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Original article

Preclinical antitumour activity and animal toxicology studies of rhizoxin, a novel tubulin-interacting agent

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Summary. Rhizoxin is a 16-membered antifungal macrocyclic lactone isolated from the plant pathogenic fungus *Rhizopus chinensis*. The compound binds to tubulin, preventing microtubule formation, and inhibiting mitosis. It possesses antitumour activity in vivo against various preclinical murine models, both leukaemias and solid tumours model, as well as in vincristine- and doxorubicin-resistant leukaemia lines. In the present study, cytotoxic activity was observed in human tumour cell lines in vitro at very low concentrations ($\pm 10^{-10}$ M) particularly against melanoma, colon, renal, non-small cell and small cell lung cancer. In vivo antitumour activity was demonstrated in murine P388 and L1210 murine leukaemias, solid tumour models B16 melanoma and M5076 sarcoma, and in 5 out of 9 human solid tumour xenografts: LOX melanoma, MX-1 breast cancer, non-small cell lung cancer A549, and small cell lung cancers LXFS 605 and LXFS 650. The absence of cross-resistance to vinca alkaloids was confirmed in vivo against the vincristine-resistant P388 leukaemia subline and the vincristine-resistant human small cell lung cancer LXFS 650. In addition, the antitumour activity of rhizoxin was improved by prolonged or repeated drug administration indicating a schedule dependency. In animal toxicology studies, transient changes in erythrocyte and leukocyte numbers, local phlebitis, diarrhea, and spermatogenic arrest were observed. The LD_{10} value of rhizoxin after a single intravenous injection was 2.8 mg/kg (8.4 mg/m^2). One-tenth of the mouse equivalent LD_{10} (0.84 mg/m^2), the starting dose for clinical phase I studies, was considered to be safe in rats. The antitumour activity of rhizoxin, its unique interactions with tubulin and the absence of non-manageable toxic effects in the animal toxicological studies have led to rhizoxin's selection for clinical trials. A phase I clinical trial has been completed showing leukopenia, mucositis and diarrhea to be the dose-limiting toxicities. In some cases phlebitis was observed. These toxicities were predicted from the animal toxicological studies. In addition, rhizoxin caused minor responses in three heavily pretreated patients with recurrent breast cancer. Phase II clinical trials will start soon within the framework of the EORTC and CRC.

Key words: rhizoxin, tubulin binder, antitumour activity, NCI in vitro screen, vinca alkaloid sensitives/resistants, animal toxicology

Introduction

Rhizoxin (NSC 332598, E87/010) is a 16-membered macrolide (Fig. 1) isolated from the plant pathogenic fungus *Rhizopus chinensis* [1] and *Rhizopus sp* No. F-360 [2] which causes rice seedling blight. It has potent antifungal activity, but weak antimicrobial activities [1, 2]. In vivo, rhizoxin exhibits moderate to good antitumour activity against various preclinical murine models such as MH134 hepatoma, B16 melanoma, L1210 and P388 leukaemia [2–4]. As compared to vincristine, the cytotoxicity of rhizoxin against murine P388 and human K562 vincristine- and doxorubicin-resistant leukaemia sublines in vitro is approximately 5–10 fold greater [4]. The lack of cross-resistance is also found in vivo in the P388 leukaemia subline resistant to vincristine [4].

Rhizoxin inhibits the mitosis of sea urchin eggs [5]. It binds rapidly to purified pig brain tubulin, preventing microtubule assembly and also causing depolymerization of microtubules [5, 6]. Further tubulin binding experiments indicate that rhizoxin and ansamitocin

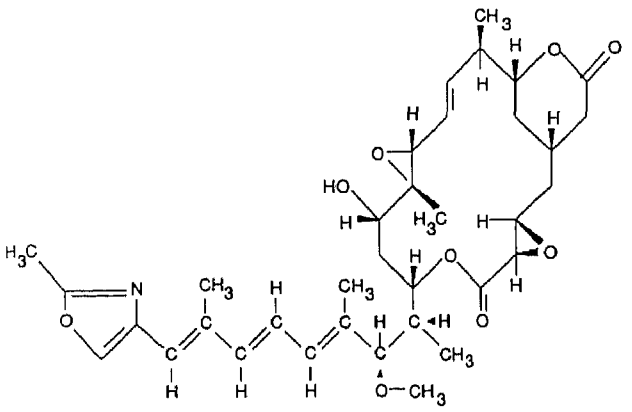

Fig. 1. Chemical structure of rhizoxin.

P-3, a maytansine analogue, share the same binding site, which is not identical to the vinblastine-binding site on tubulin, although some overlap has been observed. Furthermore, the binding site is distinct from that for colchicine. The mechanism of action of rhizoxin is very much the same as that of maytansine, another microtubule inhibitor [5–7]. Tubulin binding experiments with rhizoxin and its homologues show that the hydroxy group at C-13 might play a important role in the binding of rhizoxin to tubulin, whereas the two epoxy groups present in the rhizoxin molecule are not essential for inhibiting tubulin polymerization [6]. The IC_{50} values of rhizoxin and several homologues against P388 leukaemia in vitro [2] suggest that the same conclusion might be drawn for the two epoxy groups regarding antitumour activity. Cell cycle analysis by flow cytometry has shown that rhizoxin blocks tumour cells in the G_2-M phase in a manner similar to vinca alkaloids [4].

On the basis of its antitumour activity in leukaemia and solid tumour cell lines, particularly vincristine- and doxorubicin-resistant sublines, and its unique interactions with tubulin, rhizoxin was selected for further investigation. In this paper, preclinical antitumour activity and animal toxicological studies of rhizoxin are described.

