Pergamon

European Journal of Cancer, Vol. 35, No. 1, pp. 86–90, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00342-6

Original Paper

Antitumour Activity of Oxaliplatin in Neuroblastoma Cell Lines

A. Riccardi,[1] C. Ferlini,[2] D. Meco,[1] R. Mastrangelo,[1] G. Scambia[2] and R. Riccardi[1]

[1]Division of Paediatric Oncology; and [2]Department of Obstetrics and Gynaecology, Catholic University, Largo A. Gemelli, 8, 00168 Rome, Italy

Oxaliplatin appears non cross-resistant with cisplatin and has a comparable antitumour effect both in preclinical and clinical studies. We compared the antitumour effect of oxaliplatin with that of cisplatin in human neuroblastoma cell lines SK-N-DZ, LAN-1 and BE(2)M17 following 24 h exposure at concentrations ranging from 0.5 to 5 μM. Oxaliplatin was less potent with IC_{50} values 1.08–3.4-fold higher than cisplatin. Like cisplatin, oxaliplatin induced a cell cycle block in the G_2/M phase although to a lesser extent than that caused by cisplatin. The concomitant increase of DNA fragmentation and decrease of G_2/G_1 ratio at 72 h indicated that a fraction of blocked cells underwent apoptosis. Morphological analysis confirmed these data, although oxaliplatin appeared to be 2–3 times less potent than cisplatin in inducing apoptosis. Our results indicate that oxaliplatin is active in neuroblastoma *in vitro* and this finding warrants *in vivo* preclinical studies.

Key words: neuroblastoma, oxaliplatin, cisplatin, antineoplastic agents, apoptosis, cell cycle block
Eur J Cancer, Vol. 35, No. 1, pp. 86–90, 1999

INTRODUCTION

THE FORTUITOUS recognition of the antitumour activity of cisplatin in the late 1960s [1] was rapidly followed by clinical studies confirming the role of this new class of antineoplastic agents in the cure of testicular carcinoma and in the treatment of other neoplasms such as small cell lung carcinoma, bladder, ovarian and paediatric tumours.

The substantial toxicity of cisplatin, such as nephrotoxicity, nausea and vomiting, auditory impairment and peripheral neuropathy, prompted the development of more manageable platinum coordination compounds. Carboplatin was selected out of thousands of cisplatin analogues synthesised in search of compounds with a more favourable toxic profile. Although the use of carboplatin reduced some of the cisplatin toxicity, no increase in antitumour activity and no activity in cisplatin resistant tumours was observed [2]. Acquired drug resistance during the course of anticancer therapy is a common occurrence and represents the major obstacle to the treatment of potentially curable tumours.

A step forward in platinum therapy could come from new platinum coordination compounds with similar antitumour activity and no cross resistance with cisplatin or carboplatin. Recently, the substitution of the cisplatin amine radicals by a 1,2-diaminocyclohexane ('dach') radical resulted in a stable complex with good antitumour activity and apparently without cross resistance with cisplatin. To improve the water solubility of this virtually insoluble compound, the chloride leaving group was replaced with a variety of anionic leaving groups, including the oxalate group that is present in oxaliplatin. Further separation of the dach platinum derivatives into two geometrical isomers *cis* and *trans* followed by resolution of the *trans* isomer into two optical isomers, *trans*-D and *trans*-L, improved antitumour activity. The optical isomer *trans*-L-dach (oxaliplatin) was selected based on its potency in the L1210 leukaemia cell line and its good solubility in water. Oxaliplatin is active against a wide range of human and murine tumour cell lines [3]. In particular, oxaliplatin seems to be active in cisplatin-resistant cancer such as the cell lines A2780 ovarian carcinoma line and HT29 colon carcinoma cell line [4].

