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ORIGINAL ARTICLE

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Different microtubule network alterations induced by pachymatismin, a new marine glycoprotein, on two prostatic cell lines

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Abstract Pachymatismin is a new cytostatic factor extracted from the marine sponge *Pachymatisma johnstonii* Bowerbank. To investigate the mechanism of action of pachymatismin, we studied its effects on two human prostate cell lines (DU145 and E4) of tumor origin. Immunocytochemistry demonstrated that the drug caused depolymerization of microtubules in DU145 cells, this effect being similar to that of estramustine, known to be a microtubule-depolymerizing agent. E4 cells, described to be resistant to the microtubule-depolymerizing agent estramustine, were also found resistant to pachymatismin. Pachymatismin at the same dose that destroys microtubule organization in DU145 cells is not able to induce microtubule depolymerization in E4 cells. Compared to the estramustine- and pachymatismin-sensitive DU145 cells, E4 cells revealed an increase of βI + II, βIII, βIV isotypes as well as post-translational modifications of tubulin, such as polyglutamylation and acetylation. In addition, the level of tau protein was also enhanced in E4 cells compared to DU145 cells. The effects of pachymatismin were tested in vitro using calf brain microtubules. It was shown that the drug lowers the capacity of microtubules to reassemble in vitro. Interestingly, pachymatismin has been found to inhibit microtubule assembly less efficiently when the ratio of tau to tubulin is increased. Taken together, pachymastismin has been shown to induce in vivo microtubule depolymerization following binding to microtubule proteins. Changes in microtubule components such as tubulin isoforms or tau may be involved in a decrease of sensitivity to pachymatismin.

Key words Prostate cells · Pachymatismin · Microtubules

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Introduction

Among marine invertebrates, sponges have proved to be a rich source of pharmacological substances and for some of them various degrees of antitumor activity have been described [7, 26]. Pachymatismin, a novel glycoprotein, extracted from the marine sponge *Pachymatisma johnstonii* Bowerbank 1842, has been demonstrated to have an antiproliferative effect both in vitro [29] and in vivo [30], against a human non-small-cell line carcinoma (NSCLC-N6). According to the authors, the drug causes a cytostatic effect by inducing atypical terminal cellular differentiation. Hoping to use this drug for treatment of other solid tumors that are poorly sensitive to chemotherapy, we investigated the effects of pachymatismin on the growth of two prostate cell lines, DU145 which is very sensitive to the anti-microtubule drug estramustine [18, 23], an antimicrotubule drug used in the treatment of hormone-refractory advanced prostate cancer, and E4, a variant of DU145, which is poorly sensitive to this drug [18, 22].

In this study we demonstrated that the antiproliferative effect of pachymatismin on DU145 and E4 prostate cells is the result of its antimicrotubule effect. Microtubules are long polymers formed by the energy-dependent assembly of heterodimer subunits composed of α and β tubulins. Microtubules are present in all eukaryotic cells and are involved in a variety of cell functions. They play critical roles in mitosis, intracellular transport, cell motility, secretion and maintenance of cell polarity. It becomes increasingly clear, however, that the interactions with various specific binding proteins are of foremost

importance for microtubules to establish and perform their functions. A large and rapidly increasing number of such proteins has been identified, collectively named as "microtubule-associated proteins" (MAPs). Structure MAPs comprise a family of molecules defined by their ability to bind to tubulin and promote microtubule assembly in vitro [15]. In mammalian brain, a source rich in microtubule proteins, two groups of fibrous MAPs were initially distinguished: one included proteins of an apparent M_r of ~60 K, termed tau [27], and the other polypeptides of high M_r (~300 K), referred to as MAP1 and MAP2 [20]. The presence of these two brain MAPs has been described in cancerous cell lines and tumors [19, 25]. Another MAP of high molecular weight referred as MAP4 has also been found in numerous cell lines [16]. We examined the effect of pachymatismin on two human prostatic cell lines previously characterized by marked differences in their microtubule components [18, 19]. We found that these differences might explain the difference of sensitivity of prostatic cell lines to estramustine. In this report we established that the level of pachymatismin activity on two prostatic cell lines is also dependent on microtubule composition. In addition, analysis of pachymatismin activity on different classes of in vitro reconstituted calf brain microtubules strengthened the hypothesis of the relationship between the antimicrotubule drug activity level and the microtubule composition.

