INTERACTION OF ONCODAZOLE (R 17934), A NEW ANTI-TUMORAL DRUG, WITH RAT BRAIN TUBULIN.

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SUMMARY : Oncodazole (R 17934), methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate (I), a new synthetic drug with anti-tumoral activity, inhibits the polymerization of rat brain tubulin in vitro. It has no depolymerizing effect on preformed microtubules in vitro. Binding studies by means of molecular sieving and equilibrium dialysis indicates that the drug binds to purified rat brain tubulin in a mole to mole ratio. Finally the drug competitively inhibits colchicine binding to purified rat brain tubulin. From these results the conclusion may be drawn that oncodazole is a true microtubule inhibitor.

INTRODUCTION

Oncodazole (R 17934) (Fig. 1) has been shown to have anti-mitotic activity in vitro (1) and anti-tumoral activity in vivo (2, 3). On a cellular level the drug interferes with the structure and function of microtubules (1). The purpose of this study was to provide direct biochemical evidence that the drug acts on the tubulin molecule i.e. the protein subunit of the microtubular structure. Two properties which are common to all microtubule inhibitors were therefore investigated : their ability to inhibit the polymerization reaction of (4) and to bind on the tubulin molecule (5).

MATERIAL AND METHODS

Drugs and chemicals

Colchicine was purchased from Aldrich Europe, oncodazole (R 17934) is a product of Janssen Research Laboratories.

[^{3}H] Colchicine (3 Ci/mmol) was purchased from the Radiochemical Centre, Amerham, [^{14}C] Oncodazole (6.25 μCi/mg) was synthetized by the metabolic department of Janssen Research Laboratories.

Abbreviations used : EDTA : ethylene-diamino-tetra-acetic acid; GTP : guanosine-5'-triphosphate; Pipes : piperazine-N-N'-bis [2-ethane sulfonic acid]; EGTA : ethylene-glycol-bis (2-aminoethyl)-tetra-acetic acid

Before use colchicine was dissolved in water and oncodazole in methoxyethanol.

The other chemicals used were of reagent grade.

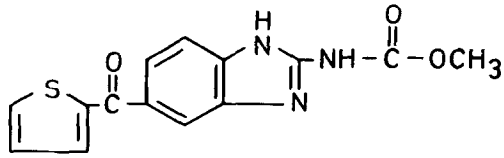

Fig. 1 : Structural formula of oncodazole (R 17934)

Turbidimetric assay of tubulin polymerization and depolymerization in vitro

Polymerization of tubulin in the supernatant of rat brain homogenate was followed turbidimetrically at 405 mm as described in a previous paper (4). The depolymerization reaction was also studied turbidimetrically under the same experimental conditions but on samples which were first incubated 30 minutes at 37°C. Polymerization and depolymerization was always monitored at the ultrastructural level in the electron microscope.

Preparation of purified rat brain tubulin

Rat brain tubulin was prepared by two polymerization-depolymerization cycles in 0.1 M phosphate buffer according to Shelanski (6). The last depolymerization was performed in the buffer used for the binding experiments. After ultracentrifugation the tubulin solution was divided into aliquots, frozen and stored in liquid nitrogen until use. Maximum storage time was 14 days.

Binding studies by means of molecular sieving

To 2.5 ml tubulin solution (1 mg/ml) in Pipes buffer 0.1 M pH = 6.4 containing 1 mM EDTA, 0.1 mM GTP and 1 mM mercaptoethanol (7) was added 0.25 ml [^{14}C] oncodazole (10^{-3}M) dissolved in methoxyethanol. At several time intervals before and during incubation at 37°C, 0.5 ml samples were removed, brought to 0°C and sieved on a 350 x 6.5 mm Sephadex G 50 column. The elution with Pipes buffer was monitored on a UV-Cord (LKB) at 280 mm and 1 ml samples were mixed with 10 ml Instagel (Packard Instruments) and counted for radioactivity in a scintillation counter (Packard Instruments).

Binding studies by means of equilibrium dialysis

Equilibrium dialysis was performed in a DIANORM equilibrium dialysis system (INNO-MED, Switzerland) consisting of 2 compartments, each with a volume of 1 ml (8). The equilibrium buffer was 0.1 M pH 6.4 phosphate containing 1 mM GTP, 1 mM EGTA, 0.5 mM $MgSO_4$ and 5 mg/ml bovine serum albumin in order to protect the tubulin from adsorption to the semi-permeable membrane. Tubulin concentration was 2 mg/ml. Ten microliters of [^{14}C] oncodazole solutions in methoxyethanol were added to both compartments. Equilibrium time was 3 h. Thereafter the contents of the compartments were mixed with 10 ml Instagel and radioactivity counted in a scintillation counter (Packard Instruments).

