Biochemical Pharmacology, Vol. 25, pp. 1383–1387. Pergamon Press, 1976. Printed in Great Britain.

MOLECULAR FEATURES OF COLCHICINE ASSOCIATED WITH ANTIMITOTIC ACTIVITY AND INHIBITION OF TUBULIN POLYMERIZATION

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(*Received* 14 *August* 1975; *accepted* 17 *October* 1975)

Abstract—Seven selected colchicine analogs have been studied for their ability to inhibit mitosis in HeLa cell culture and for their ability to inhibit tubulin polymerization in mouse brain supernatants in order to assess the molecular features of colchicine associated with these activities. A good correlation between the systems *in vivo* and *in vitro* was found for the compounds tested. It was found that for a substance to exhibit biologic activity it must contain the two aromatic ring systems and the phenyl ring must bear methoxy groups. The tropone ring is not necessary for activity in colchicine since its conversion to a benzene ring in allocolchicine is accompanied by retention of biologic activity.

The potent and specific antimitotic activity of colchicine has been known for about 40 years; the presently accepted structure of colchicine (**1**) was first proposed 30 years ago [1]. During the intervening period the relationship between the molecular structure of colchicine (**1**) and its antimitotic activity has been a subject of continuing interest [2–5]. Although many derivatives of colchicine have been prepared, the essential molecular features responsible for antimitotic activity have remained unknown because most of the structural modifications of colchicine have resulted in molecules of equivalent or greater complexity. Significant observations on analogs of simpler structures have been few; they include desacetamidocolchicine (**2**) [4] and 2-methoxytropone (**8**) [2].

Within the last few years it has become clear that the antimitotic activity of colchicine is the result of a 1:1 interaction between colchicine and tubulin, a dimeric protein which aggregates to form the microtubules of the mitotic spindle [6]. It has been suggested that this interaction disrupts a dynamic equilibrium between tubulin and its polymeric form, the microtubule. Among a series of colchicine analogs, a good correlation has been found between antimitotic activity and ability to bind to the colchicine-binding site on tubulin [7].

Although cell culture techniques for determination of antimitotic activity have been available for many years, only recently have turbidometric and viscometric techniques been described for analysis of polymerization of tubulin to form microtubules *in vitro* [8, 9]. Incubation of high-speed supernatants of mammalian brain homogenates at 37° with added GTP induces formation of microtubules from tubulin and is accompanied by an increase in viscosity and development of turbidity in the supernatants. Both parameters have been used to characterize the polymerization reaction. It has been found that formation of microtubules *in vitro* is pH and temperature dependent, colchicine sensitive and requires addition of GTP. Viscometry has been shown to be a rapid, sensitive and quantitative method for the study of tubulin aggregation [8]. It is also a simple and flexible technique and for these reasons has been used in this work.

It was the major objective of this research to determine those features of the colchicine molecule associated with antimitotic activity and ability to inhibit tubulin polymerization *in vitro*. To this end seven selected colchicine derivatives and related compounds (Fig. 1) have been evaluated for their antimitotic activity in HeLa cells and for their ability to inhibit aggregation of tubulin in mouse brain supernatants as determined by viscometry. The results show that colchicine's activities can be manifested by structures simpler than that of colchicine. In addition, a good correlation between antimitotic activity and ability to inhibit tubulin polymerization has been observed among these derivatives of colchicine.

MATERIALS AND METHODS

Preparation of brain extracts. Female CF-1 mice (20–30 g) were killed by cervical dislocation, and the brains were removed and homogenized in a cold glass homogenizer with 1.5 vol. (ml) of ice-cold 100 mM Pipes buffer (pH 6.94) containing 1 mM EGTA and 2.5 mM GTP. The homogenate was centrifuged at 20,000 rev/min (100,000 *g*) for 1 hr at 4° (Spinco-type 65 fixed angle rotor, 9.0 ml polycarbonate tubes), and the supernatant fraction (henceforth referred to as "supernatant") was used for subsequent experiments. The protein concentration (range 8–12 mg/ml) was determined by a modified biuret assay [10]. Supernatants were used within 3 hr of their preparation.

