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Antiproliferative Potential of Cytostatic Drugs on Neuroblastoma Cells *In Vitro*

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The role of single drugs in the treatment of neuroblastoma is poorly defined. We, therefore, tested neuroblastoma cell survival after a 72 h exposure to one of 19 cytostatic drugs by monolayer proliferation assay. 6 cell lines (IMR-5, Kelly, SK-N-SH, GI-CA-N, CHP-100, CHP-134) were selected on the basis of *MYCN* amplification and *PGY1* overexpression. ED_{50} drug concentrations were related to plasma levels achievable in patients during chemotherapy. More effective substances were mitoxantrone, doxorubicin, hydroxyurea, bleomycin, dactinomycin, cisplatinum, thiotepa, melphalan, carboplatinum, etoposide, vincristine, cytarabine, 6-thioguanine, cyclophosphamide, ifosfamide and zilascorb. Parental drugs (cyclophosphamide, cisplatinum) appeared more cytotoxic on a molar basis than derived drugs (ifosfamide, carboplatinum). Less effective drugs included 5-fluorouracil, 6-mercaptopurine, CCNU and procarbazine. Fractional application of a given dose was more efficient than a single dose of cyclophosphamide, ifosfamide and cisplatinum. The tested neuroblastoma cell lines showed distinct sensitivities to cytostatic drugs. Cell lines with *MYCN* amplification appeared more sensitive than *PGY1* overexpressing cells. In conclusion, comparative *in vitro* testing of cytostatic drugs may provide a rationale for their clinical evaluation. Investigation of drug combinations and application of the monolayer proliferation assay to tumour biopsy material for preclinical chemosensitivity testing are clearly warranted.

Keywords: neuroblastoma, drug screening, tumour cells cultured, alkylating agents, platinum compounds, antimetabolites antineoplastic, vinca alkaloids, etoposide, antibiotics antineoplastic
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INTRODUCTION

NEUROBLASTOMA is the most common extracranial malignant solid tumour of infancy and childhood. In contrast to the major advances which have been achieved in the treatment of other childhood cancers, the prognosis for children with metastatic neuroblastoma remains dismal, with long-term survival rates of 10% [1]. This highlights the need for more effective therapies. Polychemotherapy is considered an important treatment modality in more than 75% of neuroblastoma patients [1]. For advanced disease, most protocols include cyclophosphamide, cisplatinum, doxorubicin, vincristine and etoposide as cytostatic agents [1, 2].

However, information on the cytostatic activity of single drugs on neuroblastoma is limited. Clinical trials with monosubstances are rare and difficult to interpret because of pretreatment and resistance phenomena [3]. *In vitro* tests have not produced uniform results nor included drugs more recently introduced for the treatment of neuroblastoma, such as thiotepa [4, 5].

We, therefore, investigated neuroblastoma cell survival after exposure to antitumour drugs by monolayer proliferation assay. Cell lines were selected for *MYCN* amplification and *PGY1* overexpression. Both characteristics are associated with a poor prognosis. Drugs tested included alkylating agents (cyclophosphamide, ifosfamide, melphalan, thiotepa, CCNU, procarbazine), platinum compounds (cisplatinum, carboplatinum), antimetabolites (6-mercaptopurine, 6-thioguanine, 5-fluorouracil, cytarabine, hydroxyurea), plant alkaloids (vincristine, etoposide), antitumour antibiotics (doxorubicin, mitoxantrone, dactinomycin, bleomycin) and a protein synthesis inhibitor (zilascorb).

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MATERIALS AND METHODS

Drugs

The following drugs were tested: 4-hydroperoxycyclophosphamide, 4-hydroperoxyifosfamide (Asta Medica, Frankfurt, Germany, kindly provided by Dr Pohl), melphalan, 6-mercaptopurine (Wellcome, Burgwedel, Germany), thiotepa, mitoxantrone (Lederle, Wolratshausen, Germany), CCNU (Sigma, Munich, Germany), azoxyprocarbazine isomers (Hoffmann-La Roche, Basel, Switzerland, kindly provided by Mr Weber), 5-fluorouracil (Hoffmann La Roche, Grenzach-Wyleben, Germany), cisplatinum, carboplatinum, hydrocarbamide, etoposide (Bristol Arzneimittel, Munich, Germany), cytarabine, bleomycin (Mack, Illertissen, Germany), vincristine (Rhone-Poulenc, Cologne, Germany), doxorubicin (Farmitalia, Freiburg, Germany), dactinomycin (Merck, Sharp and Dohme,

Munich, Germany), zilascorb (Pronova, Oslo, Norway, kindly provided by Dr Dornish).

