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In Vitro and *In Vivo* Evaluation of the Indoloquinone EO-9 (NSC 382 459) Against Human Small Cell Carcinoma of the Lung*

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Abstract—*As the indoloquinone EO-9 has previously shown activity in several tumor model systems it was evaluated against four human small cell lung cancer cell lines by the clonogenic assay. In two cell lines (Nyh and Tol), exponential dose–response curves were achieved with both* 1 h *and continuous exposure, whereas no cell kill was obtained in the other two cell lines* (69 *and* 592) *when tested with* 1 h *incubation up to* 0.25 μg/ml. *When the cells were exposed to drug* in vitro, *flow cytometric DNA analysis showed perturbations in the cell cycle distribution of the most sensitive cell line (Tol) at a lower EO-9 concentration than in the less sensitive cel line* (592). *This* in vitro *predicted difference in EO-9 sensitivity between two of the cell lines* (592 *and Tol*) *was confirmed when the cell lines were heterotransplanted to nude mice.*

INTRODUCTION

In vitro testing of cytotoxic agents has been proposed as a means both of screening potential anti-cancer drugs and of assessing the activity of drugs against specific tumor types. Since *in vitro* screening tests cannot give information about the therapeutic index, the *in vitro* drug concentrations used must be related to achievable levels in animals [1], or activity demonstrated *in vitro* must be confirmed *in vivo*.

In the search for new antineoplastic agents, a series of novel indoloquinones have been synthesized. The structure of the prototype EO-9 [2] is shown in Fig. 1. These agents have the potential to act as bioreductive alkylating agents like the antitumor antibiotic mitomycin C [3]. Although most of the analogs showed no significant activity against the P 388 murine leukemia *in vivo*, significant growth delay has been demonstrated with the analog EO-9 against ovarian, breast and non-small cell lung cancer in nude mice [2].

In this study, EO-9 was tested against four cell lines of human small cell carcinoma of the lung (SCCL) *in vitro* using the clonogenic assay. Simultaneously, the ability of EO-9 to induce cell cycle perturbations was determined by flow cytometric DNA analysis.

The clonogenic assay experiments showed a differential effect among the cell lines. Based on these results, a sensitive and a less sensitive cell line were selected for heterotransplantation into nude mice, with the purpose of investigating whether the relative sensitivities found *in vitro* could be reproduced *in vivo*.

MATERIALS AND METHODS

Cell lines

The cell lines used and their source, maintenance and monitoring have been described elsewhere [4]. Briefly, the cell lines used were NCI-H69 (69), NCI-N592 (592), OC-Tol (Tol), OC-Nyh (Nyh) maintained in Roswell Park Memorial Institute medium 1640 with 10% fetal calf serum and in a 7.5% CO_2 humidified atmosphere. The cell lines were free of mycoplasm contamination and had a stable DNA content.

Drugs

EO-9 was synthesized by E.A. Oostveen (University of Amsterdam), supplied by EORTC, New Drug Development Office, Free University Hospital, Amsterdam, and dissolved to 0.5 mg/ml in isotonic

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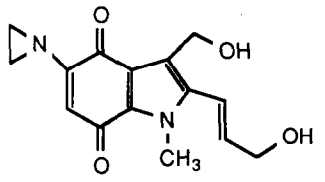

Fig. 1. Chemical structure of the indoloquinone EO-9.

saline just prior to use. This solution was further diluted with tissue culture medium for the *in vitro* experiments.

Clonogenic assay

Cell survival was assessed by colony formation in soft agar as described previously [4]. A single-cell suspension was exposed to the drug for 1 h, washed twice and plated in soft agar on top of a feeder layer. After solidification of the agar, 1 ml medium was added to prevent drying.

For continuous incubation studies, the cells were plated in agar containing three times the desired final concentration to compensate for equilibration of the drug into the feeder layer and the added medium. The feeder layers were prepared with sheep red blood cells and mercaptoethanol [4].

The colonies were counted after 3 weeks using a dissecting microscope. The surviving fractions were calculated by dividing the number of colonies on the treated plates with the number of colonies on the control plates. Corrections for lack of proportionality between the number of plated cells and the resulting number of colonies were done on basis of simultaneously performed dilution experiments [4].

Determination of drug-induced cell cycle perturbations by flow cytometric DNA analysis

The cell lines were exposed to the drug for 24 h in tissue culture flasks. After centrifugation the cells were suspended in citrate buffer, frozen on ethanol with dry ice and stored at −80°C until analysis [5]. Before analysis in a FACS III flow cytometer (Becton Dickinson, Sunnyvale, CA) the samples were stained with propidium iodide [6]. The percentage of cells in each cell cycle phase was determined by statistical analysis of the DNA distribution [7].

