The effect of Taurolidine on adherent and floating subpopulations of melanoma cells

D.P. Shrayer[a], H. Lukoff[a], T. King[b] and P. Calabresi[a]

The annual incidence of malignant melanoma is estimated at 10–12 per 100 000 inhabitants in countries of Central Europe and the US, with more recent estimates showing a dramatic upward trend. Taurolidine (Carter/Wallace, Cranberry, NJ) is a novel, potentially effective, antitumor chemotherapeutic agent. We hypothesized that Taurolidine could inhibit the growth, induce apoptosis, affect the cell cycle and change morphology of melanoma cells. We expected this process to be different in adherent and floating subpopulations that may be reflective of solid tumors and their metastases. Analysis of MNT-1 human and B16F10 murine melanoma cells showed that at 72 h the IC_{50} of Taurolidine was 25.4 ± 3.3 μM for MNT-1 human melanoma cells and 30.9 ± 3.6 μM for B16F10 murine melanoma cells. Taurolidine induced DNA fragmentation of melanoma cells in a dose-dependent manner. Taurolidine (75 and 100 μM) induced 52–97% Annexin-V binding (apoptosis), respectively. Evaluation of cell cycle after 72 h exposure to Taurolidine (0–100 μM) revealed that the percentage of melanoma cells in S phase increased from 27 to 40% in the adherent subpopulation and from 33 to 49% in the floating subpopulation. Phase contrast microscopy revealed a marked swelling of melanoma cells and decreasing cell numbers in adherent subpopulation starting at 24 h with 25 μM Taurolidine. Shrinkage of cells dominated at 75–100 μM Taurolidine. Using Cytospin assay in the floating population, we observed swelling of melanoma cells induced by 25–100 μM Taurolidine and appearance of giant (multinuclear) forms resulting from exposure to 75–100 μM Taurolidine. Some floating cells with normal morphology were observed with low concentrations of Taurolidine (0–25 μM). These data show that effects of Taurolidine may be different in adherent and floating subpopulations of melanoma cells. More importantly, floating subpopulations that may contain some viable melanoma cells, may be reflective of potential metastasis after treatment of solid tumors *in vivo*. *Anti-Cancer Drugs* 14:295–303 © 2003 Lippincott Williams & Wilkins.

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Introduction

The annual incidence of malignant melanoma is estimated at 10–12 per 100 000 inhabitants in countries of Central Europe and the US, and alarmingly there has been a dramatic upward trend. Taurolidine (Taurolin) is a novel, potentially effective antitumor chemotherapeutic agent previously used against pathogenic bacteria [1–6]. Taurolidine suppresses synthesis of interleukin-1 and tumor necrosis factor (TNF) in human peripheral blood mononuclear cells [7], and inhibits blood-clotting phenomenon [8]. Growth of colon adenocarcinoma DMD/K12/TR6 can be inhibited by Taurolidine *in vitro* and *in vivo* [9]. Taurolidine has also been shown to inhibit the growth of human ovarian, colon and prostate tumor cells with IC_{50}(s) ranging from 12.5 to 50 μM [10]. *In vivo*, Taurolidine inhibits the development of xenografts from human prostate cancer cells in nude mice [11]. The purpose of these experiments was to investigate the ability of Taurolidine to inhibit the growth of human and murine melanoma cultures *in vitro* and to investigate the mechanisms for these effects. We also wanted to study the effects of Taurolidine on the growth of melanoma cells in adherent and floating subpopulations [13]. We hypothesized that Taurolidine could inhibit the growth, induce apoptosis, affect the cell cycle and change morphology of melanoma cells, and that these effects would have different dynamics in adherent and floating subpopulations. We believe that floating subpopulations, which may contain some viable melanoma cells, may be reflective of potential metastasis after treatment of solid tumors *in vivo*.

Materials and methods

Cell culture

MNT-1 human melanoma cells and B16F10 murine melanoma cells were obtained from Dr V.H. Hearing (NCI, NIH, Bethesda, MD). Melanoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% FBS for MNT-1 and 10% fetal bovine serum for B16F10 melanoma cells, 200 mM L-glutamine,

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10 mM MEM non-essential amino acids and 100 mM sodium pyruvate (all from Gibco, Grand Island, NY). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Colonies of viable melanoma cells from floating subpopulations were stained with 0.1% Crystal Violet in 100% ethanol. Numbers of cells in adherent and floating subpopulations we separately estimated at 24–72 h using a Coulter Counter (Beckman-Coulter, Miami, FL).