Cell lines

The 6 cell lines were chosen for their presence or absence of *MYCN* amplification and *PGY1* overexpression [6]: IMR-5 (*MYCN* 25×, *PGY1* negative), CHP-134 (*MYCN* 100×, *PGY1* negative), Kelly (*MYCN* 100-120×, *PGY1* negative), SK-N-SH (*MYCN* 1×, *PGY1* positive), CHP-100 (*MYCN* 1×, *PGY1* positive), GI-CA-N (*MYCN* 1×, *PGY1* negative). They were maintained in monolayer culture in 50 ml tissue culture flasks (Falcon, Heidelberg, Germany) in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (Gibco, Eggenstein, Germany), 2 mM L-glutamine (Boehringer, Mannheim, Germany), 100 μg/ml gentamicin (Boehringer) and incubated at 37°C in 95% air/5% CO_2. Cell lines were fed twice weekly and subcultured at confluence.

Monolayer proliferation assay

The monolayer proliferation assay was performed as described by Dietel and associates [7]. In general, viable cells remain adherent to tissue culture plates, whereas non-viable cells detach from the underlying surface. To obtain a uniform single cell suspension, 1 ml of 0.05% trypsin and 0.02% EDTA without Ca^{2+} and Mg^{2+} (Boehringer) was added to the cultures for 1–5 min, and cells were separated by careful mechanical aspiration. Cells were counted visually using a standard haemocytometer. 30–40,000 cells in 750 μl of medium were seeded in each well of a 12-well tissue culture plate (Costar, Bielefeld, Germany). To assess DNA levels prior to drug incubation, one plate was rinsed with Hanks balanced salt solution (Seromed) after 72 h preincubation, and stored at −20°C until determination of the amount of DNA. Chemotherapeutic agents were added to the remaining plates at six different concentrations covering a range of 2–3 logs. The median concentrations were comparable to peak serum levels achieved in patients during chemotherapy. In addition, the total dose was divided and the fractions added to the cells at different time intervals: cyclophosphamide- 5 fractions, 2 h/fraction; ifosfamide- 5 fractions, 2 h/fraction; cisplatinum- 3 fractions, 4 h/fraction; melphalan- 3 fractions, 24 h/fraction. Cell growth was assessed microscopically after a 72 h drug incubation. The drug-containing medium was removed, the non-adherent dead cells were washed off with Hanks balanced salt solution, and the amount of DNA in the remaining adherent cells was determined. Cell growth was calculated by the difference in DNA content before and after drug incubation. The cell growth of the drug-exposed cells was defined as relative growth according to the formula:

$$\text{Relative growth (\%)} = \frac{\text{cell growth of exposed cells}}{\text{cell growth of control cells}} = \frac{DNA_{exposed} - DNA_{pre}}{DNA_{control} - DNA_{pre}}$$

For simplicity, negative growth (destruction of neuroblastoma cells) was established as 0%.

DNA determination

The amount of DNA as a function of number of adherent, living cells was determined by the method of Giles and associates using diphenylamine [8]. For standardisation, salmon sperm DNA (Boehringer) was used in triplicate (0.5–60 μg DNA). 10% perchloric acid (Merck, Darmstadt, Germany) was added to each well to cause cell lysis. The plates were then heated at 90°C for 30 min. After cooling to room temperature, 1 ml of 4% diphenylamine (Sigma) dissolved in glacial acid (Merck) and 0.016% acetaldehyde (Merck) was added for colour reaction. After a 24 h incubation at room temperature, the optical density of the solution was measured at 595 nm using a Beckman spectrophotometer model Acta CIII.

Statistics

Each drug concentration was tested in triplicate. The mean, standard deviation and variation coefficient were calculated. Regression analysis was used for the correlation of cell number and level of DNA. Dose–response curves were plotted on a semilogarithmic scale with relative growth against drug concentration. The dose effective at inhibiting 50% growth of the cell population, i.e. the ED_{50}, was obtained by interpolation from the dose–response curves.

