Biochimica et Biophysica Acta, 673 (1981) 86—92
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BBA 29512

COMPARISON OF MITOSTATIC EFFECT, CELL UPTAKE AND TUBULIN-BINDING ACTIVITY OF COLCHICINE AND COLCEMID

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(Received August 18th, 1980)

Key words: Colchicine; Colcemid; Tubulin binding; Mitosis; Cell uptake; (Microtubule)

Summary

Mitostatic action, cellular uptake and the binding of colchicine and colcemid to tubulin were compared. It was shown that mitostatic action of low doses of colchicine developed only after 24 h incubation of the drug with mouse L fibroblasts, while the colcemid-induced block of mitosis was evident after 2 h incubation. The initial rate of uptake was about 10 times greater for colcemid than for colchicine. Cellular uptake of the drugs reached an equilibrium after 2 and 15—18 h incubation for colcemid and colchicine, respectively, and the plateau values were identical. The kinetics of colchicine and colcemid binding to bovine brain tubulin was studied by the DEAE-filter binding assay. Colcemid binds to tubulin much faster than does colchicine. The rate of colcemid efflux from L cells is much higher than that of colchicine. According to the efflux data, colcemid dissociates readily from a complex with tubulin ($t_{1/2}$ = 10 min), while the colchine-tubulin complex is stable for at least 1 h. These results are consistent with previously published data (Frankel, F.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 72, 2798—2802), which showed that colcemid action on cells is more reversible than that of colchicine. We suggest that differences between colchicine and colcemid in the rate of mitostatic action and its reversibility are determined by the differences in parameters of tubulin binding.

Abbreviation: SDS, sodium dodecyl sulphate.

Introduction

Colchicine and deacetylmethylcolchicine (Colcemid) are microtubular poisons widely used for studying microtubule functions in cells. These alkaloids interact with the same site on tubulin molecules [1]; tubulin-colchicine (and, probably, tubulin-colcemid) complexes bind to microtubule ends and thus poison further assembly [2]. This leads to the disassembly of cellular microtubules and, as a result of the disassembly, to the mitotic arrest, loss of the cell polarity and to a disturbance of other cellular functions which are thought to be dependent on the microtubule system [3—6].

Although both drugs were used to disrupt microtubules in cells, their action is somewhat different. It was shown in Ref. 7 that colcemid is more active than colchicine; less colcemid than colchicine and shorter incubation time give the same results during cell treatment. As shown by immunofluorescent staining with antitubulin antibodies, colcemid-induced microtubule disassembly is readily reversible [8—10]; in colchicine-treated cells cytoplasmic microtubules appear, however, only after prolonged incubation in a drug-free medium [8,9].

In this work we examined mitostatic arrest, cellular uptake and tubulin-binding properties of colchicine and colcemid in order to explain the differences in their action on cells.

Materials and Methods

Chemicals. Colcemid was a product of CIBA, colchicine was obtained from Merck. [^{3}H]Colchicine (specific activity, 2—4 Ci/mmol) and [^{3}H]colcemid (specific activity, 8.96 Ci/mmol) were purchased from Radiochemical Centre, Amersham and New England Nuclear, respectively.

Cells. Mouse transformed L line fibroblasts were maintained in a mixture of Eagle medium and 0.5% lactalbumin hydrolysate (1 : 1), supplemented with 10% bovine serum and 100 μg/ml monomycin. Cells used in the uptake experiments were seeded at a density of (2—3) · 10^6 per 20 cm^2 flasks containing 5 ml medium. To determine the mitostatic effect of colchicine and colcemid, 4 · 10^5 cells were plated on 1.4 cm^2 cover slips in 1.8 ml of medium. The cells were used for experiments 24 h after seeding.

Evaluation of the mitostatic effect of colchicine and colcemid. The method of estimating mitostatic action of colcemid and colchicine was described previously [11]. Briefly, the cells were incubated with alkaloids at 37°C, then gently washed with warm Hanks solution, fixed and stained. The antimitotic effect of colchicine and colcemid, was measured by counting cells at various stages of mitosis. The drugs reduced the percentage of cells in postmetaphase stages of mitosis (ana- and telophases). 100 mitoses per cover slip were counted, late prophases, metaphases, anaphases, telophases and late telophases (or reconstructed nuclei) being taken into consideration.