Viscometry. Ostwald capillary viscometers (Cannon–Manning semimicro viscometers, type 100, Cannon Instrument Co., State College, Pa.) were immersed in a large water bath regulated at 37.0 ± 1°, and outflow times were measured using stopwatches calibrated to 0.5 sec. To obtain experimental data, 0.6 ml of supernatant prepared at 0–4° was placed in a viscometer equilibrated at 37°, and viscosity de-

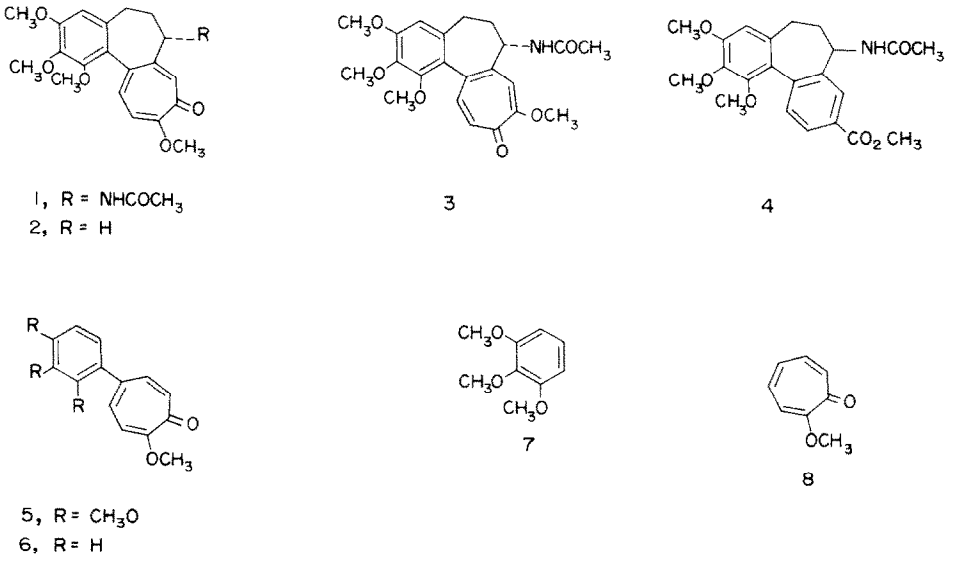

Fig. 1. Molecular structures of the compounds investigated.

velopment was followed as a function of time of incubation.

Determination of mitotic inhibition. HeLa cells purchased from Flow Laboratories (Rockville, Md.) were grown in Eagles medium with 10% fetal calf serum. Logarithmically growing cells were exposed to the compound in monolayer for 7 hr. The cells were then trypsinized off the culture dish, fixed (acetic acid–methanol; 1:3), treated with Geimsa stain and examined (500 cells/slide, two slides from separate cultures in each experiment).

Drug solutions. Whenever possible the compound to be tested was dissolved directly in buffer. However, many of the substances exhibited poor solubility in water. Such compounds were dissolved in a small amount of dimethylsulfoxide (DMSO) and then diluted with buffer. Solutions used in viscometry experiments contained not over 2% DMSO; those used in cell culture contained not over 3% DMSO.

Reagents. Colchicine was purchased from Sigma Chemical Co. Trimethoxybenzene (**7**) was purchased from Aldrich Chemical Co. Isocolchicine (**3**) [11], desacetamidocolchicine (**2**) [12], 2-methoxytropone (**8**) [13] and allocolchicine (**4**) [14] were prepared by the appropriate literature method.

Preparation of 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (**5**). Tropolone (488 mg) (Aldrich) in 10 ml H_2O was treated with a solution of sodium nitrite (552 mg) in 5 ml H_2O followed by 0·5 ml acetic acid to give 505 mg of 5-nitrosotropolone [15], reduction of which to give 5-aminotropolone was accomplished with sodium dithionite as has been described for reduction of 5-nitrotropolone [16].

A mixture of trifluoroacetic acid (342 mg) and 5-aminotropolone (274 mg) was dissolved in 2,3-dimethoxyphenol (5 g), and the resulting solution was cooled in ice, stirred and treated with isoamyl nitrite (0.2 ml) to effect diazotization. After 30 min, powdered copper (1 g) was added, and stirring was continued for 20 hr at room temperature. The mixture was diluted with dichloromethane and filtered through diatomaceous earth; the filtrate was treated with H_2S, again filtered and this filtrate, after extraction with 1% sodium bicarbonate solution, was treated overnight with diazomethane. Solvents were evaporated, and the remaining oil was chromatographed on a column of Silica gel packed in chloroform and eluted with 1% methanol in chloroform. Compound **5**, crystallized from ethyl acetate-hexane, m.p. 117–119°, was obtained in 15 per cent yield. Elemental analysis, u.v., i.r. and NMR spectra were consistent with the proposed structure.