Estimation of growth inhibition and cytotoxicity

Growth inhibition of melanoma cells by Taurolidine (Carter/Wallace, Cranberry, NJ) was estimated using a Coulter Counter (Beckman-Coulter). The growth inhibition was estimated comparing the growth of cells in the absence of Taurolidine (0% of inhibition) versus the percentage of growth inhibition in cells grown with various concentrations of Taurolidine (12.5–100 μM) [15,16]. The IC_{50} value was determined by the dose of Taurolidine which caused a 50% reduction in cell numbers compared to controls. In addition we used microscopy and counted Trypan blue-negative cells by a hemocytometer and determined percent viability by MTT assay [14].

Determination of apoptosis

Assessment of DNA integrity

The Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to isolate DNA from 2×10^6 melanoma cells growing with Taurolidine (0–100 μM) for 24, 48 and 72 h in adherent and floating subpopulations. DNA standards and DNA samples were loaded onto a 1.5% agarose gel containing Sybr Gold nucleic acid. Gel stain was purchased in 10 000 × concentrate from Molecular Probes (Eugene, OR). DNA samples were subjected to conventional agarose gel electrophoresis at 80 V for 1.5 h. The samples were then visualized and photographed under UV illumination [17,18].

Annexin-V binding

Approximately 2×10^6 melanoma cells were collected separately from adherent and floating subpopulations, washed with 1 × PBS, centrifuged at 1500 r.p.m. and resuspended in 200 µl of 1 × binding buffer. Then 5 µl of Annexin-V–FITC (final concentration 0.5 µg/µl) was added to each cell suspension and incubated for 15min in the dark [19]. Flow cytometry analysis was performed at an excitation wavelength of 488 nm. The ApoAlert Apoptosis detection protocol and reagents were purchased from Clontech (Palo Alto, CA).

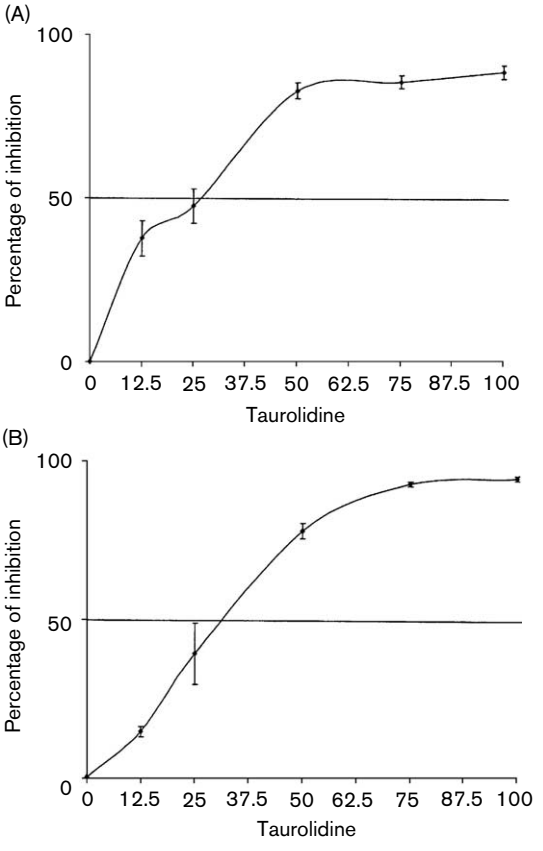
Study of cell cycle

Melanoma cells were grown for 24, 48 and 72 h in the presence of Taurolidine (0–100 μM). Culture medium was collected and spun at 1500 r.p.m. for analysis of the floating cell subpopulation. Adherent cells were detached with Trypsin/EDTA, centrifuged, washed and then resuspended in ice-cold PBS. The cells were stained for 30 min.in 0.05 mg/ml of propidium iodide, 0.1% Igepal and 0.1% sodium citrate (all from Sigma, St Louis, MO). Cell cycle profiles were obtained by FACScan (Becton Dickinson, Plymouth, UK) using the Mod Fit LT program (Becton Dickinson) [20–22].

