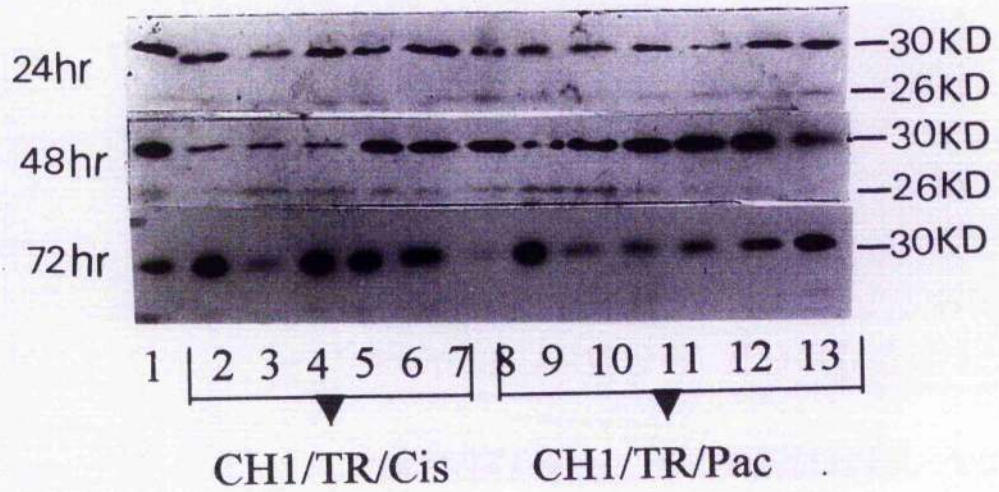


Figure 3-25 immunoblotting of bcl-2 in CH1/TR tumours following cisplatin and paclitaxel treatment at 24, 48, and 72 hrs showing the parent 26 KD bcl-2 band and related 30 KD band.

Lane 1: CH1/TR control

Lane 2-7: CH1/TR tumours treated with cisplatin

Lane 8-13: CH1/TR tumours treated with paclitaxel.

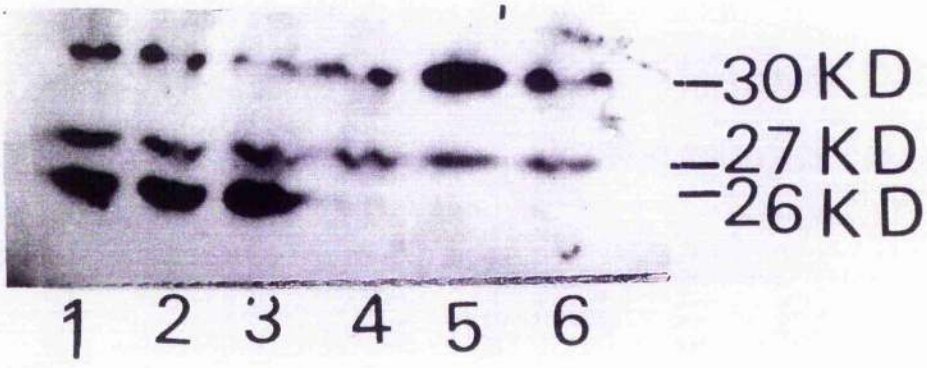


Figure 3-26: Characterization of bcl-2 related bands by addition of alkaline phosphatase in three CH1/TS tumours.

Lanes 1-3: Tumour lysates with alkaline phosphatase.

Lanes 4-6: Tumour lysates with no alkaline phosphatase.

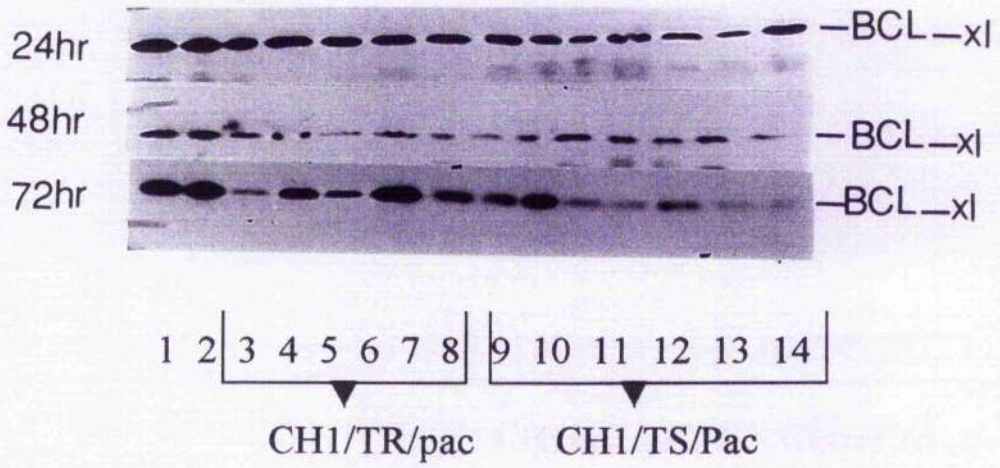


Figure 3-27: Immunoblotting of bcl-xl (31 KD) in CH1/TS and CH1/TR tumours following paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2: CH1/TS control

Lanes 3-8: CH1/TR tumours treated by paclitaxel.

Lanes 9-14: CH1/TS tumours treated by paclitaxel.

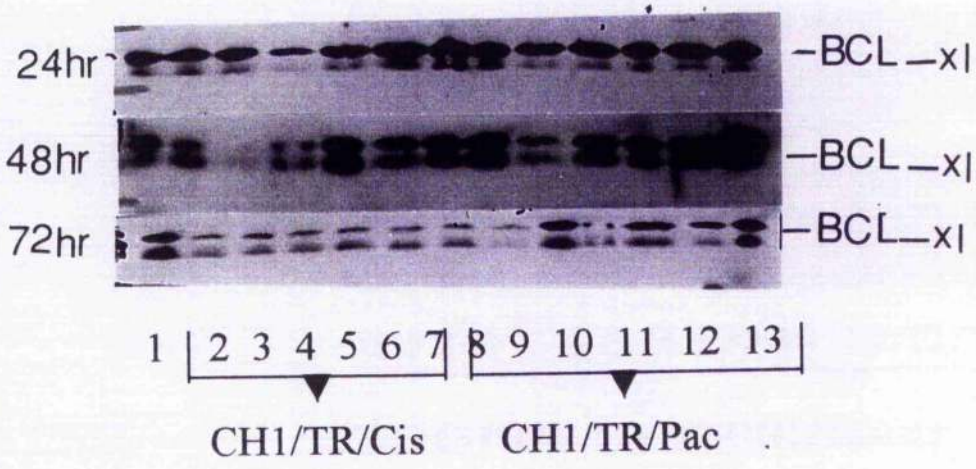


Figure 3-28: immunoblotting of bcl-xl (31 KD) in CH1/TR tumours following cisplatin and paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2-7: CH1/TR tumours treated with cisplatin

Lane 8-13: CH1/TR tumours treated with paclitaxel.

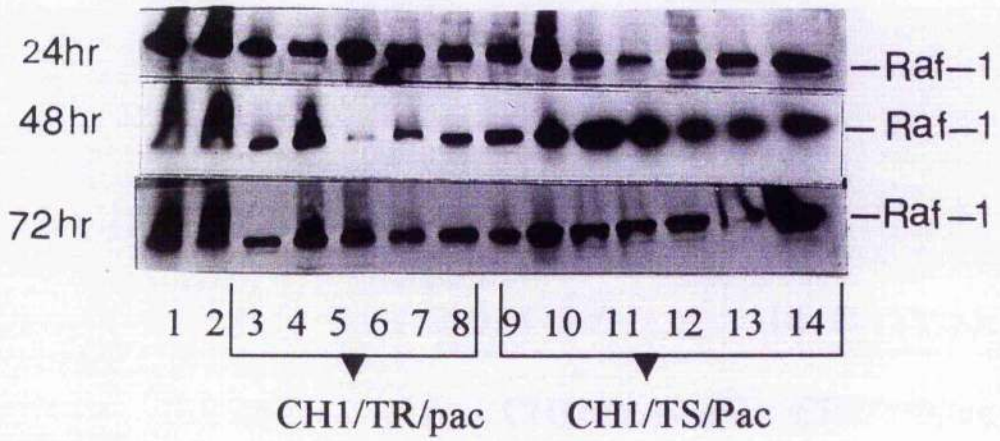


Figure 3-29: Immunoblotting of Raf-1 (74 KD) in CH1/TS and CH1/TR tumours following paclitaxel treatment at 24, 48, and 72 hrs.

Lane 1: CH1/TR control

Lane 2: CH1/TS control

Lanes 3-8: CH1/TR tumours treated by paclitaxel.

Lanes 9-14: CH1/TS tumours treated by paclitaxel.

3.6 Biological growth studies of CH1/bcl-xl xenografts

In this experiment, the growth studies of CH1/TS, CH1/bcl-xl, and CH1/ F 250 (vector control) xenografts were performed separately following paclitaxel and cisplatin treatment. The results from these three experiments are represented following paclitaxel and cisplatin in figure 3-30 and 31 respectively.

The control untreated CH1, CH1/bcl-xl, and CH1/F 250 tumours have similar growth pattern with doubling time of 2.6, 3.4, and 4.6 days respectively. The CH1 tumours displayed marked sensitivity to paclitaxel with complete disappearance of tumours 6 days post last dose of treatment and growth delay more than 66.4 days. The CH1/bcl-xl tumours showed initial partial response to paclitaxel, which was maintained for 18 days post last dose of treatment, subsequently these tumours began to grow rapidly with specific growth delay of 34.9 days. The CH1/ F 250 tumour showed a prolonged good partial response, which was maintained for 52 days post last dose of treatment and the calculated growth delay for these tumours was 51.1 days.

Similarly, cisplatin induced complete response in CH1/TS tumours with complete disappearance of all tumours 12 days post last dose of treatment and growth delay of more than 66.4 days. The CH1/bcl-xl tumours displayed a very short partial response to cisplatin, which was maintained for three days only post last dose of treatment followed by rapid growth of the tumours with specific growth delay of 24.6 days only. Interestingly, the CH1/F 250 tumours displayed some resistance to cisplatin with the good initial partial response achieved that was maintained for 22 days followed by growth of these tumours and growth delay of 26.6 days.

