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Camptothecin-induced death of amelanotic and melanotic melanoma cells in different phases of cell cycle

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Camptothecin and its analogues are used as S-phase specific antitumor drugs because of topoisomerase I inhibition providing cells to death by apoptosis. Our previous works documented that amelanotic hamster's melanoma is very sensitive to camptothecin. Because of the challenges in treating melanoma and S-phase specificity of camptothecin, we performed a study to search what melanoma cell cycle phases are susceptible to this substance.

Melanotic (Ma) and amelanotic (Ab) lines of Bomirski hamster's melanoma were used. Camptothecin cytotoxicity was determined by TUNEL method and cell cycle analysis was done by DNA staining with propidium iodide.

Camptothecin after short time killed amelanotic melanoma cells from S/G2/M phases but with extended time dying cells came from G0/G1. Melanotic melanoma had fewer cells in S/G2/M phases and 3-fold more of these cells spontaneously died in comparison to more aggressive amelanotic line. High susceptibility of amelanotic melanoma cells to camptothecin show that not only cells with proliferative activity were sensitive to this alkaloid but with extended time it killed cells from all cycle phases. High number of cells in S/G2/M phases and low rate of spontaneous death among amelanotic melanoma cells suggest that the expansive growth of this melanoma line depends mainly on the decreased ability to undergo spontaneous apoptosis. If the sensitivity of amelanotic melanoma is not only hamster's but also human melanoma feature, we can suspect that by choosing melanoma form for treatment with camptothecin we could improve effectiveness of this drug against melanoma.

Key words: amelanotic melanoma, camptothecin, cell cycle, cell death, melanoma

Despite recent advances in our understanding of melanoma, it is a disease without effective therapy. Dacarbazine, the "standard of melanoma care", has a response rate of about 10% [1]. Thus melanoma is in the first line of tumors to be examined by potential anticancer drugs. During the past two decades, camptothecin analogues have emerged as an important new class of antitumor drugs [2, 3]. Camptothecin (CPT) is a plant alkaloid produced by *Camptotheca acuminata* and some other species of angiosperms. The discovery that the main cellular target of CPT is topoisomerase I has initiated experimental works on CPT as an anticancer drug [4]. Topoisomerase I is an enzyme involved in topological changes of DNA structure; it binds to DNA single-strand breaks, allowing DNA relaxation. Topoisomerase activity is crucial during replication and transcription [2, 5]. Camptothecin-topoisomerase I-DNA complex triggers replication fork arrest and finally generates strand breaks inducing cell death. It is well established that CPT induces only/mostly death of proliferating cells [3]. The precise sequence of events that follow the CPT-induced DNA damage leading to cell death is not fully understood. The most observed way of CPT-induced death is apoptosis [3, 6]. For over forty years of CPT investigations some authors have stressed that except DNA replication it inhibits also transcription [4, 7, 8]. Camptothecin analogues, e.g. topotecan, irinotecan, are topoisomerase I-targeted drugs to various cancers (lung cancers, cervical cancer, ovarian cancer, gastric cancer, malignant gliomas) and a dozen of new CPT analogues are at various stages of clinical trials [2, 5]. There have been trials of using these compounds in metastatic melanoma treatment but without any promising effectiveness [6, 9-12].

In our hamster's melanoma model (Bomirski melanomas) camptothecin caused death of amelanotic melanoma cells (Ab line) through the apoptotic way while melanotic melanoma cells (Ma line) were insensitive to the action of this substance [13,14]. These two transplantable melanoma lines, except the presence (Ma line) or lack (Ab line) of melanin, differ in many biological features e.g. growth rate,

tumorigenicity [15]. The amelanotic form grows faster and causes animals death in a shorter time than the native melanotic line. These two types of melanoma cells: melanotic and amelanotic occur also in human melanoma, and the appearance of amelanotic cells accompanies progression of pigmented melanoma [16]. Cells' susceptibility to apoptosis induction is deeply influenced by their position in the cell cycle. Camptothecin is used as the S-phase specific inducer of death although there are works pointing out that also not proliferating cells such as neurons die under the influence of this substance [17, 18]. There is still an open question if camptothecin causes death only of proliferating melanoma cells. Recently it has been stressed that camptothecin does not only disturb replication but also production of some proteins [19]. Transcription is a process which goes on during all the cell's life, also after division.