Material and methods

Cell culture

The human prostatic carcinoma cell line (DU145) and the estramustine-resistant cell line (E4) were kindly obtained from Dr. K. D. Tew (Fox Chase Cancer Center, Philadelphia). Cells were cultured in complete media (Dulbecco's Modified Eagle Media supplemented with 4 mM glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 10% fetal bovine serum) and incubated at 37 °C in humidified, 5% CO_2 atmosphere.

Pachymatismin

The glycoprotein was purified to apparent homogeneity by anion exchange chromatography and two types of gel filtration, as previously reported [28]. The apparent molecular mass of the isolated substance was 46 kDa on a Sephacryl S-200HR column and SDS-PAGE. Pachymatismin was lyophilized and stored at −80 °C until use.

Antibodies

Anti-α tubulin monoclonal antibody was purchased from Amersham (N356, Amersham, UK). Monoclonal anti-βI + II, anti-βIII, anti-βIV tubulin isotypes and anti-acetylated tubulin were obtained from Sigma (St. Louis, Mo.). Anti-polyglutamylated tubulin monoclonal antibody, GT335, was kindly obtained from Dr. P. Denoulet (Collège de France, Paris). Polyclonal anti-tau and anti-MAP2 antibodies were prepared by our group [2, 24].

Immunocytochemistry

The cultured cells grown in monolayer in eight-well Lab-tek chamber slides were treated with pachymatismin for 2 h. Following drug treatment, cells were fixed in cold methanol. After incubation with anti-α tubulin (Amersham) at 4 °C overnight, cells were washed several times with PBS and incubated with FITC-labeled goat anti-mouse IgG (Amersham) for 30 min at room temperature. Following several rinses with PBS, coverslips were mounted using glycerol (Dako, Glostrup, Denmark). Images were acquired by a tri-CCD camera (LH750 RC3, Lhesa Electronic System, Rungis, France).

Cytotoxicity assays

A total of 2.5×10^3 cells were plated in 96-well microtiter culture plates for 96 h at 37 °C. The quantification of surviving cells in the presence of increasing concentrations of pachymatismin was determined by measurement of the endogenous enzyme hexosaminidase [10], and compared to untreated cells. Experiments were performed in triplicate, and the IC_{50}, the concentration inhibiting 50% cell growth, was determined. The average of percentage cell survival was calculated according to an Excel software program.

Protein electrophoresis and immunodetection

Samples were prepared by lysing exponentially growing cells in RIPA buffer consisting of 10 mM TRIS-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP40 and 1% sodium deoxycholate in the presence of protease inhibitors. Lysates were centrifuged at 100,000 *g* for 40 min. The supernatant was removed and protein content determined by BCA protein assay (Pierce, Rockford, Ill.). Equivalent amounts of protein from each sample were electrophoresed on 10% gradient SDS-PAGE. Rainbow molecular weight markers from Amersham were used for determination of molecular weights. Proteins were transferred onto nitrocellulose membranes and Western blots were done using anti-tubulin, anti-tau or anti-MAP2 antibodies, followed by a secondary anti-mouse IgG peroxidase conjugate and visualized by enhanced chemiluminescence (ECL, Amersham). All reactions in our experiments were expressed in the linear range.

Preparation of microtubule proteins

Microtubules were prepared from calf brain by a temperature-dependent in vitro assembly-disassembly procedure described by Shelanski et al. [20] and slightly modified [5]. Tubulin was purified by the method of Weingarten et al. [27], except that the cation exchanger Fractogel EMD SO3–650 (M) from Merck was used instead of the Phosphocellulose p11 from Whatman. Microtubules and tubulin were prepared using the buffered solution A, containing 0.1 M MES, 1 mM EGTA, 0.1 mM EDTA, 1 mM $MgCl_2$, 1 mM GTP, 1 mM 2-mercaptoethanol, 1 mM PMSF. Tau and MAP2 proteins were purified from calf brains by the microtubule thermodenaturation procedure and column gel filtration as described by Fellous et al. [5].