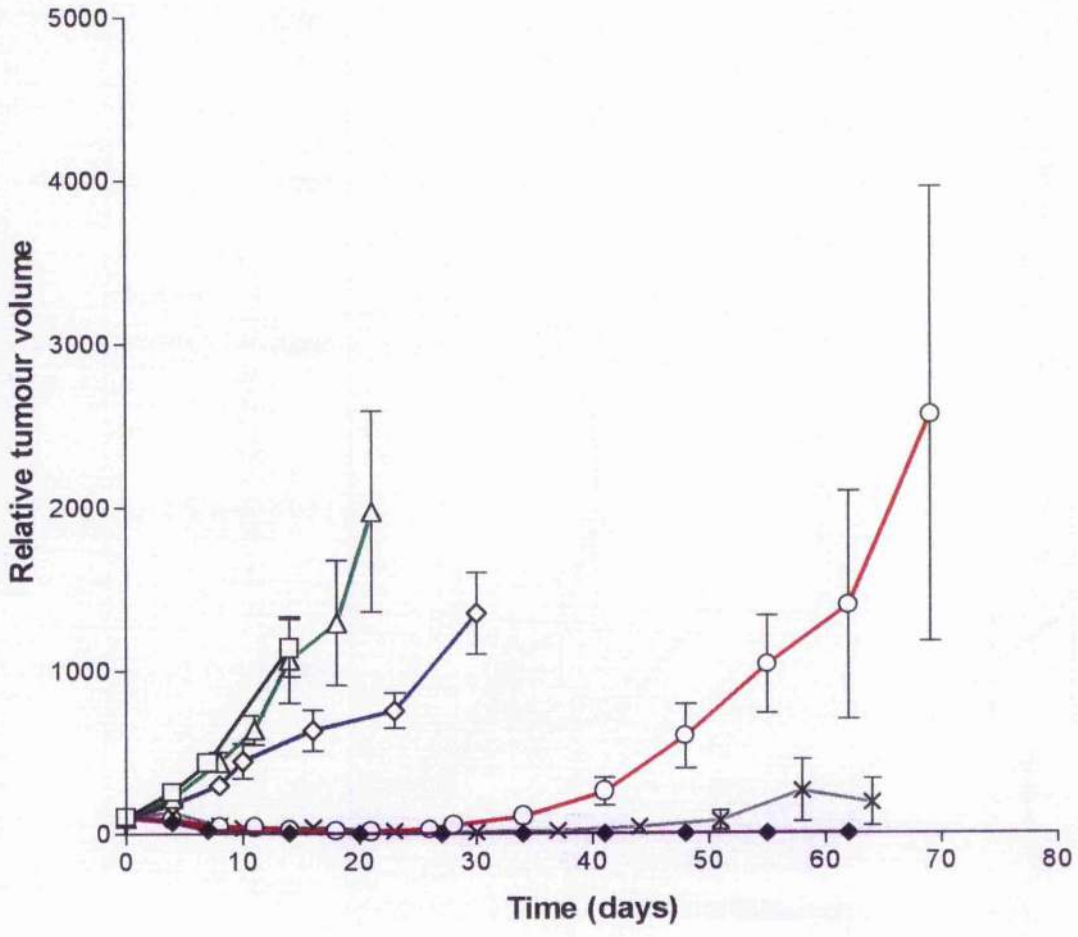


Figure 3-38: Average growth curves of CH1, CH1/bcl-xl, CH1/F250 tumours following paclitaxel (25 mg/kg i.p.) treatment. —□— CH1/control, —△— CH1/bcl-xl/control, —◇— CH1/F 250/control, —◆— CH1/paclitaxel Rx, —○— CH1/bcl-xl/paclitaxel Rx, —×— CH1/F 250/paclitaxel Rx

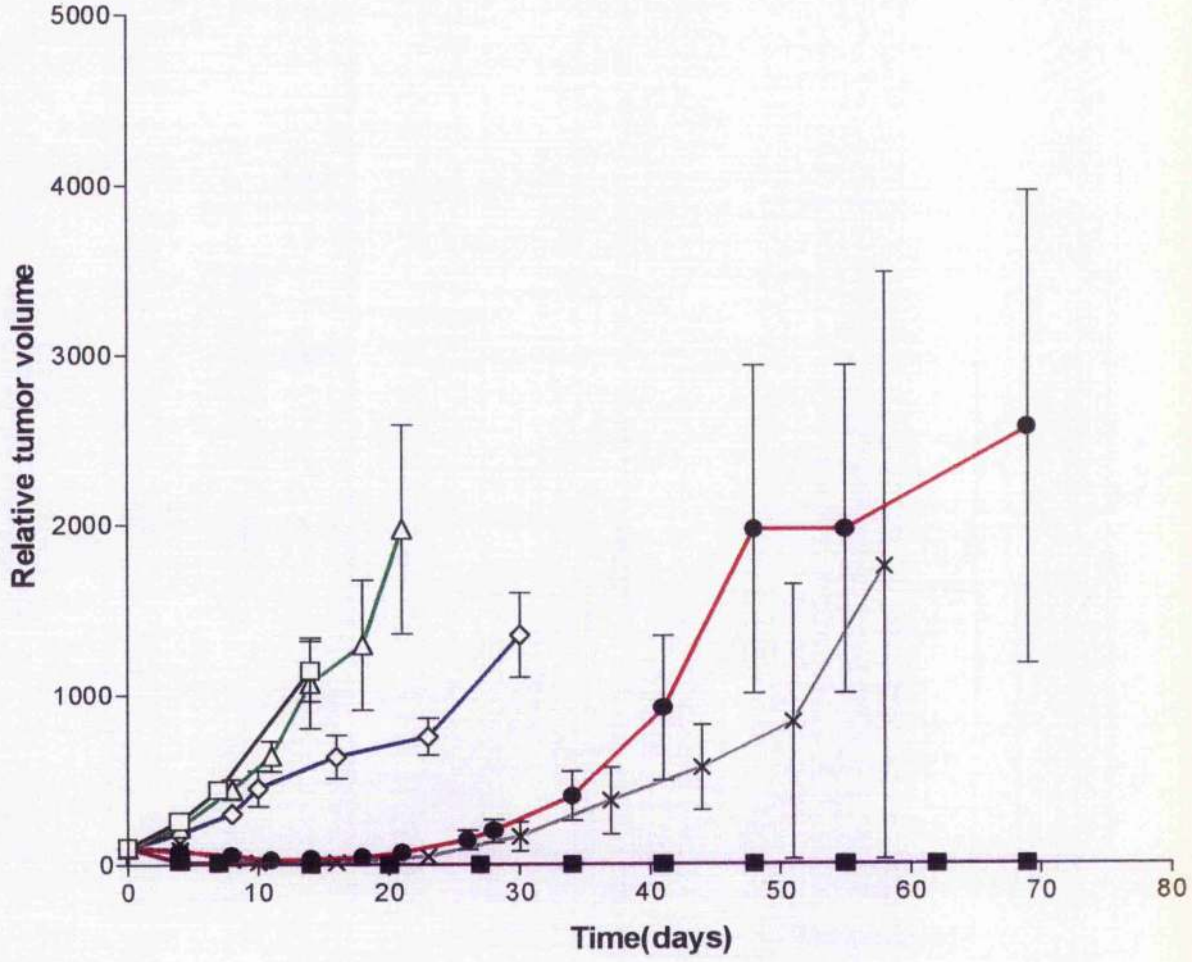


Figure 3-39: Average growth curves of CH1, CH1/bcl-xl, and CH1/F250 tumours following cisplatin treatment. —□— CH1/control —△— CH1/bcl-xl/control, —◇— CH1/F 250/control —■— CH1/cisplatin Rx, —●— CH1/bcl-xl/cisplatin Rx, —×— CH1/F 250/cisplatin Rx.

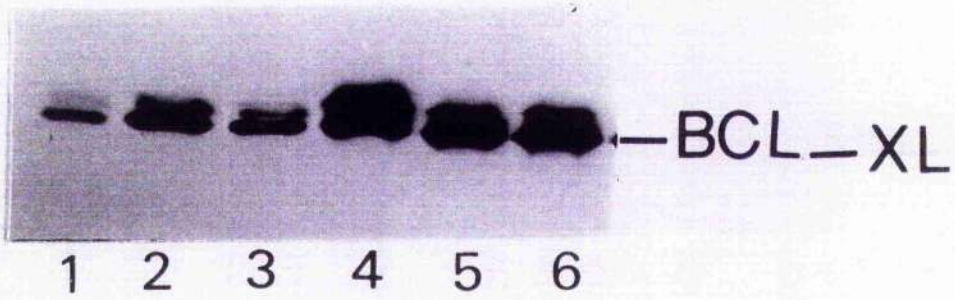


Figure 3-32: immunoblotting of bcl-xl in CH1/TS tumours and CH1/bcl-xl.

Lanes 1-3: Three CH1/TS tumours.

Lanes 4-6: Three CH1/bcl-xl tumours.

3.7 Paclitaxel uptake studies and pharmacokinetic analysis of CH1/bcl-xl tumours

This experiment was done as part of uptake study presented in section 3.2. The mean paclitaxel concentrations and concentration versus time curves of CH1/bcl-xl, and CH1/TS tumours are shown in table 3-12 and figure 3-33 respectively. There was no significant difference in mean paclitaxel concentration of CH1/TS, and CH1/bcl-xl tumours at 1, 3, 6, or 24 hours post treatment. The mean paclitaxel concentration 6 hours post treatment were almost equal and represented the mean peak values for the both types of tumours as shown table3-12. Similarly there was no significant difference in the pharmacokinetics parameters measured as shown in table 3-13.

The above results suggest that there are no major differences in either intratumour paclitaxel concentration or pharmacokinetics to account for resistant pattern to paclitaxel and cisplatin observed CH1/bcl-xl tumours as demonstrated in biological growth studies presented in section 3.6. This is consistent with in vitro studies that have shown that bcl-xl uptake does not affect paclitaxel uptake and, more importantly, does not interfere with microtubular polymerization (Ibrado AM, 1997; Liu JR, 1998). Taking together the observation from this study and other studies suggest that the expression of bcl-xl resulted in the development to chemotherapy.

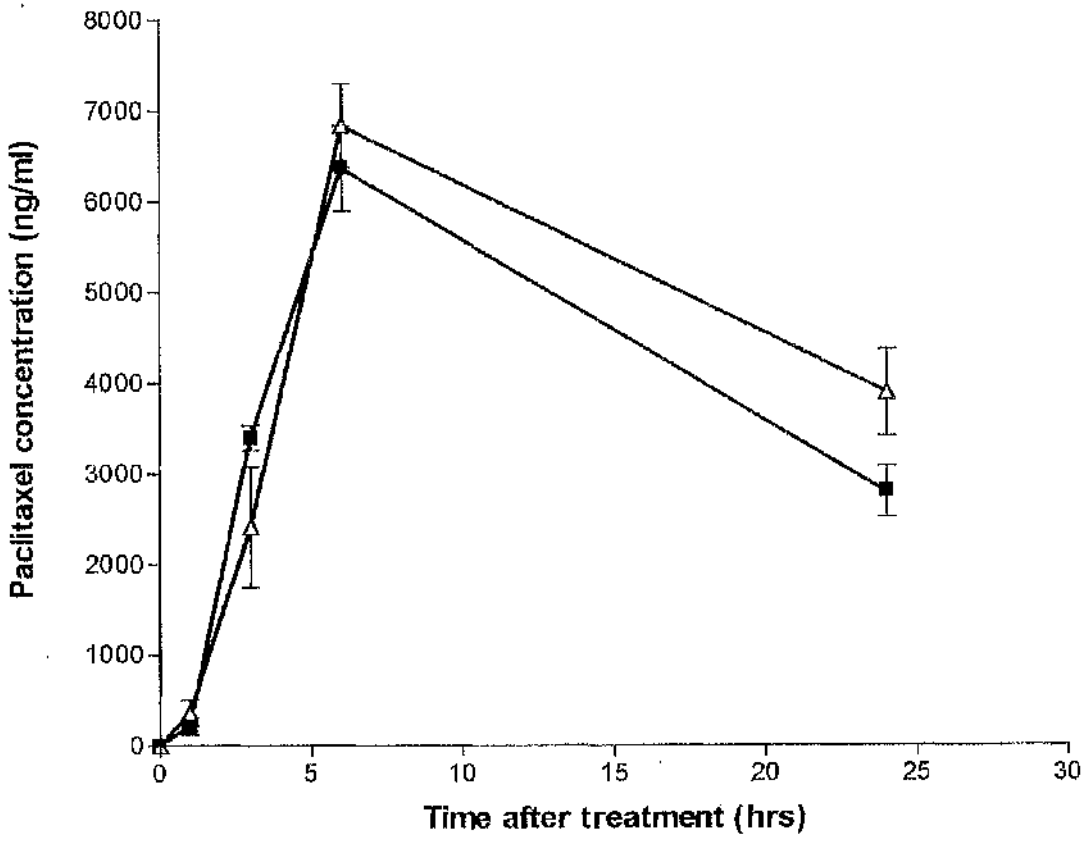


Figure 3-33: Intra-tumoural paclitaxel concentration versus time of of —△— CH1-bcl-xl and —■— CH1/TS after treatment with 25mg/kg i.p. of paclitaxel.