Thus as a continuation of our study on different susceptibility of melanotic and amelanotic melanoma cells to death, in the present work, we would like to analyze cell cycle changes and sensitivity of melanoma cells from particular phases of cell cycle to spontaneous and and CPT-induced death.

Materials and methods

Animals. Three to four months old male Syrian (golden) hamsters *Mesocricetus auratus Waterhouse*, were purchased from the Central Animal Facilities of the Silesian Medical University, Katowice, Poland. The experiment's procedures were approved by the Animal Ethics Committee at Medical University of Gdansk and conformed to the National Health and Medical Research Council's guide for the care and use of laboratory animals.

Bomirski hamster's melanoma. The original transplantable melanotic melanoma (Ma) derived from a spontaneous melanoma of the skin that had appeared in a bred of golden hamster in 1959 [20]. The amelanotic melanoma line (Ab) originated from the Ma form by a spontaneous alteration. The loss of pigment was accompanied by changes in many biological features of the amelanotic melanoma line – faster tumor growth rate, shorter animal survival, changes in ultrastructure of cells [20]. Once establish, these melanomas possessed a considerable degree of phenotypic stability over decades of transplantation [20]. Since their discovery, each melanoma line is maintained in vivo by consecutive, subcutaneous transplantations of tumor material every 21 (Ma) or 11 (Ab) days. The material from 2-3 animals was used for each experiment in the examined group of animals. This melanoma model is known as Bomirski hamster's melanomas.

Isolation of melanotic and amelanotic melanoma cells. Melanoma cells were isolated for each experiment from solid tumors by a non-enzymatic method described earlier [13]. The suspension consisted of 95 – 98% viable cells (estimated by trypan blue test).

Camptothecin [CPT] treatment. CPT is a well known inducer of apoptosis in different tumor cells also in melanoma cells [6, 10]. For induction of death, freshly isolated melanotic and amelanotic melanoma cells were incubated in medium (RPMI, 10% FBS-fetal bovine serum and antibiotics) without (spontaneous death) or with 4 μg/ml camptothecin (CPT; SIGMA Chemical Company, St Louis, USA) (CPT-induced death) for 4, 12, 24 hrs at 37°C.

Cell cycle analysis. Ethanol-fixed $1x10^6$ melanoma cells were resuspended in 1 ml of staining solution (RNaseA 200 μg/ml and PI – propidium iodide, 5 μg/ml in PBS). Then were cells incubated for 30 min at 37°C in the dark and the fluorescence was analyzed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA; Department of Pathophysiology of Medical University, Gdansk). 20 000 events were stored from each stained sample and analysed off-line using WinMDI2.6 software (obtained from J. Trotter, The Scripps Institute, La Jolla, CA, USA). Cells in S and G2/M phases of cell cycle were analyzed together and used as an indirect measure of proliferative activity of cells [15].

Estimation of dead cells by the TUNEL method. Our previous results documented that examined melanoma cells die through apoptosis way (activation of caspases, phosphatidylserine externalization, decreased mitochondrial transmembrane potential) [13-15] thus, in the present study only the TUNEL-method was chosen to estimate cells with DNA fragmentation at the final stage of death [21]. The APO-BRDU Kit (BD Bioscience Pharmingen) were used to determine dead cells and cell cycle phases they came from. Cells were prepared following manufacturer's protocol. Bivariate analysis of apoptosis (green fluorescence) and DNA content (PI) was performed using the flow cytometer.

Statistical analysis. Group data are expressed as mean ± S.D. Statistical analysis was performed using the Mann-Whitney U test with the Bonferroni correction for the level of p value; values of p lower than 0.017 (for three tests) and 0.025 (for two tests) were regarded as significant. The tests were performed using StatSoft, Inc.(2005) STATISTICA (data analysis software system), version 7.1. www.statsoft.com.