Assay of microtubule assembly

Tubulin and MAPs were incubated at 37 °C in buffer A. Microtubule assembly was measured by monitoring the changes in turbidity. Experiments to measure the rate and extent of tubulin assembly were performed in an UVICON spectrophotometer equipped with an automatic thermostated six-sample changer connected to a circulating water bath set at 37 °C or 4 °C. The optical density was determined at 345 nm every 30 s of the incubation period.

Results

Dose-dependent cytotoxicity of pachymatismin

To examine the effect of drug on microtubules in cells, DU145 were incubated with different concentrations of pachymatismin for a short period of time. Pac-

hymatismin induced a dose-dependent disassembly of microtubules. In control cells (Fig. 1a), microtubules radiated from a microtubule-organizing center and extended to the cell periphery. At 4 μM pachymatismin, the most peripheral microtubules were retracted (Fig. 1c). At 8 μM pachymatismin, a progressive depolymerization of microtubules was observed (Fig. 1e). A complete depolymerization of the microtubule network occurred at 16 μM pachymatismin (Fig. 1g). Only the microtubule-organizing center remained apparently not altered by the drug (Fig. 1g).

We further examined the mechanism of resistance to pachymatismin, by analyzing its effects on microtubules in an estramustine-resistant cell line (E4). Since estramustine is known to have the capacity to induce microtubule depolymerization in vivo [3, 18], we wished to compare the effect of pachymatismin to estramustine whose effect on microtubules and mechanism of resistance are partially understood. E4 cells were treated with increasing concentrations of pachymatismin under the same conditions as those used for DU145 cells. The microtubule network appeared well organized in E4 cells treated with 4, 8 and even 16 μM (Fig. 1b, d, f, h). The similar effects of estramustine on DU145 and E4 cell microtubule networks reported in our previous study and those of pachymatismin on the same cells in the present report suggest a similar mode of action of the two drugs.

Furthermore, the mechanism of resistance to pachymatismin appeared similar with that of estramustine. To determine the cytotoxicity of pachymatismin, DU145 and E4 cells were incubated with increasing concentrations of pachymatismin for 96 h. E4 cells presented a fourfold resistance to pachymatismin compared to DU145 cells with IC_{50} values of 0.1 ± 0.001 μM and 0.49 ± 0.022 μM, respectively (Fig. 2). The dose effect

