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The compound 14-keto-stypodiol diacetate from the algae *Stypopodium flabelliforme* inhibits microtubules and cell proliferation in DU-145 human prostatic cells

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Abstract

We investigated the effects of the drug 14-keto-stypodiol diacetate (SDA) extracted from the seaweed product *Stypopodium flabelliforme*, in inhibiting the cell growth and tumor invasive behavior of DU-145 human prostate cells. In addition, the molecular action of the drug on microtubule assembly was analyzed. The effects of this diterpenoid drug in cell proliferation of DU-145 tumor cells in culture revealed that SDA at concentrations of 5 μM decreased cell growth by 14%, while at 45 μM a 61% decrease was found, as compared with control cells incubated with the solvent but in the absence of the drug. To study their effects on the cell cycle, DU-145 cells were incubated with increasing concentrations of SDA and the distribution of cell-cycle stages was analyzed by flow cytometry. Interestingly, the data showed that 14-keto-stypodiol diacetate dramatically increased the proportion of cells in the G2/M phases, and decreased the number of cells at the S phase of mitosis, as compared with appropriate controls. Studies on their action on the *in vitro* assembly of microtubules using purified brain tubulin, showed that SDA delayed the lag period associated to nucleation events during assembly, and decreased significantly the extent of polymerization. The studies suggest that this novel derivative from a marine natural product induces mitotic arrest of tumor cells, an effect that could be associated to alterations in the normal microtubule assembly process. On the other hand, a salient feature of this compound is that it affected protease secretion and the *in vitro* invasive capacity, both properties of cells from metastases. The secretion of plasminogen activator (u-PA) and the capacity of DU-145 cells to migrate through a Matrigel-coated membrane were significantly inhibited in the presence of micromolar concentrations of SDA. These results provide new keys to analyze the functional relationships between protease secretion, invasive behavior of tumor cells and the microtubule network. (Mol Cell Biochem **187**: 191–199, 1998)

Key words: *Stypopodium flabelliforme*, 14-keto-stypodiol diacetate, DU-145 cell line, microtubule assembly, cell proliferation, plasminogen activator (u-PA), tumor invasiveness

Introduction

Microtubules, major components of the cytoskeletal network, appear to be involved in multiple cellular functions. In interphase cells, microtubules contribute to determining the cell architecture, but also participate in the intracellular transport of organelles, secretion and cell motility. Microtubules are also involved in morphogenetic events during early development

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and cell differentiation [1]. In dividing cells, microtubules are an integral part of the mitotic spindle and participate in the main cellular events that determine the segregation of chromosomes toward the cell poles during mitosis. A set of finely coordinated molecular signals regulate microtubule assembly and its dynamics in both interphase and mitotic cells. Microtubule-associated proteins are among the main modulatory systems involved in controlling microtubule assembly and organization as related to the different roles of these cytoskeletal polymers [2].

Most of the antimitotic drugs, derived from natural sources interact with the major components of microtubules [3, 4]. Within this context, an increasing family of anti-microtubule drugs have been reported, and their actions on microtubule assembly seriously perturb the cellular functions in which microtubules are implicated [5, 6]. Several of these compounds, including colchicine, nocodazole and the vinca alkaloids, that have tubulin as a molecular target, inhibit microtubule assembly and arrest mitosis. Other mitosis-arresting drugs like estramustine and its phosphorylated derivative appear to exert their inhibitory actions on microtubules by interacting with tubulin and with MAPs [7, 8]. On the other hand, there is a group of drugs of the class of taxanes, that includes taxol [9] and its analogs, and the newly reported anti-mitotic agent discodermolide [4] that associates with the tubulin molecule, blocking mitosis by a different mechanism since they promote the uncontrolled assembly of microtubules. Microtubules assembled in the presence of the latter drugs, and other anti-mitotic compounds, are significantly less dynamic [10] and insensitive to cold depolymerization.

