Eur J Cancer Clin Oncol, Vol. 24, No. 2, pp. 247–253, 1988.
Printed in Great Britain

0277-5379/88$3.00+0.00

The Cytotoxic Activity of Cisplatin, Carboplatin and Teniposide Alone and Combined Determined on Four Human Small Cell Lung Cancer Cell Lines by the Clonogenic Assay

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Abstract—*Using the clonogenic assay to compare the cytotoxic activity of cisplatin and carboplatin on four human small cell lung cancer cell lines, cisplatin was shown to be equally or more potent than carboplatin at equitoxic doses with* 1 h *incubation. Increased potency of carboplatin was revealed when the drugs were tested with continuous incubation, although cisplatin still was the most potent drug when compared on a microgram to microgram basis. This relative increase in potency of carboplatin can at least partly be explained by the development of a more reactive form of the drug when stored in tissue culture medium.*

By combining either cisplatin or carboplatin with teniposide additive cell kill was obtained. Additivity was also obtained when cisplatin was combined with carboplatin. Since the two drugs have a different toxicity pattern a clinical synergy may be obtained by combined use of these two analogues.

INTRODUCTION

THE POTENTIAL of a panel of human small cell lung cancer cell lines in the comparison of drug analogues has previously been described [1–3]. Validation of such *in vitro* methods for assessing the activity of new drugs against a given type of tumour would conceivably diminish the need for drug testing in patients [4]. Because of the high degree of clinical activity of cisplatin against a wide variety of human tumours, a search has been made both *in vitro* and *in vivo* for platin-containing compounds with a higher degree of antitumour activity [5] and/or reduced toxicity especially nephrotoxicity.

Carboplatin has been identified as such a new analogue because of increased antitumour activity in several test systems [6] and reduced toxicity in all aspects except myelosuppression [7].

In the treatment of small cell lung cancer a surprisingly high activity has been observed when cisplatin was combined with one of the most active agent, etoposide [8]. Since another epipodophyllotoxin derivative, teniposide, previously has been shown to be more potent than etoposide [2], the present study compared the cytotoxic activity of cisplatin and carboplatin alone and combined with teniposide on four human small cell lung cancer cell lines *in vitro*. Subsequently the activity of the combination of carboplatin with cisplatin was investigated. On the basis of these and previous results the relative sensitivity profiles of the panel are presented and the potential of the panel in the selection of drugs for combination chemotherapy is suggested.

Accepted 7 September 1987.
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This work was supported by grants from the Lundbeck Foundation.

MATERIALS AND METHODS

Cell lines

The cell lines, their source, maintenance and monitoring have previously been described [1]. Briefly the cell lines used, NCI-H69, NCI-N592, OC-TOL, OC-NYH were maintained in Roswell Park Memorial Institute Medium 1640 with 10% foetal calf serum and in a 7.5% CO_2 humidified atmosphere. The cell lines were free of mycoplasma

contamination, and flow cytometric DNA analysis [9] showed that they had stable DNA content.

Drugs

Cisplatin (Platinol), carboplatin (diamminecyclobutanedicarboxylatoplatinum NSC241240) and teniposide (Vumon) for infusion were obtained from Bristol-Myers. Cisplatin and carboplatin were dissolved (1 mg/ml) in sterile water, while teniposide was obtained in solution for infusion. The drugs were further diluted with tissue culture medium to 100 × final concentrations for 1 h incubation experiments and to 300 × final concentration for continuous incubation experiments. All drugs were used immediately after preparation. Based on toxicologic studies [10] carboplatin was tested at levels 15 times the cisplatin dosage used to obtain an 'equitoxic' comparison of activities.