Binding studies of colchicine in the presence of oncodazole

The [^{3}H] colchicine binding assay was performed according to Sherline et al. (9) with the modification that a phosphate buffer .1 M pH = 6.4 supplemented with 1 mM EGTA, 1 mM GTP and 0.5 mM $MgSO_4$ was used. Tubulin concentration was 1 mg/ml.

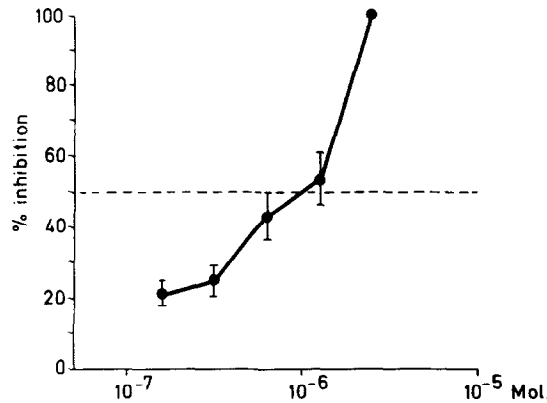

Fig. 2 : Dose response curve for oncodazole on the inhibition of the tubulin polymerization. Each point is the mean of at least two experiments. The bars respresent extreme values.

RESULTS and DISCUSSION

As shown in Fig. 2, oncodazole inhibits the polymerization of rat brain tubulin in vitro in a dose dependent way. The estimated ID_{50} is 0.63 x 10^{-6}M which corresponds well with the ID_{50} of other microtubule inhibitors (4). Indeed, oncodazole is respectively 4 and 2 times more potent than colchicine and podophyllotoxin but it is respectively 2 and 4 times less potent than the vinca alkaloids vinblastine and vincristine. Oncodazole has no effect on preformed microtubules in vitro. Neither has colchicine even at doses up to 10^{-3}M. This is in accordance with Filner and Behnke (10) showing that even 10 mM colchicine could not disrupt stabilized microtubules.

A first attempt to measure the binding of oncodazole to purified rat brain tubulin was made by means of molecular sieving as previously used in the study of colchicine binding to tubulin (11). In contrast with those colchicine binding studies, the radioactivity of labeled oncodazole elutes with the tubulin peak immediately after a minimal incubation time at 0°C (Fig. 3). The tubulin bound radioactivity was only slightly increased after 90' incubation at 37°C. The tailing-off of the radioactivity peak in the

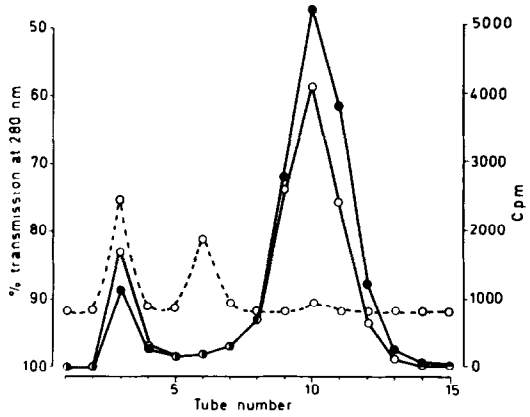

Fig. 3 : Molecular sieving on Sephadex G50 of a mixture of purified rat tubulin and oncodazole. The dotted line represents the transmission at 280 nm (the second peak is the GTP peak). Full lines indicate radioactivity (o—— o) after incubation of the mixture 90 minutes at 37°C and (●——●) immediately after mixing at 0°C.

volume towards the peak of the free drug suggests a reversible binding process.

Further binding studies were therefore done by dialysis in order to work in condition of true equilibrium. Fig. 4 a shows that the binding of oncodazole on purified rat brain tubulin is saturable and that the saturation depends on the temperature. Extrapolation of the results in a Lineweaver-Burk plot (Fig. 4b) gave the following values for the intersection of the y-axis : at 10°C 1.14, at 25°C 0.74 and at 37°C 2.62. The results at lower temperatures can be explained by the supposition that there was one binding site per mole tubulin, at higher temperature (37°C) a loss of binding sites is apparent. This is in agreement with what is known about the binding site of colchicine (11, 12, 13) as is the fact that the temperature dependency is bimodal (12) (Fig. 3a) with maximal binding at 25°C.