Taking into account that the integrity of the microtubule network is critical for maintenance of the cellular architecture and the intracellular transport, a group of drugs that inhibit the transport of tumor proteases as well as *in vitro* invasive behavior have been the subject of our previous studies [11, 12]. On the other hand, an interesting compound, stypoldione, was reported some years ago to inhibit microtubule assembly in the sea urchin and affect its assembly dynamics [13]. That compound also reacted with sulfhydryl groups in the tubulin molecule [14]. The derivative 14-keto-stypodiol diacetate (SDA), whose structure is shown in Fig. 1 is the basis for the present investigation. This compound was obtained by extraction from the algae *Stypopodium flabelliforme*, and its anti-inflammatory action was described in an earlier report [15]. In this report we analyze the effects of SDA on DU-145 cell proliferation as well as the effects on the cell's invasive behavior and its capacity to secret plasminogen activator (u-PA). The studies pointed to an antiproliferative activity of SDA and an antimitotic activity. SDA also exerted an ability to disrupt the invasive capacity of DU-145 cells and their secretion of urokinase-like plasminogen activator (u-PA). These actions were correlated at the molecular level with its inhibitory action on the assembly of microtubules *in vitro*. The present studies provide information toward the elucidation of the mechanisms of action of SDA and other stypoldione derivatives.

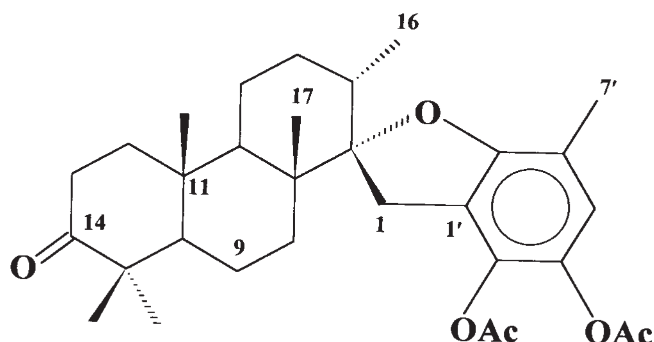

Fig. 1. Structure of 14-keto-stypodiol diacetate (SDA).

Materials and methods

Materials

Sucrose, dimethylsulfoxide, GTP type II-S, EGTA, Mes and the protease inhibitors aprotinin, soybean trypsin inhibitor and PMSF were from Sigma Chemical Co. (St. Louis, MO, USA). The monoclonal anti-β-tubulin antibody and the material for the culture media were also obtained from Sigma. The Matrigel Transwell system was from Costar. Fetal calf serum was from Gibco (Gaithersburg, MD). Culture dishes and material were from Corning (New York, USA). Phosphocellulose chomatographic grade was from Whatman (Maidstone, UK). All the other reagents and organic solvents used in the study were of the highest analytical quality.

Extraction of the compound 14-keto-stypodiol diacetate [16]

This natural product was isolated in the acetate form from the algae *Stipopodium flabelliforme*, obtained from the Easter Island located off the Pacific coast of Chile. An scheme of the extraction protocol is shown in Fig. 2. The compound was analyzed by nuclear magnetic resonance, and the purified product was used in the biological studies after solubilization in dimethylsulfoxide.

Protein purification

Bovine brain microtubular proteins were isolated by three temperature-dependent cycles of assembly-disassembly

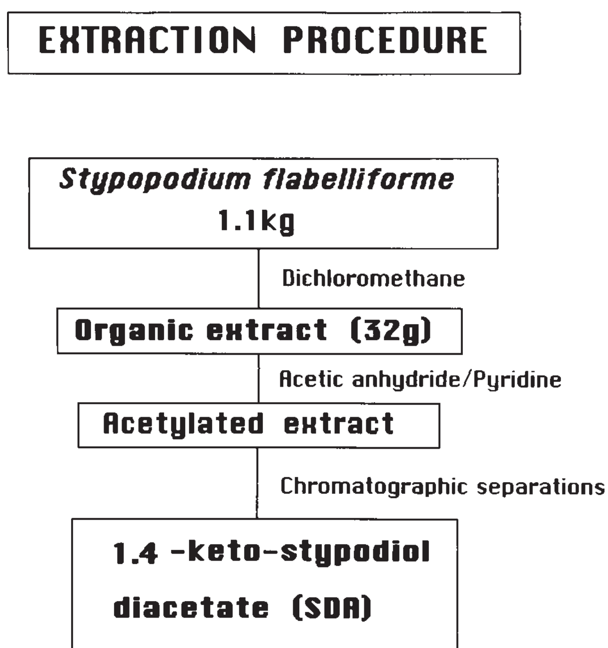

Fig. 2. Schematic representation of the extraction procedure for 14-ketostypodiol diacetate.

according with the procedure of Shelanski *et al.* [17]. The three-cycled microtubular protein preparation was re-suspended in buffer 0.02 M Mes (pH 6.5), 0.1 mM EDTA, 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM dithiothreitol and used directly in the assembly experiments, or as a source to purify tubulin by phosphocellulose chromatography [18]. This tubulin preparation (PC-tubulin) was used in the experiments where purified tubulin was required.

