Cyclin D and cisplatin cytotoxicity in primary neuroblastoma cell lines

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Cyclin D1 is a key cell cycle regulator protein with demonstrated oncogenenic activity in a variety of malignancies. Overexpression of Cyclin D1 protein has been observed in many types of tumors. We hypothesized that Cyclin D1 might be an important determinant of the sensitivity of neuroblastomas to cisplatin. Cyclin D1, D2 and D3, and Cdk4, Cdk6 and Rb protein, and Cyclin D1 mRNA expression were measured in primary patient-derived neuroblastoma cell lines. Cell cycle distribution was examined using flow cytometry. A modified MTT assay was used to determine the sensitivity of the cell lines to cisplatin. All 14 cell lines expressed Cyclin D1 protein to a variable extent (0.22–1.47 normalized to actin protein expression). All cell lines expressed Cyclin D2 and D3. There was no relationship between expression of Cyclin D1 and expression of Cyclin D2 or D3 ($p > 0.05$ and $R^2 < 0.2$ for both). All cell lines expressed Cdk4 and Cdk6 protein. In addition, Rb and two related proteins, p105 and p130, were detected in all the cell lines. The mean cisplatin IC_{50} was 19.2 μM (range 0.6–40 μM). We conclude that there was no correlation between the amount of Cyclin D1 expressed and the cisplatin IC_{50}. Our results do not support the hypothesis that Cyclin D1 expression is significantly related to cisplatin resistance. ***Anti-Cancer Drugs*** **15:883–888 © 2004 Lippincott Williams & Wilkins.**

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Introduction

Cyclin D1, also known as PRAD1 or CCND1 [1], can be induced by growth factors, associates with Cdk4 or Cdk6 [2] and participates in the regulation of proliferation through the G_1 checkpoint by regulation of pRb phosphorylation [3]. Studies in adult malignancies have suggested that Cyclin D1 overexpression is associated with advanced cancers or poor prognosis tumors [4–6]. In most cases, overexpression appears to result from amplification of chromosome 11q13, the region to which the Cyclin D1 gene maps; in some cases Cyclin D1 mRNA is overexpressed in the absence of gene amplification [5]. Tumorigenicity is believed to result from Cyclin D1's proliferative influence [5]. Cyclin D1 expression has not been widely studied in pediatric cancers.

Neuroblastoma is the most common extracranial solid malignancy of childhood [7]. This tumor is unique among childhood cancers because of its ability to regress or differentiate spontaneously, especially in infants. However, in older children, especially those with disseminated tumors or tumors with unfavorable biologic features, outcome is poor despite the use of aggressive multimodality therapy. Most children older than 1 year of age with neuroblastoma are treated with chemotherapy. Platinum-based therapy is a cornerstone of many regimens for the treatment of neuroblastoma. Cisplatin acts as a non-classical alkylating agent, covalently binding to DNA and causing interstrand and intrastrand crosslinks [8]. Although most neuroblastomas will respond initially to such therapy, those with poor prognostic features usually recur. Although dysregulation in the G_1 Cdk4/Cdk6/Cyclin D1/pRb pathway has been reported in neuroblastoma, the role of Cyclin D1 itself has not been thoroughly explored [9–11].

Expression of Cyclin D1 has been linked to tumor sensitivity to cisplatin in *in vitro* models. Increased Cyclin D1 expression is associated with increased resistance to cisplatin in fibrosarcoma cell lines [12], while use of antisense Cyclin D1 to decrease Cyclin D1 protein expression results in increased sensitivity to cisplatin [13]. Because cisplatin is an important part of chemotherapeutic regimens for neuroblastoma, we investigated the relationship between Cyclin D1 expression and cisplatin sensitivity in primary neuroblastoma tumor cell lines.

