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VINBLASTINE INHIBITS THE MATURATION OF THE PRECURSOR OF MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE. VINCRISTINE AND SIX OTHER CYTOSKELETON INHIBITORS DO NOT SHOW THIS EFFECT

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Dedicated to Professor F. Leuthardt, Zürich, on the occasion of his 80th birthday.

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Cytoskeleton inhibitors were tested in chicken embryo fibroblast cultures for possible effects on the import of the precursor of mitochondrial aspartate aminotransferase into mitochondria. Vinblastine (50 μM) increased the steady-state pool of the precursor 2.5-fold in pulse experiments with [^{35}S]methionine. If the precursor was accumulated during a pulse in the presence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) and then chased under diluting CCCP, vinblastine (50 μM) prolonged the half-life of the precursor from 0.5 min in the control to 3 min. Other cytoskeleton inhibitors, i.e. vincristine (25 to 150 μM), colchicine (50 μM), nocodazole (50 μM), podophyllotoxin (50 μM), taxol (45 μM), cytochalasin D (20 μM) and phalloidin (25 μM) did not show this effect. The observed inhibition by vinblastine does not seem to relate to its action on microtubuli.

Mitochondria have been reported to be associated with the cytoskeleton (1). Both morphological and biochemical data suggest that mitochondria interact with microtubuli (2,3), intermediate filaments (4-6) or γ-actin (7). In this study we have examined whether cytoskeleton inhibitors affect the uptake into mitochondria (for reviews, see Refs. 8-10) of a protein that is synthetized in the cytosol.

The mitochondrial isoenzyme of aspartate aminotransferase has been shown previously to be synthetized on free cytosolic polysomes as a higher molecular weight precursor (11,12). The precursor

Abbreviations used: Pre-mAspAT, the higher molecular weight precursor of mitochondrial aspartate aminotransferase; mAspAT, mitochondrial isoenzyme of aspartate aminotransferase; CCCP, carbonyl cyanide m-chlorophenylhydrazone and SDS, sodium dodecylsulfate.

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was also detected in cultured chicken embryo fibroblasts (13). Its uptake into mitochondria is a rapid process; from pulse experiments with [^{35}S]methionine a half-life of the precursor of only 0.5 min was estimated (13). Using this experimental system we have tested 8 different cytoskeleton inhibitors.

EXPERIMENTAL PROCEDURE

Materials - Colchicine was from Sandoz, cytochalasin D from Aldrich, phalloidin from Boehringer; taxol was a gift from Dr. M. Suffness, Natural Products Branch, NIH Bethesda; vinblastine sulfate and vincristine sulfate were from Eli Lilly. Stock solutions (1 mM) of colchicine, vinblastine and vincristine were dissolved in methionine-free Basal Medium Eagle, if not indicated otherwise. The concentration of aqueous solutions of the latter two compounds was checked spectroscopically. The stock solutions of all other inhibitors (10 mM) were prepared in dimethyl sulfoxide. [^{35}S]Methionine was from New England Nuclear. Potassium cyanide was from Merck, dimethyl sulfoxide from Fluka. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was a gift from Dr.P.G. Heytler, DuPont.

Cells - Chicken embryo fibroblasts were grown in Basal Medium Eagle (Gibco Europe) with Earle's salts, supplemented with 10% fetal calf serum (Gibco Europe). For the experiments tertiary cultures were used.

Pulse experiments with chicken embryo fibroblasts - The Basal Medium Eagle (10 ml per Petri dish with 100 mm diameter) was replaced by 5 ml of the same medium prepared without methionine; the Petri dishes were incubated for 10 min in order to deplete the cells of methionine. For pulsing, the medium was replaced again by Basal Medium Eagle containing 0.1 mCi ml^{-1} [^{35}S]methionine (10^{3} Ci mmol^{-1}). All incubations were carried out at 37°C. The pulse was stopped by chilling the Petri dishes on iced water.

Pulse-chase experiments with chicken embryo fibroblasts - Pre-mAspAT was accumulated in the cytosol by treating the cells with 20 μM CCCP during a pulse of 15 min with 0.1 mCi ml^{-1} [^{35}S] methionine (13). For the chase, the pulse medium was replaced by 5 ml of Basal Medium Eagle containing 10 mM unlabeled methionine and 10% fetal calf serum. All incubations were carried out at 37°C. The chase was stopped by chilling the Petri dishes on iced water.