Microtubule assembly assays

Brain microtubular protein (~88% tubulin/12% MAPs) capable of polymerizing *in vitro* was obtained after three cycles of assembly and disassembly. This protein (1.4 mg/ml) was adjusted to 0.1 M Mes (pH 6.8), 1 mM Mg^{2+}, 1 mM EGTA (buffer A) and preincubated for 10 min at 30°C in the absence (control) or presence of increasing concentrations of the 14-keto-stypodiol diacetate. Microtubule assembly was triggered by addition of GTP into the assembly system, and incubated at 37°C. The assembly was monitored turbidimetrically by following the change of absorbance at 340 nm [19].

Cell lines

The cellular studies on the effects of SDA were carried out in the carcinoma cell line, DU-145 derived from a prostatic brain metastasis [20]. The cells were grown in 150 mm culture plates (Falcon) in Dulbecco's-modified Eagles's medium (DMEM) supplemented with 10% fetal calf serum and the antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml), in a humid atmosphere at 37°C with 5% CO_2 in air. The culture medium was changed every 2 days until confluent or subconfluent cultures were obtained [21].

Cell growth inhibition studies

DU-145 cells (3×10^5) were seeded onto 10-well plates, and allowed to attach to the plates for 16 h. The medium was then removed and the cells were washed with PBS and incubated for 24 h in the presence or absence of varying concentrations of SDA (5–50 µM) in culture medium enriched with 10% fetal bovine serum. After that period, the cells were trypsinized, the viable cells were determined on the basis of the trypan blue exclusion procedure, and cell's number was plotted against the drug concentration. Additional determinations on the cytotoxic effects of SDA on these cells were performed by assessing the lactic dehydrogenase release from the cells within the same range of drug concentrations (5–60 µM SDA). In order to analyze the growth of cells continuously exposed to drug, different measurements were made on the basis of 3 experiments on each of the incubation conditions.

Flow cytometry experiments

Plates of DU-145 cells were grown in complete medium prior to treatment with the drug. Cells in log phase were seeded in plates at 3×10^5 cells per well, in a volume of 2 ml of Dulbecco's medium plus serum and allowed to attach for 24 h. Then the medium was removed and the cells were washed with phosphate buffered saline (PBS) and incubated for 48 h in medium plus serum in the absence (control) or in the presence of increasing amounts of SDA (5–30 µM) added to the cell medium. To analyze the distribution of the different cell cycle stages, three different flow cytometry measurements in a Becton-Dickinson instrument model Fax Scan were made for each experimental condition. Emission wavelength was 488 nm [22]. The data were analyzed in terms of the fraction of cells in each of the cell cycle stages for the control in the absence of the drug and cells incubated with variable drug concentrations.

u-PA secretion assays

Cells (5×10^5) were seeded onto 24-well plates for 24 h in a medium containing 10% fetal calf serum. The cell monolayer was washed two times with the medium, and incubated for an additional 24 h period with serum-free medium and

varying concentrations of SDA. At the end of the treatment, cells were washed with medium and incubated without serum in order to obtain a conditioned medium. The number of cells in both control and SDA-treated plates were counted, the medium was collected and the secreted u-PA activity was assayed by the radial caseinolytic assay [12]. In order to analyze the direct effects on protease secretion, aliquots of the conditioned media were normalized in relation to the number of cells after the SDA treatment (size of aliquots of conditioned medium were normalized to 1×10^5 cells). For the caseionolytic assay, a solution of 1% agarose and 0.5% casein in 50 mM Tris, pH 8.0, maintained a 45°C, was mixed with 20 µM plasminogen as a u-PA substrate, and 20 µM $CaCl_2$ to induce casein precipitation. The mixture was then applied to a plastic surface to allow formation of an immobilized protein coat, and finally allowed to cool to room temperature. Aliquots of u-PA containing conditioned medium, normalized with respect to number of viable cells at the end of drug treatment, were applied to the holes previously punched in the gel and incubated at 37°C for 16–24 h. As a control, plates lacking plasminogen were treated and assayed as indicated. At the end of the assay period, a solution of 0.3 M $CuSO_4$ was added to stop the reaction. To determine the relative enzyme activity, the diameters of the radial zones of caseinolysis were determined by using a micrometric Vernier instrument, and the data used to calculate the lysis area.