Materials and methods

Drug

Rhizoxin (molecular weight: 625.8) was obtained from Fujisawa Pharmaceutical Company (Osaka, Japan). Bulk material was stored at −20°C in the dark until use. Rhizoxin is chemically unstable and poorly water-soluble, but is highly soluble in organic solvents. The stability of rhizoxin in aqueous solution at 25°C is poor, $t_{90\%}$ (time for 10% decomposition to occur) is from 20 to 25 hrs, and in hydroalcoholic solutions considerably better ($t_{90\%} > 75$ hrs). Therefore, the compound was dissolved in dimethylsulfoxide, Klucel or, for toxicological studies, propylene glycol, ethanol and water (40/10/50, vol/vol) [8].

Antitumour activity

In vitro cytotoxicity studies

The in vitro antitumour activity of rhizoxin was tested in the new NCI disease-oriented screen, which included a total of 60 human tumour cell lines comprising 8 tumour types. The methodology applied for the cytotoxicity was described previously [9–11]. Briefly, the assay involves plating the cells, preincubation for 24 hours, followed by a 48-hour continuous drug exposure (at least 5 ten-fold dilutions), and a sulforhodamine B protein assay to estimate cytotoxicity. Data are expressed using three parameters: (a) the drug concentration which causes 50% growth inhibition (GI_{50}); (b) the drug concentration which inhibits growth by 100% (total growth inhibition or TGI); and (c) the drug concentration which yields a 50% cell kill as compared to the start of the test (LC_{50}), indicating a net loss of cells after drug treatment. The results were presented in the form of 'mean graphs' in which the drug effects are calculated as positive or negative deviations from the average sensitivity of all cell lines in the panel. This generates a 'fingerprint' characteristic for each individual drug. Standard chemotherapeutic agents have also been tested in this assay system, and are used as a reference database to allow comparisons of 'fingerprints' among novel and standard cytotoxic agents.

Additional in vitro screening tests were performed at the University of Freiburg. The cytotoxicity of rhizoxin was tested using cell suspensions of solid human tumour xenografts, human bone marrow (CFU-GM) and P388 murine leukaemia in a two-layer soft agar culture system [12, 13]. Tumour characteristics including histology, growth rate and chemosensitivity to standard anticancer drugs have been described previously [14]. For the experiments rhizoxin was solubilized in dimethylsulphoxide (0.1% final concentration) and applied by continuous exposure in medium. Drug effects were expressed as percentage survival obtained by comparing the mean number of colonies in the treated plates with the mean number of colonies of control plates (T/C%). Cytotoxicity was considered to be present if the compound reduced colony formation to 30% or less of the control value (T/C ≤30%). T/C% is given by the formula:

$$T/C\% = \frac{\text{Colony count drug treated group}}{\text{Colony count control group}} \times 100\% \quad (1)$$

In vivo antitumour activity studies

The in vivo preclinical antitumour activity of rhizoxin was screened at the NCI in a panel of 9 murine tumours and 7 human tumour xenografts. Details on tumour origin, mouse host strain, route of administration, schedule, and activity criteria for drug testing have been described elsewhere [15–18]. In addition, an in vivo cross-resistance profile was developed by comparing the activity of rhizoxin against a parent sensitive P388 leukaemia and sublines developed for resistance to specific agents according to previously published procedures [19, 20].

In addition, rhizoxin was evaluated against two human small cell lung cancer xenografts, LXFS 605 and LXFS 650, growing as subcutaneous implants in both flanks of nude mice [14, 21]. Tumour growth in these human tumour xenografts was assessed weekly by caliper measurements of the tumour in two dimensions. Treatment was started in a randomized fashion when tumours had reached a median tumour diameter of 6–7 mm. Each treatment group and the control group consisted of 5–6 mice bearing 6–10 evaluable tumours. Tumour volume was calculated according to the formula 0.5 × length × width^2. Relative tumour volumes (RTV) were calculated for each single tumour by dividing the tumour volume on day X by the tumour volume on day 0 at the start of treatment:

$$RTV = \frac{\text{Volume tumour day X}}{\text{Volume tumour day 0}} \times 100 \quad (2)$$

Median RTV values were used for drawing growth curves and calculating treatment results. Tumour doubling time (TD) of test and control groups was defined as the period required to reach a median RTV of 200% and 400%. Treatment efficacy was assessed by three evaluation criteria used in parallel: specific growth delay (SGD), optimimal growth inhibition (T/C%), and tumour growth curve. The SGD and T/C% were calculated as follows:

$$SGD = \frac{\text{TD treated} - \text{TD control}}{\text{TD control}} \quad (3)$$

$$T/C\% = \text{RTV treated}/\text{RTV control} \times 100\%$$

In the experiments on LXFS 605 and 650, death occurring within 2 weeks after the final drug administration were considered as toxic deaths. These mice were excluded from any evaluation in the study.

Animal toxicology studies

In order to provide a safe starting dose for phase I clinical trials and to gain an impression of the nature and reversibility of the toxicity of rhizoxin, toxicological studies in mice and rats were performed according to the EORTC guidelines [22] at the TNO-CIVO Institutes (Zeist, The Netherlands), in conformity with Good Laboratory

Practice. For these experiments rhizoxin was studied using the same formulation developed for clinical use. The vehicle of rhizoxin consisted of propylene glycol, ethanol and water (40/10/50, vol/vol) [8]. Since male animals were more sensitive than females in preliminary experiments, only males were used in subsequent studies.

