Eur J Cancer Clin Oncol, Vol. 20, No. 1, pp. 99–105, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

The Effects of Tubulazole, a new Synthetic Microtubule Inhibitor on Experimental Neoplasms

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Abstract—*Tubulazole, a new synthetic microtubule inhibitor* in vitro, *is tested* in vivo *upon three experimental neoplasms: MO_4 sarcoma, L_{1210} leukemia and TA_3 carcinoma. The compound is tested using different treatment schedules upon different inoculation routes of the cells. All trials show the compound to have distinct antineoplastic properties* in vivo *by prolonging the median survival time. The best treatment schedule seems to be an intermittent one, i.e. treatment every fourth day starting* 1 day *after tumor inoculation. Comparison with cyclophosphamide and vincristine is in favor of tubulazole for treating TA_3 mammacarcinoma, while cyclophosphamide and vincristine give somewhat better results upon L_{1210} leukemia. The effects of tubulazole and cyclophosphamide upon MO_4 fibrosarcoma are comparable, while vincristine has no effect in this system. Worthwhile noting is that all the* in vivo, *as well as* in vitro, *activity of tubulazole resides in the* cis *isomer, while the* trans *isomer has no effect at all.*

INTRODUCTION

TUBULAZOLE, or *cis*-ethyl[4-[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-ylmethylthio]phenyl]carbamate (Fig. 1), is shown to be a potent microtubule inhibitor of a class of antitumor drugs chemically unrelated to the hitherto well-known antineoplastics. It inhibits *in vitro* polymerization of rat brain tubulin ($ID_{50} = 3 \times 10^{-7}$ M) [1].

Ultrastructural investigations on mammalian cells *in vitro* clearly show the interference of tubulazole with the structure and function of microtubules both in interphase and mitotic cells. Microtubules disappear completely, followed by loss of cell polarity and directional migration. The organelle topography is disturbed, and intermediate filament bundles and annulated lamellae appear. Mitoses are blocked and multinucleation occurs [1].

The compound is also shown to interfere with malignant invasion. In a co-culture system the invasion of the chicken embryo heart by malignant cells is completely inhibited (observations by M. Mareel [1]).

Accepted 28 June 1983.
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The aim of this paper is to report on the antineoplastic properties of tubulazole on three experimental neoplasms *in vivo*: MO_4 sarcoma, L_{1210} leukemia and TA_3 carcinoma. Furthermore, a comparison is made with the activities of cyclophosphamide and vincristine sulfate.

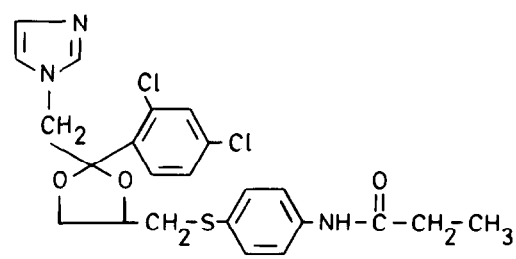

Fig. 1. Tubulazole (R 46 846).

MATERIALS AND METHODS

MO_4 is derived from the MO cell line (a C_3H embryonal cell line of epitheloid character) by transformation with the Kirsten strain of murine sarcoma virus. Injected into the syngeneic C_3H mouse, the cells produce invasively growing fibrosarcomas [2].

The cells are cultured *in vitro* in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum in a

humidified atmosphere of 5% CO_2 in air at 37°C. Prior to inoculation the cells are collected from the culture flasks, centrifuged and diluted in EMEM at a concentration of 5×10^6 cells/ml. Each mouse (DBA_2/C_3H F_1 hybrid) receives 0.2 ml suspension i.p. or s.c.

L_{1210} is a common experimental leukemia [3, 4]. The cells are transplanted weekly into DBA_2/C_3H F_1 hybrids. The cells are collected by rinsing the peritoneum with sterile saline. After centrifugation they are resuspended in EMEM to a final concentration of 5×10^6 cells/ml for i.p. injection or 5×10^5 cells/ml for i.v. inoculation. Each mouse (DBA_2/C_3HF_1 hybrid) receives a 0.2 ml cell suspension.

