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PHENYLAZOXYCYANIDE DAMAGES MICROTUBULAR PROTEIN MORE THAN ITS REFERENCE ANTIBIOTIC, CALVATIC ACID

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Abstract—The effect of phenylazoxycyanide and calvatic acid, its reference antibiotic, on some functions of tubulin obtained from different sources has been studied. Our purpose was to establish a possible correlation between the antitumour activity of these drugs and their antimicrotubular action. Microtubules are subcellular structures involved in proliferation and maintenance of the cell shape and probably in malignant transformation; indeed most antimitotic drugs influence the stability of microtubules through the interaction with tubulin, their main protein. In this work we found phenylazoxycyanide impairs, more than calvatic acid, polymerization of purified tubulin from calf brain. It also damages, in a dose-dependent manner, colchicine-binding ability of tubulin derived from rat liver and AH-130 Yoshida ascite hepatoma cells. Compounds displaying an azoxycyano group may represent new antimicrotubular agents and their effect could be modulated by the different polarity and structural characteristic of the molecule.

Phenylazoxycyanide is a calvatic acid analogue devoid of the carboxy function; calvatic acid is an antibiotic with a slight antitumour effect [1], isolated from cultures of *Calvatia lilacina* [2] and *Calvatia craniformis* [1]. Calvatic acid, or *p*-carboxyphenylazoxycyanide, is composed of an inductor of biological activity (the azoxycyanide group), a carrier of the inductor (the benzene ring), and a modifier of the induced effect (the carboxylic group), this last being absent in its derivative. Phenylazoxycyanide inhibits the colony-forming ability of cultured HeLa cells at low concentrations [3] and is active against bacteria and mycetes, with the antifungal slightly superior to the antibacterial activity [4]. Both the drugs inhibit some enzyme functions and colchicine-binding activity in isolated hepatocytes [5], with different effects probably due to their polarity characteristics. Colchicine specifically binds to tubulin, the main protein of microtubules [6, 7], which are involved in the maintenance of cell shape and are probably important in cellular transformation [8]. A relationship may exist between antitumour activity and interaction with microtubules, thus our purpose was to establish whether the azoxycyano group [—N(O)=N—CN] may represent a new cytostatic group of a potential antitumour compound and whether the carboxylic group may influence this effect.

The total synthesis of calvatic acid and phenylazoxycyanide allows an examination of structure–function relationship which may assist in the establishment of antimicrotubular and/or antineoplastic drugs. Most antimitotic drugs influence the stability of microtubules as a consequence of interactions with tubulin; consequently, new drugs that are found to interact with tubulin are of interest as potential antineoplastic agents, as well as being of use in studying the mechanism of tubulin action *in vitro* and *in vivo*. In the present study the effect of phenylazoxycyanide and its reference antibiotic on tubulin was examined.

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MATERIALS AND METHODS

The following chemicals were obtained from Sigma Chemical Co. (St Louis, MO): 2(*N*-morpholino)ethansulfonic acid (MES), ethylenglycol-bis-(β-aminoethyl ether)*N,N'*-tetra-acetic acid (EGTA), GTP. [^{3}H]colchicine was obtained from the Radiochemical Centre (Amersham, U.K.) and used 0.1 mM, 5 μCi/ml. Acrylamide and *N,N'*-methylenebis(acrylamide) were obtained from LKB; phosphocellulose and diethylaminoethyl (DEAE) cellulose filters from Whatman. Calvatic acid and its derivative were provided by the "Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino" [9, 10]. Microtubule protein was prepared and isolated from freshly obtained bovine brains by two cycles of temperature-dependent polymerization and depolymerization, using a modification of the procedure of Shelanski *et al.* [11], as glycerol was included only in the first polymerization cycle. The tubulin was further purified to remove microtubule-associated proteins (MAPs) by phosphocellulose chromatography according to the method described by Weingarten *et al.* [12]. The homogeneity of tubulin was checked electrophoretically by the method of Laemmli [13], using 8% separating gels. Protein concentrations were determined by the method of Hartree [14], using bovine serum albumin as a standard.