Morphology of melanoma cells

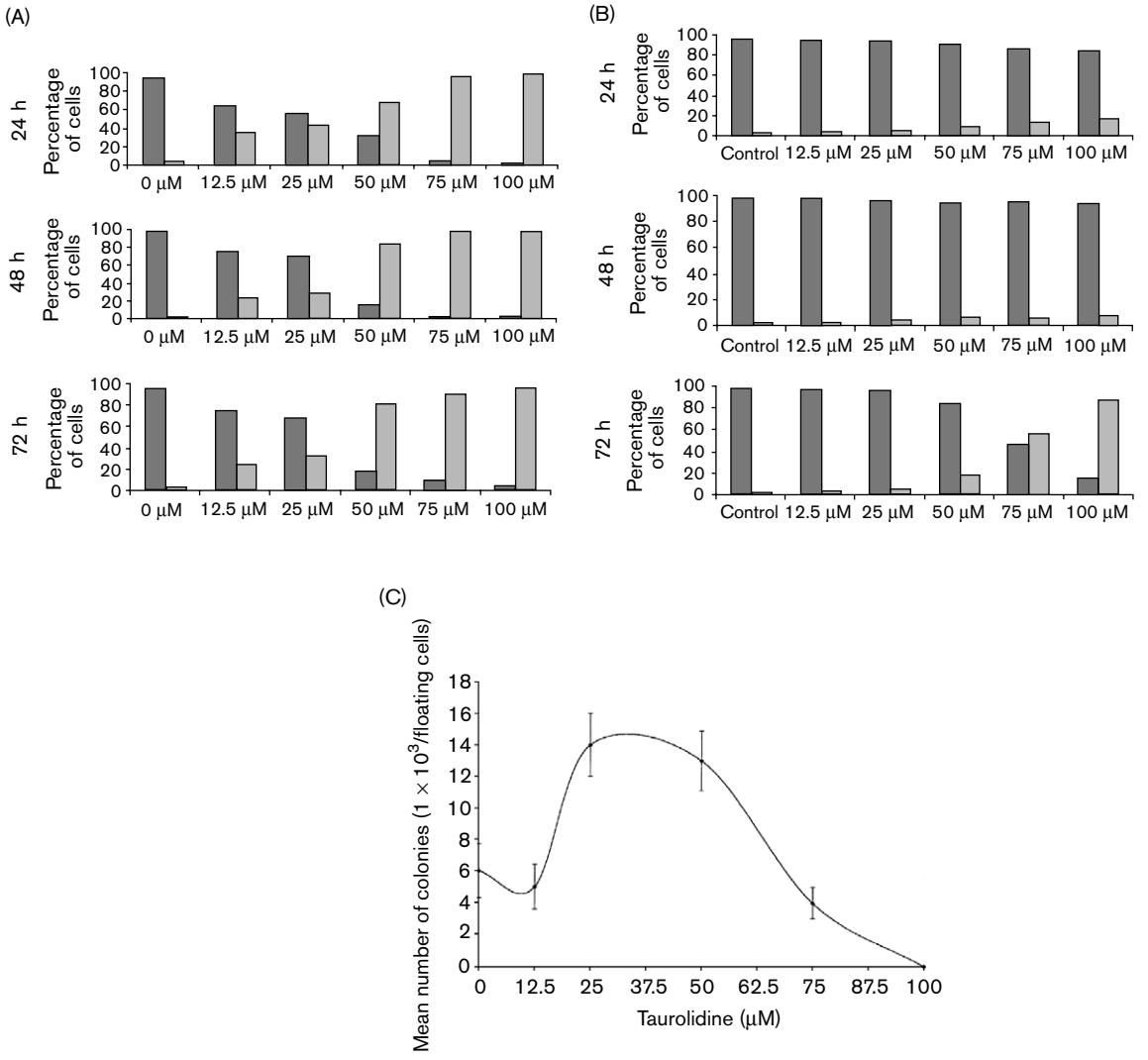
Human MNT-1 and murine B16F10 melanoma cells were incubated with various concentrations of Taurolidine (0–100 μM). The morphology of adherent melanoma cells and cells suspended in culture medium was studied by microscopy using a Nikon eclipse inverted microscope with TE phase contrast. Each image was acquired at × 20 magnification with a spot digital camera from Diagnostic Instruments (Sterling Heights, MI) in 16-bit grayscale. We also studied the morphology of adherent melanoma cells (which were detached by trypsin/EDTA) and cells suspended in culture medium (floating subpopulation). In these experiments melanoma cells were pelleted onto

Fig. 1



IC_{50} of Taurolidine versus MNT-1 human melanoma (A) and B16F10 murine melanoma (B).

Fig. 2



Amount of MNT-1 human (A) and B16F10 murine (B) melanoma cells in adherent (black bars) and floating (shaded bars) subpopulations. Melanoma cells were grown for 24–72 h in culture medium in the presence of Taurolidine (0–100 μM). The number of cells was determined by a Coulter counter. Mean number of colonies originating from floating subpopulations of MNT-1 melanoma cells which developed at 72 h in the presence of Taurolidine (0–100 μM) (C).

slides by Cytospin (Shaudon Elliot), stained by hematoxylin & eosin and studied by bright field microscopy (Nikon eclipse microscope E800), × 20 magnification [23–28].

Statistical analysis

The statistical significance of differences was analyzed by the Student's *t*-test (two-tailed). For all comparisons a two-sided p value < 0.05 was significant.

Results

Estimation of IC_{50} of Taurolidine

A 72-h exposure to Taurolidine resulted in 50% inhibition of growth of human melanoma cells at $25.4 \pm 3.3\ \mu$M (Fig. 1A) and murine melanoma cells at $30.9 \pm 3.6\ \mu$M (Fig. 1B). Trypan blue and MTT assays showed close results (data not shown).

