EFFECT OF TROPOLONE ON THE BINDING OF [^{3}H]COLCHICINE TO BRAIN TUBULIN*

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1. Introduction

Colchicine exerts its antimitotic action through its binding to tubulin, a subunit protein of spindle microtubules [1, 2]. In vitro, colchicine interacts with tubulin isolated from a wide variety of cells and tissues [3]. The subunit protein obtained from mammalian brain is a 120 000-dalton dimer [4] composed of monomers of MW 55 000–60 000 which are most probably non-identical [5]. Although there is evidence that colchicine can be linked to the subunit protein in equimolar quantities [4], the detailed mechanism of this interaction is unknown. The investigation of the sites on the colchicine molecule which are active in binding to the tubulin dimer will bring undoubtedly new insight on this matter. According to this view, it is essential to undertake the study of the biological function of the tropolone moiety of colchicine (fig. 1).

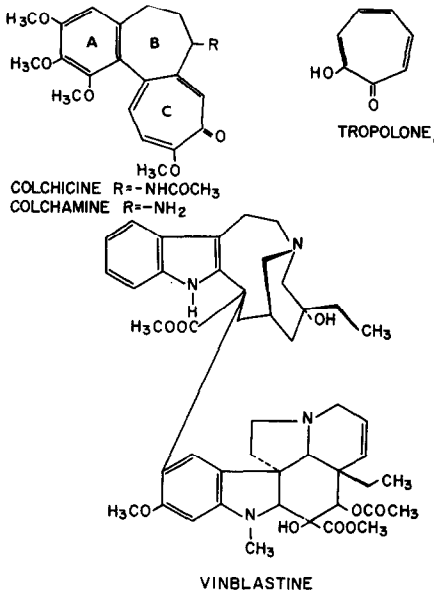

Fig. 1. Chemical structure of some mitotic spindle inhibitors.

In this connection, Fragata (unpublished data) found that tropolone suppresses the formation of colchicine-induced tumours in wheat roots. On the other hand, Benitez et al. [6, 7] showed that tropolone reverses the metaphase arrest produced by colchicine in rat fibroblast cultures. They observed, moreover, that in fibroblast cultures exposed to tropolone the mitotic spindle was sharper and clearer than in controls. These effects may share some common characteristics with the increase of birefringence of the mitotic apparatus under the action of deuterium oxide [8]. Furthermore, this is an indication that the tropolone-mediated inhibition of the colchicine effect arises primarily from an interaction with a microtubular subunit. Hence, one might expect that tropolone competes with colchicine for a closely related – if not the same – active site in the subunit protein, thereby preventing the latter substance from binding to its tubulin substrate. In this work we shall describe experiments designed to test this hypothesis. Instead, we found that tropolone may even increase the colchicine content of tubulin. The significance of these unexpected results to the elucidation of the mechanism of colchicine action will be discussed.

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2. Experimental

The calf brain tubulin was isolated at temperatures of 0°–4°C by a modified procedure of Wilson [9]. The homogenizing medium contained 100 mM sodium glutamate, 50 mM sodium phosphate (pH 6.85), 5 mM magnesium chloride and 0.1 mM guanosine triphosphate. The homogenate was centrifuged at 25 000 *g* for 30 min. The supernatant was precipitated with 32–49% saturation of ammonium sulfate and the pellet was dissolved in 2–3 ml of homogenizing medium. This fraction was filtrated through a Sephadex G-25 column (1.5 X 24 cm) to remove $(NH_4)_2SO_4$. Then, 3–4 ml of protein solution were collected and diluted with equal volume of glycerol. This mixture was stored at −20°C.

The binding of colchicine to tubulin was assayed according to Borisy and Taylor [1] and Weisenberg et al. [4]. A standard reaction mixture of 1 ml containing approximately 1 mg protein, 0.5 μM to 6.0 μM colchicine and the homogenizing medium, was incubated at 37°C for 1 hr. The protein–[^{3}H] colchicine complex was separated from free colchicine by gel filtration of 0.5 ml of the reaction mixture on Sephadex G-100 columns. A 0.1 ml sample of each fraction collected from the columns was added to 10 ml of Bray's solution [10]. The radioactivity was measured in a Packard Tri-Carb Model 2425 liquid scintillation Spectrometer. Aliquots of 0.2–0.6 ml were assayed for protein contents by the method of Lowry et al. [11] using bovine serum albumin as the standard.

For the study of the interaction of colchicine with tropolone, and with colchamine, a constant amount of these substances was incubated together with increasing concentrations of colchicine.

Chemicals: Colchicine [^{3}H]methoxy (ring C), specific activity: 5 Ci/mM, was a product of New England Nuclear. Unlabelled colchicine, GTP (Type II-S) and bovin serum albumin, were obtained from Sigma Chemical Company. Tropolone was purchased from Aldrich Chemical Co. and colchamine from K & K.

3. Results and discussion

First, we used colchamine as a model to exemplify a typical competitive inhibition of the colchicine activity. Similarly to the latter substance, colchamine is also an effective spindle poison [12]. If one takes into account that its chemical structure differs from colchicine only in a N-substitution on ring B (fig. 1), it is reasonable to predict a competitive interaction of the two ligands when incubated with the tubulin obtained from calf brain homogenates. This is what our experiments indicate (fig. 2). We proved next that the colchicine binding in the presence of tropolone deviates from this type of interaction.