Time (hrs)	CH1/TS (μg/ml)	CH1/BCL-XL (μg/ml)
1	0.22±0.09	0.36±0.14
3	3.42±0.13	2.4±0.66
6	6.36±0.47	6.84±0.46
24	2.8±0.28	3.89±0.48

Table 3-12: the inta-tumour concentration of paclitaxel in CH1/TS, and CH1/bcl-xl tumours following treatment with 25 mg/kg of paclitaxel

Parameters	CH1/bcl-xl	CH1/TS
C_{max} (ng/ml)	3417.0	3181
AUClast (ng/ml x hr)	113325.8	100798
$T_{1/2}$ Lambda z (hr)	22.1625	15.1624
AUCINF (ng/ml x hr)	118881.03	80957.95

Table 3-13: the pharmacokienitcs parameters measured in CH1/TS, and CH1/bcl-xl tumours following treatment with 25 mg/kg of paclitaxel i.p.

Chapter 4: Discussion of results.

4.1 Introduction

As outlined in section 1.5, the resistance to paclitaxel has been attributed in previous studies to three main mechanisms which include altered drug uptake due to expression of MDR phenotype, altered microtubule polymerization and content, and alteration in apoptosis regulating proteins (Bhalla K, 1994; Horwitz SB, 1993; Kavallaris M, 1997; Parekh H, 1997b). It has been suggested that understanding of resistance to chemotherapy is dependent on elucidation of the molecular mechanisms by which anti-cancer drugs induce apoptotic cell death. Over the last few years several in vitro studies have demonstrated the importance of apoptosis regulating proteins in the induction and modulation of response to paclitaxel (Liu JR, 1998; Liu QY, 1997; Strobel T, 1996; Wahl AF, 1996). The induction of cell death by paclitaxel involve these alteration of several apoptosis regulating proteins including p53, p21, the pro-apoptotic protein bax, and the anti-apoptotic proteins bcl-2 and bcl-xl (Blagosklonny MV, 1995; Blagosklonny MV, 1996). In addition, these studies demonstrated the role of the signal transduction molecule Raf-1 in connecting the polymerization of the microtubules and the alteration in apoptotis regulating proteins (Blagosklonny MV, 1997).

The present in vivo study focused on the role of the apoptosis regulating proteins in induction of cell death and acquired resistance to paclitaxel. This required a methodological approach that progressed through several steps. Firstly, establishment of successful xenografts of CH1 paclitaxel sensitive (CH1/TS) and CH1 paclitaxel resistant xenografts (CH1/TR) from their respective cell lines and the conformation of pattern of resistance and sensitivity in vivo. Secondly, the assessment of paclitaxel uptake in CH1/TS and CH1/TR tumours because differences in intra-tumoural concentration may contribute to the development of chemoresistance. Thirdly, flow cytometric analysis of cell cycle alteration and proliferation following paclitaxel treatment. Fourthly, the confirmation that paclitaxel induces apoptosis in vivo. Finally, the changes in apoptosis regulating proteins were assessed by western blotting. In all these steps the effect of paclitaxel was compared and contrasted with an important chemotherapeutic agent in management of ovarian cancer, cisplatin. This allowed an in vivo evaluation of differences in the mechanisms of action and induction of cell death between cisplatin and paclitaxel.

After the evaluation of differences in the expression of apoptosis regulating proteins following cisplatin and paclitaxel treatment between the paclitaxel sensitive and resistant tumours, the second part of the study was devoted to assess the effect of overexpression of one of these proteins (anti-apoptotic protein bcl-xl) on the response to paclitaxel and cisplatin chemotherapy in vivo.

4.2 The establishment of xenografts and confirmation of sensitivity

A previous in vitro study has shown that the CH1/TS cell line displayed sensitivity to paclitaxel and cisplatin (Rogers P, 1996). However CH1/TR cell line was resistant to paclitaxel but retained its sensitivity to cisplatin (Rogers P, 1996). In this study, the biological growth studies clearly demonstrated that the CH1/TS tumours displayed marked sensitivity to both cisplatin and paclitaxel with maintained complete responses and growth delay of more than 60.1 days (figure 3-1). In contrast, the CH1/TR tumours had a differential response to paclitaxel and cisplatin (figure 3-2). The CH1/TR tumours were resistant to paclitaxel with no responses in any tumour, while cisplatin induced partial responses in all tumours that was maintained on average for 21 days post. This represented the first demonstration that this phenotype of acquired resistance to paclitaxel with partial sensitivity to cisplatin developed in vitro could be reproduced in vivo.

4.3 Paclitaxel uptake studies

Paclitaxel is hydrophobic drug that enters the cell by passive diffusion (Parekh H, 1997a). Chemoresistance to paclitaxel has been associated with induction of multidrug resistance phenotype (MDR) and overproduction of P-glycoprotein (PGP) or overexpression of a novel 135 KD membrane glycoprotein distinct from the 170-180 KD PGP (Horwitz SB, 1993; Roy SN, 1985). Hence, it was important to first assess paclitaxel uptake in CH1/TS and CH1/TR tumours by measuring the intra-tumour concentration of paclitaxel by High Performance

Liquid Chromatography (HPLC) to rule out the presence of major drug efflux mechanisms in CH1/TR tumours.

It has been shown previously in vitro that CH1/TS and CH1/TR cell lines do not express P-glycoprotein with no major differences in paclitaxel uptake (Rogers P, 1996). In the present study, the paclitaxel uptake studies confirmed the in vitro data with no significant difference intra-tumoural concentration of paclitaxel between CH1/TS and CH1/TR tumours at 1,3,6, and 24 hours following administration of 25 mg/kg of paclitaxel intrapertionally (table 3-1). In fact, by 24 hours following paclitaxel treatment, the CH1/TR tumours were able to tolerate higher concentration of intra-tumour paclitaxel concentration suggesting that the resistance is due to other mechanisms. Similarly, there was no difference all in the pharmacokinetic parameters in both tumours (table 3-2).

Taking together the in vitro and in vivo results demonstrate no significant differences in intra-tumoural paclitaxel concentration between the CH1/TR and CH1/TS tumours to account for the changes in the growth response to paclitaxel. These observations allowed us to focus on potential distal molecular mechanisms of resistance.

4.4 Measurement of cell kinetics by flow cytometry

The Measurement of cell kinetics by bromodeoxyuridine/anti-bromodeoxyuridine method using flow cytometry provided an in vivo demonstration on different effects of paclitaxel and cisplatin on cell cycle and proliferation. In addition, it allowed a comparison of paclitaxel's effect on CH1/TS and CH1/TR tumours.

4.4.1 Comparison of mechanisms of action of paclitaxel and cisplatin in CH1/TS tumours

4.4.1.1 Paclitaxel

The cell cycle analysis results clearly demonstrate that cells are blocked in G2/M phase following the treatment of CH1/TS tumours with paclitaxel. The G2/M block was first detected at 24 hrs and became more marked at 48 and 72 hours post-treatment as shown in table 3-3 and figure 3-4. These results are consistent with the initial description G2/M phase blockage following paclitaxel treatment by Schiff et al and numerous in vitro studies that have followed (Rowinsky EK, 1988; Schiff PB, 1979; Schiff PB, 1980).

Although the CH1/TS cells treated with paclitaxel were able to traverse the S phase with no hindrance (demonstrated by the mean relative value movements (table 3-3) and green (Brd Urd uptake) versus red (DNA) bivariate histograms, figure 3-5, there was an increase in the mean percentage of cells in S phase with decrease in labelling index at 72 hours post treatment. These observations suggest that there is partial inhibition of DNA synthesis sufficient to increase percentage in S phase and decrease proliferation at 72 hours without hindering the movement through the S phase. Similar observations have been made in vitro and in a study of surgical specimens. Zaffaroni et al demonstrated an increase in S phase fraction and G2/M block following incubation of A2780 ovarian cell lines with paclitaxel for 24 hours (Zaffaroni N, 1988). Millenbaugh et al studied the effect of paclitaxel treatment on 17 three-dimensional histocultures of surgical specimens. It was demonstrated that paclitaxel produced partial inhibition of Brd Urd incorporation (LI) in about 40% of tumours where

incomplete DNA synthesis occurred with induction of apoptosis in about 90% of tumours (Millenbaugh NJ, 1998). The incomplete inhibition of DNA synthesis is also consistent with data in the literature showing that paclitaxel treated cells can proceed with DNA synthesis. The mechanism by which paclitaxel inhibits DNA synthesis is unclear, however it has been demonstrated that paclitaxel inhibits the initiation of DNA synthesis in fibroblasts stimulated by thrombin and epidermal growth factor (Crossin KL, 1981).

Wahl et al suggested that at late times after exposure to paclitaxel (> 40hr) fibroblasts with intact p53 showed an increased G1 population with decrease in Brd Urd labelled cells, suggesting that cells arrest transiently in G1 in the second cell cycle after the completion of mitosis (Wahl AF, 1996). The G1 inhibition has only been shown in normal cells, and in particular there is no evidence for its occurrence in ovarian cancer cell lines. In the present study the CH1/TS expressed wild type p53, however there was no demonstrable increase in G1 cells at any time point.

Taken together these observations suggest that paclitaxel affects all phases of cell cycle, however, maximum sensitivity appear to be at G2/M phase. Donaldson et al demonstrated by phase synchronous treatment in fibroblasts that paclitaxel sensitivity is low in G1 and increases with progress through the cell cycle with cells in G2/M being most sensitive than proliferating cells (Donaldson KL, 1994). Also, in the study by Millenbaugh et al, it was demonstrated that the tumour sensitivity to DNA inhibitory effect is opposite to apoptotic effect, suggesting that partial inhibition of DNA synthesis does not lead to the induction of apoptosis (Millenbaugh NJ, 1998). The cells held in G2/M block and mitotic arrest are most sensitive to paclitaxel and eventually undergo apoptosis (Donaldson KL, 1994).