The polymerization of tubulin or microtubule pro-

tein at 37° was monitored by measuring the change in turbidity continuously at 400 nm, at which phenylazoxycyanide absorbs minimally, on a Beckman DU-7 spectrophotometer.

Colchicine binding was studied by the standard filter assay method of Borisy [15] using Whatman DEAE cellulose DE 81 filter discs, and radioactivity was measured by liquid scintillation counting.

Male Wistar rats (about 200 g), were used, maintained on a semisynthetic diet with water *ad libitum*. AH-130 Yoshida ascite hepatoma was maintained by a weekly transplantation i.p. of a tumour inoculum of about 10^7 cells. Tumour and liver cells were treated as described elsewhere [16] in order to obtain supernatants containing soluble and microtubule-derived tubulin, which were used to measure colchicine-binding activity, according to the method of Jennett *et al.* [17].

RESULTS AND DISCUSSION

Figure 1 shows the effect of phenylazoxycyanide on the polymerization of microtubule protein: from the data there is clearly a progressive decrease in the rate of polymerization with the increasing concentration of the drug; a 1 mM concentration determined an inhibition of more than 50%. On the contrary, polymerization was found to be unaffected by the presence of calvatic acid (Fig. 2), as a maximum of 15% inhibition was reached even at 1 mM concentration and there was no relationship between the amount of drug administered and the observed effect. The percentage of microtubule formation decreased gradually with the increased concentration of phenylazoxycyanide, which appeared to be the most impairing drug. In fact, also tubulin without MAPs, when incubated in the presence of this substance decreased its ability to polymerize in a dose-dependent manner (Fig. 3), with per cent values of inhibition similar to those observed for microtubular protein. This would suggest that the interaction and the possible linkage of the drug with the protein was not influenced by MAPs, and that a stoichiometric rate in tubulin-phenylazoxycyanide binding may occur.

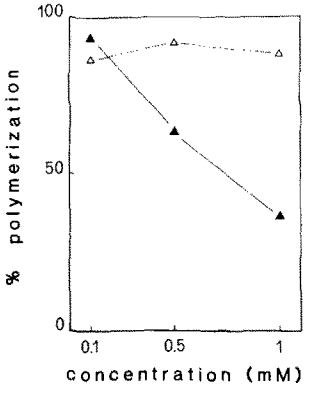

Fig. 2. Per cent polymerization with different concentrations of calvatic acid (△) or phenylazoxycyanide (▲). 100% was defined as the average maximal extent of polymerization obtained in control samples. Experimental conditions as in Fig. 1.

Two-cycles-purified tubulin was incubated with the two substances for 20 min at 20° in order to promote a linkage with them. The interaction of the drugs with tubulin apparently did not involve the colchicine binding site of the protein, as the colchicine-binding activity of microtubular protein was not significantly affected by the different doses administered with the exception of 1 mM concentration (Table 1). Similar results were obtained also without preincubation (data not shown), and this would confirm that a competitive inhibition for the colchicine-binding site did not occur.

From these data the calvatic acid derivative appeared the more effective of the drugs in altering some microtubular characteristics, so we tried to study its behaviour in different experimental conditions. The phenylazoxycyanide effect on microtubular protein was further tested, as previously done for calvatic acid [18, 19], by incubation with

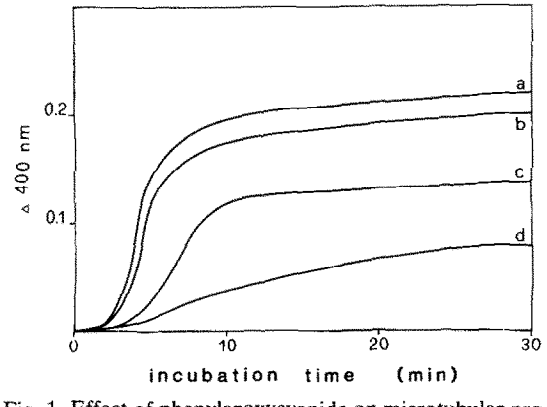

Fig. 1. Effect of phenylazoxycyanide on microtubular protein assembly: tubulin preparations (2 mg/ml) were preincubated for 20 min at 20° with different drug concentrations. Polymerization was initiated by addition of 1 mM GTP and by raising temperature to 37°; turbidity was measured at 400 nm and continuously recorded. Phenylazoxycyanide concentrations (mM): a = 0, b = 0.1, c = 0.5, d = 1.