Single dose intravenous (i.v.) lethality study in mice

The aim of these studies was to determine the LD_{10} and LD_{50} values with a single i.v. injection of the test compound. After a preliminary dose finding study, rhizoxin was administered at five appropriately spaced dose levels ranging 2.5–5.2 mg/kg to groups of 10 Swiss CD1 mice. Control mice received the vehicle in the same volume as given to the highest dose group. After dosing, animals were observed daily and mortality was recorded. Extensive macroscopic examination was performed on mice dying during the observation period of 28 days and those killed at the end of the study. Samples of thymus, spleen, gastro-intestinal tract, kidneys, heart, brain, liver, lungs, testes and tail were preserved for histology.

Single dose i.v. toxicity study in mice

In order to assess the acute toxicity of rhizoxin, a single i.v. dose at the LD_{10} value was given to a group of 20 Swiss CD1 mice. A control group of 20 mice received i.v. saline. Mice of both groups were killed at regular intervals during an observation period of 28 days after dosing. Haematology, gross pathology and organ weights were evaluated on day 0 (prior to treatment), 7, 16 and 28. Histopathology was performed on day 16 and 28. Bone marrow was collected from the femur on day 16. Cytocentrifuge preparations were prepared from the cell suspensions, and bone marrow counts were then performed.

Multiple dose intraperitoneal (i.p.) study in mice

This study was intended to investigate the toxicity of rhizoxin following multiple dosing and a subsequent 28-day recovery period. In a preliminary dose finding study, rhizoxin could not be administered i.v. for 5 consecutive days due to local toxicity at the site of injection. Therefore, i.p. injections were applied using the same schedule to select an appropriate dose level.

Next, the test group of 12 mice received rhizoxin i.p. (0.25 mg/kg) in 5 consecutive daily injections on days 0–4 and 14–18. The control group of 15 mice received saline only. At regular intervals mice were killed during the dosing period and the recovery period. An additional control group of 5 mice was used to carry out initial haematological examinations. Blood counts and bone marrow examinations, including histopathology, were performed on day 0, 21 and 46.

Single dose i.v. toxicity study in rats

In order to investigate whether the one-tenth of the mouse equivalent LD_{10}, the starting dose for clinical phase I studies, was safe in a second species, a single dose of rhizoxin was administered i.v. to 10 Wistar rats. The control group of 10 rats received saline. An additional untreated control group was used to carry out initial haematology studies. As before, blood counts and bone marrow evaluation were performed as described in the mouse toxicity studies.

Results

Antitumour activity

In vitro antitumour activity

The in vitro cytotoxicity of rhizoxin was tested by the NCI in the new disease-oriented human tumour cell line panel. Multiple tests over the range 10^{-4} to 10^{-13} M all provided essentially 'flat' dose-response curves over the entire concentration range for most of the 60 cell lines. Figure 2 gives an example of such dose-response curves for rhizoxin against leukaemia subpanel in two experiments run simultaneously from 10^{-4} to 10^{-8} and 10^{-9} to 10^{-13} M. It is not unusual for mitotic inhibitors to show this flat pattern, but the extended concentration range for rhizoxin (at least 9 log dilutions) over which this flat dose-response curve pattern occurs is not at all common and may be unique, thus far. For example, the flat dose response curve extends from approximately 10^{-4} to 10^{-8} M for taxol.

To facilitate visual scanning of the data for potential patterns of selectivity against particular cell lines or subpanels with respect to the selected response parameters, the 'mean graphs' of GI_{50}, TGI, and LC_{50} were subsequently constructed for rhizoxin. Figure 3 shows a 'mean graph' [23] of TGI results from a represensative test with rhizoxin over the range of 10^{-9} to 10^{-13} M. The graph is centered at the arithmetic mean of the logarithm of the TGI values for the entire panel. Each bar indicates whether the sensitivity of a cell line is greater (bar to the right) or less (bar to the left) than the

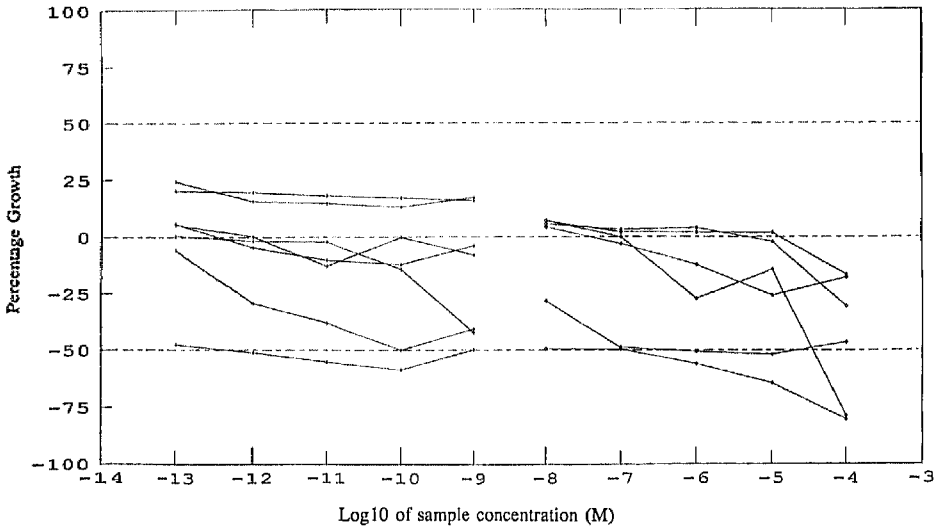

Fig. 2. Example of 'flat' dose-response curves for the cytotoxicity in vitro of rhizoxin against human tumour leukaemia lines in the NCI disease-oriented screen. Results from two experiments run simultaneously from 10^{-4} to 10^{-8} and 10^{-9} to 10^{-13} M against 6 and 7 leukaemia lines, respectively.