Clonogenic assay

Cell survival was assessed by colony formation in soft agar [1]. A single-cell suspension was exposed to cisplatin, carboplatin or both for 1 h, washed twice and plated with or without teniposide in soft agar on top of a bottom layer containing RPMI-1640 tissue culture medium with 0.33% agar. After solidification of the agar 1 ml medium was added to prevent drying. For continuous incubation the cells were plated in agar with three times the desired final drug concentrations to compensate for equilibration of the drugs into the bottom layer and the added medium. The colonies were counted after 3 weeks using a dissecting microscope and the surviving fractions were calculated by dividing the number of colonies on the treated plates with the number of colonies on the untreated control plates. Due to lack of proportionality between the number of plated cells and number of colonies, the surviving fractions should ideally be corrected with simultaneously performed dilution experiments [1]. However, since the present investigation is comparing analogues in simultaneously performed experiments, the correction would affect the dose–response curves for both analogues identically and has therefore not been applied [1].

Stability test

For testing the stability of cisplatin and carboplatin in tissue culture medium, aliquots of 3.15 ml tissue culture medium with 1.11 × desired final concentration were stored in an incubator for varying time periods. Subsequently 350 μl of a single-cell suspension were added, incubated for 1 h, washed and plated as described above.

RESULTS

Figure 1 shows the dose–response curves obtained with 1 h incubation at 'equitoxic' doses of carboplatin and cisplatin. It is seen that the tested cell lines are equally or more sensitive to cisplatin than to carboplatin. Figure 1 also shows dose–response curves for the combination of 1 h incubation with the platin analogues and subsequent continuous incubation with teniposide. The slopes of the regression lines assuming linearity are given in Table 1. The slopes are indicated as the dose increment in μg/ml needed for a 50% increment in cell kill. The acceptability of the linearity assumption is indicated by r-square above 0.9 in most cases (range 0.77–0.99). It appears that the addition of teniposide results in curves with either identical or increased slopes indicating that the resulting surviving fractions are equal to or smaller than the product of the separate surviving fractions.

In Figure 2 dose–response curves obtained with continuous incubation are shown. When the drugs are compared at 'equitoxic' doses, it appears that carboplatin is more cytotoxic than cisplatin. However, when microgram to microgram doses of the two analogues are compared cisplatin remains more cytotoxic than carboplatin.

The dose–response curves obtained with continuous incubation with teniposide and platin analogues are included in Fig. 2 while the slopes of the regression lines are described in Table 1. It is evident that with continuous incubation with platin analogues, the addition of teniposide also results in additive or more than additive cell kill.

Table 2 shows ID_{50} values obtained with 1 h incubation of NCI-H69 with cisplatin or carboplatin after storage of the drugs for 0, 12, 24 or 48 h, respectively, in tissue culture medium in an incubator. It appears that the cytotoxicity of carboplatin is increased by a factor of two whereas the cytotoxicity of cisplatin is decreased by a factor of 9 after 48 h storage.

Figure 3 shows dose–response curves obtained with 1 h incubation with cisplatin plus carboplatin compared with cisplatin alone. The slopes of the regression lines are given in Table 1. For the two most sensitive cell lines the addition of carboplatin results in a clearly additive effect, whereas only a minor increase in cell kill is achieved on the two resistant cell lines.

It should be noted that the slopes of the regression lines obtained on the individual cell lines in different experiments, performed at a distinct time, are varying with up to a factor two. This is in accordance with the previously described variation of sensitivity patterns obtained on cell lines *in vitro* [1].

This variation is insignificant since the analogues are compared in simultaneously performed experiments.

Despite the variation, a ranking of the cell lines with reference to sensitivity to platin analogue will