Invasion assays

In this assay, invasiveness was defined as the ability of the cells to penetrate an 8 µm pore polycarbonate membrane in a Transwell chamber (Costar, Cambridge MA, USA) coated with 30 µg of Matrigel (Collaborative Research, Bradford MA, USA). Different experimental sets of DU-145 cells (1×10^6) were incubated in the absence or the presence of two different concentrations of SDA for 24 h in culture medium plus fetal calf serum. Aliquots containing 10^5 cells of either control or drug-treated cells were seeded in the upper chamber of the Transwell in 200 µl of medium plus 10% serum, either in the absence of the presence of 10 and 20 µM SDA. The lower chamber was filled with 700 µl of the same medium, either with or without drug. The cells attached to the Matrigel and a fraction of cells passed through the filter. After 48 h of incubation, the cell number in both chambers was assayed. For that purpose, following an incubation (4 h) with a colorigenic substrate [23], the media from both chambers were collected and the attached cells to both sides of the filter were removed with 0.05% trypsin/0.02% EDTA in PBS solution. Cells were collected, centrifuged and the pellets were treated with a mixture of DMSO/isopropanol (3:2 v/v) in order to dissolve the formazan product. Absorbance of the colorigenic product was read at 505 nm.

Immunofluorescence studies

The intracellular patterns of microtubule cytoskeleton in DU-145 cells were analyzed by immunofluorescence using monoclonal anti-β-tubulin antibody. Cells (1000 cells/10 µl) were plated on sterile cell culture slides in Dulbecco's medium plus or minus serum in a humid atmosphere at 37°C and 5% CO_2 and allowed to attach. The medium was removed and the attached cells were treated with increasing concentrations of SDA (10–40 µM) for 24 h. The subconfluent cultures maintained over glass slides were washed in phosphate buffer saline (PBS, pH 7.2) at 37°C, and the cells subjected to permeabilization in microtubule-stabilizing buffer containing 0.5% Triton X-100 for 1.5 min. Cells were fixed with methanol at –20°C for 10 min, and washed once in saline solution. Cells were blocked with PBS containing 1% bovine serum albumin (BSA) for 10 min. Then, cells were incubated with the primary anti-tubulin antibody (dilution 1:500) for 2 h at room temperature in PBS containing 1% BSA, and the unbound antibody was removed by successive washes with PBS at room temperature. The bound antibody was detected by using rhodamine-conjugated antimouse IgG (dil. 1:100). The cells were mounted over Mowiol/DABCO and observed under a Zeiss microscope with epifluorescence attachment.

Results

We found that DU-145 cells were an appropriate system for a study on the effects of SDA, considering their proliferation in culture, secretion of plasminogen activator and their usefulness for immunofluorescence studies on drug-induced changes of the cytoskeleton. The initial studies revealed the effects of increasing concentrations of SDA on the growth of this cell line. A IC_{50} value of 24.0 ± 2.7 µM was observed (Fig. 3). The analysis by flow cytometry, based on the content of DNA, revealed an accumulation of cells in the G2/M phase of mitosis, concomitant with a significant decrease in the S-phase of cells following SDA treatment (Table 1). Immuno-fluorescence observations corroborated the findings that cells accumulated in mitosis (unpublished data). Interestingly, the GO/G1 phase also increased after the treatment with the higher concentrations of the drug (Table 1). Cytotoxicity analysis revealed that no significant change in lactic dehydrogenase release occurred in cells subjected to SDA throughout a wide range of drug concentrations (Fig. 4).