TA_3 is a mammacarcinoma derived from A mice. There are two sublines: TA_3-St, which grows only in syngeneic A strain, and TA_3-Ha, which takes in allogeneic animals, e.g. Swiss mice, rats, guinea pigs [5–7]. All experiments are carried out with the TA_3-Ha subline. The cells are handled in the same manner as the L_{1210} cells. They are transplanted once a week and diluted in EMEM, the final concentration being $5 \times 10_4$ cells/ml. Each Swiss mouse receives 0.2 ml cell suspension.

The drug tubulazole dissolves poorly in aqueous solution. It is usually given as a suspension in water, supplemented with Tween 80. For use in some trials, tubulazole is dissolved in a 20% cremophor solution acidified with lactic acid.

There are no differences in activity between the base R 45 911 or the hydrochloride R 46 846. However, most of the experiments are carried out with tubulazole hydrochloride.

Both cyclophosphamide and vincristine sulfate are prepared freshly just before administration

Table 1. Effects of tubulazole on i.p. injected MO_4 cells in DBA_2/C_3H mice

Inoculum site	Dose (mg/kg)	Scheduled day of dosing	MST %
i.p.	160	1	168
	80		159
	40		155
	20		122
i.p.	160	1, 7	146
	80		104
	40		93
	20		104
i.p.	80	1, 2, 3, 4, 7, 8, 9, 10, 11	109
	40		177
	20		132
	10		127
i.p.	80	1, 5, 9, 13	189
	40		148
	20		119
	10		100

Six mice per group each received 1×10^6 cells i.p. on day 0. Treatment with tubulazole (suspension) was given by i.p. injection on the days indicated. The median survival time of the treated animals was expressed as a percentage of the median survival time of the untreated animals (MST %). When MST was 125% or more, the compound was considered to be active.

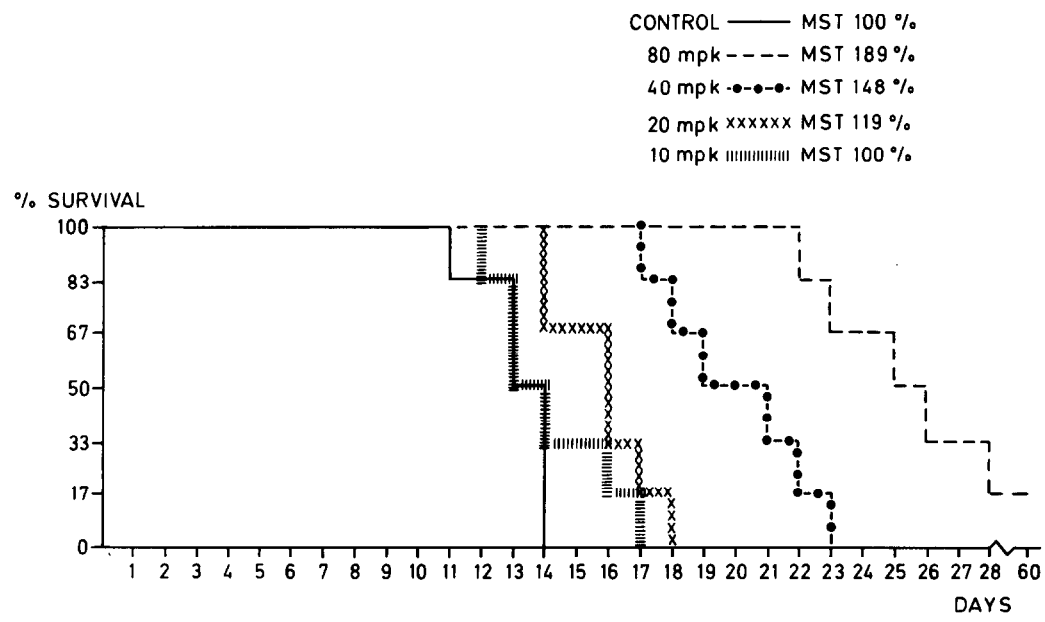

Fig. 2. Effect of tubulazole in the MO_4 system. DBA_2/C_3H F_1 hybrids are injected with 1×10^6 MO_4 cells i.p. Treatment with tubulazole is done on days 1, 5, 9 and 13 i.p. Each group consists of 6 mice. Controls receive sterile saline i.p. The percentage of surviving animals is plotted against time.