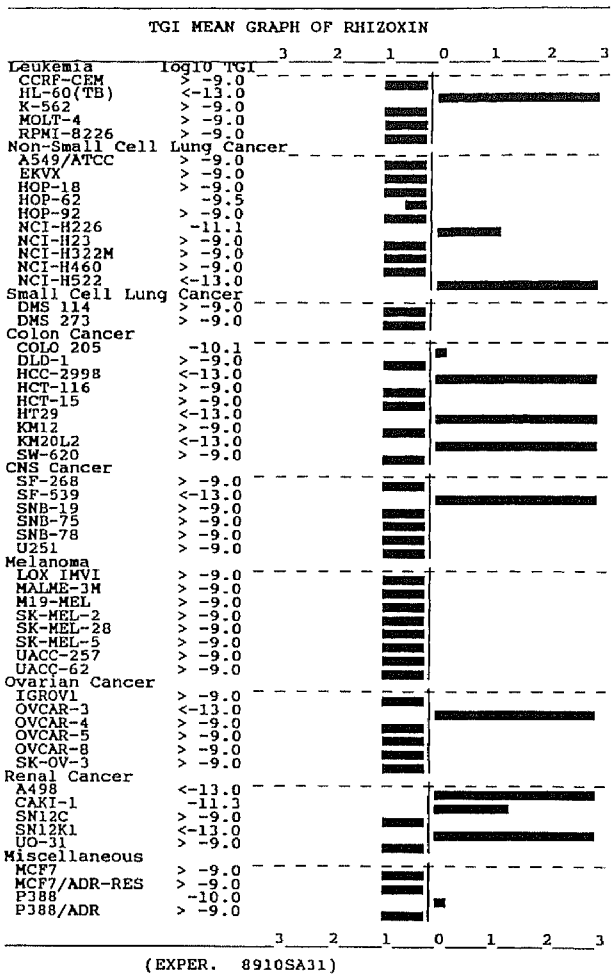

Fig. 3. 'Mean graph' of rhizoxin in the NCI disease-oriented screen of human tumour lines based on TGI values of a representative experiment.

average response. If the growth of a cell line was not inhibited by 100% (zero growth) by the highest concentration tested, TGI values are assigned as >−9.00, meaning that a concentration $>10^{-9}$ M is required to reach the TGI. If the number of cells after the test period is reduced to less than the initial number of cells by the lowest concentration used, then the TGI for that cell line is assigned the value <−13 (a concentration less than 10^{-13} M is required to reach the TGI). Since the bar scale is logarithmic, a bar extending, e.g. 3 units to the right implies, that the concentration to achieve zero growth (TGI) for the cell line is one-thousandth of the mean concentration of the entire panel. In tests with many antimitotic compounds certain cell lines are more sensitive, by far, than the average of all the cell lines. Typically sensitive cell lines include HL-60 leukaemia, non-small lung cancer H522, colon cancer KM20L2, CNS cancer SF-539, and ovarian cancer OVCAR-3. Apart from these individual tumour lines, clusters of sensitive lines corresponding to colon and renal cancer were observed. Using the 'mean graphs' for rhizoxin in comparison of the standard agent database of the NCI in the computer program COMPARE [23–25], which makes pair-wise correlations of the NCI cell line data, for which the following correlation coefficients were found between rhizoxin and other antimitotic agents: maytansine (0.911), vincristine (0.849), vinblastine (0.847) and taxol (0.748). Very poor correlations were found between rhizoxin and other drugs such as doxorubicin, cisplatin and ara-C, correlation coefficients were all below 0.2.

The in vitro cytotoxicity of rhizoxin (0.1–100 ng/ml) was also screened against a panel of 38 human tumour xenografts, human bone marrow and murine P388 leukaemia in the clonogenic assay (Table 1). It can be seen that cytotoxicity was observed in about 50% of the number of lines at 10 ng/ml ($1.6\ 10^{-8}$ M), 30% at 1 ng/ml and 20% at the lowest concentration of rhizoxin tested (0.1 ng/ml). Overall, rhizoxin had higher activity in melanoma, small cell lung cancer and non-small cell lung cancer. Human bone marrow (CFU-GM) and the P388 murine leukaemia line were also very sensitive to rhizoxin in this assay.

In vivo antitumour activity

Murine Models. Rhizoxin, following i.p. administration on daily or intermittent bolus schedules, demonstrated moderate to good activity in several murine tumour models, including the i.p. implanted B16 melanoma, M5076 sarcoma, P388 leukaemia, and the i.p. and subcutaneous (s.c.) implanted L1210 leukaemia models (Table 2). Similar activity was observed in the i.p. B16 melanoma model when the compound was given s.c. However, neither i.p. nor s.c. administrations were effective against s.c. implants of the B16 melanoma.

Rhizoxin demonstrated antitumour activity in P388

Table 1. Cytotoxicity of rhizoxin in human tumour colony forming assay (continuous drug exposure for at least 7 days), numbers of active/evaluable human tumour xenografts.