Dynamics of melanoma cells in adherent and floating subpopulations in the presence of Taurolidine

We counted the number of human and murine melanoma cells in adherent and floating subpopulations at 24, 48 and 72 h growing in the presence of different concentrations of Taurolidine. Results of these experiments are shown in Figure 2(A and B). We found time- and dose-dependent effects of Taurolidine in both human and murine melanoma cells which moved partly from adherent to floating subpopulations. Human melanoma

cells moved more actively than murine melanoma cells. At 72 h the highest concentrations of Taurolidine (50 and 100 μM) resulted in the detachment of 50–80% human cells (Fig. 2A) versus of 20–80% B16F10 murine melanoma cells (Fig. 2B). We were able to grow human melanoma colonies from pellets of floating subpopulations in the presence of 0–75 μM Taurolidine for 72 h. However, 100 μM Taurolidine had the capacity to kill all viable cells in the floating subpopulation (Fig. 2C).

Induction of DNA fragmentation by Taurolidine

DNA was isolated from both adherent and floating subpopulations of cells growing at 24 h in the presence of Taurolidine (0–100 μM). In cells treated with Taurolidine, fragmentation of nuclear DNA, which is a typical phenomenon of early apoptosis (32), was observed in a dose-dependent manner. We found 250- to 123-bp DNA fragments in DNA isolated from adherent subpopulations of melanoma cells treated with 50 and 100 μM of Taurolidine (Fig. 3, lanes 3 and 4). Most of the floating cells were in a necrotic state at 50 and 100 μM, and as a result DNA isolation predominantly looked like smears with rare fragments (Fig. 3, lanes 6 and 7.)

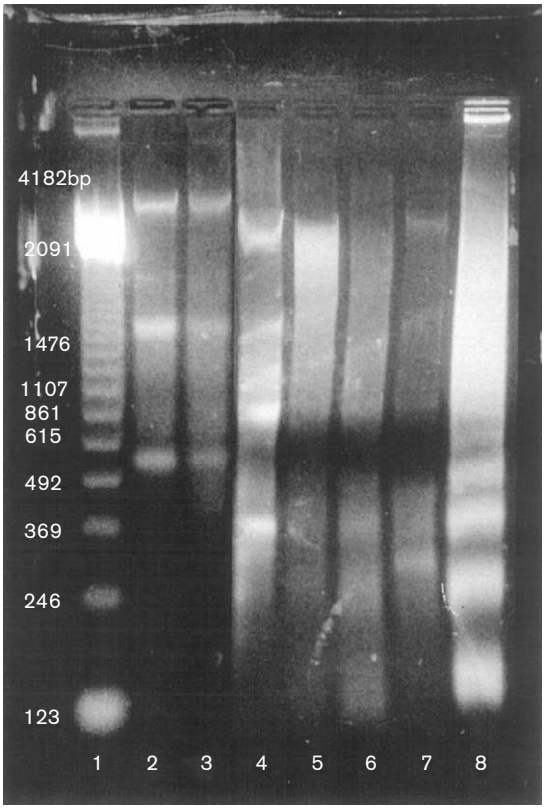
Determination of apoptosis by Annexin-V binding

In experiments with adherent subpopulations we found that binding of Annexin-V by MNT-1 human and B16F10 murine melanoma cells occurred in a dose- and time-dependent manner. Human melanoma cells exposed to 75 and 100 μM of Taurolidine for 72 h showed 52.7 and 92.6% Annexin-V binding, respectively. Murine melanoma cells exposed to Taurolidine (75 and 100 μM) for 72 h showed 19 and 47.7% Annexin-V binding, respectively (Fig. 4A and B, and Table 1). In floating subpopulations of human and murine melanoma cultures most of the cells at 72 h were in the apoptotic stage regardless independently of the presence or absence of Taurolidine (Table 1).

Effect of Taurolidine on the cell cycle of melanoma cells

In adherent subpopulations of MNT-1 melanoma cells at 24 h increased concentrations of Taurolidine resulted in a decrease in the percentage of cells in G_0/G_1 phase: 0 μM, 50.8%;. 75.0 μM, 26.8% and 100 μM, 19.8% (data not shown). After 72 h exposure with Taurolidine the percentage of melanoma cells in S phase increased from 27 to 40% in the adherent subpopulation and from 33 to 49% in the floating subpopulation (Fig. 5). Similar results were obtained when B16F10 murine melanoma cells were incubated with Taurolidine for 48 h. For the adherent subpopulations, percentages of cells in G_0/G_1 phase were as follows: 0 μM, 51.2%; 50.0 μM, 27.6%; 100.0 μM, 27.2%. In the floating subpopulations, the percentages of cells in G_0/G_1 phase were: 0 μM, 10.0%; 50.0 μM, 28.0%; 100.0 μM, 20.0% (data not shown).