Nude mice experiments

592 and Tol tumors were established by inoculation of 3–4 × 10^6 cells subcutaneously in both flanks of nude male mice (NMRI nu/nu Bomholtgaard, Ry, Denmark) and used for the i.v. drug administration studies. For the i.p. studies 1–2 mm^3 tumor pieces were implanted subcutaneously. The mice were kept under sterile conditions in laminar air-flow clean benches and allowed sterile food and water *ad libitum*. The tumors were measured at two perpendicular diameters by calipers three times per week. After six measurements, the mice were randomized into control and treatment groups. Two drug administration routes were examined. In one experiment, tumors were treated with 5 mg/kg EO-9 intravenously (i.v.) twice (in a concentration of 0.5 mg/ml) at a 7-day interval. This dose schedule was recommended by the EORTC New Drug Development Office as the optimal dosage in nude mice (Dr. M.W. Lobbezoo, personal communication). As the drug was locally irritating causing tail edema and necrosis, intraperitoneal (i.p.) injections with a more diluted drug concentration (0.2 mg/ml) was used comparing control animals with a 5 mg/kg × 2 and with a 3.5 mg/kg × 2 dose schedule. In these experiments, the injections were also given at 7-day intervals.

Evaluation of response in vivo

The mean tumor areas (product of the two measured diameters) were used to construct the mean tumor growth curves [8]. The mean tumor area A_m (t_a) equivalent to the mean double tumor volume [9] was estimated:

$$A_m(t_a) = \sqrt[1,5]{2}\, A_m(t_0)$$

where $A_m(t_0)$ is the mean tumor area at the treatment start. The mean tumor growth delays defined as the time of tumor growth to reach a tumor size equivalent to twice the mean treated tumor volume was determined from the mean tumor growth curves.

The effect of EO-9 was evaluated from the mean tumor growth curves by calculation of the mean specific growth delay (SGD_m) [8]:

$$SGD_m = \frac{TGD_m - CGD_m}{CGD_m}$$

where TGD_m is the mean growth delay of the treated tumors and CGD_m is the mean growth delay of the untreated control tumors [10].

RESULTS

In vitro *experiments*

Figure 2 depicts dose–response curves for the four cell lines obtained after 1 h incubation with EO-9. Similarly, Fig. 3 shows dose–response curves obtained with continuous incubation. EO-9 caused exponential cell kill with 1 h as well as with continuous exposure in two of the cell lines (Nyh and Tol). In the two other cell lines (69 and 592) no cell kill was obtained with 1 h incubation, and with continuous incubation only a minor cell kill was achieved in 592 at the highest concentrations used. By comparing the concentrations used for the 1 h experiments with those used for continuous incu-

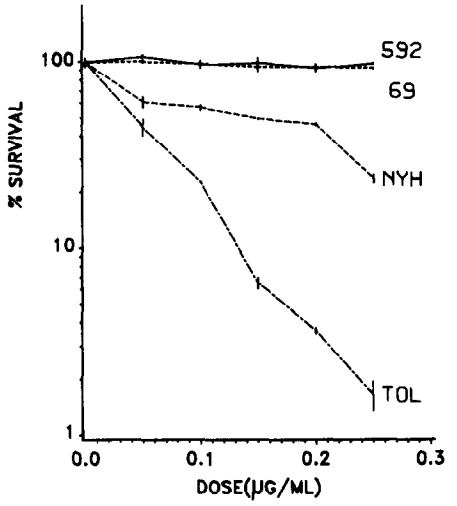

Fig. 2. Dose–survival curves for 1 h in vitro *exposure to EO-9 of four human small cell lung cancer cell lines. Bars represent two S.E.M. Each experiment was done in triplicate.*

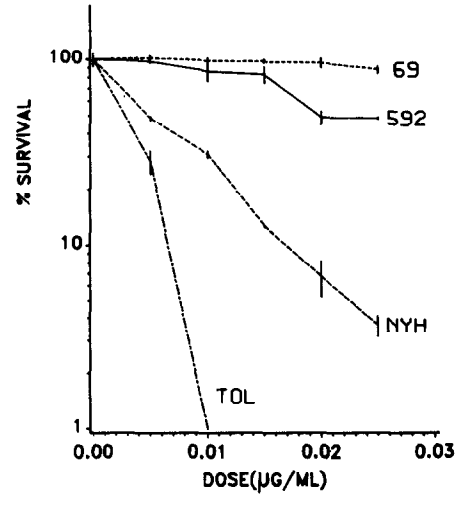

Fig. 3. Dose–survival curves for continuous in vitro *exposure to EO-9. Doses beyond* 0.01 μg/ml *caused total cell kill in Tol. Symbols as in Fig. 2.*

bation it appears that only 15–30 times higher concentrations were needed to obtain the same degree of cell kill with 1 h incubation compared with continuous incubation. The low increase in potency with increased exposure time, as well as the achievement of exponential dose–response curves without saturation, suggest that EO-9 is cell cycle non-specific [11].