RESULTS

There was a linear relationship and close correlation ($r > 0.99$) between cell number ($0.5–8 \times 10^5$) and level of DNA for all 6 cell lines (data not shown) as previously reported [4]. Reduced cell growth by monolayer proliferation assay was uniformly associated with a comparable number of dead cells by microscopic evaluation (data not shown). The inter-test variation coefficients of the monolayer proliferation assay were within small limits (4.88+/−2.6%). In all instances, S-shape dose response curves were obtained (see Figures 1–5).

Derived drugs (carboplatinum, ifosfamide) were less cytotoxic on a molar basis than parental drugs (cisplatinum, cyclophosphamide). For all cell lines, ED_{50} concentrations of carboplatinum (4.5–16 nmol/ml) were higher than those of cisplatinum (0.3–0.75 nmol/ml, Figure 1). Similarly, ED_{50} concentrations of ifosfamide (3–9 nmol/ml) were higher in comparison to those of cyclophosphamide (0.6–5 nmol/ml) (data not shown). Thiotepa showed a marked antiproliferative effect at a range of 0.75–9 nmol/ml, with IMR-5 cells being the most sensitive and CHP-100 cells the least sensitive (Figure 2). 1.5–1.8 nmol/ml 6-Thioguanine produced 50% growth inhibition in 5 of the 6 cell lines, with only GI-CA-N cells having a higher ED_{50} of 12 nmol/ml 6-thioguanine (Figure 3). Zilascorb exhibited a marked inhibitory effect on all cell lines at 150–500 nmol/ml (Figure 4).

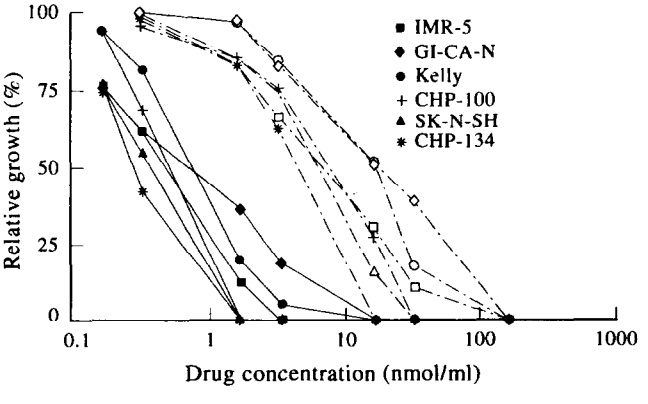

Figure 1. Dose–response curves of 6 human neuroblastoma cell lines after 72 h exposure to platinum compounds as determined by monolayer proliferation assay: cisplatinum (solid lines, closed symbols), carboplatinum (broken lines, open symbols).

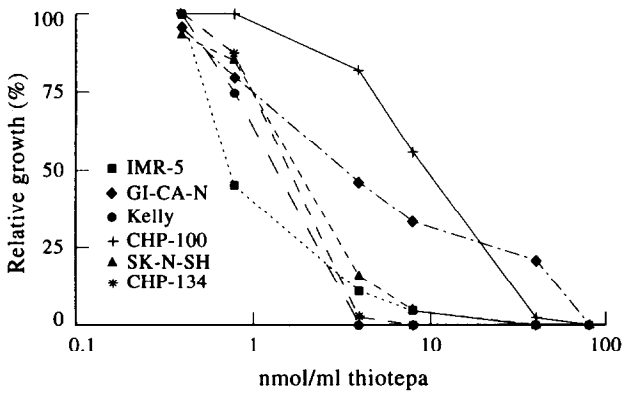

Figure 2. Dose–response curves of 6 human neuroblastoma cell lines after 72 h exposure to thiotepa as determined by monolayer proliferation assay.

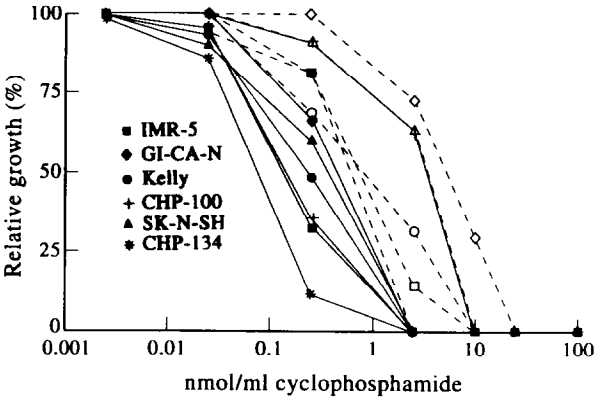

Figure 5. Dose–response curves of 6 human neuroblastoma cell lines after 72 h exposure to cyclophosphamide as determined by monolayer proliferation assay: single dose (broken lines, open symbols), fractional dose (solid lines, closed symbols).