4.4.1.2 Cisplatin

Following the treatment of CH1/TS tumours with cisplatin, there was an accumulation of cells in S phase that was apparent at 24 hours and most marked at 48 and 72 hr (table 3-4 and figure 3-6. In addition, at 72-hr post treatment there was an accumulation of cells in G2. These observations are consistent with previous in vitro studies that have demonstrated early

inhibition of DNA synthesis (12 h.), accumulation of cells in S phase, and slow-down in the traverse of cells through the S-phase of their cycle. Subsequently, there is a dose-dependent arrest in G2 (Demarcq C, 1992; Sorenson CM, 1990; Sorenson CM, 1988). It has been proposed that in G2 the cells reach a point of decision at which they overcome the G2 block, divide and then remain in cycle or remain in G2 and die (Sorenson CM, 1990; Sorenson CM, 1988).

Three tumours harvested 24 hr post-treatment and five tumours at 48 hr post-treatment had no uptake of Brd Urd (table 3-4). The tumours that took Brd Urd showed marked slow down of transition through the S phase as reflected by RM and marked decrease in LI table 3-4 and figure 3-7, which suggest that the tumours that did not take Brd Urd had complete inhibition of DNA synthesis. At 72 hrs, despite the accumulation of cells in S phase at 72 hours and the statistically significant retardation of movement through S phase, all the tumors took Brd Urd with an increase in LI. These results are consistent with in vitro data which show significant early inhibition of DNA synthesis even at the earliest time point of 12 hours, with DNA synthesis returning between 2 to 5 days when minimal (0.25-0.5 μg/ml) to intermediate (0.5-1 μg/ml) toxic concentration of platinum used (Sorenson CM, 1988).

4.4.2 The effect of paclitaxel on CH1/TR tumours

In contrast to the characteristic cell cycle effects of paclitaxel on CH1/TS tumours, there was no demonstrable cell cycle perturbation following the treatment of CH1/TR tumours with 25 mg of paclitaxel i.p. (table 3-5 and figures 3-8 and 3-9). Because there were multiple G1 peaks, it was difficult to obtain the percentage of cells in different phases of cell cycle. However, the percentage of cells in G1 and G2 phase were estimated, in order to calculate the changes in G2: G1 ratio over 72 hours following paclitaxel treatment as discussed in section 2.5.3. As shown in table 3-5, there was no difference between the control and treated CH1/TR tumors in the ratio of G2: G1 that has been shown to increase in CH1/TS tumors. Similarly, there was no alteration in DNA synthesis, movement through the S phase, or inhibition of proliferation. The inability of paclitaxel to induce any cell cycle changes is consistent with

resistance of CH1/TR tumours to paclitaxel as demonstrated by growth studies and suggest a failure of paclitaxel-microtubule interaction to trigger cell cycle arrest or apoptosis.

Interestingly, the CH1/TR tumours displayed sensitivity to cisplatin with accumulation of cells in S phase as shown in figure 3-10 and 3-11. All tumours took up Brd Urd and there was retardation of movement through S phase but there was no inhibition of proliferation as in table 3-6. These observations are suggestive of incomplete DNA synthesis at treatment dose of 4mg/kg consistent with partial response of these tumours to cisplatin as previously indicated by biological growth studies.

4.4.3 Summary

- Following paclitaxel treatment, the CH1/TS tumours displayed G2/M phase blockage with inhibition of proliferation at 72 hours. Following cisplatin treatment CH1/TS displayed accumulation of cells in S phase, accumulation of cells in G2 phase at 72 hours, and inhibition of proliferation at 24 and 48 hours.
- Following paclitaxel treatment, CH1/TR tumours there was no G2/M phase blockage or any cell cycle perturbation. Following cisplatin treatment, the CH1/TR tumours displayed accumulation of cells in S phase, but with no inhibition of proliferation and consistent partial response of these tumours to cisplatin.

4.5 Confirmation of induction of cell death

The cell cycle alterations induced by cisplatin in CH1/TS and CH1/TR tumours and paclitaxel in CH1/TS tumour are indicative of the ability of both drugs to interact with their cellular targets and induce cell cycle arrest. However, it was important to determine whether these cell cycle changes were associated with apoptosis. Apotosis was assessed by labelling of paraffin section using TUNEL assay and the results were correlated with data from DNA histograms, which estimated sub-G1 debris.

4.5.1 Induction of apotosis in CH1/TS following paclitaxel and cisplatin treatment

4.5.1.1 Paclitaxel

Numerous in vitro studies have demonstrated that paclitaxel induces both mitotic arrest followed by apoptosis (Blagosklonny MV, 1996; Poruchynsky MS, 1998) (Bhalla K, 1993). Two previous in vivo studies used murine ovarian and mammary carcinoma xenografts demonstrated that paclitaxel induced mitotic arrest and apoptosis (Milas L, 1995; Milross CG, 1995). More importantly, these two studies showed sequence dependence in that apoptosis followed mitotic arrest.

The results from the present study confirm that paclitaxel induce G2/M block (section 4.4.1.2), mitotic arrest, and apoptosis in vivo following the administration of 25 mg/kg of paclitaxel. The results in table 3-7 and figure 3- 13 show clearly that there is a statistically significant increase in percentage mean apoptotic index (A.I) at 24, 48, 72 hr. The maximum AI value was reached at 48 hr (9.3% ±0.98) which represented a slight increase from AI value at 24 hr (8.6 ±1.08 %). The difference between these two values was not statistically significant. There was a decline in Al value by 72 hr (6.5 ±1.31%) and the difference between AI value at 48 and 72 hr was statistically significant (p=0.04). These findings are consistent with observations made by Milross et al that demonstrated that following the treatment of murine ovarian cancer xenografts (OCA-1) with single dose of 40 mg/kg paclitaxel, there was

an increase in AI from control untreated levels of 2% to 12% at 24 hrs, and 10% at 48 hrs, followed by a decline to 4% at 72 hrs (Milross CG, 1995). Another study used a single 60 mg/kg paclitaxel in OCA-1 xenografts induced higher AI at 24hr (17%), followed by a decline at 48 hrs (9%) and 72 hrs (5%) (Milas L, 1995). Similarly, the sub-G1 debris values were consistent with AI values with increase in mean percentage sub-G1 debris from 3.27±0.85 (control) to 8.45±0.54, 17.62 ±3.37, 16.42 ±5.28 at 24,48, and 72 hrs.

In addition morphological evidence showed that paclitaxel effectively arrest cells in mitosis (figure 3-13). The mean percentage of cells arrested in mitosis as determined by morphology increased from control value less than 1% to a peak value of 9.6%, followed by a decline to 6.4 at 48 hr, and 4.2 at 72 hr. These results are consistent with two previous in vivo studies mentioned above that showed that peak values occur early around 9 hours post paclitaxel treatment, followed by a decline in to reach background values at 72 hrs.

The changes in apoptotic index and mean percentage cells in mitotic arrest at 24, 48, 72 hours are represented in figure 4-1. The kinetic profile of apoptosis induced by paclitaxel follows that of mitotic arrest as determined by morphology, which is consistent with previous finding suggestive that mitotic arrest precedes induction of apoptosis. However, the flow cytometric analysis of DNA content indicated that CH1/TS cells had an increasing mean G2/M content 72 hours following paclitaxel treatment. These observations suggest that some of the cells arrested in G2/M may escape paclitaxel effect without undergoing apoptosis.

4.5.1.2 Cisplatin

The present study confirms that cisplatin induces apoptosis in vivo (table 3-7, figure 3-14). There was a statistically significant increase in apoptotic index at 24, 48, 72 hrs with mean percentage values of 13.7%, 20.4%, and 19.4%. These values suggest that cisplatin is more effective than paclitaxel in inducing apoptosis. Similar results were obtained by Milross et al in OCA-1 murine tumours and demonstrated that cisplatin induces higher and more sustained apoptotic peaks at an earlier time point (Milross CG, 1995).

4.5.2 *Induction of apoptosis in CH1/TR tumours following paclitaxel and cisplatin treatment*

In contrast to CH1/TS tumours, there was no significant induction apoptosis in CH1/TR tumours (table 3-7 and figure 3-16). In addition there was no significant increase in cells in mitotic arrest from control. However, there was a statistically significant increase in sub-G1 debris as shown in table 3-8. The results of Sub-G1 debris should interpreted with caution as it may contain some necrotic cells. It is noteworthy that the mean increase in the sub-G1 debris in CH1/TR tumours was significantly less than mean increase in sub-G1 in CH1/TS tumours. These data confirm a complete failure of paclitaxel to trigger an apoptotic response in CH1/TR tumours and consistent with a lack of effect on tumour growth.

On the other hand, cisplatin was able to induce apoptosis in CH1/TR tumours, albeit less effectively than in CH1/TS tumours (table 3-7 and figure 3-17). The apoptotic index rose from 0.99% (control) to 10.4% (24hr), 7.9% (48 hr), and 7.4% (72 hr). Similarly the statistically significant increase in sub-G1 after the treatment of CH1/TR tumours with cisplatin was less marked than CH1/TS tumours. Again, these data are consistent with partial resistance observed in growth studies.

4.5.3 *Summary of apoptosis detection studies*

- Paclitaxel induced mitotic arrest and apoptosis in CH1/TS tumours and these processes probably occur in sequence. Similarly, cisplatin induced significant apoptosis in CH1/TS.
- Paclitaxel failed to induce mitotic arrest or apoptosis in CH1/TR tumour, which is consistent with the lack of response in growth studies and lack of G2/M arrest. Cisplatin induced apoptosis in CH1/TR tumours, albeit at lower levels compared to CH1/TS tumours which is consistent with the growth and cell cycle studies.

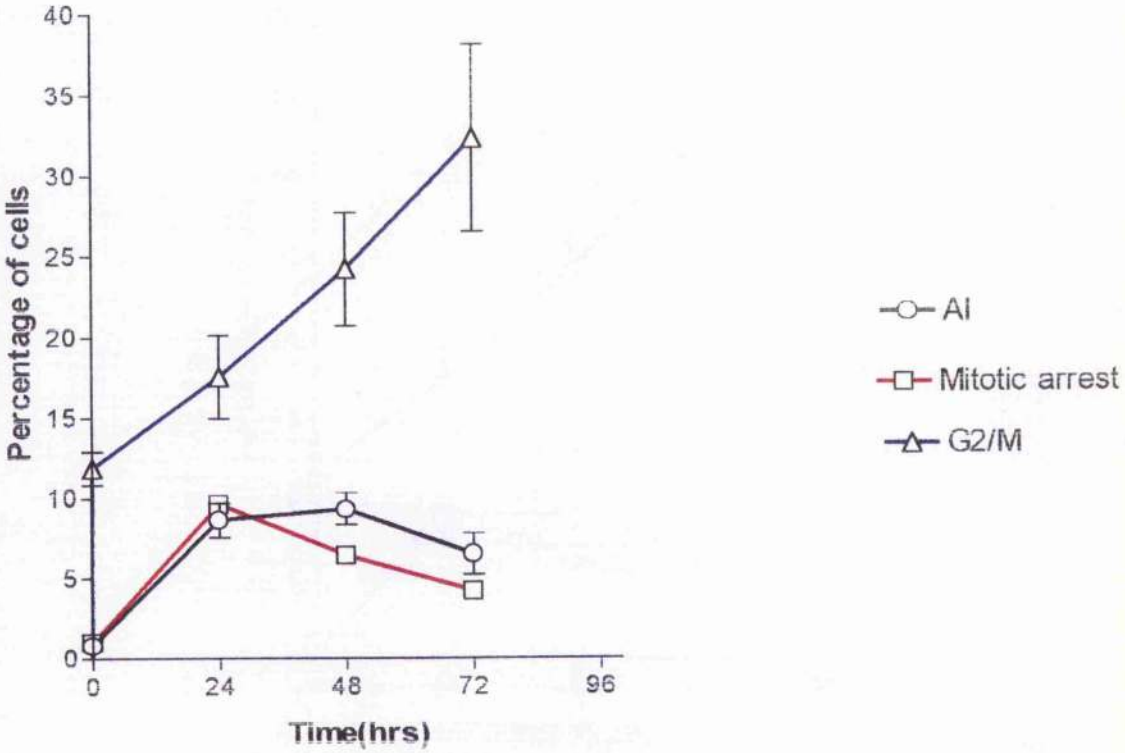


Figure 4-1: Changes in percentage of cells in mitotic arrest, G2/M block, and apoptotic index (AI) over 72 hrs following the treatment of CH1/TS tumours with paclitaxel.

