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Influence of the Synthetic Microtubule Inhibitor Erbulozole (P.I.N.N.) (R 55 104), a New Tubulozole Congener, and Gamma Irradiation on Murine Tumors *In Vivo*

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Abstract—*Erbulozole (P.I.N.N.) (R* 55 104) *is a more water soluble congener of the synthetic microtubule inhibitor tubulozole (R* 46 846) *exhibiting a reversible antimicrotubular activity* in vitro *at a dose* (1.56 × 10^{-8} M) *which is at least* 10*-fold lower. The compound also has an antiinvasive potential and shows antitumoral effects both* in vitro *and* in vivo *when administered appropriately. Eighty* mg/kg *R* 55 104, *given orally* 6 h *before or* 3 h *after radiotherapy, displays a prominent interactive effect with* 10 Gy *gamma irradiation in subcutaneous murine tumors which is similar to* 160 mg/kg *tubulozole administered* 6 h *before* 10 Gy. *The enhancing effect is also observed in a clinically relevant radiation dose fractionation schedule whereby eight fractions of* 2 Gy *each were pretreated* 2 h *before with* 40 mg/kg *R* 55 104. *Further study of this radiochemotherapeutic combination may lead to new clinical applications.*

INTRODUCTION

RECENTLY, a new class of microtubule inhibitors has been discovered, of which tubulozole (R 46 846) can be considered as the prototype [1]. As a result, the pharmacological properties of tubulozole have been intensively studied. The compound prevents the polymerization of tubulin, *in vitro* (ID_{50}* = 3.4 × 10^{-7}) [2]. It exerts a prominent antitumoral effect in different tumor systems both *in vitro* and *in vivo* [2, 3]. Inhibition of malignant invasion of tumor cells has been demonstrated *in vitro* [2] and *in vivo* [4, 5]. Tubulozole, most prominently, exhibits an enhancing effect on both gamma-irradiated tumor cells *in vitro* [6] and subcutaneous murine tumors whereby a dose–response curve has been assessed by using both different drug concentrations and doses of radiation [7, 8]. However, since the drug appears to be highly hydrophobic, it may be difficult to find a suitable clinically applicable formulation. It thus seemed worthwhile to evaluate more water soluble tubulozole congeners. Erbulozole (P.I.N.N.) (R 55 104) may be an interesting potential successor. It is more hydrophilic than tubulozole and its antimicrotubular effect on cell cultures is similar to tubulozole at a dose which is at least 10-fold lower (down to 1.56 × 10^{-8} M) [9]. Anti-invasive characteristics have been observed *in vitro* at 1.5 × 10^{-7} M [10] and an obvious antitumoral effect in different tumor systems *in vitro* and *in vivo* could be demonstrated when administered appropriately [11]. All these observations point to the existence of an antitumoral potential which is comparable to the activity of tubulozole. Erbulozole can be administered at relatively high doses before toxicity occurs (LD_{10} > 160 mg/kg in mice) [11].

The present study was exerted to evaluate the effect of the combination R 55 104 and gamma irradiation in subcutaneous murine tumors.

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*50% inhibitory dose.

MATERIALS AND METHODS

Tumor and animals

MO_4 fibrosarcoma. The MO_4 cell line has been derived from mouse embryo cells transformed by the Kirsten murine sarcoma virus [12]. MO_4 cell cultures were maintained on plastic tissue culture

substrate (Flow Laboratories Ltd., Irvine, U.K.) containing Eagle minimal essential medium (modified) with Earle's salts, antibiotics, non-essential amino acids and 0.85 g/l sodium bicarbonate without L-glutamine (EMEM, Flow Laboratories) supplemented with 1% L-glutamine, 2% sodium bicarbonate (hereafter called culture medium) and with 10% fetal calf serum. Cell cultures were held in a humidified atmosphere of 5% CO_2 in air at 37°C. MO_4 cells were subsequently trypsinized, resuspended in culture medium with 10% fetal calf serum, centrifuged and diluted in medium without fetal calf serum at a final concentration of 5×10^6 cells/ml. 10^6 MO_4 cells were injected subcutaneously into the left inguinal region of CDF_1 mice resulting in well defined tumors with reproducible growth.

Animals. Male CDF_1 (DBA/2× C3H) hybrids with a body weight of 18–22 g were obtained from Janssen Research Laboratories, Beerse, Belgium and were used throughout in all experiments.