Human tumour xenograft type	Rhizoxin concentration ng/ml			
	0.1	1.0	10.0	100.0
Human bone marrow	2/3	3/3	3/3	
Murine P388 leukaemia	1/1	1/1	1/1	
Melanoma	1/3	2/4	2/4	3/3
Breast	0/4	1/4	1/4	
Small cell lung	2/3	2/3	3/3	
Non-small cell lung	2/7	3/8	5/8	1/1
Colon	1/5	1/5	2/5	
Gastric	0/3	1/4	2/4	1/1
Head and Neck	1/1	1/1	1/1	
Mesothelioma	0/1	0/1	1/1	
Renal	0/2	0/2	1/2	
Testicular	0/2	1/2	1/2	
Miscellaneous cancers	0/4	0/4	1/4	0/1
Active/total	7/35	12/38	20/38	5/6

Colony formation was scored for each tumour line. Activity was expressed as Test/Control (T/C%) for the numbers of formed colonies. T/C ≤30% was considered to show antitumour activity.

Table 2. Summary of antitumour activity of rhizoxin in murine tumour models in vivo. Rhizoxin administered in Klucel or saline plus Tween 80.

Tumour model	Schedule	Optimal dose mg/kg/day	Activity rating (% T/C)
i.p. B16 melanoma	i.p. q1d, days 1–9	0.2	++ (214, 183)
	s.c. q1d, days 1–9	0.5, 0.1	++ (181, 154)
i.p. L1210 leukaemia	i.p. q1d, days 1–9	0.16, 0.32	+ (146, 138)
i.p. M5076 sarcoma	i.p. q4d, days 1,5,9,13	0.5, 0.25	++ (178, 166)
i.p. P388 leukaemia	i.p. q1d, days 1–5	0.08, 0.14	+ (163, 160)
s.c. B16 melanoma	i.p. q1d, days 1–9		–
	s.c. q1d, days 1–9		–
s.c. CD8F1 mamm ca	i.p. q1d, staging day		–
s.c. Colon 38 ca	i.p. q7d, days 2, 9		–
s.c. L1210 leukaemia	i.p. q1d, days 1–9	0.5, 0.25	+ (142, 130)
i.v. Lewis lung ca	i.p. q1d, days 1–9		–

Criteria for activity based on life span:
++ = reproduced activity of ≥150% T/C for B16, L1210, Lewis lung, M5076; ≥175% T/C for P388; ≤0% T/C for CD8F1, ≤10% T/C for Colon 38;
+ = reproduced activity of 125–149% T/C for B16, L1210, M5076; 120–174% for P388; 140–149% for Lewis lung and s.c. B16; 1–20% for CD8F1; 11–42% for Colon 38;
– = inactive.

Table 3. Activity of rhizoxin against P388 leukaemia and drug-resistant P388 leukaemia sublines in vivo (i.p.-i.p., q1d × 5).

Resistant	Optimal dose mg/kg/day	Parent % T/C	Resistant % T/C	Comments
P388/VCR	0.14	160	148	sensitive
	0.16	160	151	
	0.18	160	134	
P388/MTX	0.45	195	175	sensitive
	0.45	184	187	
P388/ADR	0.14	160	96	cross-
	0.2	145	102	resistant
P388/L-PAM	0.24	166	132	cross-
	0.30	158	129	resistant
P388/CISPT	0.3	165	124	cross-
	0.45	171	128	resistant
P388/ARA-C	0.3	158	133	cross-
	0.3	163	121	resistant

Table 4. Influence of schedule on the efficacy of rhizoxin given s.c. against i.p. implanted murine M5076 sarcomas.

Schedule	Dose mg/kg	Total dose mg/kg	MST[b] days	% T/C
Single bolus, day 1	1.5	1.5	24.2	110
q3h × 8, day 1	0.187	1.5	28.0	127
Single bolus, day 1–5	0.45	2.25	28.0	127
Infusion 24 hrs	0.67	0.67	26.8	122
72 hrs		2.0	33.4	152
120 hrs		3.35	30.0	136

[a] Vehicle rhizoxin: propylene glycol 40%, ethanol 10%, water 50%.
[b] Median survival time, control MST: 21.9 days.

leukaemia cell lines resistant to vincristine and methotrexate, whereas sublines resistant to adriamycin, melphalan, cisplatin and ara-C exhibited cross-resistance to the drug (Table 3).

In vivo studies using the murine i.p.-s.c. M5076 sarcoma model indicated that maximal efficacy was obtained with a 72 hr infusion of rhizoxin as compared to a single bolus, q3hx8 and q1dx5 schedules (Table 4).

Human tumour xenografts. Moderate antitumour activity of rhizoxin was shown in 3 out of 7 tumour lines implanted into nude mice either i.p., intracranial (i.c.) or under the renal capsule (s.r.c.) in a panel of human tumour xenografts (Table 5).

Rhizoxin exhibited considerable cytotoxicity in vitro at a dose level of 0.1 ng/ml in 2 out of 3 human small cell lung cancers (Table 1). The two responsive human small cell lung cancer xenografts, LXFS 605 and LXFS 650, were subsequently tested in vivo. At the maximum tolerated dose, allowing a median body weight loss of about 15% of the initial weight and toxic death in one-third of the mice per treatment group, significant antitumour activity in vivo was observed against LXFS 605 with an intermittent treatment schedule (q3dx4), whereas in LXFS 650 no activity was documented (Table 6). Studies with the same tumour LXFS 650, employing various treatment schedules, confirmed the absence of antitumour activity using the q3dx4 schedule. However, tumour regressions were observed with the two other treatment schedules (Table 6).