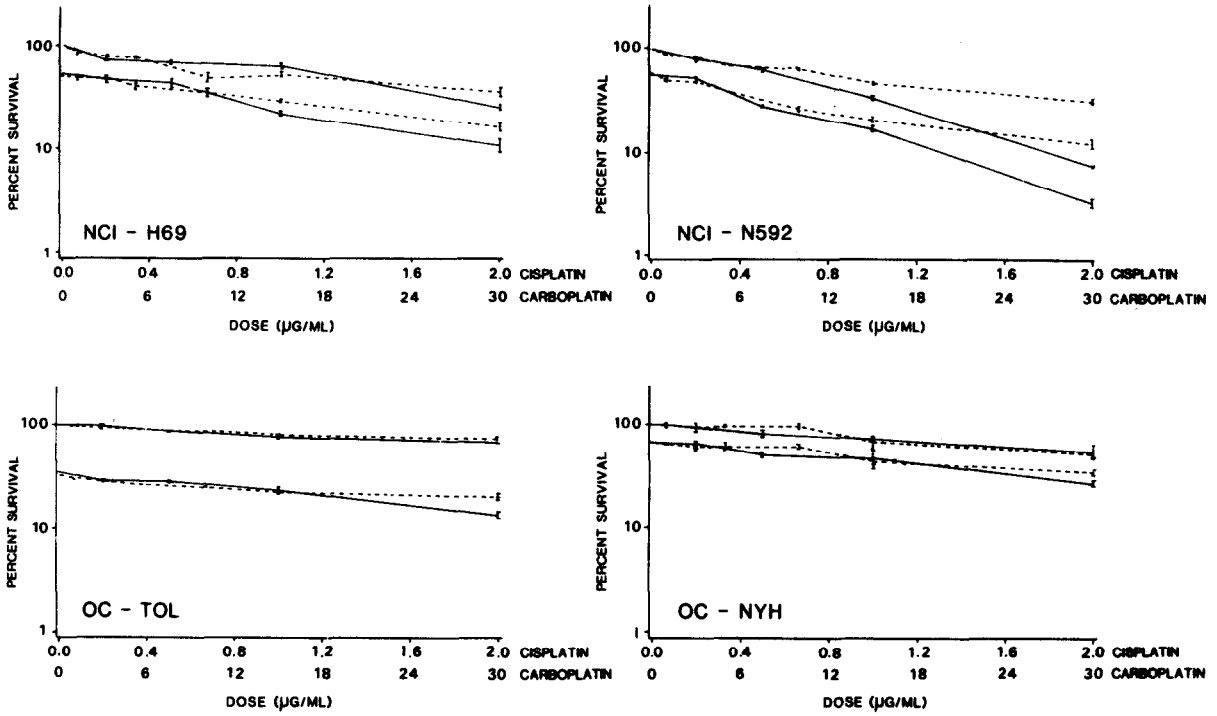

Fig. 1. *Dose-survival curves for* 1 h in vitro *exposure to either cisplatin (solid lines) or carboplatin (broken lines). The curves starting below* 100% *represent cells continuously exposed to* 0.02 *micromolar (NCI-H69, NCI-N592, OC-TOL) or* 0.01 *micromolar (OC-NYH) teniposide after exposure to platin. Note that carboplatin was used at* 15 *times the cisplatin concentrations used. Bars represent* 2 *S.E.M. Each experiment was done in triplicate. Plates without colonies have been omitted.*

Table 1. *Slopes of the regression lines expressed as the dose increment in* µg/ml *needed for* 50% *increment in cell kill* (ID_{50})

	One hour drug exposure						Continuous drug exposure			
	Data from Fig. 1				Data from Fig. 3		Data from Fig. 2			
Cell line	Cis-platin	Cisplatin + VM-26	Carbo-platin	Carboplatin + VM 26	Cis-platin	Cisplatin + carboplatin	Cis-platin	Cisplatin + VM-26	Carbo-platin	Carboplatin + VM-26
NCI-H69	1.2	0.8	23	18	0.7	0.6	0.043	0.038	0.10	0.09
NCI-N592	0.5	0.5	19	14	0.5	0.5	0.056*	0.055*	0.14	0.09
OC-TOL	3.5	1.6	70	53	1.8	1.9	0.080	0.049	0.62	0.20
OC-NYH	2.5	1.5	30	32	4.3	2.3	0.051*	0.051*	0.21*	0.21*

*Indicates that the slopes only were determined by two observations.

be identical in most of the cases and disclose two relatively sensitive lines (NCI-N592 and NCI-H69) and two more resistant lines (OC-TOL and OC-NYH).