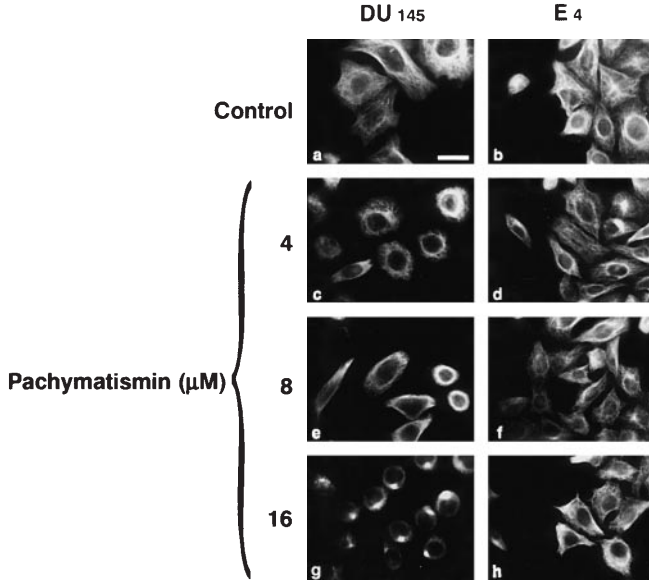
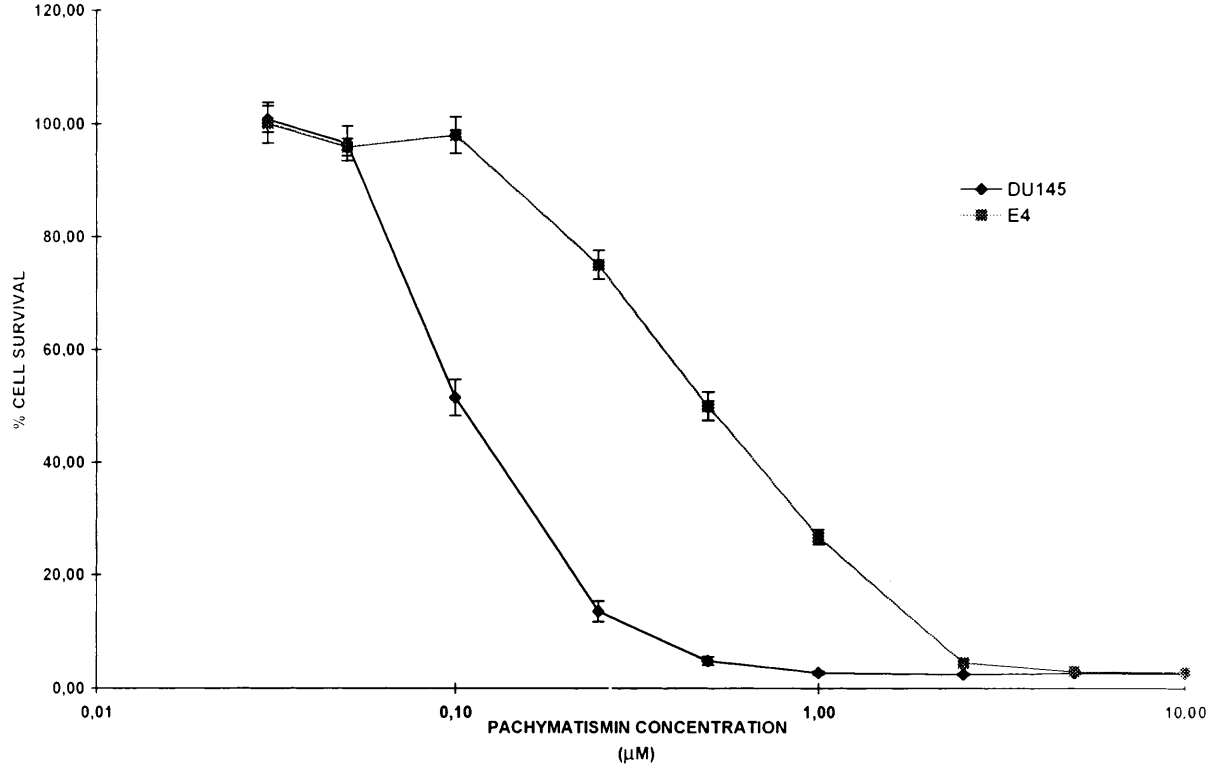


Fig. 1a–h Depolymerization of microtubules in DU145 and E4 cells. **a** Control DU145 cells; **b** control E4 cells; **c**, **e**, **g** DU145 cells treated with 4, 8, 16 μM pachymatismin, respectively; **d**, **f**, **h** E4 cells treated with the same drug concentrations. *Bar* 25 μm

Fig. 2 Dose response of DU145 and E4 cell line upon treatment with pachymatismin. Cells were plated onto 96-well plates, treated with various concentrations of the drug



represents the average of percentage cell survival of three independent experiments.

Immunodetection of microtubule components in DU145 and E4 prostate cells

Analysis of tubulin content in prostatic cells (Fig. 3A-a–e) confirmed the increase in the accumulation of βI + II, and βIII isotypes and the increase of polyglutamylation and acetylation of α tubulin [18]. In addition, we demonstrated that isotype βIV was also significantly increased in E4 cells (Fig. 3Ac). Since most microtubules in vitro are likely to be associated with MAPs, we analyzed tau and MAP2 in these two cell lines. We previously demonstrated tau overexpression in E4 cells [19]. With anti-tau antibody, we confirmed this observation (Fig. 3Ba, b). On analyzing DU145 and E4 cell extracts with a polyclonal antibody specifically directed against brain MAP2, polypeptides immunologically related to MAP2 were found as proteolytic fragments (Fig. 3Bc, d). The two cell extracts contained similar amounts of the different MAP2-related polypeptides, except that the high molecular weight MAP2 fragments was more abundant in the resistant cell line and the low molecular weight fragment was more abundant in the sensitive cell line, suggesting a higher proteolytic activity in this cell line.