Preparation of 2-methoxy-5-phenyltropone (**6**). 5-Aminotropolone (548 mg) and isoamyl nitrite (0.4 ml) were refluxed in benzene (15 ml) for 20 hr, and the resulting mixture was purified by extraction with 1% sodium bicarbonate solution. Treatment of the purified mixture with diazomethane was followed by evaporation of solvents and chromatography on a Silica gel column packed in chloroform. The desired compound was eluted with chloroform and crystallized from benzene, m.p. 139–140° (lit. [17] m.p. 140–141°) in 8 per cent yield. Elemental analysis and i.r. and NMR spectra were consistent with the proposed structure.

RESULTS

Incubation of HeLa cells with colchicine for 7 hr at 37° resulted in an accumulation of mitotic figures up to 20–25 per cent of the total number of cells. With shorter incubation times a smaller number of mitotic figures was seen; with longer incubation times other effects such as vacuolization and change in cell shape appeared, indicating that the cells had undergone degenerative alterations. All subsequent mitotic determinations were performed after 7-hr incubations. Control cultures of HeLa cells under these conditions consistently showed a mitotic index (per cent of cells in metaphase) of 4.0 ± 1.3 per cent (S. D.).

Because some of the derivatives exhibited low water solubility, it was necessary to employ DMSO to prepare solutions of adequate concentrations. Concentrations of DMSO up to 3% affected neither the control mitotic index nor the antimitotic activity of colchicine.

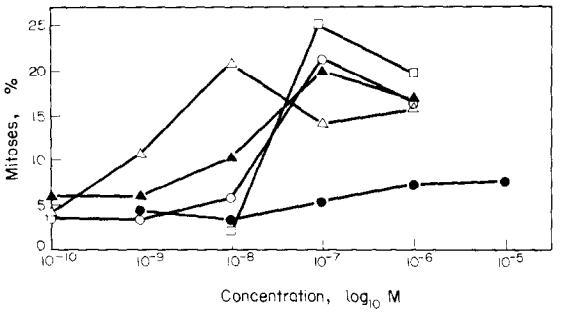

Fig. 2. Inhibition of mitosis (metaphase) by colchicine (**1**) (▲); desacetamidocolchicine (**2**) (△); allocolchicine (**4**) (□); compound **5** (○); and compound **6** (●).

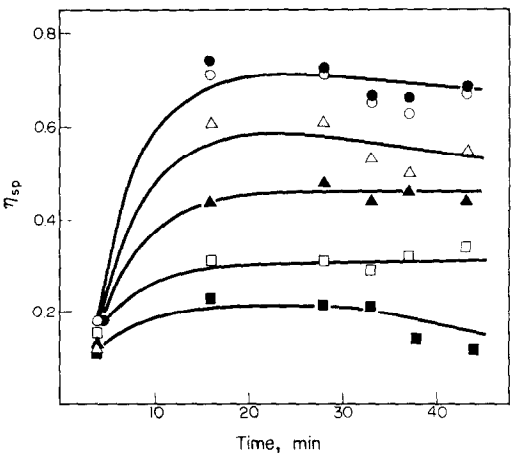

Fig. 3. Temporal profile of tubulin polymerization and effect of desacetamidocolchicine (**2**). 1.47×10^{-9} M (●); 1.47×10^{-8} M (△); 1.47×10^{-7} M (▲); 4.42×10^{-6} M (□); 1.47×10^{-5} M (■); and control (○).

Log-concentration vs mitotic index curves for compounds having observable antimitotic activity in concentrations at or below 10^{-4} M are displayed in Fig. 2. Isocolchicine (**3**) and trimethoxybenzene (**7**) showed no antimitotic activity in concentrations up to 10^{-4} M. Lack of antimitotic activity in 2-methoxytropone (**8**) in concentrations up to 7.35×10^{-4} M has previously been recorded [2].