Animal toxicology

Toxicological studies of rhizoxin were done with the clinical formulation of rhizoxin. Firstly, the toxicity of the vehicle (propylene glycol 40%, ethanol 10% and water 50%) was tested. Administration of the vehicle i.v., i.p. or orally to mice and rats (10 ml/kg) induced moderate ataxia, which disappeared within 24 hours. At autopsy, 2 weeks after dosing, no treatment related changes were found, showing that the vehicle had a very low toxicity and could be used in further studies. A summary of the toxicological data obtained is given in Table 7.

Single dose i.v. lethality studies in mice

Lethality was observed mainly from day 2 to 5 following drug administration. Survivors recovered and looked healthy throughout the remaining observation period. Treatment related changes were not found either in mice dying during the test period or in those surviving this period. The estimated LD_{50} was 3.7 mg/kg (2.8–4.8 mg/kg, 95% confidence interval) and the LD_{10} value was 2.8 mg/kg. Most mice which had received rhizoxin i.v. showed irritation at the injection site on the tail starting at days 1–2. The severity of the lesion did not appear to be dose-related, being characterized by local erythema and tissue necrosis.

Table 5. Summary of antitumour activity of rhizoxin against human tumour xenografts.

Tumour model	Schedule	Optimal dose[a] mg/kg/day	Activity rating
i.p. LOX melanoma	i.p. q1d, days 1–9	0.3, 0.44	+
s.r.c. CX-1 colon	s.c. q4d, days 1,5,9,11		–
s.r.c. LX-1 lung	s.c. q4d, days 1,5,9		–
s.r.c. A549 lung	i.p. q1d, days 1–9	0.2	+
s.r.c. MX-1 mammary	i.p. q4d, days 1,5,9 s.c. q4d, days 1,5,9	2.0, 1,0 1.0	++, + +
i.c. U-251 glioma	i.p. q1d, days 1–5		–
i.c. TE-671 medullo-blastoma	i.p. q1d, days 1–9		–

[a] Rhizoxin administered in Klucel or saline plus Tween 80. For Lox and A549 rhizoxin was administered reconstituted in ethanol/propylene glycol (1 : 4) and further diluted with ethanol/propylene glycol/water (5 : 20 : 75). i.p. = intraperitoneal; s.r.c. = subrenal capsule; s.c. = subcutaneous; i.c. = intracraneal.

Criteria for activity based on mean tumour weight (s.r.c. implants) or median survival time (i.p. and i.c. implants):
++ = activity of ≤0% T/C for s.r.c. implants;
+ = activity of 140–199% T/C for LOX; 1–20% T/C for s.r.c. implants;
– = <125% T/C for i.p. and i.c. implants; >20% T/C for s.r.c. implants.

Table 6. Antitumour activity of rhizoxin against s.c. implanted human small cell lung cancer xenografts in nude mice after i.p. injection.

Tumour line	Dose mg/kg	Schedule	Toxic death	Optimal % T/C	SGD 1–2
LXFS 605[a]	5	d1	3/5	toxic	
	2.5	d1,4,7,10	1/4	10.4	3.6
	0.5	d1–4,15–18	4/5	toxic	
	0.25	d1–4,15–18	0/5	82.4	0.1
LXFS 650[a]	2.5	d1,4,7,10	2/6	50.0	0.9
	1.25	d1,4,7,10	1/6	73.4	0.3
	0.625	d1,4,7,10	1/6	100.0	–0.3
LXFS 650[b]	2.0	d1,7	9/9	toxic	
	1.0	d1,7	3/10	6.0	5.7
	0.75	d1,4,10	0/4	34.2	1.5
	0.5	d1,4,7,10	2/7	54.1	0.9

[a] Tumours implanted into NRMI nu/nu female mice.
[b] Tumours implanted into Balb/c nu/nu female mice.

Criteria for activity based on relative tumour volume: marginal activity – % T/C <50% or SGD >1.0; active – % T/C <50% and SGD >1.0; moderate activity – % T/C <40% and SGD >1.5; good activity – % T/C <25% and SGD >2.0; strong activity – % T/C <10% and SGD >3.0.

Single dose i.v. toxicity in mice

In order to investigate the toxic effects of rhizoxin, the compound was given i.v. at the LD_{10} value (2.8 mg/kg). On the second day after dosing, 9 out of 20 mice treated with rhizoxin died. The cause of death could not be established upon autopsy. Other toxic effects included sluggishness (day 0–2), diarrhea (day 1), body weight loss during the first week after dosing, decreased weight of testicles accompanied by spermatogenic arrest, and transient changes in haematological parameters such as mild lymphocytopenia, thrombocytosis and erythropenia. The histological evaluation of bone marrow (day 16) did not reveal significant signs of drug-related toxic effects.

Multiple dose i.p. toxicity in mice

Due to local phlebitis after i.v. administration of rhi-

Table 7. Summary of animal toxicological studies of rhizoxin.