Fig. 3

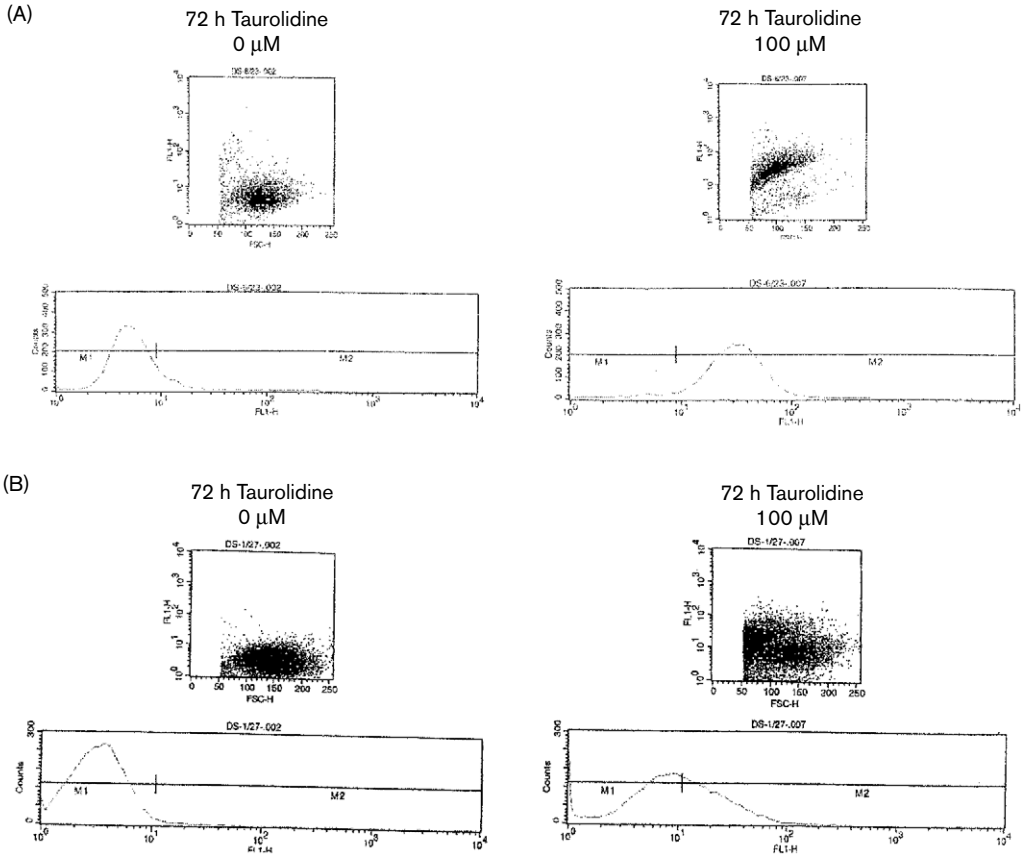


Determination of DNA fragmentation. Agarose gel electrophoresis of DNA extracted from adherent (lanes 2–4) and floating (lanes 5–7) subpopulations of MNT-1 human melanoma cells. Exposure: 24 h. Sybr stain photographed under ultraviolet light. Lane 1, 123-bp molecular weight markers (Gibco); lane 2, Taurolidine 0 μM; lane 3, Taurolidine 50 μM; lane 4, Taurolidine 100 μM; lane 5, Taurolidine 0 μM; lane 6, Taurolidine 50 μM; lane 7, Taurolidine 100 μM; lane 8, positive controls (Apoptotic DNA Ladder Kit; Roche).

Morphological changes in melanoma cells exposed to Taurolidine

Human and murine melanoma cells were grown in the presence of Taurolidine (0–100 μM). Examination of growing melanoma cells with phase contrast microscopy revealed a marked swelling of melanoma cells and decreased cell numbers starting at 24 h with 25 μM Taurolidine. The degree of swelling (oncosis) was dose dependent and reached a peak at 100 μM. Shrinkage of cells (apoptosis) dominated at 75–100 μM (Fig. 6). For murine melanoma cells similar morphologic changes were observed at 48–72 h (data not shown). Another series of morphological experiments evaluated adherent and floating subpopulations of human melanoma cells growing for 48 h in the presence of Taurolidine (0–25–50–100 μM). Melanoma cells from both subpopulations were pelleted by Cytospin and studied by bright field microscopy at $\times$20 magnification.

