Dacarbazine (DTIC) (Figure 1) is an alkylating anti-cancer pro-drug that affects the cells after previous activation in the liver by cytochrome P 450 (1, 2). DTIC is used mainly in melanoma and Hodgkin lymphoma therapy. DTIC belongs to the group of cell cycle nonspecific agents. Its activity is related to three mechanisms: alklylation by carbon ions activation, antimitabolic effect inhibiting DNA and RNA synthesis and alkylating of protein sulfhydryl groups.

The present paper supports the general concept that in vitro drug testing promises to help avoid treatment with ineffective drugs and their associated toxic side effects. In this sense, a lot of studies have investigated the in vitro sensitivity of melanoma cells to chemotherapeutic agents using several methods, such as MTT (3), tumor colony formation or plating efficiency (4) and others. However, it is important to determine the kind of death induced by the cytostatic drug at specific concentrations.

Cytotoxic activity of anticancer drugs leads to apoptosis and necrosis of cells. Apoptosis is an active process of cell death appearing in strictly indicated cells. Cells undergoing apoptosis display a characteristic pattern of structural and molecular alternations in the nucleus and cytoplasm, including cell shrinking, creation of apoptotic bodies containing cytoplasmatic fragments with or without condensed chromatin parts. Other events of early apoptosis are DNA laddering and the morphological changes occurring on the cell membrane surface (5, 6, 7).

After DNA destruction, cells die and disappear. Cell membrane loses its integrity, is fragmented and plasma membrane blebbing (zeiosis) is visible (8). The cells of this stage phase are celled sec-
ondary necrotic. The intracellular environment of apoptotic cells stays unchanged. Dead cells are quickly removed by macrophages.

Up to now a number of flow cytometry methods to identify cells with fragmented DNA have been described (9). These methods are based on determination of the phase of a cell cycle by estimating DNA content (10, 11) or by showing phosphatidylserine (PS) externalization on the cell membrane surface.

In the histograms presenting DNA content of apoptotic cells, an extensive G0/G1 peak is usually observed. This phenomenon results from a loss of DNA fragments out of the cells during the late stage of apoptosis due to reduced DNA “stainability” (12, 13).

During the early stages of apoptosis cell membrane integrity is still preserved, but the cells lose phospholipid cell membrane asymmetry. An additional change associated with beginning of apoptosis is the PS translocation from the inner to the outer leaflet of the plasma membrane (14). This event allows for specific macrophage recognition and recruit phagocytes to the site of the apoptotic lesion (7). Recognition and phagocytosis of defective cells and apoptotic bodies protect an organism from detrimental influence of cell components causing inflammatory reaction that mostly accompany necrosis (8).

Apoptotic cell death is characterized by the early exposure of PS at the outer surface of the plasma membrane (15). PS externalization was observed on the cell membrane surface of apoptotic cells following the loss of membrane integrity. This process is absent on necrotic cells. Apoptotic cells are stained with annexin V before morphological changes occur and DNA hydrolysis. Annexin V is a Ca²⁺ dependent protein, binding phospholipides and possesses an affinity to PS. Annexin V can be used as a sensitive PS exposure indicator on the outer layer of the cell membrane. It serves as a marker of the early stage of apoptosis in cells (16). Most of the results obtained from cytotoxicity assays carried out on the mouse melanoma cells in vitro can be extrapolated on their equivalents in humans. A study of influence of DTIC on cell viability and apoptosis in mouse melanoma B16 and Cloudman S91 cells in vitro was undertaken.

In vivo apoptosis quantification is almost impossible because of its asynchronism and short half-duration of apoptotic cells (17).

Using a DNA binding dye such as propidium iodide (PI) in tandem with fluorochrome-conjugated annexin V, apoptotic cells are identified and discriminated from necrotic cells (18, 19).

The estimation of cell viability and percentage of apoptotic cells after dacarbazine exposure may facilitate the choice of cytostatic drug dose. The advances in knowledge of mechanism of activity may prevent or minimize the drug toxicity.

**EXPERIMENTAL**

**Cell culture**

Parental B16 mouse melanoma cells line were obtained from the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wroclaw. The cell line was maintained through intraperitoneal transplantation and passage on C57B1/6J mice. B16 mouse melanoma cells were obtained from nodules in exudative form of mouse melanoma C57B1/6J. The cells were filtered through a steel sieve with holes 280 and 230 µm in diameter.