Evaluation of tumor growth

Tumors of approximately 1 cm^3 were obtained 14 days after injection. Tumor volumes (TV) were measured by callipers and values were obtained by multiplying the square of the smallest diameter (a) with the largest diameter (b) ($TV = a^2 \times b$). The first measurement was designated as the initial tumor volume at 'day zero' (TV_0). Individual tumors were measured on consecutive days as described above and the relative tumor growth was calculated as a percentage of the initial tumor volume. The T_d was defined as the time necessary to reach twice the initial treatment volume ($T_d = [2TV_0TV_0] \times 100\% = 200\%$) [13]. Individual T_ds were determined by linear interpolation on the plots of log tumor volume versus day of measurement. When the T_d was not achieved, the last day of observation was designated as a lower limit of the actual T_d ('censored T_d') and subsequently analyzed using 'survival data analysis methods'. This method compares the treated groups with their corresponding controls using the Peto–Peto–Wilcoxon test. Due to the 'censored' nature of the data, results were given as median values (as an index of location) and using the 25% and 75% quantiles as an index of spread. All computations were performed by means of the SAS system [14]. Two-tailed probabilities less than or equal to 0.05 ($P < 0.05$) were considered to indicate significance. 'Excess growth delay' was defined according to Barendsen and Jansc [15]. They considered 'growth delay' to be the T_d of the treated tumor, with the T_d of the untreated tumor subtracted. 'Excess growth delay' is defined as the difference between tumor growth delay of a radiochemotherapeutic combination and the sum of delays of the individual treatments.

Drugs

All concentrations of R 55 104 (erbulozole) were dissolved in 0.2 ml of an aqueous solution (less than 4 mg/ml in 1 eq. tartaric acid; more than 4 mg/ml in 20% PPG and 1 eq. formic acid). Tubulozole (R 46 846) was suspended in 0.2 ml of a 2% aqueous Tween solution. Both drugs were administered by oral gavage.

Irradiation

At day zero, tumors of unanesthetized mice were locally irradiated with collimated gamma irradiation from a ^{60}Co source. The collimator had a diameter of 2 cm and the source-to-skin distance was 13 cm resulting in a dose rate of 42.2 Gy per hour. Scatter radiation was determined by means of Fricke's dosimetry and found to be maximally 50% to the left hindleg and less than 10% to the rest of the body.

RESULTS

Comparison between the effect of R 55 104 *and* 10 Gy *gamma irradiation*

One single dose of 20, 40 or 80 mg/kg R 55 104 respectively was compared with 10 Gy alone (Fig. 1). A moderate dose-dependent effect on T_d was observed. The effect of one dose of 40 or 80 mg/kg R 55 104 on tumor growth was comparable with 10 Gy radiation.

Comparison between R 55 104 *and tubulozole in combination with* 10 Gy *radiation*

Eighty mg/kg of R 55 104 was given 2, 4 and 6 h respectively before 10 Gy radiation (Fig. 2). From this preliminary experiment it could be con-

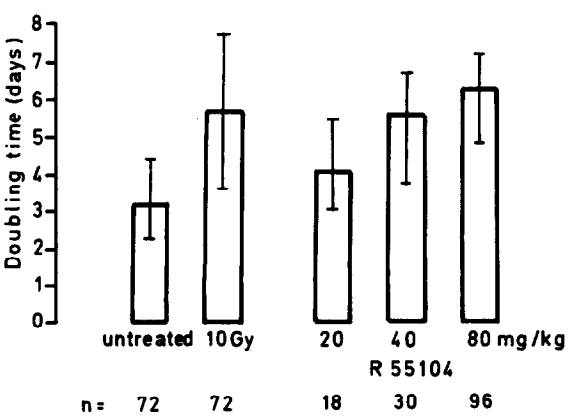

Fig. 1. Initial doubling time (days) of n MO_4 *tumors. At day* 0, *tumors approximating* 1 cm^3 *were irradiated with* 10 Gy *or treated with* 20, 40 *or* 80 mg/kg *R* 55 104 *respectively. Median and quantiles* (25% *and* 75% *are shown*).

cluded that an optimal initial MO_4 tumor regression was obtained when R 55 104 was administered 2 h before irradiation. Furthermore, the effect of this pretreatment with R 55 104 was similar to the initial tumor regression achieved with 160 mg/kg tubulozole given at the optimal pretreatment time of 6 h before 10 Gy [7] (Fig. 3).