As another approach for this analysis, the u-PA secretion in these cells was assessed by the caseinolytic assay, and the data analyzed by a dose-response plot. The culture of prostate cells maintained for a total period of 48 h in the presence of micromolar concentrations (2.5–10 µM) of SDA, resulted in a significant decrease in the active u-PA secreted to the

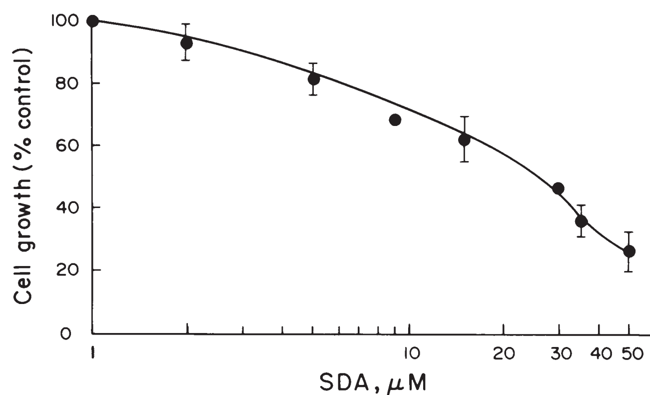

Fig. 3. Effects of increasing concentrations of SDA on DU- 145 prostate carcinoma cells. The closed circles represent the inhibition in the cells number after 24 h treatment with the drug. The experiment was repeated three times. S.E.M. represented by the vertical bars are shown.

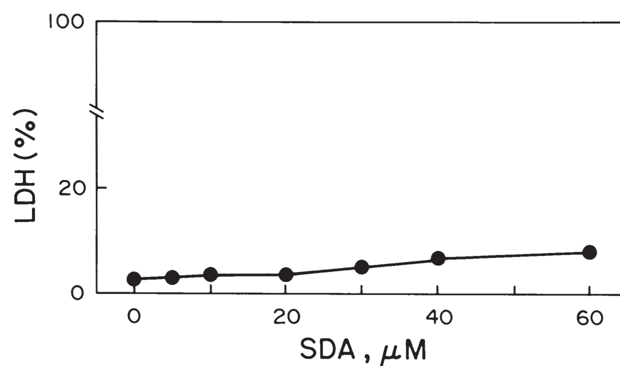

Fig. 4. Determinations of lactic dehydrogenase (LDH) release from DU-145 cells incubated with increasing concentrations of SDA, in the range from 0–60 µM. The levels of LDH were measured by enzymatic assay, and plotted as a fraction of the maximum level of LDH released under conditions of no viability (100%). The basal value obtained in the absence of SDA is also shown. Each value corresponds to averages of 3 determinations.

medium, as compared with controls cultured in the absence of drug (Fig. 5). The upper inset shows the changes in diameters of the caseinolyis areas, as related to the blockage in u-PA secretion, which result from an increase in the drug concentration. To assess whether the inhibition of u-PA secretion as well as the disruption of the cytoskeletal microtubules by SDA affected the invasive capacity of DU-145, a Matrigel-coated Transwell chamber system was used. To carry out this experiment, an evaluation of the cell growth

Table 1. SDA-induced changes in the distribution patterns of cells in the different phases of the cell cycle

SDA cone.	Phase	Av. number of cells	% of cells
Control	G0/G1	1074	15.1 ± 4.1
	S	2698	37.9 ± 3.6
	G2/M	3340	47.0 ± 2.8
Control DMSO	G0/G1	1195	16.8 ± 2.9
	S	2545	34.8 ± 4.2
	G2/M	3545	48.4 ± 1.7
5 µM SDA	G0/G1	1173	16.3 ± 3.0
	S	1619	22.5 ± 4.3
	G2/M	4405	61.2 ± 4.7
10 µM SDA	G0/G1	1610	24.0 ± 4.5
	S	740	11.0 ± 1.2
	G2/M	4372	65.0 ± 2.4
15 µM SDA	G0/G1	2095	25.7 ± 3.9
	S	591	7.3 ± 2.0
	G2/M	5458	67.0 ± 3.2
20 µM SDA	G0/G1	2193	26.3 ± 0.9
	S	674	8.1 ± 3.1
	G2/M	5468	65.6 ± 1.8

[1]The S.D. for each set of measurements are derived from the normal distribution of cells population. The average number of cells was obtained from three determinations for every cell stage, and for each experimental condition, under the same setting conditions of the flow cytometer.