Experimental design

Cell lines

This study was approved by the Institutional Review Board. After informed consent was obtained according to

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federal and institutional guidelines, tumor samples were collected from 12 patients being treated for neuroblastoma at Texas Children's Cancer Center. The samples were obtained at the time of diagnosis, second-look surgery or relapse. After surgery, the tumor tissue was placed in MEM with 10% FBS, 1% penicillin/streptomycin/fungizone and minced using disposable scalpels. The medium was carefully removed, fresh medium containing collagenase was added and cultures were incubated overnight. The next day the tumor cells were suspended by pipeting up and down repeatedly, then placed over a cell strainer to exclude large pieces. The cell strainer was washed with medium. Cells were then centrifuged, resuspended in MEM medium containing 40% FBS and cultured in uncoated flasks. For tumor derived from bone marrow specimens, the marrow samples were placed in flasks with an equal volume of complete medium and incubated overnight. The next day, the samples was placed into a new flask and the original flask washed several times with Hank's balanced salt solution before adding fresh medium. The second flask was treated in the same way the following day. Cells were confirmed to be neuroblastoma-derived on the basis of electron microscopy, immunohistochemistry, and/or expression of GD2 antigen.

Two patients' tumor samples were split, with one aliquot processed as described above and one processed as above except that tissues were left in collagenase for a maximum of 60 min and the cell lines were grown in RPMI medium with 10% fetal calf serum in EHS coated flasks. In the following tables lines P111 and GP175 were derived from the same patient sample using the two different techniques, as were lines P73 and GP150.

Expression of Cyclin D1, D2, D3, Cdk4 and Cdk6

Protein expression was measured by Western blotting. Cells were cultured at 4×10^5 per T25 flask for 72 h at 37°C in 5% CO_2. They were then lysed in ice-cold lysis buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 1% Triton X-100, 2 mM EDTA) for 30 min, the debris pelleted and the supernatant collected. The protein concentrations in these extracts were estimated by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Then, 10 µg of protein was loaded onto 12.5% SDS–PAGE gel (10% SDS–PAGE gel for Rb protein analysis). The samples were then transferred to a nitrocellulose membrane as per the manufacturer's instructions (Bio-Rad). Protein loading was visualized by Ponceau S staining. The membrane was incubated with anti-Cyclin D1, anti-Cyclin D2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cyclin D3 (Transduction, Lexington, KY), anti-Rb, anti-Cdk4 or anti-Cdk6 (Santa Cruz Biotechnology), for 2 h at room temperature. The membrane was then washed three times with TBS buffer with 0.1% Tween-20, incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature and washed again. For Cyclin D1 after hybridization with primary and secondary antibody and enhanced chemiluminescence (ECL; Amersham Pharmacia, Piscataway, NJ), the membranes were immediately exposed to film. Quantitation was by densitometry (Molecular Dynamics, Sunnyvale CA). The expressions of Cyclin D2 and D3 were developed using ECL-plus. Signals from the membranes were directly detected using a Storm imager (Molecular Dynamics). Imagequant software (Molecular Dynamics) was used for quantitation of the signal in both cases. Protein expression was normalized to actin in all experiments.

RT-PCR

Cyclin D1 mRNA was assayed by RT-PCR. Cells were cultured on a Petri dish for 72 h. The total RNA was isolated by using TRIzol reagent according to the manufacturer's instruction (GIBCO, Grand Island, NY). RNA (2 µg) was reverse-transcribed into cDNA using the Supertranscript II RNase H reverse transcriptase (GIBCO). PCR was performed using 2 µl of cDNA in a 20-µl reaction volume. The primers used were: Cyclin D1, full-length 5′-TGC CCA GGA AGA GCC CCA GCC-3′ (sense) and 5′-CCC TCA TAT GTC CAC GTC CCG C-3′ (anti-sense). The Cyclin D1 amplification was performed in 10 mM Tris–HCl (pH) 8.3, 25 mM KCl, 1.5 mM $MgCl_2$, 1% DMSO, 200 µM dNTP with 0.2 µM of each primer and 1 U Taq DNA polymerase. An initial 3-min incubation at 95°C was followed by 30 cycles of 30 s 95°C, 15 s 55°C, 30 s 72°C. PCR products were analyzed on a 1.5% agarose gel in 1 × TAE.

Cell cycle analysis

Cells in exponential growth were cultured for 72 h then washed twice with PBS, pelleted and resuspended in 100 µl of PBS followed by dropwise addition of 2 ml of 70% alcohol. Cells were frozen at –20°C overnight, then resuspended in 5 ml of cold PBS buffer and incubated on ice for 10 min. They were then stained in PBS buffer with 0.1% Triton X-100 with propidium iodide solution containing 20 µg/ml of RNase A. Cells were incubated at 37°C for 10 min. Flow cytometry analyses were performed within 2 h after cells were stained.