Repeated treatment with radiation and R 55 104 *given* 2 h *before*

Tumors were treated three times with 10 Gy and 80 mg/kg R 55 104 given 2 h before irradiation, i.e. at day 0, day 5 and day 12 respectively (Fig. 4). A marked effect on tumor regression could be seen in animals treated three times with this particular radiochemotherapeutic combination. The effect of the pretreatment with R 55 104 was also investigated in a more clinically relevant dose fractionation schedule. Tumors were irradiated with 2 Gy on 4 consecutive days and this schedule was repeated after a treatment-free interval of 4 days. All fractions (totalling 16 Gy) were pretreated with 40 mg/kg R 55 104 2 h before irradiation (Fig. 5). A prominent effect of the combined treatment on growth delay could be observed after both fractionation sessions.

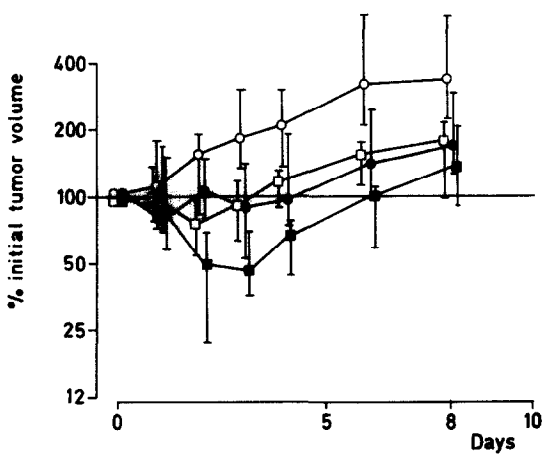

Fig. 2. Relative tumor growth (% initial tumor volume) plotted against time. Radiochemotherapeutic treatment was given at day 0. Six animals were used per treatment. Median and extreme values are shown. (○) *Untreated;* 80 mg/kg *R* 55 104 6 h (●), 4 h (□), 2 h (■) *respectively before* 10 Gy *radiation.*

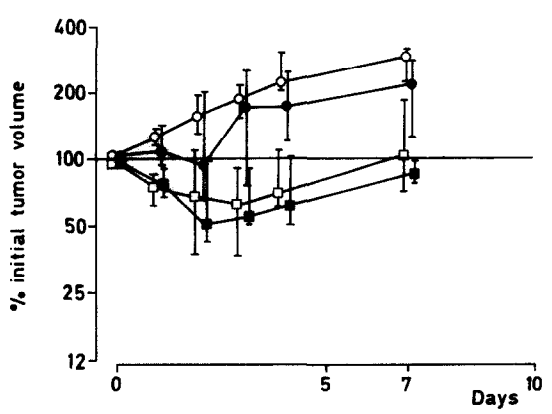

Fig. 3. Relative tumor growth (% initial tumor volume) plotted against time. Radiochemotherapeutic treatment was given at day 0. Six animals were used per treatment. Median and extreme values are shown. (○) *Untreated;* (●) 10 Gy; (□) 160 mg/kg *tubulozole* (*R* 46 846) 6 h *before* 10 Gy; (■) 80 mg/kg *R* 55 102 2 h *before* 10 Gy.

R 55 104 *in combination with* 10 Gy *radiation*

Twenty, 40 and 80 mg/kg R 55 104 respectively was given at different time intervals before or after 10 Gy radiation (Table 1). Pretreatment with 20 mg/kg R 55 104 resulted in T_ds which were higher than the sum of the T_ds of the individual drug or radiation treatment (excess growth delay). Short pretreatment times with 40 mg/kg R 55 104 also gave rise to an excess growth delay. However, posttreatment with 40 mg/kg R 55 104 2 h after 10 Gy did not differ from 10 Gy alone ($P = 0.6319$ and excess growth delay is -1.38). In the case of 80 mg/kg R 55 104, similar excess growth delays were obtained, when given up to 3 h before or after the irradiation. Longer pretreatment periods (4 or 6 h before) with 80 mg/kg R 55 104 had a better effect than 10 Gy alone ($P = 0.0044$ and $P = 0.0360$ respectively) but apparently did not enhance the effect of the irradiation (negative excess growth delays).