[^{3}H]Colchicine and [^{3}H]colcemid uptake. In the uptake experiments L fibroblasts were incubated in 20 cm^2 flasks with 4 ml of [^{3}H]colchicine or [^{3}H]colcemid solution in a growth medium at 37°C, then monolayers were washed with ice-cold saline and dissolved in 0.5 ml of Dulbecco solution containing 0.5% Triton X-100 and 0.2% trypsin. After complete dissolution of the

monolayers (usually 2 h at 37°C) samples were transferred to vials containing 15 ml of Bray's scintillator and counted on a Mark III liquid scintillation counter.

Colchicine and colcemid binding to tubulin. Microtubule protein was obtained from bovine brain by two cycles of polymerization-depolymerization [12]. Purification was performed in buffer A, containing 50 mM imidazole, pH_{20} 6.7/0.5 mM $MgCl_2$/1 mM 2-mercaptoethanol/0.1 mM EDTA/0.1 mM GTP. For the polymerization of tubulin, buffer A was supplemented with 4 M glycerol and 1 mM EGTA. This preparation contains about 90% tubulin- and 10% microtubule-associated proteins as shown by SDS-polyacrylamide gel electrophoresis [13].

Binding of alkaloids to tubulin was performed in buffer A at 37°C. Tubulin and alkaloid concentrations were 2 μM before or 0.02 μM after dilution of the mixtures (see Results). The tubulin-bound alkaloid was determined by the DEAE-filter binding assay [14]. Aliquots of incubation mixture were diluted to 10 ml with buffer A and filtered for 30 s through 20-mm DEAE-filter paper discs (Whatman DE-81). Then the filters were washed with 20 ml ice-cold buffer A 3 times and filter-bound radioactivity was determined by liquid scintillation counting. As will be shown below (see Results) colcemid easily dissociates from complexes with tubulin; thus special precautions were taken to keep application and washing time for each sample within the 80—90 s range. To determine the background binding, the mitostatics were mixed with tubulin at 0°C and immediately applied to the filter.

Results

Comparison of colchicine and colcemid on mitostatic action, cellular uptake and tubulin binding

Before studying cellular uptake of the drugs it was necessary to ascertain that differences in their mitostatic action would take place in our experimental system (mouse L fibroblasts). Table I shows that after 2 h incubation of the cells colcemid does cause full mitostatic arrest at lower concentrations than does colchicine. 0.025 μM colcemid was required for the total block of mitoses in L cells. The same effect could be caused only by 1.25 μM colchicine. The kinetic studies presented in Table II showed, however, that after 24 h incuba-

TABLE I

COMPARISON OF THE MITOSTATIC ACTION OF COLCHICINE AND COLCEMID AFTER 2 h INCUBATION

Cells were incubated with colchicine or colçemid for 2 h at 37° C. Various stages of mitosis were counted in fixed and stained cultures. Figures in the table are number of ana- and telophases per 100 mitoses. Each figure represents results obtained on a separate cover slip.

Post metaphase stages of mitosis	Drug conc. (μM)	Colcemid					Colchicine				
		0	0.01	0.02	0.2	1.0	0.01	0.02	0.1	0.2	1.0
Anaphase		57	30	0	0	0	42	47	40	37	0
Telophase		41	24	3	0	0	51	55	29	12	0

TABLE II

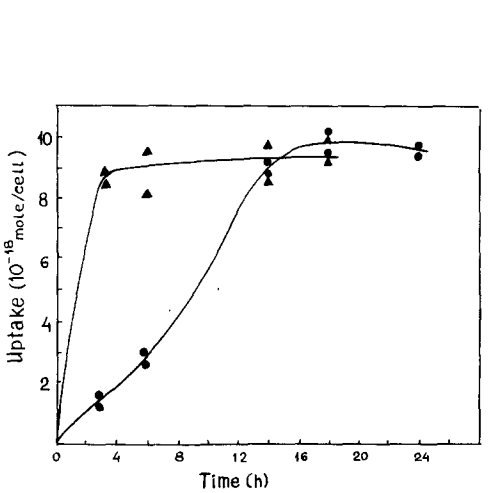
KINETICS OF MITOSTATIC ACTION OF COLCHICINE AND COLCEMID

Cells were incubated with 0.02 or 0.06 μM solution of colchicine or colcemid for 2—24 h, fixed, and stained. Figures in the table are numbers of ana- and telophases per 100 mitoses. Each figure represents the results obtained on a separate cover slip.