Evidence that oncodazole interferes with the binding site of colchicine on tubulin is shown in fig. 5 . The kinetics of colchicine binding to purified rat brain tubulin is affected in a dose-dependent way by the addition of oncodazole (5a). When the results of colchicine binding in the presence of oncodazole after 90 minutes of incubation at 37°C are expressed in a Dixon plot (14) the competitive nature of the colchicine binding inhibition is clearly demonstrated. The calculated apparent K_i value is $(0.95 \pm 0.17) \times 10^{-5}$M.

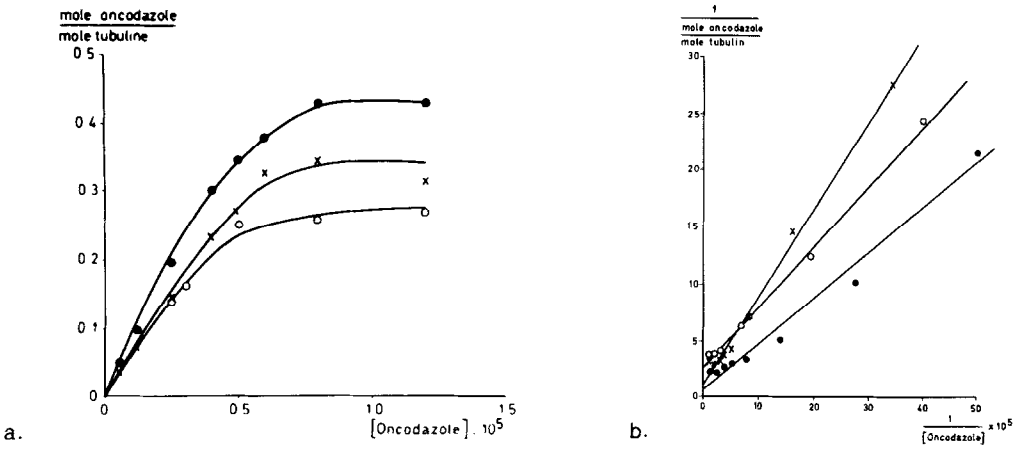

Fig. 4 : Binding of oncodazole to purified rat brain tubulin.
a) Saturation curves at three temperatures (o——o) 37°C; (●——●) 25°C and (X-X) 10°C
b) Lineweaver-Bark plot of the same points, same symbols are used.

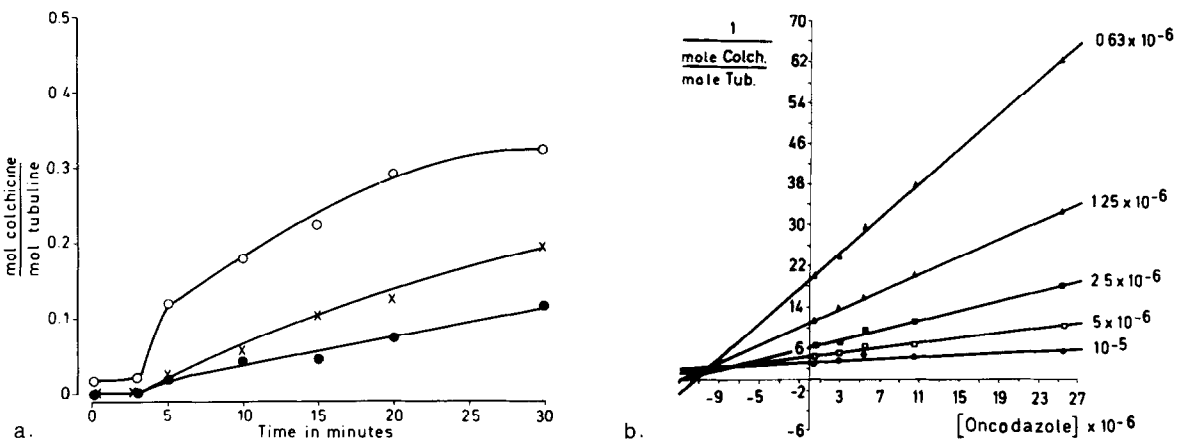

Fig. 5 : Effect of oncodazole on the binding of colchicine to purified rat brain tubulin.
a) Binding of colchicine plotted against incubation time at 37°C (o——o) in the absence of oncodazole (X-X) in the presence of 10^{-5}M oncodazole, (●——●) in the presence of 2.5×10^{-5}M oncodazole.
b) Dixon plot of colchicine binding in presence of oncodazole after 20 minutes incubation at 37°C.

From the results discussed above, the following conclusions may be drawn : first that the antimitotic activity of oncodazole can be explained

by its direct interference with the microtubule assembly process; secondly that oncodazole, whose structure is far less complex than the known microtubular inhibitors (15) provides a new means of investigating the function of microtubules in cells and organisms.

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