DISCUSSION

In the present study, we have been able to further demonstrate both the antitumoral effect and the interaction with ionizing radiation of R 55 104 (erbulozole) *in vivo* by administering the drug orally (Table 1 and Fig. 1). In drug–radiation combinations, an optimal initial MO_4 tumor regression was noticed when 80 mg/kg R 55 104 was given 2 h before 10 Gy irradiation (Fig. 2). This effect was similar to the regression seen at the optimal pretreatment time of 6 h for 160 mg/kg tubulozole (Fig. 3). The equipotent doses of 80 mg/kg R 55 104 and 160 mg/kg tubulozole may be explained by the higher antimicrotubular effect of R 55 104 in cell cultures [9]. A similar activity of R 55 104, in combination with 10 Gy radiation, was observed in subcutaneous Lewis lung carcinoma when the drug was given 2 h before 10 Gy irradiation (Distelmans W, Van Ginckel R, Vanherck W, Willebrords R, De Brabander M, unpublished results, 1988).

Repeated treatment of subcutaneous MO_4 tumors with the drug–radiation combination, as mentioned above, resulted in further tumor regression (Fig. 4). Pretreatment with R 55 104 in a more clinically relevant radiation dose fractionation schedule showed an effect on tumor regression (Fig. 5) which corroborates the previously reported

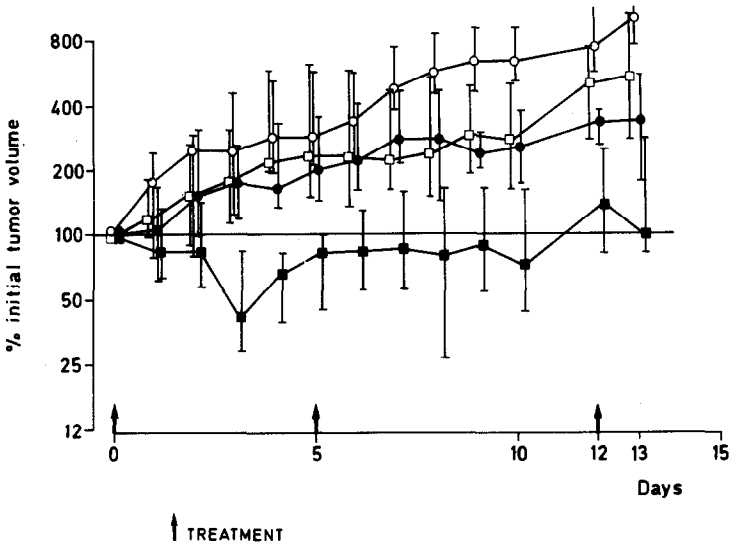

Fig. 4. Relative tumor growth (% initial tumor volume) plotted against time. Eighty mg/kg *R* 55 104 *was given* 2 h *before* 10 Gy *on days* 0, 5 *and* 12 *(arrows). Six animals were used per treatment. Median and extreme values are shown.* (○) *Untreated;* (●) *three times* 10 Gy; (□) *three times* 80 mg/kg *R* 55 104; (■) *three times R* 55 104 2 h *before* 10 Gy.

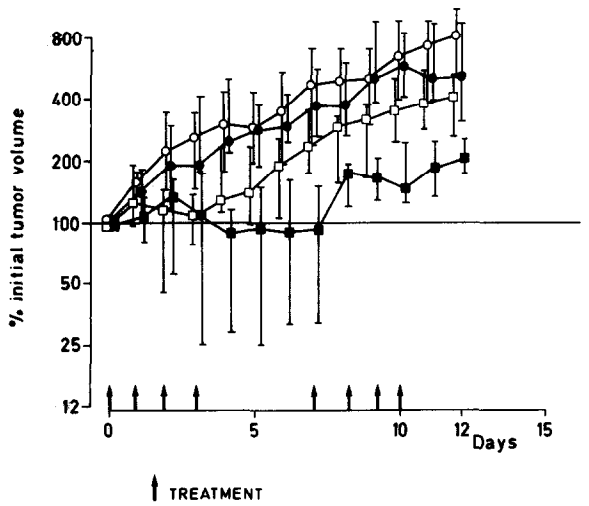

Fig. 5. Relative tumor growth (% initial tumor volume) plotted against time. Forty mg/kg *R* 55 104 *was given* 2 h *before* 2 Gy *on day* 0, 1, 2, 3 *and on day* 7, 8, 9 *and* 10 *(arrows). Six animals were used per treatment. Median and extreme values are shown.* (○) *Untreated;* (●) *eight times* 2 Gy; (□) *eight times* 40 mg/kg *R* 55 104; (■) *eight times R* 55 104 2 h *before* 2 Gy.

results obtained with tubulozole [7]. All these observations point to the existence of both a marked antitumoral effect and an interaction with gamma radiation of R 55 104 which are similar to the activity of tubulozole. However, several observations reveal slight differences between both drugs.