Currently oxaliplatin is undergoing clinical evaluation in phase II and III studies in adults. As a single agent in phase I studies it has shown activity against ovarian, breast, oesophageal and colon carcinoma, melanoma and glioma [5]. Acute toxicity of oxaliplatin is relatively mild with cumulative neurotoxicity being the dose-limiting toxicity [6]. Clinical

Correspondence to R. Riccardi, e-mail: rriccard@tin.it
Received 24 Apr. 1998; revised 7 Sep. 1998; accepted 16 Sep. 1998.

studies in advanced ovarian carcinoma confirmed the apparent lack of cross resistance of oxaliplatin with cisplatin or carboplatin, with responses in platinum-resistant patients [7]. To our knowledge, no clinical studies have been performed in children and few preclinical studies have been conducted in paediatric tumours.

Neuroblastoma is one of the most common paediatric solid tumours and in its advanced forms still carries a poor prognosis. Cisplatin is an active drug in neuroblastoma although initial responses to chemotherapy are invariably followed by the development of drug resistance and tumour progression. Thus, oxaliplatin seems a potential candidate for a phase I–II study in children with neuroblastoma especially if preclinical studies suggest activity in this tumour. To this end, using the three human neuroblastoma cell lines SK-N-DZ, LAN-1 and BE(2)M17 representative of different maturation stages evaluated according to patterns of chromaffin-related gene expression [8], we investigated the activity of oxaliplatin *in vitro* and compared all the results with those of cisplatin.

MATERIALS AND METHODS

Drugs

Oxaliplatin was a gift from Sanofi (Malvern, Pennsylvania, U.S.A.). Cisplatin was purchased from Bristol-Myers Squibb (Sermoneta, Italy).

Cell lines and culture conditions

Neuroblastoma cell lines SK-N-DZ were kindly provided by M. A. Israel (University of California, San Francisco, U.S.A.), whilst LAN-1 and BE(2)M17 were a gift from J. L. Biedler (Memorial Sloan Kettering, New York, U.S.A.). Cell lines were grown as a monolayer culture in RPMI 1640 medium (Bio Whittaker, Verviers, Belgium) with 10% heat inactivated fetal calf serum (FCS) and 2 mM L-glutamine at 37°C in humidified 5% CO_2. Cells were maintained in logarithmic growth by harvesting with trypsin-EDTA solution and seeding before cells reached confluence. Cells were seeded 48 h before an experiment to ensure exponential growth during drug exposure. At least three different experiments were performed in duplicate for each cell line and for the two platinum complexes.

Growth inhibition assay

BE(2)M17, SK-N-DZ and LAN-1 cells were cultured in the presence of cisplatin and oxaliplatin (concentration range 0.5–5 μM) for 24 h. After 24 h of culture, cells were washed and fresh medium was added to each condition. Cell counting by the Trypan Blue dye exclusion test was then performed after additional 24, 48 and 72 h of culture. The half-maximal inhibitory concentrations (IC_{50}) were calculated at each time point. Cisplatin at 5 μM was highly cytotoxic so cell cycle analysis and apoptosis evaluation were performed utilising concentrations up to 2.5 μM.

Cell cycle analysis

Progression of cells through the cell cycle was examined by flow cytometry. Cells were plated in the specific medium supplemented as above. After 48 h, the medium was replaced with fresh medium containing oxaliplatin and cisplatin (0.5–2.5 μM) or with the medium alone for 24 h. Cells were then washed and a cell cycle analysis was assessed after additional 24, 48 and 72 h of culture; cells were harvested, nuclei isolated and stained using a solution containing 0.1% (w/v) sodium citrate, 0.1% (v/v) NP40, 4 mM EDTA and 50 μg/ml of propidium iodide as a DNA dye [9]. In order to calculate DNA fragmentation, all the events falling in the subG1 region were computed and indicated as per cent of DNA fragmentation. These events were excluded from the cell cycle analysis. Incubation of the cells with the staining solution lasted a minimum of 24 h at 4°C. Flow cytometric DNA ploidy analysis was performed by acquiring a minimum of 10 000 nuclei using a Facscan flow cytometer (Becton Dickinson Immunocytometry Systems, San Josè, California, U.S.A.). DNA fluorescence was collected in linear and log modes and pulse signal processing was used to set a doublet discrimination gate. Cell cycle analysis was performed using the Multicycle software package (Phoenix, San Diego, California, U.S.A.).