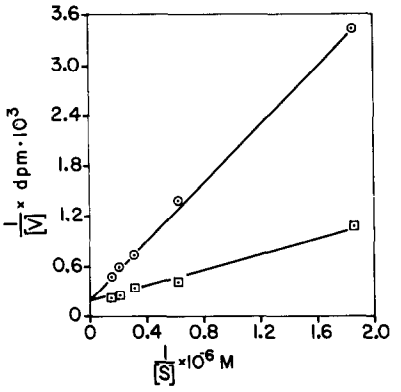

Fig. 2. Lineweaver–Burk plot of the inhibition of colchicine binding activity by colchamine. 1.0 mg of calf brain microtubular protein was incubated with colchicine (⊡) and colchicine plus 11.2 μM colchamine (⊙) at 37°C for 1 hr.

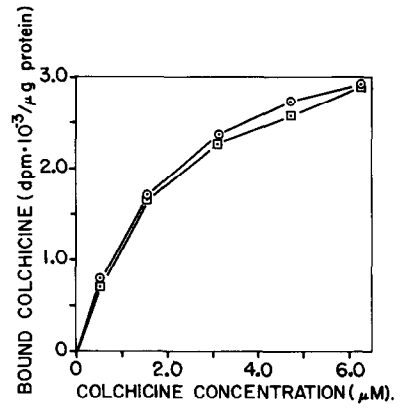

Fig. 3. Typical effect of tropolone on colchicine binding to brain microtubular protein. A reaction mixture containing 1 ml of homogenizing medium and 1.2 mg protein was incubated with colchicine (⊡) and colchicine plus 10 μM tropolone (⊙) at 37°C for 1 hr.

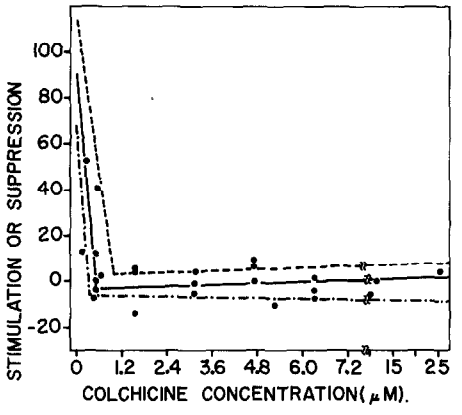

Fig. 4. Percent of tropolone-mediated stimulation or suppression of colchicine binding activity. The figure represents cumulative data from twelve experiments using 0.5 μM to 0.1 mM tropolone and 0.26 μM to 25.5 μM colchicine. The smooth line was drawn from the computer analysis by non-linear least-square curve fitting program. The dotted lines show the lower and the upper limits of error.

Table 1
Effect of tropolone on the association constant ($1/K_m$) of colchicine to calf brain tubulin

Expt. no.	$\frac{1}{K_m} \times 10^{-1}$ liters^{-1} mole	
	Colchicine	Colchicine + tropolone
1	0.35	0.30
2	0.08	0.35
3	0.38	0.45
4	0.45	0.40
5	0.13	0.58
Mean	0.278	0.416
S.D.	± 0.163	± 0.107

In every experiment a constant concentration of tropolone was incubated with increasing concentrations of colchicine (0.26 μM to 25.5 μM). However, tropolone concentration was varied in different experiments from 0.5 μM to 0.1 mM.

Fig. 3 shows the effect of tropolone on the saturation curve for tubulin as a function of the concentration of colchicine. The results demonstrate that tropolone does not hinder the binding of [^{3}H] colchicine to the calf brain protein. Instead, it is seen (fig. 4) that tropolone increases the specific activity of tubulin if the concentration of colchicine in the reaction mixture is kept low. The quantitative evaluation of K_m for the interaction was done by the Lineweaver–Burk or double reciprocal method (table 1). The table shows that tropolone enhances the value of $1/K_m$ which may be considered as a measure of the degree of association of the ligand with its substrate [13]. This effect would seem to indicate that the binding of tropolone to tubulin strengthens the linkage of colchicine with the microtubular subunit. With this view one could argue that the tropolone-induced reinforcement of the colchicine linkage excludes any possibility of competition between the two substances. This is so far an unexpected situation since it is known that tropolone antagonizes in vivo the activity of colchicine (see Introduction).

Moreover, this finding has its counterpart in the demonstration by Creasey and Chou [14] that vinblastine promoted the binding of colchicine by a soluble fraction from Sarcoma 180 cell homogenates. It has been shown that vinblastine is an inhibitor of the mitotic spindle [15]. Its chemical structure, however, does not appear to present any apparent likeness to the colchicine molecule (fig. 1). Nevertheless, Margulis [15] contended with this viewpoint and pointed out critically that the compounds referred to above have in common several methoxy substitutions on a benzenoid ring system. It was postulated furthermore that these substitutions might have a biological function. This assumption and the aforediscussed results corroborate an earlier suggestion of Fragata [16] and Fragata and Leblanc [17] that the activity of colchicine in vivo is most probably dependent on a particular orientation of its rings A and C (fig. 1) relative to the tubulin active site, or sites.

Acknowledgements

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