When the various compounds were incubated with mouse brain supernatants attainment of a lower level of maximum viscosity compared to a control incubation indicated inhibition of polymerization of tubulin. A typical curve for this process is shown in Fig. 3 for desacetamidocolchicine (**2**). For those compounds which exhibited low water solubility it was necessary to use DMSO. No brain supernatants were exposed to DMSO concentrations of greater than 2%, and this concentration affected neither tubulin polymerization as measured by viscosity nor inhibition of polymerization by colchicine. Results are expressed as specific viscosity, η_{sp}, which was calculated in terms of outflow times of buffer (OT_b) and supernatant (OT_s) by the equation:

$$\eta_{sp} = (OT_s - OT_b)/(OT_b)$$

Half-maximal inhibition values for development of viscosity were estimated from specific viscosity vs log-concentration curves and are shown in Table 1 for compounds with half-maximal inhibition values of less than 10^{-4} M. Viscosity values were taken shortly after maximal viscosity had been reached, usually at 25 or 30 min. A typical curve is shown for compound **5** in Fig. 4.

From Table 1 it can be seen that there is a good correlation between ability to inhibit mitosis and ability to block polymerization of tubulin *in vitro*. In

Table 1. Inhibition of tubulin polymerization and antimitotic activity of colchicine and related compounds

Compound	I_{50}* (M)	Mitotic inhibition: Concn of maximum inhibition (M)	Mitotic inhibition: Per cent of cells in metaphase at maximum inhibition
Colchicine (**1**)	2.3×10^{-7}	10^{-7}	20
Desacetamidocolchicine (**2**)	1.9×10^{-7}	10^{-8}	21
Isocolchicine (**3**)	$> 10^{-4}$	Control value up to 10^{-4}	
Allocolchicine (**4**)	1.9×10^{-7}	10^{-7}	21
2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (**5**)	4.0×10^{-7}	10^{-7}	22
2-Methoxy-5-phenyltropone (**6**)	†	10^{-4}	14
1,2,3-Trimethoxybenzene (**7**)	$> 10^{-4}$	Control value up to 10^{-4}	
2-Methoxytropone (**8**)	$> 10^{-4}$	‡	‡

* I_{50} is the half-maximal inhibitory concentration for tubulin polymerization.

† Less than 30 per cent inhibition of tubulin polymerization was obtained at 1.5×10^{-4} M.

‡ Lettré [2] reported lack of mitotic inhibition by this compound in chicken embryo fibroblasts at 7.35×10^{-4} M.

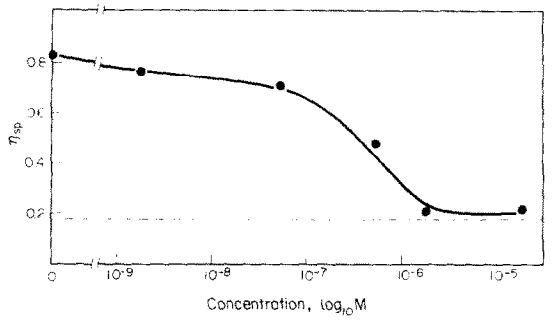

Fig. 4. Inhibition of tubulin polymerization by compound **5** as expressed by maximal specific viscosity obtained in mouse brain supernatant. Dashed line indicates initial viscosity level of the supernatant.

the case of 2-methoxy-5-phenyltropone (**6**), activity displayed in both systems was marginal. Desacetamidocolchicine appeared to be more active in cell culture than in brain supernatants relative to colchicine. The data in Table 1 also give a clear indication of the structural features associated with activity *in vitro* and *in vivo*. Removal of the 3-carbon bridge which links the phenyl ring (ring A of colchicine) and the tropone ring (ring C of colchicine), and therefore forms ring B of colchicine, gave the bicyclic derivative (**5**) which retained full colchicine-type activity in systems both *in vivo* and *in vitro*. Removal of the methoxy groups from the phenyl ring of this compound (**5**) to give **6** resulted in considerable diminution of both activities, only weak inhibition of tubulin polymerization being detected for this compound (**6**) at a concentration 375-fold greater than the half-maximal inhibitory concentration of the parent substance (**5**). Neither the trimethoxybenzene ring (**7**) nor the methoxytropone system (**8**) alone or together possessed any activity in either system at concentrations tested.

Conversion of the seven-membered tropone ring (ring C) of colchicine to the corresponding six-membered benzoate ester of allocolchicine (**4**) was accompanied by retention of potency essentially identical to colchicine in both test systems.