Cytotoxicity assay

A modified MTT assay [14,15] was used to determine the sensitivity of the cell lines to cisplatin. Aliquots of 135 µl of 1×10^5 cells/ml of each cell line was plated into 96-well microtiter plates. Twenty-four hours later cisplatin at specified concentrations was added to each well. Replicates of six wells were used at each concentration. After 72 h, 15 µl of MTT (5 mg/ml) was added to each well, the plates were incubated for 4 h at 37°C, the medium was replaced with 150 µl of DMSO to solubilize the formazan and the optical density (OD) was measured at 550 nm using a microplate spectrophotometer (Anthos

Analytical, Durham, NC). Cell survival was calculated by subtracting the background OD of media alone and then dividing the OD of test wells by the OD of the control (untreated) wells. The IC_{50}, or concentration required to inhibit 50% of control cell growth, was determined from the graph of drug concentration versus survival.

N-*myc* amplification

N-*myc* amplification was determined from the clinical laboratory reports based on biopsy specimens and not directly determined in the cultured cells.

Statistical analysis

All statistical analyses were performed using StatView (Abacus Concepts, Berkeley, CA). Correlation between Cyclin D1 expression and cisplatin IC_{50}, as well as all other correlations, were determined by linear regression.

Results

Expression of Cyclin D1, D2, D3, Cdk4/6 and pRb in NB cell lines

All 14 cell lines expressed Cyclin D1 protein (Table 1 and Fig. 1) to a variable extent (0.22–1.47 normalized to actin protein expression). All cell lines expressed Cyclin D2 and D3. There was no relationship between expression of Cyclin D1 and expression of Cyclin D2 or D3 ($p > 0.05$ and $R^2 < 0.2$ for both).

All cell lines expressed Cdk4 and Cdk6 protein. In addition, pRb and two related proteins, p105 and p130, were detected in all the cell lines (Figs 2 and 3).

Cell cycle distribution

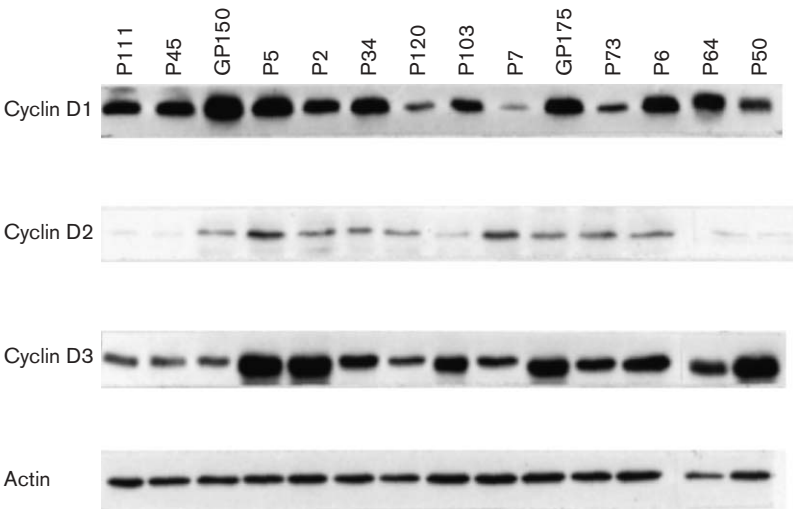
Cell cycle distribution varied among the cell lines. There was no correlation between Cyclin D1 presence or

Table 1 Cyclin D expression (normalized to actin), cell cycle distribution and cisplatin IC_{50} in 14 primary neuroblastoma cell lines

Cell line	Cyclin D1	Cyclin D2	Cyclin D3	% G_0–G_1	% S + G_2/M	Cisplatin IC_{50}	N-*myc* amplified
P111[a]	1.18	0.41	0.48	63	37	40.6	unknown
P45	0.40	0.25	0.78	63	37	30	no
GP150[b]	1.18	0.29	0.75	67	33	26.3	no
P5	0.22	0.24	0.80	66	34	22.9	no
P2	0.60	0.38	0.95	58	42	21.6	no
P34	0.27	0.42	0.50	78	22	21.6	no
P120	1.19	0.22	0.66	65	32	18.6	no
P103	0.67	0.27	0.64	62	38	17.6	no
P7	1.14	0.20	1.10	70	30	17	no
GP175[a]	1.47	0.47	0.59	52	48	15.3	unknown
P73[b]	0.94	0.21	1.43	35	65	12.6	no
P6	0.66	0.30	1.06	60	40	11.1	no
P64	1.32	0.22	0.48	73	27	10.4	yes
P50	0.56	0.26	0.65	55	45	3.7	no