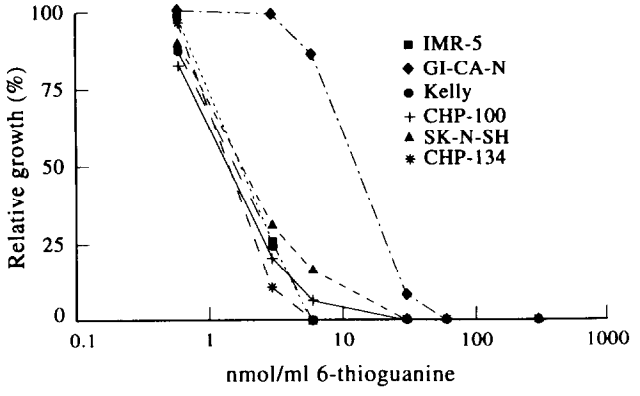

Figure 3. Dose–response curves of 6 human neuroblastoma cell lines after 72 h exposure to 6-thioguanine as determined by monolayer proliferation assay.

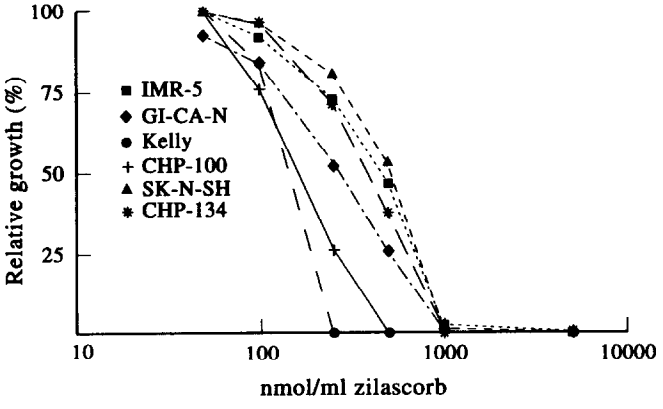

Figure 4. Dose–response curves of 6 human neuroblastoma cell lines after 72 h exposure to zilascorb as determined by monolayer proliferation assay.

The ED_{50} values of 6 human neuroblastoma cell lines following exposure to 19 cytostatic drugs are listed in Table 1. The cell lines showed distinct sensitivities to cytostatic drugs. Neuroblastoma cells with *MYCN* amplification (IMR-5, Kelly, CHP-134) appeared more sensitive having relatively low ED_{50} concentrations when compared to those for cell lines without *MYCN* amplification (SK-N-SH, CHP-100, GI-CA-N). Cells with *PGY1* overexpression (SK-N-SH) seemed to be less sensitive to cytotoxic agents, including vincristine, doxorubicin and etoposide.

Fractional application of cyclophosphamide produced an equivalent inhibition of cell growth of all cell lines at concentrations 9 times lower than single dose exposure (mean ED_{50} 0.25 fractional and 2.19 nmol/ml single dose cyclophosphamide respectively, Figure 5). Similarly, the ED_{50} concentrations were 3 times lower for ifosfamide (mean ED_{50} 1.72 nmol/ml fractional versus 5.58 nmol/ml single dose) and 2.3 times lower for cisplatinum (ED_{50} 0.23 nmol/ml fractional versus 0.52 nmol/ml single dose) when serial applications of these drugs were performed (Table 1). The fractional application of melphalan had approximately the same antiproliferative effects as a single dose (ED_{50} 2.25 fractional and 2.49 nmol/ml single dose, Table 1).

ED_{50} drug concentrations were compared with those concentrations achievable as peak plasma levels in man after conventional therapy (Table 2). More effective drugs were defined as those which showed a 50% growth inhibitory effect on neuroblastoma cells at concentrations achievable in patients. These included mitoxantrone, doxorubicin, hydroxyurea, bleomycin, dactinomycin, cisplatinum, thiotepa, melphalan, carboplatinum, etoposide, vincristine, cytarabine, 6-thioguanine, cyclophosphamide, ifosfamide and zilascorb. Less effective drugs were classified as those substances which required higher concentrations for a 50% growth inhibition than the peak plasma levels. These included 5-fluorouracil, 6-mercaptopurine, CCNU and procarbazine.

