CORRELATION BETWEEN THE IN VIVO EFFECTS OF SOME GRISEOFULVIN DERIVATIVES AND THEIR IN VITRO INTERACTIONS WITH MAMMALIAN MICROTUBULES

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1. Introduction

Griseofulvin, an antifungic metabolite produced by several species of *Penicillium* [1], is widely used in human and animal therapy [2]. Its mechanism of action remained puzzling for a long time [3]. Recently it has been shown that griseofulvin inhibits the polymerization of purified brain tubulin [4,5]. Although this property may account for its in vivo effects, this result does not allow to rule out the hypothesis that microtubule proteins may not be the main target of this drug [6]. In order to check this possibility, several griseofulvin derivatives exhibiting a lower or a higher activity than griseofulvin, were synthesized. Their in vivo effects were studied on a mouse leukemia cell line (L1210) and on a Myxomycete (*Physarum*). Their in vitro action was studied by monitoring the induced aggregation and the inhibition of polymerization of purified brain mammalian microtubule proteins [4,5]. It was found that the in vitro activities of the various griseofulvin derivatives were in correlation with their in vivo activities suggesting that microtubule proteins do represent the pharmacological target of griseofulvin.

2. Materials and methods

2.1. *Griseofulvin derivatives (fig.1)*

Griseofulvin was a gift from Imperials Chemical Industries. The other derivatives were synthesized as in [7–9]. They were dissolved in 1% final dimethyl sulfoxide for the in vivo studies.

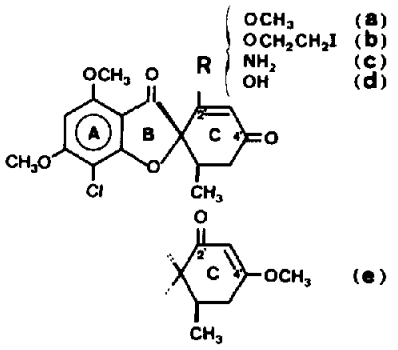

Fig.1. Structure of griseofulvin derivatives. (a) Griseofulvin; (b) 2′-(2-iodoethoxy)griseofulvin; (c) 2′-aminogriseofulvin; (d) griseofulvic acid (enol form); (e) isogriseofulvin.

2.2. *Leukemia cell culture*

Leukemia mouse cells (L1210) were grown in absence of mycoplasma, at 37°C in a liquid defined medium (RPMI 1640 Gibco) supplemented with 10% horse serum, 60 μM 2-mercaptoethanol, 2 mM L-glutamine, 50 μg/ml of streptomycin and 200 U/ml penicillin [10]. Exponential growing cultures were used (2×10^5 cells/ml). Cells were harvested 12 h and 24 h after drug addition, treated at 37°C with 0.5 M potassium chloride and fixed successively during 15 min and 30 min in acetic acid, ethanol (3 : 1) at 0°C. Nuclei were spread on microscopic slides, stained with hemalun-eosin and mounted in balsam. The nor-

Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2 ethane-sulfonic acid); EGTA, ethylene glycol-bis (β-aminoethyl ether) *N*,*N*′-tetraacetic acid

mal nuclear diameter was 12–15 μm. Nuclei having a diameter equal or lower than 6 μm were recorded as micronuclei. Nuclei which were not spherical, but which showed a bud on their surface were recorded as 'budding' nuclei.

2.3. Physarum *culture*

Physarum polycephalum microplasmodia (strain CL) were grown in liquid defined medium I [11] at 27°C under constant shaking (100 rev/min). Growth was followed by monitoring the variations of $A_{546\,nm}$.

2.4. *Preparation of brain microtubule proteins*

Microtubule proteins were purified from fresh sheep brains by 2 cycles of assembly in the presence of 4 M glycerol at 37°C and disassembly at 0°C [12]. Depolymerized material was kept at –30°C in 50% glycerol. Immediately before use, another cycle of assembly disassembly was performed. The last pellet was resuspended in 0.1 M PIPES, 2 mM EGTA, 1 mM $MgCl_2$ and 0.5 mM GTP with protein conc. 3 mg/ml. In the absence and in the presence of the drugs, protein aggregation (13°C) and tubulin polymerization (37°C) were monitored at 400 nm [13] with a spectrophotometer (Beckman Acta III) and checked by electron microscopy. Drugs were dissolved in 4% final dimethylformamide. At this concentration the solvent has no effect neither on protein aggregation nor on tubulin polymerization.