Extraction and quantitation of pre-mAspAT and mAspAT - The cells were mechanically detached, collected by centrifugation and denatured in 5% sodium dodecylsulfate (SDS). Immunoprecipitates were obtained with rabbit antiserum against SDS-denatured mAspAT from chicken as described earlier (12). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (14). Gels were stained with Coomassie blue and prepared for fluorography with Enlightning, New England Nuclear. Kodak X-1 or X-AR films were exposed for 2 to 7 days at -70°C. The relative radioactivity in the pre-mAspAT and mAspAT bands was estimated by densitometry.

Measurement of oxygen consumption of cultured chicken embryo fibroblasts - Cells from 4 Petri dishes were transferred into 4 ml of Basal Medium Eagle. Oxygen consumption was measured at 37° C with a Yellow Spring Instruments Oxygen Monitor Model 53. Vinblastine and vincristine were added from 50 mM stock solutions in dimethyl sulfoxide.

RESULTS

Effect of vinblastine on maturation pre-mAspAT - Out of 8 cytoskeleton inhibitors tested only vinblastine (50 µM) inhibited the maturation of pre-mAspAT in pulse-chase experiments (Fig.1A). Chicken embryo fibroblasts were pulsed with [^{35}S]methionine in the presence of the uncoupler CCCP (20 µM). No conversion of pre-mAspAT to mature mAspAT occurred under these conditions (lanes

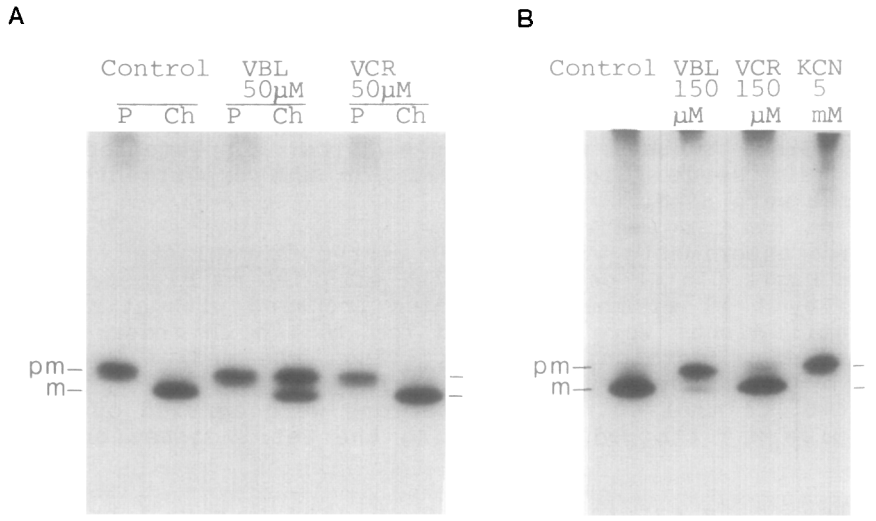

Figure 1. Effect of vinblastine on the rate of maturation of pre-mAspAT.
A) Pulse-chase experiment in the presence of cytoskeleton inhibitors with preincubation - Two Petri dishes at a time were preincubated during 1 h with vinblastine or vincristine at the indicated final concentrations. The inhibitors were also present in all subsequent stages of the experiment. The cells were then pulsed for 15 min with [^{35}S]methionine in the presence of 20 µM CCCP. The fluorograms of the immunoprecipitates after SDS-polyacrylamide gel electrophoresis are shown. Lanes P: After a 15 min pulse one Petri dish for each cytoskeleton inhibitor and one control without inhibitor were harvested without chase. Lanes Ch: The other Petri dish and one control were subjected to a 3 min chase in CCCP-free medium.
B) Pulse-chase experiment in the presence of cytoskeleton inhibitors without preincubation - The experiment was performed as in Fig.1A except that the cytoskeleton inhibitors were present in the chase medium only.
The positions of pre-mAspAT (pm, MW 47,500) and m-AspAT (m, MW 44,500) are indicated by dashes.