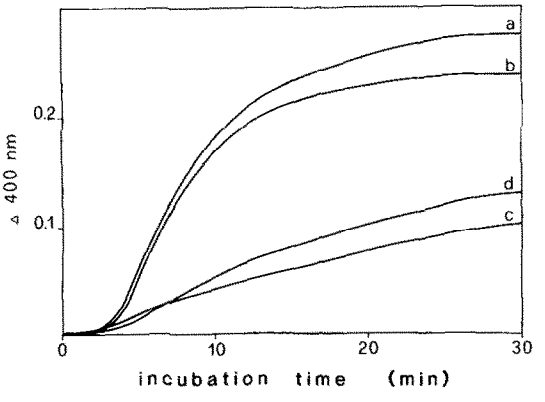

Fig. 3. Effect of phenylazoxycyanide on assembly of tubulin without MAPs. Reaction mixtures containing 5.4 mg/ml tubulin, 10% DMSO, 2.5 mM $MgCl_2$ and drug in concentrations as in Fig. 1 were incubated for 20 min at 20°. Polymerization was initiated by addition of 1 mM GTP and by raising temperature to 37°.

Table 1. Percent colchicine-binding activity of microtubular protein after incubation with calvatic acid (I) or its analogue (II)

Concentration	I	II
0.1 mM	94	96
0.5 mM	90	92
1.0 mM	88	82

Each sample was incubated for 20 min at 20° with drugs and further with [^{3}H]colchicine for 60 min at 37°. Results are expressed as per cent of control, taken as 100%.

homogenates from liver or from AH-130 hepatoma cells. In order to determine microtubular protein amounts, we used a biochemical assay, based on the ability of soluble tubulin to bind colchicine [20, 21] which does not bind to intact microtubules; the polymerized form of tubulin was estimated by measuring the tubulin disassembled from the sedimented microtubules [22]. Rat liver homogenates in a tubulin depolymerizing solution (TS) or microtubule stabilizing solution (MTS) were incubated for 30 min at 37° in the presence of different concentrations of the drug. The soluble form of tubulin was significantly impaired by concentrations of 0.5 mM and over (Fig. 4), with complete inhibition of colchicine-binding activity at 2.5 mM. The polymerized fraction of tubulin appeared to be more resistant to the damaging effects of phenylazoxycyanide, since even where a decrease occurred it was less marked than it was in the soluble form, above all at the highest concentrations. Similar behaviour was also observed after incubating the drug with tumour homogenates (Fig. 5), as the polymerized fraction of tubulin lost its ability to bind colchicine more gradually (and significantly only at 2.5 mM concentration). The amount of colchicine bound to soluble tubulin strongly decreased from 0.5 mM concentration, with a reduction of about 50%. Per cent values of colchicine-binding efficiency are shown in Fig. 6: phenylazoxycyanide was very effective at 0.5 mM and above on soluble tubulin, more so on that derived from tumour than from normal cells, with complete inhibition at 2.5 mM concentration. On the other hand, microtubules from hepatoma cells appeared more resistant to the impairing action of the drug, if compared with microtubules from liver.

Some differences between the organization of these subcellular structures in tumour cells and normal cells may thus be supposed, as a different modulation of the same protein was observed after administration of the drug.