Table 2. Effects of optimal dosages of tubulazole, cyclophosphamide (CPA) and vincristine sulfate (VCR) on MO_4 sarcoma, L_{1210} leukemia and TA_3-Ha mammacarcinoma

Drug	Dose (mg/kg)	Scheduled day of dosing	Tumor	Inoculum site	MST %	No. of survivors >60 days
VCR	1	1, 5, 9, 13	L_{1210}	i.p.	186	0
	0.5				200	0
	1	1, 5, 9, 13	MO_4	i.p.	111	0
	0.5				111	0
	1	1, 5, 9, 13	TA_3-HA	i.p.	228	0
	0.5				162	0
CPA	160	1, 5, 9, 13	L_{1210}	i.p.	220	0
	80				227	0
	160	1, 5, 9, 13	MO_4	i.p.	148	0
	80				136	0
	160	1, 5, 9, 13	TA_3-Ha	i.p.	164	0
	80				127	0
	40				105	0
Tubulazole	160	1, 5, 9, 13	L_{1210}	i.p.	88	0
	80				188	0
	40				150	0
	20				125	0
	80	1, 5, 9, 13	MO_4	i.p.	189	0
	40				148	0
	20				119	0
	10				100	0
	160	1, 5, 9, 13	TA_3-HA	i.p.	>500	5
	80				>500	6
	40				>500	5
	20				146	1

Six mice per group are injected i.p. each either with 1×10^6 MO_4 cells, 1×10^6 L_{1210} cells or 1×10^4 TA_3-Ha cells. Treatment with tubulazole (suspension) was performed i.p. on days 1, 5, 9 and 13 after tumor inoculation. The median survival time of the treated animals was expressed as a percentage of the median survival time of the control animals (MST %). When MST was 125% or higher the compound was considered active; more than four long-term survivors (for over 60 days) per group were expressed as MST >500%. VCR = vincristine sulfate; CPA = cyclophosphamide.

Table 3. Effect of tubulazole on s.c. inoculated MO_4 cells

Inoculum site	Dose (mg/kg)	Scheduled day of dosing	Tumor index (%)	Median weight of control tumors (tumor index 100%)
s.c.	320	1, 5, 9, 13	26	0.482 g
	160		44	
	80		77	
	40		91	
	20		71	

Six mice per group received each 1×10^6 MO_4 cells s.c. in the left inguinal region. Tumors were excised 14 days after inoculation and their weight determined. Tumor index (%) was calculated by dividing the median tumor weight of the treated mice by the median tumor weight of the controls, multiplied by 100. Treatment with tubulazole (suspension) was performed orally on days 1, 5, 9 and 13 after tumor inoculation.

Table 4. Combined effect of cyclophosphamide (CPA) and tubulazole on MO_4 cells

Inoculum site	Dose (drug/mg/kg)	Scheduled day of dosing	MST %
i.p.	CPA/160	1	125
	CPA/80	1	117
	CPA/160 Tubulazole/80	1 2, 6, 10, 14	196
	CPA/80 Tubulazole/80	1 2, 6, 10, 14	175
	Tubulazole/80	2, 6, 10, 14	154

Six mice per group received 1×10^6 MO_4 cells i.p. Treatment with tubulazole (suspension) and cyclophosphamide (solution in sterile saline) was performed i.p. on the days indicated. The median survival time of the treated animals was expressed as a percentage of the median survival time of the controls (MST %). When MST was 125% or higher, the compound was considered to be active.

(dissolved in sterile saline) and injected i.p. every fourth day, starting 1 day after tumor inoculation until death of the animals.

RESULTS

MO_4 trials

Control mice injected i.p. with 1×10^6 MO_4 cells normally die within 14 days. Tubulazole prolongs the median survival time (MST) regardless of the treatment schedules (see Table 1). The optimal schedule seems to be an intermittent one with injections every fourth day, starting 1 day after tumor inoculation (see Table 1 and Fig. 2). The prolongation of MST is comparable to the effect of cyclophosphamide, whilst vincristine is ineffective on MO_4 cells in our test system (see Table 2).