		Time of incubation with drug (h)		
		2	6	24
Colcemid	0.02	0	0	0
		0	1	0
	0.06	0	0	0
		0	0	0
Colchicine	0.02	24	14	3
		28	20	0
	0.06	11	6	0
		13	10	0
Without drug		57		
		41		

tion the minimal concentrations of colchicine and colcemid inducing a full block of mitosis were the same (0.025 μM). Colchicine in this concentration acts only after a prolonged incubation whereas the action of colcemid was evident after 2 h incubation; 24 h incubation did not increase its mitostatic effect. These results suggest that the stronger mitostatic action of colcemid is explained by the higher rate of its uptake.

Direct studies show that the rate of colcemid uptake is, in fact, much higher



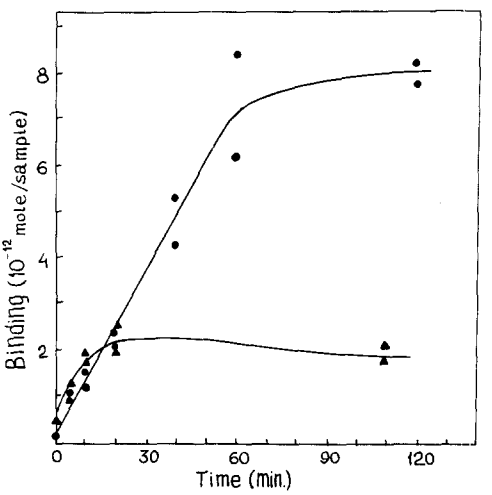


Fig. 1. Kinetics of uptake of [^{3}H]colchicine (●) and [^{3}H]colcemid (▲) by L cells. After incubation with 0.2 μM [^{3}H]colchicine or [^{3}H]colcemid, monolayers were rapidly washed with cold saline, dissolved, and cell-bound radioactivity was counted.

Fig. 2. [^{3}H]Colchicine (●) and [^{3}H]colcemid (▲) binding to tubulin. Mixtures, containing 2 μM tubulin and 2 μM [^{3}H]colchicine or [^{3}H]colcemid were incubated at 37°C. Tubulin-bound alkaloids were determined in 50-μl aliquots as described in Materials and Methods.

than that of colchicine (Fig. 1). Colcemid uptake reaches equilibrium after 2 h incubation, while intracellular colchicine concentration increases during 14–18 h. Equilibrium intracellular concentrations of both drugs are the same (about $9 \cdot 10^{-18}$ mol/cell at a 0.2 μM concentration of alkaloids in the medium).

Thus, colcemid uptake was much quicker, but after prolonged incubation intracellular levels of both antitubulins were equalized. The higher rate of colcemid uptake into the cells may be explained either by increased transport into the cells through plasma membrane or its quicker trapping by intracellular targets, i.e. the tubulin molecules. To test the latter possibility, we compared the kinetics of [^{3}H]colchicine and [^{3}H]colcemid binding to tubulin from bovine brain. As shown in Fig. 2, the rate of binding is much higher for colcemid than for colchicine. Colcemid binding reached equilibrium in 10 min, and colchicine binding increased for 60 min.

Dissociation of mitostatics from cells and from complex with tubulin

As we mentioned above, the action of colcemid on cells is much more readily reversible than that of colchicine. This may be explained by the greater efflux of the former from the cells. To show this directly, we incubated the cells with 0.2 μM [^{3}H]colchicine or 0.05 μM [^{3}H]colcemid for 2 h to achieve equal intracellular drug concentrations, and then the incubation was continued in a drug-free medium. Fig. 3 shows that the rate of colcemid efflux is much higher than that of colchicine. It is seen that after 3 h incubation of the cells without antitubulins they retain about 70% of colchicine and less than 10% of colcemid.

The rate-limiting step of drugs efflux from the cells may be either their transport from the cells through plasma membrane or dissociation of the tubulin-drug complex. If the latter possibility is correct colchicine and colcemid must