Cells of Cloudman mouse melanoma (Cloudman S91) were obtained from the Laboratory of Cytobiochemistry of the Institute of Physiological Chemistry (Philipp University, Marburg, Germany). Since 1996 they have been continuously maintained (passaged on DBA/2 mice) in the Tissue Culture Laboratory of the Department of Medical Biology at Nicolaus Copernicus University in Bydgoszcz.

Mouse melanoma B16 and Cloudman S91 were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Gibco, UK) supplemented with 4.5 g/L glucose, 10% fetal bovine serum (previously inactivated at 56°C for 30 min), 100 U/mL penicillin (Polfa Tarchomin S.A., Poland), 100 µg/mL streptomycin (Polfa Tarchomin S.A., Poland), 20 µg/mL gentamycin (Krka, Slovenia) and 0.125 µg/mL fluconazole (Pfizer, Germany). Culture medium was sterilized by passing it through 0.22 µm syringe filters (Nalgene, Denmark).

A quantity of 10⁶ melanoma cells were suspended in culture flasks (25 cm² of cell growth surface, Grainer, UK), filled with 5 mL of culture medium. The viability of cell suspension was 90 ±2% (trypane blue test, Bio Whitaker, USA). Cultures were conducted in sterile conditions in an MSC12 laminar flow unit (Jouan, France) and routinely maintained at 37°C with 5% CO₂, 98% humidity (IG150 incubator, Jouan, France).

The cells adhered to the bottom of the flask after four hours. Suspensions of investigated cells growing as monolayer, were obtained by detaching them from the culture flasks with 0.04% EDTA (Sigma, USA) in PBS Ca²⁺ and Mg²⁺ free (Serum
B16 and cloudman S91 mouse melanoma cells susceptibility...

and Vaccine Plant, Lublin, Poland) solution. The detached cells were centrifuged for 5 min at 500 \( \times \) g and washed twice with 37°C PBS. Supernatants were discarded and the cell pellets were resuspended in a small volume of culture medium. The number of cells was counted in a Neubauer’s chamber.

A cell suspension containing \( 5 \times 10^5 \) melanoma cells in 5 mL of medium (viability 90 ±2%) was used for cell culture carrying out and all studies.

Considering various growth rates of B16 and Cloudman S91 mouse melanoma cells the medium was renewed every 3 days for B16 cells and every 2 days for Cloudman S91 cells, according to the recommendations of American Type Cell Culture (B16 ñ ATCC:CRL – 6322, Cloudman S91 ñ ATCC:CCL – 53.1; Rockville, USA). A subcultivation ratio was 1:10.

Growth and morphology of cells were controlled under a phase-contrast microscope with reverse optics (Nikon TMS, Japan) before each passage.

In cytotoxicity tests, DTIC (Lachema, Czech Republic) – drug administered in treatment of human melanoma at concentrations from 0.014 M to 3.293 M was used. The drug was dissolved in PBS.

**Quantitative measurements of apoptosis in B16 and Cloudman S91 cells by flow cytometry**

In each B16 and Cloudman S91 population, the percentage of viable, apoptotic, secondary necrotic, and necrotic cells was established. The cell viability and apoptosis estimation were carried out in the control and experimental samples. 20 000 cells were counted in each sample.

10^5 cells of B16 and Cloudman S91 mouse melanoma were pre-incubated for 24 h and then different DTIC concentrations (from 0.014 M to 3.293 M) were added to the culture medium. After the next 24 h incubation melanoma cells were detached using 0.04% EDTA. Both tested cell lines in drug-free medium were used as control samples. The cells were centrifuged for 4 min at 500 \( \times \) g at 4°C, and the cell pellet was resuspended in 490 \( \mu \)L of the cold binding buffer (Immunotech, USA). Components of

![Figure 1. Structure pattern of dacarbazine.](image1)

Figure 1. Structure pattern of dacarbazine.

![Figure 2. Morphology of mouse melanoma cells stained with hematoxylin and eosin (magnification \( \times 160 \)): B16 (A) and Cloudman S91 (B) without dacarbazine treatment, B16 (C) after 1.098 M dacarbazine treatment, Cloudman S91 (D) after 0.549 M dacarbazine treatment.](image2)
The binding buffer was as follows: 10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂. The binding buffer was prepared by a 10-fold dilution of the concentrated buffer with distilled water and placed on ice.

Fluorescein isothiocyanate conjugated annexin V (Annexin V-FITC; Immunotech, USA) stock-solution was diluted 10-fold with previously prepared cold binding buffer and placed on ice. 250 µg of propidium iodide was dissolved in 1 mL of the diluted binding buffer and also placed on ice.