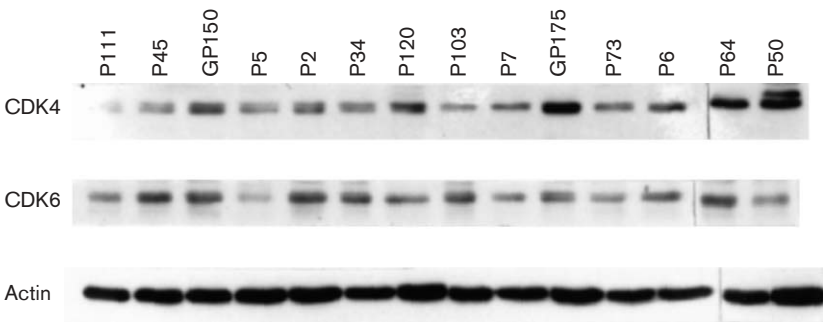
[a]P111 and GP175 were derived from the same patient sample using two different culture techniques.
[b]P73 and GP150 were derived from the same patient sample using two different culture techniques.

Fig. 1



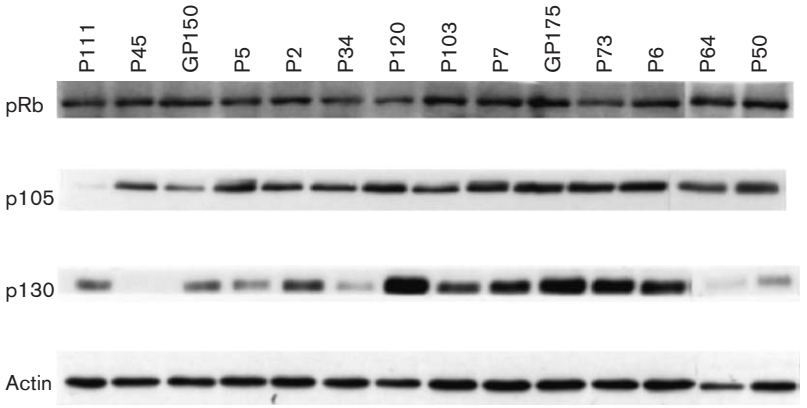
Western blot showing Cyclin D1, D2, D3 and actin expression in 14 patient-derived neuroblastoma cell lines.

Fig. 2



Western blot showing Cdk4, Cdk6 and actin expression in 14 patient-derived neuroblastoma cell lines.

Fig. 3



Western blot showing pRb, p105, p130 and actin expression in 14 patient-derived neuroblastoma cell lines.

absence or Cyclin D1 amount and cell cycle distribution. Furthermore, there was no correlation between percentage of cells in G_0/G_1 versus S + G_2/M and cisplatin cytotoxicity.

Cisplatin cytotoxicity

The IC_{50} values for cisplatin varied nearly 70- fold among the different cell lines (Table 1), with a mean IC_{50} of 19.2 μM (range 0.6–40 μM). There was no correlation between the amount of Cyclin D1 expressed and the cisplatin IC_{50}. There was a weak correlation between Cyclin D2 expression and cisplatin IC_{50} ($p = 0.049$, $R^2 = 0.20$; Fig. 4). There was no correlation between Cyclin D3 expression and IC_{50}.