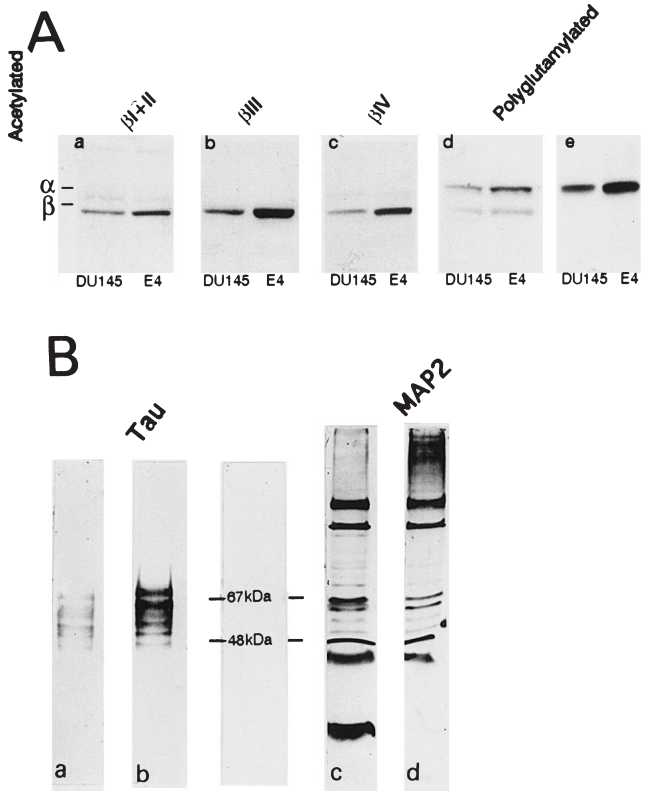


Fig. 3 **A** Tubulin isoform content in DU145 and E4. Cell lysates from log phase cells were applied to 8% polyacrylamide gels and immunostaining was performed by tubulin isoform-specific antibodies. *a* βI + II isotype; *b* βIII isotype; *c* βIV isotype; *d* polyglutamylated-tubulin; *e* acetylated-tubulin. **B** Tau and MAP2 protein revealed by using an anti-tau polyclonal antibody in DU145 (*a*) and E4 (*b*) cells and an anti-MAP2 polyclonal antibody in DU145 (*c*) and E4 (*d*) cells (*MAP2* microtubule-associated protein 2)

The effect of pachymatismin on microtubules reconstituted in vitro

To confirm the effect of pachymastismin on microtubules, in vitro experiments were performed using calf brain microtubules. Microtubules prepared according to Weisenberg's procedure are copolymers of tubulin and MAPs. Monitoring of the kinetic assembly of these microtubules showed that pachymatismin partially inhibited microtubules assembly. The percentage of inhibition at 30 min was approximately 25% (Fig. 4). To check further whether tubulin isotypes or MAPs mediate the antimicrotubule role of pachymatismin, the effect of the drug on in vitro assembly of microtubules reconstituted from tubulin and MAPs was explored. Tubulin isotype heterogeneity was not, however, investigated since the purification of each isotype of tubulin requires a complex procedure with very high quantities of antibodies. The effect of MAPs on pachymatismin activity can be checked more easily. Our investigation focused on a major set of MAPs, tau protein and the high molecular weight MAP2. Microtubule assembly in the presence of pachymatismin was investigated using various MAP/tubulin ratios. Pachymatismin (0.9 μM) induced an inhibition of microtubule assembly reconstituted from tubulin and a low concentration of tau (tau:tubulin ratio 0.07:10 μM) (Fig. 5A). However, pachymatismin failed to inhibit the microtubule assembly