DISCUSSION

The present study has shown that in a panel of human small cell lung cancer cell lines cisplatin is equally or more potent than carboplatin when 1 h incubation experiments are compared (Fig. 1). Ideally equitoxic doses of the drugs should be compared, but because of different prevailing toxicities, equitoxicity is difficult to define. In this study cisplatin has been compared to carboplatin concentrations 15 times higher, based on doses defined in beagle dogs where lethal, high toxic, low toxic and highest non-toxic doses all are approx. 15 times the corresponding doses of cisplatin [10]. Although this approach implies that carboplatin was tested at up to 10 times the maximal achievable serum concentrations in patients [11], whereas cisplatin was only tested at up to the maximal achievable concentration [12], it was chosen for maximizing the chance of carboplatin manifesting a treatment advantage. Even with this assumption, carboplatin was not superior to cisplatin when the two analogues were compared using the 1 h incubation procedure.

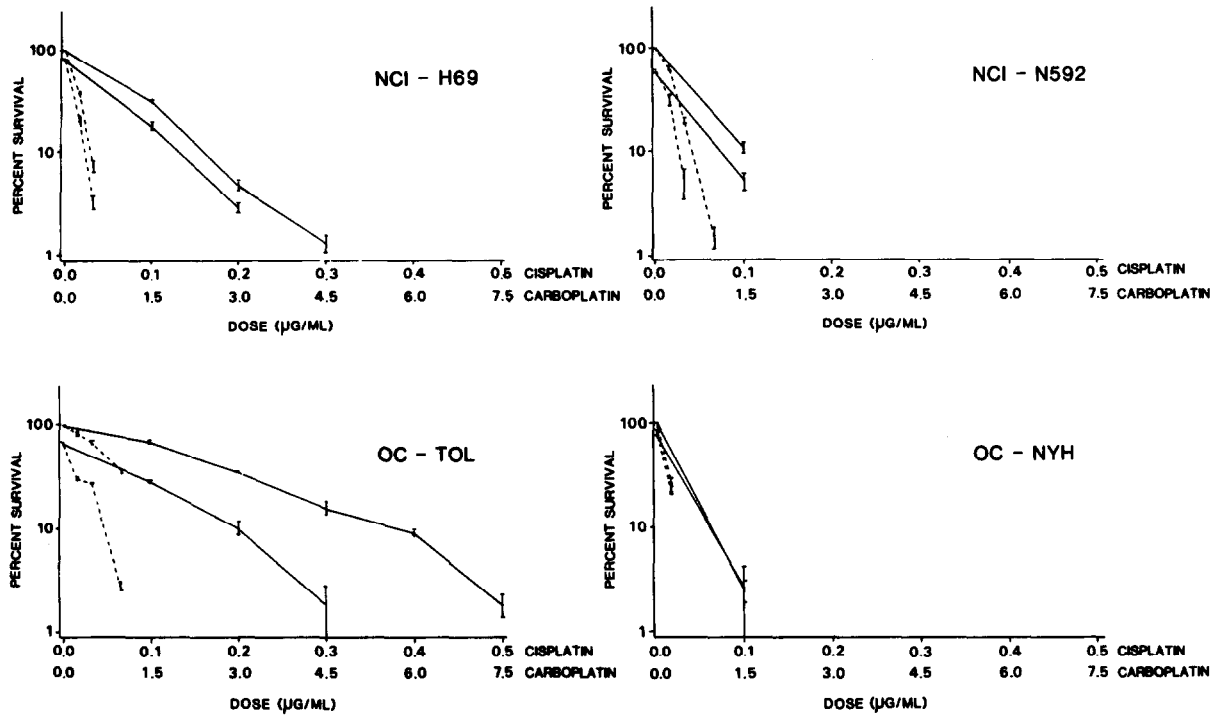

Fig. 2. Dose-survival curves for continuous in vitro *exposure to either cisplatin (solid lines) or carboplatin (broken lines). The curves starting below* 100% *represent cells concomitantly exposed to platin analogue and* 0.02 *micromolar (NCI-H69, NCI-N592, OC-TOL) or* 0.01 *micromolar (OC-NYH) teniposide. Note that carboplatin was used at* 15 *times the cisplatin concentrations used. Symbols as in Fig.* 1.