Results

Cell cycle analysis of melanotic and amelanotic melanoma cells. The cytometric analysis of cell cycle showed that cells from S and G2/M phases accounted for about 30% of melanotic melanoma cells and their number was significantly lower in comparison to less differentiated, but faster growing amelanotic melanoma, where almost 40% of cells were in S and G2/M phases ($p<0.017$; Table 1). About 51% of cells, in slowly growing and more differentiated – melanotic melanoma were in G0/G1 phases. Among amelanotic melanoma about 55% of cells were at this stage of cell cycle (Table 1). With extended time the content of amelanotic melanoma cells in particular phases did not change significantly (Figure 1B; population M1). While among melanotic melanoma cells from S/G2/M phases decreased to only 19% but from G0/G1 increased to

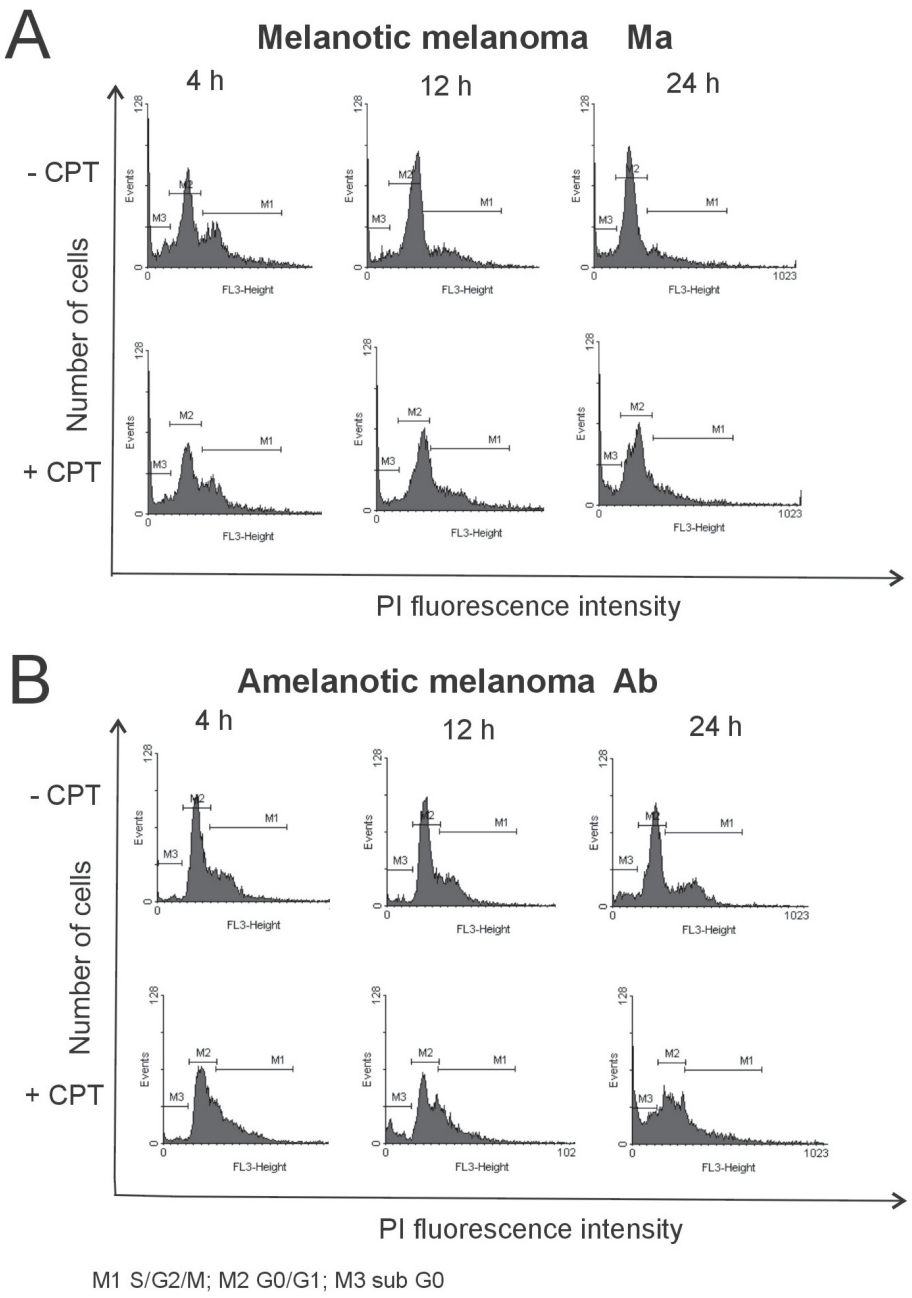

Figure 1. Cell cycle distribution in melanotic Ma (A) and amelanotic Ab (B) melanoma lines after incubation without (-CPT) and with (+CPT). The cells after isolation from the tumor mass were incubated for 4, 12, 24 hrs without (-CPT) or with (+CPT) 4μg/ml camptothecin. The DNA content in cells stained with propidium iodide was determined. Cells at S and G2/M phases were analysed together and used as an indirect measure of proliferative activity of cells (M1). Cells in subG0 include apoptotic cells in the late stage of death (apoptotic bodies, M3). Fig.1B (+CPT) illustrated cell cycle changes among Ab line under the influence of CPT – with time the content of cells in S/G2/M and G0/G1 phases decreased. One of 7 done experiments.