DISCUSSION

In vitro chemosensitivity testing can make a significant contribution to an overall drug screening programme by saving time and resources. Effective agents can be selected and further evaluated *in vivo*. Availability of a substantial number of well characterised human neuroblastoma cell lines, derived from different patients, with differing morphological, biochemical and genetic properties, permits assessment of chemotherapeutic activity against a wide spectrum of cell lines representative of the malignancy. However, for estimating the *in vitro* results, the shortcomings in simulating the *in vivo* situation have to be considered. These include differences in bioavailability of the drug due to dose, route of administration and tumour vascularis-

Table 1. ED_{50} *values (nmol/ml) of 6 neuroblastoma cell lines after 72 h drug exposure as measured by monolayer proliferation assay*

Drugs	ED_{50} range	Mean ED_{50}	IMR-5 *MYCN*$^+$ *PGY*$^-$	Kelly *MYCN*$^+$ *PGY*$^-$	CHP-134 *MYCN*$^+$ *PGY*$^-$	GI-CA-N *MYCN*$^-$ *PGY*$^-$	CHP-100 *MYCN*$^-$ *PGY*$^+$	SK-N-SH *MYCN*$^-$ *PGY*$^+$
CP	0.6–5	2.19	0.75	0.8	0.6	5	3	3
CP*	0.08–0.5	0.25	0.15	0.25	0.08	0.5	0.15	0.35
IFO	3–9	5.58	3	7	4	6	9	4.5
IFO*	0.9–2.7	1.72	0.9	2.5	0.9	2.7	1.3	2
MEL	0.6–5	2.25	0.8	0.6	1	1.1	5	5
MEL*	0.85–5	2.49	0.85	1.5	0.8	1.8	5.5	4.5
TT	0.79–9	2.93	0.75	1.4	1.6	3	9	1.8
CCNU	8.5–45	26.83	21.5	21.5	21.5	8.5	45	43
DDP	0.3–0.75	0.52	0.45	0.75	0.3	0.7	0.5	0.4
DDP*	0.18–0.3	0.23	0.2	0.3	0.18	0.26	0.22	0.22
CBDCA	4.5–16	9.25	6	16	4.5	16	7	6
PCZ	70–550	190	70	70	90	95	300	550
6-MP	8–25	18	15	22	20	25	18	8
6-TG	1.5–12	3.37	1.8	1.5	1.5	12	1.6	1.8
5-FU	0.38–6	2.4	1.7	1.5	n.d.	0.38	n.d.	6
Ara-C	0.3–2	1.63	0.5	0.7	n.d.	2	n.d.	0.3
oH-Urea	18–80	34	20	80	n.d.	18	n.d.	18
VCR	0.001–0.15	0.04	0.001	0.005	n.d.	0.001	n.d.	0.15
VP-16	0.01–0.34	0.167	0.071	0.01	n.d.	0.34	n.d.	0.25
ADR	0.0075–0.035	0.02	0.0075	0.025	n.d.	0.01	n.d.	0.035
Mitox	0.01–0.18	0.06	0.01	0.18	n.d.	0.01	n.d.	0.045
AMD	1×10^5–5×10^4	2.3×10^4	1×10^5	5×10^4	n.d.	1×10^5	n.d.	4×10^4
BLM	0.035–0.4	0.196	0.4	0.17	n.d.	0.035	n.d.	0.18
ZILA	150–500	320	450	150	400	250	160	500

*, fractional application; n.d., not determined; ED_{50}, *in vitro* concentration for 50% growth inhibition; CP, cyclophosphamide; IFO, ifosfamide; MEL, melphalan; TT, thiotepa; DDP, cisplatinum; CBDCA, carboplatinum; PCZ, procarbazine; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; 5-FU, 5-fluorouracil; Ara-C, cytarabine, oH-Urea, hydroxyurea; VCR, vincristine; VP-16, etoposide; ADR, doxorubicin; Mitox, mitoxantrone; AMD, dactinomycin; BLM, bleomycin; ZILA, zilascorb. *MYCN*$^+$, *MYCN* amplification; *MYCN*$^-$, no amplification; *PGY*$^+$, *PGY1* overexpression; PGY$^-$, no overexpression.

ation. Degradation processes *in vitro* differ substantially from *in vivo* metabolism, and, there is no tumour supporting microenvironment *in vitro*. Thus, *in vitro* results cannot simply be extrapolated to the situation in patients. The monolayer proliferation assay proved to be suitable for *in vitro* drug screening in neuroblastoma. The monolayer proliferation assay was developed by Dietel and associates, and has been successfully applied to primary cell cultures, correctly predicting tumour resistance in 94.5% and tumour sensitivity in 75.8% ($P < 0.001$) [7]. The monolayer proliferation assay is easily and quickly performed, independent from clonogenic characteristics of the tumour and permits fractional drug application.