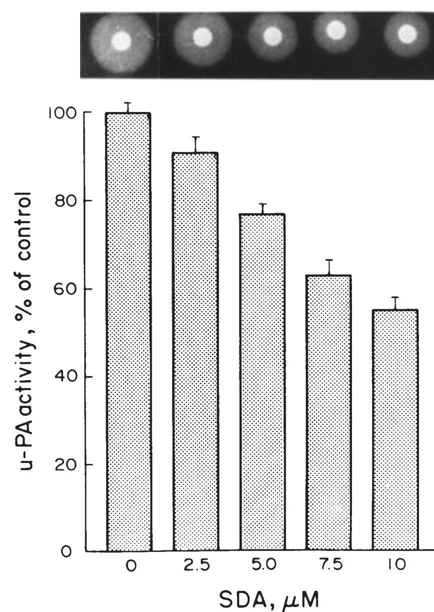

Fig. 5. Inhibition of the protease activity of plasminogen activator (u-PA) by increasing concentrations of 14-keto-stypodiol diacetate, as a percentage of control of cells incubated in the absence of the drug. The upper part shows the picture of caseinolysis zones after the treatment with equivalent amounts of the conditioned media obtained from cells incubated with variable concentrations of SDA. The S.E. corresponding to each set of data are shown (n = 3). The differences with respect to controls are significant ($p < 0.005$).

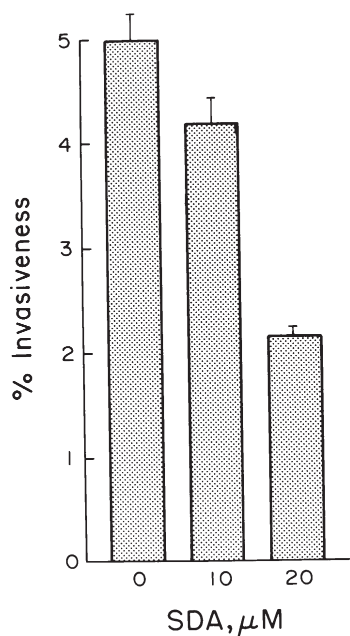

Fig. 6. Inhibition of the capacity of DU-145 prostate carcinoma cells to penetrate the reconstituted basal lamina, as a measurement of invasiveness, according to a Matrigel assay, by different concentrations of stypodiol diacetate. The average values with their respective standard errors are shown. Four sets of data ($n = 4$) for each experimental condition were obtained. The differences are highly significant ($p < 0.001$).

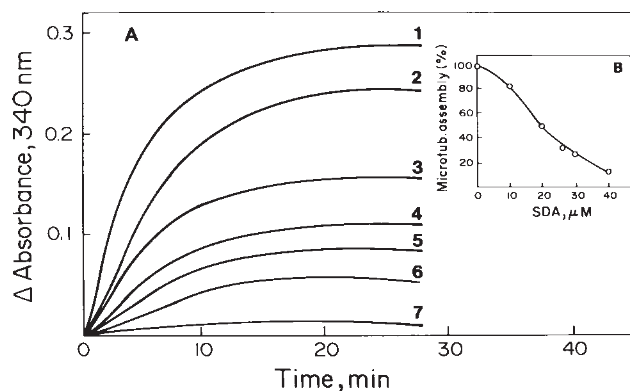

Fig. 7. Inhibition of the *in vitro* microtubule assembly by 14-keto-stypodiol diacetate at final concentrations of 10 μM (curve 2), 20 μM (curve 3), 26 μM (curve 4), 30 μM (curve 5) and 40 μM (curve 6). Three cycled microtubular protein (1.4 mg/ml) was used in all assays. A positive control in which assembly buffer was added instead of SDA (curve 1), and a control of assembly of pure PC-tubulin in the absence of MAPs (curve 7) are also shown. In the inset, the extents of microtubule assembly obtained from the assembly kinetics assays were plotted (percentage of the control) as a function of SDA concentration in the assay. Three different kinetic curves were obtained for each experimental condition ($n = 3$).

in the presence of serum was performed, determining the number of cells at the beginning and at the end of the experiment. Thus, to analyze specifically the effects of SDA on cell invasion, independently of the cell growth, experiments were carried out in the presence of 10% serum. Figure 6 shows that incubation of cells with 20 μM SDA produced around 57% decrease in the capacity of DU-145 to penetrate the reconstituted basal lamina, according to the Matrigel assay as an index of the invasive activity. A decrease of 16% in the invasive capacity was observed in the presence of 10 μM SDA.