60% (Table 1, Figure 1A; population M1). In the cytometric analysis of DNA content there were also cells with lower DNA content – located below G0/G1 (sub G0-population M3 in Figure 1), they included apoptotic cells (apoptotic bodies). In melanotic melanoma, about 12% of cells were located at sub G0 level but in amelanotic line only 4% ($p<0.017$; Table 1). With the duration of time these values increased to about 18% and 9% respectively (Table 1).

CPT-induced cell cycle changes. After 24 hrs with CPT among melanotic melanoma cells there was noticed about

Table 1. The cells cycle analysis of melanotic (Ma) and amelanotic (Ab) transplantable melanoma cells after incubation without (-CPT) and with (+CPT) camptothecin.

Melanoma cells		Percentage of cells in cell cycle phases:						Percentage of cells in sub G0		
		G0/G1			S+G2/M					
Incubation time in hours		4	12	24	4	12	24	4	12	24
Ma	- CPT	50.9±6.7	55.1±10.6	60.1±4.9	30.4±4.9	29.1±5.0	19.0±2.7	11.7±2.7	12.2±6.4	17.6±2.8
	+ CPT	51.1±6.0	52.4±4.8	52.9*±3.9	30.5±5.2	30.7±6.2	22.2±4.0	11.9±3.2	13.4±5.8	21.7*±3.1
Ab	- CPT	54.9±4.7	53.0±3.2	56.7±2.1	39.0**±4.7	37.2**±5.2	33.0**±2.8	4.4**±1.3	9.3±3.7	8.9**±3.0
	+ CPT	54.2±2.5	46.2±4.5	34.4*±6.3	38.9±3.4	33.3±6.9	25.9*±4.2	5.1±1.9	19.7±7.1	38.0*±8.1

The cells after isolation from the tumor mass were incubated for 4, 12, 24 hrs without (-CPT) or with (+CPT) and the content of cells in particular cell cycle phases was estimated; S and G2/M phases were analysed together and used as an indirect measure of proliferative activity of cells; cells in subG0 include apoptotic cells in the late stage of death (apoptotic bodies). The values are the means ± SD from 7 experiments; in each trial 20 0000 cells were analysed. Statistical analysis by Mann-Whitney U test: * p<0.017, significant differences in comparison to probes without CPT ; ** p<0.017, Ab melanoma has more cells in S/G2/M and less in sub G0 than the Ma form.