Morphological analysis of apoptosis

The ability of oxaliplatin and cisplatin to induce apoptosis was measured by the terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin *in situ* end labelling (TUNEL) assay using a commercial kit (Apoptos-I.S., Ylem, Avezzano, Italy), according to the manufacturer's instructions. Cells were exposed to cisplatin and oxaliplatin (0.5–1–2.5 μM) for 24 h, replaced with fresh medium and after additional 48 h washed with phosphate buffered saline (PBS) and centrifuged in order to obtain 10^5 cells/slide. A minimum of 500 cells were examined to score the apoptotic index. Count and image analysis were performed using the IAS2000 System (Delta Sistemi, Rome, Italy).

Statistical analysis

In some experiments the Wilks' lambda statistic was computed to evaluate differences in a multivariate analysis of variance (MANOVA). *Post-hoc* comparisons were evaluated using the Tukey-Hsd test to detect the significant interactions [10]. In other experiments the classical analysis of variance was used. A P value lower than 0.05 was considered significant. All the analyses were performed using Statistic 5.0 software package (Statsoft, Tulsa, Oklahoma, U.S.A.).

RESULTS

Growth inhibition effects

The IC_{50} calculated on the dose-survival curves in BE(2)M17, SK-N-DZ and LAN-1 cell lines are showed in Table 1. Cisplatin was always more potent than oxaliplatin (up to 3.4-fold) at all time points. In BE(2)M17 and SK-N-DZ cells both drugs induced a progressively increasing antiproliferative effect culminating after 72 h of culture. The

Table 1. *Mean IC_{50} values (μM) obtained after treatment with cisplatin (CDDP) and oxaliplatin (L-OHP) for* 24 *h and cultured in fresh medium for an additional* 24, 48 *and* 72 *h*

	BE(2)M17	SK-N-DZ	LAN-1
CDDP			
24 h	0.95	0.86	1.52
48 h	0.45	0.55	0.35
72 h	0.28	0.47	1.10
L-OHP			
24 h	1.65	1.87	1.65
48 h	0.7	1.40	1.20
72 h	0.8	1.21	2.03

Table 2. Cell cycle analysis of the three neuroblastoma cell lines after 24 h exposure to cisplatin (CDDP) and oxaliplatin (L-OHP) and 72 h of additional culture

	BE(2)M17			SK-N-DZ			LAN-1		
	G_2/M %	G_2/G_1 Ratio	*DNA fragmentation	G_2/M %	G_2/G_1 Ratio	*DNA fragmentation	G_2/M %	G_2/G_1 Ratio	*DNA fragmentation
Control	9.3	0.13	1.3	1.4	0.02	4.6	1.4	0.02	3.9
CDDP (μM)									
0.5	13.2	0.19	14.4	2.2	0.03	7.7	1.2	0.02	7.7
1.0	16.5	0.64	45.0	6.0	0.10	45.0	5.9	0.11	16.7
2.5	9.6	0.16	58.8	16.6	0.30	44.6	20.5	0.47	70.7
L-OHP									
0.5	9.6	0.14	13.0	5.5	0.09	2.8	10.8	0.20	2.8
1.0	10.5	0.16	18.6	1.4	0.03	4.8	10.8	0.21	4.8
2.5	27.7	0.72	29.8	21.2	0.51	8.8	13.1	0.28	18.6

*DNA fragmentation was excluded from cell cycle analysis.

effect on LAN-1 cells was different, with the antiproliferative effect peaking after 48 h and then partially subsided after 72 h; this finding suggests that the growth inhibition effect observed after 48 h is largely dependent on cell cycle block whilst subsequently a fraction of blocked cells re-entered into the cell cycle after G_2/M arrest. This hypothesis is also confirmed by the fact that after 72 h of culture this cell line exhibited the highest IC_{50} values with both drugs.

Cell cycle analysis

Results of the cell cycle analysis are summarised in Table 2. Treatment with both drugs induced a cell cycle block in G_2/M.