Since information on the cytostatic activity of single agents against neuroblastoma is limited, we tested various cytotoxic drugs against 6 human neuroblastoma cell lines by monolayer proliferation assay, and correlated our data with clinical pharmacokinetic data as to the possible value of these drugs as single agents in the treatment of neuroblastoma. More effective drugs included those which are commonly used in patients, i.e. cyclophosphamide, ifosfamide, cisplatinum, vincristine, etoposide, doxorubicin, carboplatinum and melphalan. Analysing retrospectively the dose intensity concept in 44 clinical trials, Cheung and associates found that the dose of cisplatinum and teniposide had the strongest impact on survival for patients with metastatic neuroblastoma, and that cyclophosphamide and doxorubicin positively affected outcome, indicating the importance of these drugs for clinical trials [23]. The *in vitro* data support this view by demonstrating a high cytotoxic activity. Thiotepa, which has been used successfully in neuroblastoma patients prior to bone marrow transplantation [24], was shown, for the time, to have antineoplastic activity against neuroblastoma cells *in vitro*.

Analogues, such as ifosfamide and carboplatinum, were less cytotoxic on a molar basis than the parental drugs cyclophosphamide and cisplatinum. Ifosfamide is usually administered at a higher dose than cyclophosphamide (1500–3000 mg/m^2 ifosfamide versus 150–600 mg/m^2 cyclophosphamide) [2, 25]. *In vivo*, ifosfamide is activated to its active metabolites more slowly than cyclophosphamide, and is also metabolised to a greater extent than cyclophosphamide to inactive compounds [11]. However, *in vitro*, the spontaneous hydrolysis of the 4-hydroperoxy compounds to the active 4-hydroxy metabolites in aqueous solution appears to follow similar kinetics for both drugs. Therefore, the different cytostatic potential of ifosfamide and cyclophosphamide on neuroblastoma cells *in vitro* may not be attributed to different pharmacokinetic properties of these drugs, but might be explained by a somewhat reduced cytotoxicity of ifosfamide. Clinical studies evaluating the activity of single agents in this tumour also showed superiority of cyclophosphamide over ifosfamide [3, 25]. Approximately 18 times more car-

Table 2. Comparison of ED_{50} *drug concentrations as determined by monolayer proliferation assay with peak plasma levels*

	Drugs	Mean ED_{50} (nmol/ml)	Plasma levels (nmol/ml)	(Ref.)	ED_{50}/plasma levels
More effective					
	Mitoxantrone	0.06	1.2–5.42	[9]	0.01–0.05
	Doxorubicin	0.02	0.4–1	[10]	0.02–0.05
	Hydroxyurea	34	300–2000	[11]	0.02–0.11
	Bleomycin	0.196	1–10	[11]	0.02–0.2
	Dactinomycin	0.00023	0.000597	[10]	0.04
	Cisplatinum	0.52	6–12	[12]	0.04–0.08
	Thiotepa	2.93	53–63	[13]	0.05–0.06
	Melphalan	2.25	32–49	[14]	0.05–0.07
	Carboplatinum	9.25	54–200	[15]	0.05–0.17
	Etoposide	0.167	3.57–11.9	[16]	0.06–0.19
	Vincristine	0.04	0.4	[11]	0.1
	Cytarabine	1.63	10	[11]	0.16
	6-Thioguanine	3.37	6–10	[17]	0.3–0.6
	Cyclophosphamide	2.19	1.4–7	[18]	0.3–1.6
	Ifosfamide	5.58	1–12	[19]	0.5–5.6
	Zilascorb	320	100–400	[20]	0.8–3.2
	5-Fluorouracil	2.4	0.5–2.5	[21]	1–4.8
	6-Mercaptopurine	18	4–8	[11]	2.25–4.5
	CCNU	26.83	4.3–8.5	[22]	3.2–6.2
	Procarbazine	190	0.3–3	[11]	63–633
Less effective					