4.6 Apoptosis regulating proteins and paclitaxel mechanisms of action and resistance

The role of apoptosis regulating proteins in the development of chemoresistance is well described (Kromer G, 1997; Reed JC, 1994; Reed JC, 1997a). However paclitaxel interaction with apoptosis regulating proteins has almost exclusively been demonstrated by in vitro studies, and little is known about its effect on these proteins in vivo. By the establishment of successful CH1/TS and CH1/TR xenografts, we were able to study the dynamic changes in apoptosis regulating protein at 24, 48,72 hr. The apoptosis regulating proteins assessed were p53, p21, bax, bcl-2, bcl-xl, and the signal transduction molecule Raf-1. Because of the contrasting response of CH1/TS and CH1/TR to paclitaxel, the effect of paclitaxel on the expression of these proteins was compared in CH1/TS and CH1/TR tumours. The CH1/TR tumours displayed a moderate sensitivity to cisplatin; hence the effect of cisplatin and paclitaxel on apoptosis regulating proteins in CH1/TR was compared. The following discussion from this point on will not include the effect of cisplatin on CH1/TS tumours.

4.6.1 P53 induction and p21

Several in vitro studies have shown that, in wild type p53 cell lines, paclitaxel induces p53 protein, which is largely mediated by an increase in p53 protein stability mediated by Raf-1 activation at high paclitaxel concentration (>9nm) and non-Raf-1 dependent mechanism at low paclitaxel concentration (Blagosklonny MV, 1995; Torres K, 1998). In the present study, p53 protein induction following paclitaxel in CH1/TS tumours was demonstrated in vivo. There was significant sustained increase in p53 protein level over 72 hours (table 3-9 and figure 3-18). In contrast, paclitaxel induced only a slight increase in p53 in CH1/TR tumours (figure 3-18). More importantly, cisplatin was able to induce significant p53 levels in CH1/TR (figure 3-19). These changes are summarized in figure 4-2. The observation that cisplatin was able to induce significant levels of p53 suggest that p53 induction pathway is intact in CH1/TR tumours and the lack of significant induction by paclitaxel is probably due to a defect proximal to p53 .The defect may be localized to microtubules or signal transduction events post-tubulin polymerization.

P53 plays a central role in the regulation of proteins involved in cell cycle arrest such as p21. In vitro studies have demonstrated that paclitaxel induces the accumulation of p21 in both p53 wild type and p53-null cancer cells (Blagosklonny MV, 1995). In the present study, we were able to demonstrate that there was down stream activation of p21 in CH1/TS tumours following paclitaxel administration. The induction of p53 by paclitaxel in CH1/TS tumours resulted in a similar rise in p21 levels (table 3-9 and figure 3-20). Paclitaxel resulted in minimal changes in p21 levels in CH1/TR tumours (figure 3-20). Although there was marked in induction of p53 protein in CH1/TR tumours by cisplatin, the level of induction of p21 was less (figure 3-21) with fold increases from control of 1.5, 1.1, and 1.9 at 24, 48, and 72 hours respectively. The changes in p21 are summarized in figure 4-3. Thus, at least in CH1/TS tumours, p21 protein appears to be p53 dependent. The lack of significant p21 induction in CH1/TR tumours following cisplatin despite the significant p53 induction may be due to defect in signaling pathway distal to p53.

The exact role of p53 and p21 in the cellular response to mitotic spindle damage by paclitaxel remain to be elucidated. P53 induced by paclitaxel has been shown to be involved in G1 and G2 checkpoints. It has been demonstrated that paclitaxel blocks cell cycle progression in G1, but only in normal cells (Wahl AF, 1996). P53 facilitates the progression of normal cells through mitosis and subsequent arrest in G1, hence it has been suggested that the disruption of wild type p53 in normal cells may result in increased in sensitivity to paclitaxel (Wahl AF, 1996). In most of the in vitro studies involving ovarian cancer cells, the disruption of p53 results in either no change or decreased sensitivity to paclitaxel (Debernardis D, 1997). In the present study there was a marked induction of p53 and p21, but there was no G1 phase arrest in CH1/TS tumours treated with paclitaxel (Wu GS, 1996).

Recently, it has been suggested that p53 may play a role during mitotic checkpoint that ensures the maintenance of diploidy, and its induction may represent a response to abnormalities in mitotic spindle such as abnormal chromosome segregation (Torres K, 1998). Cross et al has shown that mouse cells devoid of the p53-dependent spindle checkpoint are capable of completing subsequent cell cycle phases including DNA synthesis, without

complete chromosome segregation (Cross SM, 1995). On the other hand, it has been shown that p21 accumulation in both p53 wild type and p53-null cancer is not involved in mitotic arrest and –in fact- it occurs as consequence of mitotic arrest (Barboule N, 1997).

In addition, p53 has been shown to regulate other apoptosis regulating proteins. In some cell types p53 has been shown to upregulate the expression of pro-apoptotic protein bax and down regulate the anti-apoptotic proteins bcl-2 and bcl-xl (Miyashita T, 1996; Miyashita T, 1994). Thus, p53 may induce cell cycle arrest allowing repair before proceeding through cell cycle or induce apoptosis if the damage is severe. However, most of the in vitro and prognostic studies suggest that p53 is a determinant of cell induction of death or survival. This is may be particularly relevant in low clinically relevant paclitaxel concentrations where p53 induction does not occur.

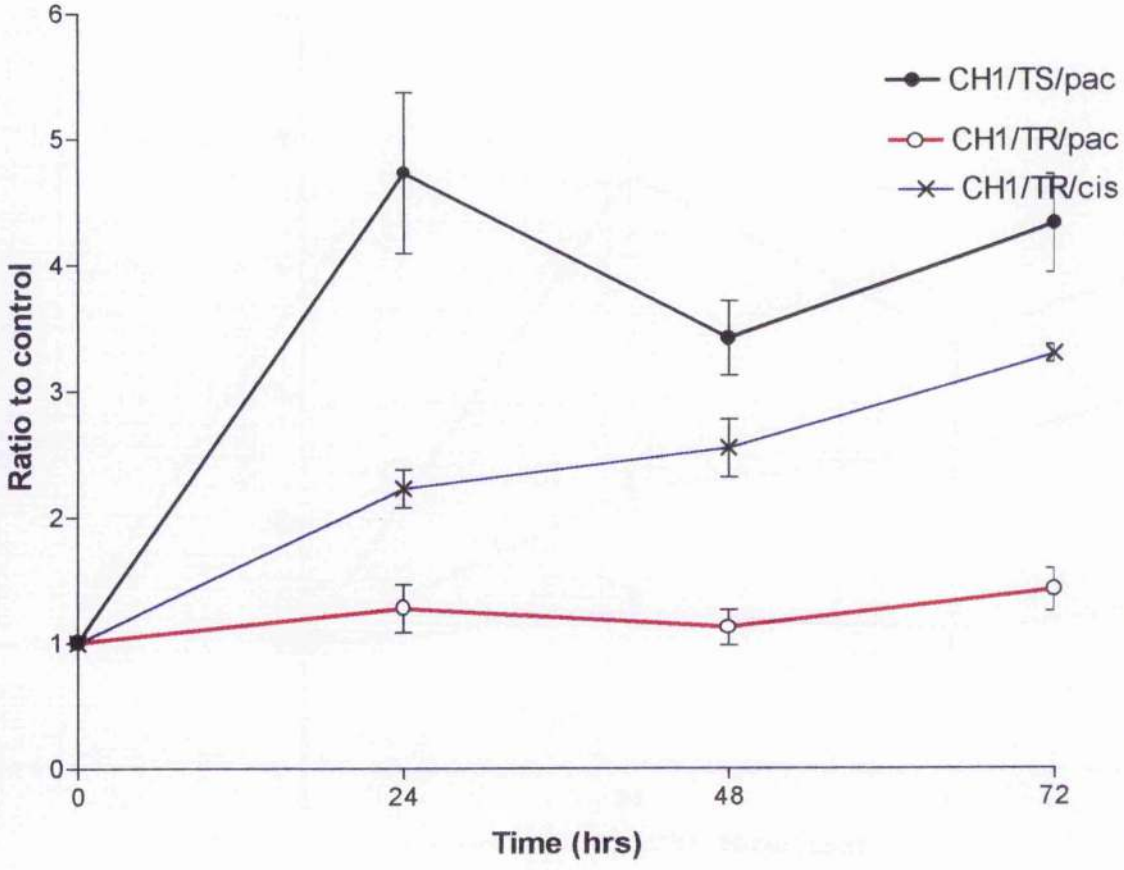


Figure 4-2: Mean p53 level changes ± SEM of CH1/TS and CH1/TR tumours following paclitaxel (pac) treatment and CH1/TR tumours following cisplatin (cis) treatment. The values at 24, 48, and 72 hrs are expressed as a ratio to control, at time 0 the ratio to control value is taken as 1.