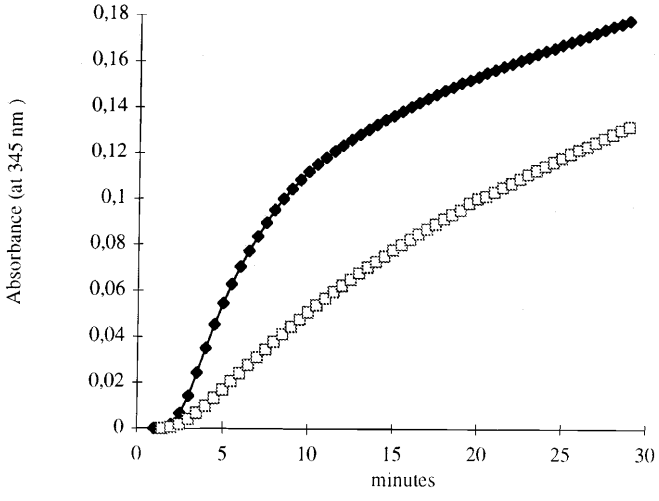


Fig. 4 Effects of pachymatismin on assembly of microtubules extracted from calf brains. Microtubules (1 mg/ml) isolated from calf brain were incubated at 37 °C in absence (◆) or presence (□) of 0.9 μM pachymastismin. The evolution of microtubule assembly was monitored by measuring light absorbance at 345 nm

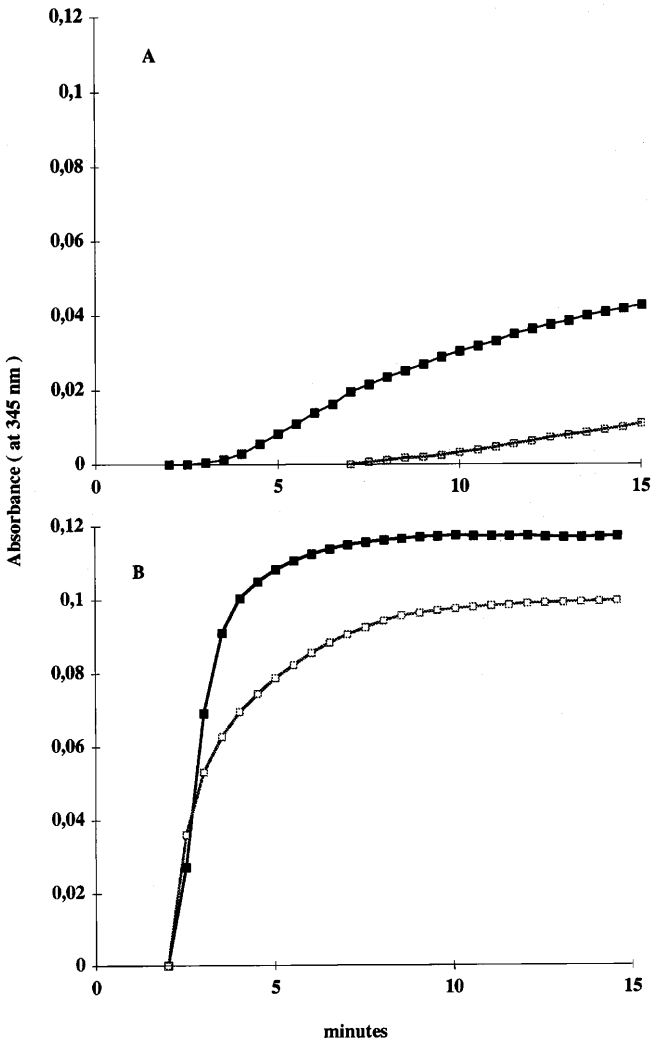


Fig. 5A, B Effect of tau:tubulin ratio on pachymatismin-induced tubulin assembly inhibition. Purified tubulin (1 mg/ml) was incubated at 37 °C in the presence of either 14 μg/ml (**A**) or 42 μg/ml (**B**) of tau in absence (■) or presence (□) of 0.9 μM pachymatismin