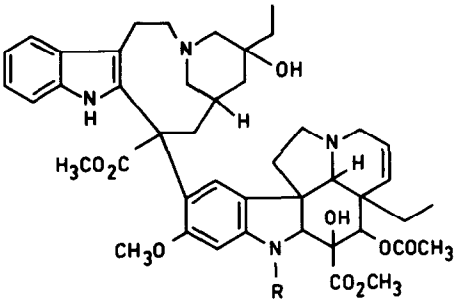

Figure 2. Structure of vinblastine (R= $-CH_3$) and vincristine (R= -CHO).

P). If the cells were chased under dilution of CCCP (lanes Ch), pre-mAspAT was rapidly processed to mature mAspAT with a half-life of about 0.5 min. However, in cells treated with vinblastine the half-life was prolonged to 3 min (Fig. 1A). The structurally closely related vincristine (50 μM; Figs. 1 and 2) as well as colchicine (50 μM), nocodazole (50 μM), podophyllotoxin (50 μM), taxol (45 μM), cytochalasin D (20 μM) and phalloidin (25 μM) showed no difference to the controls (data not shown). None of the inhibitors at the concentrations used affected the rate of protein synthesis as determined by measuring trichloroacetic acid-precipitable radioactivity. Vincristine proved also ineffective if dissolved in dimethyl sulfoxide (final concentration 1% v/v) which increases the permeability of biological membranes.

The onset of the effect of vinblastine was immediate as shown in a pulse-chase experiment where the drug was added only with the chase medium (Fig.1B). Again, vincristine at the same concentration proved ineffective. KCN (5 mM) completely inhibited the import in CCCP-pretreated cells.

In pulse experiments without CCCP, vinblastine (50 μM and 100 μM) increased the pool of pre-mAspAT 2.5-fold (Fig.3A and Table 1). At 100 μM, total protein synthesis was inhibited; at 10 μM, the steady-state pool remained unchanged. Vincristine (100 μM; not shown) or KCN (5mM) had no effect (Fig. 3B and Refs. 13,41).

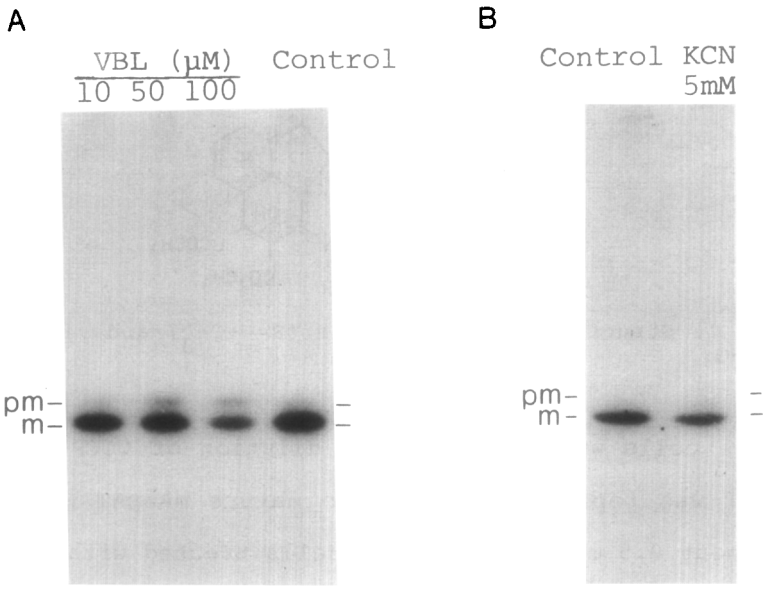

Figure 3. Effect of vinblastine on the steady-state pool of pre-mAspAT

A) Three Petri dishes were incubated with vinblastine (VBL) at the indicated final concentrations for 1 h. The cells were then pulsed with [^{35}S]methionine for 7 min. The fluorograms of the immunoprecipitates after SDS-polyacrylamide gel electrophoresis are shown. B) Cells were preincubated for 7 min in the presence of KCN.
The positions of pre-mAspAT (pm, MW 47,500) and m-AspAT (m, MW 44,500) are indicated by dashes.