Table 2. Storage stability of cisplatin and carboplatin

Storage time (h)	ID_{50} (μg/ml) Cisplatin	Carboplatin
0	1.0	47.3
12	1.3	26.6
24	4.3	23.2
48	8.8	24.2

The table indicates the ID_{50} values in μg/ml obtained from the slopes of the regression lines. NCI-H69 and 1 h exposure was used for testing the stability of the drugs for the indicated time after preparation of the solutions.

The increased potency of carboplatin with increased exposure time has previously been described. In one study a human ovarian cancer cell line showed sensitivity to cisplatin but not to carboplatin with 1 h incubation, whereas carboplatin showed efficacy similar to that of cisplatin when the period of drug exposure was lengthened to 24 h [11]. Although the present study also has revealed increased potency of carboplatin when tested with continuous exposure, the potency of carboplatin never reached that of cisplatin when compared on a microgram to microgram basis. The relative increase in potency of carboplatin, when tested with continuous incubation can at least partly be explained by the demonstrated increase in toxicity of carboplatin and the concomitant decrease in toxicity of cisplatin when stored in tissue culture medium (Table 2), although exact quantitation of this phenomenon's contribution to the increased cytotoxicity is not possible with the present data. A change in potency of carboplatin has also been described after storage in saline where 22 h storage produced a 2 log increase in cell kill [13].

Despite drug stability difficulties it may be relevant in future testing, because of substantial differences in the pharmacokinetics, to compare the two analogues *in vitro* with different periods of drug exposure, as the plasma half-life of carboplatin is longer than that of cisplatin (170 min vs. 30 min) [11, 14]. Furthermore carboplatin does not react as extensively with plasma proteins as cisplatin and there is evidence that only the free fractions are active [11].

Combination chemotherapy is used with the hope that one or more of the assets needed by drug combination to show therapeutic synergism will be present in the combination. These assets are (a) that the drugs used in the combination will be less than additive in toxicity for vital normal cells, (b) that the drugs will have different biochemical mechanisms of cytotoxic activity for drug-sensitive tumour cells, and/or (c) that tumour cells resistant to one or more drugs in the drug combination used in treatment will be sensitive to one or more other drugs in the combination. All three of these therapeutic assets are known to be obtained with a number of clinically useful drug combinations and