	Observed effects
1) Studies in CD1 mice	
Single dose i.v. lethality:	sluggishness; piloerection; local irritation; ataxia. LD_{10} = 2.8 mg/kg LD_{50} = 3.7 mg/kg
Single dose i.v. toxicity (2.8 mg/kg):	weight loss; reduced red blood cell count; slight decrease of white blood cells; mild elevation of thrombocytes; spermatogenic arrest.
Multiple dose i.p. toxicity (0.25 mg/kg d0–4, 14–18):	decrease in red blood cells, granulocytes and lymphocytes; mild elevation in thrombocytes; erythoid/myeloid hyperplasia in the bone marrow; degenerative changes in testis, kidney and liver.
2) Safety studies in Wistar rats	
Single dose i.v. (one-tenth mouse equivalent LD_{10}, 0.84 mg/m^2):	no lethality; no histopathological change; transient local irritation at the injection site; no bone marrow toxicity.

zoxin into the tail vein, it was not possible to maintain i.v. administration for 5 successive days. Attempts to prevent local irritation at the site of injection by using a modified vehicle (propylene glycol 20%, ethanol 5%, water 25% and saline 50%) or doubling the volume in which the dose of rhizoxin was given, were not successful. Thus, it was decided to administer rhizoxin i.p. in multiple dosing studies.

Considering that in a preliminary study 2 out of 5 mice died after repeated i.p. injections at the dose of 0.6 mg/kg for 5 consecutive days, a dose of 0.25 mg/kg/day was chosen for the main study with repeated i.p. injections in two periods of 5 consecutive days (day 0–4, 14–18). In this study, no mortality occurred, and a decrease in numbers of red blood cells (mean ± SD control 8.4 ± 0.6 10^{12}/L versus 7.5 ± 0.6 10^{12}/L test mice, Student t-test $P < 0.05$) and leukocytes (6.2 ± 0.9 10^9/L control versus 4.0 ± 1.2 10^9/L test mice, Student t-test $P < 0.02$) was observed on day 21. The latter was mainly due to decreased numbers of neutrophils and to a lesser extent to lymphopenia. Mild thrombocytosis was also found. On day 46, no treatment related haematological changes remained. Degenerative changes were found in testicles, liver and kidney. Bone marrow cytology revealed both erythroid and myeloid hyperplasia.

Single dose i.v. toxicity check in rats

The safety of the one-tenth of the mouse equivalent LD_{10} (0.84 mg/m^2) was tested using Wistar rats. This dose (0.14 mg/kg) failed to produce lethality when administered i.v. No treatment related macroscopic and histophatological changes were observed, except for inflammation at the site of injection on the tail in one rat. Therefore, the dose of 0.8 mg/m^2 was recommended as the starting dose in human trials.

Discussion

Rhizoxin is a novel tubulin-interacting agent, which causes inhibition of microtubule assembly. In this respect it resembles vinca alkaloids such as vinblastine and vincristine, which are widely used clinically as antitumour agents. However, rhizoxin differs from those agents in its specific binding site and interaction with tubulin, which more closely resembles that of maytansine [5–7].

Rhizoxin is a molecule that arose in Japan in the early eighties. It was further developed via the US NCI in the mid eighties, explaining the use of the older screen employing murine models, as reported here. Later again the drug was re-evaluated in Europe via the EORTC New Drug Development Office. When the new NCI disease-oriented in vitro screen became operational, the compound was also tested in this system. The idea behind the development of this new screen was that in random drug screening a disease-oriented human tumour panel might have a higher chance to discover compounds with possible clinical activity against particular clinical tumour types. Therefore, the primary screen should exploit relatively simple methods and large numbers of tumour lines representing the most frequent tumour types [9]. Based on concentrations required to inhibit growth by 100% (TGI), 4 out of 9 colon, 3 out of 5 renal and 2 out of 10 non-small cell lung tumour lines were rather sensitive to rhizoxin in this disease-oriented screen. The Freiburg panel, having less tumour lines per tumour type compared with the NCI screen, showed that melanoma, small cell lung and non-small cell lung cancers were sensitive to rhizoxin. The two in vitro sensitive small cell lung cancers responded also in the follow-up in vivo studies. Although the sensitive melanoma xenografts in the in vitro panel were not subsequently tested in vivo, rhizoxin was active in the murine B16 melanoma (i.p.-i.p., i.p.-s.c.) and the human LOX melanoma (i.p.-i.p.). The latter was not particularly sensitive to rhizoxin based on the concentration required to inhibit growth by 100% (TGI value $> 10^{-9}$ M) in the NCI human tumour cell line screen. The sensitivity of P388 leukaemia in the clonogenic and the growth inhibition assays agrees quite well. In the clonogenic assay, P388 was sensitive to 0.1 ng/ml or 1.6 10^{-10} M rhizoxin, while in the NCI screen total growth inhibition (TGI) of P388 was attained at a concentration of 10^{-10} M. Even though the P388 sensitivities in the two in vitro assays appered to be similar, it will not be expected that the results from the assays would always correlate closely because of the differences in the duration of the assays, and the need for several cell cycles to occur to manifest cell killing effects. In addition, rhizoxin at low concentrations was active against human bone marrow indicating that in clinical studies myelosuppresion may well be dose limiting, which was indeed observed in the phase I clinical trial [26]. Future phase II clinical trials will show which tumour types are responsive allowing a retrospective comparison with the preclinical data, and indicate the therapeutic index for rhizoxin.

The computer program COMPARE enables the analysis and comparison of data generated by the NCI disease-oriented screen and stored in the database. The program is capable of identifying cytotoxicity patterns produced by structurally different compounds having a common intracellular target, e.g. tubulin-interacting agents [11, 24, 25]. Analysis of the 'fingerprints' from rhizoxin obtained in the disease-orientied screen with those of other chemotherapeutic agents in the NCI database using the COMPARE program showed high correlation coefficients with other mitotic inhibitors. The rank order from high to low was: maytansine, vincristine, vinblastine, and taxol. While the precise correlation coefficients can, and do, change as new data are added to the NCI data base, the correlation coefficients reported here fit nicely with the outcome of the tubulin binding experiments [5–7] showing that maytansine has the same binding site and acts in very much the same way as rhizoxin, whereas the binding site of vinblastine

shows only some overlap and its precise mechanism of action differs from that of rhizoxin and maytansine. Taxol, on the other hand, had the lowest correlation coefficient of the tubulin interacting agents, and likewise has a quite distinct mechanism of action; promoting polymerization of microtubules [27].