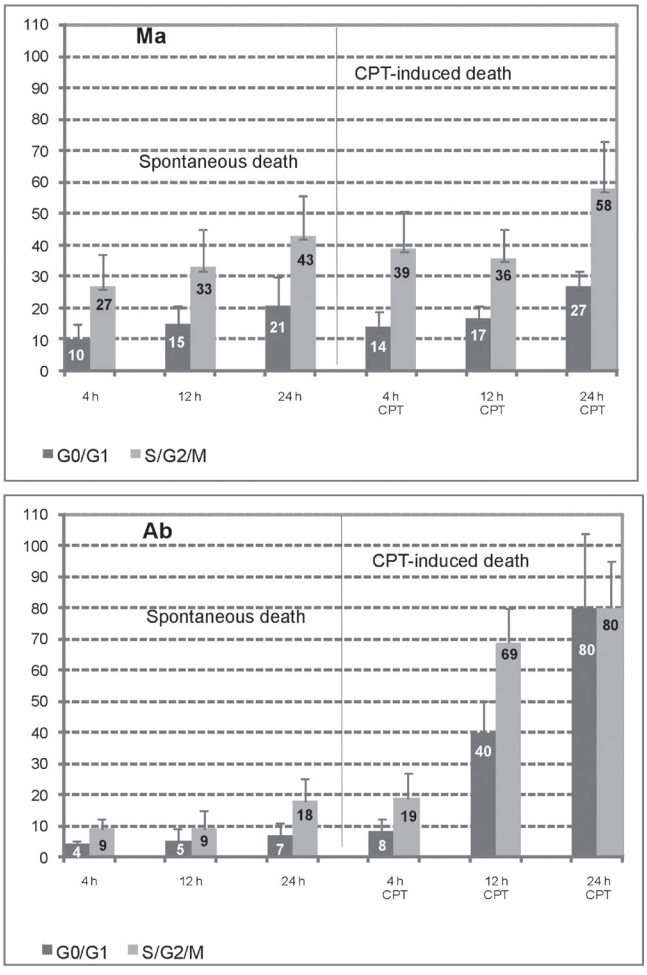

Figure 2. A graphical presentation of the percentage of TUNEL-positive melanotic (Ma) and amelanotic (Ab) melanoma cells among all cells in G0/G1 and S/G2/M phases during spontaneous and CPT-induced death. TUNEL-positive cells and the cell cycle analysis were determined as described in the Material and methods. Results are the means ± SD from 7 experiments.

a 7% decrease in the content of cells from G0/G1 while cells in S/G2/M were at the level of cells incubated without CPT (Table 1, Figure 1A). Amelanotic melanoma cells react more dramatically to CPT, just after 12 hrs it decreased the content of S/G2/M cells to 33% and G0/G1 to 46%. This tendency followed with time and after 24 hrs lowered to 26% and 34% respectively (Table 1; Figure 1B). There was also observed an increase in the number of dead cells located in the sub G0 up to 38% after 24 hrs (Table 1, Figure 1B population M3).

Spontaneous death of melanotic and amelanotic melanoma cells from particular cell cycle phases. TUNEL-positive cells from melanotic and amelanotic melanoma cells came from all cell cycle phases but there were significant differences between the examined lines (Table 2).

Among melanotic melanoma, after 4 hrs, 10% of cells from G0/G1 and 27% from S/G2/M, were TUNEL-positive, while among amelanotic melanoma cells only 4% and 9% respectively (Figure 2). With the time prolongation these values increased but more significantly in melanotic line where after 24 hrs over 40% of S/G2/M cells were TUNEL-positive (Figure 2). It is worth noticing that there were almost 3-fold more dead melanotic melanoma cells from S/G2/M phases in comparison to amelanotic ones (Table 1; Figure 2). The proportion between the percentage of TUNEL-positive cells from S/G2/M and G0/G1 to all cells in these phases was 1:4 and 1:10 for melanotic melanoma and 1:11 and 1:23 for amelanotic form respectively (Table 2). It means that proportionally more cells died from S/G2/M phases in both melanoma lines (Figure 2).

CPT- induced death of melanotic and amelanotic melanoma from particular cell cycle phases . CPT induced cells death at all cell cycle phases (Table 2). Melanotic melanoma cells did not react to CPT significantly; TUNEL-positive cells were from all cycle phases at the same level as incubated without CPT (Figure 1A); only after 24 hrs there was a slight increase in all TUNEL-positive cells (Table 2).

Among amelanotic melanoma about 19% of S/G2/M cells were TUNEL-positive already after 4 hrs and this number

Table 2. Percentage of TUNEL-positive cells and the ratio of TUNEL-positive to all cells from particular cell cycle phases (S+G2/M; G0/G1) of melanotic (Ma) and amelanotic (Ab) melanoma cells during spontaneous (-CPT) and camptothecin-induced (+CPT) death.

Melanoma cells		Percentage of TUNEL-positive cells from cycle phases Ratio of TUNEL-positive cells to all cells in cell cycle phases						Total percentage of TUNEL-positive cells		
		G0/G1			S+G2/M					
Incubation time in hours		4	12	24	4	12	24	4	12	24
Ma	- CPT	5.0±2.2 1:10	8.2±3.7 1:7	12.4±5.1 1:5	8.1±4.6 1:4	9.7±4.1 1:3	8.2±2.2 1:2	16.2±7.1	22.2±8.2	27.3±9.5
	+ CPT	6.9±2,3 1:7	8.7±1.5 1:6	14.3±2.4 1:4	12.0±5.4 1:3	11.2±2.5 1:3	12.8±4.1 1:2	23.3±7.3	24.0±5.6	37.3±7.0
Ab	- CPT	2.4±0.8 1:23	2.9±2.2 1:18	4.1**±1.8 1:14	3.7±2.3 1:11	3.5±2.4 1:11	5.9**±2.0 1:6	6.8**±1.6	10.1±4.8	13.7**±5.0
	+ CPT	4.3±2.4 1:13	18.4*±4.7 1:3	27.6*±11.7 1:1	7.3*±3.4 1:5	23.1±7.4 1:1	20.7*±5.3 1:1	14.5*±3.6	51.3*±12.1	73.5*±9.6