Aliquots of 5 µL of diluted annexin V-FITC and propidium iodide solutions were added to the tubes containing 490 µL of the B16 and Cloudman S91 melanoma cells suspensions. Then, the mixtures were vortexed for 5 seconds (Coulter vortex) and kept for 10 min on ice in the dark. After the incubation, the cell samples were analyzed by flow cytometer EPICS XL (Coulter) equipped with System IITM Software, version 1.0 (Coulter, FL, USA).

Cell morphology studies

B16 and Cloudman S91 cells were cultured on the microscope slides placed in clear culture Petri dishes with cover (Pyrex brand Petri dishes, Aldrich, Poland). Mouse melanoma cells of both cell lines were incubated with DTIC at IC₅₀ concentrations (drug inhibitory concentration – concentration inhibiting cell viability at 50%) similar to those used in apoptosis assay (Table 1). Cells were stained with hematoxilin and eosin. The morphological features of cells were evaluated under light microscopy.

**Table 1. The influence of dacarbazine on the viability of B16 and Cloudman S91 mouse melanoma cells**

<table>
<thead>
<tr>
<th>Dacarbazine concentration (M)</th>
<th>V</th>
<th>A</th>
<th>SN</th>
<th>N</th>
<th>V</th>
<th>A</th>
<th>SN</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>External control</td>
<td>98.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.6</td>
<td>98.0</td>
<td>0.9</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>0.014</td>
<td>96.0</td>
<td>3.0</td>
<td>0.9</td>
<td>0.1</td>
<td>78.3</td>
<td>21.1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>0.549</td>
<td>95.5</td>
<td>2.6</td>
<td>1.7</td>
<td>0.2</td>
<td>72.4</td>
<td>22.2</td>
<td>4.7</td>
<td>0.7</td>
</tr>
<tr>
<td>1.098</td>
<td>86.3</td>
<td>11.0</td>
<td>2.5</td>
<td>0.3</td>
<td>87.8</td>
<td>4.2</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>1.372</td>
<td>84.2</td>
<td>9.4</td>
<td>2.3</td>
<td>4.1</td>
<td>92.1</td>
<td>1.7</td>
<td>1.4</td>
<td>4.8</td>
</tr>
<tr>
<td>1.482</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>91.7</td>
<td>1.9</td>
<td>1.3</td>
<td>5.0</td>
</tr>
<tr>
<td>1.647</td>
<td>58.4</td>
<td>8.3</td>
<td>3.2</td>
<td>30.1</td>
<td>93.1</td>
<td>2.5</td>
<td>1.3</td>
<td>3.1</td>
</tr>
<tr>
<td>2.196</td>
<td>26.3</td>
<td>1.7</td>
<td>0.6</td>
<td>71.4</td>
<td>84.3</td>
<td>2.3</td>
<td>1.0</td>
<td>12.4</td>
</tr>
<tr>
<td>2.744</td>
<td>0.0</td>
<td>0.9</td>
<td>0.1</td>
<td>99.0</td>
<td>75.6</td>
<td>2.1</td>
<td>4.3</td>
<td>18.0</td>
</tr>
<tr>
<td>3.293</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>20.9</td>
<td>5.2</td>
<td>20.2</td>
<td>53.7</td>
</tr>
</tbody>
</table>

V – viable, a – Apoptotic, SN – secondary necrotic, N – necrotic
Correlation coefficient for a theoretical curve showing the percentage of viable B16 cell is 0.985 (p < 0.001) and for Cloudman S91 it is 0.970 (p < 0.001).
analyzed in fluorescent microscope equipped with dual filter set for FITC and PI (Jenalumar, Carl Zeiss, Jena, Germany). Green fluorescence of cells stained with annexin V-FITC was analyzed at wavelength $\lambda = 510$ nm. Red fluorescence of cells stained with PI was analyzed at $\lambda = 570$ nm.

**RESULTS AND DISCUSSION**

**Mouse melanoma B16 and Cl S91 cells in culture**

In comparison with primary cultures, during subculturing both B16 and Cl S91 cells gradually lost their pigmentation. B16 cells pigmentation was more intensive. B16 and Cloudman S91 in vitro had capacity to generate melanotic tumors in C57B1/6J and DBA2 mice, respectively. These tumors appeared in abdominal cavity of treated animals. B16 cells displayed a greater metastatic potential than Cloudman S91 cells (22).