In the past maytansine attracted much attention by its structure, an ansamacrolide, and good antitumour activity in murine tumours [28], but had disappointing clinical effects [29–31]. In spite of similarities in chemical structure and mechanism of action between rhizoxin and maytansine, they differ in antitumour profile. Rhizoxin is more potent in the NCI disease-oriented screen than maytansine, and has good activity against vincristine resistant lines, both in vitro and in vivo (vide infra). Maytansine is inactive against vinca resistant lines [32].

Rhizoxin showed moderate to good antitumour activity in various solid tumours in vivo in murine and human tumour models such as the murine M5076 sarcoma, and human xenografts LOX melanoma, MX-1 breast cancer, A549 non-small lung cancer, and small cell lung cancers LXFS 605 and LXFS 650. Furthermore, the activity observed previously in L1210 and P388 leukaemias and B16 melanoma [2–4] was confirmed. This was also the case for the in vivo responsiveness of the vincristine-resistant P388 leukaemia subline. Of interest is the finding that this line was sensitive to rhizoxin at doses well below the toxic dx5 dose. The absence of cross-reactivity to vinca alkaloids was further supported by the in vivo activity of rhizoxin against human tumour small cell lung cancers LXFS 605 and LXFS 650, which were vincristine-sensitive and -resistant respectively [21]. The results predict that rhizoxin might be clinically active against tumours resistant to vinca alkaloids.

With respect to the effects of various treatment schedules in M5076 sarcoma, the 72-h infusion was more efficacious than the other schedules used. This is in agreement with the data in Tables 2 and 6, demonstrating the relationship of activity to prolonged or repeated drug administration, and unpublished historical data of the NCI showing that the cytotoxicity of the compound was related to dose (0.3–10 ng/ml) and exposure time (24–48 h) in L1210 cells in vitro.

The antitumour activity of rhizoxin in both murine and human tumour models, its unique interactions with tubulin, and the absence of non-manageable toxic effects in preclinical studies have led to the selection of this agent for clinical trials. A phase I clinical study of rhizoxin has been completed by the EORTC Early Clinical Trials Group [26]. The compound was given as a single i.v. bolus administration, every 3 weeks, to patients with solid malignancies. The starting dose, 0.8 mg/m^2, was derived from the one-tenth of the mouse equivalent LD_{10}, and was shown to be safe in the first patients entered in the study. The dose limiting toxicities were leukopenia, mucositis, and diarrhea and were reversible at the maximum tolerated dose of 2.6 mg/m^2. Phlebitis at the site of injection was documented in some cases. These toxicities were predicted from animal toxicity studies. However, differences were also noted between man and mice regarding myelotoxicity and erythropenia. In patients, leukopenia was dose-related and dose limiting. Thrombocytopenia was found in one out of eighteen patients. In the single dose i.v. toxicology study in mice, however, only mild lymphocytopenia, thrombocytosis and no significant signs of bone marrow toxicity were observed. In the same animal study erythropenia was found, which was observed in one patient. These differences may be in part related to the prior therapy of the patients, but may also be due to interspecies differences in pharmacokinetics. Rhizoxin showed some clinical effects in heavily pretreated breast cancer patients in the phase I study. Phase II clinical trials will soon start within the framework of the EORTC and CRC.

Preclinical studies in mice showed that rhizoxin had an initial half-life of 4.4 minutes and a second phase half-life of 84 minutes. In addition, the pharmacokinetics in mice were nonlinear with respect to the dose: at doses of 1.2, 6, and 12 mg/m^2, which were the one tenth the LD_{10}, one- half the LD_{10}, and the LD_{10}, respectively in this pharmacokinetic study, the AUC values were 1.3, 22.4 and 70.6 μM × minute. At the maximum tolerated dose in patients (2.6 mg/m^2), only the alpha phase could be detected, ranging from 1.2–6.9 minutes which is similar to that in mice. The AUC values at this dose were 0.41–1.01 μM × minute, which is about 40–100 times lower than the comparative alpha phase AUC value of the mouse at the maximum tolerated dose [33]. The reasons for the large species differences in maximum tolerated dose and pharmacokinetics are unclear and need further investigation. Optimal antitumour activity of rhizoxin in mice bearing human tumour xenografts was seen at dose levels ranging from 1–2.5 mg/kg (3–7.5 mg/m^2). Considering that the observed LD_{10} in mice was 2.8 mg/kg (8.4 mg/m^2), this might indicate that the compound will be active at a concentration near the maximum tolerated dose. In fact, tumour responses were seen in humans near and at the maximum tolerated dose, 2.0 and 2.6 mg/m^2 respectively [26] suggesting that despite interspecies differences in pharmacokinetics, rhizoxin might exert its antitumour activity in a similar way. Pharmacokinetic studies will be performed during the phase II clinical trials.

So far, rhizoxin has been studied in humans using the standard intermittent schedule of every three weeks for cytotoxic agents having bone marrow toxicity. Considering the animal results, showing a relationship of antitumour activity to prolonged or repeated drug administration, other treatment schedules in man might improve the efficacy of the compound.

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