It was important to examine the molecular target of the drug at the level of microtubule assembly and its intracellular organization. Within this context, a study on the effects of SDA on microtubule polymerization was carried out. The data of Fig. 7 show the inhibitory effect of the drug on the assembly of microtubules, as revealed by a gradual decrease in the extent of polymerization as the drug concentration increased. The relationships between the maximum of assembly after the assays at different concentrations of the drug are shown in the inset of the figure. It was observed that 20 μM SDA produced a 50% decrease in the extent of the *in vitro* microtubule assembly. This inhibition of brain tubulin assembly produced by the drug was further analyzed within the context of the intracellular organization of the microtubule network. The immunofluorescence studies after staining with an anti-β-tubulin monoclonal antibody showed dramatic changes in cell's morphology. The rounding of cells (Fig. 8A, and B) was accompanied with a contraction of the microtubular network (Fig. 8 C, D and E).

Discussion

These studies indicate that the natural product 14-keto-stypodiol diacetate (SDA) isolated from the algae *Stypopodium flabelliforme*, inhibits microtubule assembly in a similar fashion to stypoldione, a red o-quinone isolated from a different marine product [13, 24, 25]. SDA blocks microtubule assembly *in vitro* in a concentration dependent fashion. Furthermore, SDA exhibited a significant inhibitory effect on both plasminogen activator secretion and the invasive activity of DU-145 prostate cells, actions which have not been reported for other derivatives such as stypoldione. The cellular effects of SDA are noteworthy in the sense that it affects cell cycle progression by blocking mitosis, since DU-145 tumor prostate cells treated with this compound accumulated in G2/M phase. The arrest of the cell cycle in G2/M and the consequent decrease in S-phase after treating the cells with SDA at concentrations > 10 μM also constitute relevant features of the diacetylated compound. Several microtubule-interacting drugs appear to exert their actions by interfering with mitotic progression [4, 26], with an accumulation of cells in metaphase [3, 6]. Under comparable conditions, the