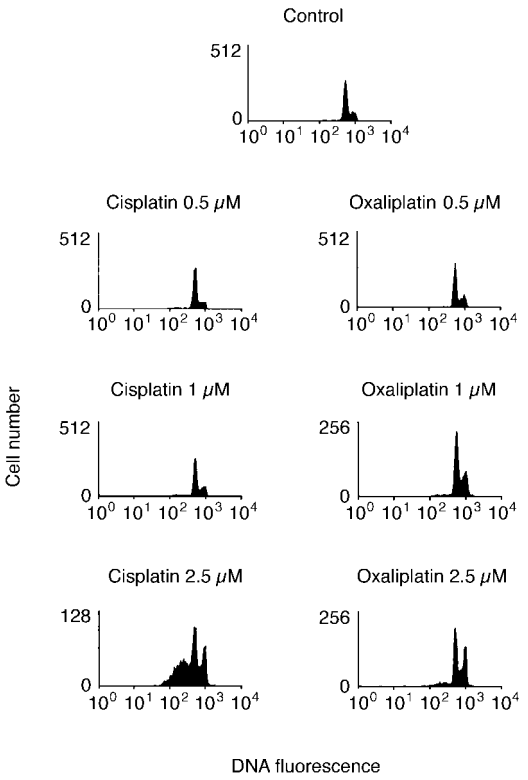

Figure 1. Cell cycle analysis of the SK-N-DZ cell line after 24 h exposure to cisplatin and oxaliplatin and 48 h of additional culture. Cell block in the G_2/M phase and increase in DNA fragmentation at 2.5 μM was observed with cisplatin and to a lesser extent with oxaliplatin. (Note different scales for the vertical axes.)

In order to perform a statistical analysis the extent of the cell cycle block was calculated using the ratio G_2/G_1, as previously described [11], whilst DNA fragmentation in the sub G_1 region was considered as an index of the presence of apoptosis. Figure 1 illustrates DNA fragmentation in SK-N-DZ cells. Statistical analysis was performed by a Manova design taking into account two independent variables (cell cycle block and DNA fragmentation) and three factors (cell

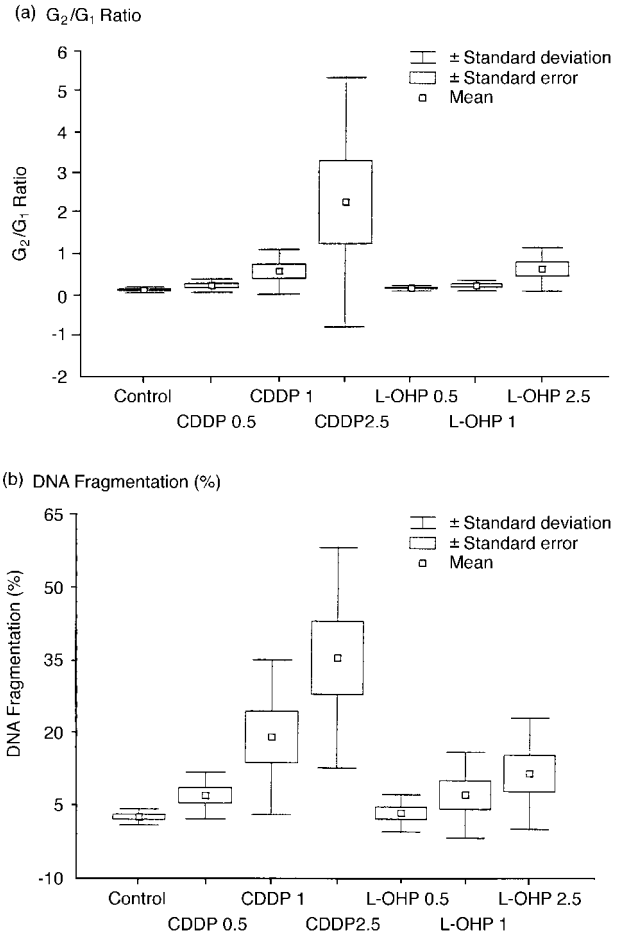

Figure 2. Mean values (squares) of G_2/G_1 ratio (a) and DNA fragmentation (b) with the standard error (boxes) and standard deviation (bars) of the three neuroblastoma cell lines at 48 h (CDDP, cisplatin; L-OHP, oxaliplatin).