**B16 and Cloudman S91 morphological changes after dacarbazine treatment**

The B16 melanoma cells after treating with dacarbazine at concentration of 1.098 M were converted to the oval shape. Characteristic for melanomas in culture spindle-like cells were absent. Lesser intracellular conjunction was observed. The cellular atypia was characterized by abnormal structure of some nuclei and strong staining. Hyperchromatosis was clearly visible. A large majority of cell nuclei were centered, but excentrically disposed nuclei were also appointed. Microscopic analysis showed polynucleosis and the chromatin-rich cells with giant nuclei, which testified a ploidy occurrence, whereas mitotic figures were not observed (Figure 2A, Figure 2C). Cytoplasm of some cells had an irregular contour and varied staining.

The Cloudman S91 cells after treatment with DTIC showed less anisocytosis when compared to B16 cells. No spindle-like, but oval Cloudman S91 cells were observed. These cells nuclei staining was similar with staining of cytoplasm. A large quantity of Cloudman S91 cells had irregular shape and uniformly disposed chromatin clusters. No mitotic figures in microscopic field were found (Figure 2B, Figure 2D).

**Quantitative measurements of apoptotic cells by flow cytometry**

The control samples revealed a low percentage of apoptotic and necrotic cells (Table 1). IC$_{50}$ value of DTIC was 1.724 M for B16 cells and 2.920 M for Cloudman S91 cells (Figure 3).

A significant diminution of B16 cell viability was found in these suspensions where dacarbazine concentration exceeded 1.373 M. The higher percentage of apoptotic B16 cells (11%) after 1.098 M DTIC exposure was identified (Table 1, Figure 4).

In the case of Cloudman S91 melanoma cells an important diminution of cell viability was observed only in cell suspensions where dacarbazine concentration exceeded 2.744 M (Table 1). Approximately 20% Cloudman S91 apoptotic cells was found only after administration of low drug concentration to the cell suspensions (from 0.137 to 0.549 M) (Figure 5). It was a highest percentage of apoptotic cells obtained during this assay. A significant decrease of Cloudman S91 mouse melanoma cells viability is connected with necrosis intensification.
Figure 4. Histograms showing flow cytometry analysis of B16 melanoma cells after dacarbazine treatment (upper row, following control sample, 0.014 M, 0.549 M, 1.098 M; lower row, following 1.372 M, 1.647 M, 2.196 M, 2.744 M).
Figure 5. Histograms showing flow cytometry analysis of Cloudman S91 melanoma cells after dacarbazine treatment (upper row, following control sample, 0.014 M, 0.549 M, 1.098 M, 1.372 M; lower row, following 1.482 M, 1.647 M, 2.196 M, 2.744 M, 3.293 M).
The B16 melanoma cells presented higher sensitivity to dacarbazine in tested range of concentration than Cloudman S91 melanoma cells.

The analysis with fluorescent microscopy method also showed the occurrence of apoptotic processes.

**B16 and Cloudman S91 mouse melanoma cell cycle evaluation based on DNA content**

The cell cycle analysis of B16 and Cloudman S91 melanoma cells after dacarbazine treatment was described by evaluation of DNA content. The per-

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Figure 6. Histograms demonstrating the number of cells at the following cell cycle phases: normal peripheral blood lymphocytes of C57B1/6J mouse (A); B16 melanoma cells (B) and Cloudman S91 melanoma cells (C) without chemotherapeutic agent. Melanoma DNA cell content after adding dacarbazine: B16 – 1.098 M (D) Cloudman S91 – 0.549 M (E).
The determination of cells sensitivity to cytostatic drugs and their susceptibility to apoptosis induction is a critical element in the control of cancer disease therapy. Considerable changes in chemosensitivity may occur when cells are brought from \textit{in vitro} to \textit{in vitro} conditions and \textit{vice versa}, and that such changes may be highly specific. Therefore, although cell lines may be useful in some respects, they should be used with caution in attempts to evaluate quantitatively the sensitivity of tumors to cancerostatic drugs (23). The chemosensitivity assay allow for the elimination of inefficient cytostatic drugs and for the indication of those which could be considered during the therapy of individual patients (24, 25, 26). Selection of chemotherapeutic drugs might increase efficacy and decrease toxicity of therapy.

Several authors showed comparable results obtained in different biochemical research undertaken on animal melanoma cell lines, especially B16 mouse melanoma, and on human melanoma tissue (27, 28, 29, 30, 31). In current studies we tested malignant melanoma cells for chemosensitivity in the experimental model.