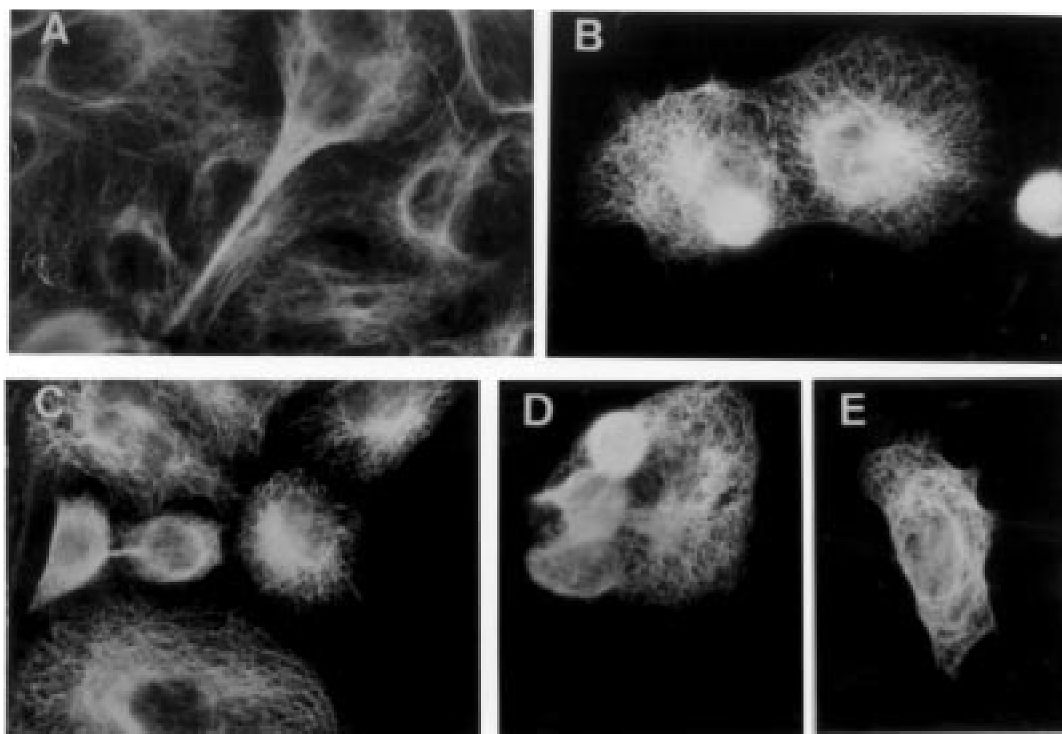

Fig. 8. Perturbation of the microtubule cytoskeletons of DU145 prostate carcinoma cells after treatment with variable concentrations of 14-ketostypodiol diacetate. Photomicrographs of the immunofluorescence patterns were obtained by using anti-β-tubulin monoclonal antibody (A–E) as primary antibodies. The panel in (A) shows the control with no drug. (B–C) Effects on cellular microtubules after the treatment with 5 and 10 µM SDA respectively, for a 24 h period. (D–E) Effects on microtubules after treatment for 24 h with 20 and 30 µM SDA respectively.

drug stypoldione also produced an accumulation in phase G2 of mitosis in the sea urchin embryos [25]. In this context, it is worth pointing out that most studies on stypoldione were carried out in sea urchin eggs, while the present studies on the effects of SDA were developed in a human cultured cell line. However, there is little information on the effects of stypoldione in tumor cells in culture. In spite of the different cell types used in these studies with both drugs, the comparison would suggest that SDA, and possibly stypoldione [25], may act by interfering with the dynamics or assembly of mitotic spindle microtubules. The *in vitro* studies on microtubule assembly and the action of SDA in accumulating cells in G2/M phase provide support for this suggestion.

There is increasing evidence for the concept that structural alterations in microtubule assembly and its dynamics produce serious alterations in the mechanisms of cell division, thus affecting cell proliferation [2]. The studies in Figs 3 and 4 indicate a gradual inhibition in cell growth as the concentration of SDA increases logaritmically, while no major changes occurred in the levels of LDH. The data suggest toward a main effect of SDA on cell proliferation. These inhibitory effects on the cell growth appears to be quite similar to those observed for stypoldione, and seem to be a result of the drug action on cell division. Besides the SDA effects on mitosis and cell proliferation, this drug also affected the microtubule cytoskeleton organization in non-dividing interphase cells. Thus, the antimicrotubular activity observed *in vitro* was corroborated at the cellular level, since SDA disrupted the normal organization of the microtubule cytoskeleton in the DU145 cell line as revealed by immunofluorescence studies. The effects of lower concentration of SDA, which produced abrupt morphological changes and a rounding of DU-145 cells could be associated to a gradual disassembly of microtubules involved in the architectural stabilization and cell morphogenesis. In this context, experiments on microtubule recovery [27] indicated that the microtubule cytoskeleton reorganized with a defined intracellular array after removing the SDA from the cell's medium. After a long exposure to the drug or in the presence of higher SDA concentrations, serious alterations in cytoskeleton organization were observed (data not shown). Especially relevant were the perturbations in microtubule arrays within the cytoplasm. These changes may be a result of the drug effects on assembled microtubules, with a consequent alteration in the distribution of the linking elements that determine the interaction patterns of the

different filaments of the cytoskeletal network, possibly via dissociation of MAPs that appear to be involved in stabilizing these interactions [20, 28].

On the other hand, an interesting feature of SDA is revealed by its strong capacity to block secretion of plasminogen activator [12] by DU-145 cells as well as the invasive activity of these cells, as shown with *in vitro* Matrigel assay. Both cellular activities are associated with the tumoral nature of DU-145 cells. The inhibition of secretion could be also related to the action of SDA on cytoplasmic microtubules that are involved along with motor systems in protein secretion [29]. Within this context, inhibition of plasminogen activator secretion mediated by microtubules was observed in the presence of the anticancer drug estramustine [12]. Moreover, the SDA analog stypoldione was reported to affect mitosis [30]. The studies reported here on the different effects of SDA, the microtubule assembly inhibition together with its cellular effects in arresting mitosis and blocking protease secretion mechanisms and cell invasion, suggest that SDA interferes with the tumoral activity of these prostatic cancer cells. It is also likely that the anti-mitotic activity be related to the SDA effects on the normal assembly and stability of the microtubule system, considering the role of these cytoskeletal polymers in the generation of the mitotic spindle and their consequent contributions to the driving force that operates in the mitotic process [31].

Acknowledgements

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