ED_{50}, *in vitro* concentration for 50% growth inhibition; More effective, drugs where mean ED_{50} < human plasma levels; Less effective, drugs where mean ED_{50} ≥ human plasma levels.

boplatinum than cisplatinum was required to achieve an equivalent cell kill, suggesting superiority of cisplatinum over carboplatinum, especially since the clinical dosage of carboplatinum is conventionally only 4 times higher than that for cisplatinum (800 mg/m^2 carboplatinum versus 200 mg/m^2 cisplatinum) resulting in approximately 10-fold higher peak plasma levels [15].

Several chemotherapeutic agents which are rarely or never used in the treatment of neuroblastoma (6-thioguanine, zilascorb, mitoxantrone, bleomycin, cytarabine and hydroxyurea) exhibited marked antineoplastic activity *in vitro*. Cytotoxic levels of 6-thioguanine and the new protein synthesis inhibitor, zilascorb, can probably be achieved *in vivo*. Neither have been tested before on neuroblastoma cells *in vitro* nor yet received clinical evaluation against this disease. Kovach and colleagues reported peak plasma levels of 6–10 nmol/ml 6-thioguanine after intravenous administration of 65 mg/m^2 [17]. Peak plasma levels of 100–400 nmol/ml zilascorb were found after a dose of 40 mg/kg was given intravenously [20]. Zilascorb is reported to be especially effective in slowly growing tumours with a low protein accumulation rate [20]. Since neuroblastoma may grow slowly and relapse late, further evaluation of zilascorb might be worthwhile in this particular tumour. Mitoxantrone, bleomycin, dactinomycin, cytarabine and hydroxyurea have been shown clinically to be of little or no benefit as single agents in the therapy of neuroblastoma [3]. 5-Fluorouracil, 6-mercaptopurine, CCNU and procarbazine, all of which proved less effective in this test system, have shown no significant activity in children with neuroblastoma [3, 26, 27].

Fractionation achieved 50% growth reduction at lower concentrations compared to single dose application, suggesting that the antineoplastic effect on neuroblastoma cells is influenced not only by drug concentrations, but also by the duration of exposure to active metabolites. The sustained activation of cyclophosphamide by the microsomal liver enzymes *in vivo* results in a 1–5 h plasma half life of the active metabolites 4-hydroxycyclophosphamide/aldophosphamide compared to an *in vitro* half life of 20 min [11, 28]. Therefore, the antiproliferative activity may be underestimated *in vitro* because the drug is added only once at the beginning of the test. Serial drug applications might better simulate the *in vivo* activity of cyclophosphamide. For ifosfamide, similar considerations apply [11]. Fractional application of melphalan achieved a comparable extent of growth inhibition when compared with single dose. According to clinical dosing intervals, melphalan was given 3 times every 24 h. Using shorter intervals (4 times every 2 h or 8 times every 1 h) Worthington-White and colleagues observed an increase in cytotoxic activity of melphalan on neuroblastoma cells [29]. Since melphalan has a short half life (approximately 1 h) *in vitro* and *in vivo* [30], shorter dosing intervals might have an impact on this drug's cytotoxicity by providing a more continuous exposure to the active compounds.

Cell lines with *MYCN* amplification appeared to be more susceptible to chemotherapy than those without *MYCN* amplification, whereas *PGY1* overexpression seemed to be associated with less sensitivity to cytotoxic agents for the two cell lines tested. Other resistance mechanisms appeared to be involved in GI-CA-N cells. In a neuroblastoma animal model, Tsuchida and

associates reported greater sensitivity of an *MYCN* amplified tumour to cyclophosphamide, cisplatinum and doxorubicin compared with a tumour without multiple *MYCN* copies [31]. The heterogeneity of drug responses between cell lines reported here emphasises the necessity of using a panel of cell lines, especially for new drug testing, to reflect the biological variability of the tumour.

In summary, the monolayer proliferation assay proved to be a reliable tool for estimating cytotoxicity of conventional and investigational chemotherapeutic agents on neuroblastoma cells *in vitro* and as a first approximation to *in vivo* conditions. Investigation of different fractionations of single drugs, use of drug combinations and application of the monolayer proliferation assay to tumour biopsy material in order to assess the drug sensitivities of individual patient's tumours are clearly warranted.

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