DISCUSSION

It is clear that colchicine-like antimitotic activity and ability to inhibit polymerization of tubulin are retained in molecules considerably simpler than colchicine itself. Inspection of the structures of the compounds investigated (Table 1) reveals that the molecular features of colchicine responsible for antimitotic activity and inhibition of tubulin *in vitro* are embodied in the bicyclic derivative, **5**. This is the simplest derivative of colchicine with similar potent activities. It remains to be seen if other ring systems or side chains can replace either ring in **5** and still retain colchicine-like activity. The conformation shown for **5** is that corresponding to colchicine but it should be noted that the two rings are free to rotate with respect to each other and can, therefore, assume an infinity of conformations. It seems likely that the active conformation would be similar to that of colchicine, since isocolchicine (**4**) is inactive in both systems (colchicine and isocolchicine can be considered semirigid analogs of the bicyclic compound, **5**).

In light of the results obtained it seems reasonable to presume that both the trimethoxyphenyl and methoxytropone systems of **5** are involved in binding to tubulin and that the binding results in inhibition of polymerization and, *in vivo*, inhibition of mitosis. This finding parallels that of Bhattacharyya and Wolff [18], who, on the basis of tubulin-induced fluorescence obtained with a series of colchicine derivatives, have suggested that the trimethoxyphenyl and methoxytropone ring systems represent at least two sites on the colchicine molecule involved in binding to tubulin.

Neither of the two ring systems (**7** and **8**) which comprise the tetramethoxy bicyclic compound (**5**), when tested individually or together, possessed any activity in tissue culture or in cell-free supernatants, thereby demonstrating the necessity for both rings to be combined in a single molecular entity in order to exhibit biological activity. Incubation of either colchicine (at 10^{-7} M) or compound **5** (at 10^{-7} M) with either **7** or **8** individually (at 10^{-4} M) or **7** and **8** together (at 10^{-4} M) in mouse brain supernatants produced no decrease in the ability of colchicine or compound **5** to inhibit tubulin polymerization as might have been expected if **7** and/or **8** could interact significantly with the binding site for colchicine and compound **5**. These results show that the combination of both rings in a single molecule (i.e. colchicine or **5**) is required for proper interaction with tubulin as well as for biological activity. It is not possible to state at present whether binding to tubulin is sufficient for colchicine and compound **5** to inhibit polymerization or whether events subsequent to binding of these compounds are more significant in this respect. Other workers [19, 20] have suggested that a conformational alteration in tubulin may be associated with the binding of colchicine.

The high activity of allocolchicine (**4**) as an antimitotic in HeLa cell culture and *in vitro* as an inhibitor of tubulin polymerization indicates that the seven-membered methoxytropone ring of colchicine is not essential for biological activity. This finding that a six-member benzene ring can replace the tropone system is consistent with the results of Zweig and Chignell [17], who reported that *N*-acetylcolchinol and *N*-acetyliodocolchinol (both of which have a phenolic ring in place of a tropone ring) effectively and competitively displaced colchicine from its binding site on tubulin. Apparently Lettré [2] recognized antimitotic activity in allocolchicine but never reported its quantitation.

The dramatic loss of biological activity which accompanies removal of the three methoxy groups from the phenyl ring of **5** to give 2-methoxy-5-phenyltropone (**6**), strongly suggests that one or more methoxy groups on the phenyl ring are required for potent inhibition of mitosis and inhibition of tubulin polymerization in compound **5** and, by analogy, in colchicine also. A requirement for methoxy groups on the phenyl ring to sustain antimitotic activity has been suggested by several investigators [1, 2, 4, 21, 22], but, until now, without direct supporting evidence. That the methoxy groups on the phenyl ring of **5** are important for binding is clear from the fact that co-addition of **5** (10^{-7} M) and **6** (10^{-4} M) to a brain supernatant did not affect the ability of **5** to inhibit polymerization of tubulin.

The data clearly suggest that, for the analogs of colchicine tested, a good correlation exists between antimitotic activity and ability to inhibit tubulin polymerization (Table 1). With regard to rank correlation, desacetamidocolchicine (**2**) presents the only exception. This molecule possesses a greater degree of hydrophobic character relative to colchicine at the seven position (the nitrogen-bearing carbon in colchicine) and is a more potent antimitotic agent not only in HeLa cell culture as demonstrated here, but in various other cell lines [4, 5, 23]. The fact that desacetamidocolchicine is only equal to colchicine in its ability to inhibit tubulin polymerization *in vitro* suggests that some feature of the intact cell may permit more ready access to or facilitate interaction with the tubulin molecule. However, presently available information does not permit emphasis of any particular hypothesis on this point.

Acknowledgements—The author wishes to express his gratitude to Bill Kerr, Jody Eaton, Doug Mayfield and Ann Veal for their valuable technical assistance. This work was supported by PHS Grant CA 16425.

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