Fig. 4



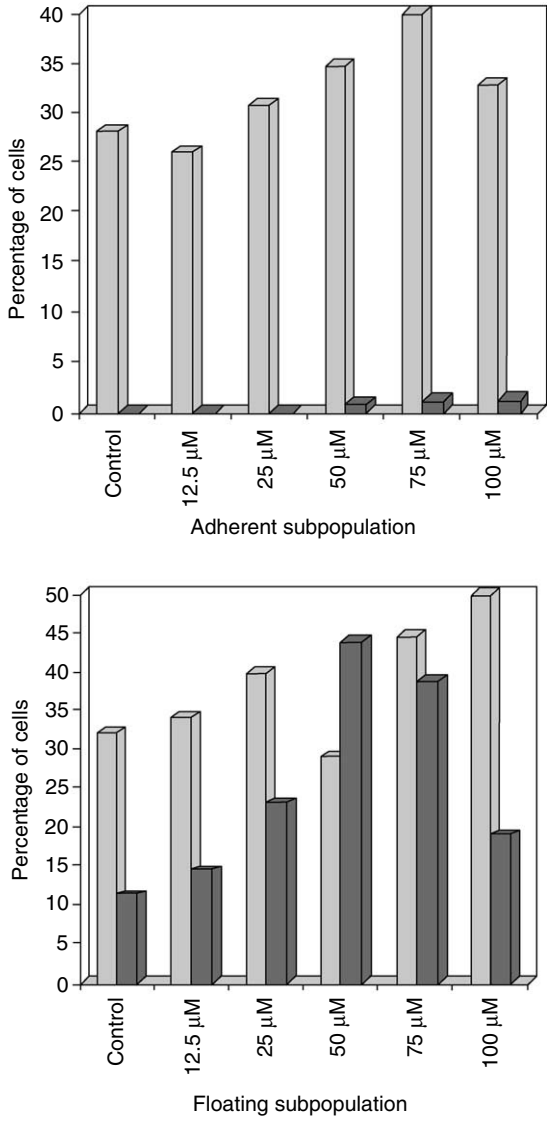
Annexin-V assay. Induction of apoptosis by Taurolidine (24–72 h). Flow cytometric detection of apoptotic cells by Annexin-V binding in adherent subpopulations of MNT-1 human (A) and B16F10 murine (B) melanoma cells.

Table 1 Percentage of gated Annexin-V$^+$ cells in the adherent and floating subpopulations various incubation times following treatment with Taurolidine

Tawolidine (μM)	24 h		48 h		72 h	
	Adherent	Floating	Adherent	Floating	Adherent	Floating
MNT-1						
0	13.7	73.6	4.8	64.2	18.2	95.1
12.5	6.9	82.1	13.7	85.9	12.3	96.7
25	5.9	94.2	10.9	88.6	16.4	96.6
50	22	78	83.4	94	24.9	96.7
75	81.8	68.5	95.1	93.2	52.7	93.2
100	97.1	82	95.1	93	92.6	95.5
B16F10						
0	5.6	80.8	4	78.2	2	96.1
12.5	6.8	83.8	4.7	81.4	5.1	95.4
25	5.8	78.9	4.3	78.6	4.3	91.5
50	3.1	76.1	6.8	66.2	13.8	82.3
75	4.8	73.5	27.9	45	19	85.8
100	14.6	57.2	80.9	75	47.3	74.1

In the adherent subpopulation we observed swelling of melanoma cells induced by 25–100 μM Taurolidine and the appearance of giant (multinuclear) forms resulting from exposure to 75–100 μM Taurolidine (Fig. 7A). Single viable cells with normal morphology were observed in floating subpopulations growing in the absence or with low concentrations of Taurolidine (0–25 μM). Numerous necrotic (eosin-stained) cells were found in floating subpopulations containing high concentrations of Taurolidine (50–100 μM) (Fig. 7B).