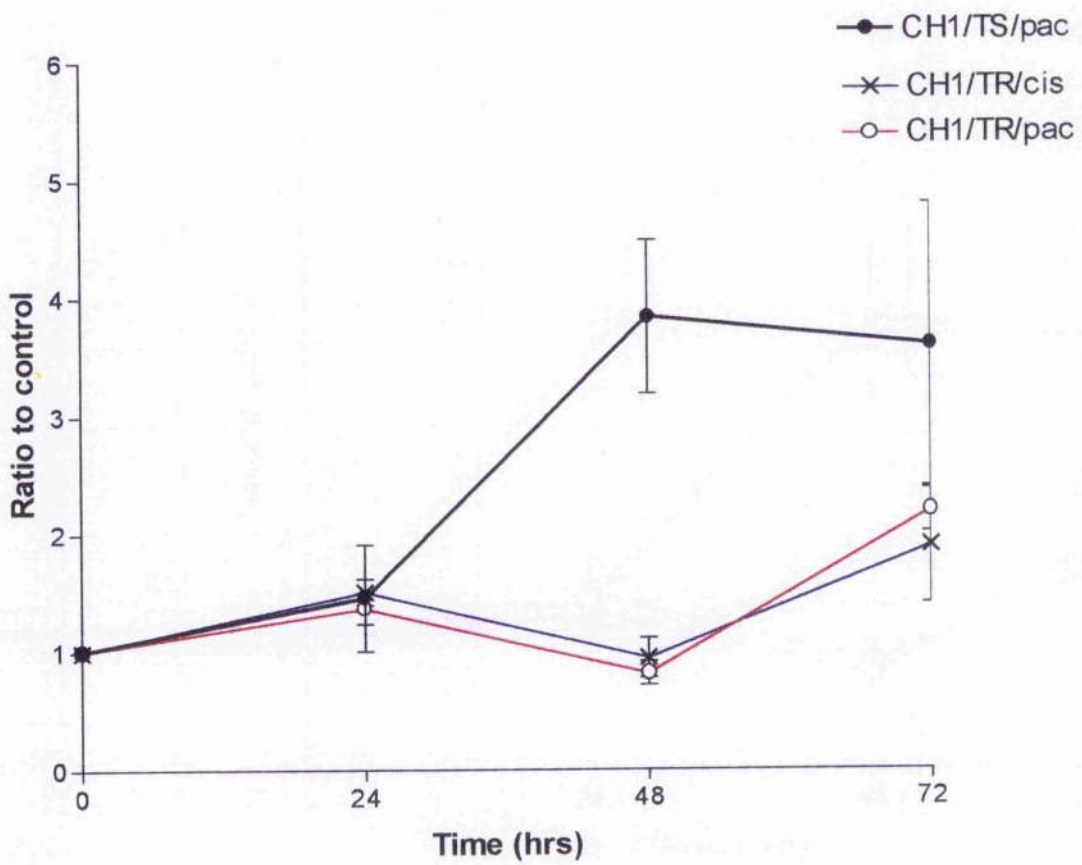


Figure 4-3: Mean p21 level changes ± SEM of CH1/TS and CH1/TR tumours following paclitaxel (pac) treatment and CH1/TR tumours following cisplatin (cis) treatment. The values at 24, 48, and 72 hrs are expressed as a ratio to control, at time 0 the ratio to control value is taken as 1.

4.6.2 The proapoptotic protein bax

Several in vitro studies have demonstrated that paclitaxel in some cell types is able to induce the pro-apoptotic protein bax through p53 induction pathway (Miyashita T, 1996; Miyashita T, 1994) (Jones NA, 1998). In contrast paclitaxel was not able to induce bax in other cell types (Liu QY, 1997). In the present study, there was marked induction of p53 proteins in CH1/TS tumours, but there was no significant increase in bax levels in CH1/TS except for mild elevation of bax protein at 48 hrs with mean fold increase of 1.21(table 3-10 and figure 3-22). In CH1/TR tumours there was no bax induction following either paclitaxel or cisplatin treatment (figure 3-23). These changes in bax levels are summarized in figure 4-4.The possibility of earlier than 24 hour bax protein peak induction can not be excluded. Similarly the possibility that other pro-apoptotic proteins such as bak or bad are induced can not be excluded.

4.6.3 The anti-apoptotic protein bcl-2

The anti-apoptotic protein bcl-2 has been shown to be phosphorylated in vitro following paclitaxel treatment leading to loss of anti-apoptotic properties and cell death (Blagosklonny MV, 1997; Blagosklonny MV, 1995; Blagosklonny MV, 1996). The molecular weight of bcl-2 protein 26 KD protein and its phosphorylated form in most cell run on SDS-PAGE under reducing condition at Mw of 27 KD.

In the present study, the immunoblotting of CH1/TS tumours revealed 26KD bcl-2 band and two other bands at MW 27 KD and 30 KD (figure 3-24). These two bands were detected in CH1/TS control tumours suggesting that these proteins are endogenously expressed, although 30 KD bcl-2 band was more prominent. It is noteworthy that the control CH1/TR tumours expressed the 26 bcl-2 faintly with no expression of 27 KD band. These observations are consistent with observation made by Tang et al and Guan et al described in section 3.5.4.

There were no major alterations in 30 KD bcl-2 band following paclitaxel treatment in CH1/TS tumours (table 3-10). However, there was decrease in intensity of 26 KD bcl-2 band

with increase in intensity of phosphorylated 27 KD band (figure 3-24). The further characterization of 27 KD and 30 KD bands by the incubation of CH1/TS protein samples with alkaline phosphatase led to attenuation of 27 KD bcl-2 band and intensification of the 26 KD bcl-2 band (figure 3-26). These observations suggest that 27 KD bcl-2 band is the phosphorylated form of bcl-2.

In contrast, the paclitaxel treated CH1/TR tumours express the 30 KD bcl-2 band prominently with no significant alteration over 72 hours following treatment (3-24). The expression of the two other bands varied over 72 hours following paclitaxel treatment. At 24 and 48 hours, there was faint expression of 26 KD bcl-2 band with no expression of 27 KD band. By 72 hours following the treatment, the CH1/TR tumours developed very prominent 26 KD bcl-2 band with faint expression of 27 KD band. Interestingly, the CH1/TR tumours that have demonstrated relative sensitivity to cisplatin showed that presence of bcl-2 30 KD and faint expression of 26 Kd bcl-2 band, with no expression 27 KD band (figure 3-25).

These observations rise several important points about bcl-2 mechanism:

(a) It remains obscure how the phosphorylated form of bcl-2 death induces cell death. Most in vitro suggest suggested that the inactivation of bcl-2 by phosphorylation result in the alteration in the balance between the pro-apoptotic and anti-apoptotic proteins (Blagosklonny MV, 1996). This result in increase of pro-apoptotic proteins such as bax with induction of bax. However, a recent study has challenged this theory and suggested that the phosphorylation of bcl-2 correlated with ability of paclitaxel to induce mitotic arrest and with apoptosis as discussed in section 1.4.2. (Ling YH, 1998)

(b) The initial low endogenous level of expression the 26 KD bcl-2 in CH1/TR tumours with marked increase by 72 hours following paclitaxel treatment and lack of significant phosphorylation may suggest that bcl-2 is functioning as survival pormoting protein which is not amenable to phosphorylation. It is not clear whether this pattern of expression in the resistant tumour is due to inherent defect at the protein level or due a more proximal defect as discussed in section 4.6.

(c) In the present study, we have demonstrated that the phosphorylated 27 KD band exist endogenously, which may reflect a degree of background apoptosis or mitotic. The

endogenous expression of phosphorylated band is consistent with previous reports. However, the administration of paclitaxel resulted in the relative expression of the unphosphorylated and phosphorylated bands.

4.6.4 The anti-apoptotic protein bcl-xl

It has been demonstrated that bcl-xl may undergo phosphorylation following paclitaxel treatment (Poruchynsky MS, 1998). However, in the present study there was no evidence of bcl-xl phosphorylation in either CH1/TS or CH1/TR tumours following paclitaxel treatment. However, there was statistically significant decline in bcl-xl levels in CH1/TS tumours following paclitaxel treatment. Following treatment with paclitaxel, the ratio bcl-xl levels to control in CH1/TS tumours was 0.63, 0.54, and 0.35 at 24, 48, and 72 hours (table 3-10, figure 3-27). In contrast, there was no similar decline in bcl-xl levels in CH1/TR tumours following paclitaxel treatment with the ratio to of bcl-xl levels to control in CH1/TR of 0.83, 0.98, and 0.83 at 24, 48, 72 hours respectively. Interestingly, Cisplatin induced a decrease in bcl-xl level in CH1/TR tumours, With ratio to control levels of 1.0, 0.73, and 0.46 at 24, 48, 72 hours respectively (figure 3-28). These changes in bcl-xl level are summarized in figure 4-5.

The decline in bcl-xl level may be due to the ability of bcl-2 family members –including bcl-xl to form heterodimers with pro-apoptotic proteins leading to the alteration of balance of these proteins (Kromer G, 1997; Reed JC, 1997a). The reduction in bcl-xl level has been previously shown to occur due to heterdimerization with pro-apoptotic proteins such as bax, bak, or bad (Ottilie S, 1997; Simonian PL, 1997; Wang K, 1998). In this study, The total levels of bax protein have been shown to be unchanged over 72 hours, however the induction of other pro-apoptotic proteins such as bik, bak, or bad can not be ruled out. In addition, the decline in total levels of bcl-xl has been shown to be due to down regulation of bcl-xl m-RNA. Paclitaxel treatment of prostate cell line (LNCaP cell) led to almost total down-regulation of bcl-xL protein in the absence of alteration of bax, bak, or bcl-2 levels, which was paralleled by a similar decrease in the level of bcl-xL mRNA, as demonstrated by reverse transcription-PCR (Liu QY, 1997).

Taken together the results from the present study and other studies suggest that the decline in bcl-xl levels may be induced by different chemotherapeutic agents such as paclitaxel and cisplatin. Of particular importance that the decline in bcl-xl levels in CH1/TR tumours occurred with cisplatin where a response has been demonstrated by growth studies, cell cycle analysis, induction of apoptosis, but not following paclitaxel administration where no such observation were made. These observations suggest that there is no inherent defect in bcl-xl protein and that the pathway involved in its down regulation was able to function following induction of cell death signal by cisplatin. Moreover, it suggests that the alteration in levels of bcl-xl may be a major determinant of cell death or survival following chemotherapy treatment.

4.6.5 Signal transduction molecule: Raf-1

It has been shown that the polymerization of microtubules leads to initiation of signaling cascade involved in alterations in p53, p21, bcl-2, and bcl-xl (Blagosklonny MV, 1997). The induction of p53 and p21 and phosphorylation of bcl-2 and bcl-xl was shown to be dependent on the signal transduction molecule c-Raf-1 (Blagosklonny MV, 1997; Blagosklonny MV, 1995; Poruchynsky MS, 1998). In the present study, there was no evidence of c-Raf-1 phosphorylation in either CH1/TS or CH1/TR tumours (figure3-29). A recent study suggested that c-Raf-1 phosphorylation may be dependent on paclitaxel concentration. The in vitro studies have shown that paclitaxel concentration greater than 9nM is needed before phosphorylation of c-Raf-1. Since a fixed dose of paclitaxel was used in this study, phosphorylation of c-Raf-1 can not ruled out with higher dose of paclitaxel, although the strain of nude mice used in this study do not tolerate significantly higher doses of drug in the schedule rate of administration used.

4.6.6 Summary of alteration in apoptosis regulating proteins following *paclitaxel treatment*

- Following paclitaxel treatment of CH1/TS tumours, there was evidence of induction of p53 and p21, bcl-xl down regulation, and phosphorylation of bcl-2. However, there was no bax or Raf-1 induction.
- Following treatment of CH1/TR tumours, there was no significant induction of p53, p21, or alterations in bcl-xl, bcl-2, bax, or Raf-1. However, cisplatin still induced p53 and down regulated bcl-xl in CH1/TR tumours with no bcl-2 phosphorylation or bax induction. These observations suggest that p53 pathway is intact in CH1/TR tumours, but paclitaxel fails to trigger the observed damage seen in CH1/TS tumours.