3. Results

3.1. *In vitro aggregation of microtubule proteins*

The addition of griseofulvin into a cold solution of tubulin prepared as in [12] induces a very rapid increase in turbidity due to the aggregation of microtubule proteins [4,5,14]. This turbidity increase followed at 13°C reached a plateau, the level of which was measured 10 min after drug addition. These values were used to determine the highest drug concentration (H_{13}) unable to induce protein aggregation (fig.2a). The various griseofulvin derivatives studied differ with their ability to induce microtubule protein aggregation (table 1). Isogriseofulvin was slightly less active than griseofulvin. 2′-Aminogriseofulvin and griseofulvic acid were 20 and 50 times less active than griseofulvin, although 2′-(2-iodoethoxy) griseofulvin was 3 times more active.

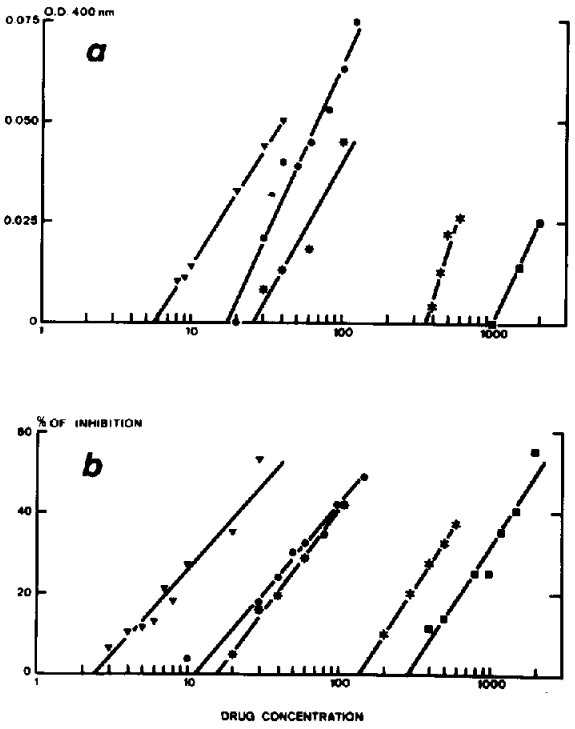

Fig.2. In vitro effects of griseofulvin derivatives on mammalian microtubule proteins. For each compound, the best curve fitting the experimental values was calculated by the method of the least squares. (a) Aggregation of microtubule proteins at 13°C. The plateau values of the increases in turbidity at 400 nm were recorded according to drug concentrations. (b) Inhibition of tubulin polymerization at 37°C. (▼) 2′-(2-iodoethoxy)griseofulvin; (●) griseofulvin; (★) isogriseofulvin; (*) 2′-aminogriseofulvin; (■) griseofulvic acid. Drug concentrations are expressed in μM.

3.2. *In vitro inhibition of microtubule polymerization*

When a solution of tubulin is shifted from 13–37°C, microtubule polymerization takes place [12,13]. In the presence of griseofulvin, a strong inhibition of the polymerization process is observed [4,5]. In order to compare the action of the various griseofulvin derivatives, the percentage of inhibition was calculated for each drug concentration. These values were then used to determine the highest drug concentration (H_{37}) unable to induce an inhibition of tubulin polymerization (fig.2b). The H_{37} values, only slightly lower than the corresponding H_{13} values, gave the same order of activity (table 1).

Table 1
Characteristic values obtained with the various griseofulvin derivatives

	H_{13}	H_{37}	MI	4m	4b
2'-(2-iodoethoxy)Griseofulvin	5.8	2.5	1	4	4
Griseofulvin	18	11	4.5	17	20
Isogriseofulvin	26	16	38	45	18
2'-Aminogriseofulvin	360	140	38	200	185
Griseofulvic acid	1000	290	no effect	no effect	no effect

H_{13} and H_{37} are respectively the highest drug concentrations unable to induce protein aggregation and to inhibit tubulin polymerization. MI is the highest drug concentration which does not affect the mitotic index. 4m and 4b are, respectively, the drug concentrations which induce the formation of 4% micronuclei and 'budding' nuclei