In Fig. 4, histograms obtained by flow cytometric DNA analysis after 24 h exposure of the tumor cells to EO-9 at different drug concentrations are shown. The results obtained with the most sensitive cell line (Tol) are shown in the upper histograms. It appears that EO-9 caused a dose-dependent accumulation of cells in the S-phase of the cell cycle and a concomitant decrease of the fraction of cells in the G_1-phase. With increasing drug concentration the accumulation was detected earlier in the S-phase, and the amount of cellular debris increased. In the lower histograms of Fig. 4, the cell cycle perturbations obtained with EO-9 in a less sensitive cell line (592) are shown. It appears that EO-9 in this cell line caused perturbations similar to the perturbations in the more sensitive cell line, but higher concentrations (approx. 10×) were needed to obtain comparable effects.

In vivo *experiments*

Based on the *in vitro* results, one of the sensitive (Tol) and one of the less sensitive cell lines (592) were chosen for *in vivo* treatment. The mean tumor growth curves are shown in Figs. 5 and 6. The mean specific growth delays (SGD_m) for the treated tumors are given in Table 1. For both cell lines, dose-dependent effects in the i.p. experiments were found. No difference was demonstrated between the two drug administration routes (i.p. vs. i.v.). The effect of EO-9 on the most sensitive tumor cell line (Tol) selected by the *in vitro* experiments was approximately twice the effect on the less sensitive cell line (592) as measured by SGD_m. Thus the *in vitro* predicted difference in EO-9 sensitivity between these two cell lines was confirmed in the *in vivo* test system.

DISCUSSION

The value of a drug screening model is ultimately established by its ability to identify compounds which are useful in clinical treatment. The human tumor colony forming assay has been shown to detect the effect of the majority of established cytotoxic agents in clinical use [12]. Since the assay only reflects the cytotoxicity of an agent it cannot predict whether the drug concentration used *in vitro* can be obtained in tumor tissue *in vivo*. By reflecting, *in vivo*, the differences in sensitivity to EO-9 *in vitro*, the present study has illustrated the feasibility of selecting the most sensitive cell line *in vitro* before testing the sensitivity in the more expensive and time consuming nude mice system.

Simultaneously, flow cytometric DNA analysis (FCM) has shown that cell cycle perturbations (Fig. 4) were encountered at lower concentrations for the sensitive (Tol) than for the less sensitive cell line (592). This indicates that flow cytometric DNA analysis can also be applied in the sensitivity testing of EO-9 as previously described for other drugs [13]. The nature of the cell cycle perturbations does not permit a distinct statement about the mode of action of EO-9, although similar perturbations have been seen with the alkylating agent BCNU [13].

The relative sensitivities of the four cell lines tested, where two cell lines were sensitive (Tol and Nyh) and the other two less sensitive (69 and 592)

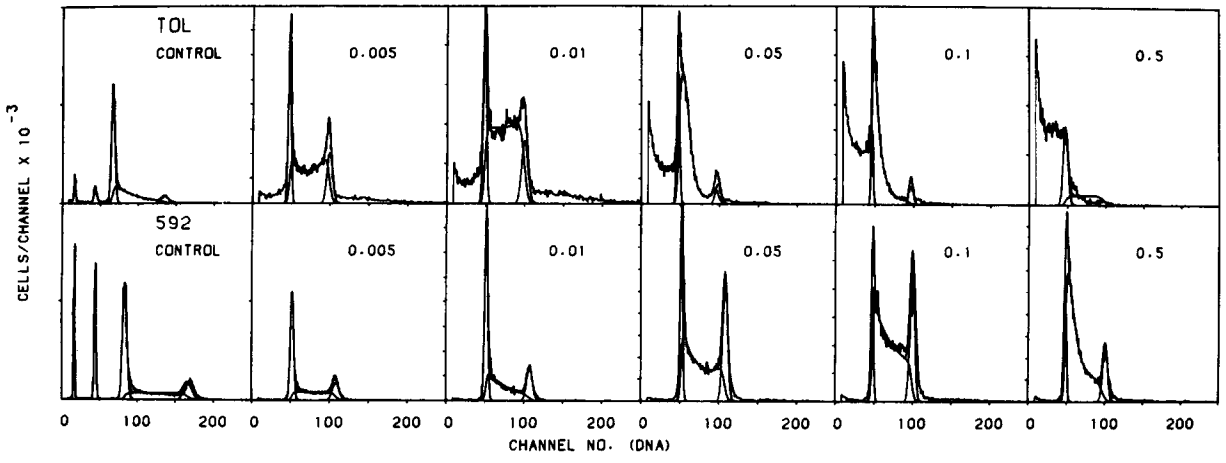

Fig. 4. Flow cytometric DNA histograms obtained after exposing one of the sensitive cell lines (Tol, upper lane) and one of the less sensitive cell lines (592, lower lane) to different EO-9 concentrations (μg/ml) for 24 h. The first two peaks in the control histograms represent internal references (chicken and trout erythrocytes) used to calculate the DNA indices.