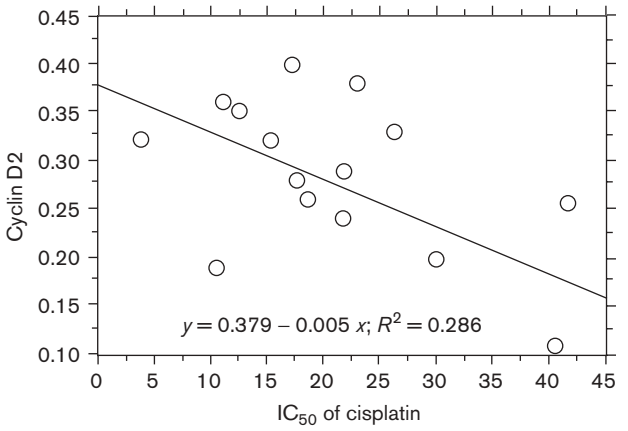
N-*myc* was not amplified in 10 patients (corresponding to 11 cell lines) and was amplified in one patient (corresponding to one cell line). N-*myc* status was unknown in one patient (corresponding to two cell lines).

Discussion

In this study, we have characterized the expression of Cyclin D1, D2 and D3, as well as Cdk4, Cdk6 and pRb in 14 primary human neuroblastoma cell lines. We found no correlation between Cyclin D1 expression and cisplatin IC_{50}, and only a weak correlation between Cyclin D2 correlation and cisplatin IC_{50}.

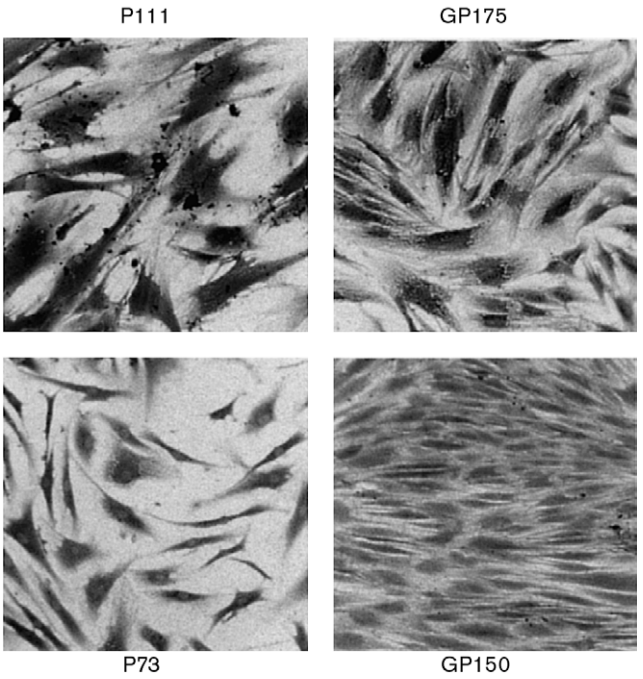
Other studies have suggested that Cyclin D1 may play an important role in tumor response to chemotherapy. Forced overexpression of Cyclin D1 in human fibrosarcoma cells resulted in an increase in the fraction of cells in S phase, increased phosphorylation of pRb, and increased dihydrofolate reductase mRNA and protein expression. These cells were relatively resistant to methotrexate, presumably as a consequence of their increased levels of dihydrofolate reductase. In contrast, there was no increase in resistance to cytarabine or paclitaxel. The authors concluded that perturbations of Cyclin D1 expression in particular tumor types could have important

Fig. 4



Poor correlation between Cyclin D2 expression and cisplatin IC_{50}.

Fig. 5



Morphologic differences in different cell lines derived from the same patient sample using two different culture techniques (P111 and GP175 were derived from the one patient sample as were P73 and GP150).

implications for the activity of specific chemotherapeutic drugs [16]. Conversely, when pancreatic tumor cells were transfected with constructs expressing Cyclin D1 antisense RNA, the antisense expressing clones contained reduced amounts of Cyclin D1 and were much more sensitive to cisplatin than the non-transfected control cells [13]. In a study involving predominately adult tumor cell lines, Warenius *et al.* investigated the relationship between endogenous Cyclin D1 expression and cisplatin and radiation cytotoxicity. High Cyclin D1 expression was correlated with cisplatin resistance, but not with radiosensitivity [12]. One critical determinant of cisplatin resistance is increased repair of DNA adducts, possibly by the 'mismatch repair' mechanism [17]. Warenius *et al.* postulated that cell lines with a higher Cyclin D1 content may traverse S phase more slowly, thus potentially allowing more time for DNA repair to take place [12].