The role of microtubules in transformed cells is controversial: according to some authors they are involved in cell transformation [23], while others did not find any relevant difference in microtubule distribution between tumour and normal cells [24]. Recently it has been shown that transformed cells present about one half of the amount of microtubules present in normal cells [8], as a consequence of the

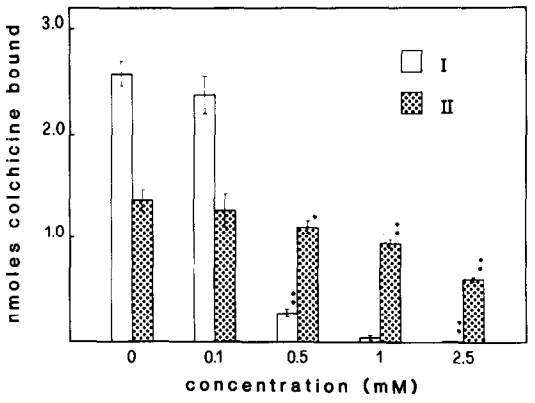

Fig. 5. Effect of phenylazoxycyanide on colchicine-binding activity of hepatoma tubulin. Experimental conditions as in Fig. 4. Values are means ± SD of at least four determinations; statistical significance of differences was evaluated by variance analysis: * $P < 0.01$, ** $P < 0.005$.

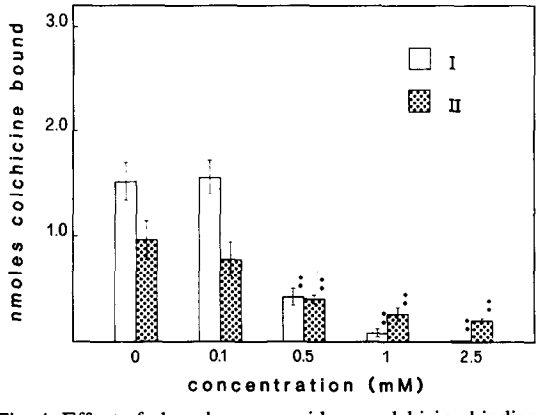

Fig. 4. Effect of phenylazoxycyanide on colchicine-binding activity of liver tubulin. Homogenates in TS or MTS were incubated for 30 min at 37° with different concentrations of drug; supernatants containing soluble (I) or microtubule-derived tubulin (II) were further incubated for 60 min at 37° with [^{3}H]colchicine. Values are means ± SD of at least four determinations; statistical significance of differences was evaluated by variance analysis: ** $P < 0.005$.

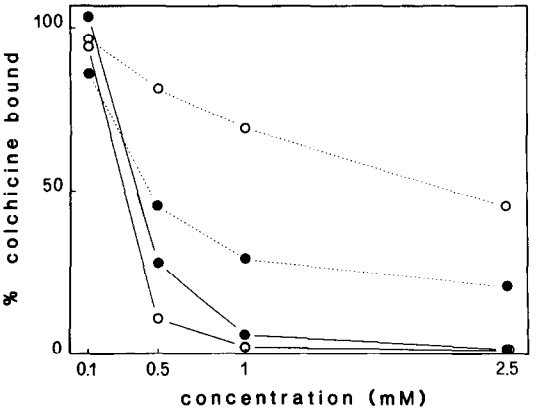

Fig. 6. Relation between colchicine-binding activity of soluble (——) and microtubule-derived (– – –) tubulin from liver (●) and hepatoma (○). Per cent values are referred to average of controls, taken as 100%.

modification of the soluble/polymerized tubulin rate, since in these cells the soluble form of tubulin increases rather than the total content of tubulin decreasing.

Tubulin is the target for different antimitotic agents, which differ in their structure and in their mechanism of action. Some of these drugs containing a cyano-group stimulate tubulin-dependent GTP hydrolysis and inhibit tubulin polymerization [25]. A simple structure compound, 2,4-dichlorobenzyl-thiocyanate (DCBT), interacts with tubulin causing reorganization of microtubules and changes in cell shape [26].

Our observation that colchicine-binding activity of purified microtubular protein was not significantly impaired by the two drugs tested, while tubulin derived from liver or ascite cells was impaired after administration of phenylazoxycyanide and calvatic acid, could be due to a promoter effect of some unknown substances present in the homogenates. Further studies will try to explain whether these drugs influence microtubular protein directly, or whether a metabolization pattern produces some more active intermediates.

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