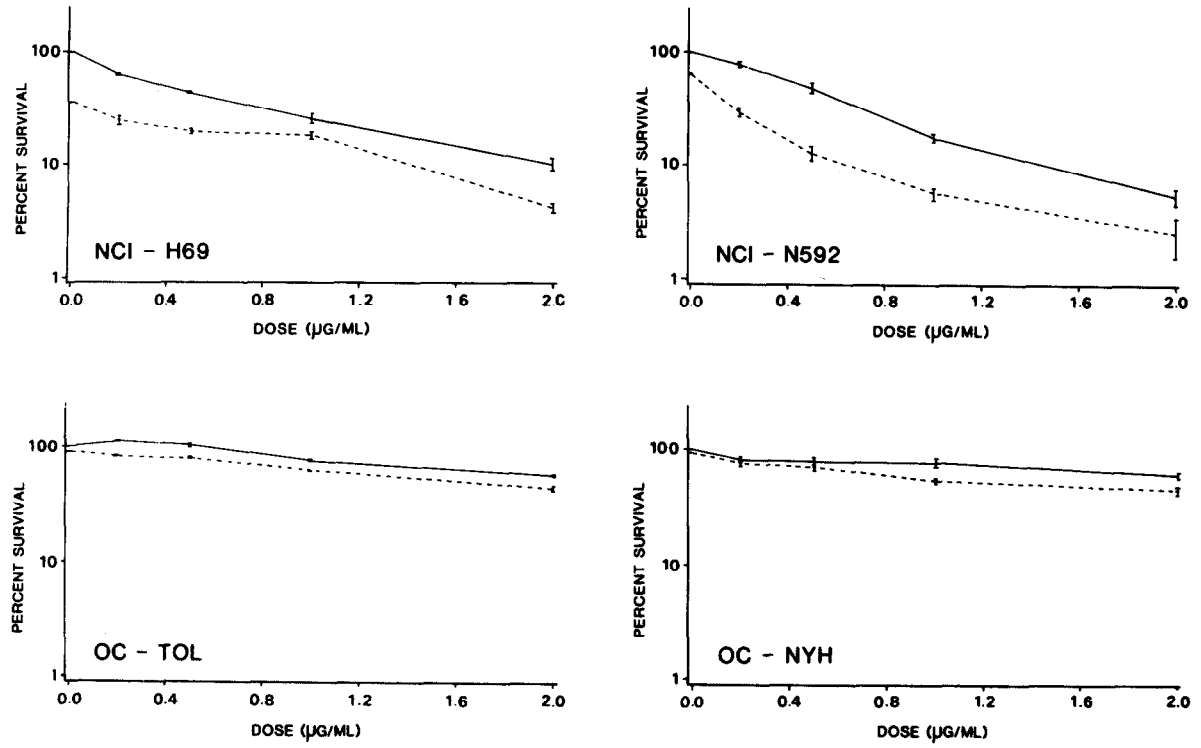

Fig. 3. Dose-survival curves for 1 h in vitro *exposure to either cisplatin alone (solid lines) or cisplatin plus* 15 μg/ml *carboplatin (broken lines). Symbols as in Fig.* 1.

are always sought with new drug combinations [15]. As a consequence of assets (a) and (c) two agents that are additive or subadditive at the cellular level can appear synergistic at the whole-organism level [16]. For the time being the selection of drugs for combination chemotherapy is based on the addition of drugs which have shown activity in clinical phase II trials. The use of a panel of cell lines in sensitivity testing makes it possible to compare the sensitivity to different drugs and thus hopefully the selection of combinations with activity against subpopulations with different sensitivity. In Fig. 4 the relative sensitivities obtained in the present investigation are compared to the results of previous experiments [1–3]. The similarity in sensitivity profile of the 'platinum' epipodophyllotoxin and nitrosourea analogues, respectively, is striking. Since cross-resistance between analogues is the rule this finding strongly supports the credibility of the results of this test system. Furthermore, the profiles of the epipodophyllotoxin derivatives seem to be complementary to those of the platinum (and to some extent the nitrosourea) analogues. It is tempting to explain the high activity of the epipodophyllotoxin derivatives against SCCL [17] by its activity against subpopulations with decreased sensitivity to other drugs. If this assumption can be confirmed when the panel of cell lines is enlarged it would further validate this approach for selecting drugs for combination chemotherapy. The final validation must await clinical trials with new combinations selected after testing in a panel of cell lines.

Clinical evidence for synergy between cisplatin and epipodophyllotoxin derivatives has been demonstrated in a trial where a 60% response rate was achieved with cisplatin and etoposide in patients with refractory recurrent SCCL previously treated with etoposide [18]. Very marked therapeutic synergism between etoposide and cisplatin have also been demonstrated in the mouse P 388 leukaemia model [15].

At the celluar level the sensitivity to cisplatin of Chinese hamster ovary cells resistant to etoposide and teniposide was found to be enhanced in comparison to the parental cells [19]. In accordance with the collateral sensitivity also demonstrated in the present investigation (Fig. 4), an additive effect of cisplatin and etoposide has been demonstrated by colony-formation technique utilizing a long-term culture of human lymphoma cells [20]. Likewise the present investigation has shown that when the cells were exposed to teniposide after exposure to either cisplatin or carboplatin, as well as concomitant exposure to a platin analogue and teniposide, at least additive cell kill was achieved. Additivity was also obtained when platin analogues were combined with etoposide (Roed *et al.*, unpublished data). Achievement of additivity in these *in vitro* conditions with both combinations indicates that the combination of epipodophyllotoxin with carboplatin could be as useful clinically as cisplatin combined with epipodophyllotoxin against SCCL. Additivity was