The content of death cells among amelanotic and melanotic melanoma after incubation for 4, 12, 24 hrs without (-CPT) or with (+CPT) was measured by TUNEL method. The values are the means ± SD from 7 experiments; in each trial 20 0000 cells were analysed. Statistical analysis by Mann-Whitney U test: * $p<0.017$ significant increase of death cells among Ab line in comparison to probes without CPT; ** $p<0.017$ significantly less cells of Ab melanoma died spontaneosly in comparison to Ma line.

increased 4-fold through additional twenty hours (Figure 2). It is worth stressing that at the same time the number of TUNEL-positive cells from G0/G1 increased 10-fold in comparison to cells incubated without CPT (Figure 2). Already after 4 hrs the ratio of TUNEL-positive amelanotic melanoma cells from S/G2/M to all cells in these phases reached the value of 1:5 and changed to almost 1:1 after 12 hrs incubation with CPT (Table 2). It nicely documented that as first cells with proliferative activity died but with extended time dying cells came from other cycle phases. Just after 4 hrs incubation with CPT the total content of TUNEL-positive amelanotic melanoma cells was the same as a spontaneous death after 24 hrs (Table 2, Figure 2).

Discussion

Ability to spontaneous death of melanotic and amelanotic melanoma cells being at different phases of the cell cycle. Over 1/3 of the examined melanotic and amelanotic melanoma were in S and G2/M phases which confirms the opinion that melanoma is a tumor with high proliferation rate [6, 22]. But our observation that the amelantic form has more cells in S/G2/M phases than the melanotic one is of special importance because there are not such data in the melanoma literature although there is a common opinion that the amelanotic form is a more aggressive, rapidly growing melanoma form [16, 23]. In both melanoma lines, TUNEL-positive cells came mainly from S/G2/M phases but there were 3-fold more of apoptotic cells from these phases among cells of the melanotic melanoma line. We can not discuss our observation with any other authors because we could not find any information in the literature from which cell cycle phases tumor cells undergoing spontaneous apoptosis come, although there are works about changes in the cell cycle under the influence of different potential chemotherapeutics [6, 24]. The special sensitivity of cells in S/G2/M phases to spontaneous apoptosis is very difficult to explain but partly it could be the result of aneuploidy of these melanomas: melanotic line is almost 4n, amelanotic line 3n [25]. Aneuploidy of tumor cells is taken into consideration as a possible reason for the spontaneous apoptotic death of tumor cells [26].

During incubation time the biological differences of both melanoma lines are nicely visible; melanotic line – less aggressive, with the lower growth rate, always has fewer proliferating cells and higher content of spontaneously dying cells. The results of other authors concerning the relationships between tumor growth and the ability of its cells to proliferate and undergo apoptosis are controversial; some authors showed that tumor growth depended mainly on cell proliferation [27], others indicated that both proliferation and apoptosis increased [28, 29], that higher proliferation with decreased ability to undergo apoptosis supported tumor progression [29]. Observations of others that human melanoma cells have very low ability to undergo spontaneous apoptosis [30] and proliferating cells accounted for 20-40% of all cells [22] led them to the conclusion that the growth of human melanoma depends mainly on cell proliferative activity [31, 32]. In contrast, our results may suggest that the expansive growth of the amelanotic melanoma line depends mainly on the decreased ability of cells in all cell cycle phases to undergo spontaneous apoptosis. According to the latest reports it seems that cell cycle regulation is an individual feature of each tumor type [33], similarly to the ability to undergo spontaneous apoptosis [34], which is in agreement with our observations.