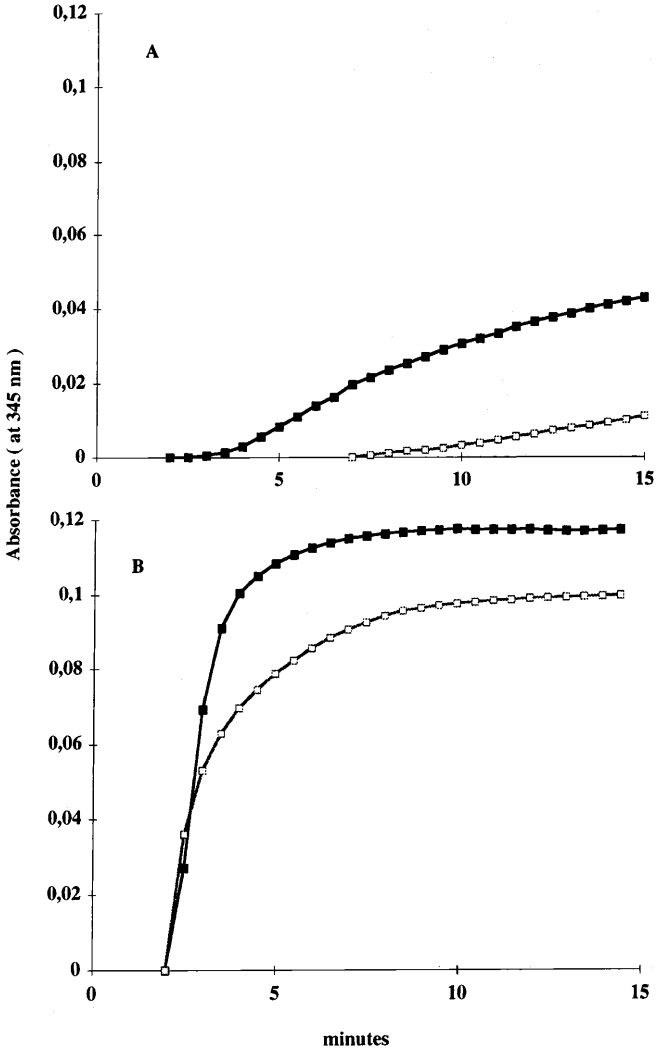


Fig. 6 Effect of tau:tubulin ratio on estramustine-phosphate-induced tubulin assembly inhibition. Purified tubulin (1 mg/ml) was incubated at 37 °C in the presence of either 14 μg/ml (**A**) or 65 μg/ml (**B**) of tau in absence (■) or presence (□) of 100 μM of estramustine phosphate

(Fig. 5B) when microtubules were reconstituted from tubulin and a higher concentration of tau (tau:tubulin ratio 0.21:10 μM). A similar experiment performed with estramustine phosphate instead of pachymatismin demonstrated that both drugs had a very close mode of action (Fig. 6). No difference in the effect of pachymatismin was observed on microtubules reconstituted from tubulin and MAP2 (data not shown), at least within the concentration range of MAP2-used. Thus, this result would suggest that tau concentration appears to be a very sensitive modulator of pachymatismin antimicrotubule activity.

Discussion

Zidane et al. [28] reported that pachymatismin induces a blockade at the G_0/G_1 and the G_2/M phase of the cell cycle in the non-small cell bronchopulmonary carcinoma line. The blockade at G_0/G_1 suggests a mechanism of terminal differentiation. The idea of 'differentiation chemotherapy' has been proposed. In this case, the effect of pachymatismin appears to be close to the effect of taxotere when this drug inhibits the proliferation and induces the differentiation of HL-60 and K562 human myeloid leukemia cells [13]. It presents also some similarity with retinoic acid-induced differentiation [8] or with platinum complex D17872-induced differentiation [14]. However, this induction of differentiation appears to occur under very precise conditions. It might be highly dependent on the drug concentration and also probably on the cells themselves, varying according to cell type, cell cycle stage or cell culture conditions. The blockade at the G2/M phase suggests a different mechanism of pachymatismin action which involves an antimicrotubule and cytotoxic effect responsible of prostate

cell growth inhibition. Microtubules are becoming an increasingly important target in cancer chemotherapy. In the prostate cell line DU145, the cytotoxicity of pachymatismin has been linked to its capacity to interact with microtubules, causing cytoskeletal dysfunction and microtubule depolymerization, as described in the same cell line for estramustine treatment. In E4 cells, pachymatismin, even at the high concentration of 16 μM failed to induce microtubule depolymerization. In contrast, in DU145 cells, the microtubule network was completely depolymerized at this concentration of 16 μM (Fig. 1). Similar observations have been reported in E4 cells resistant to estramustine [17]. This suggests that pachymatismin has a mode of action and a mechanism of resistance both similar to those described for estramustine.