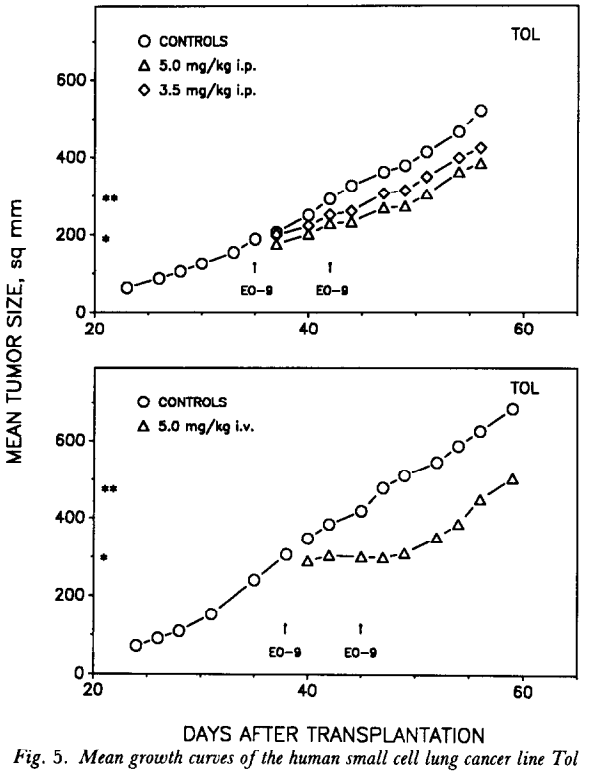

Fig. 5. Mean growth curves of the human small cell lung cancer line Tol in nude mice before and after treatment with EO-9. Top: i.p. schedule 5 mg/kg × 2 (n: 10), 3.5 mg/kg × 2 (n: 12) *and controls* (n: 9). *Bottom: i.v. schedule* 5 mg/kg × 2 (n: 8) *and controls* (n: 7). n: *number of tumors. *: Mean tumor area at treatment start. **: the calculated mean tumor area equivalent to the mean double tumor volume.*

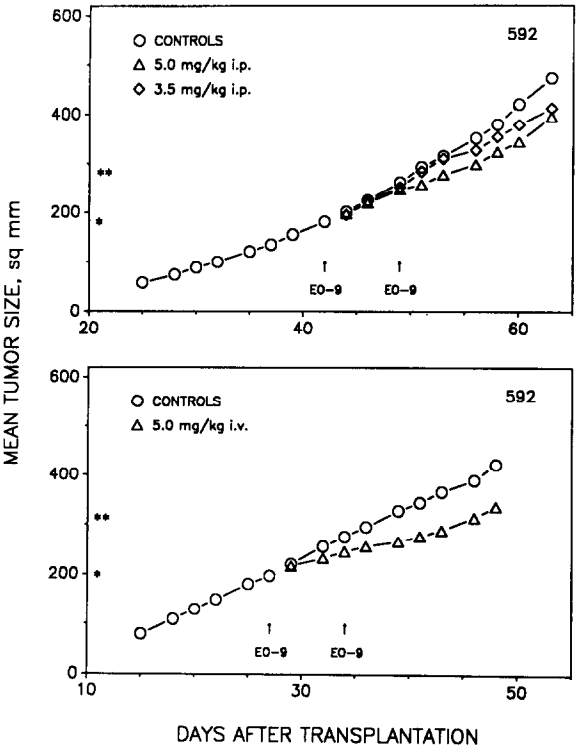

Fig. 6. Mean growth curves of the human small cell lung cancer line 592 *in nude mice before and after treatment with EO-9. Top: i.p. schedule* 5 mg/kg × 2 (n: 10), 3.5 mg/kg × 2 (n: 13) *and controls* (n: 12). *Bottom: i.v. schedule* 5 mg/kg × 2 (n: 9) *and controls* (n: 9). *Symbols as in Fig. 5.*

Table 1. Mean specific growth delays (SGD_m) for the treated tumors 592 and Tol

Tumor	Treatment schedule 5 mg/kg × 2 i.v.	5 mg/kg × 2 i.p.	3.5 mg/kg × 2 i.p.
592	0.7	0.5	0.1
Tol	1.1	1.0	0.6

is directly reverse to the findings of a previous study in which the cell lines were tested against nitrosourea [13]. This suggests collateral sensitivity between EO-9 and nitrosoureas.

These results justify further testing of EO-9 against a larger panel of SCCL cell lines. If the drug is active against a substantial number of cell lines, a phase II study in SCCL seems warranted.

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