The same MO_4 cells produce large, distinct fibrosarcoma nodules when injected s.c. (1×10^6 cells/mouse) at the left inguinal region. These nodules can be excised and their weight determined. Oral treatment with tubulazole in suspension reduces the size of the tumor burdens (see Table 3).

Synergism of tubulazole with cyclophosphamide is observed. Since cyclophosphamide given as a single injection on the first day after i.p. tumor inoculation has little effect, combination therapy with tubulazole is installed. This results in a clear prolongation of the median survival time (see Table 4).

Since all experiments showed the distinct antineoplastic effect of tubulazole on MO_4 fibrosarcoma, the compound has been run ever since as a reference drug in our current *in vivo* screening system at a dose of 80 mg/kg. Prolongation of MST has always been observed with its average being around 50%. Occasionally mice survive for over 60 days; this has never been observed with cyclophosphamide or other well-known antineoplastics.

L_{1210} trials

Treatment of leukemia induced by i.p. inoculation of 1×10^6 L_{1210} cells with tubulazole injected i.p. results in a marked prolongation of the median survival time (MST), regardless of the schedule used (see Table 5).

Cyclophosphamide and vincristine sulfate tend to be slightly more active than tubulazole (see Table 2).

Leukemia induced by intravenous inoculation of 1×10^5 L_{1210} cells is also susceptible to either oral or i.p. administration of tubulazole in suspension (see Tables 6, 7). Administration of the compound as a solution tends to increase the toxicity of the compound and to decrease the therapeutic effectiveness.

TA_3 trials

All experiments are performed with the TA_3-Ha subline. Intraperitoneal treatment with tubulazole after i.p. inoculation of the TA_3-Ha cells (1×10^4) is highly efficient in curing TA_3 mammacarcinoma since the mice survive for over 60 days. In contrast, vincristine and cyclophosphamide produce prolongation of MST but no long-term survivors (see Table 2).

In this case also, intermittent treatment every fourth day, starting 1 day after inoculation until the 13th day, is the optimal schedule.

Stereospecificity

The *trans* isomer of tubulazole, which does not affect tubulin polymerization or microtubule

Table 5. Effects of tubulazole on i.p. inoculated L_{1210} cells

Inoculum site	Dose (mg/kg)	Scheduled day of dosing	MST %
i.p.	160	1	163
	80		150
	40		138
	20		100
i.p.	160	1, 5	88
	80		188
	40		150
	20		125
i.p.	80	1, 2, 3, 4, 5, 6, 7	88
	40		100
	20		138
	10		138
i.p.	80	1, 3, 5, 7, 9, 11,	94
	40	13, 15, 17	163
	20		125
	10		119
i.p.	320	5, 10	143
	160		143
	80		143
	40		143

Six mice per group were each inoculated i.p. with 1×10^6 cells. Treatment with tubulazole (suspension) was performed i.p. on the days indicated. The median survival time of the treated animals was expressed as a percentage of the median survival time of the untreated animals (MST %). When MST was 125% or higher, the compound was considered to be active.

Table 6. Effects of tubulazole on i.v. inoculated L_{1210} cells

Inoculum site	Dose (mg/kg)	Scheduled day of dosing	MST %
i.v.	320	1	129
	160		107
	80		107
	40		93
	20		93
i.v.	320	1, 2	143
	160		114
	80		107
	40		100
	20		100
i.v.	320	1, 2, 3	114
	160		114
	80		107
	40		100
	20		86
i.v.	320	1, 5	167
	160		175
	80		167
	40		117

Six mice per group were injected i.v. each with 1×10^5 L_{1210} cells on day 0. Treatment with tubulazole (suspension) was performed i.p. on the days indicated. The median survival time of the treated animals was expressed as a percentage of the median survival time of the control mice. When MST was 125% or higher, the compound was considered to be active.

integrity in cultured cells [1], is completely devoid of antineoplastic activity in the three models used (data not shown).

DISCUSSION

Tubulazole is a potent microtubule inhibitor, chemically unrelated to other antineoplastics [1]. The present paper shows that it has also antineoplastic properties *in vivo* on MO_4 fibrosarcoma, L_{1210} leukemia and TA_3 mammacarcinoma.