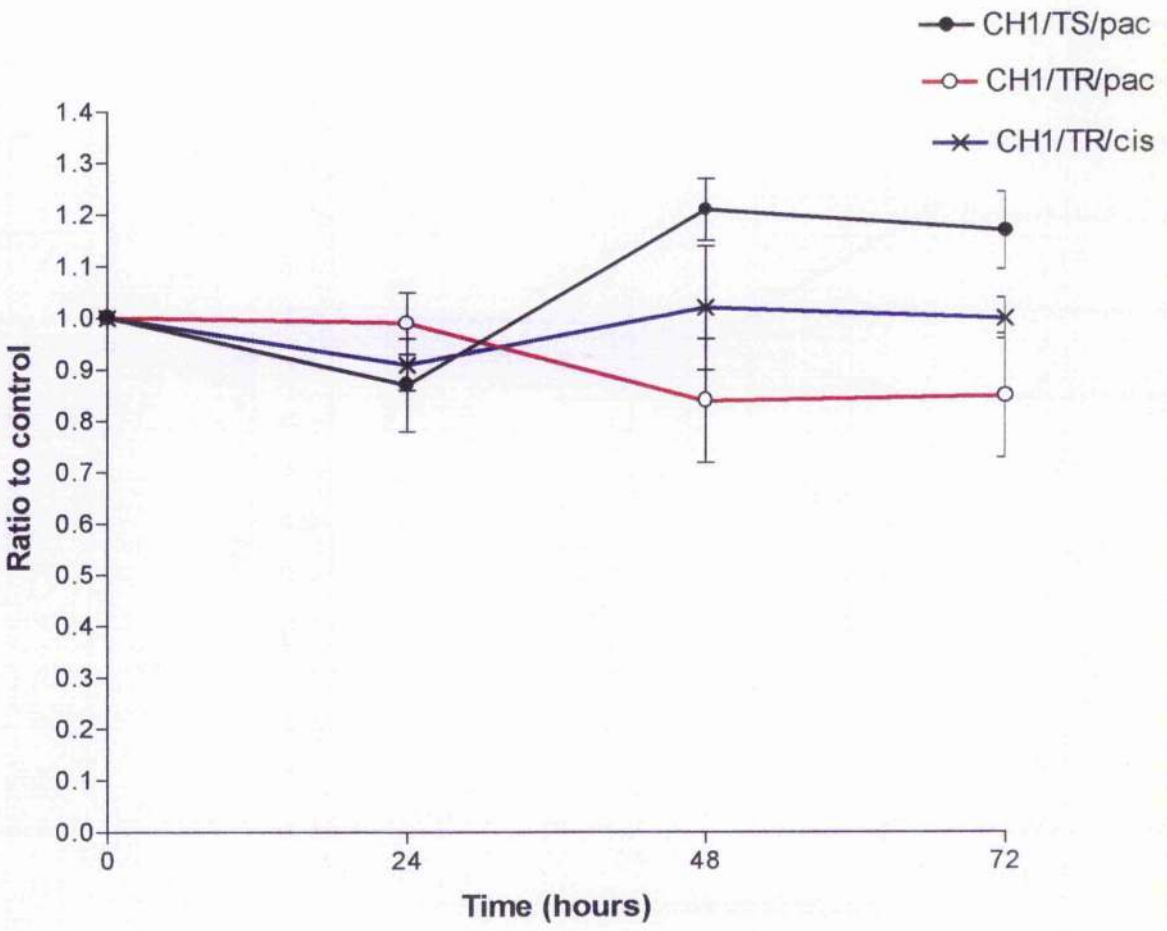


Figure 4-4: Mean bax level changes ± SEM of CH1/TS and CH1/TR tumours following paclitaxel (pac) treatment and CH1/TR tumours following cisplatin (cis) treatment. The values at 24, 48, and 72 hrs are expressed as a ratio to control, at time 0 the ratio to control value is taken as 1.

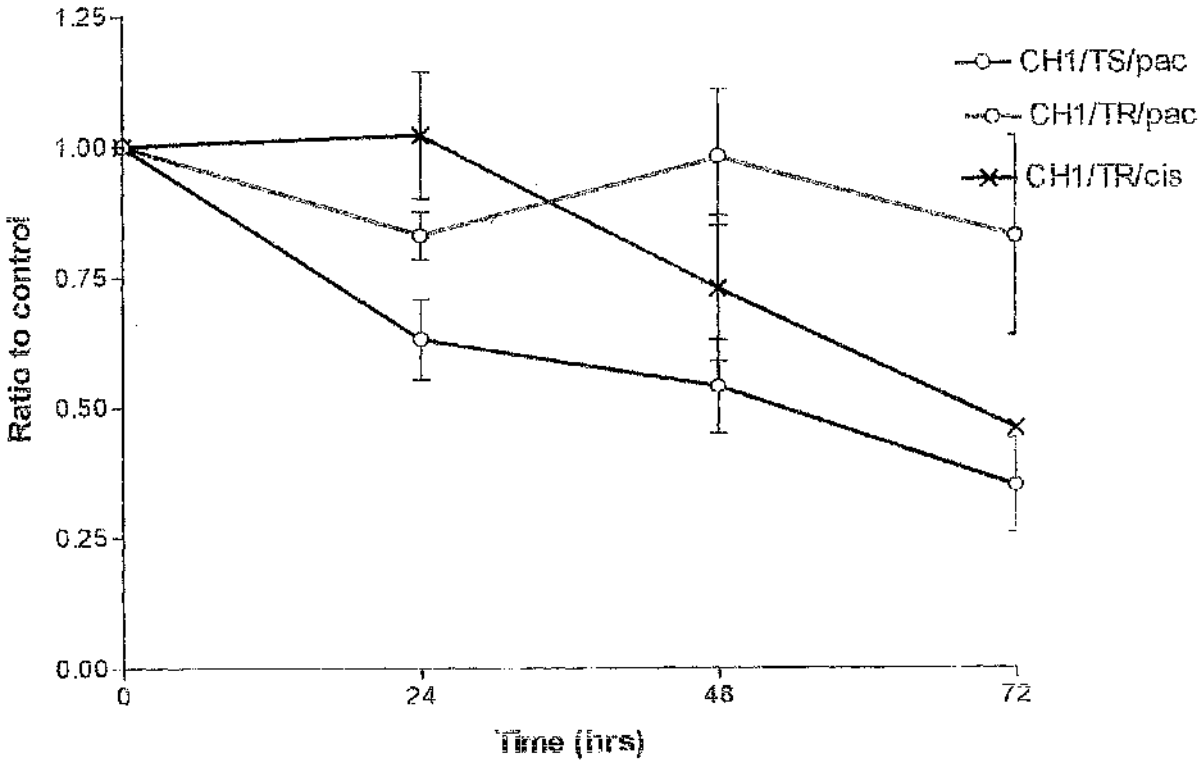


Figure 4-5. Mean bcl-xl level changes ± SEM of CH1/TS and CH1/TR tumours following paclitaxel (pac) treatment and CH1/TR tumours following cisplatin (cis) treatment. The values at 24, 48, and 72 hrs are expressed as a ratio to control, at time 0 the ratio to control value is taken as 1.

4.7 Proposed model for acquired resistance of CH1/TR tumours

Based on the observations of this in vivo study and other in vitro studies (Blagosklonny MV, 1997; Blagosklonny MV, 1995; Blagosklonny MV, 1996; Horwitz SB, 1993), a model could be proposed to explain the resistance of CH1/TR tumours. There was no difference in paclitaxel uptake between paclitaxel resistant and sensitive tumours suggesting a more distal defect to account for the resistance of CH1/TR tumours. In CH1/TR tumours, there was a failure to induce G2/M arrest and apoptosis. In vitro studies have shown that microtubules polymerization is needed for activation of a signal transduction molecule such as Raf-1 kinase, which in turn is involved in induction of p53 and p21 and phosphorylation (inactivation) of bcl-2 (Blagosklonny MV, 1997). It could be postulated that the lack of successful polymerization of microtubules may result in the lack of initiation of down stream pathways involved in apoptosis, including p53 and p21 induction, bcl-2 phosphorylation, and bcl-xl down regulation. The demonstration that these pathways can still be induced by cisplatin suggest that they remain intact, and further suggests that lack of response to paclitaxel is due to a proximal step possibly at the level of polymerization of microtubules.

4.8 Alteration of response to paclitaxel by overexpression of anti-apoptotic protein bcl-xl

Bcl-xl is a member of bcl-2 family of proteins, and shares significant homology with bcl-2 in its BH1 and BH2 regions. By alternate splicing, the gene encodes for two important protein isoforms of which the longer bcl-xl is an anti-apoptotic protein, whereas the shorter isoform bcl-xs is pro-apoptotic (Yang E, 1996).

Several in vitro studies have shown that the overexpression of the anti-apoptotic protein bcl-xl results in inhibition of apoptosis due to various stimuli including paclitaxel (Minn AJ, 1995; Tang C, 1994; Yang E, 1996). Furthermore, bcl-xl overexpression has been shown to inhibit paclitaxel-induced apoptosis in A2780ovarian cancer cell line, human acute myeloid leukaemia HL-60, prostate cell line (Ibrado AM, 1997; Liu JR, 1998). It has been postulated

that anti-apototic effect of overexpression bcl-xl is due to the formation of heterodimers between pro-apoptotic and anti-apoptotic proteins that alter their balance as discussed previously.

In the present study, we established successful xenografts of CH1 paclitaxel sensitive cell transfected with bcl-xl. In three separate experiments, the established CH1/TS, CH1/TS-bcl-xl, and CH1/TS-F250 (transfected by vector only) xenografts were treated with either paclitaxel or cisplatin. Figure 3-30 and 3-31 represents the growth curves of these three types of xenografts following paclitaxel and cisplatin. These results demonstrate that bcl-xl may modulate tumour response to paclitaxel. There was an initial partial response to paclitaxel but these tumours began to grow rapidly 12 days following last dose of treatment, while the CH1/TS and CH1/TS-250 demonstrated marked persistent sensitivity to paclitaxel. The specific growth delays were 66.4, 51.1, 34.9 days for CH1/TS, CH1/TS-F250, and CH1/bcl-xl respectively reflecting these tumours sensitivity to paclitaxel. The immunoblotting of bcl-xl protein showed that there was a three-fold increase in bcl-xl protein CH1/bcl-xl compared to CH1/TS tumours (figure 3-33). The initial response to paclitaxel may be explained by the possibility of heterogeneous expression on bcl-xl within the tumour following transfection; thus cell expressing no or small amount may undergo cell death leaving resistant focus of cells expressing of bcl-xl that grow rapidly subsequently.

The paclitaxel uptake studies showed that there was no significant difference in intra-tumour paclitaxel concentration between CH1/TS and CH1/bcl-xl, hence excluding any drug efflux mechanism contributing to resistant phenotype. These results are consistent with previous in vitro studies which demonstrated that bcl-xl overexpression does not alter paclitaxel uptake and more importantly does not affect tubulin polymerization following paclitaxel treatment (Ibrado AM, 1997). The observations from this study and previous in vitro suggest that high endogenous levels of bcl-xl may represent a distal mechanism which opposes drug induced apoptosis. The mechanisms by which bcl-xl inhibit cell death remain unclear, however it has been suggested that overexpressed bcl-xl reduce the amount of free bax induced by paclitaxel resulting prevention of cell death.