Effect of vinblastine on oxygen consumption of cultured chicken embryo fibroblasts - The oxygen consumption of chicken embryo fibroblasts was measured with an oxygen electrode. Vinblastine (100 μM) slightly reduced the oxygen consumption (to 90% of the initial value), whereas vincristine (25 to 200 μM) had no effect. CCCP stimulated the consumption of oxygen to 150% both in the absence and in the presence of vinblastine. KCN (5 mM) blocked the respiration completely.

DISCUSSION

Cytoskeleton inhibitors have been reported to interfere with intracellular protein traffic, e.g. sorting of proteins (15-17), export of proteins (18-21) and the mobility of membrane proteins (22,23). To our knowledge, cytoskeleton inhibitors have not been

Table 1 - The relative radioactivity of the bands of pre-mAspAT and mAspAT in the fluorogram of Fig. 3A was estimated by densitometry. Fluorograms of different exposure times gave similar results.

Vinblastine μM	pre-mAspAT (pm)	mAspAT (m)	ratio pm/m
0	1	13.5	0,07
10	1.1	12.3	0.09
50	2.8	12.5	0.22
100	2.4	8.2	0.29

tested to date as to their effects on the import of cytosolically synthetized proteins into mitochondria. The exact nature of the interaction of the cytoskeleton with mitochondria still being unknown, we have used four different types of cytoskeleton inhibitors (for reviews, see Refs. 24-26): 1) Microtubuli polymerization inhibitors, i.e. colchicine, nocodazole (27), podophyllotoxin, vinblastine and vincristine; 2) the microtubuli stabilizing agent taxol (28); 3) the microfilament polymerization inhibitor cytochalasin D (29,30); 4) the microfilament stabilizing agent phalloidin (31,32). The preincubation times (usually 1 h, or up to 24 h with taxol) were long enough and the concentrations of the cytoskeleton inhibitors sufficiently high to ensure an effect on the cytoskeleton (27,30,33,34).

Only vinblastine inhibited the import of pre-mAspAT into mitochondria (Figs. 1 and 3). This inhibition is probably not mediated by the cytoskeleton: All other microtubuli disrupting agents proved ineffective. Consistent with this conclusion, the concentration of vinblastine needed (50 μM) was higher than that necessary for inhibition of polymerization of microtubuli in vitro (25), mitotic arrest (25,35,36) or inhibition of the microtubuli-dependent amino acid uptake into isolated rat hepato-

cytes (37). However, another effect of vinblastine and vincristine on microtubuli, i.e. the formation of paracrystals (38-40), occurs in the concentration range effective on import.

The import of proteins into mitochondria depends on the electrochemical gradient of the mitochondrial inner membrane (41,42). Inhibition of oxygen consumption by vinblastine was reported for certain cultured cells, while in other cell types no inhibition was found (43, 44). We have measured a slight decrease in oxygen consumption (to 90% of the initial value) in chicken embryo fibroblasts treated with vinblastine. This minor effect most likely cannot explain the observed inhibition of the import. KCN (5 mM) which completely blocks respiration, inhibited the import of pre-mAspAT when the precursor had been accumulated under CCCP (Fig. 1), but had no effect on the steady-state pool of pre-mAspAT in the absence of CCCP (Fig.3B and Refs. 10 and 41). Oxidative phosphorylation seems to be necessary for regenerating the electrochemical gradient after dissipation by CCCP, but its blockage does not inhibit the import in steady-state experiments without CCCP (Fig. 3B). Apparently, vinblastine which not only affected the maturation of pre-mAspAT in pulse-chase experiments (Fig.1A) but also increased its steady-state pool (Fig. 3) acts not through inhibition of the respiratory chain.

Possible explanations for the observed effect of vinblastine include interference with the translocation system of the mitochondrial envelope, an inhibition of the processing protease (45), or an interaction with the precursor molecule itself. We concider the first possibility the most likely one, because vinblastine in chicken embryo fibroblasts inhibits another function of the mitochondrial membrane, i.e. the oxygen consumption. Vinblastine is more hydrophobic than vincristine (Fig. 2) which affected neither the maturation of pre-mAspAT, nor the oxygen consumption.

Conceivably, vinblastine might be inserted into the mitochondrial membranes.

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