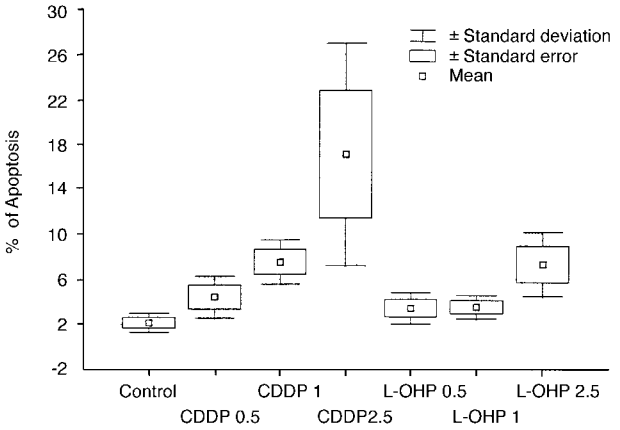

Figure 3. Mean values (closed squares) with standard error (boxes) and standard deviation (bars) of the apoptosis rate (CDDP, cisplatin; L-OHP, oxaliplatin). Cells were exposed to cisplatin or oxaliplatin for 24 h and then cultured in fresh medium for an additional 48 h.

line, i.e. BE(2)M17, SK-N-DZ and LAN-1; culture time, i.e. 24, 48 and 72 h; treatment, i.e. control, cisplatin 0.5 μM, 1 μM, 2.5 μM, oxaliplatin 0.5 μM, 1 μM, 2.5 μM). Albeit LAN-1 cells showed the highest cell cycle block (and this result is in accordance with the ability to recover from the G_2/M arrest), no significant differences were detected between the three cell lines ($\lambda = 0.923$; P = n.s., data not shown). The culture time had a significant effect ($\lambda = 0.758$; $P = 0.002$). A *post-hoc* analysis demonstrated that this effect was mainly dependent on the increase in DNA fragmentation after 72 h of culture ($P = 0.011$), whereas the increase in cell cycle block after 24 h was not significant (data not shown). As expected, drug treatment had the most significant effect ($\lambda = 0.4$; $P < 0.001$). This effect was evident in both cell cycle block and DNA fragmentation (Figure 2).

Evaluation of morphological apoptosis

As DNA fragmentation *per se* can not be considered sufficient to ascertain the presence of apoptosis, a morphological analysis was performed using the TUNEL technique in order to quantify the cells undergoing apoptosis. A one-way analysis of variance was performed using apoptosis as the dependent variable and the cell line and drug treatment as singular factors. Again, no significant differences were found between the three cell lines ($F = 1.58$; P = n.s.). Drug treatment significantly affected the presence of apoptosis ($F = 4.66$; $P = 0.008$). As shown in Figure 3, the highest dose of cisplatin produced an apoptosis rate of up to 2.67-fold higher than that of oxaliplatin, thus, confirming data obtained by flow cytometric DNA fragmentation.

DISCUSSION

Oxaliplatin has already been tested in a few early clinical trials in adult cancer patients [5, 7, 12, 13]. This cisplatin analogue appears active in ovarian cancer and colorectal cancer. In France oxaliplatin is now commercially available and used as a second-line treatment for colorectal cancer patients relapsing after 5-fluorouracil (5-FU) treatment. Oxaliplatin has a particularly favourable toxic profile with respect to cisplatin or carboplatin. Only a mild to moderate myelosuppression has been reported with no cases of grade 3–4 neutropenia. In heavily pretreated ovarian cancer patients acute neurotoxicity was observed in the form of cold-triggered dysaesthesia in 80% of patients although only mild or moderate in 60%. Remarkably, due to the high water solubility of the drug, renal toxicity is negligible.