Compared to DU145 cells, E4 showed an increase in the accumulation of βI + II, βIII, βIV, polyglutamylated and acetylated tubulin as well as tau protein (Fig. 3). In our previous study, we reported that resistance to estramustine was associated with modified pattern of tubulin expression [18]. Thus, the mechanism of resistance to pachymatismin could be explained with the same hypothesis. Although it is not well established how microtubules, tubulin isotypes and MAPs composition affect drug sensitivity, several in vitro studies have demonstrated the role of microtubules heterogeneity on drug action. For example, microtubules composed of αβIII and αβIV isotypes were four times less sensitive to inhibition of microtubules dynamic by taxol [4]. Lu and Luduena [12] have shown that microtubules assembled from βIII-depleted tubulin were shorter and more resistant to podophyllotoxin and colchicine compared with microtubules from unfractionated tubulin. In this study, microtubules enriched in βIII may have a lower affinity for pachymatismin. It has been demonstrated by others authors that βIII binds poorly estramustine [9]. It has also been shown that the post-translational modifications of tubulin such as polyglutamylation and acetylation have a role on microtubule stability and dynamics and on drug binding, so that they may have a role on drug action. In a previous study we also reported that estramustine does not bind to the most acidic post-translationally modified forms of α tubulin [18]. Thus, a cell might be more or less sensitive to estramustine according to its content in tubulin isotypes. It is possible to propose the same hypothesis for pachymatismin. Using MAP-depleted tubulin, we demonstrated that tubulin is the target of pachymatismin. The drug strongly inhibited the formation of microtubules promoted by MAP-depleted tubulin and 16 μM Mg^{2+} (data not shown).

In this report we have concentrated on the role of MAPs in drug activity. Tau is normally "specifically" expressed in neurons, where it represents a marker of differentiation. We previously reported that tau was shown to be expressed in the two prostate cancer cell lines DU145 and E4 [19], but at a higher level in E4 cells. The function of tau is generally thought to involve microtubule stabilization. The binding of tau to microtubules could well be regulated by post-translational modifications of tubulin. It has been shown that the post-translational polyglutamylation of tubulin could regulate the binding of tau and MAP2 as a function of chain length [1]. Lee and Rook [11] demonstrated that the presence of tau correlated with acetylation of all cellular microtubules, indicating enhanced stability for microtubules. The significant elongation of the polyglutamyl chain of tubulin in E4 cells could increase their affinity for MAPs and, thus, increase microtubule stability [18]. In a previous report [25], we suggested that tau-stabilized microtubules might be responsible of the higher sensitivity of tumor cells to taxotere. Alternatively, tau-stabilized microtubules may be less sensitive to depolymerization by pachymastismin. The results from in vitro studies showed that pachymatismin and estramustine phosphate were less efficient in inhibiting microtubule assembly when the tau:tubulin ratio is increased. This is in agreement with the fact that tau is more abundant in E4 than in DU145 cells, and also with the fact that E4 cells are more resistant to both pachymatismin and estramustine phosphate than DU145 cells.

We have not been able to demonstrate that MAP2 also regulates the level of pachymatismin activity. MAP2 is known to stabilize microtubules less efficiently than tau. However, we cannot exclude that MAP2, under other conditions, may also interfere with pachymatismin activity. For example, proteolytic fragments of MAP2 may have properties different from those of non-degraded MAP2, as suggested previously [6]. It is noteworthy that, in DU145 cells, MAP2-like proteins were more proteolytically degraded, with a major fragment of about 25 kDa. Some low molecular weight MAP2 fragments may have an effect on pachymatismin affinity to tubulin in DU145 cells.

In conclusion, the present study demonstrates that pachymatismin is able to cause depolymerization of microtubules in vitro and in living cells, and that also the drug resistance is associated with changes in microtubule composition that may alter both the stability of microtubules and their capacity to bind the drug.

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