Fig. 5



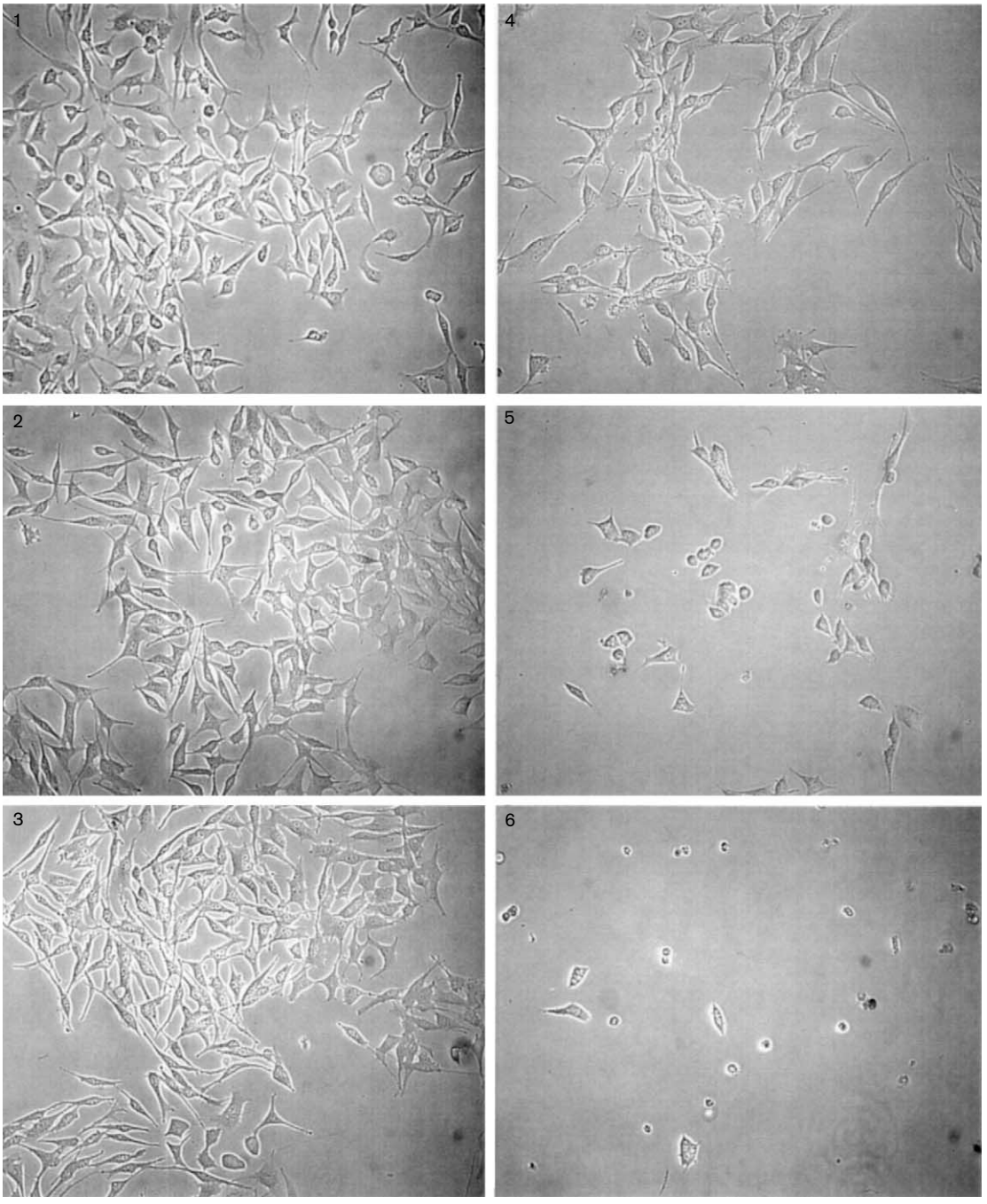
Cell cycle. Adherent and floating subpopulations of MNT-1 human melanoma cells in S phase (light bars) and apoptotic (dark bars) stage at 72 h.

Discussion

We hypothesize that the adherent subpopulation of melanoma cells may represent a model of primary melanoma tumor and that floating subpopulations of viable cells may represent a model of metastatic cells developing in the presence of Taurolidine. We found that Taurolidine was able to inhibit the growth of human and murine melanoma cells. However, in the presence of Taurolidine, melanoma cells actively moved from adherent to floating subpopulations in a time- and dose-dependent fashion. Taurolidine showed the capacity not only to inhibit the growth of melanoma cells, but also to induce DNA fragmentation, to stimulate Annexin-V binding at 72 h consistent with apoptosis and to induce cell cycle arrest of melanoma cells [29–39]. The most dramatic effects were observed after 72-h exposure to Taurolidine. Inhibition of melanoma cell growth by Taurolidine, DNA fragmentation, cell cycle arrest and Annexin-V binding were accompanied by a marked swelling of melanoma cells and decreased cell population. The degree of swelling (oncosis) was time and dose dependent; shrinkage (apoptosis) and necrosis of melanoma cells was also dose and time dependent. Some investigators describe these morphological changes as separate processes. Death by swelling (oncosis) [26,27] may be a process that is not connected with apoptosis (cell shrinkage, appearance of budding and giant forms) [23–28]. Changes in morphology of apoptotic cells differ from necrosis when cells lose nuclear structures [24–26,28]. Analysis of our data shows that Taurolidine induces different morphologic changes, which are concentration/time dependent. We found that the cytotoxic capacity of Taurolidine is likely due to the induction of apoptosis for most of the melanoma cells exposed to this agent. All stages of cell death start with oncosis, go through apoptosis (shrinker cells, budding and giant forms) and finish with necrosis. Special attention should be paid to the finding of viable cells (growth of colonies) in floating subpopulations of melanoma cells which developed in the presence of lower doses of Taurolidine (0–75 μM) at 72 h (Fig. 2C). These single cells may escape apoptosis [35] after treatment with subtherapeutic concentrations of Taurolidine. In our experiments only the highest concentration of Taurolidine (100 μM) was capable of eliminating the growth of colonies originating from viable cells in the floating subpopulations (Fig. 2C).