3.3. *In vivo induced nuclear abnormalities in L1210 mouse leukemia*

Mouse leukemia was used to study the cytological action of griseofulvin derivatives. After adding griseofulvin an increase of the percentage of mitotic nuclei and 2 types of nuclear abnormalities (micronuclei and 'budding' nuclei) were observed. The mitotic index increased with the addition of the various griseofulvin derivatives (fig.3a) except with griseofulvic acid. Even at the highest concentration (400 μM) of griseofulvic acid, the percentage of mitotic nuclei remained normal. For the other derivatives the maximum drug concentrations (MI) which did not affect the mitotic index were 1 μM for 2'-(2-iodoethoxy)griseofulvin,

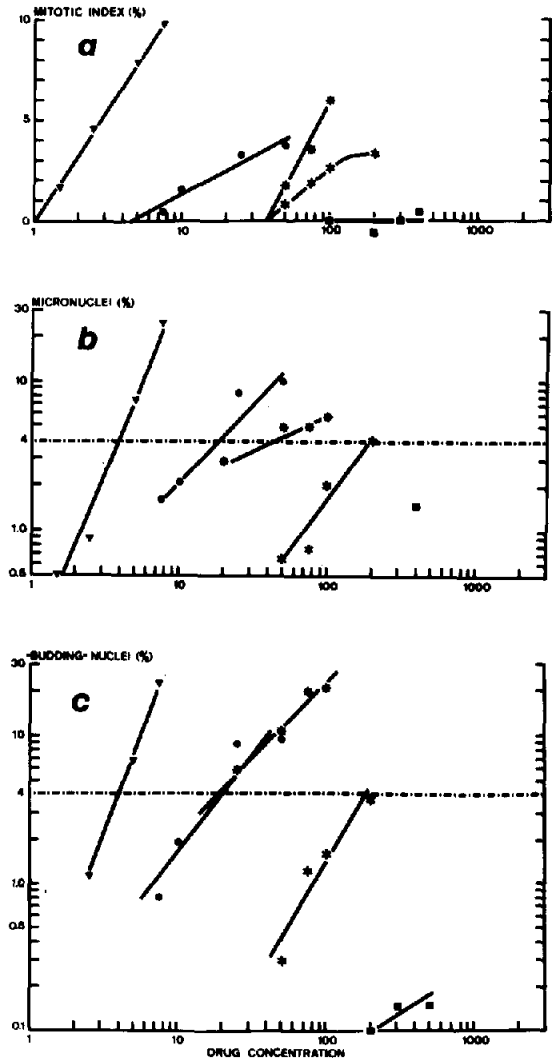

Fig.3. In vivo effects of griseofulvin derivatives on leukemia cells. (a) Mitotic index after 12 h (% nuclei showing condensed chromosomes). (b) Micronuclei after 12 h (%). (c) 'Budding' nuclei after 24 h (%). In each case, the values observed with the controls (3.4–4.2% for mitotic index, 0–15% for micronuclei and 0–0.3% 'budding' nuclei) have been substracted from the percentages obtained on 2000 nuclei. For each compound the best curve fitting the experimental values was calculated by the method of the least squares. In these experiments, the % growth inhibition after 24 h were the following: 2' (2-iodoethoxy) griseofulvin, 2%, 26%, 25%, 48% for 1 μM, 2.5 μM, 5 μM, 7.5 μM; griseofulvin, 2%, 4%, 28%, 41% for 7.5 μM, 10 μM, 25 μM, 50 μM; isogriseofulvin, 33%, 59%, 64%, 69% for 25 μM, 50 μM, 75 μM, 100 μM; 2'-aminogriseofulvin, 3%, 0%, 0%, 7% for 50 μM, 75 μM, 100 μM, 200 μM; griseofulvic acid, 6%, 0%, 0%, 0% for 100 μM, 200 μM, 300 μM, 400 μM, respectively. (▼) 2'-(2-iodoethoxy)griseofulvin; (●) griseofulvin; (★) isogriseofulvin; (✱) 2'-aminogriseofulvin; (■) griseofulvic acid. Drug concentrations are expressed in μM.

4.5 μM for griseofulvin and 38 μM for isogriseofulvin and 2′-aminogriseofulvin. The same overall picture was obtained with the variations of the micronuclei or the 'budding' nuclei percentages (fig.3b,c). Griseofulvic acid was almost completely inactive: less than 2% micronuclei were produced after 12 h and only 0.25% 'budding' nuclei after 24 h in the presence of the highest available drug concentration (400 μM). In order to compare the action of the other compounds we have used the drug concentrations leading to 4% of micronuclei (4m) and 4% of 'budding' nuclei (4b) (table 1).