Susceptibility of melanotic and amelanotic melanoma cells at different cell cycle phases to camptothecin-induced death. During spontaneous death mainly cells in S/G2/M phases die but with extended time of incubation with CPT a high increase in the content of cells from G0/G1 phase was observed, so after 24 hrs there was equal proportion

of TUNEL-positive cells from S/G2/M and G0/G1 phases among amelanotic melanoma. This observation is of special importance because in the characteristics of camptothecin it is stressed that this alkaloid kills only cells being in the S phase of cycle, also melanoma cells [8, 10]. This S-phase-sensitivity is the result of DNA replication disturbances caused by the formed complex of CPT with topoisomerase I, an enzyme involved in the replication and transcription [7]. Although some authors noticed that CPT causes apoptotic death of cells in other than S phases [18] and also not proliferating cells e.g. neurons [17], the mechanism of killing cells from other than S phases is still not well understood. Some recent findings have stressed that CPT influences DNA replication and transcription separately and the disturbances of the last process cause death of not proliferating cells [19, 35]. Translation follows transcription so we could suspect inhibition of proteins production after CPT action on cells and there are such observations on fibroblasts and neurons [36, 37] but not on melanoma or cells from other tumors.

According to the aforementioned results we observed that after 24 hrs incubation of amelanotic melanoma cells with CPT, when 74% of cells were TUNEL-positive (the late apoptotic stage), there was 2.5-fold decrease in the protein content estimated by Bradford assay (unpublished data). The same analysis also shows that at the beginning of the experiment melanotic melanoma cells have 7.3±2.9 mg of proteins in ml of lysate while amelanotic melanoma cells 11.3±3.4 mg/ml (unpublished data). Thus, we can conclude that the level of transcription/translation is higher in the amelanotic line and could influence the G0/G1 cells susceptibility to CPT-induced death apart from other possible explanations of special sensitivity of hamster's amelanotic melanoma cells to CPT discussed in the previous works [13, 14]. Therefore not only the content of cells with proliferative activity but also metabolic activity e.g. ability to protein synthesis could determine the susceptibility to CPT action although the mechanism is not understood. Camptothecin antitumor activity depends on many extracellular and intracellular interactions [38] and probably there are other than topoisomerase I targets for it [37]. It should be stressed that the most important difference between melanotic and amelanotic melanoma is the presence or absence of melanogenesis, a specific process of melanin production ongoing inside special organelles melanosomes. Melanogenesis, as the process of cell differentiation, takes place after cell division – not in the S phase of cycle. Melanotic and amelanotic lines of Bomirski melanomas have many structural and metabolic differences [20].

Our earlier data show that amelanotic melanoma cells have higher plasma membrane fluidity in comparison to melanotic melanoma cells [39]. The structure of lipid bilayer influences CPT stability and penetration to the cell [40]. Camptothecin is a lipophilic substance and much effort has been done to improve water solubility of its analogues to make them less toxic. However, lipophilicity has been reported to improve the stability of the active form (with the lactone ring) and to favour rapid uptake and intracellular accumulation [40, 41]. On the basis of these observations, we can suppose that higher membrane fluidity allows better influx and accumulation of CPT in amelanotic melanoma cells which already after 4 hrs incubation show signs of apoptotic death [14, 15].

According to the recently published data that the side of camptothecins accumulation inside the cell is important for cytotoxicity [41], it is possible that melanosomes, presented only in the melanotic melanoma cells, could interfere with the sensitivity of these cells to CPT, what is suggested that even to other drugs [42-44].

Camptothecin undergoes a pH-dependent reversible interconversion between the lactone (active) and the ring-opened carboxylate forms [38] thus Bomirski amelanotic melanoma cells' ability to environmental pH modulation (acidification of medium during incubation) could also influence CPT cytotoxicity against these cells.

After over forty years of experimental examination and proved cytotoxicity against different tumors the question about the mechanism of tumor cells sensitivity to camptothecins has still no clear answer. Thus our observation that amelanotic melanoma cells with extended time of incubation with CPT died in all cell cycle phases could open up the possibility of using camptothecin analogues in the treatment of melanoma of this type. In the literature concerning biology of melanoma the information about the degree of melanization is rarely mentioned, so only through a detailed examination of studies it is possible to find out some data that this special sensitivity to CPT could not be only a feature of Bomirski amelanotic melanoma line but also human: Me26414 [11], A-375 [12] and mouse B16F10 [45] melanoma lines. If sensitivity to camptothecins is a feature of the amelanotic melanoma cells, the degree of melanoma cells melanization should be taken into account by biologists and chemists during the examination of camptothecin analogues as antitumor drugs. The amelanotic melanoma is biologically different than the melanotic melanoma which is not always taken into consideration during experimental results interpretation. Melanoma resistance is a major problem in treating patients with different types of conventional therapies.

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