Optimal results—marked prolongatin of MST with a relatively broad range of active dosages—are obtained following an intermittent treatment schedule, i.e., treatment every fourth day, starting 1 day after tumor inoculation.

The antineoplastic activity of tubulazole has been confirmed using other transplantable neoplasms, i.e., 3-methylcholanthrene-induced fibrosarcoma (Meth-1), Lewis lung carcinoma and a vinca-resistant leukemia P 388 [Ashirawa and Morimoto, Kyowa Hakko, Japan, personal communication]. Although tubulazole has a poor aqueous solubility, aqueous solutions are not an absolute prerequisite for antineoplastic activity since the best results are obtained with the suspension formulation given orally in the antileukemic L_{1210} trials. Furthermore, a direct *in situ* contact between compound and tumor cells is not required since (1) the optimal schedule for L_{1210} leukemia is oral treatment with the drug for intravenous inoculation of the leukemic cells; (2) orally administered tubulazole reduces the size of nodules, resulting from subcutaneous inoculation of MO_4 cells.

Comparative experiments with cyclophosphamide and vincristine show that tubulazole is more effective against MO_4 sarcoma and TA_3 mammacarcinoma, while its activity against L_{1210} leukemia is less. The activities of tubulazole, as well as those of the vinca alkaloids, can be explained by their antimicrotubular action. Cell division, invasion and metastasis formation are all dependent upon the microtubular apparatus of the malignant cells [8]. This is further substantiated by the inactivity of the *trans* isomer, which is devoid of microtubule inhibiting properties. Although tubulazole and the vinca alkaloids probably have the same mode of action, some notable differences exist in their activity *in vivo*. Tubulazole is more active on solid tumors, while vincristine is superior in a leukemia model. This may indicate a more pronounced effect of

Table 7. Effects of tubulazole on i.v. inoculated L_{1210} cells

Inoculum site	Dose (mg/kg)	Scheduled day of dosing	Suspension or solution	MST %
i.v.	160	1, 3, 5, 7, 9	suspension	158
	80			175
	40			150
	20			117
i.v.	160	1, 2, 3, 4, 5, 6, 7	suspension	150
	80	8, 9, 10		158
	40			133
	20			100
i.v.	320	1, 5	suspension	167
	160	single dose		150
	80			158
	40			100
	3 × 107	1, 5	suspension	167
	3 × 53	divided dose		142
	3 × 27			150
	3 × 13			117
i.v.	320	1, 5	solution	33
	160	single dose		108
	80			125
	40			133
	3 × 107	1, 5	solution	33
	3 × 53	divided dose		67
	3 × 27			142
	3 × 13			125

Six mice per group received each 1×10^5 cells i.v. Treatment with tubulazole (suspension or soution) was performed orally on the days indicated. The compound was given as a single dose or divided in three doses (3×...). The median survival time of the treated animals was expressed as a percentage of the median survival time of the controls. When MST was 125% or higher, the compound was considered to be active.

tubulazole *in vivo* on the process of malignant invasion and metastasis, and a stronger effect of vincristine on cellular proliferation. Differences in pharmacokinetics or distribution of the compound may be involved in their preferential activity. Another contributing factor may be the easy reversibility of tubulazole on a cellular basis [1]. Vincristine, on the other hand, co-precipitates with tubulin intracellularly and may thus have a longer biological half-life. The compounds may have a different impact on the host's immune system. The obtention of a high percentage of cured animals in the allogeneic TA_3-Ha system does indicate that, at therapeutic dosages, tubulazole, unlike cyclophosphamide or vincristine, does not prohibit the animals from mounting an effective immune response, resulting in the rejection of the residual tumor burden. During the course of our experiments with tubulazole and its preliminary toxicological studies (unpublished observations) we did not note any sign of neurotoxicity (head jerking, paralysis), which is one of the major drawbacks, prohibiting the prolonged use of vinca alkaloids. The existence of an active and inactive enantiomer of tubulazole should be useful for further investigations into the biological effects of microtubule inhibitors, in particular when questions about specificity are involved.

In conclusion, tubulazole, a new synthetic microtubule inhibitor, is shown to be active against experimental neoplasms *in vivo*. Further studies are warranted to investigate the possible clinical usefulness.

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