R 55 104 should be given within shorter time intervals with respect to the irradiation (Table 1). Eighty mg/kg R 55 104 does not enhance the effect of radiation when administered 4 or 6 h before (negative excess growth delay), the latter being the optimal time interval for tubulozole. The difference in optimal pretreatment time may be explained by the higher water solubility of R 55 104 which might influence its pharmacokinetic properties.

Forty mg/kg R 55 104 given 2 h after 10 Gy does not exert more antitumoral effect than 10 Gy alone (marked negative excess growth delay; $P = 0.6319$). In contrast with tubulozole however, the highest dose (80 mg/kg) of R 55 104 also appeared to further enhance the effect of radiation when given after the radiotherapy. We previously suggested that the effect of pretreatment with tubulozole could only partly be explained by conditioning the cells prior to irradiation, e.g. by accumulating radiosensitive mitotic cells at the time of irradiation [7, 8]. We also mentioned the possible existence of an additional 'cytotoxic effect' of these drugs. There may be interference with enzymatic repair mechanisms of radiation damage whose transport or activity may be mediated by an intact microtubular system in both interphase and mitotic cells. The distinct activity of R 55 104 at 40 mg/kg and 80 mg/kg (radiosensitizing activity only when administered before radiation and radiosensitizing effect when given shortly before or after irradiation respectively) may sustain this hypothesis. It is conceivable that the 'additional cytotoxic effect' plays a greater role at increasing doses.

It is well known that the clinical use of a particular drug–radiation combination is predominantly

Table 1. *Median and quantiles of* T_d* *(days) of* n *subcutaneous* MO_4 *tumors treated with* 20, 40 *or* 80 mg/kg *R* 55 104 *respectively and* 10 Gy *gamma-irradiation*

Treatment	*n*	Treatment time (h)	Median	Quantiles 25%	75%	Excess growth delay	*P* values†
None	72	—	3.23	2.30	4.46	—	
10 Gy	72	—	5.72	3.59	7.77	—	
20 mg/kg	18	—	4.10	3.10	5.46	—	0.0240
40 mg/kg	30	—	5.58	3.76	6.92	—	0.0000
80 mg/kg	96	—	6.34	4.85	7.22	—	0.0000
20 mg/kg + 10 Gy	6	−3	8.86	6.21	10.12	2.27	0.0500
	6	−2	9.30	8.81	9.55	2.71	0.0101
	6	−1	8.10	7.08	10.61	1.51	0.0480
40 mg/kg + 10 Gy	6	−3	14.40	7.84	>15	6.33	0.0042
	12	−2	9.10	7.98	10.96	1.03	0.0011
	12	−1	11.36	10.03	12.05	3.29	0.0002
	6	+2	6.69	4.7	7.75	−1.38	0.6319
80 mg/kg + 10 Gy	12	−6	8.78	6.33	10.93	−0.05	0.0360
	12	−4	8.65	8.01	9.02	−0.18	0.0044
	6	−3	11.31	11.15	11.78	2.48	0.0012
	36	−2	9.99	7.71	11.30	1.16	0.0000
	12	−1	12.63	9.72	13.35	3.80	0.0001
	12	−0.5	10.29	8.75	11.85	1.46	0.0007
	6	+1	11.82	11.60	13.33	2.99	0.0018
	12	+2	11.12	10.45	13.21	2.29	0.0061
	6	+3	11.38	7.56	11.75	2.55	0.0069

*Time to reach twice the initial treatment volume of approximately 1 cm^3.
†Compared to corresponding controls (none: no drug; 10 Gy only).

dependent on its concurrent toxicity on normal tissues [16]. However, preliminary results with tubulozole revealed a radioprotective effect of the compound when given before total body irradiation [8]. Therefore, further studies of the antitumoral effects of R 55 104 and radiotherapy should focus on 'normal tissue effects' which may lead to new interesting applications.

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