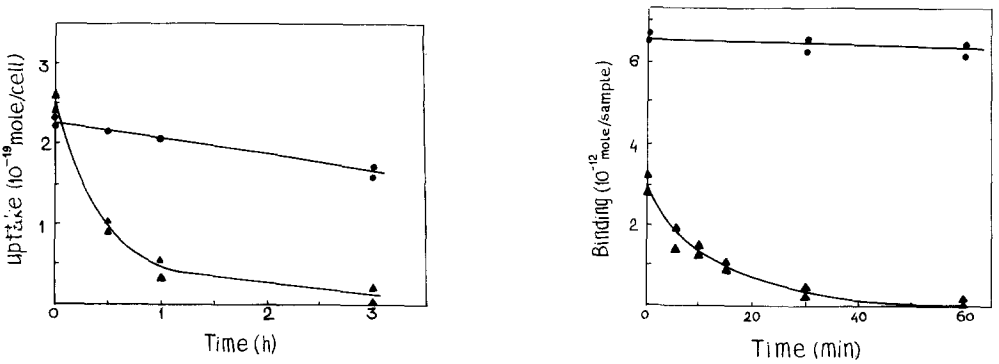


Fig. 3. Efflux of [^{3}H]colchicine (●) and [^{3}H]colcemid (▲) from L cells. Cells were incubated with 0.2 μM [^{3}H]colchicine or 0.05 μM [^{3}H]colcemid for 2 h at 37°C, then washed and further incubated in an alkaloid-free medium. Cell-bound radioactivity was determined at different times after transfer to a medium without drugs.

Fig. 4. Dissociation of [^{3}H]colchicine (●) and [^{3}H]colcemid (▲) from complex with tubulin. 2 μM tubulin was incubated with 2 μM [^{3}H]colchicine or [^{3}H]colcemid for 1 h at 37°C, then incubation mixtures were diluted 100-fold with the buffer. Tubulin-bound alkaloids were determined in 5-ml aliquots taken at different times after dilution.

differ in the stability of the complexes they form with tubulin. To measure the rates of dissociation of complexes we incubated 2 μM [^{3}H]colchicine or [^{3}H]-colcemid with 2 μM tubulin for 1 h at 37°C and then diluted the incubation mixtures with a 100-fold volume of buffer A. Such a dilution completely inhibits further binding because incubation of 0.02 μM colchicine or colcemid with 0.02 μM tubulin does not result in any binding above the background level. In these conditions the kinetics of tubulin-bound radioactivity presented in Fig. 4 reflects that of dissociation of the tubulin-drug complex. One may see that 60 min after dilution is sufficient for complete dissociation of the colcemid-tubulin complex whereas the amount of tubulin-bound [^{3}H]colchicine does not change significantly for that time. Half-dissociation of colcemid-tubulin complex in ionic conditions of buffer A takes about 10 min. Dissociation obeys first-order kinetics (not shown). The time of half-dissociation of the colchicine-tubulin complex in this experiment is difficult to measure because it is much higher than 60 min, and after prolonged incubation it is the irreversible decay of colchicine-binding activity of tubulin which contributes significantly to the dissociation of the complex.

Discussion

The present study reveals correlation between the mitostatic action of colchicine and colcemid and the amount of these preparations taken up by the cells. The cellular uptake of colchicine is a slow process and the full mitostatic action of this drug is manifested only after 24 h. The rate of colcemid uptake is much higher and, therefore, colcemid-induced mitotic arrest is evident after as little as 2 h. Reversibility of colchicine and colcemid action also correlates with the rate of efflux of the drugs from the cells.

It is clear that the net uptake of the mitostatics by the cells depends on both the transport of the drugs through plasma membrane and their trapping by tubulin molecules in the cytoplasm. In order to show which of them determines the differences in the biological action of colchicine and colcemid we studied the binding of the drugs to tubulin and dissociation of the preformed complexes. We used the method of Borisy [14] to determine the alkaloid binding. According to this method, aliquots of the incubation mixture were filtered through DEAE-paper which adsorbs tubulin (and tubulin-bound alkaloids) and free alkaloids were washed out with a buffer. The washing procedure, however, strongly restricts the use of this method for studying colcemid-binding reaction because it is readily reversible and colcemid probably partially dissociates from tubulin during washing. This probably explains why in our experiments tubulin binds more colchicine than colcemid (Fig. 2). Although one cannot use Borisy's method for estimating the stoichiometry of colcemid binding it can be used to measure the time required for a binding reaction equilibrium to be attained as well as for the time of half-dissociation of the complex. Thus the method can be applied to compare the kinetics of colchicine and colcemid binding to tubulin.

These experiments show that colcemid binds to tubulin more rapidly than colchicine. The rate of its dissociation from the complex is also much higher. Similar results were obtained recently by another technique [15].

Thus, it appears that the differences in the action of colchicine and colcemid on cells are determined by the rate binding to tubulin.

Acknowledgements

The authors are indebted to Professor Ju.M. Vasiliev and Dr. A.A. Stavrovskaya for their support and interest and to Dr. V.A. Rosenblat for discussions. We are also grateful to Carol MacPherson for correction of English in this paper.

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