The evaluation of antineoplastic agents’ effectiveness is based on different kinds of tests. The viability of cells, their metabolism and mitotic division capacities were analyzed. The apoptosis estimation by flow cytometry is a multiparameter method that allows the determination of both cell viability and pathway of cell death. Quantification of apoptotic and necrotic cell populations by flow cytometry can replace the other chemosensitivity tests. This method gives more advantages than the ones used before. The other tests, i.e. the MTT assay, permit only relative quantification of viable cells but do not show qualitative image.

The sensitivity of flow cytometry is high due to an individual multiparameter analysis of single cells and accuracy of detection systems and signal amplification.

In our study the B16 and Cloudman S91 mouse melanoma cell lines were tested for sensitivity to dacarbazine. The apoptosis process running in cell populations exposed to DTIC gives the knowledge which can be useful in finding out the appropriate dose for a cytostatic effect. The convenient dacarbazine dose induces programmed cell death without features of necrosis, i.e. lysis. The necrosis of the cell is often the result of cytostatic drugs overdosage.

\textit{In vitro} conditions are a new environment for melanoma cells where their vital functions proceed rather differently than \textit{in vivo}. It has been described that sometimes cultured cells showed an increased drug sensitivity which did not correlate with drug sensitivity of the same fresh or cryopreserved tumor or with clinical response (32). It should be emphasized that cell clones from different patients can revealed distinct cell chemosensitivity on the same drug especially \textit{in vivo}. The investigated mouse melanoma cell lines are able to grow autonomicaly under a lack of growth factors and hormones although they are sensitive to shortage of nutrients (33).

The apoptosis investigation in control samples of B16 and Cloudman S91 cells using flow cytometry showed a high percentage of viable cells. The percentage of viable cells that retain membrane integrity was 98.5% for B16 cells and 98% for Cloudman S91 cells.

Dacarbazine (DTIC) is the most commonly used drug in human melanoma therapy. Its primary mode of action is cell disruption by nucleic acids alkylation. It has been described that certain cytotoxic drugs with subtoxic concentrations, by apoptotic induction, sensitize melanoma cells to CTL lysis (34).

The \textit{in vitro} mouse melanoma cells viability tests after DTIC addition showed the necessity of drug dose increase to achieve the effects comparable with IC$_{50}$ = 0.274 M DTIC on the leukemic cell lines. IC$_{50}$ were 6-fold higher for B16 cells and 10-fold for Cloudman S91 cells. High dacarbazine doses decreased cell viability to 20% in B16 cells (2.196 M) and Cloudman S91 cells (3.293 M). However, apoptosis was revealed to occur at lower
drug doses and is more intensive in the case of Cloudman S91 cells. The maximal percentage of apoptotic cells was 11% and 22.2% in B16 and Cloudman S91 cells, respectively. The resistance of Cloudman S91 and to a lesser degree of B16 cells after dacarbazine treatment was observed. These results may suggest that the inefficient therapy of human malignant melanoma can be connected to very low apoptotic number after DTIC treatment.

It is supposed that melanoma cells resistance to alkylating agents (cisplatin, dacarbazine) is correlated with an increase of intracellular drugs detoxification by glutathione-S-transferase (GST). TST is a system interacting with reduced glutathione (GSH) machinery. The alteration of reduced glutathione intracellular levels can modify cell proliferation and detoxification and thus may determine cell sensitivity to cytostatic drugs (35).

DTIC addition to the culture medium in concentrations 1.098 M for the B16 cells and 1.647 M for the Cloudman S91 cells exerted a direct effect on DNA synthesis during the S phase of cell cycle. DTIC caused also cell arrest in G2/M cell cycle phase. Higher concentrations of dacarbazine induced only a block in G2/M phase. Dacarbazine affects melanoma cells during more than one cell cycle phase just like other cycle-nonspecific agents.

Probably the basic mechanism of cell death after exposure to cytostatic drugs is apoptosis triggering. One drug can induce both apoptosis and necrosis, depending on cell type and experiment conditions (36). The simple transfer of results observed in vitro to much more complexed in vivo conditions is, however, more complex. Additionally, some drugs induce more often apoptosis in vivo than the others (37). An in vivo examination of malignant cells sensitivity to action of chemotherapeutic agents should be a continuation of studies described above.

DTIC act on malignant melanoma by induction of apoptosis as a basic mechanism. However, effectiveness of this cytostatic drug varies in different kinds of melanoma cells. Then, although chemotherapy has had limited success in the treatment of melanoma, it is clear that combination of cytotoxic therapy and immunotherapy can enhance rates of tumor response.

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