Although the cisplatin IC_{50} varied 70-fold among the cell lines we studied, the Cyclin D1 expression normalized to actin varied less than 10-fold (0.22–1.47). It is possible that we did not see a correlation between Cyclin D1 and cisplatin IC_{50} simply because our cell lines did not exhibit enough variability in Cyclin D1 expression to affect cisplatin IC_{50}. If this is the case, then there still could be a correlation and the expression of Cyclin D1 in neuroblastoma cells could be clinically relevant. In addition, the effect of cell culture technique on the differentiation and maturation of cell lines is well described [18,19]. We noted that cell lines derived from the same patient but cultured differently demonstrated differences in Cyclin D1 expression and cisplatin sensitivity, as well as obvious morphologic differences (Fig. 5). These results suggest that culture techniques must be standardized if *in vitro* testing for gene expression and chemosensitivity is to be used as a diagnostic or therapeutic tool. At present, however, our results do not support the hypothesis that Cyclin D1 expression is significantly related to cisplatin resistance in neuroblastoma.

References

1 Sherr C. Mammalian g1 cyclins. *Cell* 1993; **73**:1059–1065.
2 Hirama T, Koeffler H. Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood* 1995; **86**:841–854.
3 Beijersbergen R, Bernards R. Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. *Biochim Biophys Acta* 1996; **1287**:103–120.
4 Bartkova J, Lukas J, Muller H, *et al.* Cyclin d1 protein expression and function in human breast cancer. *Int J Cancer* 1994; **57**:353–361.
5 Hall M, Peters G. Genetic alterations of cyclins, cyclin dependent kinases, and cdk inhibitors in human cancer. *Adv Cancer Res* 1996; **68**: 67–108.
6 Motokura T, Arnold A. Cyclins and oncogenesis. *Biochim Biophys Acta* 1993; **1155**:63–78.
7 Brodeur G, Castleberry R. Neuroblastoma. In: Pizzo P, Poplack D (editors): *Principles and Practice of Pediatric Oncology*. Philadelphia, PA: Lippincott-Raven; 1997. pp. 761–797.
8 Balis F, Holcenberg J, Poplack D. General principles of chemotherapy. In: Pizzo P, Poplack D (editors): *Principles and Practice of Pediatric Oncology*. Philadelphia, PA: Lippincott-Raven; 1997. pp. 215–272.
9 Easton J, Wei T, Lahti J, *et al.* Disruption of the cyclin D/cyclin-dependent kinase/ink4/retinoblastoma protein pathway in human neuroblastoma. *Cancer Res* 1998; **58**:2624–2632.
10 Diccianni M, Omura-Minamisawa M, Batova A, *et al.* Frequent deregulation of p16 and the p16/g1 cell cycle regulatory pathway in neuroblastoma. *Int J Cancer* 1999; **80**:145–154.

11 Takita J, Hayashi Y, Nakajima T, *et al.* The p16 (cdkn2a) gene is involved in the growth of neuroblastoma cell and its expression is associated with prognosis of neuroblastoma patients. *Oncogene* 1998; **17**:3137–3143.
12 Warenius H, Seabra L, Maw P. Sensitivity to *cis*-diamminedichloroplatinum in human cancer cells is related to expression of cyclin D1 but not c-raf-1 protein. *Int J Cancer* 1996; **67**:224–231.
13 Kornmann M, Arber N, Korc M. Inhibition of basal and mitogen-stimulated pancreatic cancer cell growth by cyclin D1 anitsense is associated with loss of tumorigenicity and potentiation of cytotoxicity to cisplatinum. *J Clin Invest* 1998; **101**:344–352.
14 Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer* 1987; **56**:279–285.
15 Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**:55–63.
16 Hochhauser D, Schneidrs B, Ercikan-Abali E, *et al.* Effect of cyclin D1 overexpression on drug sensitivity in a human fibrosarcoma cell line. *J Natl Cancer Inst* 1996; **88**:1269–1275.
17 Reed E, Kohn K. Platinum analogues. In: Chabner B, Collins J (editors): *Cancer Chemotherapy*. Philadelphia, PA: Lippincott; 1990, pp. 465–490.
18 Baringa M. Forging a path to cell death. *Science* 1996; **273**: 735–737.
19 Ishihara M, Saito Y, Yura H, *et al.* Heparin-carrying polystyrene to mediate cellular attachment and growth via interaction with growth factors. *J Biomed Mater Res* 2000; **50**:144–152.