Currently no information on the preclinical activity of oxaliplatin is available in paediatric solid tumours and, in particular, no data on the cytotoxic effect of this new cisplatin analogue have been reported for neuroblastoma. Neuroblastoma is one of the most common paediatric solid tumours and in advanced stages in children over 1 year of age the prognosis is dismal despite aggressive treatment [14]. The mainstay of therapy for children with stage III and IV disease is multidrug high-dose chemotherapy which includes platinum compounds. This clinical approach is effective in inducing complete remission in 40–60% of patients with advanced disease [15]. Unfortunately, tumour recurrence is a frequent event and relapsing patients are often resistant to previous chemotherapy including cisplatin and carboplatin. New platinum coordination compounds, such as oxaliplatin, a member of the 'dach' family, appear to have a similar antitumour activity with no cross resistance with cisplatin or carboplatin.

We compared the antineoplastic activity of oxaliplatin and cisplatin in three neuroblastoma cell lines. A dose-dependent antitumour activity was observed for both cisplatin and oxaliplatin in all the three cell lines. Cisplatin appeared more potent than oxaliplatin not only in terms of growth inhibition, but also in cell cycle perturbations and, consequently, DNA fragmentation and apoptosis. Cell lines showed a different sensitivity to oxaliplatin in this order BE(2)M17 > SK-N-DZ > LAN-1.

The difference in sensitivity to antineoplastic agents of the neuroblastoma cell lines is a relatively frequent finding [16] reflecting the heterogeneity of the human neuroblastoma cell lines [8]. Both drugs were able to block the cell cycle at the G_2/M checkpoint. During the time course a large part of the blocked cells underwent apoptosis, as demonstrated by the increase in DNA fragmentation when the G_2/M arrest subsided. Considering the overall data, oxaliplatin showed antitumour activity albeit to a lesser extent compared with cisplatin. The antitumour effect was achieved at oxaliplatin concentrations ranging from 0.5 to 2.5 μM.

In adult patients with relapsed gastrointestinal tract tumours, 130 mg/m^2 given every 3 weeks is the recommended dose for oxaliplatin. To our knowledge, no phase I study with oxaliplatin in children has been conducted and, therefore, the maximum tolerated dose (MTD) has not yet been determined. Since in adults cisplatin is usually given at a dose of 60–75 mg/m^2 every 3 weeks, it appears that the slightly lower antitumour activity of oxaliplatin compared with cisplatin may well be compensated by the possibility of administering oxaliplatin at double the cisplatin dose. This may not be the case in children with neuroblastoma in which doses up to 200 mg/m^2 of cisplatin are utilised.

Previous reports have shown that the amount of DNA platination following cisplatin treatment exceeds by $\geq$2-fold that observed with equimolar doses of oxaliplatin [17, 18]. This difference could explain the lower potency of oxaliplatin observed in tumour growth inhibition. In addition to this mechanism, according to previous reports in other cellular models [19], it is reasonable to hypothesise that DNA platination patterns obtained by oxaliplatin could differ partially from those induced by cisplatin treatment. Studies are now in progress in our laboratory to characterise the quantitative and

qualitative differences in DNA platination that could be responsible for the different potency of cisplatin and oxaliplatin in the three neuroblastoma lines.

In summary, our results suggest that oxaliplatin is active against neuroblastoma *in vitro*. Nevertheless, before this compound can be utilised in early clinical trials in children, experimental studies on murine models are needed in order to confirm the antitumour activity of oxaliplatin in neuroblastoma *in vivo*.