The hypothesis that bcl-xl may represent a distal mechanism of resistance is strengthened by results following treatment of CH1/bcl-xl by cisplatin treatment of treatment. The CH1/bcl-xl tumours displayed a very short partial response to cisplatin, which was maintained for three days only post last dose of treatment followed by rapid growth of the tumours with growth delay of 24.6 days only. The CH1 tumours showed marked sensitivity to cisplatin with growth delay of more than 66.4 days. However, CH1/F 250 tumours displayed some resistance to cisplatin with a good initial partial response achieved that was maintained for 22 days followed by growth of these tumours and growth delay of 26.6 days. The partial resistance displayed by CH1/F250 may be explained on the basis that cisplatin act on DNA where the DNA viral vector is incorporated; hence it may influence the response to cisplatin.

Overall these in vivo results are consistent with previous in vitro results suggesting that the overexpression of anti-apoptotic protein bcl-xl may result in modulation of response to chemotherapy – in particular paclitaxel- by acting through a distal mechanism. This observation could gain clinical significance because of demonstration that ovarian cancer cells with de novo in previous in vitro studies high levels of bcl-xl, which may represent a target for modulation.

Chapter 5: Conclusion and clinical implications

5.1 Paclitaxel and apoptosis: Implications for resistance

Paclitaxel has established an evolving role in the management of ovarian cancer. The demonstration in this in vivo study and several in vitro studies that cisplatin and paclitaxel have different cellular targets suggest that the combination of both drugs may overcome the resistance to either of them. The biological growth studies have demonstrated that cisplatin induced response in CH1/TR tumours where there was resistance to paclitaxel. Similarly, the demonstration in vitro that p53 status influences the outcome in terms of response and survival when cisplatin used, but not with paclitaxel suggest that combination chemotherapy may be more useful. In fact, at least two randomized trails using combination cisplatin and paclitaxel adjuvant treatment have shown survival advantage for patients treated with combination chemotherapy, further strengthening this argument (McGuire WP, 1996; Stuart G, 1998). However, the emergence of resistance to paclitaxel is likely to become an increasing problem, and thus a reason for failure of the therapy of ovarian cancer. Understanding the molecular pathways involved in the development of resistance to paclitaxel may provide suitable targets for novel therapies to help to circumvent resistance to paclitaxel.

Previous in vitro studies have identified three main mechanisms of resistance to paclitaxel. The first two mechanisms operate proximally and include the expression of MDR phenotype and the alterations in polymerization of the microtubules. The third mechanism operates distally, involving an expanding family of apoptosis regulating proteins which include p53, the pro-apoptotic protein bax, and the anti-apoptotic proteins bcl-2 and bcl-xl. The ability of paclitaxel to induce bcl-2 phosphorylation, induce bax expression, and down regulate bcl-xl has been shown to induce cell death in vitro. Hence, these apoptosis regulating proteins were the focus of the present in vivo study as they may either represent oncogenes responsible for acquired resistance, or alternatively potential targets for molecular intervention to circumvent resistance to paclitaxel.

5.1.1 Apoptosis regulating proteins: Changes with acquired resistance

Despite the abundance of in vitro studies, there is a paucity of information in vivo regarding paclitaxel's mechanism of action and the basis for acquired resistance. An ideal scenario would be to study xenografts established from cells of patients before and following the development de novo resistance to avoid cell selection pressures induced by in development of acquired resistance in vitro (Kaye SB, 1996a). However, this approach is hindered by difficulty of obtaining adequate samples, propagation of cells, and establishing successful xenografts. Nevertheless, the present study provided an insight to mechanism of action and resistance of paclitaxel using xenografts of cell lines which had acquired resistance in vitro.

In the present study, we demonstrated that following intraperitonial delivery sufficient intra-tumoural paclitaxel concentrations were achieved to induce cytotoxicity, with no significant uptake difference between the sensitive and resistant tumours. In this model therefore, an MDR-like phenotype is not responsible for resistance to paclitaxel.

The cell cycle analysis and proliferation studies provided an insight into the mechanisms of action of paclitaxel and cisplatin in vivo. In CH1/TS tumours paclitaxel induced G2/M arrest with inhibition of proliferation at 72 hours following treatment. In contrast, cisplatin induced accumulation of cells in S phase with an early inhibition of cell proliferation. These observations reflect the damage induced by paclitaxel and cisplatin on their known targets, the microtubules and DNA, respectively, and are consistent with in vitro studies (Demarcq C, 1992; Ormerod MG, 1996; Rowinsky EK, 1990). Furthermore, it was demonstrated that in paclitaxel resistant tumours (CH1/TR) there were no cell cycle alterations following paclitaxel, although accumulation of cells in S phase was still obsereved following cisplatin. This would be consistent with a phenotype of acquired resistance to paclitaxel where the drug for whatever reason, failed to induce G2/M arrest, yet the same cells remained sensitive to DNA damage with capacity to induce cell cycle arrest.

More importantly, we demonstrated that the cell cycle alterations induced in CH1/TS tumours following paclitaxel and cisplatin treatment, and in CH1/TR tumours following

cisplatin treatment only, were accompanied by significant apoptosis. The failure of paclitaxel to induce G2/M arrest and apoptosis in CH1/TR tumours despite adequate levels of the drug are suggestive of an acquired inability to trigger apoptosis.

The contribution of apoptosis regulating proteins to the induction of cell death and resistance by paclitaxel was evaluated. We demonstrated that following paclitaxel treatment of CH1/TS tumours, there was significant induction of p53, evidence bcl-2 phosphorylation, and down regulation of bcl-xl. Again, these observations are consistent with in vitro studies that are associated with an ability of paclitaxel to induce cell death. Following paclitaxel, the CH1/TR tumours failed to demonstrate significant p53 induction, bcl-2 phosphorylation, or down regulation of bcl-xl. This raises a question as to whether these differences between sensitive tumours in expression of apoptosis regulating proteins following paclitaxel are due to an upstream defect or an inherent defect in expression of these proteins. The demonstration that cisplatin was able to induce significant levels of p53, down regulate bcl-xl, and induce cell death in CH1/TR tumours suggests that the acquired resistance to paclitaxel is not associated with an inherent defect in the distal pathways, but instead due to an upstream defect possibly at the level of microtubules. It is known that successful polymerization of microtubules is required for induction of cell death, induction of p53 and p21 and phosphorylation of bcl-2 (Blagosklonny MV, 1997).

It has been suggested by several in vitro studies that induction of p53 by paclitaxel may result in significant induction of the pro-apoptotic protein bax, and that this may contribute to a p53-dependent mechanism of cell death (Miyashita T, 1996; Miyashita T, 1994). However, in the present study, we observed no significant induction of bax despite a significant induction of p53. A similar observation has been made in vitro where induction of other pro-apoptotic proteins other than bax were found, such as bak (Liu QY, 1997). The role of p53 in regulating pro-apoptotic genes in response to paclitaxel remains an area for future study.

There has been recent debate about the role of phosphorylation of the anti-apoptotic protein bcl-2 following paclitaxel, and in particular whether this inactivates

bcl-2 and contributes directly to cytotoxic drug-induced cell death. While we detected evidence for phosphorylation of bcl-2 in CH1/TS cells after paclitaxel, we failed to see significant phosphorylation (activation) of Raf-1 kinase which is thought to be the mediator of paclitaxel induced phosphorylation of bcl-2. An absence of phosphorylated bcl-2 was seen in control untreated CH1/TR resistant versus CH1/TS sensitive cells, with a complete failure of paclitaxel to trigger subsequent phosphorylation of bcl-2. This may relate to differences in endogenous expression of bcl-2 which could account for a failure to trigger apoptosis. Alternatively, there may be a difference in cell kinetics in the resistant tumours with a failure to undergo any mitotic arrest which could be related to an acquired tubulin defect, resulting in a failure to see any phosphorylated bcl-2. This would be the case if as Ling et al suggest that the phosphorylation of bcl-2 is a marker of M-phase events and not a determinant of apoptosis.

Overall, in this model, we failed to see significant endogenous differences in apoptosis regulating proteins in the CH1/TR tumours to account for acquired resistance to paclitaxel. Indeed, their continued expression in response to cisplatin further supports the argument that acquired resistance to paclitaxel occurs because of a failure to trigger changes in levels of these proteins, rather than inherent differences in their expression. The likely level of this could be at the microtubules where an acquired defect in tubulin structure, such as overexpression or point mutations of β-tubulin isotypes which have been recognized (Giannakakou P, 1997; Horwitz SB, 1993; Kavallaris M, 1997), fails to allow a cytotoxic message to be delivered to cells. Assays are available to measure the polymerization of microtubules in vitro (Blagosklonny MV, 1997; Kavallaris M, 1997), and studies are now undergoing to see if polymerization of CH1/TR Cells is in any way altered compared with CH1 cells.

5.1.2 Apoptosis regulating proteins: Overexpression modulating resistance

In the second part of this study, we demonstrated that the overexpression of the anti-apoptotic protein bcl-xl resulted in development of resistance both to paclitaxel and cisplatin. This observation was particularly important because it demonstrated that these proteins do control a common pathway controlling cell death, and further underline their importance as possible targets for molecular therapy.

Other potential molecular therapeutic interventions may include:

(1) Downregulation of anti-apoptotic proteins such as bcl-xl and bcl-2 by the development of antisense oligonucleotide to bcl-2 or bcl-xl. The aim of this approach would be to reduce the balance of anti-apoptotic proteins, thus rendering the cell more susceptible to an apoptotic stimulus, either endogenously or following chemotherapy. In fact, the antisense bcl-2 oligonucleotide is already in clinical trails and has shown promising results in lymphoma (Reed JC, 1997b; Webb A, 1997).

(2) Overexpression of pro-apoptotic proteins such bax or bad using gene therapy. Such an approach has already been shown to improve chemosensitivity to paclitaxel in vitro (Strobel T, 1996; Strobel T, 1998b). It is proposed to conduct more experiments where by the pro-apoptotic protein bax is overexpressed in both CH1/TR and CH1/bcl-xl cells to see whether the previously demonstrated resistance in these tumours. This is particularly important because if the overexpression of bax were able reverse resistance to paclitaxel in both CH1/TR cells (where there is no endogenous difference in levels of anti-apoptotic proteins, except perhaps an absence in levels of phosphorylated bcl-2), and in CH1/bcl-xl cells (where overexpression of anti-apoptotic protein induced resistance to paclitaxel). If resistance were overcome in both cells, this would support a strategy of up regulating tumour levels of pro-apoptotic proteins as a general approach to bypass multiple mechanisms of resistance.

Finally, the signal transduction pathway between microtubule polymerization and alteration in apoptosis regulating proteins remains obscure. Ultimately this may provide a clue as to the basis of acquired resistance to paclitaxel in CH1/TR cells, and further studies of Raf-1 kinase are merited. A better understanding of this pathway of may provide important new targets for molecular intervention.

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