The progression of tumors is believed to be attributed to their ability to produce micrometastases in spite of chemotherapy and radiotherapy [41,42]. The detection of circulating malignant melanoma cells has been the subject of numerous investigations in recent years [43–45], yet the biology of micrometastases remains unclear [46]. It is likely to be related to the selection of single melanoma cells with different levels of adhesion [47] and invasion [48]. In our experiments the adherent subpopulation may be a model of primary melanoma tumor and the floating subpopulations of viable cells a model of metastatic cells developing in the presence of Taurolidine. Whereas large numbers of cells from a primary tumor may gain access to the circulation, few of them will give rise to metastases. The mechanism of elimination of these tumor cells, often termed 'metastatic inefficiency', is not clear [49]. Treatment of patients with metastasizing melanoma, still one of the most deadly diseases in modern medicine [50], could be successful only by reaching doses of chemotherapeutic agents which would result in 100% mortality of tumor cells. These experiments show that when a concentration of 100 μM

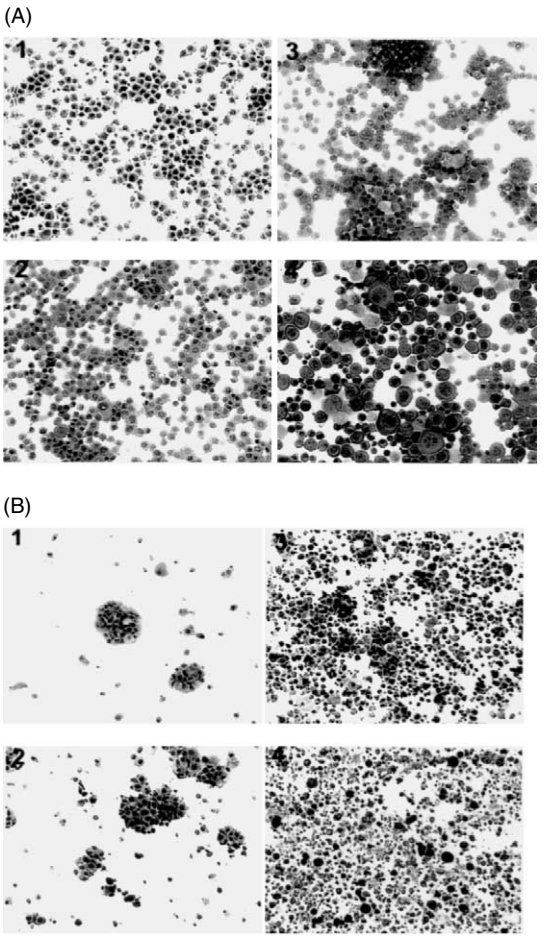
Fig. 6



Morphological changes in MNT-1 melanoma cells induced by Taurolidine. Cells were incubated for 24 h with Taurolidine (1, 0 μM; 2, 12.5 μM; 3, 25 μM; 4, 50 μM; 5, 75 μM; 6, 100 μM). Phase contrast microscopy of cells studied at $\times$ 20 magnification. Marked swelling of melanoma cells and decreased amount of cells in the population began at 25 μM. The degree of swelling (oncosis) was dose dependent and reached a peak at 100 μM Taurolidine. Shrinkage of cells (apoptosis) dominated at 75–100 μM Taurolidine.

Taurolidine is used, we can totally inhibit the growth of human melanoma cells in adherent and floating subpopulations. Our hypothesis is that in spite of the capacity of a chemotherapeutic agent (Taurolidine) to inhibit the growth, to induce apoptosis, to affect the cell cycle and to change morphology of melanoma cells, some tumor cells from the floating subpopulations could survive. We believe that these melanoma cells may be reflective of potential metastasis after treatment of solid tumors *in vivo*.

Fig. 7



Morphology of MNT-1 human melanoma cells in adherent (A) and floating (B) subpopulations at 48 h. Melanoma cells were pelleted by Cytospin, stained by hematoxylin & eosin and studied by bright field microscopy at $\times 20$ magnification. Taurolidine concentrations (1, 0 μM; 2, 25 μM; 3, 50 μM; 4, 100 μM). Swelling of melanoma cells (A2, 3 and 4) and appearance of giant forms (A4). A small amount of cells in floating subpopulations corresponded with absence or low concentration of Taurolidine (B1 and 2). Domination of apoptotic or necrotic cells was found in culture medium containing high concentrations of Taurolidine (B3 and 4).

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