1. Rosemberg B, Van Camp L, Trosko JE, Mansour VH. Platinum compounds: a new class of potent antitumor agents. *Nature* 1969, **222**, 385–386.
2. Ozols FR, Ostchega Y, Curt G, Young RC. High-dose carboplatin in refractory ovarian cancer patients. *J Clin Oncol* 1987, **5**, 197–201.
3. Rixe O, Ortuzar W, Alvarez M, *et al*. Oxaliplatin, tetraplatin, cisplatin and carboplatin: spectrum of activity in drug-resistant cell lines and in cell lines of the National Cancer Institute's anticancer drug screen panel. *Biochem Pharmacol* 1996, **52**, 1855–1865.
4. Pendyala L, Creaven PJ. *In vitro* cytotoxicity, protein binding, red blood cell partitioning and biotransformation of oxaliplatin. *Cancer Res* 1993, **53**, 5970–5976.
5. Soulie P, Raymond E, Brienza S, Cvitkovic E. Oxaliplatin: the first DACH platinum in clinical practice. *Bull Cancer* 1997, **84**, 665–673.
6. Extra JM, Espie M, Calvo F, Ferme C, Mignot L, Marty M. Phase I study of oxaliplatin in patients with advanced cancer. *Cancer Chemother Pharmacol* 1990, **25**, 299–303.
7. Chollet P, Bensmaine MA, Brienza S, *et al*. Single agent activity of oxaliplatin in heavily pretreated advanced epithelial ovarian cancer. *Ann Oncol* 1996, **7**(10), 1065–1070.
8. Cooper MJ, Hutchins GM, Cohen PS, Helman LJ, Israel MA. Neuroblastoma cell lines mimic chromaffin neuroblastoma maturation. *Prog Clin Biol Res* 1991, **366**, 343–350.
9. Ferlini C, Di Cesare S, Rainaldi G, *et al*. A flow cytometric analysis of the early phases of apoptosis by cellular and nuclear techniques. *Cytometry* 1996, **24**, 106–115.
10. Lund RE, Lund JR. Probabilities and upper quantiles for the studentized range. *Applied Statistics* 1983, **32**, 204–210.
11. Ferlini C, Scambia G, Distefano M, *et al*. Synergistic antiproliferative activity of tamoxifen and docetaxel on three oestrogen receptor-negative cancer cell lines is mediated by the induction of apoptosis. *Br J Cancer* 1997, **75**(6), 884–891.
12. Degardin M, Cappelaere P, Krakowski I, Fargeot P, Cupissol D, Brienza S. Phase II trial of oxaliplatin (L-OHP) in advanced, recurrent and/or metastatic squamous cell carcinoma of the head and neck. *Eur J Cancer B Oral Oncol* 1996, **32B**(4), 278–279.
13. Machover D, Diaz-Rubio E, de Gramont A, *et al*. Two consecutive phase II studies of oxaliplatin (L-OHP) for treatment of patients with advanced colorectal carcinoma who were resistant to previous treatment with fluoropyrimidines. *Ann Oncol* 1996, **1**, 95–98.
14. Evans AE, D'Angio GJ, Propert K, Anderson J, Hann HW. Prognostic factors in neuroblastoma. *Cancer* 1987, **59**, 1853–1859.
15. Stram DO, Matthay KK, O'Leary M, *et al*. Consolidation chemoradiotherapy and autologous bone marrow transplatation versus continued chemotherapy for metastatic neuroblastoma: a report of two concurrent children's cancer group studies. *J Clin Oncol* 1996, **14**, 2417–2426.
16. Riccardi A, Servidei T, Tornesello A, *et al*. Cytotoxicity of paclitaxel and docetaxel in human neuroblastoma cell lines. *Eur J Cancer* 1995, **31A**(4), 494–499.
17. Los G, Mutsaers PH, Ruevekamp M, *et al*. The use of oxaliplatin versus cisplatin in intraperitoneal chemotherapy in cancers restricted to the peritoneal cavity in the rat. *Cancer Lett* 1990, **51**(2), 109–117.
18. Woynarowsky JM, Chapman WG, Napier C, Raymond E. Oxaliplatin (OxPt) effects on naked and intracellular DNA. *Proc Amer Assoc Cancer Res* 1997, **38**, 311(abstr 2083).
19. Fink D, Nebel S, Zheng H, *et al*. The role of mismatch repair in platinum drug resistance. *Cancer Res* 1996, **56**(21), 4881–4886.

Acknowledgements—Supported by AIRC, CNR Acro grant n. 96.00429.PF39 and FOP. We thank Dr N. Maggiano for his help in detection of apoptosis by the TUNEL technique. Oxaliplatin was kindly supplied by Sanofi (Malvern, Pennsylvania, U.S.A.).