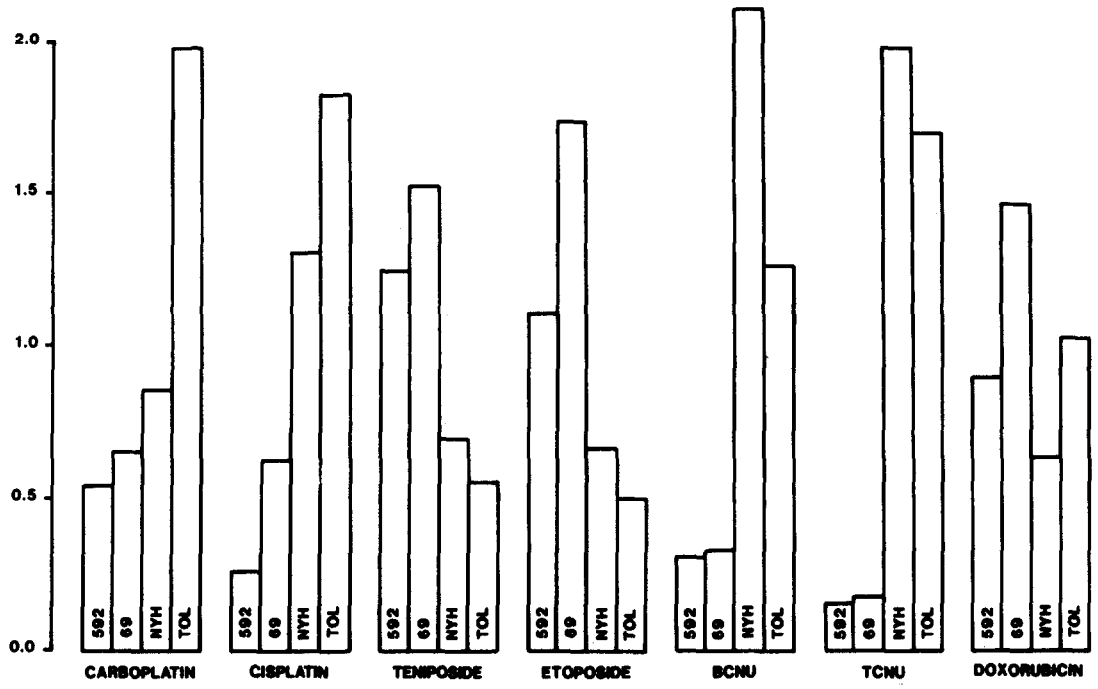

Fig. 4. Relative sensitivities of four SCCL cell lines to seven anticancer drugs. The sensitivities are depicted as the doses needed to obtain 50% *cell kill* (ID_{50}) *normalized with the mean* ID_{50} *values obtained with the individual drugs. Such a depiction implies that the cell lines most sensitive to a particular drug will have values below* 1.0.

also obtained when cisplatin was combined with carboplatin. Since the two drugs have different prevailing toxicities, this additivity will imply a clinical synergy if the two drugs can be combined without reducing their dose levels to a greater extent [21]. If these *in vitro* results mimic the clinical behaviour of SCCL it can be concluded that (1) cisplatin is equi- or more potent than carboplatin; (2) combination of platin analogue with teniposide results at least in additive cell kill *in vitro* which could imply a clinical synergism; (3) cisplatin and carboplatin could be a clinically useful combination.

If these assumptions can be confirmed in clinical trials already initiated they would validate the use of *in vitro* trials and thus diminish the need for testing drugs in patients [4].

Acknowledgements—Annette Nielsen and Eva Høj are thanked for excellent technical assistance, and Merete Jacobsen, Annemarie Schultz and John Post for preparing the manuscript.

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