3.4. *Toxicity on* Physarum *microplasmodia*

Physarum polycephalum is a Myxomycete sensitive to the antifungic action of griseofulvin. Griseofulvin specifically disturbed mitotic microtubules leading to abnormal nuclei and to a growth arrest after 24 h [15]. We found that 2′-(2-iodoethoxy) griseofulvin was more potent (10 μM) than griseofulvin (100 μM) to inhibit plasmodial growth but 2′-aminogriseofulvin and griseofulvic acid were inactive at the highest available concentrations (250 μM and 1000 μM, respectively).

4. Discussion

These results indicate that in vivo, several derivatives of griseofulvin are either less active than griseofulvin, like 2′-aminogriseofulvin and griseofulvic acid, or more active like 2′-(2-iodoethoxy) griseofulvin in the case of a Myxomycete and mammalian cells. This is in agreement with [16,17] obtained on various parasitic fungi. All these compounds induce the same nuclear phenotype on mammalian cells which can be explained by an action on mitotic microtubules [18–21]. We have found that 2′-(2-iodoethoxy)griseofulvin, the most active compound in vivo, was also the most active in vitro on microtubule protein aggregation and on tubulin polymerization. Likewise, the 2 less active derivatives in vivo, 2′-(2-aminogriseofulvin and griseofulvic acid, were found less active in vitro. Isogriseofulvin showed in vivo a comparable activity with griseofulvin, except in the case of the mitotic index, but in vitro was slightly less active than griseofulvin. Contrary to what was observed in vitro, the slopes of the curves obtained from the in vivo

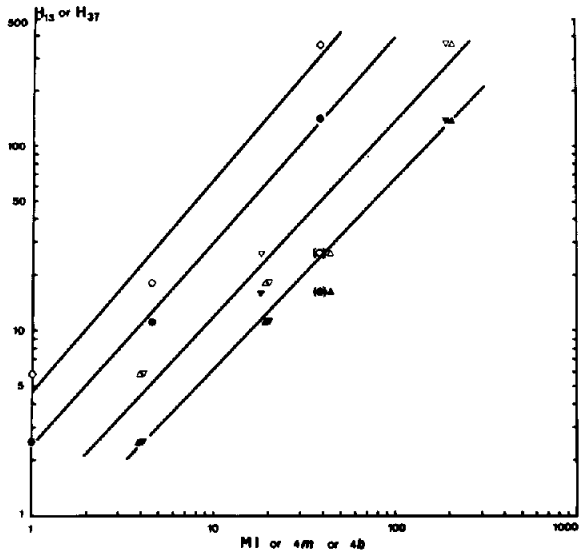

Fig.4. Correlation between in vivo and in vitro effects. Values obtained for griseofulvic acid have been omitted because at the highest available concentration, this derivative showed no increase of the mitotic index and an increase in the percentage of micronuclei and 'budding' nuclei lower than 4%. Drug concentrations are expressed in μM. (○,●) H_{13} and H_{37} values versus the highest drug concentrations unable to increase the mitotic index (MI); points shown between parentheses correspond to isogriseofulvin. (△,▲) H_{13} and H_{37} values versus drug concentrations inducing 4% micronuclei (4m). (▽,▼) H_{13} and H_{37} values against drug concentrations inducing 4% of 'budding' nuclei (4b).

results were different for several compounds. A difference in permeation or in metabolization can be hypothesized to explain such discrepancies. The order of activity of the 5 griseofulvin derivatives found in vitro was the same as the order found in vivo. The probability to find such a distribution is equal to 1/5! i.e., 8.4×10^{-3}.

Thus a correlation seems to exist between the in vivo activity of these compounds and their in vitro action, both on cold microtubule protein aggregation and on tubulin polymerization (fig.4). These observations strongly support the hypothesis that microtubule proteins are the pharmacological target of griseofulvin and of some of its derivatives. This conclusion implies that the different biological activity of these compounds is at least partially due to their differential effect on the microtubular system. The existence of such a correlation suggests also that the

in vitro interaction of drugs with microtubule protein could be used as a screening procedure in order to find more active compounds.

Acknowledgements

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